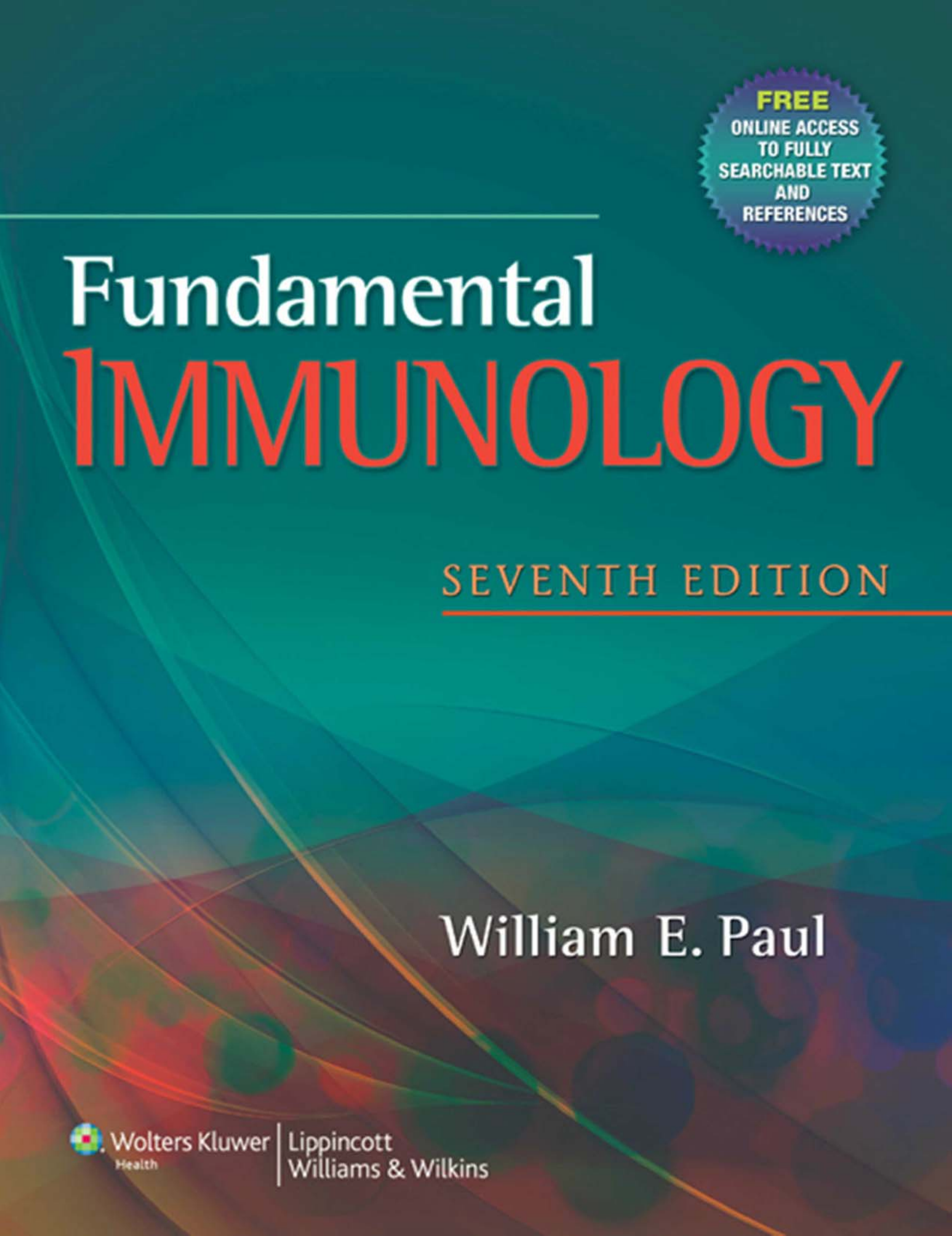
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SEVENTH EDITION
William E. Paul
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Fundamental Immunology

SEVENTH EDITION

Fundamental Immunology

SEVENTH EDITION

EDITOR

William E. Paul, MD

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For Charlotte, Gloria, Lucy, Jenna, Silvie, and Jake—and for Julien

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PREFACE

Immunology is the quintessential medical science. Indeed, no branch of the medical sciences has improved the health of people more than the application of immunologic principles to prevention of disease. Smallpox has been eliminated from the planet as a natural infection, as has poliomyelitis from the western hemisphere. Hepatitis B vaccine has prevented more cancers than any intervention other than smoking cessation. The human papilloma virus vaccine promises to cut strikingly the toll of cervical cancer.

The continued need for progress in immunology is clear. The epidemic of human immunodeficiency virus roars on. Glimmers of hope from vaccine trials have led to a redoubling of effort, and the struggle to design effective vaccines for the great infectious scourges goes on, with encouraging results but no breakthroughs yet. Highly effective therapeutic vaccines for cancers still elude us, but some immunologic therapies for cancer have met with encouraging results. We hope for much more as we marshal the tools coming from the study of the innate immune system, regulatory T cells, and lymphocyte differentiation and effector function.

Understanding the basis of inflammation and the cytokine world has given us effective drugs to treat rheumatoid arthritis and a growing number of other autoinflammatory/autoimmune diseases. The value of the interventions based on this knowledge, such as the use of tumor necrosis factor, interleukin-6, and interleukin -1 blockers, is now established. The application of anti–CD20 in the treatment of autoimmune disorders shows great promise. Even more promising strategies are on the horizon.

Fundamental Immunology has the goal of aiding in the education of a new generation of immunologists who can both probe more deeply into the organizing principles of the immune system and can translate this new information into effective treatments and preventatives that will extend and enlarge on the record of immunologic science in bettering the lot of human kind.

Were I beginning the task of preparing a comprehensive text of immunology today, I might have titled it *Immunology, Endless Fascination.* Certainly that describes my own view of this science over the 30 years that I have been working on the seven editions of *Fundamental Immunology.* I had believed that scientific progress was marked by periods of intense creativity, during which new concepts were established, followed by longer periods of consolidation, when work that made important but anticipated advances would dominate. Perhaps that will prove to be true of modern immunology as well when it is looked at by a disinterested observer, but for one in the midst, the pace of discovery seems to speed up with each passing year. *Endless fascination* certainly describes my experience of immunology.

I hope that this seventh edition will convey the dynamism and creativity of modern immunology and provide the reader with a solid introduction to our field and a picture of much of the very latest that has been achieved. As with each of the previous editions, most of the chapters are entirely new and not simple reworkings of the chapter in the previous edition. In order to contain the seventh edition within one volume, a decision was made to cite references in the printed text but only to provide the detailed citations in the online version. However, the references will be linked to PubMed so cited information can be easily obtained. The electronic version can be accessed at www.fundamentalimmunology.com.

As before, this edition begins with an introductory section consisting of the chapters "The Immune System" and "History of Immunology." These give an overview of modern immunology and of its origins, and provide those new to the field with the basis to go on to the subsequent chapters. This is followed by an "expanded introduction" provided by the sections "Organization and Evolution of the Immune System," "Immunoglobulins and B-Lymphocytes," and "T-Lymphocytes." These are followed by the two core "basic" immunology sections: "The Intersection of Innate and Adaptive Immunity" and "Induction, Regulation, and Effector Functions of the Immune Response." The book concludes with sections devoted to the immune system's role in protection against pathogenic microorganisms, "Immunity to Infectious Agents," and to how the immune system is involved in a variety of human disorders, "Immunologic Mechanisms in Disease."

I repeat a word of caution that has been in the Preface to each edition. Immunology is moving very fast. Each of the chapters is written by an expert in the field, but in some areas there may be differences of opinion expressed by equally accomplished authors. I ask the reader to take note of the differences and to follow developments in the field.

William E. Paul
Washington, DC

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The preparation of the seventh edition required the efforts of many individuals. I particularly wish to thank each of the authors. Their contributions, prepared in the midst of extremely busy schedules, are responsible for the value of this book. Leanne Vandetty of Lippincott Williams & Wilkins saw that the process of receiving, editing, and assembling the chapters went as smoothly as possible. Without her efforts, the completion of this edition would have been immeasurably more difficult. Frances DeStefano's counsel was of utmost importance in planning this edition and when she had to leave the project, Julie Goolsby took over and played a key role in making important publication decisions. I wish to gratefully acknowledge the efforts of each of the members of the editorial and production staffs of Lippincott Williams & Wilkins who participated in the preparation of this edition.

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Nothing is as powerful as an idea whose time has come.

Paraphrased from Victor Hugo

Everything should be made as simple as possible, but not simpler.

Albert Einstein

From my teachers I have learned much, from my colleagues still more, but from my students most of all.

The Talmud

Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

Albert Szent-Gyorgyi

. . . the clonal selection hypothesis . . . assumes that . . . there exist clones of mesenchymal cells, each carrying immunologically reactive sites . . . complementary . . . to one (or possibly a small number) of potential antigenic determinants.

Frank Macfarlane Burnet

In the fields of observation, chance favors the prepared mind.

Louis Pasteur

In all things of nature there is something of the marvelous.

Aristotle

SECTION I

Introduction to Immunology

CHAPTER 1

The Immune System

William E. Paul

The immune system is a remarkable defense mechanism. It makes rapid, specific, and protective responses against the myriad potentially pathogenic microorganisms that inhabit the world in which we live. The tragic examples of acquired immunodeficiency syndrome (AIDS) and the inherited severe combined immunodeficiencies graphically illustrate the consequences of a nonfunctional adaptive immune system. Patients with AIDS and children with severe combined immunodeficiency often fall victim to infections that are of little or no consequence to those with normally functioning immune systems. The immune system also has a role in the rejection of tumors and, when dysregulated, may give rise to a series of autoimmune diseases, including insulin-dependent diabetes mellitus, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, among others.

Fundamental Immunology has as its goal the authoritative presentation of the basic elements of the immune system, of the means through which the mechanisms of immunity act in a wide range of clinical conditions, including recovery from infectious diseases, rejection of tumors, transplantation of tissue and organs, autoimmunity and other immunopathologic conditions, and allergy, and how the mechanisms of immunity can be marshaled by vaccination to provide protection against microbial pathogens.

The purpose of the opening chapter is to provide readers with a general introduction to our current understanding of the immune system. It should be of particular importance for those with a limited background in immunology, providing them with the preparation needed for subsequent chapters of the book. Rather than providing extensive references in this chapter, each of the subject headings will indicate the chapters that deal in detail with the topic under discussion. Those chapters will not only provide an extended treatment of the topic but will also furnish the reader with a comprehensive reference list that can be found in the online version of *Fundamental Immunology.*

KEY CHARACTERISTICS OF THE IMMUNE SYSTEM

Innate Immunity (Chapters 15, 17, 19, and 20)

Powerful nonspecific defenses prevent or limit infections by most potentially pathogenic microorganisms. The epithelium provides both a physical barrier to the entry of microbes and produces a variety of antimicrobial factors. Agents that penetrate the epithelium are met with macrophages and related cells possessing "microbial sensors" that recognize key molecules characteristic of many microbial agents. These "pattern recognition receptors" include several families of molecules, of which the most intensively studied are the toll-like receptors (TLRs) and the nucleotide oligomerization domain–like receptors. Each TLR recognizes a distinct substance (or set of substances) associated with microbial agents; for example, TLR4 recognizes lipopolysaccharides, TLR3, double-stranded ribonucleic acid, and TLR9, unmethylated CpG-containing DNA. Because the recognized substances are generally indispensable to the infectious agent, microbial sensors provide a highly efficient means to recognize potential pathogens.

The interaction of a TLR with its ligand induces a series of intracellular signaling events, of which activation of the NF-κB system is particularly important. Macrophage activation with enhancement of the cell's phagocytic activity and the induction of antimicrobial systems aid in the destruction of the pathogen. The induction of an inflammatory response as a result of the activation of the innate immune system recruits other cell types, including neutrophils, to the site. The innate system can provide an effective means to control or eliminate pathogens. Indeed, life forms other than vertebrates rely on the innate immune system to allow them to deal with microbial infection.

In vertebrates, the innate immune system also acts to recruit antigen-specific immune responses, not only by attracting cells of the immune system to the site of infection, but also through the uptake of antigen by dendritic cells (DCs) and its transport by these cells to lymphoid tissues where primary immune responses are initiated. Activated DCs express cell surface costimulatory molecules and produce cytokines that can regulate the quality of the immune response so that it is most appropriate to combating the particular infectious agent, be it a virus, bacterium, or parasite.

Primary Responses (Chapters 10 and 14)

Primary immune responses are initiated when a foreign antigenic substance interacts with antigen-specific lymphocytes under appropriate circumstances. The response generally consists of the production of antibody molecules specific for the antigenic determinants of the immunogen and of the expansion and differentiation of antigen-specific helper and effector T-lymphocytes. The latter include cytokine-producing cells and killer T cells, capable of lysing infected cells. Generally, the combination of the innate immune response and the primary adaptive response are sufficient to eradicate or to control the microbe. Indeed, the most effective function of the immune system is to mount a response that eliminates the infectious agent from the body, so-called sterilizing immunity.

Secondary Responses and Immunologic Memory (Chapters 10, 14, 29, and 31)

As a consequence of initial encounter with antigen, the immunized individual develops a state of immunologic memory. If the same (or a closely related) microorganism is encountered again, a secondary response is made. This generally consists of an antibody response that is more rapid, greater in magnitude, and composed of antibodies that bind to the antigen with greater affinity and are more effective in clearing the microbe from the body. A more rapid and more effective T-cell response also ensues. Thus, an initial infection with a microorganism often initiates a state of immunity in which the individual is protected against a second infection. In the majority of situations, protection is provided by high-affinity antibody molecules that rapidly clear the re-introduced microbe. This is the basis of most licensed vaccines; the great power of vaccines is illustrated by the elimination of smallpox from the world and by the complete control of polio in the western hemisphere.

The Immune Response is Highly Specific and the Antigenic Universe is Vast

The immune response is highly specific. Primary immunization with a given microorganism evokes antibodies and T cells that are specific for the antigenic determinants found on that microorganism but that generally fail to recognize (or recognize only poorly) antigenic determinants expressed by unrelated microbes. Indeed, the range of antigenic specificities that can be discriminated by the immune system is enormous.

The Immune System is Tolerant of Self-Antigens (Chapters 32 and 33)

One of the most important features of the immune system is its ability to discriminate between antigenic determinants expressed on foreign substances, such as pathogenic microbes, and potential antigenic determinants expressed by the tissues of the host. The failure of the system to make full-blown immune responses to self-antigens is referred to as *immunologic tolerance*. Tolerance is a complex process that actually involves several distinct processes. One element, perhaps the most important, is an active process involving the elimination or inactivation of cells that can recognize self-antigens. In addition, there are mechanisms through which cells that encounter antigens (such as self-antigens) in the absence of cues from the innate immune system may fail to make a response, may make a minimal response, or may be inactivated through a process referred to as *anergy*. Finally, a specialized set of T cells exist designated regulatory cells that actively *suppress* responses against self-antigens. Indeed, individuals who have mutations in the key transcription factor *Foxp3* expressed by the regulatory cells develop severe multiorgan autoimmunity (Immunodysregulation polyendocrinopathy, enteropathy X-linked syndrome). The critical necessity to control self-reactivity is clearly shown by this multilayered system that involves elimination, inactivation, and suppression.

Immune Responses Against Self-Antigens can Result in Autoimmune Diseases (Chapter 44)

Failure in establishing immunologic tolerance or unusual presentations of self-antigens can give rise to tissue-damaging immune responses directed against antigenic determinants on host molecules. These can result in autoimmune diseases. As has already been mentioned, a group of extremely important diseases are caused by autoimmune responses or have major autoimmune components, including systemic lupus erythematosus, rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, myasthenia gravis, and inflammatory bowel disease. Efforts to treat these diseases by modulating the autoimmune response are a major theme of contemporary medicine.

Acquired Immunodeficiency Syndrome is an Example of a Disease Caused by a Virus That the Immune System Generally Fails to Eliminate (Chapter 42)

Immune responses against infectious agents do not always lead to elimination of the pathogen. In some instances, a chronic infection ensues in which the immune system adopts a variety of strategies to limit damage caused by the organism or by the immune response. Indeed, herpes viruses, such as human cytomegalovirus, frequently are not eliminated by immune responses and establish a chronic infection in which the virus is controlled by immune responses. One of the most notable infectious diseases in which the immune response generally fails to eliminate the organism is AIDS, caused by the human immunodeficiency virus (HIV). In this instance, the principal infected cells are those of the immune system

itself, leading to an eventual state in which the individual can no longer mount protective immune responses against other microbial pathogens. Indeed, under the assault of HIV, control of viruses such as cytomegalovirus is lost and they may cause major tissue damage.

Major Principles of Immunity

The major principles of the immune response are:

- Elimination of many microbial agents through the nonspecific protective mechanisms of the innate immune system
- Cues from the innate immune system inform the cells of the adaptive immune system as to whether it is appropriate to make a response and what type of response to make
- Cells of the adaptive immune system display exquisitely specific recognition of foreign antigens and mobilize potent mechanisms for elimination of microbes bearing such antigens
- The immune system displays memory of its previous responses
- Tolerance of self-antigens.

The remainder of this introductory chapter will describe briefly the molecular and cellular basis of the system and how these central characteristics of the immune response may be explained.

CELLS OF THE IMMUNE SYSTEM AND THEIR SPECIFIC RECEPTORS AND PRODUCTS

The immune system consists of several distinct cell types, each with important roles. The lymphocytes occupy central stage because they are the cells that determine the specificity of immunity. It is their response that orchestrates the effector limbs of the immune system. Cells that interact with lymphocytes play critical parts both in the presentation of antigen and in the mediation of immunologic functions. These cells include DCs and the closely related Langerhans cells, monocyte/macrophages, natural killer (NK) cells, neutrophils, mast cells, basophils, and eosinophils. In addition, a series of specialized epithelial and stromal cells provide the anatomic environment in which immunity occurs, often by secreting critical factors that regulate migration, growth and homeostasis, and gene activation in cells of the immune system. Such cells also play direct roles in the induction and effector phases of the response.

The cells of the immune system are found in peripheral organized tissues, such as the spleen, lymph nodes, Peyer's patches of the intestine, and tonsils, where primary immune responses generally occur (see Chapter 3). Many of the lymphocytes comprise a recirculating pool of cells found in the blood and lymph, as well as in the lymph nodes and spleen, providing the means to deliver immunocompetent cells to sites where they are needed and to allow immunity that is initiated locally to become generalized. Activated lymphocytes acquire the capacity to enter nonlymphoid tissues where they can express effector functions and eradicate local infections. Some memory lymphocytes are "on patrol" in the tissues, scanning for reintroduction of their specific antigens. Lymphocytes are also found in the central lymphoid organs, the thymus, and bone marrow, where they undergo the developmental steps that equip them to mediate the responses of the mature immune system.

Individual lymphocytes are specialized in that they are committed to respond to a limited set of structurally related antigens. This commitment exists before the first contact of the immune system with a given antigen. It is expressed by the presence on the lymphocyte's surface of receptors specific for determinants (epitopes) of the antigen. Each lymphocyte possesses a population of receptors, all of which have identical combining sites (this is a slight oversimplification as occasionally T cells and less frequently B cells may express two populations of receptors). One set, or clone, of lymphocytes differs from another clone in the structure of the combining region of its receptors and thus in the epitopes that it can recognize. The ability of an organism to respond to virtually any non–self-antigen is achieved by the existence of a very large number of different lymphocytes, each bearing receptors specific for a distinct epitope. As a consequence, lymphocytes are an enormously heterogeneous group of cells. Based on reasonable assumptions as to the range of diversity that can be created in the genes encoding antigen-specific receptors, it is virtually certain that the number of distinct combining sites on lymphocyte receptors of an adult human can be measured in the millions.

Lymphocytes differ from each other not only in the specificity of their receptors but also in their functions. There are two broad classes of lymphocytes: the B-lymphocytes, which are precursors of antibody-secreting cells, and the T (thymus-derived)-lymphocytes. T-lymphocytes express important helper functions, such as the ability to aid in the development of specific types of immune responses, including the production of antibody by B cells, the increase in the microbicidal activity of macrophages, and the recruitment of granulocytes to sites of infection. Other T-lymphocytes are involved in direct effector functions, such as the lysis of virus-infected cells or certain neoplastic cells. Regulatory T-lymphocytes have the capacity to suppress immune responses.

B-LYMPHOCYTES AND ANTIBODY

B-Lymphocyte Development (Chapter 8)

B-lymphocytes derive from lymphoid progenitor cells, which in turn are derived from hematopoietic stem cells (Fig. 1.1). A detailed picture has been obtained of the molecular mechanisms through which committed early members of the B lineage develop into mature B-lymphocytes. These events occur in the fetal liver and, in adult life, in the bone marrow. Interaction with specialized stromal cells and their products, including cytokines such as interleukin (IL)-7 and BAFF, are critical to the normal regulation of this process.

The key events in B-cell development involve commitment to the B lineage and repression of the capacity to differentiate to cells of other lineages. In pro-B cells and pre-B cells, the genetic elements that encode the antigen-specific receptors are assembled. These receptors are im-

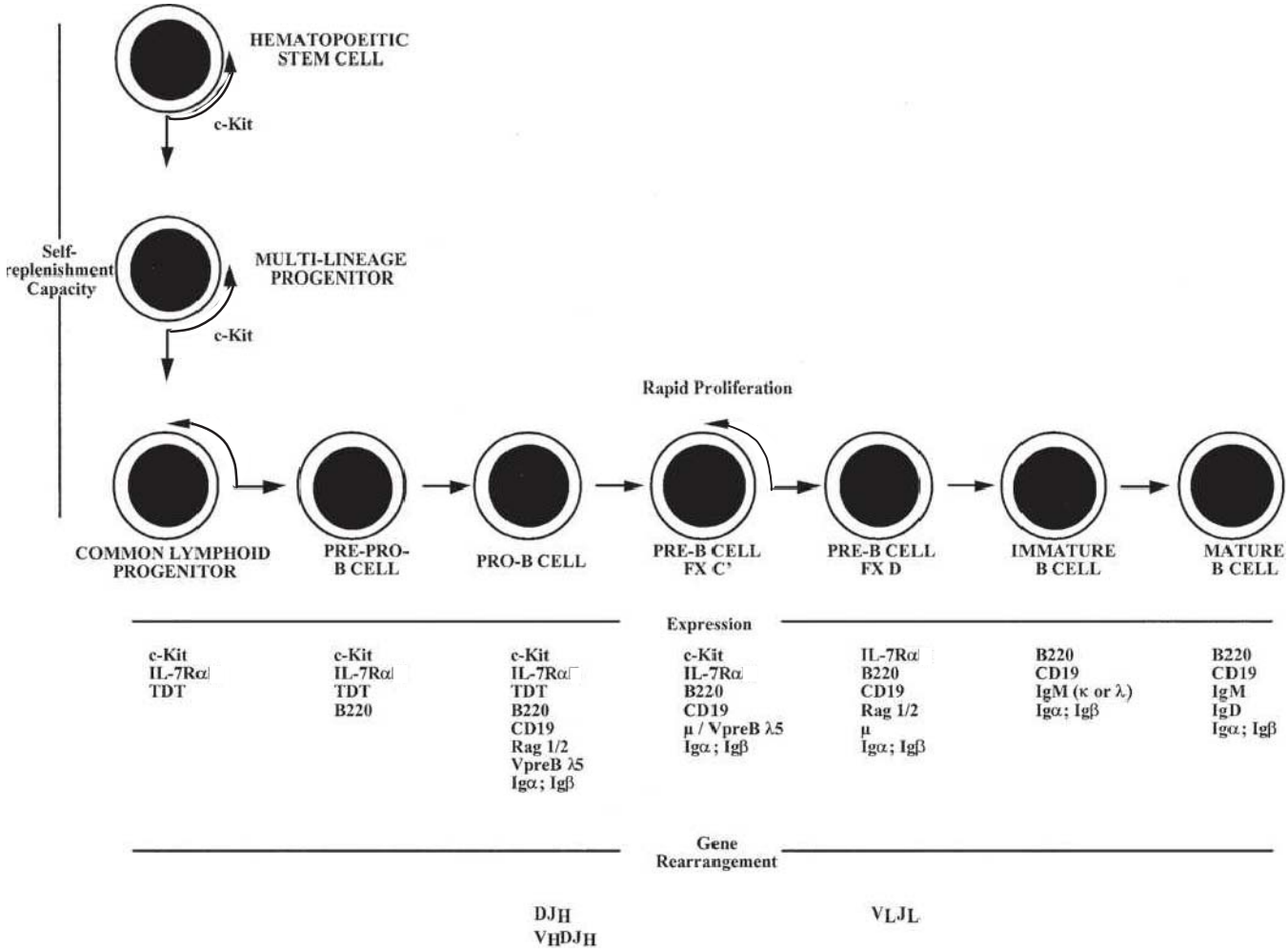

FIG. 1.1. The patterns of gene expression, timing of gene rearrangement events, and capacity for self-replenishment and for rapid proliferation of developing B lymphocytes are indicated. (Adapted from Hardy RR, Hayakawa K. B cell development pathways. *Ann Rev Immunol.* 2001;19:595–621.)

munoglobulin (Ig) molecules specialized for expression on the cell surface. Igs are heterodimeric molecules consisting of heavy (H) and light (L) chains, both of which have variable (V) regions, which are responsible for the binding of antigen and that differ in sequence from one Ig molecule to another (see Chapters 5, 6, and 7) (Fig. 1.2) and constant (C) regions.

The genetic elements encoding the variable portions of Ig H and L chains are not contiguous in germline DNA or in the DNA of nonlymphoid cells (see Chapter 6) (Fig. 1.3). In pro- and pre-B cells, these genetic elements are translocated to construct an expressible V-region gene. This process involves a choice among a large set of potentially usable V, diversity (D), and joining (J) elements in a combinatorial manner and depends upon the recombinating activating gene (RAG) proteins, RAG1 and RAG2. Such combinatorial translocation, together with the addition of diversity in the course of the joining process, results in the generation of a very large number of distinct H and L chains. The pairing of H and L chains in a quasirandom manner further expands the number of distinct Ig molecules that can be formed.

The H-chain V region is initially expressed in association with the product of the μ C-region gene. Together, these elements encode the μ IgH chain, which is used in Igs of the IgM class.

The successful completion of the process of Ig gene rearrangement and the expression of the resultant IgM on the cell surface marks the transition between the pre-B– and B–cell states (see Fig. 1.1). The newly differentiated B cell initially expresses surface Ig solely of the IgM class. The cell completes its maturation process by expressing on its surface a second class of Ig composed of the same L chain and the same H chain V (VDJ) region but of a different H chain C region; this second Ig H chain is designated δ, and the Ig to which it contributes is designated IgD, so that the mature naïve B cells express both IgM and IgD surface molecules that share the same V region.

The differentiation process is controlled at several steps by a system of checks that determines whether prior steps have been successfully completed. These checks depend on the expression on the surface of the cell of appropriately constructed Ig or Ig-like molecules. For, example,

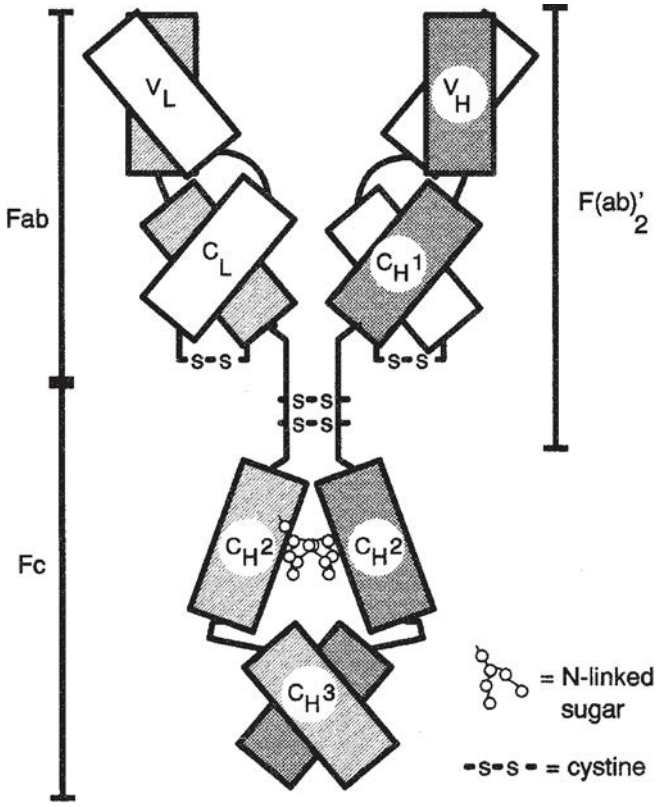

FIG. 1.2. A schematic representation of an immunoglobulin molecule indicating the means through the variable regions and the CH1 and CL regions of heavy and light chains pair with one another and how the CH2 and CH3 regions of the heavy chains pair.

in the period after a μ chain has been successfully assembled but before an L chain has been assembled, the μ chain is expressed on the cell surface in association with a surrogate L chain, consisting of VpreB and λ5. Pre-B cells that fail to express this μ/VpreB λ5 complex do not move forward to future differentiation states or do so very inefficiently.

B-Lymphocyte Activation (Chapter 9)

A mature B cell can be activated by an encounter with antigen-expressing epitopes that are recognized by its cell surface Ig (Fig. 1.4). The activation process may be a direct one, dependent on cross-linkage of membrane Ig molecules by the antigen (*cross-linkage–dependent B-cell activation*), or an indirect one, occurring most efficiently in the context of an intimate interaction with a helper T cell, in a process often referred to as *cognate help*.

Because each B cell bears membrane Ig molecules with identical variable regions, cross-linkage of the cell surface receptors requires that the antigen express more than one copy of an epitope complementary to the binding site of the receptor. This requirement is fulfilled by antigens with repetitive epitopes. Among these antigens are the capsular polysaccharides of many medically important microorganisms such as pneumococci, streptococci, and meningococci. Similar expression of multiple identical epitopes on a single immunogenic particle is a property of many viruses because they express multiple copies of envelope proteins on their surface. Cross-linkage–dependent B-cell activation is a major protective immune response mounted against these microbes. The binding of complement components (see Chapter 36) to antigen or antigen/antibody complexes can increase the magnitude of the cross-linkage–dependent B-cell activation due to the action of a receptor for complement, which, together with other molecules, increases the magnitude of a B-cell response to limiting amounts of antigen.

Cognate help allows B cells to mount responses against antigens that cannot cross-link receptors and, at the same time, provides costimulatory signals that rescue B cells from inactivation when they are stimulated by weak cross-linkage events. Cognate help is dependent on the binding of antigen by the B cell's membrane Ig, the endocytosis of the antigen, and its fragmentation into peptides within the endosomal/lysosomal compartment of the cell. Some of the resultant peptides are loaded into a groove in a specialized set of cell surface proteins, the class II major histocompatibility complex (MHC) molecules (Fig. 1.5). The resultant class II/

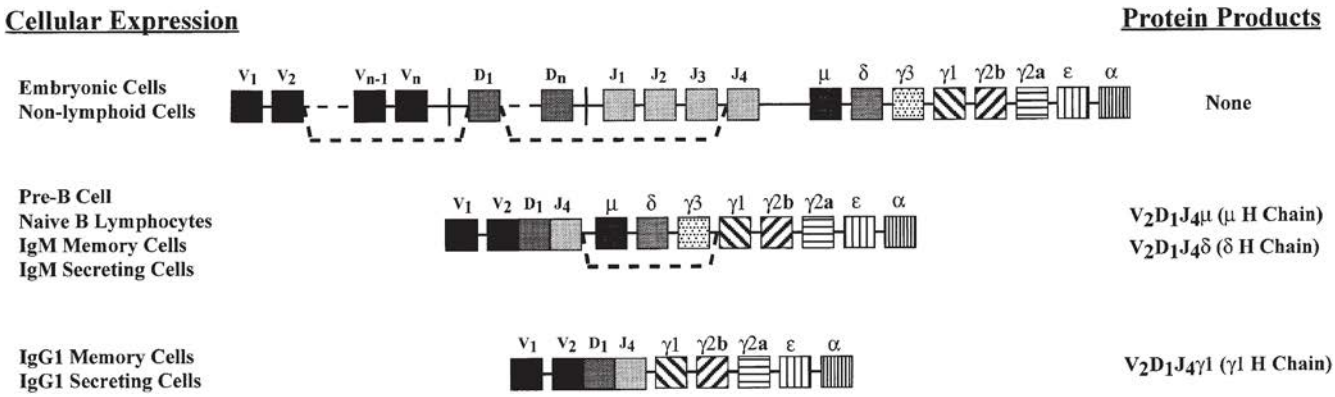

FIG. 1.3. Organization and translocation of mouse immunoglobulin (Ig)H genes. IgH chains are encoded by four distinct genetic elements: Igh-V (V), Igh-D (D), Igh-J (J) and Igh-C. The variable (V), diversity (D), and joining (J) genetic elements together specify the variable region of the heavy chain. The Igh-C element specifies the constant (C) region. The same V region can be expressed in association with each of the C regions (μ, δ, γ3,γ1,γ2b, γ2a, ε, and α). In the germline, the V, D, and J genes are far apart and there are multiple forms of each of these genes. In the course of lymphocyte development, a VDJ gene complex is formed by translocation of individual V and D genes so that they lie next to one of the J genes, with excision of the intervening genes. This VDJ complex is initially expressed with μ and δ C genes but may be subsequently translocated so that it lies near one of the other C genes (e.g., γ1) and in that case leads to the expression of a VDJ γ1 chain.

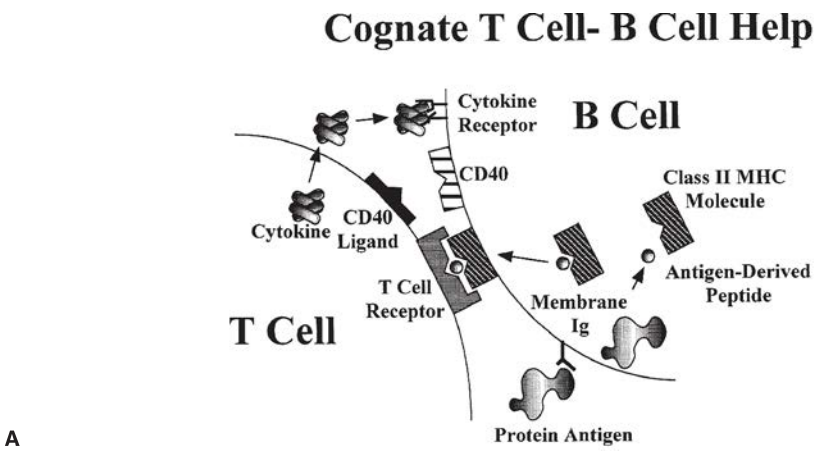

FIG. 1.4. Two Forms of B-Cell Activation. A: Cognate T cell/B cell help. Resting B cells can bind antigens that bear epitopes complementary to their cell surface Ig. Even if the antigen cannot cross-link the receptor, it will be endocytosed and enter late endosomes and lysosomes where it will be degraded to peptides. Some of these peptides will be loaded into class II major histocompatibility complex molecules and brought to the cell surface, where they can be recognized by CD4-positive T cells that bear receptors specific for that peptide/class II complex. This interaction allows an activation ligand on the T cells (CD40 ligand) to bind to its receptor on B cells (CD40) and to signal B-cell activation. In addition, the T cells secrete several cytokines that regulate the growth and differentiation of the stimulated B cell. **B:** Cross-linkage–dependent B cell activation. When B cells encounter antigens that bear multiple copies of an epitope that can bind to their surface immunoglobulin, the resultant cross-linkage stimulates biochemical signals within the cell leading to B-cell activation, growth, and differentiation. In many instances, B-cell activation events may result from both pathways of stimulation.

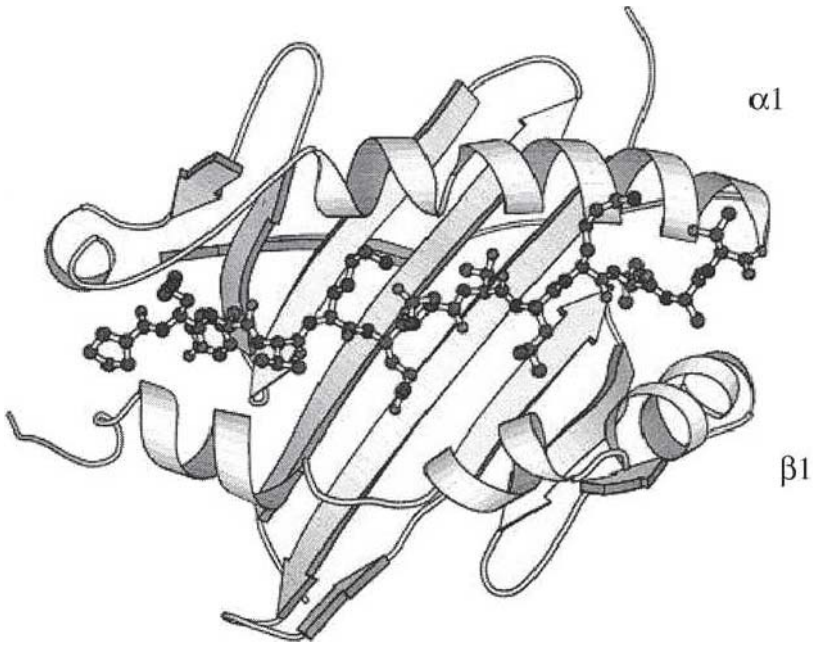

FIG. 1.5. Illustration of the structure of the peptide binding domain (α1 and β1) of a class II major histocompatibility complex molecule bound to an antigenic peptide from influenza hemagglutinin (adapted by D.H. Margulies from Stern LJ, Brown JH, Jardetzky TS, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature*. 1994;68[6468]:215–221.)

peptide complexes are expressed on the cell surface. As will be discussed subsequently, these complexes are the ligands for the antigen-specific receptors of a set of T cells designated CD4 T cells. CD4 T cells that have receptors specific for the class II/peptide complex expressed on the B-cell surface recognize and interact with that B cell. That interaction results in the activation of the B cell through the agency of cell surface molecules expressed by the T cells (e.g., the CD40 ligand [CD154]) and cytokines produced by the T cell (see Fig. 1.4). The role of the B-cell receptor for antigen is to create the T-cell ligand on the surface of antigen-specific B cells; activation of the B cell derives largely from the action of the T cell. However, in many physiologic situations, receptor cross-linkage stimuli and cognate help synergize to yield more vigorous B-cell responses. Recently, it has been shown that the association of ligands for TLRs with antigen will strikingly enhance B cell responses.

B-Lymphocyte Differentiation (Chapters 9 and 10)

Activation of B cells prepares them to divide and to differentiate either into antibody-secreting cells or into memory cells, so that there are more cells specific for the antigen used for immunization. Those cells that differentiate into antibody-secreting cells account for primary antibody responses. Some of these antibody-secreting cells migrate to the bone marrow where they may continue to produce antibody for an extended period of time and may have lifetimes very long.

Memory B cells give rise to antibody-secreting cells upon rechallenge of the individual. The hallmark of the antibody response to rechallenge (a secondary response) is that it is of greater magnitude, occurs more promptly, is composed of antibodies with higher affinity for the antigen, and is dominated by Igs expressing γ, α, or ε C regions (IgG, IgA, or IgE) rather than by IgM, which is the dominant Ig of the primary response.

Division and differentiation of cells into antibody-secreting cells is largely controlled by the interaction of the activated B cells with T cells expressing CD154 and by their stimulation by T-cell–derived cytokines.

The differentiation of activated B cells into memory cells occurs in a specialized microenvironmental structure in the spleen and lymph nodes: the germinal center. The increase in antibody affinity also takes place within the germinal center. This process, designated *affinity maturation*, is dependent on somatic hypermutation. The survival of B cells within the germinal center depends on their capacity to bind antigen so that as the amount of antigen diminishes, B cells that have higher affinity receptors, either naturally or as a result of the hypermutation process, have a selective survival and growth advantage. Thus, such cells come to dominate the population.

The process through which a single H-chain V region can become expressed with genes encoding C regions other than μ or δ is referred to as Ig class switching. It is dependent on a gene translocation event through which the C-region genes between the genetic elements encoding the V region and the newly expressed C gene are excised, resulting in the switched C gene being located in the position that the Cμ gene formerly occupied (see Fig. 1.3). This process also occurs mainly in germinal centers. Both somatic hypermutation and immunoglobulin class switching depend upon the action of activation-induced cytidine deaminase (AID) that plays an important role in the breakage and repair of DNA, which is essential for recombination events.

B1 and Marginal Zone B-Lymphocytes (Chapters 8 and 10)

B lymphocytes consist of at least three distinct populations: conventional B cells, B1 B cells, and marginal zone B cells. B1 B cells were initially recognized because some express a cell-surface protein, CD5, not generally found on other B cells. In the adult mouse, B1 B cells are found in relatively high frequency in the peritoneal cavity but are present at low frequency in the spleen and lymph nodes. B1 B cells are quite numerous in fetal and perinatal life and appear to be self-renewing, in contrast to conventional B cells, in which division and memory are antigen driven.

Marginal zone B cells are localized in a distinct anatomical region of the spleen (the marginal zone) that represents the major antigen filtering and scavenging area. Like B1 B cells, marginal zone B cells express a repertoire biased toward bacterial cell wall constituents and senescent self-components. Marginal zone and B1 B cells respond very rapidly to antigenic challenge, likely independently of T cells. Uniquely, among all populations of B cells, marginal zone B cells are dependent on Notch-2 signaling for their development.

B1 B cells and marginal zone B cells are responsible for the secretion of the serum IgM that exists in nonimmunized mice, often referred to as natural IgM. Among the antibodies found in such natural IgM are molecules that can combine with phosphatidylcholine (a component of pneumococcal cell walls) and with lipopolysaccharide and influenza virus. B1 B cells also produce autoantibodies, although they are generally of low affinity and in most cases not pathogenic. There is evidence that B1 B cells are important in resistance to several pathogens and may have a significant role in mucosal immunity.

B-Lymphocyte Tolerance (Chapter 32)

One of the central problems facing the immune system is that of being able to mount highly effective immune responses to the antigens of foreign, potentially pathogenic agents while ignoring antigens associated with the host's own tissues. The mechanisms ensuring this failure to respond to self-antigens are complex and involve a series of strategies. Chief among them is elimination of cells capable of self-reactivity or the inactivation of such cells. The encounter of immature, naïve B cells with antigens with repetitive epitopes capable of cross-linking membrane Ig can lead to elimination of the B cells, particularly if no T-cell help is provided at the time of the encounter. This elimination of potentially self-reactive cells is often referred to as clonal elimination. Many self-reactive cells, rather than dying upon encounter with self-antigens, undergo a further round of Ig gene rearrangement. This *receptor editing* process allows a self-reactive cell to substitute a new receptor and therefore to avoid elimination.

There are many self-antigens that are not encountered by the developing B-cell population or that do not have the capacity to cross-link B-cell receptors to a sufficient degree to elicit the receptor editing /clonal elimination process. Such cells, even when mature, may nonetheless be inactivated through a process that involves cross-linkage of receptors without the receipt of critical costimulatory signals. These inactivated cells may be retained in the body but are unresponsive to antigen and are referred to as anergic. When removed from the presence of the anergy-inducing stimulus, anergic cells may regain responsiveness.

Immunoglobulins

Structure (Chapter 5)

Igs are the antigen-specific membrane receptors and secreted products of B cells. They are members of a large family of proteins designated the Ig supergene family. Members of the Ig supergene family have sequence homology, a common gene organization, and similarities in three-dimensional structure. The latter is characterized by a structural element referred to as the Ig fold, generally consisting of a set of seven β-pleated sheets organized into two opposing layers (Fig. 1.6). Many of the cell surface proteins that participate in immunologic recognition processes, including the T-cell receptor (TCR), the CD3 complex, and signaling molecules associated with the B-cell receptor (Igα and Igβ), are members of the Ig supergene family.

The Igs themselves are constructed of a unit that consists of two H chains and two L chains (see Fig. 1.2). The H and L chains are composed of a series of domains, each consisting of approximately 110 amino acids.

The L chains, of which there are two types (κ and λ), consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the C region. As already discussed, the amino terminal domain varies from L chain to L chain and contributes to the binding site of antibody. Because of its variability, it is referred to as the V region. The variability of this region is largely concentrated in three segments, designated the hypervariable or complementarity-determining regions (CDRs). The CDRs contain the amino acids that are the L chain's contribution to the lining of the antibody's combining site. The three CDRs are interspersed among four regions of much lower degree of variability, designated framework regions.

The H chains of Ig molecules are of several classes determined by their constant regions (μ, δ, γ [of which there are several subclasses], α and ε). An assembled Ig molecule, consisting of one or more units of two identical H and L chains, derives its name from the constant region of the H chain that it possesses. Thus, there are IgM, IgD, IgG, IgA, and IgE antibodies. The H chains each consist of a single amino terminal V region and three or four C regions. In many H chains, a hinge region separates the first and second C regions and conveys flexibility to the molecule, allowing the two combining sites of a single unit to move in relation to one another so as to promote the binding of a single antibody molecule to an antigen that has more than one copy of the same epitope. Such divalent binding to a single antigenic structure results in a great gain in energy of interaction (see Chapter 7). The H-chain V region, like that of the L chain, contains three CDRs lining the combining site of the antibody and four framework regions.

The C region of each H-chain class conveys unique functional attributes to the antibodies that possess it. Among the distinct biologic functions of each class of antibody are the following:

- IgM antibodies are potent activators of the complement system (see Chapter 36).
- IgA antibodies are secreted into a variety of bodily fluids and are principally responsible for immunity at mucosal surfaces (see Chapter 34).
- IgE antibodies are bound by specific receptors (FcεRI) on basophils and mast cells. When cross-linked by antigen, these IgE/FcεRI complexes cause the cells to release a set of mediators responsible for allergic inflammatory responses (see Chapter 45).
- IgD antibodies act virtually exclusively as membrane receptors for antigen.

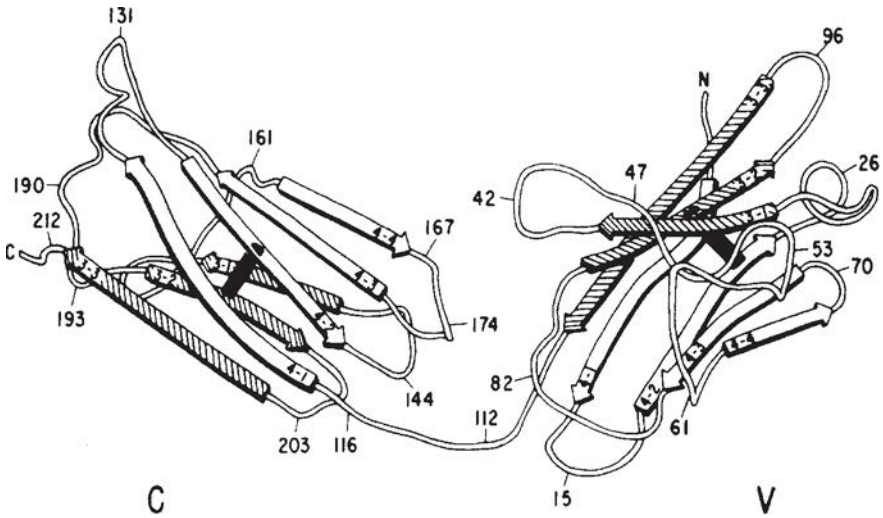

FIG. 1.6. Schematic Drawing of the Variable and Constant Domains of an Immunoglobulin Light Chain Illustrating the "Immunoglobulin Fold." The β strands participating in the antiparallel β-pleated sheets of each domain are represented as *arrows*. The β strands of the three-stranded sheets are *shaded*, whereas those in the four-stranded sheets are *white*. The intradomain disulfide bonds are represented as *black bars*. Selected amino acids are numbered with position 1 as the N terminus. (Reprinted with permission from Edmundson AB, Ely KR, Abola EE, Schiffer M, Panagiotopoulous N. Rotational allomerism and divergent evolution of domains in immunoglobulin light chains. *Biochemistry*. 1975;14:3953–3961).

- IgG antibodies, made up of four subclasses in both humans and mice, mediate a wide range of functions including transplacental passage and opsonization of antigens through binding of antigen/antibody complexes to specialized Fc receptors on macrophages and other cell types (see Chapters 19, 20, and 24).

IgD, IgG, and IgE antibodies consist of a single unit of two H and L chains. IgM antibodies are constructed of five or six such units, although they consist of a single unit when they act as membrane receptors. IgA antibodies may consist of one or more units. The antibodies that are made up of more than a single unit generally contain an additional polypeptide chain, the J chain, that appears to play a role in the polymerization process. In addition, secreted IgA expresses a chain, a secretory piece, that is derived from the receptor for polymeric IgA, which plays a role in the transport of IgA through the cells lining the lumen of the gut.

Each of the distinct Igs can exist as secreted antibodies and as membrane molecules. Antibodies and cell surface receptors of the same class made by a specific cell have identical structures except for differences in their carboxy-terminal regions. Membrane Igs possess a hydrophobic region, spanning the membrane, and a short intracytoplasmic tail, both of which are lacking in the secretory form.

Immunoglobulin Genetics (Chapter 6)

The components of the Ig H-chain gene have already been alluded to. To reiterate, the IgH chain gene of a mature lymphocyte is derived from a set of genetic elements that are separated from one another in the germline. The V region is composed of three types of genetic elements: V_H, D, and J_H. More than 100 V_H elements exist; there are more than 10 D elements and a small number of J_H elements (4 in the mouse). An H-chain V_HDJ_H gene is created by the translocation of one of the D elements on a given chromosome to one of the J_H elements on that chromosome, generally with the excision of the intervening DNA. This is followed by a second translocation event in which one of the V_H elements is brought into apposition with the assembled DJ_H element to create the V_HDJ_H (V region) gene (see Fig. 1.3). Although it is likely that the choice of the V_H, D, and J_H elements that are assembled is not entirely random, the combinatorial process allows the creation of a very large number of distinct H-chain V-region genes. Additional diversity is created by the imprecision of the joining events and by the deletion of nucleotides and addition of new, untemplated nucleotides between D and J_H and between V_H and D, forming N regions in these areas. This further increases the diversity of distinct IgH chains that can be generated from the relatively modest amount of genetic information present in the germline.

The assembly of L-chain genes follows generally similar rules. However, L chains are assembled from V_L and J_L elements only. Although there is junctional diversity, no N regions exist for L chains. Additional diversity is provided by the existence of two classes of L chains, κ and λ.

An Ig molecule is assembled by the pairing of an IgH-chain polypeptide with an IgL-chain polypeptide. Although this process is almost certainly not completely random, it allows the formation of an exceedingly large number of distinct Ig molecules, the majority of which will have individual specificities.

The rearrangement events that result in the assembly of expressible IgH and IgL chains occur in the course of B-cell development in pro-B cells and pre-B cells, respectively (see Fig. 1.1). This process is regulated by the Ig products of the rearrangement events. The formation of a μ chain signals the termination of rearrangement of H-chain gene elements and the onset of rearrangement of L-chain gene elements, with κ rearrangements generally preceding λ rearrangements. One important consequence of this is that only a single expressible μ chain will be produced in a given cell, as the first expressible μ chain shuts off the possibility of producing an expressible μ chain on the alternative chromosome. Comparable mechanisms exist to ensure that only one L-chain gene is produced, leading to the phenomenon known as allelic exclusion. Thus, the product of only one of the two alternative allelic regions at both the H- and L-chain loci are expressed. The closely related phenomenon of L-chain isotype exclusion ensures the production of either κ or λ chains in an individual cell, but not both. An obvious but critical consequence of allelic exclusion is that in most cases an individual B cell makes antibodies, all of which have identical H- and L-chain V regions, a central prediction of the clonal selection theory of the immune response. During receptor editing, secondary rearrangements occur. Receptor editing is induced when the initial membrane Ig is capable of self-reactivity. As a consequence of the resultant secondary rearrangement, Ig of a different specificity is expressed, usually no longer self-reactive.

Class Switching (Chapter 6)

An individual B cell continues to express the same IgH-chain V region as it matures but it can switch the IgH-chain C region it uses (see Fig. 1.3). Thus, a cell that expresses receptors of the IgM and IgD classes may differentiate into a cell that expresses IgG, IgA, or IgE receptors and then into a cell secreting antibody of the same class as it expressed on the cell surface. This process allows the production of antibodies capable of mediating distinct biologic functions but that retain the same antigen-combining specificity. When linked with the process of affinity maturation of antibodies, Ig class switching provides antibodies of extremely high efficacy in preventing re-infection with microbial pathogens or in rapidly eliminating such pathogens. The associated phenomena of class switching and affinity maturation account for the high degree of effectiveness of antibodies produced in secondary immune responses.

The process of class switching is known to involve a genetic recombination event between specialized switch (S) regions, containing repetitive sequences, that are located upstream of each C region genetic element (with the exception of the δ C region). Thus, the S region upstream of the μ C_H region gene (Sμ) recombines with an S region upstream of a more 3′ isotype, such as Sγ1, to create a chimeric Sμ/Sγ1

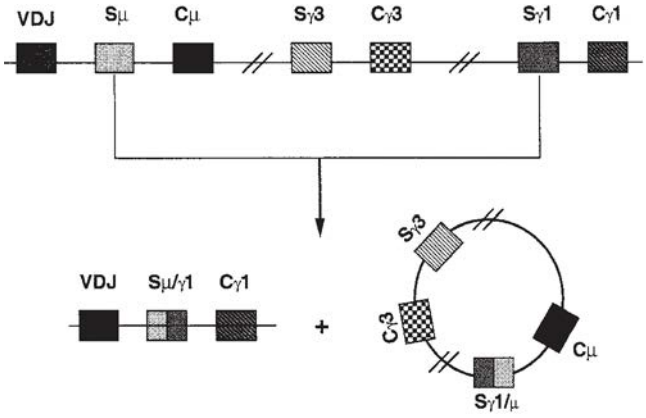

FIG. 1.7. Immunoglobulin Class Switching. The process through which a given VDJ gene in a stimulated B cell may switch the constant region gene with which it is associated from μ to another, such as γ1, is illustrated. A recombination event occurs in which DNA between a cleavage point in Sμ and one in Sγ1 forms a circular episome. This results in Cγ1 being located immediately downstream of the chimeric Sμ/γ1 region, in a position such that transcription initiating upstream of VDJ results in the formation of VDJCγ1 mRNA and γ1 H-chain protein.

region and in the deletion of the intervening DNA (Fig. 1.7). The genes encoding the C regions of the various γ chains (in the human γ1, γ2, γ3, and γ4; in the mouse γ1, γ2a, γ2b, and γ3), of the α chain, and of the ε chain are located 3′ of the Cμ and Cδ genes.

The induction of the switching process is dependent on the action of a specialized set of B-cell stimulants. Of these, the most widely studied are CD154, expressed on the surface of activated T cells, and the TLR ligands such as bacterial lipopolysaccharide. The targeting of the C region that will be expressed as a result of switching is largely determined by cytokines. Thus, IL-4 determines that switch events in the human and mouse will be to the ε C region and to the γ4 (human) or γ1 (mouse) C regions. In the mouse, interferon-gamma (IFN-γ) determines switching to γ2a and transforming growth factor-beta (TGF-β) determines switching to α. A major goal is to understand the physiologic determination of the specificity of the switching process. Because cytokines are often the key controllers of which Ig classes will represent the switched isotype, this logically translates into asking what regulates the relative amounts of particular cytokines that are produced by different modes of immunization.

As already noted, both the switching process and somatic hypermutation depend upon the AID. Mice and humans that lack AID fail to undergo both immunoglobulin class switching and somatic hypermutation.

Affinity Maturation and Somatic Hypermutation (Chapters 6 and 10)

The process of generation of diversity embodied in the construction of the H- and L-chain V-region genes and of the pairing of H and L chains creates a large number of distinct antibody molecules, each expressed in an individual B cell. This primary repertoire is sufficiently large so that most epitopes on foreign antigens will encounter B cells with complementary receptors. Thus, if adequate T-cell help can be generated, antibody responses can be made to a wide array of foreign substances. Nonetheless, the antibody that is initially produced usually has a relatively low affinity for the antigen. This is partially compensated for by the fact that IgM, the antibody initially made, is a pentamer. Through multivalent binding, high avidities can be achieved even if individual combining sites have only modest affinity (see Chapter 7). In the course of T-cell–dependent B-cell stimulation, particularly within the germinal center, a process of somatic hypermutation is initiated that leads to a large number of mutational events, largely confined to the H-chain and L-chain V-region genes and their immediately surrounding introns.

During the process of somatic hypermutation, mutational rates of 1 per 1,000 base pairs per generation may be achieved. This implies that with each cell division close to one mutation will occur in either the H- or L-chain V region of an individual cell. Such a high rate of mutation creates an enormous increase in antibody diversity. Although most of these mutations will either not affect the affinity with which the antibody binds its ligand or will lower that affinity, some will increase it. Thus, some B cells emerge that can bind antigen more avidly than the initial population of responding cells. Because there is an active process of apoptosis in the germinal center from which B cells can be rescued by the binding of antigen to their membrane receptors, cells with the most avid receptors should have an advantage over other antigen-specific B cells and should come to dominate the population of responding cells. Thus, upon rechallenge, the affinity of antibody produced will be greater than that in the initial response. As time after immunization elapses, the affinity of antibody produced will increase. This process leads to the presence in immunized individuals of high-affinity antibodies that are much more effective, on a weight basis, in protecting against microbial agents and other antigen-bearing pathogens than was the antibody initially produced. Together with antibody class switching, affinity maturation results in the increased effectiveness of antibody in preventing reinfection with agents with which the individual has had a prior encounter.

T-LYMPHOCYTES

T-lymphocytes constitute the second major class of lymphocytes. They derive from precursors in hematopoietic tissue, undergo differentiation in the thymus (hence the name thymus-derived [T]-lymphocytes), and are then seeded to the peripheral lymphoid tissue and to the recirculating pool of lymphocytes (see Chapters 13 and 14). T cells are subdivided into two distinct classes based on the cell surface receptors they express. The majority of T cells express TCRs consisting of α and β chains. A second group of T cells express receptors made up of γ and δ chains. Among the α/β T cells are two important sublineages: those that express the coreceptor molecule CD4 (CD4 T cells) and those that express CD8 (CD8 T cells). These cells differ in how they recognize

antigen and mediate different types of regulatory and effector functions.

CD4 T cells are the major *helper* cells of the immune system (see Chapter 29). Their helper function depends both on cell surface molecules, such as CD154, induced upon these cells when they are activated and on the wide array of cytokines they secrete upon stimulation. CD4 T cells tend to differentiate, as a consequence of priming, into cells that principally secrete the cytokines IL-4, IL-13, IL-5 and IL-6 (T_{H2} cells) into cells that mainly produce IFN-γ and lymphotoxin (T_{H1} cells), or into cells that produce IL-17 and related cytokines (T_{H17} cells). T_{H2} cells are very effective in immunity to helminthic parasites, T_{H1} cells are effective inducers of cellular immune responses, involving enhancement in the microbicidal activity of monocytes and macrophages and consequent increased efficiency in lysing microorganisms in intracellular vesicular compartments, while T_{H17} cells are efficient recruiters of granulocytes and other cells of the inflammatory system and play a major role in responses to extracellular bacterial pathogens. CD4 T cells can also acquire the capacity to enter B-cell follicles and help B cells develop into antibody-producing cells and undergo immunoglobulin class switching and affinity maturation; the cells are referred to as T follicular helper (T_{fh}) cells. Another possible fate of naïve CD4 T cells is to differentiate into induced regulatory T cells (iT_{regs}). However, most T_{regs} develop as a independent lineage of CD4 T cells. T_{regs} express the transcription factor Foxp3 and many express large amounts of the α chain of the IL-2 receptor (CD25).

T cells mediate important effector functions. Some of these are determined by the patterns of cytokines they secrete. These powerful molecules can be directly toxic to target cells and can mobilize potent inflammatory mechanisms. In addition, T cells, particularly CD8 T cells, can develop into cytotoxic T-lymphocytes (CTLs) capable of efficiently lysing target cells that express antigens recognized by the CTLs.

T-Lymphocyte Antigen Recognition (Chapters 11, 21, and 22)

T cells differ from B cells in their mechanism of antigen recognition. Ig, the B-cell's receptor, binds to individual antigenic epitopes on the surface of native molecules, be they on cell surfaces or in solution. Antibody and B-cell receptors evolved to bind to and to protect against microorganisms in extracellular fluids.

By contrast, T cells invariably recognize cell-associated molecules and mediate their functions by interacting with and altering the behavior of such *antigen-presenting cells* (APCs). Indeed, the TCR does not recognize antigenic determinants on intact, undenatured molecules. Rather, it recognizes a complex consisting of a peptide, derived by intracellular proteolysis of the antigen, bound into a specialized groove of a class II or class I MHC protein. Indeed, what differentiates a CD4 T cell from a CD8 T cell is that the CD4 T cells recognize peptide/class II complexes whereas the CD8 T cells recognize peptide/class I complexes.

The TCR's ligand (i.e., the peptide/MHC protein complex) is created within the APC. In general, class II MHC molecules bind peptides derived from proteins that have been taken up by the APC through an endocytic process (Fig. 1.8). These endocytosed proteins are fragmented by proteolytic enzymes within the endosomal/lysosomal compartment. The resulting peptides are loaded into class II MHC that traffic through this compartment. Peptide-loaded class II molecules are then expressed on the surface of the APC where they are available to be bound by CD4 T cells that have TCRs capable of recog-

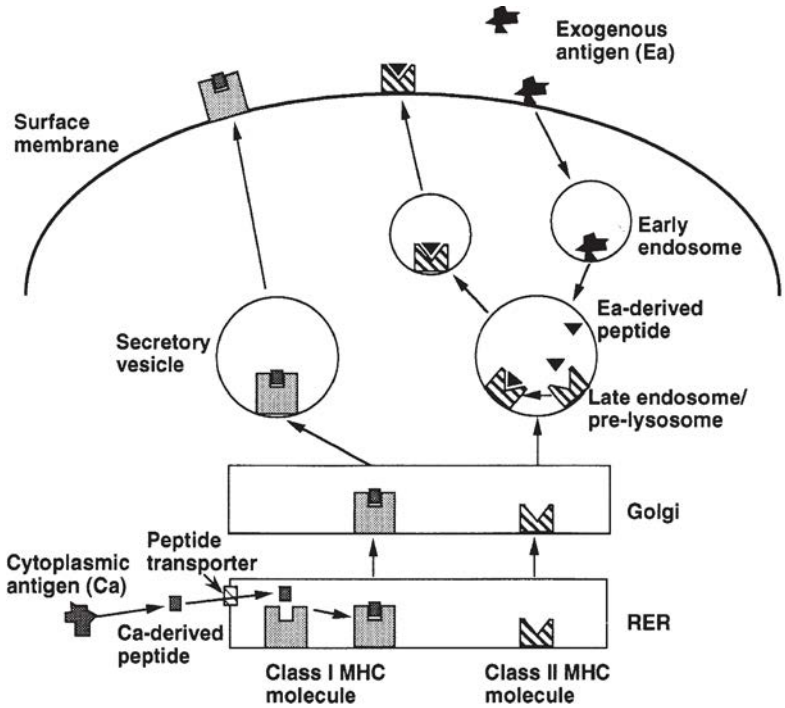

FIG. 1.8. Pathways of Antigen Processing. Exogenous antigen (Ea) enters the cell via endocytosis and is transported from early endosomes into late endosomes or prelysosomes, where it is fragmented and where resulting peptides (Ea-derived peptides) may be loaded into class II major histocompatibility complex (MHC) molecules. The latter have been transported from the rough endoplasmic reticulum (RER) through the Golgi apparatus to the peptide-containing vesicles. Class II MHC molecules/Ea-derived peptide complexes are then transported to the cell surface, where they may be recognized by T-cell receptor expressed on CD4+ T cells. Cytoplasmic antigens (Ca) are degraded in the cytoplasm and then enter the RER through a peptide transporter. In the RER, Ca-derived peptides are loaded into class I MHC molecules that move through the Golgi apparatus into secretory vesicles and are then expressed on the cell surface where they may be recognized by CD8+ T cells (Reprinted with permission from Paul WE. In: Gallin JI, Goldstein, I, Snyderman, R, ed. *Inflammation.* New York: Raven,;1992:776.)

nizing the expressed cell surface peptide/MHC protein complex. Thus, CD4 T cells are specialized to largely react with antigens derived from extracellular sources.

In contrast, class I MHC molecules are mainly loaded with peptides derived from internally synthesized proteins, such as viral gene products. These peptides are produced from cytosolic proteins by proteolysis within the proteasome and are translocated into the rough endoplasmic reticulum. Such peptides, generally nine amino acids in length, are bound by class I MHC molecules. The complex is brought to the cell surface, where it can be recognized by CD8 T cells expressing appropriate receptors. This property gives the T-cell system, particularly CD8 T cells, the ability to detect cells expressing proteins that are different from, or produced in much larger amounts than, those of cells of the remainder of the organism (e.g., viral antigens [whether internal, envelope, or cell surface] or mutant antigens [such as active oncogene products]), even if these proteins, in their intact form, are neither expressed on the cell surface nor secreted.

Although this division of class I–binding peptides being derived from internally synthesized proteins and class II–binding peptides from imported proteins is generally correct, there are important exceptions to this rule that are central for the function of the immune system. The most effective priming of naive CD8 T cells occurs in response to peptide/MHC-I complexes expressed by DCs and yet many viruses do not infect these cells but rather target other cell types. Viral antigens produced by infected cells can be taken up by specialized DCs and loaded into class I molecules in a process referred to as cross-presentation.

T-Lymphocyte Receptors (Chapter 11)

The TCR is a disulfide-linked heterodimer (Fig. 1.9). Its constituent chains (α and β, or γ and δ) are Ig supergene

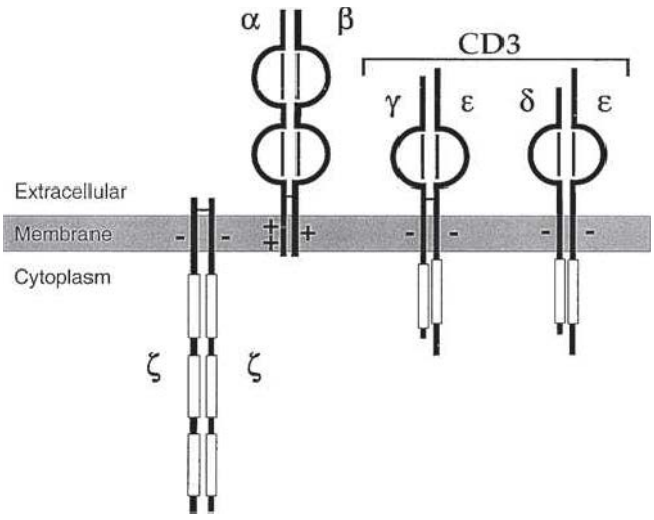

FIG. 1.9. The T-Cell Antigen Receptor. Illustrated schematically is the antigen binding subunit comprised of an αβ heterodimer and the associated invariant CD3 and ζ chains. Acidic (–) and basic (+) residues located within the plasma membrane are indicated. The *open rectangular boxes* indicate motifs within the cytoplasmic domains that interact with protein tyrosine kinases.

family members. The TCR is associated with a set of transmembrane proteins, collectively designated the CD3 complex, that play a critical role in signal transduction. The CD3 complex consists of γ, δ (note that the CD3 γ and δ chains and the TCR γ and δ chains are distinct polypeptides that, unfortunately, have similar designations) and ε chains and is associated with a homodimer of two ζ chains or a heterodimer of ζ and η chains. CD3 γ, δ, and ε consist of extracellular domains that are Ig supergene family members. The cytosolic domains of CD3 γ, δ, and ε and of ζ and η contain one or more copies of the immunoreceptor tyrosine–based activation motif (ITAM) (D/ExxYxxLxxxxxxxYxxL/I) that is found in a variety of chains associated with immune recognition receptors. This motif appears to be important in the signal transduction process and provides a site through which protein tyrosine kinases can interact with these chains to propagate signaling events.

The TCR chains are organized much like Ig chains. Their N-terminal portions are variable and their C-terminal portions are constant. Furthermore, similar recombinational mechanisms are used to assemble the V-region genes of the TCR chains. Thus, the V region of the TCR β chain is encoded by a gene constructed from three distinct genetic elements (V_β, D, and J_β) that are separated in the germline. Although the relative numbers of V_β, D, and J_β genes differ from that for the comparable Ig H variable region elements, the strategies for creation of a very large number of distinct genes by combinatorial assembly are the same. Both junctional diversity and N-region addition further diversify the genes and their encoded products. TCR β has fewer *V* genes than IgH but much more diversity centered on the *D/J* region, which encodes the equivalent of the third CDR of Igs. The α chain follows similar principles, except that it does not use a *D* gene.

The genes for TCR γ and δ chains are assembled in a similar manner except that they have many fewer V genes from which to choose. Indeed, γ/δ T cells in certain environments, such as the skin and specific mucosal surfaces, are exceptionally homogeneous. It has been suggested that the TCRs encoded by these essentially invariant γ and δ chains may be specific for some antigen that signals microbial invasion and that activation of γ/δ T cells through this mechanism constitutes an initial response that aids the development of the more sophisticated response of α/β T cells.

T-Lymphocyte Activation (Chapter 12)

T-cell activation is dependent on the interaction of the TCR/CD3 complex with its cognate ligand, a peptide bound in the groove of a class I or class II MHC molecule, on the surface of a competent APC. Through the use of chimeric cell surface molecules that possess cytosolic domains largely limited to the ITAM signaling motif alluded to previously, it is clear that cross-linkage of molecules containing such domains can generate some of the signals that result from TCR engagement. Nonetheless, the molecular events set in motion by receptor engagement are complex ones. Among the earliest steps is the activation of tyrosine kinases leading to the tyrosine phosphorylation of a set of substrates that control

several signaling pathways. Current evidence indicates that early events in this process involve the src family tyrosine kinases $p56^{lck}$, associated with the cytosolic domains of the CD4 and CD8 coreceptors, and $p59^{fyn}$, and ZAP-70, a Syk family tyrosine kinase, that binds to the phosphorylated ITAMs of the ζ chain. The protein tyrosine phosphatase CD45, found on the surface of all T cells, also plays a critical role in T-cell activation.

A series of important substrates are tyrosine phosphorylated as a result of the action of the kinases associated with the TCR complex. These include a 1) set of adapter proteins that link the TCR to the Ras pathway; 2) phospholipase Cγ1, the tyrosine phosphorylation of which increases its catalytic activity and engages the inositol phospholipid metabolic pathway, leading to elevation of intracellular free calcium concentration and activation protein kinase C; and 3) a series of other important enzymes that control cellular growth and differentiation. Particularly important is the phosphorylation of LAT, a molecule that acts as an organizing scaffold to which a series of signaling intermediates bind and upon which they become activated and control downstream signaling.

The recognition and early activation events result in the reorganization of cell surface and cytosolic molecules on the T cell, and correspondingly, on the APC to produce a structure, the *immunological synapse*. The apposition of key interacting molecules involving a small segment of the membranes of the two cells concentrates these interacting molecules in a manner that both strengthens the interaction between the cells and intensifies the signaling events. It also creates a limited space into which cytokines may be secreted to influence the behavior of the interacting cells. The formation of the immunological synapse is one mechanism through which the recognition of relatively small numbers of ligands by TCRs on a specific T cell can be converted into a vigorous stimulatory process.

In general, normal T cells and cloned T-cell lines that are stimulated only by TCR cross-linkage fail to give complete responses. TCR engagement by itself may often lead to a response in which the key T-cell–derived growth factor, IL-2, is not produced and in which the cells enter a state of anergy such that they are unresponsive or poorly responsive to a subsequent competent stimulus (see Chapter 32). Full responsiveness of a T cell requires, in addition to receptor engagement, an accessory cell–delivered costimulatory signal. The engagement of CD28 on the T cell by CD80 and/or CD86 on the APC (or the engagement of comparable ligand/receptor pairs on the two cells) provides potent costimulatory activity. Inhibitors of this interaction markedly diminish antigen-specific T-cell activation in vivo and in vitro, indicating that the CD80/86–CD28 interaction is physiologically important in T-cell activation (see Chapters 12 and 14).

The interaction of CD80/86 with CD28 increases cytokine production by the responding T cells. For the production of IL-2, this increase appears to be mediated both by enhancing the transcription of the IL-2 gene and by stabilizing IL-2 mRNA. These dual consequences of the CD80/86–CD28 interaction cause a striking increase in the production of IL-2 by antigen-stimulated T cells.

CD80/86 has a second receptor on the T cell, CTLA-4, that is expressed later in the course of T-cell activation. The bulk of evidence indicates that the engagement of CTLA-4 by CD80/86 leads to a set of biochemical signals that terminate the T-cell response. Mice that are deficient in CTLA-4 expression develop fulminant autoimmune responses and anti–CTLA-4 antibodies are used as drugs to enhance antitumor immune responses.

T Lymphocyte Development (Chapter 13)

Upon entry into the thymus, T-cell precursors do not express TCR chains, the CD3 complex, or the CD4 or CD8 molecules (Fig. 1.10). Because these cells lack both CD4 and CD8, they are often referred to as double-negative cells. Thymocytes develop from this double-negative pool into cells that are both CD4+ and CD8+ (double-positive cells) and express low levels of TCR and CD3 on their surface. In turn, double-positive cells further differentiate into relatively mature thymocytes that express either CD4 or CD8 (single-positive cells) and high levels of the TCR/CD3 complex.

The expression of the TCR depends on complex rearrangement processes that generate TCR α and β (or γ and

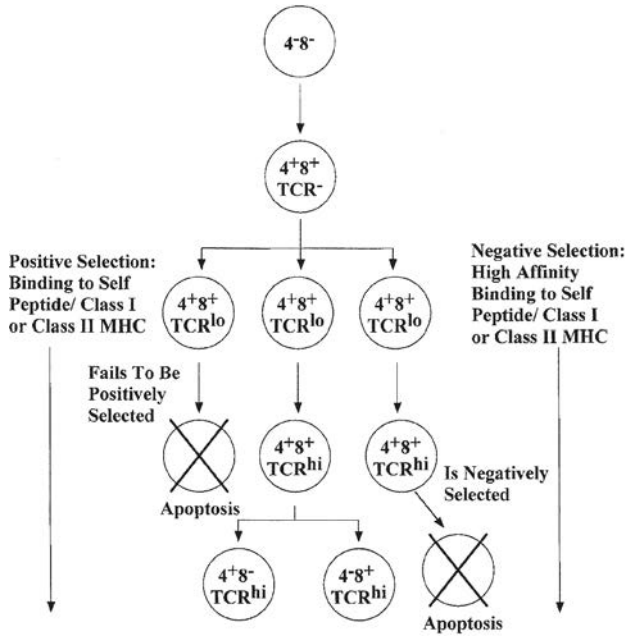

FIG. 1.10. Development of α/β T Cells in the Thymus. Double-negative T cells (4^-8^-) acquire CD4 and CD8 (4^+8^+) and then express α/β TCRs, initially at low levels. Thereafter, the degree of expression of T-cell receptiors increases and the cells differentiate into CD4 or CD8 cells and are then exported to the periphery. Once the T cells have expressed receptors, their survival depends upon the recognition of peptide/major histocompatibility complex (MHC) class I or class II molecules with an affinity above some given threshold. Cells that fail to do so undergo apoptosis. These cells have failed to be *positively selected*. Positive selection is associated with the differentiation of 4^+8^+ cells into CD4 or CD8 cells. Positive selection involving peptide/class I MHC molecules leads to the development of CD8 cells whereas positive selection involving peptide/class II MHC molecules leads to the development of CD4 cells. If a T cell recognizes a peptide/MHC complex with high affinity, it is also eliminated via apoptosis (it is *negatively selected*).

δ) chains. Once TCR chains are expressed, these cells undergo two important selection processes within the thymus. One, termed *negative selection*, is the deletion of cells that express receptors that bind with high affinity to complexes of self-peptides with self-MHC molecules. This is a major mechanism through which the T-cell compartment develops immunologic unresponsiveness to self antigens (see Chapters 13 and 32). In addition, a second major selection process is *positive selection*, in which T cells with receptors with "intermediate affinity" for self-peptides bound to self-MHC molecules are selected, thus forming the basis of the T-cell repertoire for foreign peptides associated with self-MHC molecules. T cells that are not positively selected are eliminated in the thymic cortex by apoptosis. Similarly, T cells that are negatively selected as a result of high-affinity binding to self-peptide/self-MHC complexes are also deleted through apoptotic death. These two selection processes result in the development of a population of T cells that are biased toward the recognition of peptides in association with self-MHC molecules from which those cells that are potentially autoreactive (capable of high-affinity binding of self-peptide/self-MHC complexes) have been purged.

One important event in the development of T cells is their differentiation from double-positive cells into CD4+ or CD8+ single-positive cells. This process involves the interaction of double-positive thymocytes with peptide bound to class II or class I MHC molecules on accessory cells. Indeed, CD4 binds to monomorphic sites on class II molecules, whereas CD8 binds to comparable sites on class I molecules. The capacity of the TCR and CD4 (or of the TCR and CD8) to bind to a class II MHC (or a class I MHC) molecule on an accessory cell leads either to the differentiation of double-positive thymocytes into CD4+ (or CD8+) single-positive T cells or to the selection of cells that have "stochastically" differentiated down the CD4 (or CD8) pathway.

Less is understood about the differentiation of thymocytes that express TCRs composed of γ/δ chains. These cells fail to express either CD4 or CD8. However, γ/δ cells are relatively numerous early in fetal life; this, together with their limited degree of heterogeneity, suggests that they may comprise a relatively primitive T-cell compartment.

T-Lymphocyte Functions (Chapters 14, 29, and 33)

T cells mediate a wide range of immunologic functions. These include the capacity to help B cells develop into antibody-producing cells, the capacity to increase the microbicidal action of monocytes/macrophages, the inhibition of certain types of immune responses, direct killing of target cells, and mobilization of the inflammatory response. In general, these effects depend on their expression of specific cell-surface molecules and the secretion of cytokines.

T Cells that Help Antibody Responses (Chapter 13)

Helper T cells, T_{FH} cells, can stimulate B cells to make antibody responses to proteins and other T-cell–dependent antigens. T-cell–dependent antigens are immunogens in which individual epitopes appear only once or only a limited number of times so that they are unable to cross-link the membrane Ig of B cells or do so inefficiently. B cells bind the antigen through their membrane Ig, and the complex undergoes endocytosis. Within the endosomal and lysosomal compartments, the antigen is fragmented into peptides by proteolytic enzymes and one or more of the generated peptides are loaded into class II MHC molecules, which traffic through this vesicular compartment. The resulting complex of class II MHC molecule and bound peptide is exported to the B-cell surface membrane. T cells with receptors specific for the peptide/class II molecular complex recognize that complex on the B cell.

B-cell activation depends not only on the binding of peptide/class II MHC complexes on the B cell surface by the TCR but also on the interaction of T-cell CD154 with CD40 on the B cell. T cells do not constitutively express CD154; rather, it is induced as a result of an interaction with an activated APC that expresses a cognate antigen recognized by the TCR of the T cell. Furthermore, CD80/86 are generally expressed by activated but not resting B cells so that interactions involving resting B cells and naïve T cells generally do not lead to efficient antibody production. By contrast, a T cell already activated and expressing CD154 can interact with a resting B cell, leading to its upregulation of CD80/86 and to a more productive T-cell/B-cell interaction with the delivery of cognate help and the development of the B cell into an antibody-producing cell. Similarly, activated B cells expressing large amounts of class II molecules and CD80/86 can act as effective APCs and can participate with T cells in efficient cognate help interactions. Cross-linkage of membrane Ig on the B cell, even if inefficient, may synergize with the CD154/CD40 interaction to yield vigorous B-cell activation.

The subsequent events in the B-cell response program, including proliferation, Ig secretion, and class switching, either depend on or are enhanced by the actions of T-cell–derived cytokines. Thus, B-cell proliferation and Ig secretion are enhanced by the actions of several type I cytokines including IL-2 and IL-4. Ig class switching is dependent both on the initiation of competence for switching, which can be induced by the CD154/CD40 interaction, and on the targeting of particular C regions for switching, which is determined, in many instances, by cytokines. The best studied example of this is the role of IL-4 in determining switching to IgG1 and IgE in the mouse and to IgG4 and IgE in the human. Indeed, the central role of IL-4 in the production of IgE is demonstrated by the fact that mice that lack the IL-4 gene or the gene for the IL-4 receptor α chain, as a result of gene knockouts, have a marked defect in IgE production. Similarly, IFN-γ determines switching to IgG_{2a} in the mouse. The relationship between T_{FH} cells that produce IL-4 and T_{H2} cells and those that produce IFN-γ and T_{H1} cells is still uncertain.

Induction of Cellular Immunity (Chapters 14 and 19)

T cells also may act to enhance the capacity of monocytes and macrophages to destroy intracellular microorganisms. In particular, IFN-γ enhances several mechanisms through which mononuclear phagocytes destroy intracellular bacteria and parasites, including the generation of nitric oxide and induction of TNF. T_{H1} cells are particularly effective in

enhancing microbicidal action because they produce IFN-γ. By contrast, three of the major cytokines produced by T_{H2} cells, IL-4, IL-13, and IL-10, block these activities; IL-4 and IL-13 induce an alternative gene activation program in macrophages resulting in *alternatively activated macrophages*, characterized (in the mouse) by the expression of arginase 1 and chitinase. Thus, T_{H2} cells often oppose the action of T_{H1} cells in inducing cellular immunity and in certain infections with microorganisms that are intracellular pathogens of macrophages; a T_{H2}-dominated response may be associated with failure to control the infection.

Regulatory T Cells (Chapter 33)

There has been a longstanding interest in the capacity of T cells to diminish as well as to help immune responses. Cells that mediate such effects are referred to as T_{regs}. T_{regs} may be identified by their expression of Foxp3 and of CD25, the IL-2 receptor alpha chain. These cells inhibit the capacity of both CD4 and CD8 T cells to respond to their cognate antigens. The mechanisms through which their suppressor function is mediated are still somewhat controversial. In some instances, it appears that cell/cell contact is essential for suppression, whereas in other circumstances production of cytokines by T_{regs} has been implicated in their ability to inhibit responses. Evidence has been presented for both IL-10 and TGF-β as mediators of inhibition.

T_{regs} have been particularly studied in the context of various autoimmune conditions. In the absence of T_{regs}, conventional T cells cause several types of autoimmune responses, including autoimmune gastritis and inflammatory bowel disease. T_{regs} express cell surface receptors allowing them to recognize autoantigens; their responses to such recognition results in the suppression of responses by conventional T cells. Whether the receptor repertoire of T_{regs} and the conventional T cells are the same has not been fully determined, although there is increasing evidence that T_{regs} derive from a thymic CD4 T-cell population with relatively high affinity for self-antigen. As noted previously, iT_{regs} can be derived in the periphery from naive CD4 T-cell populations. This is seen when naive cells are stimulated by their cognate ligands in the presence of TGF-β and IL-2.

Cytotoxic T Cells (Chapter 37)

One of the most striking actions of T cells is the lysis of cells expressing specific antigens. Most cells with such cytotoxic activity are CD8 T cells that recognize peptides derived from proteins produced within the target cell and bound to class I MHC molecules expressed on the surface of the target cell. However, CD4 T cells can express CTL activity, although in such cases the antigen recognized is a peptide associated with a class II MHC molecule; often, such peptides derive from exogenous antigens.

There are two major mechanisms of cytotoxicity. One involves the production by the CTL of perforin, a molecule that can insert into the membrane of target cells and promote the lysis of that cell. Perforin-mediated lysis is mediated a series of enzymes produced by activated CTLs, referred to as granzymes. Many active CTLs also express large amounts of fas ligand on their surface. The interaction of fas ligand on the surface of the CTL with fas on the surface of the target cell initiates apoptosis in the target cell.

CTL-mediated lysis is a major mechanism for the destruction of virus-infected cells. If activated during the period in which the virus is in its eclipse phase, CTLs may be capable of eliminating virus and curing the host with relatively limited cell destruction. On the other hand, vigorous CTL activity after a virus has been widely disseminated may lead to substantial tissue injury because of the large number of cells that are killed by the action of the CTLs. Thus, in many infections, the disease is caused by the destruction of tissue by CTLs rather than by the virus itself. One example is hepatitis B, in which much of the liver damage represents the attack of hepatitis B virus–specific CTLs on infected liver cells.

It is usually observed that CTLs that have been induced as a result of a viral infection or intentional immunization must be reactivated in vitro through the recognition of antigen on the target cell. This is particularly true if some interval has elapsed between the time of infection or immunization and the time of test. This has led to some question being raised as to the importance of CTL immunity in protection against re-infection and how important CTL generation is in the long-term immunity induced by protective vaccines. On the other hand, in active infections, such as seen in individuals with HIV, CTL that can kill their target cells immediately are often seen. There is much evidence to suggest that these cells play an active role in controlling the number of HIV-positive T cells.

CYTOKINES (CHAPTERS 25 TO 28)

Many of the functions of cells of the immune system are mediated through the production of a set of small proteins referred to as cytokines. These proteins can now be divided into several families. They include the type I cytokines or hematopoeitins that encompass many of the interleukins (i.e., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21, IL-23, and IL-27), as well as several hematopoietic growth factors; the type II cytokines, including the interferons and IL-10; the TNF-related molecules, including TNF, lymphotoxin, and Fas ligand; Ig superfamily members, including IL-1, IL-18, IL-33, IL-36, and IL-37; and the chemokines, a large family of molecules playing critical roles in a wide variety of immune and inflammatory functions. IL-17 and its congeners, including IL-25, constitute a structurally unique set of cytokines.

Many of the cytokines are T-cell products; their production represents one of the means through which the wide variety of functions of T cells are mediated. Most cytokines are not constitutive products of the T cell. Rather, they are produced in response to T-cell activation, usually resulting from presentation of antigen to T cells by APCs in concert with the action of a costimulatory molecule, such as the interaction of CD80/86 with CD28. Although cytokines are produced in small quantities, they are potent, binding to their receptors with equilibrium constants of approximately 10^{10} M^{-1}. In some instances, cytokines are directionally secreted into the immunological synapse formed between a T cell and an APC. In such cases, the cytokine acts in a paracrine manner. Indeed, many cytokines

have limited action at a distance from the cell that produced them. This appears to be particularly true of many of the type I cytokines. However, other cytokines act by diffusion through extracellular fluids and blood to target cells that are distant from the producers. Among these are cytokines that have proinflammatory effects, such as IL-1, IL-6, and TNF, and the chemokines, that play important roles in regulating the migration of lymphocytes and other cell types.

Chemokines (Chapter 28)

A large family of small proteins that are *chemotactic cytokines* (chemokines) have been described. While members of this family have a variety of functions, perhaps the most dramatic is their capacity to regulate leukocyte migration and thus to act as critical dynamic organizers of cell distribution in the immune and inflammatory responses. The receptors for chemokines are seven transmembrane-spanning, G-protein coupled receptors.

The chemokines are subdivided based on the number and positioning of their highly conserved cysteines. Among chemokines with four conserved cysteines, the cysteines are adjacent in one large group (the CC chemokines), whereas in a second large group they are separated by one amino acid (CXC chemokines). There are also rare chemokines in which the cysteines are separated by three amino acids (CX3C) or in which there are only two conserved cysteines (C chemokines).

Individual chemokines may signal through more than one chemokine receptor, and individual receptors may interact with more than one chemokine, producing a complex set of chemokine/chemokine receptor pairs and providing opportunities for exceedingly fine regulation of cellular functions.

THE MAJOR HISTOCOMPATIBILITY COMPLEX AND ANTIGEN PRESENTATION (CHAPTERS 21 AND 22)

The MHC has already been introduced in this chapter in the discussion of T-cell recognition of antigen-derived peptides bound to specialized grooves in class I and class II MHC proteins. Indeed, the class I and class II MHC molecules are essential to the process of T-cell recognition and response. Nonetheless, they were first recognized not for this reason but because of the dominant role that MHC class I and class II proteins play in transplantation immunity (see Chapter 46).

When the genetic basis of transplantation rejection between mice of distinct inbred strains was sought, it was recognized that although multiple genetic regions contributed to the rejection process, one region played a dominant role. Differences at this region alone would cause prompt graft rejection, whereas any other individual difference usually resulted in a slow rejection of foreign tissue. For this reason, the genetic region responsible for prompt graft rejection was termed the *major* histocompatibility complex.

In all higher vertebrates that have been thoroughly studied, a comparable MHC exists. The defining features of the MHC are the transplantation antigens that it encodes. These are the class I and class II MHC molecules. The genes encoding these molecules show an unprecedented degree of polymorphism. This, together with their critical role in antigen presentation, explains their central role as the target of the immune responses leading to the rejection of organ and tissue allografts.

The MHC also includes other genes, particularly genes for certain complement components. In addition, genes for the cytokines TNF-α and lymphotoxin (also designated TNF-β) are found in the MHC.

Class I MHC Molecules (Chapter 21)

Class I MHC molecules are membrane glycoproteins expressed on most cells. They consist of an α chain of approximately 45,000 daltons noncovalently associated with α2-microglobulin, a 12,000-dalton molecule (Fig. 1.11). The gene for the α chain is encoded in the MHC, whereas that for β2-microglobulin is not. Both the α chain and β2-microglobulin are Ig supergene family members. The α chain is highly polymorphic, with the polymorphisms found mainly in the regions that constitute the binding sites for antigen-derived peptides and that are contact sites for the TCR.

The class I α chain consists of three extracellular regions or domains, each of similar length, designated α1, α2, and α3. In addition, α chains have a membrane-spanning domain and a short carboxy-terminal cytoplasmic tail. The

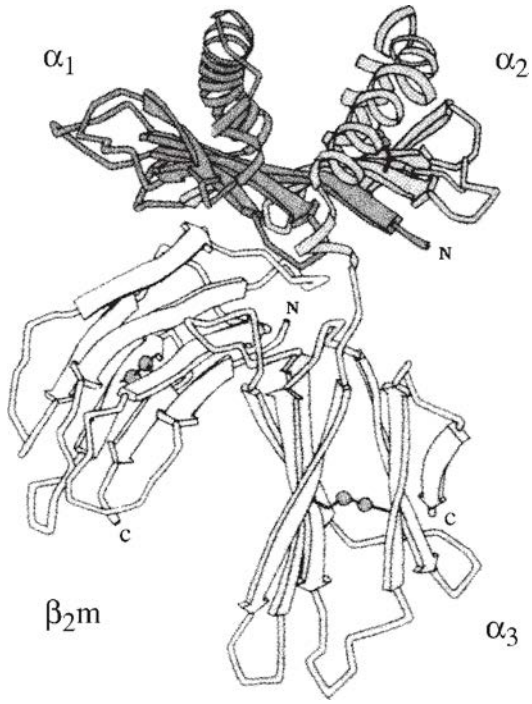

FIG. 1.11. Model of the Class I HLA-A2 Molecule. A schematic representation of the structure of the HLA-A2 class I major histocompatibility complex (MHC) molecule. The polymorphic α1 and α2 domains are at the top. They form a groove into which antigen-derived peptides fit to form the peptide/MHC class I complex that is recognized by T-cell receptors of CD8 T cells. (Reprinted from Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class II histocompatibility antigen, HLA-A2. *Nature*. 1987;329:506–512).

crystal structure of class I molecules indicates that the α1 and α2 domains form a site for the binding of peptides derived from antigens. This site is defined by a floor consisting of β sheets and bounded by α-helical walls. The polymorphisms of the class I molecule are mainly in these areas.

In the human, three loci encoding classical class I molecules have been defined; these are designated human leukocyte antigen (HLA)-A, HLA-B, and HLA-C. All display high degrees of polymorphism. A similar situation exists in the mouse. In addition, there are a series of genes that encode class I–like molecules (class Ib molecules). Some of these have been shown to have antigen-presenting activity for formylated peptides, suggesting that they may be specialized to present certain prokaryotic antigens. The class Ib molecule CD1 has been shown to have antigen-presenting function for mycobacterial lipids, providing a mechanism through which T cells specific for such molecules can be generated. CD1d, presenting certain endogenous or exogenous phospholipids, is recognized by a novel class of T cells (NK T cells) that produce large amounts of cytokines upon immediate stimulation.

Class II MHC Molecules (Chapter 21)

Class II MHC molecules are heterodimeric membrane glycoproteins. Their constituent chains are designated α and β; both chains are immunoglobulin supergene family members, and both are encoded within the MHC. Each chain consists of two extracellular domains (α1 and α2; β1 and β2, respectively), a hydrophobic domain, and a short cytoplasmic segment. The overall conformation of class II MHC molecules appears to be quite similar to that of class I molecules. The peptide-binding site of the class II molecules is contributed to by the α1 and β1 domains (see Fig. 1.5); it is within these domains that the majority of the polymorphic residues of class II molecules are found.

A comparison of the three-dimensional structures of class I and class II molecules indicates certain distinctive features that explain differences in the length of peptides that the two types of MHC molecules can bind. Class I molecules generally bind peptides with a mean length of nine amino acids, whereas class II molecules can bind substantially larger peptides.

In the mouse, class II MHC molecules are encoded by genes within the I region of the MHC. These molecules are often referred to as I region–associated (Ia) antigens. Two sets of class II molecules exist, designated I-A and I-E, respectively. The α and β chains of the I-A molecules (Aα and Aβ) pair with one another, as do the α and β chains of I-E (Eα and Eβ).

In the human, there are three major sets of class II molecules, encoded in the DR, DQ, and DP regions of the HLA complex.

Class II molecules have a more restricted tissue distribution than class I molecules. Class II molecules are found on B cells, DCs, epidermal Langerhans cells, macrophages, thymic epithelial cells, and, in the human, activated T cells. Levels of class II molecule expression are regulated in many cell types by interferons and in B cells by IL-4. Indeed, interferons can cause expression of class II molecules on many cell types that normally lack these cell surface molecules. Interferons also can cause striking upregulation in the expression of class I MHC molecules. Thus, immunologically mediated inflammation may result in aberrant expression of class II MHC molecules and heightened expression of class I molecules. Such altered expression of MHC molecules can allow cells that do not normally function as APCs for CD4 T cells to do so and enhances the sensitivity of such cells to CD8 T cells. This has important consequences for immunopathologic responses and for autoimmunity.

Antigen Presentation (Chapter 22)

As already discussed, the function of class I and class II MHC molecules is to bind and present antigen-derived peptides to T cells whose receptors can recognize the peptide/MHC complex that is generated. There are two major types of antigen-processing pathways, specialized to deal with distinct classes of pathogens that the T cell system must confront (see Fig. 1.8).

Extracellular bacteria and extracellular proteins may enter APCs by endocytosis or phagocytosis. Their antigens and the antigens of bacteria that live within endosomes or lysosomes are fragmented in these organelles, and peptides derived from the antigen are loaded into class II MHC molecules as these proteins traverse the vesicular compartments in which the peptides are found. The loading of peptide is important in stabilizing the structure of the class II MHC molecule. The acidic pH of the compartments in which loading occurs facilitates the loading process. Once the peptide-loaded class II molecules reaches neutral pH, such as at the cell surface, the peptide/MHC complex is stable. Peptide dissociation from such class II molecules is very slow, with a half-time measured in hours. The peptide/class II complex is recognized by those CD4 T cells that have complementary receptors. As already pointed out, the specialization of CD4 T cells to recognize peptide/class II complexes is partly due to the affinity of the CD4 molecule for monomorphic determinants on class II molecules. Obviously, this form of antigen processing can only apply to cells that express class II MHC molecules. Indeed, APCs for CD4 T cells principally include cells that normally express class II MHC molecules, namely DCs, B cells, and macrophages.

T cells also can recognize proteins that are produced within the cell that presents the antigen. The major pathogens recognized by this means are viruses and other obligate intracellular (nonendosomal/nonlysosomal) microbes that have infected cells. In addition, proteins that are unique to tumors, such as mutant oncogenes, or are overexpressed in tumors also can be recognized by T cells. Endogenously produced proteins are fragmented in the cytosol by proteases in the proteasome. The resultant peptides are transported into the rough endoplasmic reticulum through the action of a specialized transport system. These peptides are then available for loading into class I molecules. In contrast to the loading of class II molecules, which is facilitated by the acid pH of the loading environment, the loading of class I molecules is controlled by interaction of the class I α chain

with β2-microglobulin. Thus, the bond between peptide and class I molecule is generally weak in the absence of β2-microglobulin, and the binding of β2-microglobulin strikingly stabilizes the complex. (Similarly, the binding of β2-microglobulin to the α chain is markedly enhanced by the presence of peptide in the α chain groove.) The peptide-loaded class I molecule is then brought to the cell surface. In contrast to peptide-loaded class II molecules that are recognized by CD4 T cells, peptide-loaded class I molecules are recognized by CD8 T cells. This form of antigen processing and presentation can be performed by virtually all cells because, with a few exceptions, class I MHC molecules are universally expressed.

Although the specialization of class I molecules to bind and present endogenously produced peptides and of class II molecules to bind and present peptides derived from exogenous antigens is generally correct, there are exceptions, many of which have physiologic importance. Particularly important is the capacity of some DCs to load peptides from exogenous antigens into class I MHC molecules, allowing sensitization of CD8 T cells to the antigens of pathogens that infect cells other than DCs.

T-Lymphocyte Recognition of Peptide/MHC Complexes Results in MHC-Restricted Recognition (Chapter 11)

Before the biochemical nature of the interaction between antigen-derived peptides and MHC molecules was recognized, it was observed that T-cell responses displayed *MHC-restricted antigen recognition*. Thus, if individual animals were primed to a given antigen, their T cells would be able to recognize and respond to that antigen only if the APCs that presented the antigen shared MHC molecules with the animal that had been immunized. The antigen would not be recognized when presented by APCs of an allogeneic MHC type. This can now be explained by the fact that the TCR recognizes peptide bound to an MHC molecule. MHC molecules display high degrees of polymorphism, and this polymorphism is concentrated in the regions of the class I and class II molecules that interact with the peptide and that can bind to the TCR. Differences in structure of the MHC molecules derived from different individuals (or different inbred strains of mice) profoundly affect the recognition process. Two obvious explanations exist to account for this. First, the structure of the grooves in different class I or class II MHC molecules may determine that a different range of peptides are bound or, even if the same peptide is bound, may change the conformation of the surface of the peptide presented to the TCR. Second, polymorphic sites on the walls of the α-helices that are exposed to the TCR can either enhance or diminish binding of the whole complex, depending on their structure. Thus, priming an individual with a given antigen on APCs that are syngeneic to the individual will elicit a response by T cells whose TCRs are specific for a complex consisting of a peptide derived from the antigen and the exposed polymorphic residues of the MHC molecule. When the same antigen is used with APCs of different MHC type, it is unlikely that the same peptide/MHC surface can be formed, and thus the primed T cells are not likely to bind and respond to such stimulation.

Indeed, this process also occurs within the thymus in the generation of the T-cell repertoire, as already discussed. T cells developing within the thymus undergo a positive selection event in which those T cells capable of recognizing MHC molecules displayed within the thymus are selected (and the remainder undergo programmed cell death). This leads to the skewing of the population of T cells that emerges from the thymus so that the cells are specialized to respond to peptides on self-MHC molecules. One of the unsolved enigmas of positive selection within the thymus is how the vast array of T cells with receptors capable of reacting with a very large set of foreign peptides associated with self-MHC molecules are chosen by self-MHC molecules that can only display self-peptides. It is believed that a high degree of cross-reactivity may exist so that T cells selected to bind a given class I (or class II) molecule plus a particular self-peptide can also bind a set of other (foreign) peptides bound to the same MHC molecule.

Furthermore, the affinity of an interaction required for positive selection in the thymus appears to be considerably lower that that required for full activation of peripheral T cells. Thus, thymocytes selected by a given self-peptide/self-MHC complex will generally not mount a full response when they encounter the same peptide/MHC complex in the periphery, although they will respond to a set of foreign peptide/MHC complexes to which they bind with higher affinity. Recognition of the self-peptide/self-MHC complex in the periphery nonetheless is important in sustaining the viability of resting T-lymphocytes.

Our modern understanding of T-cell recognition also aids in explaining the phenomenon of immune response gene control of specific responses. In many situations, the capacity to recognize simple antigens can be found in only some members of a species. In most such cases, the genes that determine the capacity to make these responses have been mapped to the MHC. Such immune response gene control of immune responses is based on the capacity of different class II MHC molecules (or class I MHC molecules) to bind different sets of peptides. Thus, for simple molecules, it is likely that peptides can be generated that are only capable of binding to some of the polymorphic MHC molecules of the species. Only individuals that possess those allelic forms of the MHC will be able to respond to those antigens. Based on this, some individuals are nonresponders because of the failure to generate a peptide/MHC molecule complex that can be recognized by the T-cell system.

This mechanism also may explain the linkage of MHC type with susceptibility to various diseases. Many diseases show a greater incidence in individuals of a given MHC type. These include reactive arthritides, gluten-sensitive enteropathy, insulin-dependent diabetes mellitus, and rheumatoid arthritis (see Chapter 44). One explanation is that the MHC type that is associated with increased incidence may convey altered responsiveness to antigens of agents that cause or exacerbate the disease. Indeed, it appears that many of these diseases are due to enhanced or inappropriate immune responses.

Antigen-Presenting Cells (Chapter 16)

T cells recognize peptide/MHC complexes on the surface of other cells. Such cells are often referred to as APCs. Although

effector cells can mediate their functions by recognizing such complexes on virtually any cell type, naïve cells are most efficiently activated by a set of specialized APC, the DCs. DCs are a multimember family with distinctive locations and functions. Among them are the plasmacytoid DCs that are the principal source of type I interferons in viral infections.

In general, in their immature form, DCs are resident in the tissues where they are efficient at capturing and endocytosing antigen. Their antigen capture activity is dependent upon expression of several surface receptors including Fc receptors, receptors for heat shock proteins, and C-type lectins. If they receive signals, such as various inflammatory stimuli, often mediated by TLRs, they downregulate the expression of these molecules but increase their expression of surface MHC molecules and various costimualtory molecules such as CD80/86. In addition, such stimulation induces expression of chemokine receptors such as CCR2 and CCR7. The latter allow stimulated DCs to follow signals from the chemokines SLC and ELC, and to migrate into the T-cell zone of lymph nodes. As part of the maturation process, they may also acquire the capacity to produce cytokines and express surface molecules that can aid in determining the polarization of T-cell priming. This includes the production of IL-12, IL-23, IL-6, and IL-10, and the expression of inducible costimulator ligand and of Notch ligands. Interaction of naïve T cells with immature DCs may induce a state of peripheral tolerance.

EFFECTOR MECHANISMS OF IMMUNITY

The ultimate purpose of the immune system is to mount responses that protect the individual against infections with pathogenic microorganisms by eliminating these microbes or, where it is not possible to eliminate infection, to control their spread and virulence. In addition, the immune system may play an important role in the control of the development and spread of some malignant tumors. The responses that actually cause the destruction of the agents that initiate these pathogenic states (e.g., bacteria, viruses, parasites, tumor cells) are collectively the effector mechanisms of the immune system. Several have already been alluded to. Among them are the cytotoxic action of CTLs, which leads to the destruction of cells harboring viruses and, in some circumstances, expressing tumor antigens. In some cases, antibody can be directly protective by neutralizing determinants essential to a critical step through which the pathogen establishes or spreads an infectious process. However, in most cases, the immune system mobilizes powerful nonspecific mechanisms to mediate its effector function.

Effector Cells of the Immune Response

Among the cells that mediate important functions in the immune system are cells of the monocyte/macrophage lineage, NK cells, mast cells, basophils, eosinophils, and neutrophils. It is beyond the scope of this introductory chapter to present an extended discussion of each of these important cell types. However, a brief mention of some of their actions will help in understanding their critical functions in the immune response.

Monocytes and Macrophages (Chapter 19)

Cells of the monocyte/macrophage lineage play a central role in immunity. One of the key goals of cellular immunity is to aid the macrophages in eliminating organisms that have established intracellular infections. In general, non-activated macrophages are inefficient in destroying intracellular microbes. However, the production of IFN-γ and other mediators by T cells can enhance the capacity of macrophages to eliminate such microorganisms. Several mechanisms exist for this purpose, including the development of reactive forms of oxygen, the development of nitric oxide, and the induction of a series of proteolytic enzymes, as well as the induction of cytokine production. Macrophages can act as APCs and thus can enlist the "help" of activated, cytokine-producing CD4+ T cells in regulating their function.

Although macrophages function as APCs for attracting activated T cells, they do not appear to be particularly effective in the activation of naïve CD4 T cells. In instances in which they are the site of infection or have phagocytosed infectious agents or their proteins, antigens from these agents may be transferred to DCs. In such cases, the DCs would be the principal APCs that activate naïve or possibly resting memory CD4 T cells. Such activated T cells would then be available to help infected macrophages.

Natural Killer Cells (Chapter 17)

NK cells play an important role in the immune system. Indeed, in mice that lack mature T and B cells due to the *scid* mutation, the NK system appears to be highly active and to provide these animals a substantial measure of protection against infection. NK cells are closely related to T cells. They lack conventional TCR (or Ig) but express two classes of receptors. They have a set of activating receptors that allow them to recognize features associated with virally infected cells or tumor cells. They also express receptors for MHC molecules that shut off their lytic activity. Thus, virally infected cells or tumor cells that escape the surveillance of cytotoxic T cells by downregulating or shutting off expression of MHC molecules then become targets for efficient killing by NK cells because the cytotoxic activity of the latter cells is no longer shut off by the recognition of particular alleles of MHC class I molecules.

In addition, NK cells express a receptor for the Fc portion of IgG (FcγRIII). Antibody-coated cells can be recognized by NK cells, and such cells can then be lysed. This process is referred to as antibody-dependent cellular cytotoxicity.

NK cells are efficient producers of IFN-γ. A variety of stimuli, including recognition of virally infected cells and tumor cells, cross-linkage of FcγRIII, and stimulation by the cytokines IL-12 and IL-18, cause striking induction of IFN-γ production by NK cells.

Mast Cells and Basophils (Chapters 20 and 45)

Mast cells and basophils play important roles in the induction of allergic inflammatory responses. They express cell surface receptors for the Fc portions of IgE (FcεRI) and for certain classes of IgG (FcγR). This enables them to bind antibody to

their surfaces, and when antigens capable of reacting with that antibody are introduced, the resultant cross-linkage of FcεRI and/or FcγR results in the prompt release of a series of potent mediators such as histamine, serotonin, and a variety of enzymes that play critical roles in initiating allergic and anaphylactic-type responses. In addition, such stimulation also causes these cells to produce a set of cytokines, including IL-3, IL-4, IL-13, IL-5, IL-6, granulocyte-macrophage colony-stimulating factor, and TNFα, that have important late consequences in allergic inflammatory responses.

Granulocytes (Chapter 20)

Granulocytes have critical roles to play in a wide range of inflammatory situations. Rather than attempting an extended discussion of these potent cells, it may be sufficient to say that in their absence it is exceedingly difficult to clear infections with extracellular bacteria and that the immune response plays an important role in orchestrating the growth, differentiation, and mobilization of these crucial cells. Recent work indicates that T_{H17} cells are particularly important because of their role in recruiting granulocytes to sites of immune responses.

Eosinophils (Chapters 20 and 45)

Eosinophils are bone marrow–derived myeloid cells that complete their late differentiation under the influence of IL-5. They migrate to tissue sites in response to the chemokine eotaxin and as a result of their adhesion receptors. Because T_{H2} cells can produce IL-5 and stimulate the production of eotaxin, eosinophil accumulation is often associated with T_{H2}-mediated inflammation. Eosinophils store a series of proteins in their secondary granules including major basic protein, eosinophil cationic protein, and eosionphil peroxidase. When released, these proteins are responsible for much of the damage that eosinophils mediate both to helminthic parasites and to the epithelium. Eosinophils have been implicated as important in protective responses to helminths and in the tissue damage seen in allergic inflammation in conditions such as asthma.

The Complement System (Chapter 36)

The complement system is a complex system of proteolytic enzymes, regulatory and inflammatory proteins and peptides, cell surface receptors, and proteins capable of causing the lysis of cells. The system can be thought of as consisting of three arrays of proteins. Two of these sets of proteins, when engaged, lead to the activation of the third component of complement (C3) (Fig. 1.12). The activation of C3 releases proteins that are critical for opsonization (preparation for phagocytosis) of bacteria and other particles, and engages the third set of proteins that insert into biologic membranes and produce cell death through osmotic lysis. In addition, fragments generated from some of the complement components (e.g., C3a and C5a) have potent inflammatory activities.

The Classical Pathway of Complement Activation

The two activation systems for C3 are referred to as the classical pathway and the alternative pathway. The classical pathway is initiated by the formation of complexes of antigen with IgM or IgG antibody. This leads to the binding of the first component of complement, C1, and its activation, creating the C1 esterase that can cleave the next two components of the complement system, C4 and C2.

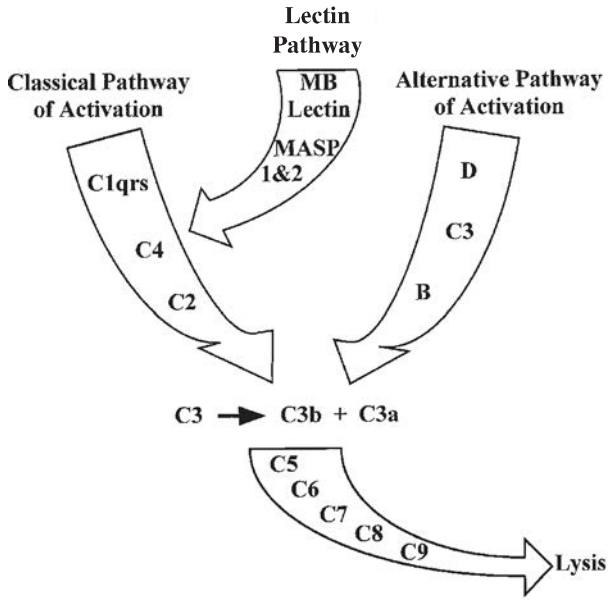

FIG. 1.12. The Complement System. The classical pathway of complement activation, usually initiated by the aggregation of C1 by binding to antigen/antibody complexes, resulting in the formation of an enzyme, a C3 convertase, that cleaves C3 into two fragments, C3b and C3a. The classical pathway can also be initiated by the aggregation of mannan-binding lectin as a result of binding sugars expressed in the capsules of many pathogenic microbes. The components of the lectin pathway appear to mimic the function of C1qrs. The alternative pathway of complement activation provides a potent means of activating complement without requiring antibody recognition of antigen. It results in the formation of a distinct C3 convertase. The fragments formed by cleaving C3 have important biologic activities. In addition, C3b, together with elements of the classical pathway (C4b, C2a) or the alternative pathway (Bb, properdin), form enzymes (C5 convertases) that cleave C5, the initial member of the terminal family of proteins. Cleavage of C5 leads to the formation of the membrane attack complex that can result in the osmotic lysis of cells.

C4 is a trimeric molecule, consisting of α, β, and γ chains. C1 esterase cleaves the α chain, releasing C4b, which binds to surfaces in the immediate vicinity of the antigen/antibody/C1 esterase complex. A single C1 esterase molecule will cause the deposition of multiple C4b molecules.

C2 is a single polypeptide chain that binds to C4b and is then proteolytically cleaved by C1 esterase, releasing C2b. The resulting complex of the residual portion of C2 (C2a) with C4b (C4b2a) is a serine protease whose substrate is C3. Cleavage of C3 by C4b2a (also referred to as the classical pathway C3 convertase) results in the release of C3a and C3b. A single antigen/antibody complex and its associated C1 esterase can lead to the production of a large number of C3 convertases (i.e., C4b2a complexes) and thus to cleavage of a large number of C3 molecules.

The components of the classical pathway can be activated by a distinct, non–antibody-dependent mechanism, termed the lectin pathway. The mannose-binding lectin (MBL) is

activated by binding to (and being cross-linked by) repetitive sugar residues such as N-acetylglucosamine or mannose. The activation of MBL recruits the MBL-associated serine proteases MASP-1 and MASP-2, which cleave C4 and C2 and lead to the formation of the classical pathway C3 convertase. Because the capsules of several pathogenic microbes can be bound by MBL, the lectin pathway provides an antibody-independent mechanism through which the complement system can be activated by foreign microorganisms.

The Alternative Pathway of Complement Activation

Although discovered more recently, the alternative pathway is the evolutionarily more ancient system of complement activation. Indeed, it, and the MBL activation of the classical pathway, can be regarded as important components of the innate immune system. The alternative pathway can be activated by a variety of agents such as insoluble yeast cell wall preparations and bacterial lipopolysaccharide. Antigen/antibody complexes also can activate the alternative pathway. The C3 convertase of the alternative pathway consists of a complex of C3b (itself a product of cleavage of C3) bound to the b fragment of the molecule factor B. C3bBb is produced by the action of the hydrolytic enzyme, factor D, that cleaves factor B; this cleavage only occurs when factor B has been bound by C3b.

Apart from the importance of the alternative pathway in activating the complement system in response to nonspecific stimulants, it also can act to amplify the activity of the classical pathway because the C3 convertase of the classical system (C4b2a) provides a source of C3b that can strikingly enhance formation of the alternative pathway convertase (C3bBb) in the presence of factor D.

The Terminal Components of the Complement System

C3b, formed from C3 by the action of the C3 convertases, possesses an internal thioester bond that can be cleaved to form a free sulfhydryl group. The latter can form a covalent bond with a variety of surface structures. C3b is recognized by receptors on various types of cells, including macrophages and B cells. The binding of C3b to antibody-coated bacteria is often an essential step for the phagocytosis of these microbes by macrophages.

C3b is also essential to the engagement of the terminal components of the complement system (C5 through C9) to form the membrane attack complex that causes cellular lysis. This process is initiated by the cleavage of C5, a 200,000-dalton two-chain molecule. The C5 convertases that catalyze this reaction are C4b2a3b (the classical pathway C5 convertase) or a complex of C3bBb with a protein designated properdin (the alternative pathway C5 convertase). Cleaved C5, C5b, forms a complex with C6 and then with C7, C8, and C9. This C5b/C9 complex behaves as an integral membrane protein that is responsible for the formation of complement-induced lesions in cell membranes. Such lesions have a donut-like appearance, with C9 molecules forming the ring of the donut.

In addition to the role of the complement system in opsonization and in cell lysis, several of the fragments of complement components formed during activation are potent mediators of inflammation. C3a, the 9,000-dalton fragment released by the action of the C3 convertases, binds to receptors on mast cells and basophils, resulting in the release of histamine and other mediators of anaphylaxis. C3a is thus termed an anaphylotoxin, as is C5a, the 11,000-dalton fragment released as a result of the action of the C5 convertases. C5a is also a chemoattractant for neutrophils and monocytes.

Finally, it is important to note that the process of activation of the complement cascade is highly regulated. Several regulatory proteins (e.g., C1 esterase inhibitor, decay accelerator factor, membrane cofactor protein) exist that function to prevent uncontrolled complement activation. Abnormalities in these regulatory proteins are often associated with clinical disorders such as hereditary angioedema and paroxysmal nocturnal hemoglobinuria.

CONCLUSION

This introductory chapter should provide the reader with an appreciation of the overall organization of the immune system and of the properties of its key cellular and molecular components. It should be obvious that the immune system is highly complex, that it is capable of a wide range of effector functions, and that its activities are subject to potent, but only partially understood, regulatory processes. As the most versatile and powerful defense of higher organisms, the immune system may provide the key to the development of effective means to treat and prevent a broad range of diseases. Indeed, the last two sections of this book deal with immunity to infectious agents and immunologic mechanisms in disease. The introductory material provided here should be of aid to the uninitiated reader in understanding the immunologic mechanisms brought into play in a wide range of clinical conditions in which immune processes play a major role either in pathogenesis or in recovery.

CHAPTER 2

History of Immunology

Steven Greenberg

INTRODUCTION

There comes a time during every argument at which the two opposing parties reach a critical juncture: either resolution or impasse. Variations on this essentially Socratic theme have played out in all spheres of human intellectual activity. The dialectic of science ranges from incremental and relatively harmonious shifts in key, to a few abruptly dissonant ones, taking the form of what Thomas Kuhn would refer to as "paradigm shifts."[1] Such is the case with immunology, a field distinguished by more than its fair share of paradigm shifts. Arguably, its first dialectic, between the "cellularists" and the "humoralists," did not result in an early synthesis, but was characterized by partisan and often entrenched positions. Ultimately, the two parallel paths of cellular and chemical immunology converged, but it was not until the latter half of the 20th century that the two paths became one. How the paths were forged in the first place was an amalgam of the cultural institutions of the time, the creative output of the scientists themselves, and the imperatives of devising effective strategies to combat infection and contagion.

ANTECEDENTS TO THE GERM THEORY OF DISEASE

Ancient Theories of Disease Causation

Religious beliefs in ancient Greece drew contrasts between the sacred or the pure (*katharos*) and the polluted (*miaros*).[2] Pollution, or *miasma*, was blamed for many ancient transgressions, from the petty and personal, to the gravest, most famously embodied in the Oedipus myth. To remove the stain of *miasma*, the transgressor must undergo rites of purification (*catharsis*). To the ancient Greeks in the age of Homer, these were deeply ingrained beliefs that were essentially religious in nature. Because *miasma* was viewed as a source of suffering, it is not surprising that *miasma* was implicated in disease, as described by Hippocrates in his treatise "On Air, Water, and Places" in which *miasma* was associated with "unhealthy vapors."[3,4] Hippocrates is credited with being the first to recognize the potential of disease to arise from the environment and not as a result of religious superstition. *Mal aria*, which is Old Italian for "bad air," was one of many diseases thought to be caused by *miasma*. The concept that *miasma* was the source of disease persisted through the millennia and was a leading theory of how contagious diseases were transmitted up until the time of Pasteur.

Much of what we know about the medicine of ancient Greece is codified in *The Hippocratic Corpus*, a collection of more than 60 volumes of text. Its authorship is disputed, but it is generally recognized as a compilation of works by Hippocrates himself as well as his students and intellectual heirs. One of his students, as well as son-in-law, Polybus, was credited as the author of *De Natura Hominis* (*On the Nature of Man*), the earliest known text describing the ancient Greek conceptual basis for disease pathogenesis, as embodied in the four humors: black bile, yellow bile, phlegm, and blood.[5]

> The body of man has in itself blood, phlegm, yellow bile and black bile; these make up the nature of his body, although these he feels pain or enjoys health. Now he enjoys the most perfect health when these elements are duly proportioned to one another in respect of compounding, power and bulk, and when they are perfectly mingled. Pain is felt when one of these elements is in defect or excess, or is isolated in the body without being compounded with all the others.

The Greek view that disease arose from an imbalance of the four humors did not supersede the *miasma* theory of disease, but was rather a more general view of disease causation, compared with the subset of apparently communicable diseases best explained by *miasma*.

The Romans developed and refined Greek concepts of disease. Marcus Terentius Varro (116 to 27 BCE), referred to as "the most learned of all Romans" by the Roman rhetorician Quintilian,[6] was a prolific Roman scholar, estimated to have written more than 600 volumes. During the civil war of the first century, he served as Pompey's legate in Spain and fought at Pharsalus against Caesar but ultimately reconciled with Caesar, who appointed him director of the public library in 47 BCE. Varro is perhaps best known for his only complete extant work, *Rerum Rusticarum Libri Tres* (*On Agricultural Topics*), in which he so presciently anticipated the existence of disease-causing microbes that seemed to Varro to be the immediate cause of diseases[7]:

> Precautions must also be taken in the neighbourhood of swamps, both for the reasons given, and because there are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there cause serious diseases.

Thus, Varro provided a mechanistic basis for disease that was consistent with the prevailing belief of *miasma* as the source of illness.

The Greek medical tradition was carried on for generations and was ultimately passed on to Claudius Galinus

(Galen), the Greek expatriate who was its greatest explicator. Galen was born in Pergamum in Asia Minor in 130 CE. After beginning his medical training in Pergamum at the behest of his father, he traveled widely in pursuit of "postgraduate" medical training in Smyrna, Corinth, and Alexandria. He returned to Pergamum and practiced surgery on gladiators, which provided a unique opportunity to deepen his knowledge of human anatomy and perfect his surgical technique.[8] Following an outbreak of plague among the Roman troops in Aquileia in 168 CE, he was summoned by the Emperor Marcus Aurelius and was appointed personal physician to his son, Commodus.[8] Galen's view of medicine was based on Hippocrates' *Corpus*. His output was prodigious more than any other ancient author of medical texts. He distinguished symptoms from diseases and offered explanations of the former that were consistent with his interpretations of disease pathogenesis; thus, tertian fever was the result of an "imbalance of yellow bile," quartan was caused by "too much black bile," and quotidian by "an excess of phlegm." Vomiting was viewed as the body's attempt to expel poisons, and the prescription of bleeding was to rid the body of "corrupt humors."[9] Galen's view of medicine remained the dominant one until the 17th century.

Early Concepts of Immunity

The term "immunity" itself is derived from the Latin practice of "exemption" from taxes or public service that normal citizens had to discharge, a favor bestowed by the emperor to meritorious individuals or entire communities.[10] However, the concept of immunity dates back at least as far as Thucydides, who described the plague of Athens of 430 BCE that was responsible for the death of more than a quarter of the Athenian population[11]:

> Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what I had from experience and had now no fear for themselves; the same man was never attacked twice-never at least fatally.

The traditional, religious view of the plague was that it is the work of Apollo who was held responsible for earlier plagues (eg, the plague on the Greek army in Troy because the Greek general Agamemnon abducted the daughter of Apollo's priest, Chryses). The religiously inclined could take some refuge in appealing to Apollo's son, Asklepios, who was revered as the god of healing, as were his daughters Hygeia (Hygiene), Iaso (Remedy), Akæso (Healing), Aglæa (Healthy glow), and Panakeia (Cure-all). In contrast, Thucydides characteristically did not offer facile religious explanations for sickness or recovery. In fact, he described the futility of the Athenians' plight in stark terms[11]:

> Neither the fear of the gods nor laws of men awed any man, not the former because they concluded it was alike to worship or not worship from seeing that alike they all perished, nor the latter because no man expected that lives would last till he received punishment of his crimes by judgment.

It seemed inevitable that in seeking an explanation for why some developed disease and others did not, many others would rely on a moralistic or religious view. In a remarkable passage from Galen's *On the Different Types of Fever*, not only does he set forth an explanation of how disease is transmitted through the air, and using the same term as Fracastoro would, "seeds," some 1300 years later, but he also blames a licentious lifestyle for susceptibility to the plague:

> Suppose, for example, that the circumambient air carries certain seeds of plague, and that of the bodies which share [breathe] it, some are full of various residues which are soon to become putrefied in themselves, while others are clean and free of such residues. Assume also that in the former there is a general blockage of their pores, a so-called plethora, and a life of ease devoted to gluttony, drink and sex, with all their necessarily concomitant digestive disorders. The others, which are clean and lack these residues, as well as being fine in themselves, have all a wholesome transpiration through pores that are neither blocked nor constricted; they take appropriate exercise and lead a temperate life. Assuming all this, which of these bodies is most likely to be affected by the rotting air they inspire?

This passage has been analyzed extensively by Nutton, who questioned the extent to which "seed" is used metaphorically[12]; if so, it is particularly apt.

Religious explanations for disease and immunity persisted throughout history. Particularly during the growth of Christianity during the Middle Ages, disease and sin were linked, though not inextricably; the great theologian Thomas Aquinas provided this distinction between sin and other causes of diseases[13]:

> . . . we need to consider that sin consists of a disorder of the soul, just as physical disease consists of a disorder of the body. And so sin is a disease of the soul, as it were, and pardon is for sin what healing is for disease.

Yet for many, a disease as dire as the plague would continue to be viewed as divine retribution for sin; for others, it was the result of astrological phenomena, while for still others, it was the result of a "conspiracy plotted by Jews to poison wells."[14] With the exception of the latter, which at least offered a proximate physical cause of disease, regardless of the lack of evidence, there was an abstract quality to these explanations that were shrouded in belief and superstition but lacking in substance. Further theories of disease pathogenesis would have to await the 16th century.

Fracastoro's Seeds

Girolamo Fracastoro (1478 to 1553) was an Italian who would have met most definitions of a Renaissance scholar: an accomplished physician, poet, mathematician, botanist, and astronomer. Educated by his father in Verona, and later at the University of Padua, he became an instructor in logic at the University of Padua in 1501 and in anatomy in 1502. He left Padua in 1508 and returned to Verona, where he

dedicated himself to his studies and his medical practice. In 1546, he proposed that disease was caused by *seminaria* ("seeds") that could be transmitted by three ways: direct contact from one person to another; through "fomites," or articles of clothing or dirty linen; and through the air. Although Nutton[12] has pointed out that Galen and Lucretius also used the term "seeds" to describe the transmission of illness through the air (see previous discussion), Fracastoro was the first to make them the focal point for disease transmission and to describe their predilection for certain organs. Like the 10th century Arab physician Rhazes, who believed smallpox to have an affinity for blood, and specifically for the traces of menstrual blood that were believed to taint everyone in utero, as later suggested by Avicenna, Fracastoro offered the following explanation for immunity to smallpox: Following infection by smallpox seminaria, the menstrual blood would putrefy, rise to the surface, and force its way out via the smallpox pustules.[15] "Hence when this process has taken place, the malady usually does not recur because the infection has already been secreted in the previous attack."

Fracastoro's *seminaria* theory remained influential for nearly three centuries, in many ways serving as an early template for the germ theory of disease.

THE GERM THEORY OF DISEASE AND DEVELOPMENT OF VACCINES

Until the mid-19th century, the Galenic view of disease origins was the dominant one. Not only was the etiology of diseases misunderstood but also was the origin of life itself. The theory of spontaneous generation, which arose from Aristotle, held that life originated spontaneously from inanimate matter. The first experimental evidence against spontaneous generation came from Franceso Redi, the head physician in the Medici court, who in 1668 provided early evidence against the theory.[16*] Nevertheless, no overarching theory was proposed to replace it. Neither the mindset nor the necessary technology were available until the latter part of the 17th century, when an apprentice in a dry goods store, Anton van Leeuwenhoek, began a lifelong obsession with grinding the perfect lens. Leeuwenhoek's lenses were tiny but were ground with high degree of curvature, enabling him to visualize the hitherto undiscovered word of microbes. On September 17, 1683, Leeuwenhoek wrote a letter to the Royal Society, which was the first description of living, motile bacteria obtained from the plaque of his own teeth.[17] Leeuwenhoek was not the first to build a microscope (which was used by, among others, Redi), but his was far superior to existing multilensed or compound microscopes. Other scientists of the time, notably Robert Hooke, also observed microorganisms, and it was Hooke who was the first to publish the first image of a microorganism (the fungus *Mucor*) in 1665.[18] Some 150 years later, Dutrochet and then later Schwann, Schleiden, and Virchow, taking advantage of the microscopes of the their time, advanced the concept that "all living things are composed of cells and cell products."[19] Virchow took this one step further by declaring *omnis cellula e cellula* or "all cells develop only from existing cells." Whether Virchow rejected the germ theory or not is a matter of debate, but it is more likely that Virchow's underlying emphasis was not on external causes, but disease mechanisms, as he wrote in 1885: "First the discovery of the parasite, then the investigation of its etiology, then the question: how does it give rise to the disease."[20] Although it is hard to escape the possibility that a certain degree of professional jealousy may have played a role in Virchow's refusal to embrace the germ theory of disease, his viewpoint is one of but many examples of apparently strict dichotomies in science that would ultimately undergo revision and later synthesis. This is a theme that was to be recapitulated many times in the history of immunology.

The Conceptual Basis for the Germ Theory of Disease

Between Leeuwenhoek's technical breakthroughs in lens design in the late 17th century and the work of Pasteur and Koch in the late 19th century, several individuals endorsed Fracastoro's "seed" theory, which gained new relevance when bacteria were first visualized. Among these was Jacob Henle, a German pathologist who was later to become Koch's teacher. Henle wrote a treatise that not only laid out the germ theory of disease in great detail but also arguably articulated an early version of what would later be known as "Koch's postulates"[21]: "Before microscopic forms can be regarded as the cause of contagion in man, they must be constantly found in contagious material. They must be isolated from it and their strength tested." However, Henle's essay was a theoretical one and Henle himself never provided any experimental evidence in support of it. In the same year, decades before Pasteur and Koch would even begin to describe the germ theory of disease, Henry Holland, a Scottish-trained physician to Queen Caroline, who traveled extensively and was acquainted with the scientific luminaries of the time, including Davie, Gay Lussac, Berthollet, and Laplace, wrote a treatise in which he stated,[22]

> The question is, what weight we may attach to the opinion that certain diseases, and especially some of epidemic and contagious kind, are derived from minute forms of animal life, existing in the atmosphere under particular circumstances; and capable, by application to the lining membranes or other parts, of acting as a virus on the human body.

In a footnote, he cited others, including Kircher, and particularly Johannes Nyander, who wrote nearly a century earlier[23]:

> . . . it may be an easy matter for very minute insects to be the causes of diverse contagious diseases," of which he included plague, measles, smallpox, and syphilis.

Nyander himself credits Lynceus Leuwenhoekius ("lynx-eyed" or "keen-eyed" Leuwenhoek) as the first to have seen such

*Much later, Spallanzani and Pasteur provided key experimental evidence against spontaneous generation, a belief that some scholars still held in the late 19th century, when Pasteur, for example, showed that broth in swan-neck-bent, but not unbent or broken flasks, failed to support microbial growth.

"animalcules." Thus, it is fair to say that the germ theory of disease itself had been "germinating" for some time prior to its scientific proof by Pasteur and Koch.

Experimental Evidence for the Germ Theory of Disease

The honor of the first experimental demonstration for the germ theory of disease may belong to two students of Francesco Redi. In 1687, soon after Redi had offered proof against spontaneous generation, two of his students, Bonomo and Cestoni, went on to observe the causative agent of scabies using the newly developed microscope and were able to transfer disease from person to person.[24] Perhaps the first demonstration of the bacterial pathogenesis of diseases in animals and humans was by Casimir Davaine, a French scientist who provided essentially the same evidence that Pasteur and Koch would years later that anthrax was caused by *bacteridies*. In 1865, Davaine was awarded the Prix Bréant by the Académie des Science for his work.[25]

Davaine's compatriot, Louis Pasteur, was the consummate experimentalist. A chemist by training, his interest in infectious disease began with his study of fermentation. He made important contributions to the science of fermentation and his work led to many practical benefits to the beer and wine industry of France. His interest in microbes began with his speculation that the same type of microbe required for fermentation was likely responsible for transmitting disease. In 1865, he was asked to investigate a disease called pébrine that affected the silk worm industry. Within a year, Pasteur had established that pébrine was caused by a microbe, which provided further proof for the germ theory of disease. Some 14 years later, his expertise was again sought out of economic interests, in this case by farmers whose poultry stocks were diminished by chicken cholera. In a famous example of scientific serendipity, his assistant, Charles Chamberland, failed to inoculate chickens with cultures of chicken cholera bacilli, as instructed by Pasteur, but instead went on vacation. Upon returning several weeks later, he inoculated chickens with the old bacterial cultures, but the chickens didn't die as expected. Rather than disregard the experiment as a failure, "chance had favored the prepared mind" of Pasteur, who had his assistants inject fresh cholera into the same hens that had previously been injected; now none of the hens became ill. Pasteur had surmised that the bacterial cultures had become weakened by extended culture.[26] Thus began the use of attenuated strains of microbes to immunize against disease,[27] and the birth of the science of vaccination. The term "vaccine," derived from the Latin *vaccus* for cow, was, coined by Pasteur in honor of Jenner. Attenuation as a strategy of developing vaccines would prove to be enormously valuable, leading to development of the first rabies vaccine by Pasteur himself, a vaccine against the viral causative agent of yellow fever by Theiler,[28] and the Bacillus Calmette-Guérin vaccine at Pasteur's institute.[29]

Although Robert Koch is credited as the originator of "Koch's Postulates," it may come as a surprise that the essence of the postulates were first formulated by Koch's teacher, Jacob Henle (see previous discussion) and his contemporary, Edwin Klebs.[30] However, neither Henle nor Klebs applied their theories to any practical benefit, which is why Koch received credit for the postulates. Koch was the first to articulate then systematically apply them to prove that *Bacillus anthracis* was the causative agent of anthrax. Koch was a country physician living in Prussia, whose scientific career began unceremoniously with a gift of a microscope from his wife.[31] His first series of investigations began with observing the blood of a dead cow that succumbed to anthrax. Confirming the observation of Davaine and others before him, he observed filamentous bacteria in the blood. Not content merely with the observation, he began a series of technically challenging experiments, necessitating the development of many novel techniques used in microbiology laboratories even today, such as the use of solid medium to grow individual clones or colonies of bacteria. He proved that the filamentous bacteria were present only in infected animals and were capable of reproducing the disease when injected into healthy animals. This was the first systematic application of the eponymous postulates, which has since become the *sine qua non* of disease causation by infectious agents. His work was published in 1876,[32] the first of many groundbreaking publications. Koch's most profoundly important contribution to medicine was the discovery of the causative agent of tuberculosis. The lecture at which he announced his findings, on March 24, 1882, described later by Ehrlich as "the most important experience of his scientific life," is considered by many to be the single most important lecture in medical history. Koch described the invention of novel staining methods to detect the tubercle bacillus and presented tissue dissections from guinea pigs that were infected with tuberculous material from the lungs of infected apes and humans who had died from the disease.[33] For "his investigations and discoveries in regard to tuberculosis," Koch was awarded the Nobel Prize in 1905.

The Unhealthy Rivalry Between Pasteur and Koch, and its Lasting Effects

The Franco-Prusssian war of 1870, the culmination of years of tension between France and Prussia, resulted in a Prussian victory and unity among the German states under King Wilhelm of Prussia. The Treaty of Frankfurt left a unified Germany the city of Strasbourg as well as possession of Alsace and the northern part of Lorraine, which was thought to contribute to further resentment of the Germans by the French and public support for World War I. It is against this backdrop that the relationship between Pasteur and Koch must be viewed. Koch had served in the Prussian army, and Pasteur's son was a conscript fighting for the French. Furthermore, there was intense professional rivalry between the two, especially over their work on anthrax pathogenesis. According to a letter from Charles Ruel, former *privat docent* at the University of Geneva, Koch was in the audience when Pasteur spoke on attenuation and vaccination at the fourth International Congress of Hygiene and Demography held in Geneva in September 1882. Pasteur spoke repeatedly about

German collected works (*recueil Allemand*). According to the letter[34]:

> Koch and his friend Prof. Lichtheim, were sitting side by side; they knew French only imperfectly and both mistook the word pride (orgueil) for collection (recueil). They felt their self-respect profoundly wounded and interpreted the words German pride as a grave insult.

This is but one ironic example of the level of rancor and misunderstanding between the two great men. It is said that Pasteur and Koch underwent some form of reconciliation later in their lives. Regardless, the aftermath of their rivalry, in many ways personifying the bitter relations between France and Germany, had a lasting effect on the evolution of the nascent field of immunology. In the years to come, the intellectual heirs of Pasteur and Koch would reenact the lifelong competitive tensions that characterized their relationship.

The Germ Theory of Disease: A Summation

In many ways, the "germ theory of disease" really did not begin with Pasteur and Koch, but rather by their predecessors, Henle, Klebs, and Davaine, who in turn owed credit to Fracastoro, and ultimately to Galen and Varro, some 1,600 years earlier. What enabled Pasteur and Koch to firmly establish the germ theory of disease began as a thought process that over time became distilled to something tangible: First, the idea that invisible "seeds" might propagate disease; second, the advent of an optically superior microscope, by Leuwenhoek, which enabled scientists their first glimpses at the "minute creatures" postulated by Varro; third, the growing evidence against spontaneous generation that began with Redi, thus opening the door for a new theory of disease; and finally, the inductive genius and careful experimental techniques of Pasteur and Koch.

The Long History of Vaccination: Success and the Unprepared Mind

Vaccination did not originate with Pasteur; its practice had been carried out for centuries without any fundamental understanding of its basis. Probably the earliest recorded example of intentionally inducing immunity to an infectious disease was in the 10th century in China, where smallpox was endemic.[35] The process of "variolation" involved exposing healthy people to material from the lesions caused by the disease, either by putting it under the skin, or, more often, inserting powdered scabs from smallpox pustules into the nose. Variolation was known and practiced frequently in the Ottoman Empire, where it had been introduced by Circassian traders in the 17th century.[35] Unfortunately, because there was no standardization of the inoculum, variolation occasionally resulted in death or disfigurement from smallpox, thus limiting its acceptance. Variolation later became popular in England, mainly due to the efforts of Lady Mary Wortley Montague. Lady Montague was the wife of the British ambassador to the Ottoman court who herself had contracted a severe case of smallpox. While in Istanbul, Lady Montague observed the practice of variolation. Determined not to have her family suffer as she had, she directed the surgeon of the embassy to learn the technique and, in March 1718, to variolate her 5-year-old son. After her return to England, she promoted the technique and had her surgeon variolate her 4-year-old daughter in the presence of the king's physician. The surgeon, Charles Maitland, was given leave to perform what came to be known as the "Royal Experiment," in which he variolated six condemned prisoners who later survived. By these and other experiments, the safety of the procedure was established, and two of the king's grandchildren were variolated on April 17, 1722. After this, the practice of variolation spread rapidly throughout England in the 1740s and then to the American colonies.[35,36]

It is difficult to say what influence the English country physician Edward Jenner (1749 to 1823) had on Pasteur's later discovery of attenuation of bacterial cultures and its application to vaccination. Regardless, it is fair to say that Jenner had a major influence on public health, as he was the first to publish the development and use of a safe alternative to variolation.[37] Although Jenner is rightly celebrated for his development of cowpox as a safe vaccine for smallpox, he was not the first to make use of a relatively nonpathogenic virus to induce immunity. Twenty years earlier, Benjamin Jesty, an English farmer, inoculated his wife and two sons with material taken from the cowpox lesion of the udder of a cow in his neighbor's herd.[36] In 1796, Jenner inoculated James Phipps, an 8-year-old boy, with material obtained from a cowpox lesion that appeared on the hand of a dairymaid (Fig. 2.1). Six weeks later, he inoculated the experimental subject with smallpox without producing disease. Further studies by Jenner established the efficacy of his vaccination procedure. For this feat, Jenner received a cash prize of £30,000 and election to nearly all of the learned societies throughout Europe.[38]

EMERGENCE OF IMMUNOLOGY AS A DISCIPLINE

The Cellularists versus the Humoralists

The international renown of Pasteur and Koch led to the establishment of research institutes that bore their names. Many talented young scientists were drawn to these institutes whose research missions should have been complementary, but were not, and the partisan battle that began with Pasteur and Koch would soon be reenacted on a larger scale.

Metchnikoff and the Birth of Cellular Immunology

Elie Metchnikoff (1845 to 1916), an ambitious student at the University of Kharkoff ("I have zeal and ability, I am naturally talented—I am ambitious to become a distinguished investigator"),[31] borrowed a professor's microscope and began a lifelong quest to understand the cellular basis for immunity. A comparative zoologist by training, his early academic focus was on understanding the development of metazoans. Heavily influenced by Darwin's publication of *The Origin of Species* in 1859,[39] he viewed early embryologic development as a competition among specialized cell

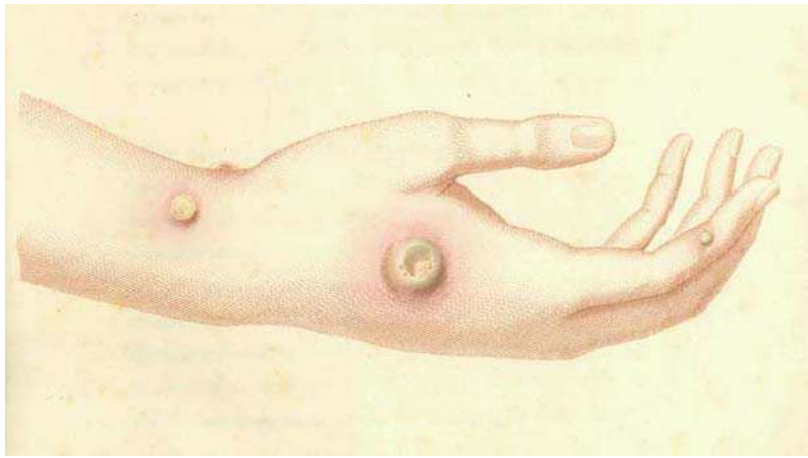

FIG. 2.1. Cowpox Pustule on the Arm of Sarah Nelmes. Reprinted with permission from Jenner[37]; courtesy of Dr Jenner's House: Birthplace of Vaccination, Gloucestershire, UK.

types.[40] Ontogeny was seen as a set of "interactions of cell lineages with each other to limit self-replication by any one component in favour of the interests of the organism as a whole."[40] He focused his interest on an amoeboid marker cell of the mesoderm, which was dubbed "phagocyte" (devouring cell) by a contemporary of Metchnikoff's, the Viennese zoologist, Carl Claus. In a justly famous passage from Olga Metchnikoff's biography of her husband, she describes the observation that became the defining moment of his scientific career[41]:

> One day when the whole family had gone to a circus to see some extraordinary performing apes, I remained alone with my microscope, observing the life in the mobile cells of a transparent star-fish larva, when a new thought suddenly flashed across my brain. It struck me that similar cells might serve in the defense of the organism against intruders. Feeling that there was in this something of surpassing interest, I felt so excited that I began striding up and down the room and even went to the seashore in order to collect my thoughts. I said to myself that, if my supposition was true, a splinter introduced into the body of a star-fish larva, devoid of blood-vessels or of a nervous system, should soon be surrounded by mobile cells as is to be observed in a man who runs a splinter into his finger. This was no sooner said than done. There was a small garden to our dwelling, in which we had a few days previously organised a "Christmas tree" for the children on a little tangerine tree; I fetched from it a few rose thorns and introduced them at once under the skin of some beautiful star-fish larvae as transparent as water. I was too excited to sleep that night in the expectation of the result of my experiment, and very early the next morning I ascertained that it had fully succeeded. That experiment formed the basis of the phagocyte theory, to the development of which I devoted the next twenty-five years of my life.

Contrary to popular belief, Metchnikoff was not the first to visualize and describe phagocytosis, nor was he the first to suggest that it played a role in host defense. In a historical recount of the history of phagocytosis,[42] Stossel argues that a Lutheran pastor, Johann August Ephraim Goeze, was the first to describe phagocytosis by microscopic observations of cells derived from hay infusoria in 1777. Much later, German pathologists of the mid-19th century, including Lieberkühn, Henle, and Vogel, drew connections between "pus corpuscles" of wounds and blood corpuscles.[42] Others at the time, particularly the English physicians William Addison and Augustus Waller, observed leukocyte migration through capillaries in response to local injury, and Ernst Haeckel, a German marine biologist who was later to oppose Metchnikoff in an early theory of gastrulation, described molluscan leukocytes ingesting India ink particles in 1862.[43]

A handful of scientists of the time made the conceptual link between phagocytosis and host defense, and Metchnikoff himself cites a few: "When (the phagocytosis theory) is once firmly established, it will be time enough to determine each part taken in its foundation by workers such as Panum, Gaule, Roser, etc. . ."[44] However, it seems that none of these workers seized upon this concept and appreciated its importance to the degree that Metchnikoff had; certainly, none had further developed and tested what in retrospect was the correct interpretation of the defensive function of phagocytosis.

Following the key experiment in 1882, described previously, Metchnikoff performed many others to test the "phagocytosis theory," such as the observation of infection in water fleas, which he viewed as a Darwinian struggle between pathogen and host[45]:

> Once they have insinuated themselves into the organism's inmost part, the spores cause an accumulation of the mobile cells round them, which correspond to the white corpuscles in human blood. A battle takes place between the two elements.

Drawing the analogy with host defense in higher organisms, Metchnikoff described the killing of the spores by "mobile cells," thus ensuring immunity for the organism. His view of phagocytosis expanded to encompass not only host defense but also organismal development, which he viewed in a teleologic context; he cited the dissolution of the tadpole's tail by the "pervasion of phagocytes."[46] By the time he moved to Paris to become *Chef de Service* at the Pasteur Institute in

1888, Metchnikoff had already formulated and tested what had become, according to his view, a cornerstone of the science of immune system. Perhaps what he had not appreciated at the time was that his move to Paris would come to signify to the Germans a complicit alliance with the French. He thus had unwittingly entered a battle that not only had been fought in the political arena but in the laboratory as well.

The Ascendance of Humoral Immunity

Several related developments in the new field of immunology occurred at the end of the 19th century that would seal the fate of cellular immunology for at least 50 years: The discovery by Roux and Yersin[47] that toxins alone derived from diphtheria bacilli could reproduce the symptoms of diphtheria; the discovery by von Behring and Kitasato in 1890[48] of humoral immunity to diphtheria and tetanus and the passive transfer of immunity to diphtheria in animals by von Behring and Wernicke in 1892[49]; and the discovery of alexins (Greek for "without a name") by Hans Buchner in 1899[50] and Jules Bordet at about the same time.[51] Alexin was renamed "complement" by Ehrlich, as it "complemented" the activity of antibodies. Indeed, the ability of humoral components alone to lyse bacteria (the Pfeiffer phenomenon) or erythrocytes in the absence of phagocytosis[51] provided strong independent evidence supporting the humoralists' claims. The discovery of complement also had practical uses, as complement fixation became the basis of a widely used serologic test for the diagnosis of syphilis, the so-called Wasserman test.[52] The collective discoveries of the "humoralists" would lead to the successful treatment of a number of previously intractable diseases, such as diphtheria. Indeed, the first Nobel Prize in physiology of medicine was awarded to von Behring in 1901, "For his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and death." Bordet himself would later be awarded the Nobel Prize "for his studies in regard to immunity." Among Bordet's contributions was the development of the complement fixation test together with his brother-in-law, Octave Gengou. This formed the basis of what Bordet termed "serodiagnosis."

Although the lines in the sand had already been drawn by the mostly Prussian humoralists led by Ehrlich on the one hand and the cellularists led by Metchnikoff on the other, it was Metchnikoff who wrote a letter to von Behring, proposing a scientific "truce"[53]:

> I now believe . . . we can calmly work side by side. We can mutually support one another, just like the phagocytes and antitoxins, since . . . the phagocytes receive considerable assistance from the antitoxic property, just as the phagocytes . . . render great assistance to the organism or respectively its antitoxic powers, since they capture and destroy . . . bacteria.

In fact, von Behring did not seem rigidly against the cellular school, and Metchnikoff viewed him as supporting the view that "active immunity requires some type of cellular basis."[53,54] It is difficult to know what to make of this passage; it sounds vague and seems to state the obvious, yet it does suggest a degree of flexibility that more intransigent proponents of the humoralist camp seemed to lack at the time. Regardless, scientific reconciliation would not be forthcoming until many years later, although phagocytosis theory was granted a temporary reprieve by the British physician Almoth Wright, who demonstrated the phenomenon of opsonization of bacteria. Wright was the first to describe a mechanism by which humoral and cellular components of immunity cooperate to kill bacteria.[55] Wright is possibly best known in his incarnation as Sir Colenso Ridgeon in Shaw's "The Doctor's Dilemma." Ridgeon defined "opsonin" as "...what you butter the disease germs with to make your white blood corpuscles eat them."[56] In what was viewed as a well-deserved but partly symbolic gesture, nevertheless, Metchnikoff and Ehrlich, two exemplars of the opposing schools of immunity, were awarded the Nobel Prize in 1908 "in recognition for their work on immunity." It would not be until 40 years later that another cell type of the immune system would be first identified as being the source of antibody[57] and 70 years later when dendritic cells (DCs) would be first identified as being the key phagocytic leukocyte responsible for initiating the immune response.[58]

Paul Ehrlich: The Cellularist's Humoralist

Paul Ehrlich embodies a pivotal figure in the history of immunology. Although he would make many practical discoveries in his long research career, his greatest contribution to immunology, like Jerne's over a half century later, was a conceptual breakthrough that served to stimulate the field of immunology for years to come. Although I am tempted to consider his "side chain theory" of antibody formation a paradigm shift, that would presume that there was a preexisting paradigm to shift from, when in fact there was no paradigm of antibody specificity to begin with.

Ehrlich began his research career as a medical student. One of his professors was the pathologist Wilhelm von Waldeyer, who introduced the young Ehrlich, who already showed a strong interest in chemistry, to histologic methods for staining cells and tissues. Following further training in several medical schools, he was influenced by the chemist von Bayer and the pathologists Cohnheim, Haidenhain, and Weigert (his cousin). He presented his thesis on histologic staining in Leipzig at the age of 24, in which he was the first to describe mast cells. As noted by Silverstein, the opening sentence of the thesis gave an inkling of his approach to science[59]:

> While in the modern histological literature, directions on tintorial method are already so numerous, and still increase from day to day, yet their theoretical basis has had only a very negligible consideration.

The same year, he was appointed senior physician in the Department of Internal Medicine at the Charité-Hospital in Berlin. The head of the clinic, Friedrich Frerichs, encouraged Ehrlich to continue his histochemical investigations, which led to the identification of neutrophils, eosinophils,

and basophils as well as the diagnosis of his own case of pulmonary tuberculosis.[60]

It is notable that the term "side-chain" (*seitenketten* in German) was a chemical term in use at Ehrlich's time meaning much the same as it does today. It is inescapable to conclude that Ehrlich's focus and interest in chemistry would be the driving force behind his thinking about how antibody is formed, and "selected for," by antigen. The essence of Ehrlich's side chain theory, first proposed in 1897 (Fig. 2.2) is well articulated in Ehrlich's Nobel lecture and is paraphrased here[61]:

1. "The relationship between toxin and antitoxin are *strictly specific*—tetanus antitoxin neutralizes exclusively tetanus toxin, diphtheria serum only diphtheria toxin . . ."
2. "For this reason it must be assumed that the (toxin and antitoxin) enter into a *chemical bond* . . . fitting each other 'like lock and key.'"
3. "The group in the protoplasm, the *cell receptor*, must be identical with the *antitoxin* which is contained in solution in the serum of immunized animals."
4. "As these receptors, which may be regarded as *lateral chains* ("seitenketten") of the protoplasm...become occupied by the toxin, the relevant normal function of this group is eliminated...the deficiency is not merely *exactly compensated*, but *made up to excess* (hyperregeneration)."

What Ehrlich proposed purely on theoretical grounds is a brilliant argument based on a combination of inductive and deductive reasoning. Characteristic of Ehrlich, it is lucid, logical, and profound. Beginning with a consideration of a simple chemical bond, Ehrlich somehow ends up with an antibody-producing cell; hence, "the cellularist's humoralist."

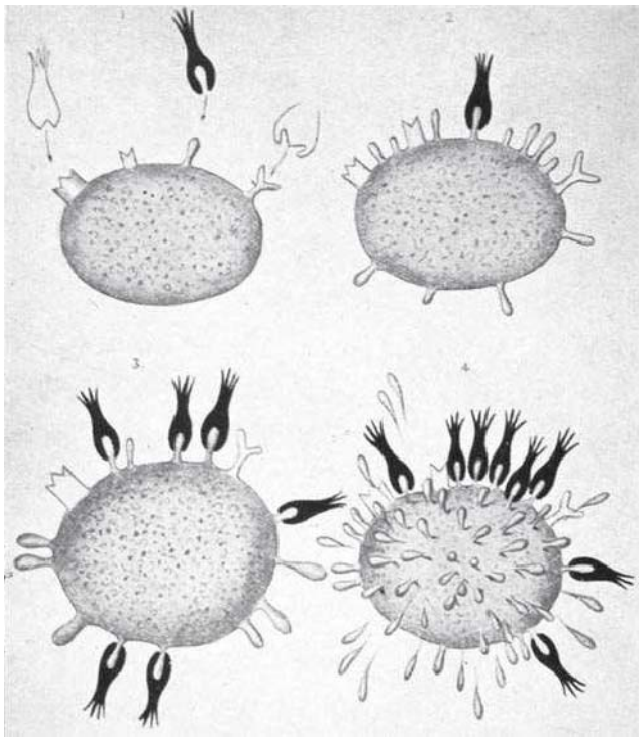

FIG. 2.2. Ehrlich's "Seitenkette" (Side Chains). From Ehrlich.[171]

Not every biologist was enamored of Ehrlich's model, and soon after he proposed it, it came under attack, most notably by Jules Bordet. Bordet objected to Ehrlich's insistence that the specificity of the antigen–antibody reaction required an irreversible bond, whereas Bordet, whose views seemed to be more deductively grounded in his immediate line of investigation (eg, precipitin reactions and complement fixation) thought that adsorptive interactions between antigen and antibody were sufficient to account for specificity.[15,60] Although Ehrlich's side-chain theory provided a logically consistent mechanism to account for antibody specificity, it would later be criticized for failing to account for antibody diversity. That problem would not be conquered for another 60 years in yet another "paradigm shift" when Talmage, Burnet, and Lederberg proposed the clonal selection theory. However, the concept of clonality was not yet conceived of in 1897, and in any case, it is hard to envision how a clonal selection theory could have been developed without the prior description by Ehrlich of antibody selection itself.

IMMUNOLOGY BRANCHES OUT: BENEFICIARIES OF THE EARLY FOCUS ON HUMORAL IMMUNITY

"Man built most nobly when limitations were at their greatest."
–Frank Lloyd Wright

Immunology during the early part of the 20th century was heavily influenced by the early victories of the humoralists. Aside from the spectacular practical implications of the work of von Behring, Bordet, and Ehrlich, among others, the immunologist's toolkit of the early 20th century immunologist was very limited. Advanced microscopic techniques were not yet available, and cell fractionation techniques had not yet been invented. Given these limitations, it is hard to envision how the gap between antibody and cell could have been bridged any closer than Ehrlich had managed, at least on a theoretical level. However, the focus on humoral immunity did allow for the rapid development of several fields, most notably immunochemistry as well as hematology and allergy. It would be some time before cellular immunology would catch up.

Karl Landsteiner and the Birth of Transfusion Medicine and Autoimmunity

As described by Silverstein, "No single individual contributed as importantly to so many different areas of immunology as did Karl Landsteiner."[15] Landsteiner was born in Baden in 1868 and attended Vienna Medical School. He began his training in internal medicine and studied chemistry with Emil Fischer, who would receive the Nobel Prize in chemistry in 1902. He transferred to the Department of Pathological Anatomy, home to Erdheim, Billroth, Escherich, and other accomplished scientists, where he remained until

1907.[62] Landsteiner's first major accomplishment was the discovery of the human ABO blood group antigens at the turn of the century.[63,64] His motivation was succinctly described in his Nobel lecture speech[65]:

> . . . proteins in individual animal and plant species differ and are characteristic of each species . . . The problem raised by the discovery of biochemical specificity peculiar to a species . . . was to establish whether the differentiation extends beyond the species and whether the individuals within a species show similar though smaller differences.

His experiment was a simple one, in which he applied the sera of six heathy men, including himself, to red blood cells of each, and noted that the sera of the men reacted differently with each other—no serum reacted with that same individual's red blood cells. At the end of the paper, Landsteiner noted that the results could account for the variable clinical consequences of human blood transfusion—and thus was borne the discovery of the ABO red blood cell antigens that would later become useful in blood typing prior to transfusions. Many years later, Landsteiner would discover the M, N, P isoantigens in 1927[66] and the Rh system in 1940[67]).

Landsteiner's next major contribution was the codiscovery with Donath of the first autoimmune disease, paroxysmal cold hemoglobinurea,[68] which challenged Ehrlich's dictum that such a situation could not occur.[69]

> The organism possesses certain contrivances by means of which the immunity reaction . . . is prevented from acting against the organism's own elements and so giving rise to autotoxins . . . so that one might be justified in speaking of a "horror autotoxicus" of the organism.

The nature of the contrivances was thought to be of "the greatest importance" by Ehrlich, who later stated[70]: "According to our present investigations either the disappearance of receptors or the presence of autoantitoxin is foremost among these contrivances." Depending on how this statement is interpreted, it could be viewed as Ehrlich's formulation of either clonal deletion or anti-idiotypes. Two years after the discovery of paroxysmal cold hemoglobinurea, an Italian ophthalmologist, who observed sympathetic ophthalmia, a disease in which damage to one eye leads to inflammation of the opposite eye, speculated that this disease was due to "autocytotoxins."[71] Autoimmunity research was taken up by a few others, but perhaps owing to either a misinterpretation of Ehrlich, or possibly due to deference to his authority in the field, progress was slow. It would not be years later, until the discovery of the role of sensitization of the newly discovered Rh antigen as an etiology of erythroblastosis fetalis,[72,73] that autoimmunity became an active area of research for immunologists. The first time that autoimmunity was first associated specifically with arthritis was 1957, when Kunkel and colleagues discovered what came to be called "rheumatoid factor," large complexes of immunoglobulin (Ig)M directed against IgG in the sera of some patients with rheumatoid arthritis.[74] This observation fundamentally changed the field of rheumatology.[75]

Landsteiner received the Nobel Prize in 1930 "for his discovery of the human blood groups." Ironically, upon receiving the prize, it is said that he felt the prize should have been awarded for his work on haptens, which would play a great role in the development of the growing field of immunochemistry.

Discovery of Hypersensitivity: The "Other Work"

In 1901 to 1902, Paul Portier and Charles Richet were attempting to raise tolerance in laboratory animals to actinotoxin, an uncharacterized toxin derived from tentacles of sea anemones. Their experiments appeared to be unsuccessful, and, in some cases, it appeared that the animals actually became sensitized to the antigen. They repeated their studies using dogs and obtained completely unanticipated results: All eight dogs collapsed and died within minutes after receiving a relatively small dose. First thinking the results were due to experimental error, they later realized that the sensitized animals had all been exposed to antigen 14 to 23 days previously.[76] They proposed the name "aphylaxis" (against protection), a term later changed to "anaphylaxis."[77] Richet continued his investigations on anaphylaxis and was awarded the Nobel Prize in 1913 for his work. Soon after Portier and Richet made their seminal discovery, Maurice Arthus was able to induce a localized form of anaphylaxis (swelling and ultimately gangrene) by repeated subcutaneous injections of horse serum, considered fairly nontoxic.[76] Yet, a third type of hypersensitivity was described by the pediatricians von Pirquet and Schick,[78] who noted that vaccinated children occasionally developed fever, joint pains, rash, diarrhea, and hypotension. They concluded that the clinical features of what is now called "serum sickness" were not a direct result of the injection of antiserum, but the outcome of "when antigen and antibody meet." As von Pirquet later explained,[79]

> We are able to observe the effects of the toxic body formed when antigen and antibody meet, that is, the serum disease. We see that at the time when the antibody arises, and therefore the antigen disappears, symptoms of general disease occur. The supposed connection is that these symptoms are due to toxic bodies formed by this digestion of the allergen through the antibody.

Although von Pirquet does not precisely define what he meant by "toxic body," some have interpreted this to mean antigen–antibody complexes. It is possible that he was reluctant to be more specific as he did not actually have a way of observing or quantifying the complexes; it is also possible that he did not have a ready explanation for how such a complex, if formed, could lead to the symptoms and signs of serum sickness. It would not be until many years later that Frank Dixon and colleagues would precisely delineate the nature of the immune complexes.[80] Nevertheless, it is clear that von Pirquet and Schick viewed this phenomenon as

lying along a continuum with the normal immune response, or rather being a necessary component of it. As they later stated,[81]

> The conception that the antibodies, which should protect against disease, are also responsible for the disease, sounds at first absurd. . . One forgets too easily that the disease represents only a stage in the development of immunity, and that the organism often attains the advantage of immunity only by means of disease.

It is von Pirquet and Schick who coined the term "allergy" (from the Greek *allos*, other, and *ergon*, work).[79] By highlighting the role of tissue injury in promoting immunity, they closed the loop that Metchnikoff had begun at the end of the 19th century. This theme would be revisited and expanded upon in the latter part of the 20th century when it was discovered that the inflammation that accompanied the innate immune response was a necessary prequel to the acquired immune response.

THE LONG JOURNEY FROM THE DAWN OF IMMUNOCHEMISTRY TO THE STRUCTURE OF IMMUNOGLOBULINS

Challenges to Ehrlich: The Problem with the "Keys"

One of the difficulties that Ehrlich faced was bridging the gap between the conceptual basis of antibody specificity and the actual basis of antibody specificity. If his side-chain theory was correct, then every cell involved in antibody production would be capable of reacting against every possible antigen it might encounter. Even without considering the cellular origins of antibodies, Ehrlich's critics, such as the Viennese Max von Gruber, raised the question of how the astonishingly large number of different specificities of the antibody molecules themselves could be generated.[15] The size of the repertoire seemed impossibly large if every "lock" had a unique "key." Landsteiner became von Gruber's assistant at the University of Vienna in 1896, and he inherited von Gruber's critical view of Ehrlich's theory. Landsteiner's early approach to this problem was to adopt Bordet's "colloid" explanation of the antigen–antibody interaction,[15] which rejected covalent interactions in favor of multiple weaker interactions, a theme that was later taken up by Pauling, with some interesting consequences. Later, stimulated by the work by Obermeyer and Pick, who described chemical modifications of proteins,[82] Landsteiner used structurally related reactive chemicals to derivatize proteins. He showed that antisera raised against one of the chemically modified proteins would react to varying degrees with proteins modified by structurally similar, but not identical, reactive molecules. These results were interpreted as being incompatible with Ehrlich's "lock and key" specificity but called for a more nuanced view of antigen–antibody specificity. It became immediately apparent that the size of the repertoire could be greatly enhanced if one allowed for such graded degrees of binding affinities.

Immunoglobulin Structure: The "Keys" to the Problem

It was clear that further progress on defining the physicochemical nature of the antigen–antibody reaction required the development of specific tools that were unavailable at the turn of the century. Antibodies, whose chemical structures were unknown at the time, were considered "colloids" (from Greek *kolla*, glue), a suspension of particles suspended in a continuous phase of another component. In 1924, the Swedish chemist Svedberg designed a centrifuge based on a modified milk separator. The centrifuge could develop a centrifugal field of up to 5000 g and enabled Svedberg to measure the molecular mass of hemoglobin[83]; he was the first to determine the molecular mass of macroglobulins, derived from a patient with Waldenström macroglobulinemia, which we now know as IgM. A student in Svendberg's laboratory, Arne Tiselius, developed gel electrophoresis,[84] which allowed for the separation of molecules based on charge and size. These tools enabled Michael Heidelberger to establish the field of "immunochemistry." Heidelberger devoted nearly his entire research career pursuing the implications of a simple but profound discovery he made with Oswald Avery in 1923 that type-specific antigens of pneumococcus bacteria are complex polysaccharides. Over the next three decades, this discovery enabled him to determine, for the first time, the exact weight and chemical composition of antibodies, antigens, and complement. He showed that antibodies are multivalent proteins and used these insights to devise a simple vaccine against pneumonia whose effectiveness was first proven in soldiers who fought in World War II.[85]

The Chemical Nature of Immunoglobulin Molecules

By 1950, it was appreciated that antibodies were proteins of 150,000 molecular mass containing bivalent antigen-combining sites. Based on Porter's observation that Igs could be split into smaller products by enzymes such as papain, yielding Fab fragments that bind antigen and Fc (crystallizable) portions that do not, Edelman and Poulik further defined Ig structure by hypothesizing that Bence-Jones proteins, derived from myeloma cells, were free light chains of Ig molecules.[86] As this hypothesis appeared to be correct, this provided a means of obtaining a ready supply of homogeneous Ig subunits. Reduction of disulfide bonds led to still different products, enabling them to propose that the Ig molecule consists of two light chains and two heavy chains, and that the antigen-binding site consisted of contributions from both heavy and light chains.[86] Further refinement of techniques in protein chemistry allowed Edelman and Porter to work out the primary structure of Ig molecules, for which they received the Nobel Prize in 1972.

CONFRONTING THE SIZE OF THE REPERTOIRE

The value of a large repertoire from which to select is appreciated by any performing musician. Yet in the 1950s, the repertoire problem was unsolved, leading to competing theories of how a large repertoire of diverse antibodies are generated. There were two mutually exclusive leading schools of thought: "instruction" theories and "selection" theories.

Instruction Theories of Antibody Diversity

As noted by Silverstein,[15] the first description of antigen as template was by Bail and Tsuda, who proposed in 1909 that antigen persists after its encounter with antibody, and that by so doing, it leaves an impression on the antibody.[87] This concept was further refined by Breinl and Haurowitz, who suggested that antigen is carried to the site of protein formation, where it would serve as a template to instruct antibody formation.[88] Finally, in 1940, Linus Pauling provided a chemical explanation for antigen-directed instruction: that "antibodies differ from normal serum globulin only the way the ends of the polypeptide chain is coiled" as a result of their amino acid sequence, and that "they have accessible a very great many configurations with nearly the same stability." Under the "influence" of antigen, they "assume configurations complementary to surface regions of the antigen," forming two active ends, and that after "freeing one end and the liberation of the central part of the chain, it folds up to form the central part of the antibody molecule, with two oppositely directed ends able to attach themselves to two antigen molecules." This interaction would be further stabilized by weak interactions between antigen and antibody.[89] Assuming a degree of degeneracy in the initial antigen–antibody interaction, these theories were consistent with the findings of Landsteiner, who showed that antigen–antibody interactions were not absolutely specific, as envisioned by Ehrlich (Fig. 2.3). In retrospect, these theories had great chemical appeal; however, a central weakness is that if the initial interactions between antigen and antibody are degenerate, that implies that the interactions cannot be of high affinity (otherwise they would not be degenerate). Yet, if the initial interactions are weak, how would they occur in the first place? At some level, there has to be a certain degree of preexisting antibody specificity, which implies a preexisting repertoire. Although the instruction theories of antibody selectivity helped explain some of the specificity of the antigen–antibody interactions, they could not account for all of them. It is notable that the underlying basic principles have been since invoked for other encounters in the immune system. For example, a "bar code model" of interactions between the T-cell receptor (TcR) and peptide-major histocompatibility complex (MHC) (pMHC) has recently been proposed, in which the initial encounter between TcR and pMHC is governed by strong interactions, followed by "scanning" the epitope and modest changes in conformation of the TcR, leading to strengthening of the TcR–pMHC association.[90]

Jerne's Natural Selection Theory of Antibody Formation

Instruction theories of antibody specificity persisted through the 1950s, when they were modified to include participation of enzymes to act as intermediaries between antigen and antibody as well as the newly discovered structure of deoxyribonucleic acid (DNA). In 1955, Niels Jerne published a highly influential paper in which he proposed a new theory of antibody formation that he described as the "natural selection theory of antibody formation."[91]

> The antigen is solely a selective carrier of spontaneously circulating antibody to a system of cells which can reproduce this antibody. Globulin molecules are continuously being synthesized in an enormous variety of different configurations...among which... will be fractions possessing affinity toward any antigen to which the animal can respond.

Jerne referred to these preexisting antibodies "natural antibodies," and went on to state that antigens selectively attach to those globulin molecules that happen to have a complementary configuration. According to Jerne, once the interaction occurs, the antigen–antibody complexes may be engulfed by a "phagocytic cell," at which point the antigen is eliminated. The antibody within the phagocytic cell can remain or be transferred to another cell, which is the signal for reproduction of the same specific antibodies. More antibody is released into the circulation, resulting in a larger percentage of specific circulating antibody. Jerne states that "the reproduction need not be highly faithful; copying mistakes will be harmless and may occasionally produce an improved fit."[91] These are remarkable concepts, as Jerne appeared to have invoked Metchnikoff, a Darwinian interpretation of antibody selectivity at the organismic level, as well

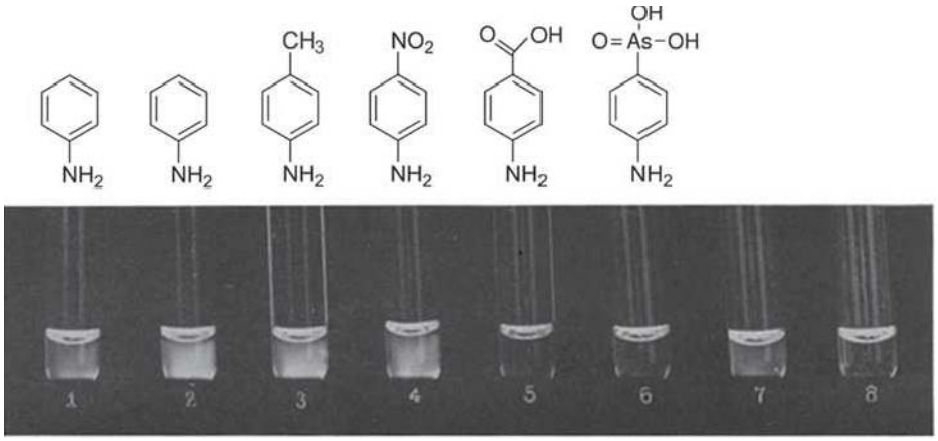

FIG. 2.3. Demonstration of Serologic Specificity by Landsteiner and Scheer. Reactions from immune serum for aniline with various azoproteins and with unchanged horse serum reading after 15 minutes. *(1)* azoprotein from chicken serum and aniline, *(2)* azoprotein from horse serum and aniline, *(3)* azoprotein from horse serum and para-toluidine, *(4)* azoprotein from horse serum and para-nitroaniline, *(5)* azoprotein from horse serum and para-aminobenzoic acid, *(6)* azoprotein from horse serum and para-arsanilic acid, *(7)* unchanged horse serum, *(8)* saline control. Modified from Landsteiner and van der Scheer.[486]

as to have anticipated affinity maturation. It is easy to see why Jerne's ideas were so influential. However, the central problem, the lack of an explanation for the huge existing repertoire, which hampered the instruction theories of antibody diversity, as well as Ehrlich's side-chain theory was still unanswered. Jerne admitted this weakness and speculated that the "spontaneous production of globulin molecules of a great variety of random specificities" may reside in a ". . . specialized lymphoid tissue, such as that of the thymus."[91] Perhaps a more existential problem was that the flow of information was from the existing preformed antibody to more antibody without any genetic intermediary. What was lacking was a specific mechanism to transfer information between a specific antibody and the specific synthesis of that same antibody. Jerne admitted to this problem and nominated ribonucleic acid (RNA) as a key template; he then stated " . . . a protein molecule may determine the order of the nucleotides in the synthesis of RNA," citing a paper that does not actually make this assertion.[91]

What accounted for this conceptual block that had persisted for more than half a century? On one level, it was ignorance of the "fundamental dogma of molecular biology," which had not yet been articulated.[92] But on another level, it perhaps can be traced back to the decisive victory of the humoralists over the cellularists. So long as the focus was on the antigen–antibody interaction, there was no way to invoke a biologically plausible mechanism of generating a preexisting Ig repertoire and of selectively expanding a specific portion of that repertoire.

Development of the Clonal Selection Theory

In 1957, the American immunologist David Talmage published a review whose focus was allergy; however, in the review, Talmage drew an analogy between natural selection, in which there is "selective multiplication of a few species out of a diverse population" and antibody production. In a very succinct but remarkably insightful passage, he lays out the essence of the clonal selection theory[93]:

> As a working hypothesis it is tempting to consider that one of the multiplying units in the antibody response is the cell itself. According to this hypothesis, only those cells are selected for multiplication whose synthesized product has affinity for the antigen injected.

At about the same time, Burnet published his own paper outlining the key aspects of the clonal selection theory[94] (Fig. 2.4). In a later interview, Talmage discussed how he provided a preprint of his review to Burnet before he published his landmark paper on the clonal selection theory, but both he and Nossal stated that Burnet developed the theory independently. Talmage thought that the two papers were similar in substance, but that "Burnet had the good fortune to make an analogy of the idea to the clones that grow in bacteria and he gave it a very catchy name, 'clonal selection.'"[95] Among the ideas that led both immunologists to propose the clonal selection theory was the known logarithmic rise in antibody titer during the primary immune response "as if it is a product of some multiplying substance."[95] Other experiments demonstrated that lymphoid cells obtained from previously immunized rabbits that were then transferred to x-radiated recipients were sufficient to induce a recall response in the recipients.[96]

Although we now know that the clonal selection theory is correct, it took more than 10 years for clonal selection to be widely accepted, according to Talmage.[95] Among the key pieces of evidence to prove the theory was the demonstration of antibody production from single cells, by Nossal and Lederberg,[97] and the later finding that cognate antigen was capable of aggregating all surface Ig on individual cells,[98]

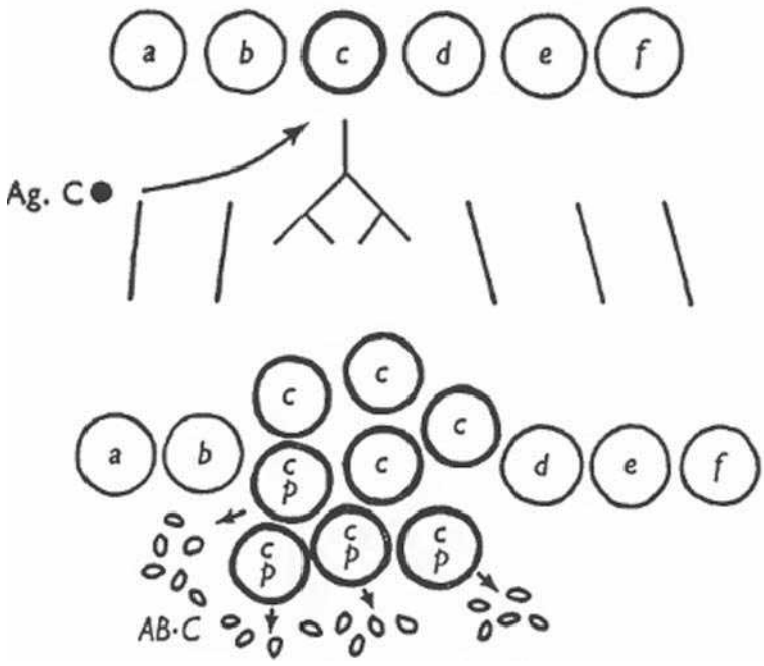

FIG. 2.4. In this diagram, antigen C (Ag.C) is recognized by clone "c," triggering this clone, but not others, to proliferate. Antibody against Ag.C (AB.C) is indicated at the bottom. From Burnet.[246] Copyright ©1959 Sir MacFarlane Burnet. Reprinted with the permission of Cambridge University Press.

which corroborated Nossal and Lederberg's results. Finally, in 1975, Köhler and Milstein demonstrated the production of monoclonal antibodies from single clones of immortalized plasma cells.[99] Along with Jerne, they shared the Nobel Prize in 1984.

The Structural Basis of Antibody Diversity: The Dialectic Revisited

By the mid-1970s, the puzzle of antibody diversity was far from solved—many of the pieces were still missing. The conceptual basis for the clonal selection theory was laid in 1957 by redirecting the focus of investigation from immunochemistry to cell biology. However, what was needed to actually prove the theory was a delineation of the molecular basis for the enormous size of the repertoire. Two lines of investigation converged to provide this evidence: the sequencing of Ig proteins followed by the sequencing of Ig genes. In 1965, Hischmann and Craig sequenced two Bence-Jones proteins and found that there was conservation of the amino acid sequence at the C-termini but considerable diversity at the N-termini.[100] In the ensuing years, sequence data on a number of myeloma proteins became available, and in 1970, Kabat and Wu applied statistical criteria to analyze sequencing data from 77 Ig chains. They identified three regions within the 107 residues comprising the light chain variable region that demonstrated a still further degree of variability (ie, hypervariability). They hypothesized that these regions of extreme diversity represented the complementarity-determining residues and suggested by analogy with prokaryotes that they arose through episomal incorporation into the light chain locus by a recombination event.[101] If this, indeed, was the underlying explanation for antibody diversity, then there would have to be a very large number of episomal elements to account for a diverse repertoire. This was a different view than the one taken by Dreyer and Bennet 5 years earlier, who proposed that variable region genes that had undergone duplication and spontaneous mutation throughout evolution underwent a "genetic scrambling" event, resulting in their juxtaposition to the constant regions of the Ig genes. They even suggested the involvement of enzymes involved in DNA repair as contributing to such an event.[102] Thus, two opposing viewpoints began to emerge to account for the generation of diversity (or GOD, as playfully described by Richard Gershon): somatic mutation of a few genes, as suggested originally by Burnet,[94] and supported by Weigert and Cohn's sequencing data of the mouse λ light chain locus,[103] or somatic recombination among many duplicated genes within the germline, as proposed by Dreyer and Bennet[102] and later refined by Edelman and Gally[104] and Hood and Talmage.[105] In the years that followed, various teleologic arguments were proposed to support one theory over the other. In 1976, at least a partial resolution was provided by Hozumi and Tonegawa, who provided evidence that the V_κ and C_κ loci from embryonic DNA rearrange to form a contiguous polypeptide in mature lymphocytes.[106] The advent of molecular cloning led to direct proof that the variable and constant regions of the light chain gene had undergone rearrangement at the DNA level.[107,108] This was followed by the demonstration by Weigert et al.[109] that the Ig V_κ region in the mouse is encoded by multiple V, J, and C regions joined at the DNA level during differentiation of individual lymphocytes. Finally, in 1980, Early et al.[110] demonstrated how V, D, and J regions of the Ig locus could recombine to generate a virtually unlimited combination of antibody specificities.

In the ensuing years, the molecular mechanisms governing VDJ recombination were uncovered. These involved recognition of conserved sequences flanking germline V, D, and J segments, introduction of double-strand breaks, potential loss or gain of nucleotides at the coding junctions, and polymerization and ligation to complete the joining process. Many talented scientists contributed to these discoveries, including Alt, Yancopoulos, Blackwell, and Gellert.[111] This culminated in the isolation of the recombinase activating genes (RAG) by Baltimore's group.[112, 113] The dominant view that emerged from these studies largely favored the "germline-ists," reinforced by Tonegawa's receiving the Nobel Prize in 1984. However, in yet another example of a synthesis of two apparently contradictory approximations of the truth, evidence for somatic hypermutation began to emerge.[114,115] Its importance was established when it was causally linked to the generation of B cells with very high affinity antibodies,[116] a phenomenon termed "affinity maturation."[117]

SPECIALIZATION WITHIN THE IMMUNE SYSTEM

Division of Labor: Discovery of T and B Cells

The first person to demonstrate delayed type hypersensitivity may have been Robert Koch in 1882. On his quixotic pursuit of developing a vaccination against tuberculosis, he injected himself with spent medium from cultures of human tubercle bacilli and noted a particularly severe reaction, including systemic effects.[118] Although he was not successful in developing a vaccine against tuberculosis, he recognized the diagnostic potential of this procedure. It was not until 1942, and then later in 1945, that Landsteiner and Chase demonstrated that cells from guinea pigs previously immunized with *Mycobacterium tuberculosis* or hapten would transfer reactivity to a naïve recipient when challenged with the immunogen.[119,120] This was the first demonstration that cells, rather than antibody, transmitted specific immunity, a finding that in some ways vindicated Metchnikoff's cellular focus.

The identity of the cells mediating the transferred hypersensitivity was unknown. Based on experiments performed decades earlier, as Silverstein has noted,[121] James Murphy at the Rockefeller Institute argued that lymphocytes were important in the host resistance to tuberculous infection. Murphy used mice exposed to x-rays or splenectomized to manipulate lymphocyte numbers and showed that conditions that would have been predicted to deplete lymphocytes resulted in early death of the mice due to disseminated tuberculosis.[122] In earlier papers, Murphy also showed more directly that lymphocytes were important in graft rejection in transplanted chick embryos. Why were these seemingly important observations ignored? Was it because the

experiments relied to a certain extent on inference, rather than direct proof that lymphocytes were key mediators of tuberculous immunity? Most likely, it was a combination of events: The lingering vestiges of the confrontation between the cellularists and the humoralists and the fact that there was little conceptual basis for understanding how a small innocuous-appearing cell type could participate in immunity.

In a completely independent line of investigation, it was known for some time that certain strains of mice had a high spontaneous rate of developing lymphocytic leukemia. In attempting to explain the finding that thymectomy of 2-month-old mice failed to develop leukemia, Jacques Miller found that neonatally thymectomized mice appeared ill and revealed a marked deficiency of lymphocytes in blood and lymphoid tissues. Furthermore, these mice failed to reject allografts or xenografts.[123] However, not all areas within lymphoid organs were depleted of lymphocytes, consistent with "thymic-dependent" (paracortical areas of the lymph nodes and periarteriolar sheaths of the spleen) and "thymic-independent" regions (follicles and medullary cords). Miller concluded that the thymus was important for the development of a subset of lymphocytes important in allograft rejection.

The involvement of lymphoid cells in antibody production was considered likely in the 1940s, mainly due to "guilt by association." For example, Erich and Harris injected antigens, such as typhoid vaccine and sheep erythrocytes, into the feet of rabbits, and then compared the formation of antibody to histologic changes in the draining lymph nodes.[124] Similar experiments were performed using intravenous injection of antigen, and the appearance of antibody and plasma cells in the spleen appeared to be correlated.[125] However, actual proof for the involvement of B cells in antibody production was quite accidental.[126] In 1952, Bruce Glick, a young doctorate student at Ohio State University, was investigating the function of an obscure avian organ, the "bursa of Fabricius." He removed the organ, which did not result in a discernable phenotype. A fellow graduate student asked to use one of Glick's birds to develop an antibody against *Salmonella* and found that the bursectomized chicken failed to make antibodies. This led to the publication that eventually appeared in *Poultry Science* describing the role of the organ in the generation of bursa-derived or "B" cells.[127] As there was no anatomic equivalent of the bursa in mammals, an exhaustive search finally revealed the bone marrow origin of these cells. It was also found that thymus-derived cells, later named "T cells," were needed to "help" B cells produce antibody.[128–130] These distinctions were further clarified when Cooper et al.[131,132] showed that T cells were required for delayed-type hypersensitivity and graft versus host reaction. Thus was borne one of many central dichotomies that Mazumdar[133] has argued drives the field of immunology.

In 1957, Gowans[134] cannulated the thoracic duct of rats and measured the rate of flow of the lymph. He suggested that "the continuous entry of living lymphocytes into the blood may be essential for maintaining the output of lymphocytes from the thoracic duct." He later showed that intravenous transfusion of radiolabeled lymphocytes resulted in appearance of the radiolabeled cells into the thoracic duct, thus defining the continuous recirculation of lymphocytes from the lymphatics to the blood.[135] In retrospect, these experiments were critically important in understanding how lymphocytes are constantly patrolling the lymphatics, vigilantly on the lookout for antigen.

TRANSPLANTATION BIOLOGY AND THE PURSUIT OF IMMUNOLOGIC TOLERANCE

The history of transplantation began many hundreds of years ago and was vigorously pursued by surgeons and tumor researchers during the early part of the 20th century. These events are well summarized in several texts, notably Brent's *A History of Transplantation Immunology* and Silverstein's *A History of Immunology*. The influence of these early workers, particularly Carrel's, on the surgical aspects of transplantation is clear, but their impact on the field of transplantation immunology was limited because the conceptual framework of immunology was still rudimentary. Analogous to the role that smallpox had in catalyzing early vaccine development, large-scale bombing campaigns in World War II resulted in many civilian and military burn victims who required skin grafting, compelling surgeons to develop better techniques to avoid homograft rejection. The British zoologist Peter Medawar was assigned to the War Wounds Committee of the Medical Research Council. In 1943, Medawar and Gibson[136] published "The fate of skin homografts in man" based on a single burn victim who received multiple "pinch grafts" of skin. The authors concluded that autografts succeed, whereas allografts fail after an initial take, and that the destruction of the foreign epidermis is brought about by a mechanism of active immunization. Medawar returned to Oxford University to study homograft rejection in laboratory animals and proved that this was an immunologic phenomenon. Medawar[137] concluded that the mechanism by which foreign skin is eliminated belongs to the category of "actively acquired immune reactions." Shortly after this publication, Ray Owen[138] published the provocative finding that dizygotic twin calves, who share a common circulatory system in utero, exhibit chimaerism with respect to their twin's erythrocytes and fail to produce antibodies against each other's erythrocytes. This led Burnet and Fenner[139] to predict that introduction of a foreign antigen early enough in life would fail to elicit an immune response. Medawar reasoned that the successful exchange of skin grafts between dizygotic calves would verify this hypothesis. Together with his postdoctoral fellow Rupert Billingham, he performed a series of grafting experiments that provided direct support for this model.[140]

> Mammals and birds never develop, or develop to only a limited degree, the power to react immunologically against foreign homologous tissue cells to which they have been exposed sufficiently early in fœtal life . . . this phenomenon is the exact inverse of "actively acquired immunity", and we therefore propose to describe it as "actively acquired tolerance."

At the same time, Milan Hašek[141] in Prague demonstrated successful parabiosis of chick embryos derived from two

different strains. Hašek's stated motivation to perform the experiment was to determine whether exchange of blood in the different chick strains induced a "mutual metabolic assimilation between parabionts," rather than to induce a state of immune tolerance. It has been suggested that Hašek's real motivation for the experiment was to advance the Lysenkoist genetic doctrine to appease the local communist regime in Czechoslovakia.[142] Regardless, the result has been reinterpreted as an example of the induction of immunologic tolerance. In 1960, Medawar shared the Nobel Prize with Burnet "for the discovery of acquired immunologic tolerance."

Mechanism versus Metaphor: "Self/Nonself" Discrimination

Medawar's experiments and Burnet's formulation of the clonal selection theory occurred at a critical juncture in the history of the evolving field of immunology. Had their development been dyssynchronous, it is doubtful that much progress would have been made on either front. Indeed, transplantation experiments had been performed earlier in the century with little insight as to why allografts failed. The viewpoint that Burnet espoused, that the function of the immune system is to distinguish "self" from "nonself," has proved to have enormous heuristic value ever since its formulation more than 60 years ago. Is this distinction mostly metaphorical, as suggested by Tauber,[143] or does it reflect a more concrete generative reality? To begin to address this question, it is necessary to briefly review a parallel development in immunology, the discovery of the components of the immune system that define molecular self-hood.

Looking Under the Hood: The Discovery of the Major Histocompatibility Complex

The clonal selection theory represented a conceptual breakthrough in the history of immunology, but it did not explain how lymphocytes actually recognize antigen. These insights would eventually come from three sources over the span of 20 years: studies of the genetics of graft rejection in inbred strains of mice by George Snell in the 1940s, studies of the agglutination of white blood cells by sera from transfused patients by Jean Dausset in the 1950s, and studies of the immune response to simple antigens in guinea pigs by Baruj Benacerraf in the 1960s. Snell was interested in identifying genes that controlled the ability of mice to resist tumor transplants. He pioneered the use of congenic mice, which are genetically identical except for a single region or locus. Snell observed that tumor grafts were accepted between inbred mice but not between mice of different strains. The same was true for normal tissues. Snell termed the underlying genes "histocompatibility" genes. In collaboration with Peter Gorer, who had prepared antisera that reacted with cells from one mouse strain but not another, Snell established that the major histocompatibility locus corresponded to a reactivity that Gorer had designated antigen II, renamed locus histocompatibility 2 or H-2.[144] Two loci within this region, designated K and D, carried genes specifying antigens involved in triggering graft rejection. In the 1950s, Dausset observed that patients who had received many blood transfusions produced antibodies that could agglutinate white blood cells from donors but not the patient's own cells. Several of the patients produced antibodies against the same antigen.[145] Subsequent family studies indicated that a genetically determined system, named "human leukocyte antigen" (HLA) system, was found to be the ortholog of H-2 in the mouse. Dissection of the human system would take many years, during which time transplantation surgeons made use of the emerging findings to assist in tissue typing. In the 1960s, Baruj Benacerraf, an immunologist working at New York University, noticed that some outbred guinea pigs responded to simple antigens by developing delayed hypersensitivity reactions upon challenge while others did not, and that this was under genetic control. He termed these genes "immune response genes." McDevitt and Sela observed similar genes in mice, and McDevitt showed that they are encoded in the MHC.[146] In 1980, Benacerraf, Snell, and Dausset shared the Nobel Prize "for their work on genetically determined structures of the cell surface that regulated immunologic reactions."

Of course, the identification of the HLA region and the subsequent cloning of the genes encoded in this region proved to be landmarks in the history of immunology. Besides providing a molecular identity to the key orchestrators of antigen presentation to T cells, the identification of specific MHC alleles with autoimmune diseases has led to important insights into their pathogenesis. Ironically, the first recognized HLA association with human disease, HLA-B27, which was associated with the disease ankylosing spondylitis,[144,147] has generated perhaps more heat than light, as there is no definitive mechanism that explains how HLA-B27 is linked to disease pathogenesis, underscoring that the HLA locus, which took so many years to uncover at the genetic level, still has much to teach us.

The Discovery of Major Histocompatibility Complex Restriction as the Molecular Basis for "Self/Nonself" Discrimination

It was known for several years that cooperation between T and B cells occurred in syngeneic or H-2–compatible animals.[148–150] In 1972, Kindred and Shreffler[151] showed that even in nude mice, cooperation between T and B cells required H-2 compatibility; however, the exact role of that H-2 molecules played in this process and the nature of the T- and B-cell interactions remained a mystery. A valuable clue was provided by experiments by Rosenthal and Shevach,[152] who demonstrated that efficient presentation of antigen by antigen-pulsed macrophages to T cells also required histocompatibility matching. In 1974, Doherty and Zinkernagel sought to understand the role of T cells in the immune response to viral meningitis. They theorized that it was the strength of the immune response that caused the fatal destruction of brain cells infected with this virus. To test this theory, they mixed virus-infected mouse cells with T lymphocytes from other infected mice. The T lymphocytes did destroy the virus-infected cells, but only if the infected cells and the lymphocytes came from a genetically identical strain of mice. T lymphocytes would

ignore virus-infected cells that had been taken from another strain of mice.[153] Further experiments strongly suggested that the same TcR that recognizes viral antigen also recognizes the MHC molecule.[154] The implications of the Zinkernagel-Doherty experiment were profound. First, it established the principle of MHC restriction: T cells recognize antigen only in the context of MHC molecules. Second, the experiment established that cytotoxic TcR-bearing cells must recognize two separate signals on an infected cell before they can destroy it. One signal is a fragment of the invading virus that the cell displays on its surface and the other is a self-identifying tag from the cell's MHC molecules; it was felt likely that the same TcR probably recognizes both. Thus, the experiment pointed to the identity of the molecular structure that constituted immunologic "self"—it is the MHC molecule—and therefore a virus-infected cell bearing MHC molecules was likely to constitute "altered" or "nonself." Finally, the fact that MHC is highly polymorphic implies that any given allelic product will be capable of forming a different altered self from other MHC allelic products; thus, the specific identity of the MHC molecule itself determines the strength of the immune response.

Although the Zinkernagel-Doherty experiment answered many questions, the steps between encounter of antigen by an antigen-presenting cell (APC) and presentation of that antigen to a T cell was somewhat of a "black box." It had long been thought that intact antigen was presented to T cells, but it was not until 1981 that Ziegler and Unanue[155] showed that an antigen processing event was necessary for I-region (MHC class II)-restricted antigen presentation to T cells. This appeared to require antigen processing in a lysosomal-like compartment.[156] Peptide loading is a complicated affair, involving prior binding of MHC class II by an "invariant chain" to prevent premature loading of incompletely folded proteins in the endoplasmic reticulum.[157,158] Peptide loading requires proteolysis of the invariant chain.[159,160] Mellman and colleagues would go on to show that the actual compartment in which antigen loading occurred is uniquely specialized for antigen presentation in B cells[161] and DCs.[162] In a landmark paper, Unanue and colleagues purified MHC class II molecules from 10^{11} B cells and showed a 1:1 binding with peptide and MHC class II I-A^d, but not I-A^k,[163] thus demonstrating MHC restriction at the biochemical level.

The processing events required for MHC class I restriction seemed more elusive, as some of the molecular components required for this were not known at the time. It was suspected that an intracellular proteolytic event was needed to process antigen, but that was not proven until 1994 when Rock et al.[164] showed that proteasomal inhibitors blocked degradation of most cell proteins and subsequent generation of peptides presented on MHC class I molecules. The actual mechanism by which peptides generated in the cytosol gained entry into the secretory compartment was provided in 1990, when four groups announced the identification of a member of a family of ABC transporters, called "TAP," which provided this function.[165–168] Finally, in 1987, Bjorkman et al.[169,170] demonstrated that the antigen in question was a peptide actually bound to the groove of the MHC class I molecule.

SPECIALIZATION WITHIN THE IMMUNE SYSTEM II.

Discovery of B- and T-Cell Antigen Receptors

The discovery of the B-cell receptor (BcR) for antigen began with Ehrlich,[171] when he proposed that cells that produced "amboceptors" expressed them at their surfaces; in fact, Ehrlich's drawings of amboceptor-producing cells emphasized this point (see Fig. 2.2). Evidence for the existence of surface Ig was provided by indirect immunofluorescence and autoradiography, in which immunoreactivity against a single class, IgM, was observed in 1970.[172–175] The function of the BcR was uncovered by Rock and Lanzavecchia, who showed that MHC class II–restricted antigen presentation by hapten-specific B cells was enhanced 10^3- to 10^4-fold by specific binding, endocytosis, and loading of peptide antigen onto MHC class II molecules.[176,177] In 1952, Colonel Ogden Bruton at the Walter Reed Army Hospital was caring for the 8-year-old son of a general. The boy had had recurrent pneumococcal infections, including bacteremia, but he recovered with antibiotics. Bruton noted that the boy did not mount an antibody response to pneumococcal vaccination and upon testing his serum using Tiselius apparatus, the electrophoretic pattern revealed a complete absence of γ-globulins.[178] Other cases appeared, and it was soon clear that the defect was X-linked. This is often cited as the first description of a primary immunodeficiency. It was not until 1993 that the molecular defect of agammaglobulinemia was uncovered. It was due to deficient expression of a tyrosine kinase, named *Bruton tyrosine kinase*.[179,180] This discovery highlights another function of the BcR, which is to help provide signals for B-cell maturation. Without its expression, B-cell development is blocked beyond the pre-B-cell to immature B-cell stage.[181]

The discovery of the TcR for antigen was likened to the hunt of the apocryphal Snark,[182] according to Mak,[183] except the TcR was finally captured after a hunt that lasted over 20 years or more, depending on one's perspective. The hunt was characterized by extended periods of uncertainty, when it was not clear whether the same receptor bound to antigen and MHC simultaneously, whether two separate TcR proteins bound MHC and antigen separately (the "intimacy model"), or whether one TcR molecule bound MHC molecules altered by antigen. It was also not known what form the antigen would actually take, and many considered it likely that the binding was strictly analogous to the binding of antibody and antigen, amounting to an "IgT-antigen" interaction.[184] The existence of the TcR was deemed within reach when a clone-specific monoclonal antibody (mAb) against a murine lymphoma was isolated.[185] Finally, in 1984, using differential hybridization approaches, Davis and Mak independently announced the cloning of the β-subunit of the mouse and human TcR, respectively.[186,187] This was soon followed by the cloning of the α-subunits of the mouse and human TcRs,[188–191] and the identification of other subunits of a different type of TcR, the γδ TcR.[192] Eventually, the crystal structures of pMHC combinations demonstrated that contact between TcR and pMHC was dominated by direct TcR-MHC contacts, rather than TcR-peptide interactions.[193–195] Beyond the initial recognition stage, signaling through the

TcR, as in the BcR, is a highly regulated process tuned to recognize cues from pMHC that govern positive and negative selection.[196] There is little doubt that had Medawar survived, he would have appreciated how the clonal selection theory has remained a driving force underlying the molecular details of antigen receptor signaling.

Discovery of Distinct Immunoglobulin Classes

Ehrlich's terminology for antibodies changed depending on the context; he sometimes referred to "amboceptors," or immune bodies, and at other times he used the more familiar term "antikörper." Regardless, he did not know the chemical makeup of antibodies, though he recognized that they must contain separate binding sites for antigen and complement. Elucidation of the actual structure of antibodies would have to await the seminal work of Porter and Edelman in the 1960s; however, the actual discovery of specific antibody classes, or isotypes, took place over many years. The first class of antibodies to be discovered was IgG. In 1939, Tiselius and Kabat immunized rabbits with ovalbumin, then absorbed a portion of the resulting antiserum with ovalbumin. When they applied samples of the unabsorbed and antigen-absorbed antisera to electrophoresis, they observed a marked decrease in the amount of protein that migrated in the γ region of electrophoretic mobility, farthest away from the fast-migrating albumin peak. They called the antibody that corresponded to this fraction "γ-globulin."[197]

The next antibody class to be discovered was a result of an observation by the Swedish oncologist Jan Waldenström. He described two patients with oronasal bleeding, lymphadenopathy, low serum fibrinogen, and increased lymphoid cells in the bone marrow.[198] With the help of a colleague in Svedberg's laboratory, he noted that serum from these patients contained an abnormally large amount of homogeneous globulin with sedimentation coefficients corresponding to a molecular weight of more than one million. Waldenström thought this corresponded to a preformed large molecule, which became known as macroglobulin. Proof that a macroglobulin possessed antibody activity was finally provided in 1967,[199] which ultimately led to its current name, IgM.

IgA was discovered by Gugler et al.[200] who isolated IgA from human milk, and Heremans et al.[201] who isolated IgA from human serum. It was later found in high concentrations in all exocrine secretions[202] and further characterized by Tomasi and Zigelbaum,[203] who suggested that IgA plays an essential role in mucosal immunity. It was not until 1984 that the mechanism of secretion of IgA and IgM across epithelial barriers was uncovered; it was shown to depend on interactions of an Ig-associated polypeptide, the J chain, with a glycoprotein on the surface of epithelial cells, referred to as the "polymeric IgA receptor."[204] This receptor mediated transcytosis of secretory IgA and IgM.

The discovery of IgE had a particularly long gestation period and is well summarized in a review.[205] It began with a report by Prausnitz, in 1921, who injected his forearm with serum from a coworker allergic to fish (and coauthor on the ensuing paper); this was followed by a wheal and flare upon further injection of fish extract.[206] This property was expected for a class of antibodies termed "reagins" (from German *reagieren*, to react). IgE was identified as a specific immunoglobulin class by Teruka and Kimishige Ishizaka in 1966[207,208] and was shown to be increased in sera from asthmatics.[209] In 1993, Kinet and colleagues demonstrated that its high affinity receptor, FcεRI, was required for anaphylaxis.[210]

IgD is the most recent Ig subclass to be recognized. It was discovered from a patient with multiple myeloma.[211] Surface IgD is coexpressed with IgM on mature B cells. The function of IgD is not well understood, although the abundance of IgM- IgD+ B cells in the human upper respiratory mucosa suggests that it is likely involved in mucosal immunity.[212]

Because IgA and IgE were isolated based on their predominant location (IgA) or function (IgE), their role in the immune response was relatively easy to decode, but the relationships between the different Ig classes was unknown. It was discovered in 1963 that the early immune response was initiated by the rapidly sedimenting (19S) antibody, later shown to be IgM, followed by the production of a more slowly sedimenting (7S) antibody, now known to be IgG.[213,214] Although production of the different Ig classes was initially interpreted as being due to the participation of different cells, it was later shown that single clones of B cells were capable of producing multiple isotypes[215–217] in a process called class switch recombination (CSR). The enzymology of CSR was worked out much later by Alt, Nussenzweig, Honjo, and others, who showed that it required DNA repair enzymes[218,219] and activation-induced cytidine deaminase (AID). AID is mutated in an autosomal recessive form of the primary immunodeficiency, hyper-IgM type 2, in which afflicted patients are deficient in CSR and somatic hypermutation, consistent with an important role for AID in both processes.[220–222]

Discovery of Antibody Effector Functions: Fc Receptors

Almoth Wright was the first to recognize the importance of antibody-mediated effector functions other than neutralization or complement-mediated lysis. His studies were ignored for a long time, and it would take many years to appreciate the importance of the "other end" of the antibody molecule. Since the 1970s, studies on the effector functions of the Fc portion of IgG made extensive use of several in vitro model systems, often involving phagocytosis.[223–232] The eventual cloning of receptors for the Fc portion of IgG (FcγRs) by Ravetch and et al.[233] revealed many similarities and some differences, but these early studies did not reveal how the receptors transduced signaling events, such as phagocytosis. It was not until 1989 that Michael Reth[234] noted a short tyrosine-containing sequence in common with several antigen receptors, including subunits of the TcR, the BcR,[235] and Fcγ and Fcε receptors[236] that the signaling function of FcγRs was understood in a larger context. Following receptor engagement, tyrosine residues within this consensus sequence, subsequently named "immunoreceptor tyrosine-based activation motif" (ITAM), become phosphorylated

by Src family tyrosine kinases and serve as docking sites for Syk tyrosine kinase,[237] or ZAP-70, in the case of the TcR. The membrane-associated tyrosine kinases become activated and phosphorylate substrates that further convey downstream signals. Absence of ZAP-70 leads to a severe combined immunodeficiency disease (SCID).[238] These studies uncovered the central role of nonreceptor tyrosine kinases in the immune system, which appeared to explain in large part how antigen and Fc receptors evoke calcium signaling,[239] degranulation,[240] phagocytosis,[229,241,242] and antibody-mediated cellular cytotoxicity.[243] Furthermore, the ITAM-containing γ-chain[244] that is associated with FcγRs is required for immune complex-mediated glomerulonephritis in mice.[245] Thus, it is likely that the hypersensitivity phenomena originally observed by von Pirquet at the beginning of the 20th century, and later attributed to immune complexes by Dixon et al.,[80] has a similar pathophysiology, at least in part.

THE HERMENEUTICS OF LYMPHOCYTE ACTIVATION

Evolution of Early Lymphocyte Activation Models

By the late 1950s, it became clear that lymphocytes were highly adept at interpreting or translating environmental cues and responding by maintaining a state of activation or tolerance. The focus of immunology had shifted from identifying the relevant cell types involved in the immune response to discovering what can be viewed as the "hermeneutics" (a term derived from the Greek ἑρμηνεύω, "translate" or "interpret") of lymphocyte activation. Based on the clonal selection theory, Burnet[246] proposed that activation or tolerance was the result of an antigen stimulating a single cell, depending on the age of the organism. This was in keeping with experiments in which tolerance was induced by exposing antigen during fetal life or shortly thereafter.[138,140,141] In 1959, Lederberg[247] modified this model to describe activation or tolerance as occuring depending on the age of the cell rather than the organism.

> If an antigen is introduced prior to the maturation of any antibody-forming cell, the hypersensitivity of such cells, while still immature, to an antigen-antibody reaction will eliminate specific cell types as the arise by mutation, thereby inducing apparent tolerance to that antigen.

However, the model could not account for several observations, among which were that the dosage and specific form of the immunogen, such as a hapten, could also influence the outcome of the encounter. This led Talmage and Pearlman[248] to propose an alternative model in which antigen alone would induce tolerance, but aggregated antigen, perhaps associated with complement, could induce an additional nonspecific stimulus to trigger clonal expansion. The suggestion of the need for a second "nonspecific" stimulus, and particularly complement, was remarkably prescient in light of the finding that complement can provide exactly such a stimulus to amplify immunogenicity by 10^3- to 10^4-fold.[249] In 1968, and later revised in 1970, Bretscher and Cohn[250,251] provided a variation on this model, in which a thymus-derived antigen-sensitive cell-bound antigen containing at two separate sites, one for a receptor on the "antiantigen-sensitive" cell and another bound to a "carrier" antibody. If the second site on the antigen was occupied by antibody, then that would deliver a signal to the "antiantigen-sensitive" cell to induce immunity. In contrast, if the site remained unbound, the antigen would induce tolerance. In this model, two cells were involved, but they were separated in time and space. Among the problems with the model was its circularity: What would provide the stimulus for the production of the carrier antibody in the first place? Other theories were proposed to explain the phenomenon of alloreactivity. The most intriguing of these was provided by Lafferty and Cunningham,[252] who proposed the existence of an "antigen bridge," which was now provided by another cell to provide "Signal 2." When MHC restriction was described in 1977, this model was modified somewhat to include the provision that the second signal was triggered by MHC on the stimulator cell[253] (Table 2.1).

Discovery of Costimulation: T Cells as Beneficiaries

By 1975, models for lymphocyte activation had matured, but many details remained sketchy, particularly the identity of the elusive Signal 2. In 1987, Jenkins and Schwartz utilized a system designed to "convert" Ag-specific T-cell clones into suppressor cells. They used a model system in which T-cell clones against a pigeon cytochrome peptide were incubated with ethylene carbodiimide-fixed Ag-pulsed splenocytes as APCs. They obtained the surprising result that the T-cell clones became unresponsive to subsequent restimulation with untreated APCs plus peptide, although they remained viable and proliferated in response to interleukin (IL)-2.[254] This showed that Ag-specific unresponsiveness could be induced in the absence of suppressor cells and that stimulation with cognate antigen alone was insufficient to induce proliferation; the missing Signal 2 was defined as "costimulation" and ultimately identified as cluster of differentiation (CD)28-mediated signaling by Allison and colleagues.[255] Since this seminal discovery, an entire family of CD28-like molecules in T cells and their cognate ligands on APCs has been identified,[256,257] which includes an inhibitory member of the family, cytotoxic T-lymphocyte antigen-4.[258] This has led to the clinical development of cytotoxic T-lymphocyte antigen-4–Ig, an inhibitor of CD28,[259] which was approved for the treatment of rheumatoid arthritis by the U.S. Food and Drug Administration in 2006 and by the European Medicines Agency in 2007.

Discovery of the Mechanism of B-Cell Help

It was known since the 1960s that T cells were needed to "help" B cells produce antibody, but the molecular nature of that help was unknown.[129,260] Experiments had suggested that T-cell help required cell-to-cell contact, implying the existence of the involvement of cell surface receptor–ligand interactions. Agonistic antibodies against CD40 drove B cells into cell cycle, raising the possibility that a cognate ligand on T helper cells participates in the mechanism of

TABLE 2.1 Different Models of Lymphocyte Activation

Basis of Immune Recognition	Signals	Components	Conditions for Tolerance	Authors
Self versus nonself	1	B cell/Ag	Immature organism	Burnet, 1959[230]
	1	B cell/Ag	Immature Ab-forming cell	Lederberg, 1959[231]
	2	B cell/Ag–Ab + complement	Absence of bound Ab/complement	Talmage and Pearlman, 1963[232]
	2	Humoral Ag-sensitive responder cell/bivalent Ag/carrier antigen-sensitive thymus-dependent cell	Absence of carrier Ab or lack of	Bretscher and Cohn, 1968,[234] 1970[235]
	2	Signal 1: Ab-bound responder cells + Ag Signal 2: Stimulator cell + Ag	Absence of Signal 2	Lafferty and Cunningham, 1975[236]
Noninfectious self versus infected nonself	2	Signal 1: Lymphocyte + MHC/Ag Signal 2: PAMP+ APC	Absence of Signal 2	Janeway, 1989[390]
Danger	3+	"Signal 0": Alarm Signal 1: (Th cell-APC–Ag; Tc-infected cells; B cell/Ag; Signal 2: Help (Th to B) or costimulation (APC to T)	Absence of "Signal 0"	Matzinger, 1994[451]

Abbreviations: Ag, antigen; Ab, antibody; APC, antigen-presenting cells; MHC, major histocompatibility complex; PAMP, pathogen-associated molecular pattern; Th, T helper.

T-cell help. Using a CD40–Ig fusion protein, murine CD40L was cloned.[261,262] Use of a mAb against the human CD40L confirmed that expression was restricted to mantle and centrocytic zones of lymphoid follicles and the spleen periarteriolar lymphoid sheath in association with CD40+ B cells,[263] which is the distribution that might be expected for a highly localized signal to provide T-cell help to B cells. Indeed, in 2000, the specific type of T cell that provided CD40L-mediated help to B cells, now called a T follicular helper cell, was identified by its unique anatomic location and expression of a homing receptor, CXCR5.[264,265] In 1993, four groups independently announced that defects in the CD40 ligand gene are responsible for X-linked hyper-IgM syndrome in which affected adults express elevated levels of IgM and defects in class switching.[266–269]

T-Cell Subsets and T-Cell Signaling Paradigms

The widespread use of mAbs since their discovery by Köhler and Milstein resulted in the production of many antibodies directed against T cells that had nonoverlapping patterns of expression. Much of this early work was done by Schlossman, Reinherz, and colleagues.[270–275] In most cases, the antibodies themselves identified T cells with specific functions; and in 1987 and 1988, two groups found that the molecules recognized by two of these mAbs, denoted by their cluster designations, CD8 and CD4, mediated adhesion to MHC class I and II, respectively.[276,277] CD4 and CD8 were shown to bind to nonpolymorphic regions of MHC molecules,[277] suggesting that they might stabilize otherwise weak interactions between TcR and pMHC. Later experiments demonstrated that the cytosolic domains of these coreceptors were able to bind, and recruit, the nonreceptor Src family tyrosine kinase Lck.[278,279] It was not known at the time what the relevant substrates of Lck might be, but in 1989, at about the same time that the ITAM consensus sequence was identified, two groups showed that Lck was capable of phosphorylating subunits of the CD3 complex and the ζ subunits of the TcR.[280,281] Following the discovery of another key tyrosine kinase associated with the ζ subunit of the TcR, ZAP-70, by Chan and Weiss,[238] a complicated cell signaling paradigm began to emerge in which "Signal 1," delivered by pMHC expressed on either APCs or virus-infected cells, is conveyed by a series of phosphorylation events, leading to phosphorylation of phospholipase C-γ, triggering increases in cytosolic calcium and dephosphorylation of a key transcription factor, NFAT. The elusive machinery of "store-operated calcium channels" required for calcium-based signaling[282] was finally elucidated in 2006.[283–286] Defects in this pathway in lymphocytes lead to a form of SCID.[286] Dephosphorylated NFAT, alone or in conjunction with AP-1, translocates to the nucleus where it binds to sites on the promoters of key genes such as IL-2, culminating in T-cell proliferation.[287] The initial interaction between TcR-bearing T cells and pMHC-bearing APCs induces a cooperative series of protein–protein interactions in a spatially delimited fashion (the "immunologic synapse") that facilitates signaling events.[288] We now know that signal transduction by the TcR shares common elements, and in some cases identical kinases and substrates, with signaling by other ITAM-containing receptors, such as the BcR, and Fc receptors or IgG and IgE.[289–291] Recent data suggest that similar mechanisms governing "immunologic synapses" in T cells may also be characteristic of "phagocytic synapses,"[292] underscoring a further degree of conservation of ITAM-based pathways. It is ironic that lymphocytes, which had appeared morphologically uninteresting to so many biologists until the latter part of the 20th century, would share so many characteristics in common with their visually more intriguing cousins.

Cell to Cell Communication: Mice Have a Tale to Tell

The first cytokine to be characterized is credited to Isaacs and Lindenmann,[293] who studied an influenza virus-induced factor from chick chorioallantoic membranes that blocked a second viral infection; this factor was called "interferon" (IFN). It was not purified to homogeneity until 20 years later.[294,295] Type I IFNs, which include IFNα and IFN-β, and type II IFN, IFNγ, bind to distinct receptors. A major advance in our understanding of cytokine signaling occurred in 1992, when Schindler et al.[296] demonstrated the tyrosine phosphorylation and nuclear translocation of a complex of four proteins, named IFNα stimulated gene factor 3. IFNα stimulated gene factor proteins were isolated from nuclear extracts derived from over 10^{10} cells, followed by sequencing and cloning of their genes; two of these proteins are members of what are now known as STAT proteins, which were shown to be substrates for members of the Janus-associated kinase family.[297–303] The most common severe immunodeficiency attributable to defective cytokine signaling is X-linked SCID, which is due to mutation in the common γ_c gene that is shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.[304] Mutations in Janus-associated kinase 3 were found in two patients with a severe form of SCID indistinguishable from X-linked SCID.[305,306] Dominant-negative mutations in STAT3, which is important in the signal transduction of IL-6 and IL-10, were found to be responsible for hyper-IgE syndrome, a disease associated with highly elevated serum IgE, recurrent staphylococcal skin infections and pneumonia, and skeletal abnormalities.[307,308]

Much of our knowledge of this pathway, as well as many other components of the immune system, is due to the use of gene targeting in mice. In 1985, Smithies et al.[309] introduced a short DNA sequence from the human beta-globin locus into an erythroleukemia cell line and were able to detect a specific exchange of the beta-globin gene with the homologous sequence in about 1 in 10^3, demonstrating the feasibility of gene targeting. At the same time, Capecchi introduced DNA directly into the nucleus of a cell using a microelectrode. Capecchi noted that multiple copies of the introduced gene were integrated into the host cell's chromosome through homologous recombination. These studies established the potential for homologous recombination in somatic cells. The next major step was based on the ability of using blastocyst-derived embryonic stem cells to introduce genes into the germline of the mouse.[310,311] By injecting blastocysts with cultured embryonic stem cells that were infected with a retrovirus, Evans and colleagues generated chimeric mice in which retroviral DNA was detectable in both somatic and germ-line cells.[311] Eventually, Evans, Smithies, and Capecchi refined these techniques, which led to the first knockout mouse, in which the gene encoding hypoxanthine guanine phosphoribosyl transferase was deleted. The resultant phenotypic resembled that of the Lesch-Nyhan syndrome, which is characterized by mental retardation and self-mutilation.[312,313] Today, gene targeting techniques are used worldwide to study the effects of deletion or overexpression of genes important in immunity and any disease that can be reproduced in the mouse and in other species in which the technique has been used. In 2007, Smithies, Capecchi, and Evans shared the Nobel Prize "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells."

Chemokines

If cytokines determine what cells do, then chemokines determine where cells go. The first chemokines purified were derived from platelets, although neither protein (platelet factor 4 [CXCL4][314,315] and β-thromboglobulin [CXCL7][316]) was recognized to be chemotactic when first identified. IL-8 (CXCL8) was the first chemokine to be purified and sequenced based on its chemotactic function for neutrophils,[317] and its receptors were cloned in 1991.[318,319] The word "chemotaxis" was coined by Pfeffer in 1884, who observed spermatids migrating toward a pipette containing maleic acid salts.[320] It is not clear who first explicitly observed chemotaxis of leukocytes, although several scientists, notably Schultze, Lieberkühn, Davaine, and Wharton Jones described amoeboid movement in leukocytes in the middle of the 19th century.[321] Since then, chemotaxis has been studied in vitro using various techniques, including specialized chambers that facilitate this process.[322]

The effects of chemokines on cells are complex, but a major function is to orchestrate the process of diapedesis, the process of transendothelial migration first described by Dutrochet in 1824.[323] In 1979, Hayward et al.[324] described six infants from two families whose umbilical cords were still attached at 3 weeks of age. Five of these developed severe local and disseminated infections from which four died. The molecular defect was identified by Springer et al.[325] in 1984, who showed that leukocytes from patients with the disease lacked all known β_2 integrin adhesion receptors. This was eventually traced to absent or abnormal β_2 subunits (CD18). Marlin and Springer[326] later described the requirement of the β_2 integrin, LFA-1, to bind its counter-receptor, ICAM-1, on endothelial surfaces for diapedesis to occur normally. The function of chemokines is to provide chemotactic gradients as well as activate the integrins for tight adhesion prior to the active participation in endothelial cells in diapedesis.[327,328] Interestingly, LFA-1 was shown to be required as an accessory molecule during interaction of T cells with their cognate targets.[329]

Chemokines are critical for initiating the primary immune response as APCs, such as DCs. DCs and naïve T cells both share a common chemokine receptor, CCR7. T cells migrate through high endothelial venules, which secrete the chemokine SLC (CCL21). SLC is needed for integrin-mediated adhesion of the T cells.[330] Mice lacking expression of SLC have defects in lymphocyte and DC localization to the T-cell zone of secondary lymphoid organs.[331] The chemokine-directed "patrolling" of naïve T cells in the lymph nodes is probably the major mechanism by which the very few lymphocytes with the relevant TcR specificity remain there to interact with the antigen-loaded APCs during an immune response. Thus, chemokines are essential components of the "clonal selection" of lymphocytes.

T-Cell Polarization: The Power of Dichotomy

In 1986, Coffman and Mosmann at DNAX Research Institute were interested in determining factors that differentiated different types of T helper cell lines. One such line was capable of inducing a 100-fold increase in IgE secretion from mouse splenocytes, which was potently blocked by IFNγ.[332] When the team characterized other cloned T-cell lines, it became clear that there were very specific patterns of secretion: Some clones, which they called T_H1 cells, produced IL-2 and IFNγ, while other clones, which they called T_H2 cells, produced factors that stimulated B cells (B-cell–stimulating factor-1), mast cells, other T cells, and IgE and IgG1 secretion. All clones produced IL-3.[333] Further work, in collaboration with Paul, who first identified B-cell–stimulating factor-1,[334–336] clarified that B-cell–stimulating factor-1 was the same factor that stimulated mast cells and T cells, later renamed IL-4. Coffman and Mosmann[337] defined the now well-established paradigm of polarized T helper cell secretion; they demonstrated that IL-4 and IFNγ reciprocally inhibit the outgrowth of T_H1 and T_H2 cells, respectively. The later identification of transcription factors that are selectively expressed in these cells (GATA-3 and T-bet, respectively) and can even reprogram the cells to transdifferentiate toward the "opposite" phenotype further substantiated this dichotomy.[338,339] These findings had profound implications for immunologists, who began to classify diseases according to their predominant T_H cytokine profiles. Thus, tuberculosis and most bacterial and fungal infections produced a T_H1 pattern of cytokines, whereas asthma and other allergic diseases produced a T_H2 pattern of cytokines. While this reductionist view of cytokine production is no doubt an oversimplification, this simple dichotomy has proved quite robust and has helped guide the development of novel therapeutics for various diseases.

There is, however, a danger to oversimplification[340]—sometimes it is necessary to be a "fox" rather than a "hedgehog."[341] Some diseases, such as Crohn disease, an inflammatory bowel disease that was thought to be driven by the T_H1-polarizing cytokine, IL-12, is more likely driven by a related cytokine, IL-23, with which IL-12 shares a common subunit; indeed, a genome-wide association study in 2006 revealed a highly significant association of the gene encoding a subunit of the IL-23 receptor and early-onset Crohn disease.[342] Ustekinumab, a mAb against the IL-12/IL-23 p40 common subunit was approved by the U.S. Food and Drug Administration for the treatment of moderate-to-severe Crohn disease in 2006 and for moderate-to-severe plaque psoriasis in 2009.

The Language of Immunoregulation: Explaining the "Contrivances"

The concept of immunoregulation is implicit in the term "immunity" (exempt), and the history of how different immunologists have viewed immunoregulation, depending on their perspectives, is worthy of a chapter in itself. Jerne[343] hypothesized that antibodies can act as antigens and elicit an immune response against their idiotypes, which would then serve to regulate the immune response. While appealing, efforts to prove the importance of this concept have achieved limited success. In 1970, Gershon and Kondo[344] found that thymus-derived cells could specifically induce tolerance, and they and others spent many years trying to isolate antigen-specific T suppressor cells but were unable to do so. In the 1990s, the field shifted away from clonotypic suppressor cells toward T cells secreting specific tolerogenic factors, such as IL-10–secreting "Tr1 cells" and transforming growth factor-β–secreting "Th3 cells."[340,345] This was followed by the identification of cell surface markers of a subset of CD4+ cells that were required to suppress autoimmunity in mice. These turned out to be CD25 and CD5 in a CD4+ population of cells in the mouse, cells which were later called regulatory T cells. In 2001, the *FoxP3* gene was identified as the gene that was mutated in Scurfy mice, which develop severe autoimmunity as a result of a single gene mutation.[346] Similarly, mutations in the human homolog of *FoxP3* were found to be associated with the disease called IPEX (*i*mmune dysregulation, *p*olyendocrinophathy *e*nteropathy, *X*-linked syndrome).[347–349] Analogous to the capacity for T-bet and GATA3 to drive T_H1 and T_H2 differentiation, respectively, expression of FoxP3 was sufficient to drive the differentiation of T helper cells to a CD4+ CD25+ regulatory T phenotype.[350,351]

Central tolerance, sometimes referred to as "negative selection," is a means of shaping the repertoire that was thought likely to exist following the discovery of the thymus as an immune organ by Miller in 1961; however, proof for this was lacking. How could one prove the existence of a phenomenon that predicted the absence of a cell, rather than its presence? It was not until 1987 that Kappler et al.[352] used a mAb against a specific TcR Vβ segment to show that the mAb recognized immature thymocytes but not mature thymocytes or T cells, thus proving that negative selection occurs. Von Boehmer demonstrated a similar phenomenon using a TcR transgenic mouse model.[353] Negative selection of immature B cells was also demonstrated in the classic experiments of Nossal and Pike,[354] Sidman and Unanue,[355] and Raff et al.[356]

Clearly, the field of immunoregulation is still evolving. Novel mechanisms of immunoregulation will surely be discovered, and novel regulatory interactions will be uncovered. The role of tolerogenic DCs,[357,358] CD8+ T cells,[359,360] and myeloid-derived suppressor cells[361–363] are likely to play important roles and some of these will likely find their way to the clinic. Perhaps, however, we have made some progress in support of Ehrlich's contention[364]:

> The organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from working against the organism's own elements.

Immune Subversion: The Case of Tumors

The idea that the immune system is capable of responding to tumors dates back to Ehrlich,[365] who predicted that cancer would occur at a high frequency in the absence of

an immune response. This theme was recapitulated by others, notably Burnet,[366] who argued that T cells would be prominent in what he termed "immune surveillance." The field enjoyed a resurgence in 1994 when Schreiber and colleagues demonstrated that tumors expressing dominant-negative IFNγ receptors demonstrated enhanced tumorigenicity in syngeneic mice.[367] This was supported by studies using IFNγ receptor-deficient or STAT1-deficient mice.[368] Additional evidence for an active immune response against tumors is the spontaneous development of autoimmunity in some individuals with tumors; for example, paraneoplastic cerebellar degeneration occurs in individuals with a cytotoxic T-cell response to a shared antigen on tumor cells and neuronal cells.[369] The immune response to tumors is complex, with participation from cytotoxic T cells, natural killer (NK) cells, DCs, and myeloid cells.[370] The "counter-response" by the tumors, which has been termed "escape" or "evasion," is equally complex.[370] While it is beyond the scope of this chapter to discuss any of these in detail, several relevant points of historical interest should be noted. In 1976, a postdoctoral fellow named Mike Bevan was studying alloreactive cytotoxic T-cell responses and was able to demonstrate that spleen cells from an H-2^b/H-2^d–restricted mouse primed with H-2^b cells that differed in minor histocompatibility loci contained increased numbers of both H-2^b– and H-2^d–restricted cytotoxic CD8 T cells.[371,372] Bevan interpreted this as evidence of "cross-priming," in which ingestion of cell-associated antigens by phagocytosis led to cytosolic entry of antigens and loading onto MHC class I molecules and then presentation to cytotoxic T cells. It was completely novel and counterintuitive, but subsequent studies validated this interpretation.[373] Recent progress has been made in our understanding mechanisms by which antigens gain entry into the cytosol from phagosomes.[374] These studies were based on key findings by Desjardins and colleagues who first demonstrated recruitment of endoplasmic reticulum membrane to nascent phagosomes.[375,376] Mice that lack CD8α+ DCs, a cell type that is adept at cross-priming, were unable to demonstrate cross-priming and were incapable of generating a cytotoxic response against West Nile virus and a highly immunogenic fibrosarcoma tumor.[377] Thus, cross-priming is important in tumor immunity.

Tumor cells are capable of circumventing the host immune response in various ways, including downregulation of MHC class I molecules, although this would potentially render them susceptible to NK-mediated killing, according to the "missing self" hypothesis.[378] Tumors elaborate a host of cytokines and growth factors that directly inhibit various components of the immune system.[370] An additional means of tumor evasion specifically bears on the meaning of "immunologic self." Most cells in the body express a cell surface protein, CD47, which is a ligand for a receptor present on macrophages and DCs, SIRPα.[379] SIRPα interacts with tyrosine phosphatases via its cytosolic domain[380]; therefore, corecruitment of SIRPα to tyrosine kinase-coupled signaling scaffolds is predicted to inhibit kinase-mediated signaling events. Lindberg and colleagues showed in 2000 that CD47 on erythrocytes in mice prevented their phagocytosis until levels fell below a certain level, thus CD47 served as an aging "clock" that signified a "don't eat me" signal.[381] In 2010, in a remarkable study, expression levels of CD47 on non-Hodgkin lymphoma (NHL) cells were negatively correlated with survival. Blocking anti-CD47 antibodies preferentially enabled phagocytosis of NHL cells and synergized with rituximab. Treatment of human NHL-engrafted mice with an anti-CD47 antibody reduced lymphoma burden and improved survival, while combination treatment with rituximab (anti-CD20) led to elimination of lymphoma and cure.[382] In these settings, CD47, rather than MHC proteins, serve as a marker of "self." It is somewhat counterintuitive that, at least in the case of NHL, tumor masquerading as self is one mechanism by which tumors evade the immune system. Ironically, downregulation of MHC class I molecules, the classic marker of self, is yet another mechanism of how tumor evasion of immunity.[370]

Immune Hijacking: The Discovery of Human Immunodeficiency Virus

The first description of what would come to be known as the "acquired immunodeficiency syndrome" appeared in 1981.[383] Initially described in five homosexual men, the disease soon was apparent in Haitians, transfusion recipients, infants, Africans, and female sexual contacts of infected men.[384] In just 2 years, Montagnier's team published the first paper demonstrating the presence of retroviral particles from diseased patients,[385] and a year later, Gallo's group published five papers in *Science* providing convincing evidence that this retrovirus was the cause of acquired immunodeficiency syndrome.[386–390] Barré-Sinoussi and Montagnier won the Nobel Prize for their discovery of human immunodeficiency virus (HIV), along with zur Hausen, for demonstrating that human papillomavirus can cause cervical cancer. Many felt that Gallo should have been awarded the prize as well.

Pneumonia due to *Pneumocystis carinii* (later re-named *P. jerovici*) was common in initial cases of acquired immunodeficiency syndrome, but patients soon presented with an array of opportunistic infectious diseases, malignancies (eg, Kaposi sarcoma and lymphoma), and even autoimmune diseases. In 1986, Maddon et al.[391] demonstrated that CD4 is an essential receptor that mediates HIV-1 entry into lymphocytes; this was followed in 1996 by the demonstration of CXCR4 and CCR5 as coreceptors for HIV-1 entry.[392] Since then, many laboratories worldwide have dedicated their efforts to uncovering HIV pathogenesis.[393,394] One of the major challenges to the immune system as well as the development of an HIV-1 vaccine is the enormous plasticity of the viral sequence due to the high error rate of reverse transcription.[393]

Intensive efforts in academia and the pharmaceutical industry have resulted in highly active antiretroviral therapy based on targeting multiple steps of viral replication, mostly focusing on reverse transcriptase and protease. A disease that was once virtually 100% fatal is now manageable, resulting in an 80% to 90% decrease in mortality rates in the United States and Europe.[393] Among the major

challenges facing immunologists and virologists is eliminating the latent reservoir of virus in resting memory CD4+ lymphocytes, developing an effective HIV-1 vaccine, and providing treatment for the 90% of infected individuals worldwide who reside in developing countries and have poor access to antiretroviral therapy.

METCHNIKOFF'S LEGACY

The Rediscovery of Innate Immunity

Arguably, the most important advance in immunology in the last 15 years has been the "rediscovery" of innate immunity. The closest term to "innate immunity" that was used at the turn of the century was "natural immunity." When the cellularists and the humoralists were debating the relative importance of cells and soluble antikörper at the turn of the century, much of the phenomena that Metchnikoff observed under the microscope represented different aspects of innate immunity. However, Metchnikoff distinguished enhanced immunity due to vaccination as a result of what we would call today an acquired immune response: "an agglutinative substance...in the...fluids of the body becomes much more developed in those of immunised animals."[395] He thought that infections could lead to an acquired immune response, but the nature of that response was to foster an enhanced response in phagocytic cells.[395]

> In certain infective diseases terminating fatally a very marked phagocytosis is observed even in susceptible animals . . . The acquisition of immunity against microorganisms is, therefore, due not only to the change from negative to positive chemiotaxis, but also to the perfecting of the phagocytic and digestive powers of the leucocytes.

In 1932, Fleming[396] recounted the discovery of lysozyme, which he made in 1921, "because its importance in connection with natural immunity does not seem to be generally appreciated." Although the discovery has been cited to represent another example of scientific serendipity, resulting from accidental dripping of nasal secretions from Fleming himself onto a culture plate,[397] Fleming wrote that "cultures of nasal mucus were made from a person suffering an acute cold" when the bacterolytic phenomenon due to lysozyme was observed.[396] Fleming would go on to discover penicillin, which was a bona fide example of scientific serendipity, and receive the Nobel Prize for his discovery.

Of course, lysozyme is but one of many innate immune molecules important in the early phase of host defense. Other cationic proteins include defensins, which are secreted by leukocytes and epithelial cells, and chemokines, whose tertiary but not primary structures are related to defensins. Some members of both classes have the dual role of recruiting inflammatory cells to sites of infection and killing bacteria.[398–401] Other examples of components of innate immunity include "innate immune lymphocytes," such as NK cells, NK T cells, and γδ T cells. Although it is beyond the scope of this chapter to describe these cell types except in passing, the discovery of NK cells in 1975[402–404] provided a cellular mechanism to account for the observation that some host immune cells recognize and kill virus-infected or neoplastic cells lacking expression of MHC class I molecules. NK cells also provide an early, immediate source of cytokines, allowing them to rapidly respond to injury prior to maturation of the primary immune response.[405]

The "modern era" of the study of innate immunity began in 1989. In a characteristic example of his remarkable insight, Janeway[406] published an article in which he described a new model for immune recognition. Janeway argued that rather than distinguish "self" from "nonself," the immune system had evolved to distinguish "noninfectious self from infected nonself" (see Table 2.1). Janeway compared the innate and acquired immune systems. He emphasized that the former evolved to respond rapidly to "pathogen associated molecular patterns" by as yet unidentified receptors in a nonclonal fashion. Janeway was acutely aware of the significance of Freund's discovery of adjuvant[407] and the implications this had for the primary immune response. What was remarkable about Janeway's hypothesis is its intuitive appeal and its Darwinian flavor, in some ways analogous to Metchnikoff's view of phagocytosis. In addition, it had great predictive power. In a completely independent line of investigation, Jules Hoffmann became interested in exploring inflammatory pathways in *Drosophila*. Hoffman was aware of Baltimore's discovery of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), a transcription factor, originally identified as a lipopolysaccharide (LPS)-inducible transcription factor in B cells in 1986.[408] Mice rendered deficient in NF-κB demonstrated multifocal defects in immune responses, including those to LPS.[409,410] Based on his observation of similarities between the cytokine-induced activation cascade of NF-κB in mammals and the activation of the morphogen dorsal in *Drosophila* embryos, Hoffmann demonstrated that the dorsoventral signaling pathway and an extracellular toll ligand control expression of antifungal peptide gene expression. Mutations in the toll signaling pathway dramatically reduced survival after fungal infection.[411] Until that time, there was only a limited understanding of pathogen recognition in eukaryotes; the only receptor that was known to participate in the recognition of LPS was CD14, a molecule that is expressed predominantly on macrophages.[412] Shortly after publication of Hoffmann's landmark study, Janeway and Medzhitov published an article in *Nature* that described the cloning of a human homolog of *Drosophila* toll. A constitutively active mutant of human toll transfected into human cell lines induced the activation of NF-κB and the expression of the inflammatory cytokines IL-1, IL-6, and IL-8 as well as the expression of the costimulatory molecule B7.1 "which is required for the activation of naïve T cells."[413] The choice of these proteins was not accidental, and it was clear that Medzhitov and Janeway had Janeway's hypothesis of 1989 in mind. Meanwhile, Beutler et al.[414] were zeroing in on the genetic identification of a mutation in a mouse strain that was incapable of responding to LPS, a component of the cell walls of all gram-negative bacteria. Beutler and Cerami were the first to show that immunization against tumor necrosis factor, the prototypic cytokine

that is produced following administration of LPS, protected mice from lethal shock.[414] Beutler demonstrated that the codominant Lpsd allele of C3H/HeJ mice corresponded to a missense mutation in the toll-like receptor-4 gene.[415] Since these publications, a family of innate immune receptors has been defined, each responding to different ligands encountered by hosts during infection.[416] Initial interactions of innate immune receptors on APCs with their ligands encounters are necessary for the upregulation of costimulatory molecules, without which tolerance would occur.[417] Janeway's prediction in 1989 was borne out, providing strong empirical evidence in support of his model for immune recognition (see Table. 2.1). In 2012, Beutler and Hoffman, together with Steinman, received the Nobel Prize.

The field of innate immunity has exploded since the key observations of Hoffman, Beutler, Janeway, and Medzhitov. Many other receptors and components of the innate immune system have been discovered. These include components of the complement system,[418] cell surface lectins,[419] collectins such as lung surfactant proteins[420] and mannose-binding proteins,[421] scavenger receptors,[422,423] and pentraxins.[424] Most of these participate in recognition of pathogens, including fungi,[425] bacteria,[426] and viruses such as HIV.[427] In some instances, the phagocytic cells themselves serve as the source of opsonins, such as complement.[428] A variety of innate immune receptors also recognize apoptotic and necrotic cells.[429,430] Secretion of opsonins, such as milk fat globule-EGF factor 8, can enhance uptake of targets such as apoptotic cells,[431] and the absence of this protein has been linked to autoimmunity in mice.[432] Similar results were found in other mouse strains engineered to be deficient in clearance of apoptotic cells.[433,434] The conceptual basis of these experiments was built on earlier work of Savill, Henson, and others who showed that the immunologically "silent" disposal of apoptotic debris is an active process serving to divert self-antigens toward a nonphlogistic mode of phagocytosis.[435–437] In the context of resolution of acute infections, a similar function is provided by the production of omega-3 polyunsaturated fatty-acid-derived "anti-inflammatory" lipids ("resolvins"), first identified by Serhan et al. in 2000.[438,439]

Cytosolic Components of Innate Immunity: Discovery of the Nicotinamide Adenine Dinucleotide Phosphate-Oxidase

In 1957, Good and colleagues described an X-linked disease in which children succumbed to chronic suppurative and granulomatous infiltrations and chronic infections.[440] Neutrophils isolated from these children showed decreased bactericidal activity, although they demonstrated normal phagocytosis.[441] However, they showed decreased hydrogen peroxide production and hexose monophosphate shunt activity.[442] In 1974, Curnutte et al.[443] identified defective superoxide anion production in children with this syndrome who also failed to reduce the dye, nitroblue tetrazolium.[444] This simple test has been widely used to diagnose what came to be called "chronic granulomatous disease." In 1978, Segal et al.[445,446] identified the molecular defect in X-linked chronic granulomatous disease as the absence of cytochrome b. Since then, all the components of this multiprotein enzyme complex have been identified.[447–452] Of note is that the oxidase responsible for X-linked chronic granulomatous disease, now referred to as NOX2, is but one of a family of oxidases that are widely expressed and have been implicated in various disease.[453] We now know that there are multiple reactive oxygen species, many of which combine with other reactive molecules, such as nitric oxide, to generate reactive nitrogen species. Nathan and colleagues demonstrated that mice deficient in inducible nitric oxide synthase proved highly susceptible *Mycobacterium tuberculosis*, resembling wild-type littermates immunosuppressed by high-dose glucocorticoids.[454]

In addition to its role in microbial killing, NOX2 is recruited to early phagosomes in DCs and mediates the sustained production of low levels of reactive oxygen species, causing active and maintained alkalinization of the phagosomal lumen. DCs lacking NOX2 show enhanced phagosomal acidification and increased antigen degradation, resulting in impaired cross-priming.[455]

Cytosolic Components of Innate Immunity Signal Danger

There are many additional cytosolic proteins important in innate immunity. Of particular interest is the discovery of a family of pattern recognition receptors, most commonly referred to as nucleotide oligomerization domain–like receptors.[456] In 2000 and 2001, a number of genetic studies linked defects in *NLR* genes to inflammatory diseases, including Crohn disease,[457] Blau syndrome,[458] Muckle-Wells syndrome,[459] and familial Mediterranean fever.[460] In 2002, Tschopp and colleagues described a multiprotein complex that they called the "inflammasome," which included members of the nucleotide oligomerization domain–like receptors family and caspase-1.[461] They showed that activation of the inflammasome leads to generation of IL-1β, a cytokine that was first cloned by Dinarello and colleagues in 1984,[462] representing one of the key "endogenous pyrogens" first detected in 1953.[463,464] In 2006, Tschopp showed that the NLRP3 inflammasome was activated by monosodium urate crystals, implicating this pathway in gout.[465] From a historical perspective, gout is among the earliest diseases to be described. It was identified by the Egyptians in 2640 BCE and was later recognized by Hippocrates in the fifth century BC, who referred to it as "the unwalkable disease." Leeuwenhoek was the first to observe urate crystals from a tophus.[466] Recently, an advisory panel of the U.S. Food and Drug Administration recommended against approval of canakinumab, a humanized mAb against IL-1, for the treatment of gout; although it was effective, it was not deemed safe due to increased risk of serious infections.

In 1994, Matzinger[467] proposed a new model of immune recognition. She proposed that APCs are activated by danger/alarm signals from injured cells (see Table 2.1). Although this was a purely theoretical model, since then, there have been numerous instances in which injured or

damaged components of the host have been recognized to trigger inflammation. As Matzinger explains,[468]

> Although this may seem to be just one more step down the pathway of slowly increasingly complex cellular interaction, this small step drops us off a cliff . . . in which the "foreignness" of a pathogen is not the important feature that triggers a response, and "selfness" is no guarantee of tolerance.

Thus, release of uric acid crystals can be viewed as an example of a "danger signal." Similarly, release of intracellular stores of adenosine triphosphate from dying cells as a trigger for signaling cellular injury, predicted in 1988,[469] was shown to be capable of activating the NLRP3 inflammasome.[470] Although Matzinger's model shares features in common with Janeway's, the emphasis on endogenous danger signals rather than direct engagement of pattern recognition receptors by pathogens is novel. The fact that pathogens and "endogenous danger signals" share common receptors and signaling pathways is consistent with the idea that either exogenous or endogenous triggers of innate immune receptors accomplish the same thing: to facilitate the primary immune response.

Cytosolic Components Maintain Safety: The Role of Autophagy

There are numerous examples of microbes that are ingested by phagocytosis but either remain viable within acidic organelles or escape into the host cytoplasm. Macroautophagy is an evolutionarily conserved process in which cytoplasmic components are sequestered by a double membrane sac, eventually acquiring endosomal and lysosomal characteristics.[471] In 2004, Deretic and colleagues showed that IFNγ induced autophagy in macrophages, which contributed to suppression of intracellular survival of mycobacteria.[472] In the same year, Nakagawa et al.[473] demonstrated that autophagy was necessary for killing of group A *Streptococcus*, which had escaped into the host cytoplasm. Since then, many publications have confirmed the importance of autophagy in both innate and acquired immunity.[471]

The Renaissance of Cellular Immunology: Discovery of Dendritic Cells

Although we tend to take for granted the concept that APCs are necessary to process antigen, this was not firmly established until 1967. Mosier used a method pioneered by Mishell and Dutton for measuring in vitro antibody production with sheep erythrocytes as antigen.[474] Mosier separated mouse spleen cells into an adherent fraction and a nonadherent fraction. The adherent cells, which were phagocytic, were deemed "macrophage rich," and the nonadherent cells were deemed "lymphocyte rich." Both populations together, but neither alone, were needed for an antibody response, and Mosier[475] concluded that antibody production required "antigen phagocytosis by macrophages and macrophage lymphocyte interactions," although he did not know the nature of the interactions. Further experiments established that exceedingly few adherent cells were needed for antibody production, perhaps as few as 1 in 10^4 adherent cells.[476] In the mid-1970s, Zanvil Cohn and a postdoctorate in his laboratory at Rockefeller University, Ralph Steinman, were characterizing a novel adherent population of cells from spleen.[477–479] The cells had an unusual "tree-like" morphology, prompting the name "dendritic cell."[477] These cells proved difficult to purify, necessitating Steinman and Cohn to develop a rather laborious density gradient technique for cell purification.[480] In 1978, they used this technique to show that DCs could stimulate a primary mixed leukocyte reaction—an index of lymphocyte proliferation—and showed that DCs are at least 100 times more effective than B cells and macrophages for this function.[58] This landmark paper was the first demonstration of the unique capability of DCs to efficiently present antigen. Since then, many DC subsets have been described with critical functions in shaping the immune response. For example, Banchereau and colleagues showed that one subtype of DC, a "plasmacytoid" DC, is the principal IFNα-secreting cell in systemic lupus erythematosus with the capacity to induce plasma cell differentiation.[481,482] Other DC subsets can respond to cues from epithelial cells, which secrete the cytokine TSLP, and shape T-cell polarization toward a T_H2 phenotype.[483,484] This may be a critical pathway in promoting allergic inflammation in asthma.[485] Since the initial discovery of DCs in 1973, Steinman and his many colleagues and collaborators have uncovered novel functions of DCs in triggering and shaping immunity as well as inducing tolerance. Many laboratories worldwide are using DCs in both capacities, and Steinman himself was the recipient of a DC-based vaccine against his own pancreatic cancer. Steinman was awarded the Nobel Prize, posthumously, in 2012—tragically, just days after he succumbed to cancer. The Nobel was a fitting tribute to a consummate cellular immunologist whose scientific curiosity and pedigree could be traced to Metchnikoff.

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SECTION II

Organization and Evolution of the Immune System

CHAPTER 3

Lymphoid Tissues and Organs

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INTRODUCTION

The mammalian immune system defends against invading pathogens, by both the innate and adaptive mechanisms. Although cells that can respond to pathogens are scattered in tissues throughout the body, the optimal structures for the response to antigens are organized, compartmentalized cellular aggregates that facilitate antigen concentration and presentation to a large repertoire of antigen-specific lymphocytes. The primary lymphoid organs, the fetal liver, thymus, and bone marrow, are the sites where diverse populations of naïve lymphocytes mature to disperse throughout the body to await foreign invaders. This remarkable differentiation process occurs in a foreign antigen-independent fashion. The secondary lymphoid organs, including the lymph nodes, spleen, Peyer's patches, and other mucosal-associated lymphoid tissues (MALTs), are discrete sites in which naïve, antigen-specific T- and B-lymphocytes encounter invaders to generate an adaptive response. The lymph nodes and spleen have been considered to be somewhat static structures, while, in fact, they are responsive to environmental influences and undergo remarkable changes in the course of antigenic challenge. Precise programs control the development of the spleen, lymph nodes, Peyer's patches, tonsils and adenoids, and (in the mouse and rat) the nasal-associated lymphoid tissue (NALT). Somewhat less anatomically restricted tissues that are even more sensitive to the environment facilitate and include accumulations of lymphoid cells are organized, but less discretely defined: the bronchus-associated lymphoid tissues (BALTs) and inducible lymphoid follicles (ILFs). Tertiary lymphoid organs, or more accurately, tertiary lymphoid tissues, are accumulations of lymphoid cells that arise ectopically in sites that are not anatomically restricted and are not regulated by developmental programs. Tertiary lymphoid tissues respond to environmental stimuli and arise during chronic inflammation subsequent to microbial infection, graft rejection, autoimmunity, or cancer by the process of lymphoid neogenesis.[1,2]

In this chapter, the structure, function, trafficking patterns, and developmental signals that regulate the hierarchy of lymphoid organs will be described.

PRIMARY LYMPHOID ORGANS

The primary lymphoid organs are the sites where pre-B- and pre-T-lymphocytes mature into naïve B and T cells in the absence of foreign antigen. Each T cell or B cell expresses a unique receptor that can recognize and respond to exogenous antigen and, in most cases, discriminate between self- and foreign antigens. Naïve cells leave the primary lymphoid organs having received and responded to developmental cues that result in the rearrangement of their genetic material to generate a repertoire capable of recognizing and responding to a wide variety of foreign antigens. In the course of maturation in the primary lymphoid organs, the naïve lymphocytes express various chemokine receptors and adhesion molecules that direct them to secondary lymphoid organs.

Fetal Liver

The earliest lymphoid cell precursors derive from self-renewing hematopoietic precursors called hematopoietic stem cells (HSCs). During ontogeny, these cells occupy several niches. In the fetal mouse, the first wave of hematopoiesis occurs in the yolk sac and aorta-gonad-mesonephros region at E10.5.[3] The placenta also contains HSC activity.[4–6] Cells leave these tissues and migrate to the fetal liver, and then the bone marrow and thymus and spleen under the influence of chemokines and adhesion molecules (Fig. 3.1). The cells in the fetal liver respond to CXCL12 (stromal cell derived factor) and, in contrast to those in the bone marrow, also respond to Steel factor[7] (Table 3.1). The fetal liver is also the source of CD4+CD3− lymphoid tissue inducer cells that express lymphotoxin (LT)α (also called tumor necrosis factor [TNF] β) and LTβ. The requirement of inducer cells for the

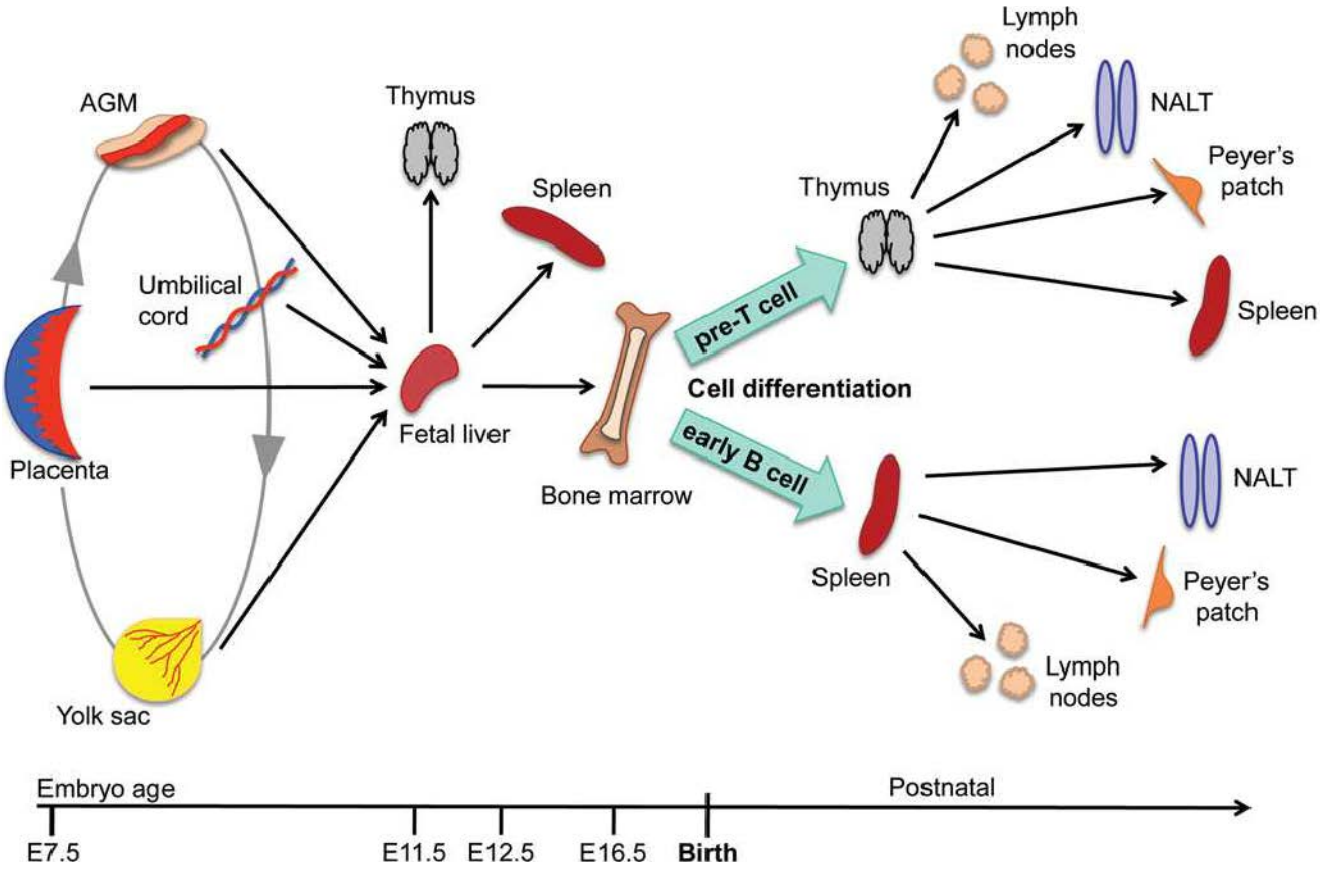


FIG. 3.1. Embryonic and Postnatal Development of T and B Cells in Mouse Primary and Secondary Lymphoid Organs. Embryonic diagram based on information summarized in Medvinsky et al.[6]

development of secondary lymphoid organs is described in the following. Differentiation of HSCs to B cells occurs in the fetal liver, but does not require interleukin (IL)-7. B-cell development is described in detail elsewhere in this volume. Of the several different subsets of B cells, those generated by the fetal liver HSCs are somewhat limited and are of the B-1 subset.[8] They do not give rise to cluster of differentiation (CD)5 B cells, predominately B1-B cells, and do not express terminal deoxynucleotidyl transferase and myosin-like light chain.[8,9] It is not known whether these differences are intrinsic to the cells or are due to differences in the cytokine environment of fetal liver and bone marrow. The ligands for P-selectin, E-selectin, and vascular cell adhesion molecule (VCAM)-1 are required for the cells to leave the fetal liver and home to the bone marrow.[10,11]

TABLE 3.1 Chemokines Implicated in Lymphoid Organ Development and Maintenance

Standard Name	Common Names	Receptor
CXCL12	SDF-1	CXCR4
CXCL13	BCA-1, BLC	CXCR5
CCL17	TARC	CCR4
CCL19	ELC, MIP-3β	CCR7
CCL20	MIP-3α	CCR6
CCL21	SLC, 6Ckine	CCR7
CCL25	TECK	CCR9
CCL28	MEC	CCR10

BCA, B-cell chemoattractant; BLC, B lymphocyte chemoattractant; ELC, EBI-1 ligand chemokine; MEC, mucosae as-associated epithelial chemokine; MIP, macrophage inflammatory protein; SDF-1, stromal cell derived factor; SLC, secondary lymphoid tissue chemokine; TARC, thymus and activation regulatory chemokine; TECK, thymus-expressed chemokine.

Bone Marrow

Functions

The bone marrow is the source of self-renewing populations of stem cells. These cells include hematopoietic precurscor cells, HSCs, and endothelial progenitor cells, which may derive from a single precursor.[12] The adult bone marrow contains stem cells that can differentiate into adipocytes, chondrocytes, osteocytes, and myoblasts.[13] Collectively, these cells are called hematopoietic/stem progenitor cells. In the adult, cells leave the leave the bone marrow and seed the thymus where they undergo differentiation to naïve T cells. Additional factors enable the differentiation of immature B cells from HSCs.

In addition to serving as a primary lymphoid organ where B-cell differentiation and development occur, the bone marrow is also a home for antibody secreting cells.[14] After B cells

have interacted with antigen in the secondary lymphoid organs, such as the lymph nodes, spleen, and Peyer's patches, they enter the bloodstream and travel to the marrow. Thus, this organ not only serves as a primary lymphoid organ, but also as a reservoir for fully differentiated plasma cells.

Architecture: Cellular and Functional Niches

The microenvironment of the bone marrow, contained in the central cavity of bone, is a complex three-dimensional structure, with cellular niches that influence B cells during their development and later, as plasma cells, as they return to the bone marrow. The bone marrow has a rich blood supply with a nutrient artery that branches into ascending and descending arteries further dividing into cortical capillaries, periosteal capillaries, and endosteal capillaries, finally merging into a sinus.[15] Previously, the prevailing understanding of B-cell differentiation in the bone marrow was that primitive HSCs were located in close contact with the endosteum near osteoblasts (the "endosteal niche"). During the course of differentiation into mature B cells, they moved into the central region of the bone marrow cavity (the "vascular niche").[16] The former niche was identified as the location of HSC; the latter, as the site of B-cell differentiation.[17] This anatomic concept has been challenged, as it has been reported that HSCs are found throughout the bone marrow. More recently, a reticular niche has been described that includes CXCL12 abundant reticular cells.[18] Growth factors and cytokines produced by different stromal cells influence cells at different stages in their differentiation. Thus, it is more appropriate to consider functional or cellular, rather than anatomical, niches.[15,19] Once the HSCs differentiate into immature B cells expressing cell surface immunoglobulin (Ig) M, they undergo processes of negative selection and receptor editing, leave the bone marrow, and travel through the blood stream to the secondary lymphoid organs where they complete their differentiation.

Several cytokines and chemokines influence B-cell differentiation in the bone marrow. Flt-3 ligand (also called Flk-2L) signals B-cell differentiation and growth and synergizes with several other hematopoietic growth factors.[20] Its receptor, Flt-3 (also known as Flk-2), expressed by primitive HSCs, is a member of the class II tyrosine kinase family. In contrast to fetal liver, HSCs from adult bone marrow do not respond to Steel factor. Chemokines contribute to B-cell differentiation in the bone marrow and define the functional niches. For excellent reviews of this topic, see Nagasawa,[15] Heissig et al.,[18] and Mazo et al.[21] Many of these factors affect other lymphoid cells, such as dendritic cells (DCs) and T cells. Several of these factors, whose functions have been identified in gene deletion studies in mice, in morphologic analysis, and in cell culture studies, play roles in multiple aspects of lymphoid organ development; their activities, though important in the bone marrow, are not limited to that organ. CXCL12, also known as stromal cell derived factor, is a chemokine that is crucial for recruitment of HSCs to the bone marrow. It is widely expressed by osteoblasts, reticular cells,[19] and endothelial cells.[22] In fact, the interaction of HSCs expressing CXCR4, the receptor for CXCL12, with that chemokine on the endothelial surface is the first step in the HSC's exit from the circulation into the marrow.[23] CXCL12 is also essential for the earliest stage of B-cell development (pre-pro-B cells). Its receptor, CXCR4, is expressed on early B cells and is downregulated in pre-B cells. CXCR4 remains at low levels in immature B cells and mature B cells in secondary lymphoid organs, but is upregulated after B cells interact with antigen and differentiate into plasma cells.[14] This explains the propensity of antibody-secreting cells to return to the bone marrow. Once a pre-pro-B cell has interacted with CXCL12, it moves on to a different cell expressing IL-7. In B-cell development, IL-7 acts later than CXCL12 in a narrow window between pro-B cells and immature B cells in the scheme proposed by Nagasawa.[15]

Traffic In and Out: Chemokines and Adhesion Molecules

The extensive vascularization of the bone marrow allows entrance of hematopoietic precursors and plasma cells and egress of mature cells. Hematopoietic progenitor recruitment to the bone marrow in the mouse is dependent on the interaction of a variety of chemokines, integrins, and selectins, and their receptors, counter receptors, and vascular cell adhesion molecules. These include α_4 integrin (VLA4 or $\alpha_4\beta_1$), VCAM-1,[24] P-selectin glycoprotein ligand-1, E-selectin,[25] $\alpha_4\beta_7$, and mucosal addressin cell adhesion molecule (MAdCAM-1).[26] A small subpopulation of newly formed B cells in the bone marrow that expresses L-selectin has been described,[27] suggesting a mechanism for entrance into lymph nodes (see subsequent discussion).

In addition to the acquisition of Ig expression that occurs in the bone marrow under the control of stromal cells, B cells express various chemokine receptors and adhesion molecules in the process of differentiation. In a study of human bone marrow B cells, it was determined that pro-B and pre-B cells migrate toward CXCL12, but not toward a wide range of other chemokines, including CCL19 and CCL21, though they do express low levels of CCR7, the receptor for those ligands. However, mature bone marrow B cells do respond to CCL 19 and CCL 21,[28] CXCL13 (indicating a functional CXCR5), and CCL20 (MIP3α). On the other hand, CCR6 expression and responsiveness to CCL20 are only seen in mouse B cells after they have migrated into the periphery during the process of maturation and are in the circulating B cell pool.[29]

Thymus

Functions

The thymus is defined as a primary lymphoid organ due to its inimitable role in T-cell development. Indeed, a mutant known as the "nude" (*nu/nu*) mouse, which lacks a normal thymus, is completely devoid of mature T cells.[30] Although some studies suggest that T cells can develop extrathymically in organs such as the "cervical thymus"[31] and the gut epithelium,[32] the thymus remains the main site for T-cell maturation, education, and selection. T-cell precursors represent more than 95% of total cells in the thymus and give rise to mature T cells. These cells are crucial components of the adaptive immune system in that they are highly specific in their ability to recognize a nearly infinite number of antigens owing to their diverse repertoire.[30,33]

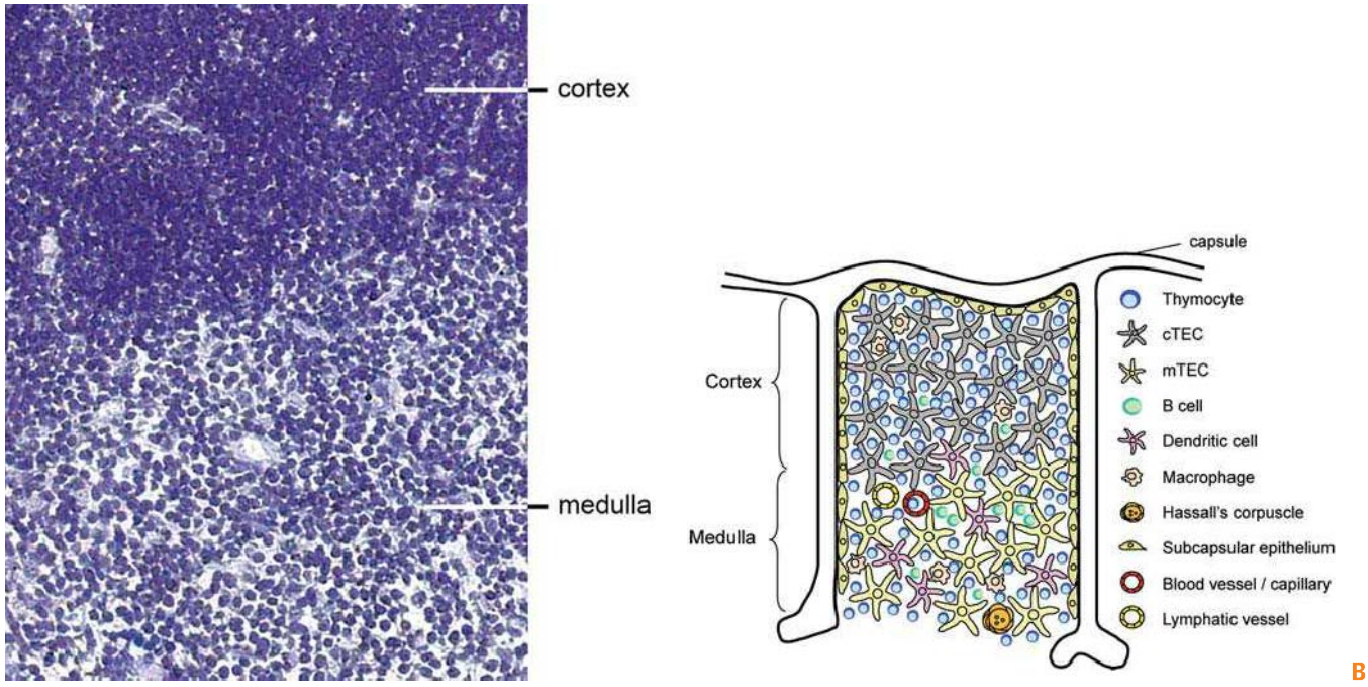


FIG. 3.2. A: Thymic structure. Low power magnification of a hematoxylin-stained frozen section of a mouse thymus. The cortex is well populated with lymphocytes; the medulla is less tightly packed and thus stains less intensely. **B:** Diagram of mouse thymic cellular populations. Precursor T cells enter through blood vessels at the corticomedullary junction. They progress to the medulla where they undergo differentiation from double negative to double positive (DP) cells expressing T-cell receptors. Thymic stromal cells provide growth factors. DP cells undergo positive selection, under the influence of cortical thymic epithelial cells. Single positive (SP) cluster of differentiation (CD)4 or CD8 cells migrate into the medulla where they undergo negative selection, mainly through autoimmune regulator expressing medullary thymic epithelial cells and dendritic cells. SP cells, having undergone differentiation, exit through blood vessels (not shown) to the periphery.

The T-cell repertoire is shaped during development in the thymus by the processes of positive and negative selection. Negative and positive selection are so stringent that nearly 95% of all T-cell precursors are deleted in the thymus.[34] Negative selection ensures that T cells, which are capable of recognizing the organism's self-antigens presented on major histocompatibility complex (MHC) class I or II molecules with high affinity, are eliminated prior to their export to the periphery. In contrast, positive selection, which requires a higher degree of T-cell receptor (TCR) avidity than negative selection,[35] allows for T cells recognizing self-antigen with low to medium affinity to leave the thymus and eventually protect the organism against invading pathogens.

While negative selection is highly efficient in eliminating the majority of self-reactive T cells, some of these cells do escape the thymus and exit to the periphery. These autoreactive cells impose a threat to various organs as their activation may result in the development of autoimmune diseases. The immune system has evolved several ways to prevent the activation of autoreactive T cells in the periphery. One mechanism that protects against autoimmunity is the generation of a T-cell population capable of suppressing activation of self-reactive T cells. These thymus-derived protective T cells, known as regulatory cells (T_{reg}), recognize self-antigens with relatively high affinity.

Architecture

The thymus in the mouse consists of two symmetric lobes located above the heart, while in humans the thymus is multilobed. Each lobe can be divided into three distinct regions: capsule, cortex, and medulla. The latter two regions harbor thymocytes at various maturation stages. Although maturing T cells constitute the majority of cells in the thymus, other cell types such as macrophages, DCs, B cells, and epithelial cells are also present.[36] Histologic analysis of the thymus reveals a clear distinction between the thymic cortex and medulla, which are separated by a corticomedullary border. The thymic cortex appears darker and more densely populated with T-cell precursors, whereas, the medulla appears considerably lighter and contains smaller numbers of T cells relative to other cell types (Fig. 3.2A). Blood vessels and small blood capillaries are found throughout the thymus. The fact that T-cell progenitors are found in the more highly vascularized corticomedullary border suggests that blood vessels in this region facilitate the entry of T-cell progenitors into the thymic parenchyma.[37] In the thymic medulla, the close association between medullary thymic epithelial cells and thymic blood vessels[38] suggests that these vessels may act as organizers of the medullary thymic compartment. Lymphatic vessel distribution coincides with that of blood vessels and capillaries.[39] The majority of lymphatic vessels are located in the thymic medulla, though some can also be found in the cortex. The role of lymphatic vessels in thymic function is unclear, although it has been proposed that these vessels deliver extrathymic antigens into the thymus or export mature T cells from the thymus into the circulation.

Cellular Composition and Functions

T-cell precursors at various stages of differentiation represent the majority of cells in the thymus. Antigen-presenting cells (APCs) of either hematopoietic or stromal origin mediate the education and selection of T cells in the thymus. The different stages in T-cell selection and maturation that take place in distinct regions of the thymus (Fig. 3.2B) are discussed at length elsewhere in this volume. Briefly, lymphoid progenitors enter the thymus at the corticomedullary border.[37] Following their entry, these cells, identified as double negative (DN) T cells due to a lack of expression of the cell surface molecules CD4 and CD8, undergo four maturation steps termed DN1 to DN4, which are distinguished by the expression of two additional cell surface molecules: CD25 and CD44.[40,41] DN3 cells migrate to the subcapsular zone while rearranging their TCRβ chain and expressing it in combination with a surrogate α chain. Those cells that have successfully rearranged the genes for α and β chains of the TCR become double positive (DP) cells, and express both CD4 and CD8 surface markers. In the cortex, DP cells undergo negative and positive selection.[42,43] Positively selected DP cells further differentiate into single positive (SP) cells expressing either CD4 or CD8. Following their differentiation, SP cells relocate to the medulla where they mature and undergo further rounds of deletion. SP cells that do survive are then exported out of the thymus.[44] DN precursors can give rise to an additional T-cell population expressing the γδTCR. These T cells are distinct from TCRαβ T cells in their tissue distribution and recognition of antigens. TCRαβ DP cells control the development of TCRγδ cells via the production of LTβ.[45] T_{reg} development in the human thymus occurs at the DP stage allowing for the production of CD4 and CD8 positive T_{reg}s.[46]

The thymic parenchyma consists of a complex three-dimensional structure supported by thymic epithelial cells (TECs). TECs in the thymic cortex and thymic medulla are phenotypically and functionally distinct and support different stages of T-cell maturation. Several cell surface markers are used to distinguish medullary TECs (mTECs) from cortical TECs (cTECs) in the mouse. Among these markers are the cytokeratins K5 and K8, the adhesion molecule Ep-CAM, and the glycoprotein Ly-51. K8 and Ly-51 are expressed by cTECs, while K5 and Ep-CAM are expressed by mTECs.[47,48] TECs are unique in that they express MHC II constitutively, similarly to professional APCs. The role of TECs in T-cell selection was recently elucidated by their expression of the transcription factor, autoimmune regulator (AIRE). This transcription factor plays an important role in T-cell selection and prevention of autoimmunity, as illustrated by the fact that humans with a mutated form of the *AIRE* gene exhibit polyendocrine autoimmunity due to inadequate T-cell selection.[49,50] It has become clear that AIRE controls the expression of certain tissue-restricted antigens in the thymus, such as insulin, a protein that is unique to the β cells of the islets of Langerhans in the pancreas.[48,51] The expression of tissue-restricted antigens in the thymus facilitates the negative selection of maturing T cells, which would otherwise be allowed to leave the thymus. The thymic medulla includes distinct structures also known as Hassall corpuscles. Originally described by Arthur Hill Hassall in 1849, this structure consists of concentric stratified keratinizing epithelium. Hassall corpuscles are implicated in several processes of the thymus,[52] including the expression of tissue-restricted antigens, such as insulin (a key antigen in type 1 diabetes),[53] Igs, and filaggrin (key antigens in rheumatoid arthritis),[54,55] and serving as a prominent site for T-cell apoptosis.[56]

DCs are professional APCs that are found in the thymus. Thymic DCs can be divided into two distinct populations. The first population originates from a thymocyte precursor,[57,58] whereas the second population is derived from partially mature peripheral DCs that continuously enter the thymus from the circulation.[59] DCs do not appear to be involved in positive selection, but do contribute to negative selection.[60–63] They have also been implicated in the selection of T_{reg}s in humans,[64] and recent reports in the mouse suggest that peripheral DCs can migrate to the thymus and serve as efficient inducers of T_{reg}s.[65] Activation of thymic DCs can be mediated in part by the IL-7–like cytokine, thymic stromal lymphopoietin. In the human thymus, Hassall corpuscles produce thymic stromal lymphopoietin, and thymic stromal lymphopoietin–activated DCs can alter the fate of self-reactive T cells from deletion to positive selection of T_{reg}s.[66] More specifically, thymic stromal lymphopoietin expression in a subset of DCs, plasmacytoid DCs, can induce the generation of particularly potent T_{reg}s from CD4+CD8−CD25− thymocytes.[67] These findings highlight the heterogeneity of DCs and emphasize their ability to fulfill different roles during T-cell development in the thymus.

Macrophages and B cells are additional hematopoietic-derived professional APCs in the thymus. In contrast to DCs, thymic macrophages are located throughout the thymus and do not play a significant role in T-cell selection.[68] B cells are detected in human and mouse thymus at relatively low numbers[69,70] and are characterized by the expression of the cell surface molecule CD5. They are capable of producing antibodies of several different isotypes.[71] It has been suggested that thymic B cells induce negative selection in developing thymocytes,[69] although B cell–deficient mice show a limited T-cell repertoire when compared with normal mice, also suggesting a role in positive selection.[72] Recent data consistent with a role for B cells in thymic negative selection have emerged. Mice whose B cells were experimentally manipulated to express a myelin oligodendrocyte glycoprotein peptide show deletion of myelin oligodendrocyte glycoprotein–specific T effector cells.[73] One mechanism for the effect of B cells on T-cell selection is suggested by the observation that B cells play a role in the control of tissue-restricted antigens. Studies done in mice deficient in B cells showed fewer thymic epithelial cells with reduced expression of insulin and myelin oligodendrocyte glycoprotein, native antigens of the pancreas and brain, respectively.[74]

Traffic In and Out: Adhesion Molecules and Chemokines

Adhesion molecules mediate the extravasation of leukocytes from blood and lymphatic vessels into the thymus. These molecules also play an important role in facilitating lymphocyte homing into various regions of the thymus. Indeed,

thymic blood vessels express the adhesion molecules intercellular adhesion molecule (ICAM)-1, VCAM-1, CD34, peripheral node addressin (PNAd) and vascular adhesion protein-1.[75] The expression of high levels of ICAM-1, VCAM-1, and vascular adhesion protein-1 on venules near the corticomedullary border suggests that these molecules may play a role in the recruitment of thymocyte progenitors. More specifically, vascular adhesion protein-1, which is restricted to the venules surrounding the sites of progenitor homing, can mediate the extravasation of leukocytes. The regional distribution of these adhesion molecules further illustrates the importance of a distinct anatomical separation between cortex and medulla and represents their individual functions.

Chemokine-mediated T-cell migration and traffic to and within the thymus is crucial for normal T-cell selection. The compartmentalization of the thymus is orchestrated by a milieu of chemokines. The thymic cortex is involved in the maturation of DN cells to DP cells and by positively selecting DP T cells capable of recognizing MHC:peptide complexes and eliminating the cells that are not. The thymic medulla acts as a site of negative selection of SP T cells based on TCR recognition avidity to self-antigens and possibly positive selection of T_{reg}. Various chemokines that are produced by the thymic cortex and medulla allow T cells expressing different chemokine receptors to home to specific regions of the thymus. This differential expression of chemokines is complemented by the fact that T cells at different maturation states express different chemokine receptors. During development, the entry of lymphoid progenitors into the thymus is highly dependent on CCL21 and CCL25, which bind the chemokine receptors CCR7 and CCR9, respectively. Mice lacking CCR7 or CCL21[76,77] show a transient delay in thymus colonization by lymphocytes (day 14.5), and this delay is further extended (day 17.5) in mice lacking CCR9.[78] Lymphoid progenitors enter the thymus at the corticomedullary border and commence their migration outwards toward the subcapsular region of the cortex as DN3 cells. The expression of the chemokine receptors CXCR4 and CCR7 by DN cells is important in directing cell migration.[79,80] In the subcapsular region, DN thymocytes that have successfully rearranged their TCRαβ chains progress to the DP cell stage. Positively selected DP cells move inwards toward the thymic medulla for further differentiation into SP cells. The ligands for CCR7 are crucial in mediating the migration of positively selected DP cells into the medulla as illustrated by the fact that a deficiency in CCR7 or its ligands, CCL19 or CCL21, prevents DP cell relocation from the cortex to the medulla resulting in abnormal central tolerance.[81,82] This abnormal tolerance is associated with a reduction in thymic B-cell numbers and reduced expression of tissue-restricted antigens.[74] Interestingly, antigen itself can control T-cell migration speed from the cortex to the medulla. In the presence of a negative selection ligand, T cells slow down considerably and are limited to a confined zone 30 μ in diameter allowing for a more prolonged selection and induction of developmental arrest.[83]

The export of positively selected SP T cells out of the thymus is also dependent on chemokines. Chemokines involved in T-cell emigration are CXCL12 and its receptor CXCR4, which repel SP cells out of thymus,[84] and CCL19, which promotes T-cell emigration from the thymus of newborn mice.[85] The chemoattractant, sphingosine 1-phosphate (S1P), is an additional mediator of T-cell emigration. SP T cell express a S1P receptor ($S1P_1$) and are attracted to the high levels of S1P present in the serum promoting their egress.[86,87]

While the role of different thymic compartments and chemokines in the maturation of naïve SP T cells has been extensively studied, the thymic regions and chemokines that control the selection of regulatory T cells remain largely unknown. It may be that chemokines produced by both cTECs and thymic DCs play a role in regulatory T-cell selection, albeit during different stages of maturation.

Development

The initial development of the thymus at midgestation in the mouse is independent of vascularization or bone marrow–derived cells. In the mouse, the thymus rudiment is first evident on day 11 of gestation as it evolves from the endoderm of the third pharyngeal pouch.[88] This gives rise to the thymic lobes as well as to the parathyroid gland. On day 12.5 of gestation, a separation of the primordium is observed and by day 13.5 a distinct thymus is apparent. Evidence has suggested that not only the pharyngeal pouch endoderm but also the ectoderm may also be involved in thymic development.[89,90] More specifically, it was suggested that the pharyngeal pouch ectoderm contributes to the development of cTECs whereas the endoderm contributes solely to the development of mTECs. This "dual origin" model was mainly supported by histological data as well as data collected from thymi of *nu/nu* mice.[91–93] More recently, it was shown that both thymic cortex and medulla are derived solely from the pharyngeal pouch endoderm[94]; although the endoderm and ectoderm are found in close proximity between gestational day 10.5 and 11, only the endoderm actively contributes to thymic development.[95] These findings support the so-called single origin model of thymus development.

The contribution of lymphocytes to the normal development of thymic cortex and medulla is well recognized. In models of T-cell deficiency, cTEC and mTEC development is halted at different stages depending on the stage of T-cell arrest. If T-cell development is arrested at the DN stage, as in the recombination activating gene–deficient mouse, the thymic medulla is greatly reduced while the thymic cortex remains unaffected.[96,97] A more severe phenotype is observed in transgenic mice that overexpress the human CD3 signaling molecule. In these mice, T-cell arrest occurs earlier than in recombination activating gene knockout mice, leading to a loss of both cortex and medulla and to a shift from a three-dimensional to a two-dimensional structure of the thymic epithelia.[97]

Recently, LTα and LTβ have been identified as master regulators of mTEC development and expression of tissue-restricted antigens. LTα and LTβ are members of the TNF superfamily and mediate the processes of secondary lymphoid organ development and inflammation.[1,98] In the absence of LT, tissue-restricted antigen and AIRE expression are reduced and certain mTEC subpopulations fail to develop.[99,100] T cells, owing to their vast numbers, appear

to be the main source for LT production in the thymus[45]; however, additional resident cells also serve as a source of LT. Recently, resident thymic B cells were identified as the highest source of LTα and LTβ on a per cell basis,[74] revealing yet another aspect of thymic B cells in thymus development and function.

The development of the thymus in humans closely follows the model of thymic development in the mouse and bird. Similar to the mouse, human thymic colonization by hematopoietic stem cells occurs relatively early, at week 8.2 of gestation. During this stage, the thymic medulla and cortex are organized, suggesting that thymocytes are required for normal thymic development. Between gestation week 9.5 and 10, the first signs of thymocyte negative selection are evident, and by gestation week 10 to 12.75 the gradual onset of positive selection is detected.[101]

SECONDARY LYMPHOID ORGANS

Naïve cells express their receptors for specific antigen, leave primary lymphoid organs, circulate through the bloodstream, migrate into the tissues, and lodge in secondary lymphoid organs. The frequency of naïve cells specific for an individual antigen is quite low (estimates range from 1 in 10^5 to 1 in 10^6). Thus, the chance that an individual T or B cell will encounter its specific antigen in the circulation is rather low. Secondary lymphoid organs are strategically located in anatomically distinct sites where foreign antigen and APCs efficiently concentrate and activate rare antigen-specific lymphocytes, thus leading to the initiation of adaptive immune responses and generation of long-lived protective immunity. These organs include highly organized, compartmentalized, and mostly encapsulated tissues such as lymph nodes, spleen, appendix, tonsils, murine NALTs, and Peyer's patches. Naïve cells are also primed in less discrete tissues throughout the body, including the BALTs, cryptopatches, and ILF.

Lymph Nodes

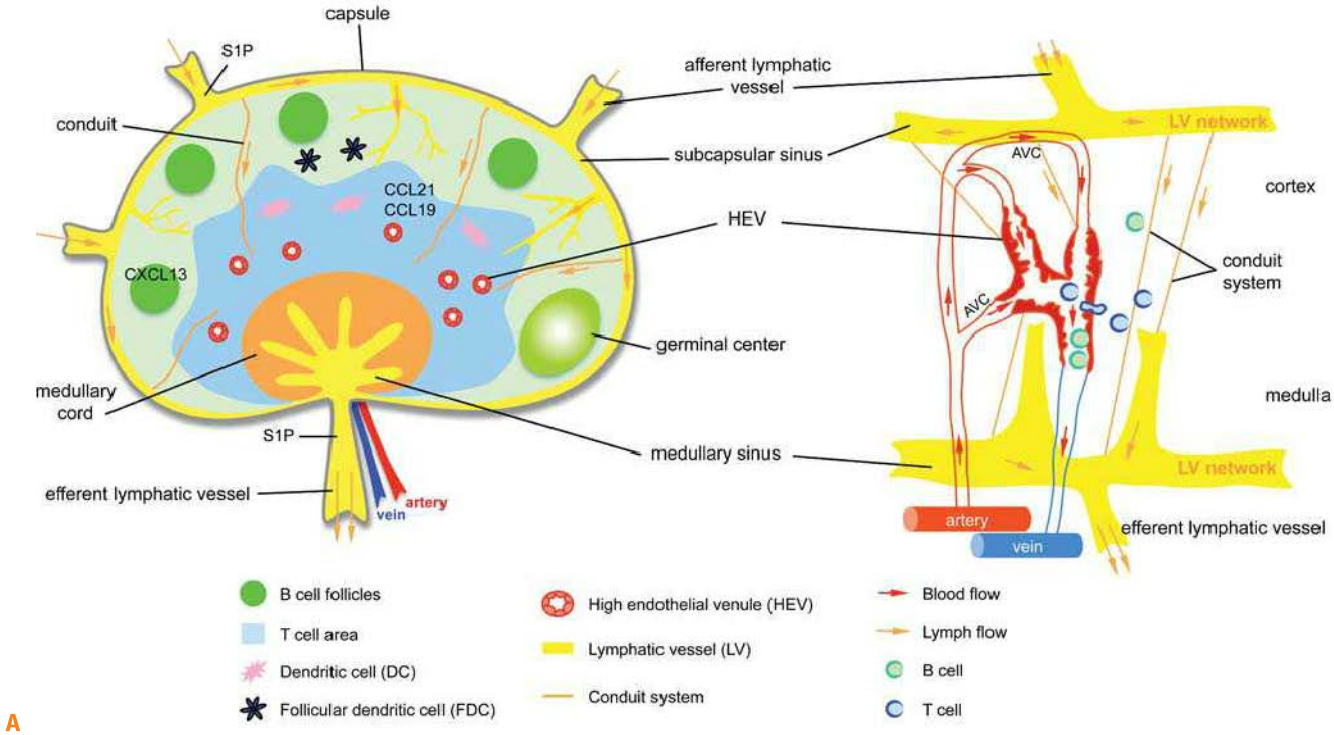
Lymph nodes are bean-shaped structures dispersed along lymphatic vessels. The lymphatic vessel system plays important roles in tissue fluid balance, fat transport, and the immune response. In contradistinction to blood vessels, which form a closed recirculating system, lymphatic vessels comprise a blind-end, unidirectional transportation system. The absorbing lymphatic vessels, or lymphatic capillaries, remove interstitial fluid and macromolecules from extracellular spaces and transport the collected lymph through the primary collector. The collected lymph and its cellular contents are transported into the thoracic duct and returned back to blood circulation. In humans, lymph collected from the entire lower body region, the left head, and left arm region accumulates in the thoracic duct and returns to blood circulation via the left subclavian vein; lymph collected from right head and right arm region returns to blood via the right subclavian vein.

Lymph nodes, usually embedded in fat, are located at vascular junctions, and are served by lymphatic vessels that bring in antigen, and connect them to other lymph nodes. Though most lymph nodes are classified as peripheral lymph nodes, a few (cervical, mesenteric, and sacral), termed mucosal nodes, express a slightly different complement of endothelial adhesion molecules, cooperate with the mucosal system, and are regulated somewhat differently in development (see following discussion). Although all lymph nodes are vascularized, and thus can receive antigens from the bloodstream, they are also served by a rich lymphatic vessel system and are thus particularly effective in mounting responses to antigens that are present in tissues. These antigens may be derived from foreign invaders that are transported by APCs or can be derived from self-antigens. Thus, lymph nodes extend the role of the primary lymphoid organs and discriminate between dangerous foreign antigens and benign self-antigens. This capacity relies on the APCs and their state of activation in the lymph node, and the recognition capacity of the naïve T and B cells.

Lymph nodes can also function as niches for generating peripheral tolerance, an additional mechanism to minimize the effects of those self-reactive T-cells that escape central tolerance in the thymus.[102] DCs constitutively sample self-antigens and migrate to draining lymph nodes even in the steady state.[103–105] Because most self-antigen–bearing DCs in lymph nodes are immature[106] and have low levels of costimulatory molecules, they are not effective at activating naïve cells. They regulate self-reactive T cells by inducing anergy, clonal deletion, and/or expanding T_{regs}.[105–109] Several groups have recently described populations of cells in secondary lymphoid organs that can present tissue-restricted antigens to CD8 cells.[110–114] These include two different populations of AIRE-expressing stromal cells, DCs, and AIRE-negative lymphatic endothelial cells. These cells may play a role in self-tolerance, or perhaps they can prime for autoimmunity in inflammatory conditions.

Structure and Organization

A collagen capsule surrounds the highly compartmentalized lymph node (Fig. 3.3). The cortical region includes discrete clusters called primary follicles consisting of densely packed naïve B cells and follicular DCs (FDCs). After B cells encounter their cognate antigen, they are activated. They then proliferate secondary follicles and germinal centers develop. T cells and DCs distribute in the paracortex. Macrophages reside in the subcapsular zone and in the medullary area, and those in the subcapsular zone appear to be particularly adept at presenting antigen-antibody complexes to B cells.[115] Follicular DCs, a population of mesenchymal origin, support B-cell follicles or germinal centers under stimulation.[1] Plasma cells are also concentrated in the medulla as they prepare to leave the lymph node and circulate to the bone marrow. A network composed of reticular fibers, fibrous extracellular matrix bundles, and another mesenchymal cell population, the fibroblastic reticular cells, supports the entire lymph node.[116] Compartmentalization of cells in the lymph node is orchestrated by lymphoid chemokines CCL19, CCL21, and CXCL13. Stromal cells in the paracortical region produce CCL19 and the protein is transported to the surface of high endothelial venules (HEVs).[117] CCL21 is encoded by several genes[118]; CCL21-leu is expressed by lymphatic vessels



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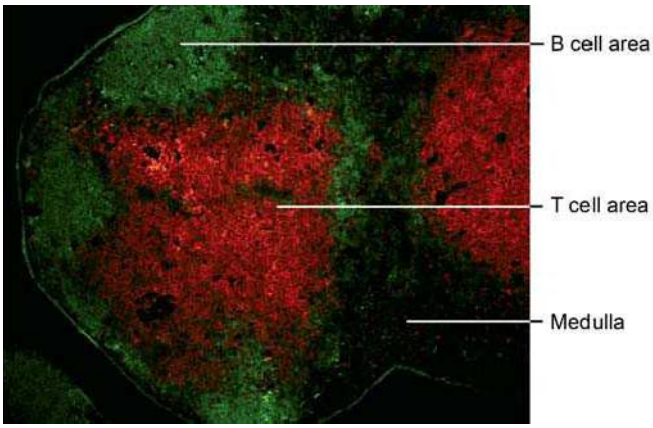


B

FIG. 3.3. A: Lymph node structure and functional regions. The lymph node is divided into an outer cortex and inner medulla surrounded by a capsule and lymphatic sinus. The cortex includes B cells and follicular dendritic cells. The paracortical region includes T cells and dendritic cells. Macrophages are found in the subcapsular sinus and medullary cord. Lymphocytes enter into the lymph node through an artery at the hilus region, and into the parenchyma through high endothelial venules (HEVs) expressing peripheral node addressin and CCL19 and CCL21. Stromal cells also produce these chemokines. T cells and dendritic cells (DCs) are directed to the paracortical region by CCL19 and CCL21. B cells are directed to the cortex by CXCL13. After interaction with antigen and T cells, germinal centers develop. Antigen and DCs drain into the lymph node from the tissues through afferent lymphatic vessels. Antigen continues to percolate through the node via a conduit system. Activated cells leave through efferent lymphatic vessels. Diagram of the blood network of a rat lymph node (*right*) is adapted from Anderson and Anderson.[125] Note the arteriovenous communications, the venous sphincters, and cells leaving the HEVs into the parenchyma. **B:** Lymph node compartmentalization. Immunofluorescent staining of B cells (anti-B220, *green*) in follicles in the cortex and T cells (anti-cluster of differentiation 3, *red*) in the paracortical area. The medulla is unstained.

outside the lymph node[119]; CCL21-ser is made by stromal cells and HEVs in the lymph node. CCL19 and CCL21 recruit CCR7-expressing cells across the HEVs to the paracortical region. CXCL13, produced by stromal cells in the B-cell follicles, attracts CXCR5 expressing B cells.[120] After naïve T and B cells encounter antigen, they undergo extensive changes in expression of chemokine receptors and adhesion molecules that result in their movement to different areas of the lymph node or leaving it all together.[121,122] $S1P_1$ facilitates lymphocyte egress from lymph nodes as they move toward the ligand S1P in the lymph,[123,124] as discussed in more detail subsequently.

Lymph Node Vasculature: Blood Vessels and Lymphatic Vessels

Soluble antigen and APCs enter into lymph nodes via afferent lymphatic vessels. After surveying antigens in the lymph

nodes, lymphocytes leave those organs via efferent lymphatic vessels that can connect to the next lymph node in the chain and finally return to blood circulation.[125–127] In this manner, HEVs and lymphatic vessels maintain lymphocyte homeostasis during the steady state.

Blood endothelial cells play a crucial role in lymphocyte trafficking in the lymph node. One or two arteries enter the lymph node at the hilus. These arteries branch and pass through the medulla area, enter the cortex, and sometimes continue in the subcapsular area. Beneath the subcapsular sinus, the branching capillaries form loops and some of them become arteriovenous communications. Arteriovenous communications become HEVs in the cortex area and occasionally extend from the subcapsular sinus to the medulla. HEVs constitute a specialized postcapillary network in the lymph node, playing a critical role in lymphocyte recirculation. Each main HEV trunk receives three to five branches lined with high endothelial cells and two or three branches lined with flat endothelial cells. The luminal diameters of HEVs progressively increase from cortex to medulla. Finally, HEVs merge into segmental veins in the medulla area and join larger veins in the hilus[125] (see Fig. 3.3A). Intravital microscopy has revealed that the entire venular tree consists of five branching orders with the higher orders in the paracortex and the lower orders located in the medulla and hilus areas. Only the higher order venules, located in the T-cell area, are specialized into HEVs and are recognized by the monoclonal antibody MECA-79.[126,127] Recirculating naive lymphocytes leave the bloodstream via HEVs, specialized vessels with a high cuboidal endothelium, and migrate into the lymph node parenchyma. PNAd, defined by the MECA-79 antigen, is an L-selectin ligand, and is a characteristic HEV adhesion molecule. PNAd is composed of a variety of core glycoproteins, including GlyCAM-1, CD34, Sgp200, and podocalyxin; these proteins must be sialylated, sulfated, and fucosylated to become functional L-selectin ligands. The several enzymes that mediate these posttranslational modification events include FucT-IV, FucT-VII, and GlcNAc6ST2 (also called HEC-6ST, LSST, GST-3, HEC-GlcNAc6ST, gene name *Chst4*),[128–132] which with the exception of a population of cells in the intestine[133,134] is uniquely expressed in high endothelial cells. Together with another sulfotransferase, GlcNAC6ST1, that is expressed at sites in addition to HEVs, it sulfates glycoproteins in the Golgi apparatus[135] to generate the MECA-79 epitope. PNAd, expressed on the endothelial surface, slows down (tethers) naïve L-selectin^{hi} lymphocytes in their progress through the blood vessels. After this initial interaction, CCL19 and CCL21 on the HEVs are instrumental in activating the lymphocyte integrin lymphocyte function–associated antigen (LFA)-1. This results in tight binding of LFA-1 to ICAM-1 on the HEV, facilitating diapedesis of lymphocytes that migrate between or through the endothelial cells toward the chemokines located in the paracortical region (T cells, DCs) or cortex (B cells). Data suggest that the HEV-lymphocyte interaction is not random in different lymphoid tissues in the mouse.[136,137] T-lymphocytes adhere preferentially to peripheral lymph node HEVs, B cells prefer to adhere to HEVs in Peyer's patches, and T and B cells exhibit an intermediate pattern of adhesion to mesenteric lymph node HEVs.[34,136,138] The selective adhesion of naïve lymphocytes to HEVs is at least partially controlled by the differentially expressed adhesion molecules in different lymphoid organs. PNAd rapidly replaces MAdCAM-1 after birth in mouse peripheral lymph nodes,[139] but is expressed in mucosal lymph nodes together with MAdCAM-1, the ligand for the integrin $\alpha_4\beta_7$.

Lymphatic vessels also play critical roles in the immune response. The collected lymph and cell contents enter the lymph node via several afferent lymphatic vessels and filter through the node where they again are concentrated in the medullary sinus. Lymphatic vessels are concentrated in the subcapsular sinus and medullary area.[125] Factors from afferent lymph can be either transported deep into the lymph node cortex or move via the subcapsular sinus and leave the lymph node through efferent lymphatic vessels.[116] In this manner, soluble antigen and APCs from peripheral tissues are efficiently concentrated in the draining lymph node and initiate an adaptive immune response. Lymphocytes can also enter lymph nodes through afferent lymphatic vessels and leave via efferent vessels and move to the next lymph node in the chain. This is accomplished in part through a gradient of S1P, which is at a high concentration in the lymph and low concentration in the lymph node. Lymphocytes in the lymph node downregulate this receptor ($S1P_1$) and then upregulate it as they prepare to leave and migrate toward the higher concentration in the efferent lymph.[123,124] Human and murine lymphatic vessels express several characteristic markers: lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), Prox-1, podoplanin, CCL21, the vascular endothelial growth factor receptor-3 (VEGFR-3), and neuropilin-2.[140]

Conduit System

A conduit system in the lymph nodes physically connects the lymphatic sinus with the walls of blood vessels and enables the incoming factor(s) from lymph to move rapidly deep into the paracortical area.[116,125] The conduit system consists of four layers: 1) a core of type I and type III collagen bundles; 2) a microfibrillar zone composed largely of fibrillins; 3) a basement membrane abundant with laminins 8 and 10, perlecan, and type IV collagen that provides a supportive structure; and 4) fibroblastic reticular cells that embrace the entire conduit system.[141,142] This conduit system enables incoming lymph to penetrate deep into the T-cell area. A special subset of immature DCs, called conduit-associated DCs, can take up and process antigens moving along the conduit.[142] In this manner, the conduit system probably provides a physical support for rapid initiation of adaptive immune responses after immunization.

The Intimate Relationship Between Lymph and High Endothelial Venules

Incoming lymph is necessary for the maintenance of HEV phenotype and function. After afferent lymphatic vessels are severed, dramatic changes occur in HEVs. These include flattening of the endothelium, a decrease in the uptake of ^{35}S-sulphate[143,144] (a functional marker of GlcNAC6ST-2), a reduction of lymphocyte adherence to the vessels,[145–147]

and decreased expression of PNAd and the HEV genes, *Glycam-1* and *Fuc-TVII*.[146–148] An increase in MAdCAM-1 expression[146] suggests that these events are not simply due to a general downregulation of blood vessel gene expression and indicates that continual accumulation of afferent lymph factor(s) in lymph nodes is necessary for HEV maintenance. The nature of such lymph factor(s) is unknown.

Topographic relations between HEVs and the lymphatic sinus have been described in the rat mesenteric lymph nodes. Some HEVs are located in the medulla area and positioned closely in relation to the lymphatic sinus.[125] Occasionally, HEVs are separated from an adjacent lymphatic sinus by only a thin layer of collagen bundles. The closely apposed HEV and lymphatic sinus in the medulla provide the physical support for the intimate relationship between lymph and HEV. However, most HEVs are located in the paracortical area and are separated from the lymphatic sinus by lymphocytes. The conduit system physically connects the subcapsular sinus and HEVs and allows incoming lymph factor(s) to migrate rapidly to the wall of HEVs.[125,149,150] Low-molecular-weight fluorescent tracers (below 70 kD) move rapidly via the conduit system and lead directly to the wall of HEVs. In this manner, low-molecular-weight tracers can migrate with lymph within minutes to HEVs and enter the HEV lumen.[116] Lymph-borne chemokines likely adopt this route to regulate HEV function. IL-8 administration via afferent lymph increases lymphocyte HEV transmigration within minutes.[125,151] In addition, lymph-borne chemokines, such as MIP-1α, also enter the conduit system and move rapidly to HEVs.[116] These data suggest that lymph factor(s) can quickly access and regulate HEVs.

Development

Despite their distinctions in the adult with regard to morphology and expressed genes, a close association of blood vessels and lymphatic vessels is seen during embryogenesis. The generation of embryonic lymphatic vessels from preexisting veins in pig embryos was first described in the early 1900s by Sabin and has recently been molecularly defined.[140] A variety of transcription factors have been identified that contribute to the lymphatic specification and maintenance of the lymphatic vessels phenotype.[152] As early as mouse E9, expression of Sox18 and CoupTFII is apparent in the cardinal vein. These induce Prox1 in the dorsolateral side of the vein resulting in polarization and lymphatic-biased endothelial cells. Prox1 generates a feedback signal for the further maintenance of budding and migration of endothelial cells. Targets of Prox1 include Prox1 itself, podoplanin, VEGFR3, integrin-α, and Nrp-2. Prox1 expression in blood endothelial cells also represses expression of markers of those cells. NfatC1, Foxc2, and Tbx1 are additional transcription factors that are expressed later in lymphatic vessel development and contribute to patterning, pericyte covering of lymphatic vessels, lymphatic vessel valves, and lymphatic vessel maturation. At E11.5-12.0, CCL21 is expressed in these lymphatic-biased endothelial cells, as is VEGFR-3, which is reduced in blood endothelial cells. The endothelial cells expressing LYVE-1, Prox1, VEGFR-3, and CCL21 become irreversibly committed toward a lymphatic pathway.[153,154] The separation of lymphatic endothelial cells from venous endothelium requires a Syk/SLP-76 signal[155] that is provided by platelets.[156,157] Thus, during early lymphangiogenesis, some endothelial cells express both blood vessel and lymphatic vessel markers, indicating the close association of these two vascular systems. Mesenchymal lymphangioblasts may also contribute to early lymphangiogenesis.[158] The lymphatic venous junction remains in adults in only limited regions but plays an essential role in connecting the function of the two vascular systems.

Studies in the mouse have taken advantage of transgenic, knockout, and imaging studies to provide a mechanistic understanding of the process of lymph node development. Although lymph sacs per se do not appear to be essential for the initiation of lymph nodes, a lymphatic vessel system is necessary for the later development and cellular population of lymph nodes.[159] Furthermore, an interaction between mesenchymal cells and primitive endothelial cells is apparent in the early lymph node anlage.[160]

Cytokines, Chemokines, and Transcription Factors in Lymphoid Organogenesis

The TNF-/LT-receptor family members play key roles in secondary lymphoid organ development. $LT_{\alpha 3}$, signaling through TNFRI and TNFRII, and membrane bound $LT_{\alpha 1\beta 2}$, signaling through the LTβ receptor (LTβR) have been implicated. Mice deficient in LTα lack all lymph nodes and Peyer's patches, and exhibit a disorganized spleen and severely disorganized NALT (see subsequent discussion).[161–163] Mice deficient in LTβ lack peripheral lymph nodes but retain mesenteric, sacral, and cervical lymph nodes.[164–166] *Ltβr−/−* mice have a phenotype similar to that of *Ltα−/−* mice.[167] LTβR is also recognized by LTβ-related ligand, LIGHT, which also binds to the herpes virus entry mediator. LIGHT-LTβR signaling does not appear to play an essential role during lymphoid organogenesis, as no significant defect is observed in *Light−/−* mice[168]; however, mice doubly deficient in LIGHT and LTβ have fewer mesenteric lymph nodes than mice deficient in LTβ alone, indicating a cooperative effect of the two LTβR ligands. Treatment of pregnant females with an inhibitory soluble protein of LTβR (LTβR and human IgG Fc fusion protein, LTβR-Ig) inhibits most lymph nodes in the developing embryos, depending on the time of administration. Mesenteric lymph nodes are not inhibited by this treatment. These studies indicate that individual lymph nodes differ in the nature and time of cytokine signaling during development.[169] Several additional cytokine and chemokine-receptor pairs are crucial for lymphoid organogenesis. Mice deficient in IL-7 or IL-7R,[170,171] or TRANCE or TRANCER, also called RANKL and RANK,[172,173] exhibit defects in lymph node development. CXCR5- or CXCL13-deficient mice[174,175] lack some lymph nodes and almost all Peyer's patches.[98] The relative importance of the different cytokines in the development of individual lymphoid organs has been recently summarized.[176]

The NF-κB signaling pathways, downstream of the TNF family receptors, play important roles in lymphoid organ development.[177] The alternative pathway, characterized by NF-κB–inducing kinase (NIK) and IKKα is particularly

important. *aly/aly* mice, which have a point mutation in *Nik*, lack all lymph nodes and Peyer's patches.[178,179] In these mice, LTβR, but not TNFR, mediated signaling between NIK and members of the TRAF family appears to be disrupted.[179–181] LTβR signaling induces gene expression via both the classical and alternative NF-κB pathways in mouse embryo fibroblasts. The classical pathway mediated by p50:p65 heterodimers induces expression of proinflammatory genes (*Vcam-1*, *Mip1b*, *Mip2*). Intraperitoneal injection of an agonistic LTβR antibody induces splenic chemokines (CCL19, CCL21, and CXCL13) and requires NIK activity and subsequent p100 processing.[182] IKKα is a critical component in alternative NF-κB pathway. Mice with a mutated form of the *Ikka* gene have reduced HEV expression of HEC-6ST (GlcNAc6ST2) and GlyCAM-1, further confirming that the LTβR signal regulates HEVs through the alternative NFκB pathway.[183] Several other signaling pathways that contribute to lymphoid organogenesis include the helix-loop-helix transcription factor inhibitor (Id2) and retinoid acid-related orphan receptors (RORs) RORγ and RORγt.[184–186]

Studies of lymph node anlage formation reveal the mechanisms by which cytokines trigger and coordinate lymphoid organogenesis. There is likely an initiating factor, though this has not been definitively identified. In the case of Peyer's patches, a cell producing the receptor tyrosine kinase has been implicated.[187,188] Recent data indicate that the earliest stages of lymph node development appear to be dependent on retinoic acid that may be produced by nerves in the vicinity of the developing node.[189] Circulating CD4+CD3−CD45+ RORγt+ hematopoietic progenitor cells called lymphoid tissue–inducer cells, derived from fetal liver progenitors,[98,185,190–192] provide crucial signals in lymph node organogenesis. These lymphoid tissue–inducer cells accumulate in the developing lymph node, forming clusters with resident stromal organizer cells, to initiate a cascade of intracellular and intercellular events that lead to the maturation of the primordial lymph node.[1,98] During this early step, a positive feedback loop involves several signaling pathways, including LTαβ/LTβR, IL-7R/IL-7, CXCR5/CXCL13, and RANK/RANKL, are expressed on the lymphoid tissue–inducer cells and the stromal organizer cells. The prolonged interaction between lymphoid tissue–inducer cells and stromal organizer cells promotes the development of HEVs, which support the entry of naïve lymphocytes.[193] It is unclear how HEVs differentiate from the flat blood vessels during early lymphoid organogenesis. At birth, HEVs of all lymph nodes express MAdCAM-1, which is replaced in the first few days in peripheral lymph nodes by PNAd.[194] Both MAdCAM-1 and PNAd are expressed in mucosal lymph nodes. LTα alone can induce MAdCAM-1, but PNAd requires LTαβ.[2,195,196] In the remaining mesenteric lymph nodes of *Ltβ*−/− mice, PNAd expression is impaired,[196] indicating that optimal lymph node HEV PNAd expression requires $LT\alpha_1\beta_2$ signaling through the LTβR and the alternative NF-κB pathway.[183] Because the maturation of HEVs is coincident with further development of the lymph node,[139,197] the homing of LT-expressing lymphocytes most likely contributes to HEV maturation. Continual signaling through the LTβR is necessary for maintenance of HEV gene expression.[198,199] Recent data suggest that the actual physical presence of lymphocytes in the close vicinity of HEVs contributes in important ways to the physical cobblestone appearance of these vessels.[200]

Changes in Lymph Nodes after Immunization

Lymph nodes undergo dramatic changes and remodeling after immunization. Early after a variety of immunogenic exposures, such as skin painting with oxazolone, injection of ovalbumin or sheep red blood cells in adjuvant, or bacterial or viral infection, remodeling occurs. This remodeling is apparent as a complex kinetics of changes in lymph flow, lymph cell content, blood flow, HEV gene expression, and lymphatic vessels.[199] Afferent lymph flow and lymph cell content increase soon after initial inflammation, and eventually return to preimmunization levels.[201–205] Lymph node lymphangiogenesis occurs, which eventually resolves.[199,206] Blood flow and lymphocyte migration into lymph nodes peak at 72 to 96 hours,[166,204,207,208] accompanied by an increase in HEV number and dilation,[209,210] accounting for the significant lymph node enlargement apparent at 72 to 96 hours.[207] Efferent lymph flow also increases soon after immunization, but lymph cell content in the efferent lymph drops during the first several hours, indicating the first wave of accumulation of lymphocytes in the draining lymph node. The cell content of the efferent lymph later increases and peaks at 72 to 96 hours.[211] These events, taken together, contribute to the significant enlargement of the draining lymph node at day 4 after immunization. During the early times after immunization, despite the increase in the number of HEVs, the expression of genes that contribute to L-selectin ligand, including *Fuct-vii*, *Glycam-1*, *Sgp200*, and *Chst4*, is initially downregulated followed by a recovery.[148,199,212] However, despite the downregulation of chemokines CCL21 and CXCL12, some genes such as those encoding CXCL9, CCL3, and E-selectin are upregulated,[213] as is MAdCAM-1,[199] suggesting a reversion to an immature phenotype before the eventual recovery of the mature phenotype.

In rodents, HEV maturation is coincident with the continuing development of the lymph node and population of lymphocytes after birth, indicating that the mature HEV phenotype relies on the lymph node microenvironment.[139,197] Plasticity of the mature lymph node is also seen after injection of LTβR-Ig. LTβR-Ig treatment reduces lymph node cellularity, reverts the HEV phenotype to the immature state, inhibits FDC function, and disrupts immune responses to foreign antigens.[198,199,214]

Spleen

Function

The spleen is a large reddish organ located beneath the diaphragm close to the stomach and the pancreas. It is the main filter of the blood and integrates the innate and adaptive responses. Its structure in well-defined different compartments, red pulp and white pulp, determines a variety of functions. The red pulp is a source of hematopoiesis in the embryo that can continue in adult life under stress. Clearance of blood-borne damaged platelets, aged

erythrocytes, and dead cells also occurs in that region. An important function, consequent to its destruction of effete erythrocytes, is its role in iron recycling. In some species, such as horses or dogs, the spleen stores erythrocytes that can be released after stress. The white pulp and marginal zone constitute the highly organized lymphoid compartment of the spleen. Due to this organized lymphoid structure and the special vasculature and circulation, the spleen is a crucial site for blood-borne antigen clearance and presentation to T and B cells. The spleen is crucial in defense against blood-borne pathogens and contributes most significantly to defense against bacterial and fungi infections.[215]

Architecture and Cellular Composition

A fibrous capsule and trabeculae of fibrous connective tissue maintain the structure of the spleen. The general principles of splenic organization are similar across species, though specifics may differ.[216–220] Classically, the spleen can be divided into two areas: red pulp and white pulp. A transit area, the marginal zone (MZ), surrounds the white pulp (Fig. 3.4). These compartments are anatomically and functionally distinct. The red pulp, in its activities as a hematogenous organ, removes damaged cells and acts as a site for iron storage and turnover. The white pulp is an organized lymphoid structure. The complex structure of the spleen is directly related to the complexity of the vasculature of this organ. The afferent splenic artery branches into central arterioles, surrounded by white pulp areas, and end in cords in the red pulp. Blood then collects in venous sinuses that determine the engulfment of erythrocytes by red pulp macrophages. Finally, the sinuses empty into the efferent splenic vein. Some small arterial branches end in the MZ, which demarcates the red and white pulp. The MZ structure differs somewhat between humans and rodents. A perifollicular zone surrounds the human MZ, which consists of inner and outer marginal zones, whereas the rodent MZ is

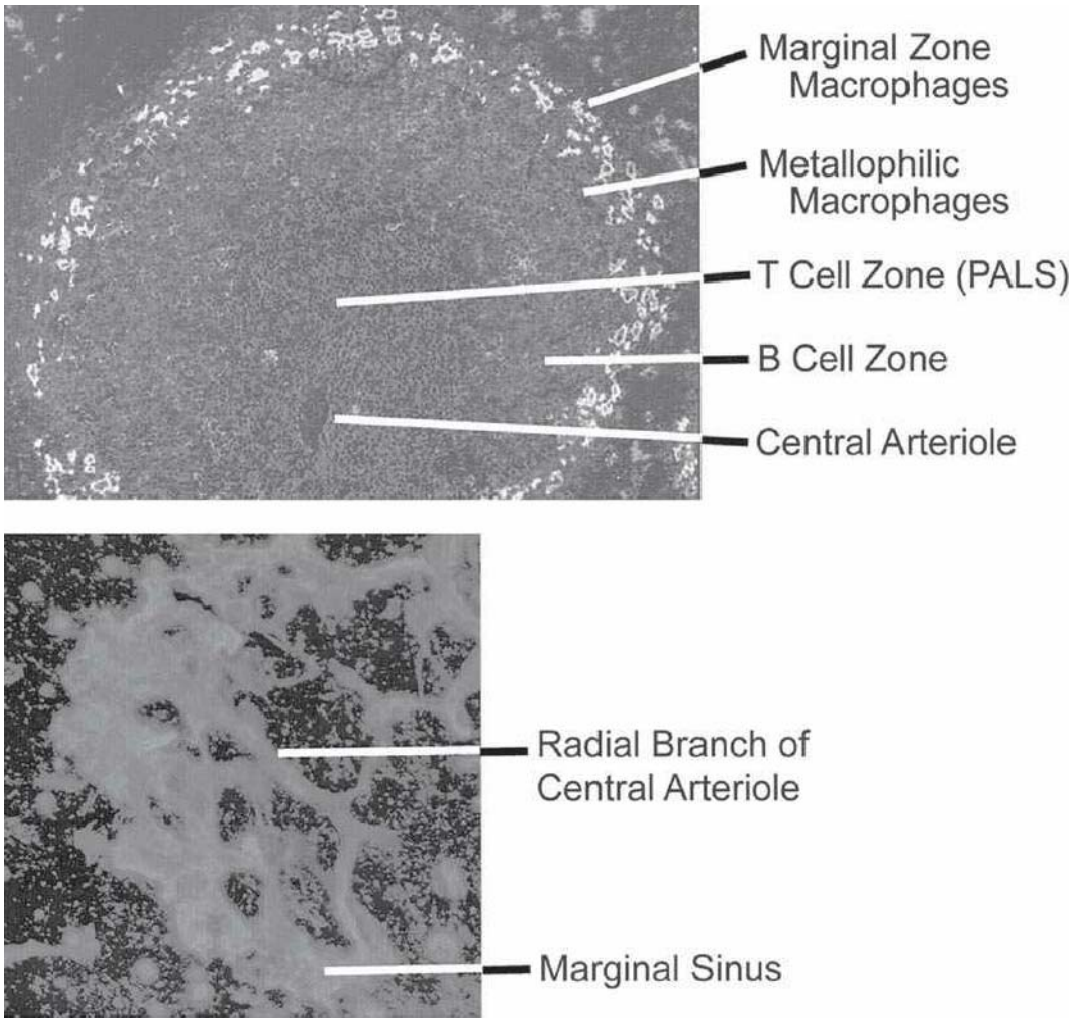


FIG. 3.4. Organization of the Spleen White Pulp. Immunofluorescence staining of a white pulp unit in the mouse spleen. T cells (anti- cluster of differentiation [CD]4+anti-CD8+, *red*) are localized around the central arteriole. B cells (anti-immunoglobulin M, *green*) are localized in follicles around the T-cell area, surrounded by a layer of metalophilic macrophages (labeled with monoclonal antibody-1) and a more peripheral layer of marginal zone macrophages (labeled with monoclonal antibody ERTR9, *orange*). The marginal zone is located between the metalophilic macrophage and the marginal zone macrophage layers (not shown).[357] From Chaplin[358] with permission.

a single structure and has no perifollicular zone. The MZ is the transition between the innate and acquired immune systems and is an important transit area for cells reaching the white pulp. It also contains a large number of resident cells. There are two specialized macrophage populations: the MZ macrophages and MZ metallophilic macrophages. MZ macrophages have a high phagocytic activity and are phenotypically different from other macrophage populations in the spleen as exemplified by the expression of SIGNR1 and macrophage-associated receptor (MARCO) with collagenous structure, molecules implicated in the recognition of pathogens. SIGNR1, a C-type lectin in the mouse that is a homologue of human DC–specific intercellular adhesion molecule,[221] is also found in medullary and subcapsular macrophages in the lymph node. MZ macrophages are crucial Marco for the capture of a wide variety of pathogens, including yeast, bacteria, and viruses.[222,223] MARCO, expressed constitutively on MZ macrophages,[224] is a class A scavenger pattern recognition receptor. In addition to the recognition of blood-borne pathogens, MARCO interplays with MZ B cells, modulating their migration to the white pulp.[225] MZ metallophilic macrophages are located at the inner border of the MZ, in contact with marginal sinus lining cells, stromal cells that express MadCAM-1.[226] MZ metallophilic macrophages differ from the MZ macrophages and red pulp macrophages in that they express the sialic acid-binding Ig-like lectin sialoadhesin (Siglec-1), which binds to oligosaccharide ligands present on many cells.[227] Little is known about the specific roles of this population of macrophages during the immune response; however, the phenotype of mice deficient in sialoadhesin implies a role of MZ metallophilic macrophages in T-cell activation.[228,229] Recent studies confirm that MZ metallophilic macrophages are essential for cross-presentation of blood-borne adenovirus antigens to splenic CD8 DCs, activating cytotoxic T-lymphocytes.[230] The MZ also contains a specialized subset of B cells that differ phenotypically and functionally from follicular B cells; they can be considered as a bridge between the innate and adaptive immune systems. They express higher levels of IgM low levels of IgD and the molecule CD1d. These B cells bind antigen in the MZ directly and/or through interactions with MZ macrophages[231] and migrate to the white pulp, where they present blood-borne antigens.[232] The organization of the remainder of the white pulp of the spleen is similar to that of the other secondary lymphoid organs with compartmentalized B- and T-cell areas. The white pulp consists of a central arteriole that is surrounded by T cells, also known as the periarteriolar lymphoid sheath, which is surrounded by B-cell follicles (see Fig. 3.4). T cells interact with DCs and B cells. B cells migrate to the follicles where they interact with FDCs. At the T:B-cell border, T cells, especially T follicular helper cells,[233] interact with B cells. Germinal centers are the site of somatic hypermutation and Ig class switching. Plasma cells are found mainly in the red pulp.

The spleen does not have an afferent lymphatic system, and initial antigen transport must occur through the blood vasculature. A conduit system has been described that allows antigens and chemokines to be transported through the white pulp in a manner similar to that described for the lymph node.[234] This conduit differs from that in the lymph node with regard to the identity of the transported molecules by the fact that it contacts the blood rather than the lymphatic system.

Traffic In and Out: Chemokines and Adhesion Molecules

Cell trafficking in the spleen is similar to that which occurs in the lymph node in some respects, and differs in others. The MZ is the main transit area for blood cells that enter the white pulp. This process seems to be controlled by marginal sinus lining cells[235] and chemokines, involving signaling through G-couple protein receptors.[236] Stromal cell–produced lymphoid chemokines CXCL13, CCL19, and CCL21 control the migration of lymphocytes and their organization in the various compartments in the white pulp. CXCL13 is essential to positioning of B cells in the follicles, whereas T cells respond to CCL19 and CCL21. After the immune response, germinal center B cells differentiate into plasma cells in the white pulp, then migrate to the red pulp or return the bloodstream to migrate to peripheral tissues. In the migration to the red pulp, plasma cells upregulate CXCR4, a receptor that binds the chemokine CXCL12, which is expressed in the red pulp.

The role of adhesion molecules with regard to lymphocyte traffic in the spleen is not fully well understood. Lymphocytes enter into the white pulp through the marginal sinus and cells lining that structure express MAdCAM-1 and ICAM-1.[226,237] MadCAM-1 expression in these cells seems to be involved in splenic structure development and therefore migration of B and T cells.[235] However, treatment with anti-MAdCAM-1 or anti-$\alpha 1\beta 7$ antibodies does not totally inhibit homing to the spleen, suggesting that this ligand-receptor pair is not the only receptor required for lymphocyte entrance into the white pulp. Early studies indicating that treatment with an antibody that blocks the αL-integrin chain of LFA-1 inhibits its homing by only 20%, and that lymphocytes deficient in LFA-1 enter the white pulp,[237] suggested that LFA-1 is not absolutely essential for entry of all cells into the white pulp. However, both $\alpha_4\beta_1$ and LFA-1 integrins are necessary for B-cell retention in the MZ,[238] indicating a role for VCAM-1 and ICAM-1 in cell trafficking in spleen. Once lymphocytes have encountered antigen, they most likely undergo changes in chemokine receptor and adhesion molecule expression similar to those noted in the lymph nodes, leave the white pulp and enter the red pulp (plasma cells) or the circulation. The mechanisms by which lymphocytes leave the white pulp are unclear. The observation of channels that bridge into the MZ[239] suggests one route of egress of lymphocytes. In the lymph nodes, the process of egress of activated lymphocytes through the efferent lymphatic vessels is mediated by upregulation the expression of $S1P_1$. In the spleen, $S1P_1$ is required for B-cell localization in the MZ, and the interplay with CXCR5 regulates the constant trafficking of these cells between the MZ and the white pulp,[232,240] as MZ B cells from $S1P_1$-deficient mice are not found in the MZ but are found in the follicles.

Although it is still unclear which molecules are involved in the egress of lymphocytes from the spleen, and splenic egress cannot be compared to egress in the lymph nodes,

recent studies attribute an important role to the transcription factor Nkx2.3 in the regulation of spleen vasculature and homing of lymphocytes.[241,242]

Development

Because the spleen has characteristics of both a hematopoietic organ and a secondary lymphoid organ, the genes that regulate its development include, in addition to LT, others that are concerned with patterning and hematopoiesis. Several genes that contribute to the development of the spleen are also crucial for normal development of other nonlymphoid organs. The first sign of splenic development in the mouse occurs at E10.5-11. Segregation of red and white pulp starts to be regulated during embryogenesis and continues after birth. Some structures, such as the MZ, develop during the 3 first weeks after birth. At E10.5-11, progenitor cells begin to condense within the dorsal mesogastrium, adjacent to the stomach and dorsal pancreas. The spleen and pancreas are so intimately associated that it is difficult to distinguish between them at these early stages. In fact, many genes that affect splenic development also contribute to pancreatic development. Several of these are homeobox genes and transcription factors that are expressed in the splenopancreatic mesenchyme at E10.5.[215] The effects of just one of these genes, *Hox11* (now called *Tlx1*[215]), are on the spleen. The others affect multiple organs. *Tlx1* was originally described as an oncogene in T-cell childhood acute leukemias involving the (10;14) translocation breakpoint.[243] *Tlx1*-deficient mice are asplenic,[244] and the product of this gene is a cell survival factor.[245] Several additional genes that are important for development of lymph nodes, NALT, and Peyer's patches also contribute to splenic development. Mice deficient in members of the LT/TNF ligand receptor family and their downstream signaling molecules in the classical and alternative pathways exhibit defects in splenic development. However, none of these molecules is necessary for the early splenic anlagen, as mice deficient in any of the chemokines or cytokines retain a spleen. Recent studies address a role for Glce, an enzyme that modifies heparan sulfate, in early lymphoid tissue morphogenesis, as fetal spleen in Glce deficient mice exhibit a reduced size.[246] The changes in lymphoid tissue organization in mice deficient in LT/TNF ligand receptor family members are due in part to a reduction or near absence of lymphoid chemokines (CXCL13, CCL19, CCL21).[247] Lymphoid chemokine messenger ribonucleic acid (mRNA) expression is reduced in the spleens of mice deficient in TNFR1, TNF, LTα, or LTβ, though CXCL12 mRNA levels are normal. However, treatment with an agonistic LTβR antibody induces expression of the lymphoid chemokines and CXCL12, suggesting that signaling through both the classical and alternative NF-κB pathways are responsible for organization and maintenance of splenic white pulp architecture. Mice deficient in LTα exhibit a disorganized white pulp with loss of T- and B-cell compartmentalization, MZ macrophages, metallophilic macrophages, MZ B cells, MAdCAM-1 sinus lining cells, and germinal centers. *Lt*β−/− mice exhibit similar characteristics except that the disorganization is somewhat less pronounced. However, these lymphoid cytokines seem to be dispensable during fetal spleen development, when segregation between white and red pulp begins, but essential for maintenance of this structure during postnatal development of the white pulp.[248] *Tnf*−/− and *Tnfr1*−/− mice show defects similar to lymphotoxin deficient mice, with the exception of MZ B cells. T cells, B cells, and CD4+CD3− cells produce the cytokines necessary for maintenance of splenic architecture.[249,250]

Plasticity after Virus Infection

Though much is known regarding changes in the lymph node after immunization, the spleen has not been studied as extensively in this regard. However, after infection with cytomegalovirus, white pulp T:B compartmentalization is disrupted.[251] The spleens of *Lt*α−/− deficient mice exhibit a marked reduction in expression of CCL21-ser. This is even further reduced in cytomegalovirus infection, indicating that, in the adult, LT-independent pathways can contribute to maintenance of expression of lymphoid chemokines.

Mucosal-Associated Lymphoid Tissues

General Features

The MALT covers all mucosal surfaces not only in the gut, but also the oropharyngeal and lacrimal mucosae, the nasal and bronchial airways, and the genitourinary tracts. MALT protects a huge surface area and contains approximately half of the lymphocytes of the entire immune system.[252] MALT is considered to be the body's gatekeeper because it is in intimate contact with the commensal flora at the mucosal surfaces.

Although the location of some mucosal lymphoid tissues like the palatine tonsils and appendix are fixed, all MALT are somewhat plastic because of their constant exposure to environmental antigens that induces them to change and remodel. Tonsils, adenoids, and their equivalent in rodents, the NALT, have a fixed location, whereas the location, number, and size of Peyer's patches in the small intestine vary according to antigen exposure. The BALT in the lung and ILFs in the colon are the most plastic MALTs, and their number and location are subject to change by environmental influences.

The mucosal epithelium that surrounds MALT is populated by a dense network of DCs, plasma cells, and intraepithelial lymphocytes that helps to maintain the epithelial barrier. These intraepithelial leukocytes provide retinoic acid and cytokines like IL-10 and TGF-β that condition the gut to become tolerant of antigens produced by the harmless commensal bacteria.[253,254] They are also responsible for inducing and maintaining tolerance to food antigens and commensal bacteria.[255]

The large MALT structures like the tonsils, NALT, and Peyer's patches share many features with lymph nodes. Like lymph nodes, MALT contains HEVs for the entry of naïve lymphocytes, and stromal cells that secrete chemokines that direct lymphocyte traffic in the MALT (CCL19, CCL21 and its receptor CCR7[256]; CCL25 and CCL28 and their receptors CCR9 and CCR10[257]; and CXCL13 and its receptor CXCR5[258,259]). MALT also contains separate T- and B-cell compartments with B-cell follicles, FDCs, germinal centers, and interfollicular T cells and DCs.[260]

MALT differs importantly from other secondary lymphoid organs with regard to its capsules and afferent lymphatic vessels. Unlike the spleen and lymph nodes, which are surrounded by a dense fibrous capsule, MALT often has no capsule, with the exception of the tonsil that has a partial capsule that separates it from the pharyngeal constrictor muscles. Because MALT can sample antigens directly at the epithelial surface, it has no afferent lymphatics. MALT epithelium, especially that of the Peyer's patch, is replaced by specialized lymphoepithelial cells called microfold (M) cells, which because of their high transcytotic capacity, transport antigens to the underlying lymphoid tissue. Tonsils and adenoids are covered by a squamous epithelium and have few M cells. They can sample surface antigens using epithelial DCs that push dendrites through the epithelial surface.[261,262] M cells have not been characterized in human BALT.[263]

The proximity of MALT to the epithelium is an important point, because it helps differentiate MALT from tertiary lymphoid organs (see following discussion). MALT can be defined as organized lymphoid structures in the mucosa that are in direct contact with the epithelium. Using these criteria, a lymphoid aggregate in the submucosa that is not directly in contact with the epithelium (ie, below the lamina propria) is more correctly defined as a tertiary lymphoid organ.[264]

The Mucosal-Associated Lymphoid Tissue Immune Response: Inductive and Effector Sites

MALT can simultaneously be a site for the induction of an immune response and an effector organ. The MALT surface containing M cells and DCs efficiently samples and transports antigens across the epithelium. Immediately below this single-cell epithelium is a dense network of DCs that can extend dendrites through the epithelium to grasp antigen to present to lymphocytes. Two distinct types of DC have been identified in MALT: CD103+ DC and CX3CR1+ DC.[265] Both types of DC produce tolerogenic responses to commensal bacteria by enhancing the differentiation of Foxp3+ T_{reg}s and inhibiting that of inflammatory Th17 cells. CD103+ DCs express αE integrin, make retinoic acid, induce T_{reg}s, and increase T-cell expression of the two gut homing receptors CCR9 and $\alpha_4\beta_7$. CD103+ DCs exit MALT via the lymphatics and present antigen in the draining lymph nodes. CX3CR1+ DCs that express the chemokine receptor for fractalkine do not migrate to the lymph node. CX3CR1+ DCs are longer-lived mucosal resident DCs that produce less retinoic acid than CD103+ DCs.[265]

MALT has three effector roles: local antibody secretion, systemic antibody secretion, and effector lymphocyte dispersal. MALT plays an important role in defense against pathogens by generating cells that migrate to other sites. MALT can dispatch lymphocytes to lymph nodes, the spleen, and plasma cells to the bone marrow. MALT also sends lymphocytes to other MALT effector sites including the salivary and lacrimal glands, the lactating breast,[266] and the vagina. There are some differences between the effector cells made at different MALT sites. NALT-derived B cells express CCR7 and do not home back to the gut. Instead, cells induced in the NALT home to the salivary glands and the vaginal mucosa lymph nodes,[267] whereas lymphocytes leaving the Peyer's patch express $\alpha_4\beta_7$ and migrate to the gut mucosa. These "preferred pathways" for effector cells coming out of the different MALT has led to the suggestions that MALT is compartmentalized and cells circling in the BALT are in a separate compartment from the GALT. It is more likely that the nature of antigen influences the homing of effector cells. There needs to be flexibility, and there is much overlap and redundancy in the system. For example, after the removal of the tonsils and adenoids in humans, or the NALT in the mouse, the cervical lymph node can act as an inductive site.[268] Likewise, the mouse BALT can mount an immune response in the absence of secondary lymphoid organs.[269,270]

Tonsils and Adenoids

Waldeyer's ring is a group of lymphoid tissues encircling the wall of the throat that are the first defense against pathogens entering through the mouth or nose.[271] Humans have several tonsils with indistinct borders: one pharyngeal tonsil (adenoid), two tubal, two palatine, and one lingual. In addition to T cells, the tonsils contain a large complement of B cells, many of which are positive for IgA.[271] The tonsil epithelium makes the polymeric IgA receptor, or secretory component,[272] crucial for transport of IgA dimers across the epithelium. Secreted IgA provides an early form of defense against pathogens and toxins. Overall, the adenoids produce more secreted IgA than the tonsils.

The common cold virus uses ICAM-1 as receptor to invade the nasal mucosa. ICAM-1 is also an expressed HEV in tonsils.[271] As noted previously, HEVs are specialized vessels that express adhesion molecules that allow naïve lymphocytes enter the tonsil. Naïve L-selectin^{hi} lymphocytes adhere to PNAd on HEVs.[258,259] L-selectin^{hi} cells also bind to MAdCAM-1 predominantly found in the gut HEVs. Considering the "mucosal name" MAdCAM-1, it may be surprising to discover that tonsil HEVs express MAdCAM-1 only weakly or not at all.[273] In mice, the HEVs also manufacture the chemokine CCL21 that can recruit naïve T cells and mature DCs that express CCR7. Human tonsil HEVs do not manufacture CCL21 but are able to display chemokines that have been made and secreted by fibroblasts.[274] During tonsillitis, inflammatory chemokines, cytokines, and adhesion molecules such as CCL19,[256] VCAM-1, and E- and P-selectin are upregulated.[165]

Nasal-Associated Lymphoid Tissue

The NALTs are a pair of lymphoid organs above the soft palate in mice and rats that are considered analogous to Waldeyer's ring.[275] Despite being anatomically separate from the genitourinary tract, NALT has an important "effector" role concerning the generation of immune responses in the genitourinary tract.[276] After nasal immunization with human papillomavirus 16 or ovalbumin, human papillomavirus 16–specific or ovalbumin-specific IgA is detected in vaginal washes,[163,277,278] and cytotoxic T cells are found in vaginal draining lymph nodes.[267] However, the NALT itself is clearly an inductive site for both humoral and cellular immune responses,[279] and supports class switching to IgA.[280]

The stromal cells in murine NALT produce both CCL19 and CXCL13, whereas, in contrast to lymph nodes, only HEVs transcribe CCL21 mRNA.[163] NALT HEVs express high levels of luminal and abluminal PNAd and HEC-6ST.[163] Similar to the tonsils, the major homing receptor-ligand pair in NALT is L-selectin and PNAd, rather than MAdCAM-1 as might have been expected of this mucosal-associated tissue.[281]

Development

Human NALT and tonsils appear early in fetal development, are cellular, and have primary follicles. After birth, secondary follicles and germinal centers appear in response to bacterial antigens.[266] By contrast, the NALT in the mouse and the rat is hypocellular at birth and undergoes dramatic changes after weaning, strongly suggesting that bacterial colonization is important for NALT development in these species. Id2 is required for initiation of the rodent NALT,[282] although RORγT, LTα, or LTβ are not required for this step.[162] The expansion of rodent NALT at weaning includes changes in the expression of LTα and LTβ and lymphoid chemokines, leading to T- and B-cell compartmentalization and HEV maturation.[162,163] The alternative NF-κB pathway is required for the expression of chemokines and the HEV genes *glycam-1* and *chst4* in NALT.[183] LTα, LTβ, IL7R, and the NIK signaling pathways are required for NALT organization and function, and mice that are deficient in these cytokines have a hypocellular NALT.[163,183,283,284]

Bronchus-Associated Lymphoid Tissue

BALT is less organized but more responsive to environmental antigens than the NALT. The number of lymphoid aggregates varies depending on the level of microbial exposure and germ-free pigs have no BALT.[252,285] BALT is found commonly in rabbits and rats, is less frequent in guinea pigs and pigs, and is absent in cats.[264] BALT is not a prominent structure in the laboratory mouse, and its presence varies by strain and age.[286]

An inducible form of the BALT (iBALT) has been described in *Lt*α*−/−* mice after infection with influenza.[287] The term iBALT is misleading because these aggregates have no contact with the bronchial lumen and cannot sample inspired antigens. Therefore, iBALT should be considered as a tertiary lymphoid organ.[288] Splenectomized, lethally irradiated LTα−/− mice reconstituted with normal bone marrow have no lymphoid organs. However, these mice can generate immunological memory in the iBALT.[269,270]

BALT is rare in normal adult human lungs, but tertiary lymphoid organs are common in chronic pulmonary diseases.[283] BALT is frequent in children and is found in fetuses after infections in utero.[289,290] Like NALT, the HEVs in human BALT express PNAd and not MAdCAM-1.[291]

Gut-Associated Lymphoid Tissue

Gut-associated lymphoid tissue is the largest immune system in the body. The gut-associated lymphoid tissue in the small intestine includes the Peyer's patches, smaller isolated lymphoid follicles, and cryptopatches. In the large intestine, there is the appendix, caecal patches, and lymphoglandular complexes.[292]

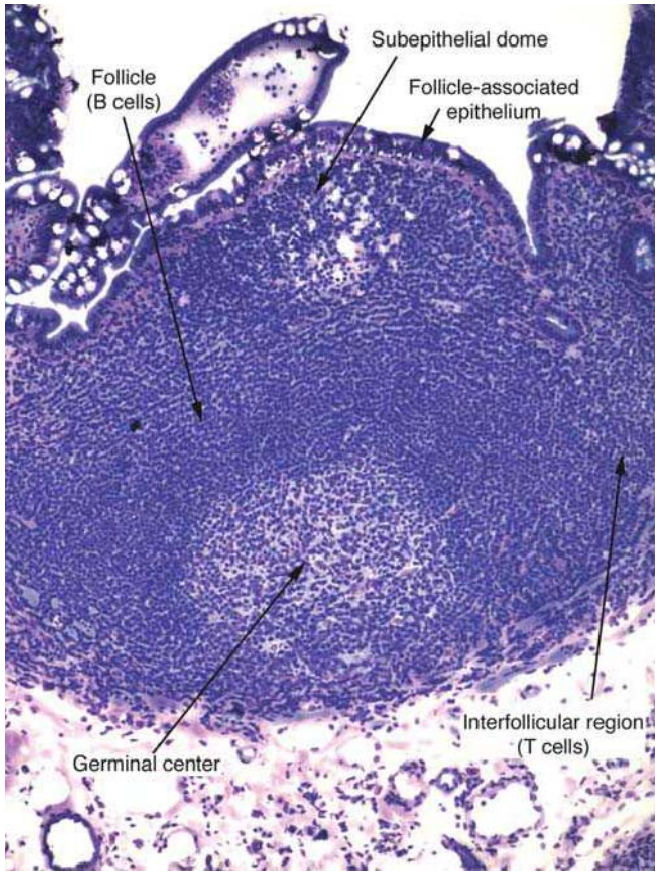
Peyer's Patches

Peyer's patches are lymphoid aggregates aligned on the antimesenteric border of the small intestine (Fig. 3.5A). They are present in most species, though their number and location vary. They are dome shaped and are covered by a specialized epithelium that lacks surface microvilli and goblet cells; they have numerous M cells. Because Peyer's patches lack afferent lymphatic vessels, these M cells are critical transporters of antigen. Pathogens, including human immunodeficiency virus and salmonella take advantage of the M cells to facilitate their own invasion.[293,294] Below the epithelium is a diffuse area, the subepithelial dome, divided into three to six parts. Peyer's patch organization is similar to that of the lymph node (see Fig. 3.5). Peyer's patches contain 6 to 12 basally located germinal centers, but the B-cell areas are larger with a T:B cell ratio of 0.2; CD4+ cells predominate over CD8+ cells.[295] Although M cells transport antigen across the epithelial barrier, they are not believed to have a crucial role in processing or presenting antigen. Peyer's patches are populated by several subsets of DCs that can carry out these functions.[296,297]

Trafficking In and Out

Cells enter Peyer's patches through HEVs. In contrast to peripheral lymph nodes and the NALT, the HEVs of Peyer's patches express MAdCAM-1,[298] and homing depends on the interaction of MAdCAM-1 with the integrin $\alpha_4\beta_7$ on lymphocytes. Luminal PNAd is rarely found in Peyer's patch HEVs; only occasional abluminal expression is detected. This pattern is identical to that seen in lymph node HEVs in *chst4−/−* mice.[128] However, lymphocytes from mice that lack both L-selectin ligand and $\alpha_4\beta_7$ home less efficiently to Peyer's patches than do lymphocytes from mice that lack only one or the other of the ligands,[299,300] suggesting that an L-selectin ligand does contribute to homing to Peyer's patches. Even though P-selectin is expressed only weakly on HEVs in Peyer's patches, cells from mice deficient in P-selectin show reduced rolling and adhesion in vivo. This suggests that P-selectin, as well as MAdCAM-1, is important for homing to Peyer's patches.[299]

Lymphoid chemokines CCL19, CCL21, and CXCL13 are found in the T- and B-cell areas[301] (see Fig. 3.5B). Stromal cells make CXCL12 mRNA, but the protein is also found on HEVs, presumably transported in a manner similar to CCL19.[117] The CXCL16/CXCR6 chemokine/receptor pair is important in Peyer's patches but not in lymph nodes. Even under germ-free conditions, CXCL16 is constitutively produced by the dome epithelium.[295] CD4+ and CD8+ cells that express CXCR6 are found in Peyer's patches and require CXCL16 to home to the subepithelial dome. CCL20 and its receptor CCR6 are also important in Peyer's patches. CCL20 is made by the intestinal epithelium and plays a crucial role in dendritic cell trafficking to Peyer's patches.[302] Peyer's patch HEVs express CCL25, the ligand for CCR9, which has been defined as a mucosal homing chemokine receptor for intraepithelial lymphocytes and plasma cells.[78]



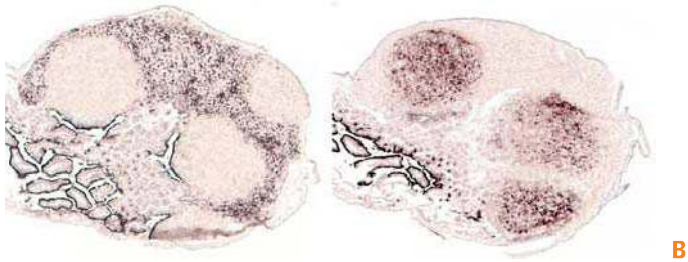


FIG. 3.5. Organization of Peyer's Patches. **A:** Photomicrograph of Peyer's patch. Peyer's patches are located in the intestine near intestinal villi. The follicle associated epithelium (FAE) is in contact with the gut lumen. M cells (not shown) in the FAE transport antigen into the subepithelial dome, populated by dendritic cells and T cells. The interfollicular T-cell–rich region surrounds the B-cell follicle and germinal center. Courtesy of A. Iwasaki (Yale University School of Medicine, New Haven, CT). **B:** In situ hybridization of chemokine messenger ribonucleic acids (mRNAs). On the *left*, CCL21 mRNA defines the T-cell zone and high endothelial venules; on the *right*, CXCL13 defines the B-cell zone.

Development

Several cytokines and chemokines have been shown to be crucial for Peyer's patch development. *Lt*α−/− and *Lt*β−/− mice completely lack Peyer's patches,[161,303] as do *Cxcr5*−/− mice.[174,175,301] Because mice deficient in the LTβR also lack Peyer's patches,[304] these effects are mediated in large part through $LT\alpha_1\beta_2$. However, some *Tnfr1*−/− mice lack organized Peyer's patches, suggesting that either LTα3 or TNFα also influences generation or later stages in maintenance of Peyer's patches.[305] Mice deficient in IL-7, RORγt, Id2, NIK, and factors in the classical and alternative NFκB pathways[170,177,304] lack Peyer's patches, although RANKL is not required.[172]

A model for the embryonic development of Peyer's patches[306–308] provided the framework for studies in that organ. The first sign of Peyer's patch development at E15.5 in the mouse is the appearance of regions on the small intestine that stain positively with antibodies to ICAM-1, VCAM-1, and LTβR. The cells in these aggregates are called the lymphoid tissue organizer cells and express CXCL13, CCL19, CCL21, and IL-7. At E17.5, clusters of IL-7R+CD4+CD3+ inducer cells are found. These express LTα and LTβ, CXCR5 and CCR7, Id2, and RORγ. They also express $\alpha_4\beta1$ integrin activated by CXCR5[309] that allows interaction with the VCAM-1+ organizer cells. At E 18.5, mature T and B cells enter through HEVs, CCL20 is produced by the FAE, and DCs expressing CCR7 and CCR6 are found. By day 4, the typical microarchitecture is apparent, with M cells and T- and B-cell compartmentalization.[310]

Lymphocytes influence the maintenance of Peyer's patches. Mice that lack mature T and B cells have either undetectable or small Peyer's patches that lack follicles and germinal centers.[311,312] B cell–deficient mice retain some M cells, suggesting that T cells may regulate M-cell maintenance. When B cell–deficient mice are reconstituted by a membrane IgM transgene, M cells are found at levels comparable to those of normal mice,[311] indicating that cells in addition to CD4+CD3+ lymphoid tissue–inducer cells are crucial for the maintenance of mature, functioning Peyer's patches.

Cryptopatches and Isolated Lymphoid Follicles

The small intestine of a mouse contains 100 to 200 isolated lymphoid follicles and more numerous cryptopatches. The cryptopatches in the lamina propria containing lymphoid tissue–inducer cells and DCs can be precursors of ILFs. The cryptopatches are composed of lin−c-kit+ cells, DCs, and VCAM-1+ stromal cells with few or no mature T and B cells. The cryptopatch cells express RORγt, IL-7R, and CCR6,[313] and their development is dependent on IL-7, CCR6, and its receptor CCL20. Cryptopatches are quite plastic, and although the LT family is necessary for their development, they can be restored by administration of wild-type bone marrow to adult *Lt*α−/− mice.

After mice are exposed to microbes or during some forms of autoimmunity, cryptopatches give rise to ILFs,[314,315] which resemble small Peyer's patches and usually only have a single

dome. ILFs require B cells that express LT, but not T cells, for their formation.[316]. In contrast to lymph nodes, they also require the TNFR1.[317] The location of ILFs is antigen driven, and they resolve completely after mice are treated with antibiotics or cytokine inhibitors.[121,185,314,316,317]

Appendix

Both rabbits and humans have an appendix,[318] a vestigial organ distal to the ileocecal junction that divides the small intestine from the colon. The appendix has epithelial M cells and dense lymphoid accumulations and germinal centers that are similar to Peyer's patches. Similar to the tonsils and adenoids, the appendix involutes and regresses after puberty.

Colon Lymphoglandular Complexes

The colon has the highest bacterial load of any of the MALTs, and the distribution of the MALT is more random than in the small intestine. Lymphoglandular complexes are smaller than Peyer's patches. As their name suggests, the lymphoid cells of the lymphoglandular complexes surround the mucus glands.[292]

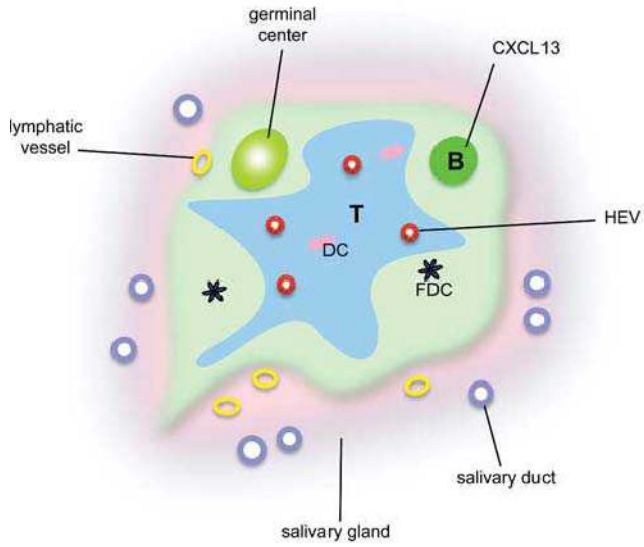


FIG. 3.6. Tertiary Lymphoid Tissue. Schematic diagram of Sjögren syndrome salivary gland. Note similarities with the organization of a lymph node.

TERTIARY LYMPHOID TISSUES

Similarities to Secondary Lymphoid Organs

Tertiary lymphoid tissues, also termed tertiary lymphoid organs, are ectopic accumulations of lymphoid cells that arise in nonlymphoid organs during chronic inflammation through a process termed "lymphoid neogenesis" (also "lymphoid neo-organogenesis").[2] The iBALT could be considered as either a secondary or tertiary lymphoid tissue. This semantic issue epitomizes the plasticity of all lymphoid organs and the fact that lymphoid organ regulation represents a continuum from ontogeny through chronic inflammation. A notable difference between secondary lymphoid organs and tertiary lymphoid tissues is the fact that the latter can arise in almost any organ in the adult. Nonetheless, tertiary lymphoid tissues exhibit remarkable morphologic, cellular, chemokine, and vasculature similarities to secondary lymphoid organs. They exhibit T- and B-cell compartmentalization; naïve T and B cells; DCs; FDCs; germinal centers; plasma cells; lymphoid chemokines CCL19, CCL21, and CXCL13[1]; HEVs[135,196,319]; and conduits.[320] Lymphatic vessels have also been noted in tertiary lymphoid tissues in chronic graft rejection[321,322] and mouse models of Sjögren syndrome[134] (Fig. 3.6) and Type 1 diabetes.[323] Although it is not completely clear whether they function as afferent and/or efferent vessels, the fact that the tertiary lymphoid tissues respond to S1P inhibitors suggest that their lymphatic vessels function similarly to those in lymph nodes.[323]

Lymphoid neogenesis has been noted in humans in autoimmunity, microbial infection, and chronic allograft rejection. A few examples are shown in Table 3.2 and are described in more detail in Drayton et al.[1] These accumulations also occur in atherosclerotic plaques with FDCs, organized B-cell follicles, HEVs (HECA-452), and CCL19 and CCL21 in addition to those chemokines more often associated with acute inflammation.[324] Tertiary lymphoid tissues have also been noted in non–small-cell lung cancer[325] and many other solid tumors.[326] The tendency for tertiary lymphoid tissues to develop into lymphomas has also been noted in many studies.[327]

Ectopic lymphoid tissues are also apparent in several animal models. The pancreatic infiltrates in early stages of diabetes in the nonobese diabetic mouse express lymphoid chemokines[328] and HEVs expressing MAdCAM-1,[329,330] PNAd, and HEC-6ST.[135] The brain in experimental autoimmune encephalomyelitis, a model of multiple sclerosis, has HEVs, CCL19, CCL21, CXCL13, and FDCs.[331–333] The thyroid in the BioBreeding (BB) rat has T- and B-cell compartmentalization and DCs,[334] and the gut in autoimmune gastritis in the mouse has HEVs and CXCL13.[335] Atherosclerotic plaques of apoprotein-E–deficient mice exhibit a marked increase in T and B cells, expression of CCL19 and CCL21, and PNAd+ HEVs.[336–338] Lymphoid neogenesis also occurs in chronic mouse heart allograft rejection.[339,340] Tertiary lymphoid tissues have been induced in several transgenic mouse models with the use of tissue-specific promoters driving the expression of inflammatory cytokines or lymphoid chemokines.[1] These mouse models, in addition to serving as examples of human disease, have provided valuable insight into the regulation of secondary lymphoid organ development.

From Chronic Inflammation to Organized Lymphoid Microenvironments

Data generated from analyzing the cellular and molecular requirements for secondary lymphoid organ development have provided a paradigm for understanding the development of tertiary lymphoid tissues in chronic inflammation. This paradigm proposes that the processes and molecules governing secondary lymphoid organs are also the basis of tertiary lymphoid tissue development,[2] and informs understanding of both secondary and tertiary lymphoid tissues. The physiologic event(s) that precipitate lymphoid neogenesis remain unclear. Data obtained from experiments in knockout and transgenic

TABLE 3.2 Lymphoid Neogenesis in Human Autoimmunity, Infectious Diseases, and Graft Rejection

Disease	Affected Tissue	Characteristics
Autoimmunity		
Rheumatoid arthritis	Synovial membrane	T cells and B cells, plasma cells, GCs, FDCs, CXCL13, CCL21, HEVs (PNAd, HEC-6ST)
Sjögren syndrome	Salivary glands	T cells and B cells, plasma cells, GCs, FDCs, CCL21, CXCL12, CXCL13 HEVs (PNAd)
Myasthenia gravis	Thymus	T cells and B cells, GCs, FDCs
Hashimoto thyroiditis	Thyroid	T cells and B cells, GCs, FDCs CCL21, CXCL13, CXCL12, plasma cells, HEVs (PNAd)
Grave disease	Thymus	T cells and B cells, GCs, FDCs, CCL21 CXCL13, CXCL12, HEVs (PNAd)
Multiple sclerosis	Brain	Lymphatic capillaries, B-cell follicles and centroblasts, GCs, CCL19, CCL21, CXCL12, CXCL13
Ulcerative colitis	Colon	CXCL13
Inflammatory bowel disease (Crohn disease)	Bowel	T cell-B compartments, LVs, HECA-452+ HEV
Psoriatic arthritis	Joint	T cells and B cells, CXCL13, CCL19, CCL21, HEVs (PNAd)
Infectious Diseases		
Borrelia burgdorferi/Lyme disease	Joints	T and B cells, FDCs, HEVs
Borrelia burgdorferi/ neuroborreliosis	Central nervous system/ cerebrospinal fluid	CXCL13
Hepatitis C virus	Liver	T-cell and B-cell compartments, MAdCAM-1
Bartonella henselae/cat scratch disease	Granuloma	CXCL13
Graft Rejection		
Organ		
Heart		GCs
Kidney		GCs, LVs

FDC, follicular dendritic cell; GC, germinal center; HEV, high endothelial venule; LV, lymphatic vessel; MAdCAM-1, mucosal addressin cell adhesion molecule; PNAd, peripheral node addressin. Original references are in Drayton et al.[1]

mice and clinical observations indicate that cooperative activities of TNF/LT family members and the lymphoid chemokines play central roles in this process.

Inflammation is a localized response to tissue injury, irritation, or infection often marked by tissue damage. Acute inflammation is an early innate immune response that is generally short-lived and self-limiting. However, in some situations, acute inflammation transitions to a chronic inflammatory response that is long-lived and self-perpetuating. Tertiary lymphoid tissues arise under conditions of constitutive cytokine and/or chemokine expression, but the precise signal(s) that initiates their development is unknown. By integrating studies of lymphoid neogenesis in human pathologies and in animal models, it is becoming clear that at least three critical events promote tertiary lymphoid tissue formation: inflammatory (eg, TNF/LT) cytokine expression, lymphoid chemokine production by stromal cells, and HEV development. It is not known if lymphoid tissue–inducer cells are necessary for lymphoid neogenesis, though such cells have been noted in the mouse models of lymphoid neogenesis,[341] and lymphoid tissue–inducer-like cells have been described in several instances.

Functions of Tertiary Lymphoid Tissues

Ectopic accumulation of lymphoid cells has been considered the hallmark of destructive inflammation. Indeed, some tertiary lymphoid tissues are accompanied by tissue damage. However, it is likely that tertiary lymphoid tissues in chronic inflammation have roles in addition to tissue destruction. In the case of microbial infection, it is likely that lymphoid neogenesis occurs as a way to sequester pathogens and to prevent their access to the other parts of the body. This may represent a primitive form of immunity. Local antigen presentation within the tertiary lymphoid tissue itself likely prevents bacteremia or viremia. Enhanced long-term survival has been noted for those individuals whose lung or breast tumors included tertiary lymphoid tissues.[325,326] The propensity for tertiary lymphoid tissues to develop into lymphomas, as noted previously, the ability of lymphatic vessels to serve as conduits for tumor metastasis, and the ability of tertiary lymphoid tissues to serve as sites of prion accumulation are obvious manifestations of detrimental functions.[342] Furthermore, the development of tertiary lymphoid tissues in autoimmunity may perpetuate clinical disease through epitope spreading.

Data from human and mouse studies provide compelling evidence that tertiary lymphoid tissues are permissive microenvironments for the induction of antigen-specific immune responses. Extensive immunohistochemical analyses of tertiary lymphoid tissues in autoimmunity and other chronic inflammatory states have established the presence

of germinal centers and FDC networks in these tissues; several groups have demonstrated that tertiary lymphoid tissue germinal centers can support B-cell differentiation. Microdissection of discrete lymphocytic foci and subsequent deoxyribonucleic acid sequence analysis of germinal center B cells from the inflamed synovial tissue of patients with rheumatoid arthritis revealed a restricted number of Vκ gene rearrangements, a result consistent with oligoclonal B-cell expansion in the synovial tissue.[343] Somatic hypermutation is apparent in synovial germinal center B cells.[344] Furthermore, synovial B cells exhibit a limited number of heavy and light chain gene rearrangements consistent with local clonal expansion of these cells. The molecular analysis of tertiary lymphoid tissue germinal centers from the salivary glands of patients with primary Sjögren syndrome or the thymus of patients with myasthenia gravis demonstrates oligoclonal B-cell proliferation in these tissues in addition to somatic hypermutation of Ig variable genes.[345–347] Together, these studies indicate that tertiary lymphoid tissue germinal centers in several autoimmune pathologies can support antigen-driven clonal expansion and extensive diversification.

Another important hallmark of antigen-driven B-cell responses is the terminal differentiation of activated B cells into Ig-secreting plasma cells. Plasma cells have been detected in tertiary lymphoid tissues associated with germinal centers in rheumatoid arthritis[348] and in Sjögren syndrome. Mice expressing LTα under the control of the rat insulin promoter (RIPLTα) exhibit tertiary lymphoid tissue at the sites of transgene expression (pancreas, kidney, and skin). After immunization with sheep red blood cells, evidence of isotype switching is apparent in these cellular infiltrates.[2] Although the presence of plasma cells in tertiary lymphoid tissues is consistent with local antigen presentation, it is occasionally unclear whether these cells develop in the tertiary lymphoid tissues themselves or migrate from canonical secondary lymphoid tissues. Nonetheless, taken together, these studies indicate that tertiary lymphoid tissues in several human pathologies and animal models support antigen-driven B-cell differentiation marked by somatic hypermutation of Ig variable genes, affinity maturation, isotype switching, and terminal differentiation into antibody-secreting plasma cells.

T-cell priming occurs in tertiary lymphoid tissues as suggested by the presence of isotype switched plasma cells in the RIPLTα tertiary lymphoid tissues,[2] accelerated graft rejection,[349] and T-cell epitope spreading in the central nervous system during experimental allergic encephalomyelitis.[350] The restricted TCR repertoire in a melanoma-associated tertiary lymphoid tissue[351] further supports the concept that tertiary lymphoid tissues can act as priming sites. The demonstration of naïve T-cell proliferation in the islets of nonobese diabetic mice after surgical removal of pancreatic lymph nodes[352] suggests, together with evidence noted previously, that tertiary lymphoid tissues present antigen to naïve cells at the local site and generate an immune response. Determinant or epitope spreading, a phenomenon that arises in several autoimmune diseases, occurs when epitopes other than the inducing antigen become major targets of an ongoing immune response. It is considered to occur subsequent to the tissue damage induced by the initiating autoreactive T cells and therefore is the result of the presentation of new antigens.[353] Data generated in murine models of central nervous system inflammation support the possibility that intermolecular and intramolecular epitope spreading occur in tertiary lymphoid tissues in the central nervous system.[350] More recently, it has become apparent that T_{reg}s can populate tertiary lymphoid tissues,[338] again suggesting that manipulating the immune response at the local site is an avenue of control.

Plasticity and Adaptability of Tertiary Lymphoid Tissues

Tertiary lymphoid tissues are the most plastic and adaptable of the lymphoid tissues. First, lymphoid neogenesis can be induced by a variety of stimuli. Their nimbleness in this regard suggests that they might represent the most primitive tissues in the immune system. Tertiary lymphoid tissues can be "turned off" (ie, resolve) upon removal of the initial stimulus or after therapeutic intervention. The destruction of the islets of Langerhans β cells in type I diabetes mellitus is an example of a situation in which removal of the antigen stimulus is accompanied by tertiary lymphoid tissue resolution. Antibiotic treatment results in the resolution of tertiary lymphoid tissues and even MALT lymphomas.[354] Treatment has been shown to resolve some established tertiary lymphoid tissues, reversing insulitis and protecting against diabetes in nonobese diabetic mice.[355] Such treatment can also "turn off" established tertiary lymphoid tissues in a mouse model of collagen-induced arthritis.[356] These studies are similar to those described previously regarding the plasticity of lymph nodes after mice are immunized or treated with LTβR-Ig,[198,199] again emphasizing the commonality of these tissues.

CONCLUSION

The immune system depends on a remarkable organization of tissues and cells. The organs have defined functions that include generation of an immune repertoire (primary lymphoid organs) and responding to antigen (secondary lymphoid organs and tertiary lymphoid tissues). Development of primary and secondary lymphoid organs depends on precisely regulated expression of cytokines, chemokines, and adhesion molecules. Similar signals regulate the transition from inflammation to tertiary lymphoid tissues. Chemokines and adhesion molecules regulate trafficking in and out of lymphoid organs. Secondary lymphoid organs are remarkably plastic in their response to antigenic assault and adapt with changes in expression of chemokines and adhesion molecules to maximize encounter of antigen with antigen-specific cells. Tertiary lymphoid tissues, characteristic of many pathologic states, may actually represent the most primitive form of lymphoid tissues in their even greater plasticity and ability to develop directly at the site of antigen exposure.

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CHAPTER 4

Evolution of the Immune System

Martin F. Flajnik • Louis Du Pasquier

INTRODUCTION

Defense mechanisms are found in all living things, even bacteria, where they are surprisingly elaborate. Although new adaptive or adaptive-like (somatically generated) immune systems have been discovered in invertebrates and the jawless fish, adaptive immunity based upon immunoglobulin (Ig), T-cell receptors (TCRs), and the major histocompatibility complex (MHC) is only present in jawed vertebrates (gnathostomes); because of clonal selection of lymphocytes, positive and negative selection in the thymus, MHC-regulated initiation of all adaptive responses, etc., the major elements of the adaptive immune system in gnathostomes are locked in a coevolving unit that arose in concert over a short period of evolutionary time.[1–3] In addition, a large cast of supporting players, including a large array of cytokines and chemokines, adhesion molecules, costimulatory molecules, and well-defined primary and secondary lymphoid tissues, evolved in the jawed vertebrates as well. This scheme was superimposed onto an innate system inherited from invertebrates, from which many innate molecules and mechanisms were coopted for the initial phase of the adaptive response and others for effector mechanisms at the completion of adaptive responses. Over the last 10 years, we have learned that various components of the innate immune system are also incredibly complex and locked as well in a coevolving unit.[4,5]

In each group of organisms, one can detect a basic set of immune functions, and these are employed in different ways in representative species. For example, we detect that in the jawed vertebrates fine tuning, or adaptations, or even degeneration of molecules/mechanisms in each group (Taxon) are observed, and not a steady progression from fish to mammals as is documented for most other physiologic systems.[6] Given that all the canonical adaptive immune system features are present in cartilaginous fish and apparently none were lost (except in particular groups of organisms that will be discussed), differential utilization of defense molecules rather than sequential installation of new elements is observed. In this chapter, there are only isolated cases of increasing complexity in immune systems, but many examples of contractions/expansions of existing gene families; thus, "more or less of the same" rather than "more and more new features" is the rule. We observe a bush growing from a short stem rather than a tall tree with well-defined branches, and thus deducing the primitive, ancestral traits is not clear cut.

GENERAL PRINCIPLES OF IMMUNE SYSTEM EVOLUTION

The Common Ancestor Hypothesis

Figure 4.1 displays the extant animal phyla ranging from the single-celled protozoa to the metazoan protostome and deuterostome lineages. It is often suggested or assumed by those unfamiliar with thinking in evolutionary terms that molecules or mechanisms found in living protostomes, like the well-studied arthropod *Drosophila*, are ancestral to similar molecules/mechanisms in mouse and human. While this is true in some cases, one must realize that *Drosophila* and humans have taken just as long (over 900 million years) to evolve from a common ancestral triploblastic coelomate (an animal with three germ layers and a mesoderm-lined body cavity, features shared by protostomes and deuterostomes) that looked nothing like a fly or a human, and thus *Drosophila* is not our ancestor (ie, the manner by which flies and humans utilize certain families of defense molecules may be quite different and both may be disparate from the common ancestor). Thus, understanding of how model invertebrates and vertebrates perform certain immune tasks is an important first step in our understanding of a particular mechanism, but we only deduce what is primordial or derived when we have examined similar immune mechanisms/molecules in species from a wide range of phyla. We will touch upon each of the defense molecule families and will emphasize those which have been conserved evolutionarily and those that have evolved rapidly.

Rapid Evolution of Defense Mechanisms

Immune systems are often compared to the Red Queen in *Alice in Wonderland*, (Red Queen's Hypothesis[7,8]) who must continually keep moving just to avoid falling behind. Because of the perpetual conflict with pathogens, the immune system is in constant flux. This is exemplified by great differences in the immune systems of animals that are even within the same phylogenetic group (eg, mosquito [*Anopheles*] and fruitfly [*Drosophila*], both arthropods, or human and mouse, both mammals). In fact, in contrast to what was believed previously, defense mechanisms are extremely diverse throughout the invertebrate phyla, and Kepler et al. have aptly and succinctly described the situation in the title of a recent review: "not homogeneous, not simple, not well understood."[9] In the jawed vertebrate (or *gnathostomes*), the most rapidly evolving system is an innate system, natural killer (NK) cell recognition, governed by different classes (superfamilies) of receptors in mice and humans, and also extremely plastic even

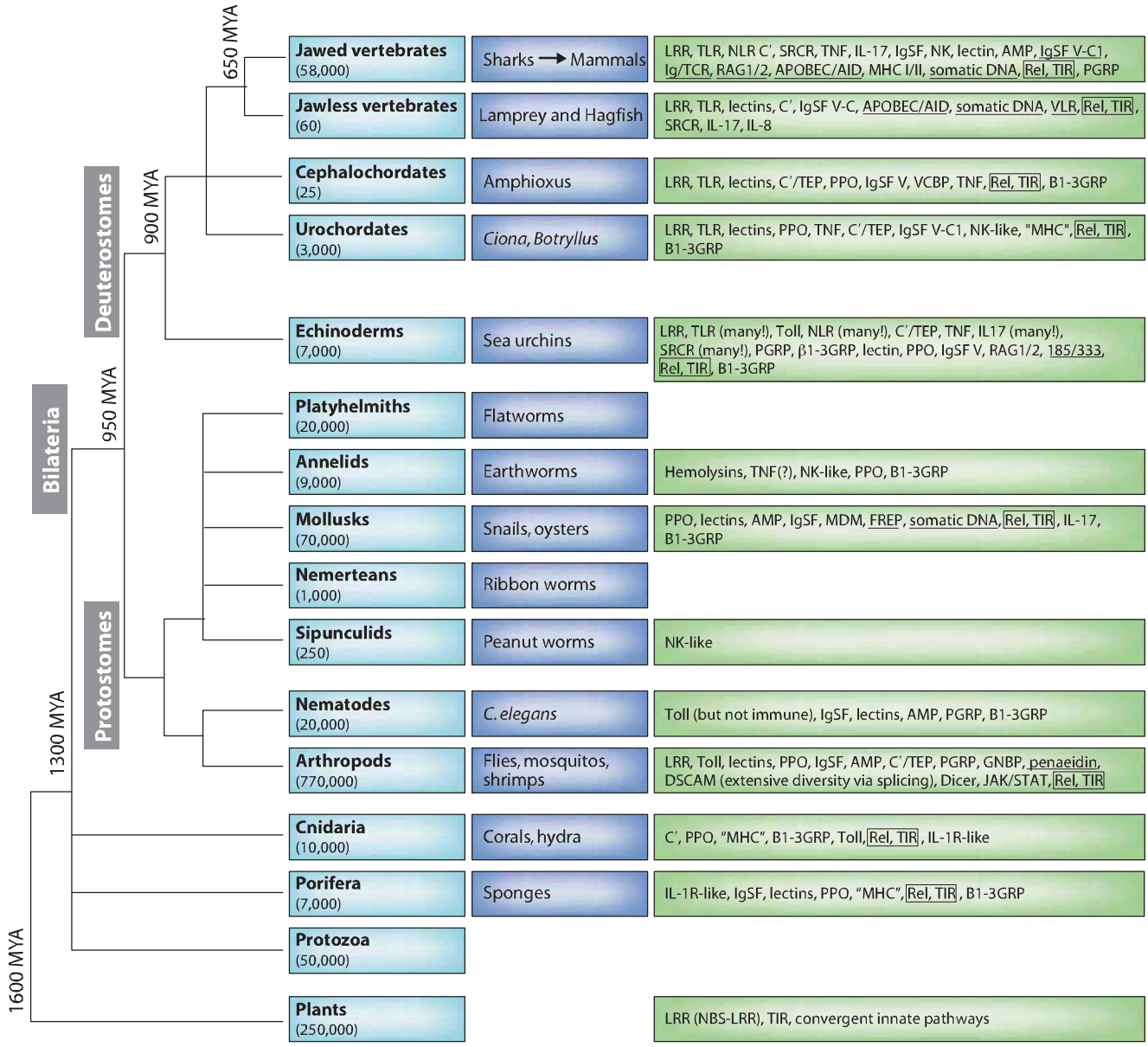

FIG. 4.1. Major Animal Groups and Immune Mechanisms/Molecules Described to Date in Each Group. The first box on the left in each row describes the animal taxon and the approximate number of species in that group. The next box shows specific examples of species or subgroups. The third box lists molecules/mechanisms found in each group: *underlined terms* indicate somatic changes to antigen receptors or secreted molecules. Figure modified from Hibino et al.[48] and Flajnik and Du Pasquier.[61] See Table 4.1 for definition of the acronyms.

within the same species, exemplified by the large number of killer immunoglobulin superfamily (KIR) haplotypes found in humans.[10] Studies of Ig superfamily (SF) genes expressed in the nervous system and immune system showed definitively that the immune system molecules evolve at a faster rate.[11] Finally, rapid evolution of immune system molecules and mechanisms is the general rule, but molecules functioning at different levels of immune defense (recognition, signaling, or effector) can evolve at widely varying rates.

Conservation of Defense Mechanisms

While the immune system is the most rapidly evolving physiologic system, nevertheless there is also deep conservation of defense families and mechanisms. Klein[11a] has compared this dichotomy to the two-headed god Janus, the major idea being that certain basic mechanisms/functions are obligatory for immune systems to function, but they still must evolve rapidly to avoid pathogen subterfuge. For example, MHC class I molecules have similar structure/function/features in all gnathostomes, but even within groups of primates class I genes are not orthologous (ie, they can be derived from totally different ancestral class I genes[10]). So, the idea is to preserve vital immune functions but rapidly modify the gene or pathway to outwit the pathogen. Additionally, certain features are conserved (eg, development and function of conventional αβ T cells), but a second, similar system, can be exploited in very different ways in closely related

species (eg, the function[s] of γδ T cells). The "Janus paradigm," therefore, can be quite useful when examining any pathway in the immune system.

Convergent Evolution

Early on in the comparative study of immunity, it was assumed that the same features appearing in different taxa proved that they were present in the common ancestor as well (ie, they were submitted to divergent evolution). While this dictum still holds true and establishes one of the dogmas of comparative immunology, later we discovered, because of the aforementioned rapid evolution of immune systems, *convergence* of similar functions has occurred in evolution (ie, the same function or even molecular conformation has arisen independently in different organisms, sometimes in species that are relatively closely related). While we will discuss several cases of convergent evolution throughout the chapter, for frame of reference, the NK cells, which use different receptor families in primates and rodents to achieve precisely the same ends of recognizing polymorphic MHC class I molecules, is a striking example of convergence.[12] Additionally, the emergence of a lymphocyte-based somatic generation of two entirely different receptor families for the same function in jawless and jawed vertebrates is another remarkable illustration of convergent evolution.[13] Finally, in innate immunity, the cytosolic nucleotide-binding domain leucine-rich repeat (NLR) proteins, despite their striking similarity in deuterostomes and plants, arose (at least) twice in evolution.[14]

Multigene Families

Genes involved in immunity are often found in clusters, with extensive contraction and expansion via so-called birth and death processes.[15] It is well known that such gene clusters can change rapidly over evolutionary time due to unequal recombination crossovers and gene conversion (and not only in the immune system, but in any *cis*-duplicating gene family). Often, families of related immune genes—especially those involved in recognition events—are found near the telomeres of chromosomes, presumably because this further promotes gene-shuffling events. Nonclassical MHC class I loci, NK receptors, and NLRs are conspicuous examples of this phenomenon, again believed to be a consequence of the race against pathogens. We will discuss many examples of how such multigene families have been exploited in different species throughout the chapter.

Gene duplication, either in the clusters mentioned above or as a consequence of en bloc duplications, certainly has been a major feature of immune system diversity and plasticity. The two types of duplications are not equivalent, the former being more taxon-specific and the latter (en bloc) having a lasting impact on the entire system. It is now universally accepted that two genome-wide duplications (the so-called 2R hypothesis[2,16]; see Fig. 4.13) occurred early in vertebrate history, tracking very well with the emergence of the Ig/TCR/MHC-based adaptive immune system.[2,17] This theory forms the basis for much that will be discussed concerning the evolution of the vertebrate adaptive immune response as one can track the emergence of new immune mechanisms, as well as fine tuning of old ones, by examining the paralogous syntenic groups of genes. Our view is that these genomewide duplications were as crucial as the "RAG transposon"[18–20] in the development of the Ig/TCR/MHC-based adaptive immunity. In addition, the common ancestor of bony fish (teleosts) underwent a third round of genomewide duplication, which many believe to have played the major role in these fishes' unique outlier status regarding immune system genetics and physiology.[2,21]

Polymorphism

In addition to gene duplications, polymorphism also augments the diversity of immune recognition within a population. It can be generated any time during the history of a gene family of either receptor or effector molecules: MHC, toll-like receptors (TLRs), Ig, TCRs, NK receptors (NKRs) and related molecules, and antimicrobial peptides (AMPs) are just a few examples. Polymorphism, either within the gene itself or in its regulatory elements, provides populations with flexibility in function of the changing pathogenic environment. This subject, central to the studies on MHC, leukocyte receptor complex (LRC), and NK cell complex (NKC), is becoming well documented for immunity-related genes in insects as well. In *Drosophila*, polymorphism in regulatory networks is indeed expected as parasites often target their elements.[22] In humans, there are two major NK cell haplotypes found in all subpopulations, which are under "balancing selection." In such a case, the polymorphisms presumably adopt a division of labor required for the maintenance of the species: one haplotype is believed to be involved in protection from virus and the other perhaps for promoting reproduction.[23]

Somatic Generation of Diversity

Somatic modifications can take place at multiple levels to generate immune system diversity. Long believed to be the sole domain of jawed vertebrates, modifications at the deoxyribonucleic acid (DNA) level via somatic hypermutation, gene conversion, and rearrangement (primary and secondary [eg, receptor editing]) irreversibly modify genes within an individual. The well known V(D)J joining, class switch recombination (CSR), and somatic hypermutation (SHM) are examples of this processes in the IgSF receptors of jawed vertebrates, but modifications to genomic DNA can also occur in the jawless fish and some invertebrates.[7,13] The list of organisms undergoing such diversity of germline immune genes will only grow as more organisms are examined and more genome and expressed sequence tags (EST) sequencing projects are undertaken (see Fig. 4.1).

Alternative splicing can be a source of tremendous diversity in some gene families encoding receptors involved in immunity in insects and crustaceans. The Down syndrome cell adhesion molecule (DSCAM) gene in several arthropods (described in detail in the following) was shown to generate enormous diversity via ribonucleic acid (RNA) processing.[7,24] In the vertebrates, this mechanism is important in

determining the function of different molecules, best known for the Igs (transmembrane [TM] versus secreted forms, as well as inflammatory versus neutralizing forms in non-mammalian vertebrates). Further diversity can be obtained by the assembly of multichain receptors in which different components are combined. The classical example in the jawed vertebrate adaptive immune system is that of Ig light (L) and heavy (H) chains of antibodies but similar combination can occur with insect peptidoglycan-recognizing proteins (PGRP), vertebrate TLRs, and many others.

The study of the evolution of immunity has resulted in a fundamental appreciation of the heart of immunity, both innate and adaptive. Especially now, with studies in many plants and in both invertebrate and vertebrate animals, we can see what features have been conserved and when they arose. "Simple" genetic models such as *Drosophila* and *Candida elegans* provide a glimpse into these elemental mechanisms and also allow us to remove the clouds that surround studies of mouse and human, with so many interconnected pathways. As mentioned, examination of the well-studied mammalian models in combination with studies of invertebrates allows us to deduce the condition of the common ancestor. Interestingly, major pathways of defense known to all immunologists, such as the ones involving TLR, JAK-signal transducer and activator of transcription (STAT), NOTCH, and tumor necrosis factor (TNF) pathways, clearly arose in an early animal ancestor and have been perpetuated in derived fashions in all major taxa. We shall see how these pathways are manipulated in different animals, always drawing upon the best-known mammalian model as a foundation (whenever possible).

MAJOR GENE FAMILIES INVOLVED IN IMMUNITY

Defense molecules can be composed of a very large number of protein folds, some of which are clearly some used to a large extent.[25–27] Some of the most common families (Fig. 4.2) are IgSF, leucine-rich repeats (LRRs), C-type lectins, and the TNF family, and certain other domains in immune recognition (eg, scavenger receptor cysteine-rich [SRCR]). All of the domains discussed in this chapter are found in Table 4.1 and a few are displayed in Figure 4.2. As a means of introduction, two of these families, which constitute the "top two" quantitatively, will be described in the following.

Leucine-Rich Repeats

LRRs consist of 2 to 45 motifs of 20 to 30 amino acids in length (XLXXLXLXXNXHXXHXXXXFXXLX) that fold into an arc shape (see Fig. 4.2).[28] Both the concave and convex parts of the domain have been shown to interact with ligands. Molecular modeling suggests that the conserved pattern LxxLxL is sufficient to impart the characteristic horseshoe curvature to proteins with 20- to 30-residue repeats. LRRs are often flanked by cysteine-rich domains. LRRs occur in proteins ranging from viruses to eukaryotes and are found most famously in the toll/TLRs, as well as tyrosine kinase receptors, cell-adhesion molecules, resistance (R) factors in plants found at the cell surface, and in the cytosol, extracellular matrix (ECM)-binding glycoproteins (eg, peroxidasin), and are involved in a variety of protein-protein interactions: signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, apoptosis, and the immune response. LRR-containing proteins can be associated with a variety of other domains, whether they are extracellular (LRR associated with IgSF or fibronectin [FN] type III) or intracellular (caterpillar family LRR associated with a variety of effector domains; see subsequent discussion). In these chimeric molecules, the LRR moiety is involved in recognition, most likely due to its extraordinarily malleable structure. There are at least six families of LRR proteins, characterized by different lengths and consensus sequences of the repeats.[29] Repeats from different LRR subfamilies never occur simultaneously and have most probably evolved independently in different organisms.

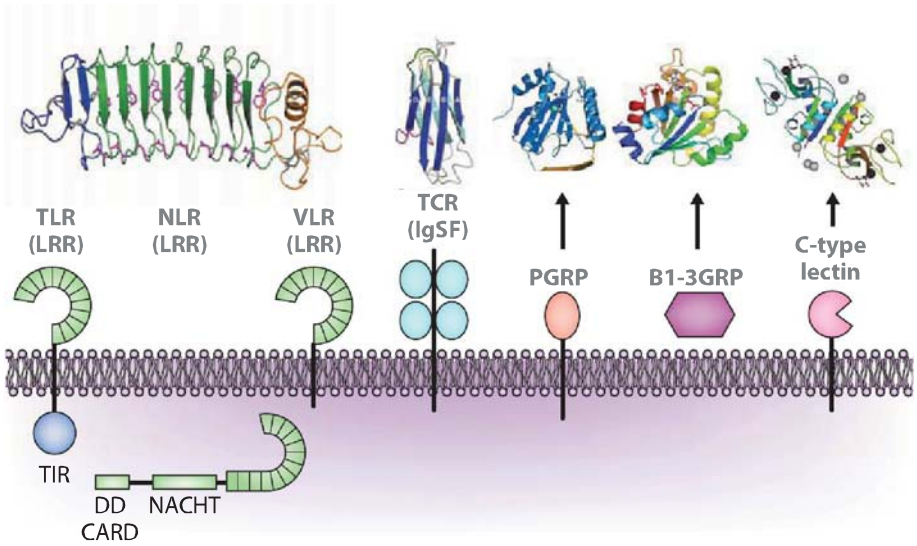

FIG. 4.2. Major Molecular Families Described in the Text and Representatives of Each Family: Leucine-Rich Repeats (LRRs), Immunoglobulin Superfamily (IgSF), Peptidoglycan-Recognition Protein (PGRP), β1-3 Glucan Recognition Protein (β1-3GRP), and C-Type Lectins. Representative structures are shown above for LRR (toll-like receptor, nucleotide-binding domain leucine-rich repeat [NLR], variable lymphocyte receptor), IgSF, PGRP, β1-3GRP, and C-type lectin. Other acronyms are defined in Table 4.1. For the NLR model, the echinoderms have N-terminal death domains, whereas all other animals have caspase-recruitment domains. Figure modified from Hibino et al.[48]

TABLE 4.1 Molecules and Abbreviations Found Throughout the Text

Acronym/Defense Molecule	Full Name	Function
AID	Activation-induced cytidine deaminase	SHM/gene conversion/CSR
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide	Innate immunity (antiviral)
AGM	Aorta/gonad/mesonephros	Intraembryonic origin of hematopoietic cells
AMP	Antimicrobial peptide	Innate immunity (eg, defensins)
APAR	Agnathan paired antigen receptor	Similarities to Ig/TCR and NKRs
AVR	Avirulence protein	Pathogen effector recognized by plant NLR
Bf	Factor B	Enzyme of C′ cascade
B1-3GNP	Beta 1-3 glucan-recognizing protein	Binds to gram-negative bacteria
C′	Complement	Innate/adaptive immunity
CARD	Caspase-recruitment domain	Domain in intracellular defense molecules
CATERPILLER or CLR	CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats	Apoptosis/immunity/inflammation
CDR	Complementarity-determining region	Portion of Ig/TCR that binds to antigen
CSR	Class switch recombination	Adaptive humoral immunity modification
DD	Death domain	Cytosolic interacting domain
DSCAM	Down syndrome cell adhesion molecule	Insect immune (adaptive?) defense and neuron specification
ECM	Extracellular matrix	
ETI	Effector-triggered immunity	Immunity in plants triggered by NLR
FBA	F box–associated domain	Intracellular domain
FcRN	Fc receptor neonatal	MHC-like FcR
FN3	Fibronectin type III repeat	Domain found in many innate molecules
FREP	Fibrinogen-related protein	Mollusk (adaptive?) defense
FuHC	Fusion histocompatibility	Histocompatibility locus in tunicates
GALT	Gut-associated lymphoid tissue	
GPI	Glycophosphatidylinositol	Lipid linkage to cell membrane (eg, VLR)
Hemolysin		Cell lysis
ICE	Interleukin-converting enzyme	IL-1β processing
Ig	Immunoglobulin	Adaptive immunity
IgSF	Immunoglobulin superfamily	Innate/adaptive immunity
IFN	Interferon	Innate (type I)/adaptive (type II) immunity
IMD	Immune deficiency	Insect innate defense
IRF	Interferon regulatory factor	Innate (transcription factor)
IRG	Immunity-related GTPases	Innate immunity
ITAM	Immunoreceptor tyrosine-based activation motif	Signaling motif for NK and antigen receptors
ITIM	Immunoreceptor tyrosine-based inhibitory motif	Signaling motif for NK and antigen receptors
JAK	Janus kinase	Signaling molecule associated with cytokine receptors
KIR	Killer IgSF receptor	NK cell receptor
lectins	For example, galectin, C-type, S-type	Many (eg, NKRs, selectins)
LITR	Leukocyte immune-type receptors	Fish NK-like receptors of the IgSF
LMP	Low-molecular-weight protein	Proteasome subunit
LRC	Leukocyte receptor complex	Gene complex containing KIR and many IgSF molecules
LRR	Leucine-rich repeat	Innate/adaptive immunity module
MAC	Membrane-attack complex	C′, pore-forming
MACPF	MAC-perforin domain	Potential pore former
MASP	MBP-associated serine protease	Lectin C′ pathway
MBP (or MBL)	Mannose-binding protein (lectin)	Lectin C′ pathway
MDM	Mollusk defense molecule	IgSF defense molecule
MHC	Major histocompatibility complex	T-cell recognition; innate immunity
MIF	Macrophage inhibitory factor	Innate immunity; inflammation
MyD88 (also dMyD88)	(*Drosophila*) Myeloid differentiation primary response gene 88	TLR adaptor
NITR	Novel immune-type receptors	Teleost fish NK-like receptors of the IgSF
NK cell	Natural killer cell	Vertebrate innate cellular immunity
NKC	Natural killer cell complex	Gene complex with many C-type lectin genes (especially NK cells)
NKR	Natural killer cell receptor	Receptor on NK cells

(continued)

TABLE 4.1 Molecules and Abbreviations Found Throughout the Text *(Cont.)*

Acronym/Defense Molecule	Full Name	Function
NALP	NACHT leucine-rich repeat and PYD-containing protein	Intracellular PRR
NBD-LRR	Nucleotide-binding domain LRR	Motif of intracellular defense molecules
NFκB	Nuclear factor-κB (Rel homology domain)	Evolutionarily conserved transcription factor
NLR	NACHT leucine-rich repeat protein	Intracellular PRR
NOD	Nucleotide oligomerization domain protein	Intracellular PRR
NOS	Nitric oxide synthase	Intracellular killing innate defense molecule
PAMP	Pathogen-associated molecular pattern	Conserved target epitopes on pathogens
PCD	Programmed cell death	Many pathways
Penaedins		Defense molecule in shrimp
PGRP	Peptidoglycan-recognition protein	Gram-positive bacteria defense family; receptor and effector
PPO	Propolyphenol oxidase	Plant/invertebrate defense (melanization)
PRR	Pattern-recognition receptor	Recognize PAMP, innate/adaptive immunity
PMSB	Proteasome subunit beta subunit	Proteolytic member of 20S proteasome
Polμ	DNA polymerase μ	Error-prone polymerase (related to TdT)
PPO	Prophenoloxidase	Invertebrate defense molecule
PYD	Pyrin domain	Domain in intracellular defense molecules
RAG	Recombination-activating gene	Ig/TCR rearrangement
RFP-Y	Restriction fragment polymorphism-Y	Chicken nonclassical MHC gene cluster
RFX	Regulatory factor X	Transcription factor, class I regulation
RIG	Retinoic acid-inducible gene	Intracellular double-stranded RNA recognition
RSS	Recombination signal sequence	DNA element next to Ig/TCR gene segments necessary for RAG-mediated rearrangement
RXR	Retinoid X receptor	Transcription factor encoded in MHC
SHM	Somatic hypermutation	Adaptive humoral immunity
SPE	Spaezle-processing enzyme	Insect defense molecule in toll cascade
SRCR	Scavenger receptor cysteine-rich	Innate immunity recognition molecule
TAK	TGF-β activated kinase	ubiquitin-dependent kinase of innate pathways
TAP (and TAP-L)	Transporter associated with antigen processing	Rransports peptides from cytosol to ER lumen
TAPBP	TAP-binding protein	Tethers TAP to class I
TCR	T-cell receptor	Adaptive defense
TdT	Terminal deoxynucleotidyl transferase	Involved in Ig/TCR rearrangement
TEP	Thioester-containing protein	Opsonization (like C3)
TGF	Transforming growth factor	Immunosuppressive cytokine
TNF	Tumor necrosis factor	
UPD	Unpaired	Protostome cytokine induced by viral infection
TLR	Toll-like receptor	Innate receptor on the cell surface or in endosomes
TM	Transmembrane	
TNF	Tumor necrosis factor	Proinflammatory cytokine (and family)
TRIM	Tripartite motif-containing proteins	Large family of cytosolic innate defense molecules
V-, C1-, C2-, I-	Variable, constant 1 and 2, intermediate IgSF domain	IgSF domain types
VAV	Guanine exchange factor, the "onc F" proto-oncogene	Encoded in MHC, involved in adaptive signaling pathways
VCBP	Variable domain chitin binding	Amphioxus defense molecule
VLR	Variable lymphocyte receptor	Agnathan adaptive defense molecule
WKRY		Plant transcription factor used to upregulate defense genes (analog of NF-κB)
XMIV	*Xenopus* MHC-linked IgSF V region	*Xenopus* MHC-linked NKR-like genes
XNC	*Xenopus* nonclassical	*Xenopus* class Ib cluster
185/333	Sea urchin defense molecule	(Adaptive?) Defense

DNA, deoxyribonucleic acid; ER, endoplasmic reticulim; IL, interleukin; mRNA, messenger ribonucleic acid; RNA, ribonucleic acid.

LRR-containing proteins are involved in immunity from plants to animals. The functions in the immune systems range from control of motility of hemocytes and lymphocytes[30] to specific recognition of antigens via a novel system of gene rearrangement (the variable lymphocyte receptors [VLR] described in the following; see Fig. 4.9). LRRs can occur in soluble forms, the ECM, in the cytosol, or as TM forms, either integral membrane proteins or glycophosphatidylinositol (GPI)-anchored. The bottom line is that because of its basic structure and malleability, the LRR module was locked in early in evolution as an ideal motif for recognition of essentially any ligand.

Immunoglobulin Superfamily

IgSF domains are encountered in a very large number of molecules in the animal kingdom (see Fig. 4.2).[31] They are found intracellularly (eg, connectin) or as cell adhesion molecules, many of which are in the nervous system (eg, the neural cell adhesion molecule, NCAM), coreceptors and costimulatory molecules of the immune system (eg, cluster of differentiation [CD]79, CD80), molecules involved in antigen presentation to lymphocytes (eg, class I molecules), certain classes cytokine receptors (eg, interleukin [IL]-1R), and of course Ig (and TCR), where they were first characterized and were bestowed with their name (Ig). They can be associated with other domains such as FN (eg, titin and FREP) and LRRs,[32] or they can be the sole constitutive elements of the polypeptide chain often associated to a transmembrane segment and a cytoplasmic tail (or GPI-linked). The β barrel IgSF structure was adopted independently in other families such as cadherins, calycins, lipocalin, etc., and the super (or über) family has hundreds of members and has been selected for several different functions. These functions are somehow related, almost all involved in protein-ligand interactions. The vertebrate lymphocyte surface can express 30 different IgSF members simultaneously.

IgSF domains are commonly classified according to different domain constitution in their β strands and loops.[31,33] All conform to the stable shape of a β barrel consisting of two interfacing β sheets, usually linked by a disulfide bridge. There are three types of domains: variable (V), and two types of constant (C1 and C2); the so-called I set domain is intermediate between the C1 and C2. The V domain is most complex with more strands (C′ and C″), which make up complementarity determining region (CDR) 2 in conventional Igs and TCRs. C1 domains lack these strands entirely, and C2/I domains have varying sizes in the C′/C″ region. V domains, either alone (eg, the new antigen receptor [NAR]) or in association with another V domain (eg, Ig H/L), recognize the antigenic epitope and are therefore the most important elements for recognition. Domains with the typical V fold, whether belonging to the true V-set or the I-set, have been found from sponges to insects (eg, amalgam, lachesinm and fascicilin) and even in bacteria. The mollusk fibrinogen-related proteins (FREPs, described in the following) have one or two V-like domains at their distal end, associated with a fibrinogen-like domain. For V domains, the interface between dimers is the beta strand bearing the C, C′, C″, F, and G strands, so that in Igs the CDR3 are in the center of the binding site; for C domains, the other beta strand bearing the A, B, D, and E strands forms the interface.

The binding capacities of V domains in molecules besides Ig/TCR can reside in different areas of the molecule (interstrand loops, A-A′ strand, F strand), while in Ig/TCR, CD8, and certain NKR, these regions are the targets for variation in shape and charge. The binding capacities can be modulated whether one domain acts as a single receptor unit (eg, IgNAR) or whether it is associated with a contiguous domain (eg, KIR, variable domain chitin-binding protein [VCBP]) or with another polypeptide chain (eg, TCR, Ig). In the case of a dimer, the binding capacity can again be modulated by the presence or the absence in the G strand of a diglycine bulge, which can modify the space between the faces of the Ig domain. In several cases, the sites responsible for binding are known (KIR, Ig, TCR); in many other cases, they are not known but inferred from crystal structures and/or variability plots (leukocyte immune-type receptor [LITR], chicken Ig-like receptor [CHIR], triggering receptor expressed on myeloid cells [TREMs], DSCAM, hemolin).

HEMATOPOIEIS AND CELL TYPES IN THE INVERTEBRATES

Invertebrate Cell Types

Examples of conservation of fundamental mechanisms of genetic control of developmental pathway between protostomes and deuterostomes, even in the absence of homology of the cells or organ considered, are accumulating: the organization and expression of the homeotic gene clusters and eye formation through the function of a complex of proteins including Pax-6.[34] The cell types involved, besides direct interaction with the external layer of cells on the skin, or external teguments, have been specialized cells of mesodermal origin devoted to defense. This is true for all coelomates where effector cells have been identified, but recent data have shown that cnidarian diploblastic organisms that lack mesoderm also have many of the same genetic systems as the coelomates[35] (see Fig. 4.1). The cells can be circulating or sessile, and often are found associated with the gut. Several morphologically distinct hemocyte types in insects cooperate in immune responses: they attach to invading organisms and isolate them, trapping larger organisms in nodules or forming large multicellular capsules around them. Indirect evidence for the role of hemocytes in immune responses can be derived by contrasting properties of such cells in healthy and parasitized animals (ie, modifications in adherence and opsonic activity).

All animals show heterogeneity of the free circulating cells, generically called hemocytes (arthropods), coelomocytes, amebocytes (annelids, mollusks, and echinoderms), or leukocytes (sipunculids). However, the repertoire of insect "blood cells" is clearly less heterogeneous than that of vertebrates. Basically, three or four types of cell lineages can be identified in *Drosophila* (Fig. 4.3[36]): plasmatacyte, crystal cells, and lamellocytes, and an equivalent number in *Lepidoptera* (butterflies). The functional roles they play consist of immune defense, disposing of apoptotic and other debris, contributing to the ECM, and modeling of the

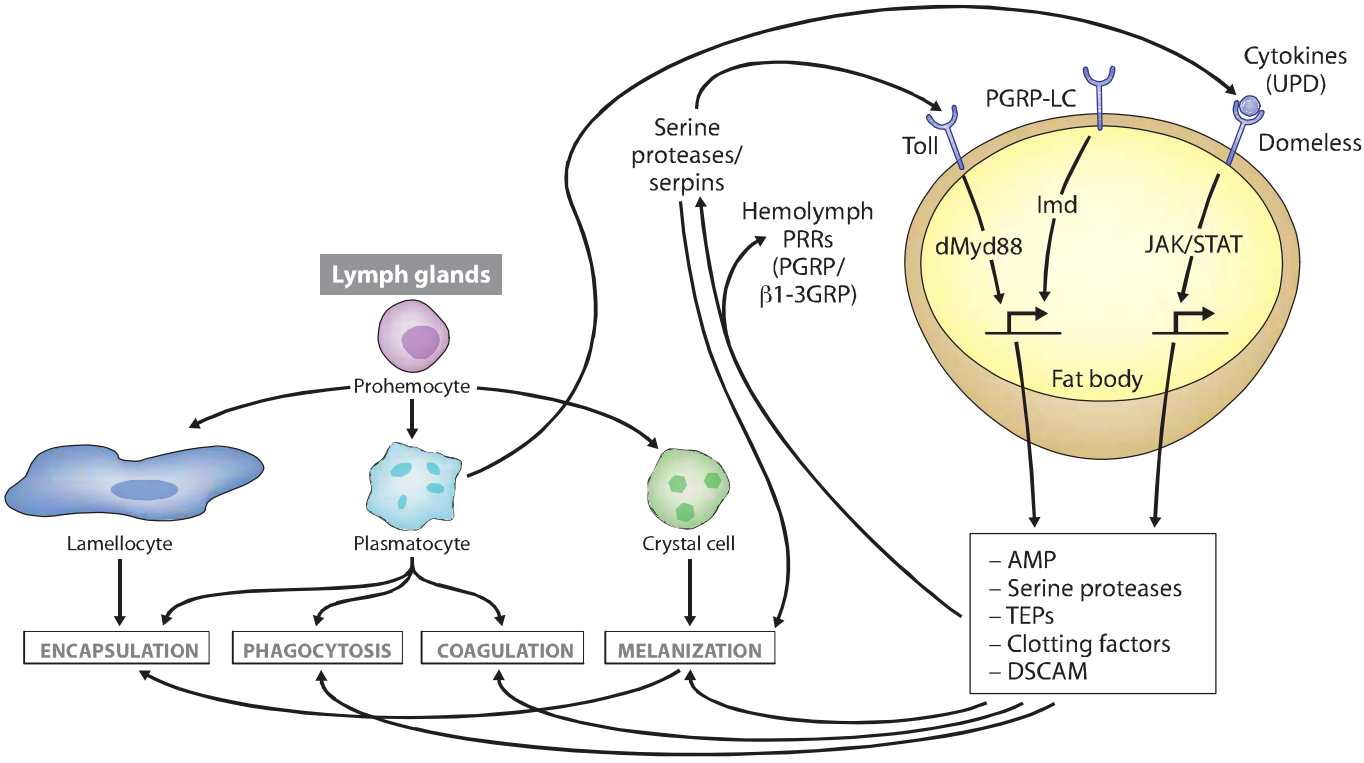

FIG. 4.3. Types of Immune Responses and Cells in Insects, with *Drosophila* as the Prototype. Secreted defense molecules are made by fat body cells in response to pathogens, which either act as direct effector molecules or feed back on hemocytes to stimulate their defense functions. Unpaired (UPD) is produced after virus infection, stimulating defense molecule upregulation via the JAK/signal transducer and activator of transcription pathway. Not shown is the RNAi pathway, also induced upon virus infection. Details on stimulation of the toll and immune deficiency pathways are found in Figure 4.4. This figure was modified from Lemaitre and Hoffmann.[36]

nervous system. The immunity role encompasses phagocytosis, encapsulation, and sometimes production of effector molecules (see Fig. 4.3[36]). These roles all require recognition of pathogen-associated molecular patterns (PAMPs) or self-derived defense molecules (ie, opsonization) at the cell surface.[37–40]

Only in a few organisms has the characterization of hemocyte lineages gone beyond morphologic or basic physiologic functions. Among these free circulating cells are always one or more types that can undergo phagocytosis. Different cells participate in encapsulation, pinocytosis, and nodule formation, and can upon stimulation produce a great variety (within an individual and among species) of soluble effector molecules that may eliminate the pathogen. In an attempt to integrate all of the data available in invertebrates, Hartenstein has proposed a unified nomenclature of four basic types: prohemocytes, hyaline hemocytes (plasmatocytes or monocytes), granular hemocytes (granulocytes), and eleocytes (chloragocytes).[37] These designations will be found in the following description of the blood cell types.

Earthworm (annelid) coelom-tropic coelomocytes are called eleocytes. They contain glycogen and lipid and are considered of the same lineage as the chloragocytes involved in the production of immune effector molecules such as fetidin or lysenin. The phagocytic cells of annelids are apparently granular "leukocytes" derived from the somatopleura and involved in wound healing, whereas the ones derived from the splanchnopleura participate in immunity. Heterogeneity of annelid coelomocytes is not encountered in primitive oligochaetes or in hirudinae (leeches). Phagocytic coelomocytes show an acid phosphatase activity and a beta glucoronidase activity.[41] The large coelomocytes and free chloragocytes (eleocytes) in the typhlosole of *Eisenia foetida* appear to produce the bacteriolytic and cytolytic factor lysenin.[42] From electron microscopy studies, macrophage-like cells seem to be involved in graft rejection. In the closely related sipunculid phylum, two main cell types can be identified in the blood: erythrocytes (a rare occurrence in invertebrates) and granular leukocytes. The latter are capable of cytotoxicity and even have dense granules reminiscent of vertebrate "NK cells."[43]

Two developmental series have been described in mollusks, the hyaline and granular cells, but cephalopods seem to have only one lineage. They participate in encapsulation, with hemocytes adhering around the foreign body like *Drosophila* lamellocytes. Phagocytosis is carried out by the wandering granular cells, which resemble vertebrate monocytes/macrophages. In oysters, electron microscopy revealed different types of circulating hemocytes, including granular hemocytes resembling the granulocytes of sipunculus mentioned previously.[44,45] In crustaceans, the situation is similar to that in mollusks, with three main populations identified based again on the presence of granules in the cytoplasm.

The hyaline cells are involved in the clotting process and the granular cells in phagocytosis, encapsulation, and the prophenoloxidase (PPO) pathway. The hematopoietic organ is located on the dorsal and dorsolateral regions of the stomach.[39] Crustacean hemocytes can now be cultured and their response to virus can be examined,[46] and markers of the three hematopoietic lineages are available.[47]

In insects, the so-called prohemocytes are believed to be stem cells. They are only found in the embryonic head mesoderm and the larval lymph glands but not in the hemolymph. However, prohemocytes are frequent in both the hemolymph and hematopoietic organs of the lepidopteran *Bombyx* (silkmoth). Plasmatocytes of *Drosophila* have a phagocytic function. This type of hemocyte is equivalent to the granulocytes of *Bombyx*, which play a key role in phagocytosis in larvae. Lamellocytes seem to be unique to *Drosophila*, but they are probably the equivalent of the lepidopteran plasmacytoid cells. Their precursors reside in the larval lymph gland, where they differentiate in response to macroscopic pathogens, following a brief phase of mitosis linked to the presence of the pathogens and under hormonal control via ecdysone. The transcription factors (GATA, Friend-of-GATA, and Runx family proteins) and signal transduction pathways (toll/NF-κB, Serrate/Notch, and JAK/STAT) that are required for specification and proliferation of blood cells during normal hematopoiesis, as well as during hematopoietic proliferation that accompanies immune challenge, have been conserved throughout evolution. The specific differentiation of lamellocytes requires the transcription factor Collier. The mammalian early B-cell factor, an ortholog of Collier, is involved in B-cell differentiation in mice. The *Drosophila* crystal cells are responsible for melanization through the PPO system (see subsequent discussion). In silkworm oenocytoids, crystal-like inclusions are also found, but they disappear later after bleeding.[36,37,40]

Echinoderm coelomocytes express a diversity of effector functions, but no studies of lineages have been performed. In echinoderms, the number of different coelomocytes may vary according to the particular family. The sea urchin is endowed with at least four cell types, only one of which only is phagocytic and corresponds to the bladder or filiform forms. Another type is described as the round vibrating cell involved in clotting. Pigment cells (red spherule cells) have been detected ingesting bacteria; the morphology of phagocytic cells can vary enormously, precluding any easy classification.[48]

In tunicates, amoeboid cells circulate in the blood and are involved in a large number of processes, such as clotting, excretion, nutrition budding, and immunity. Large numbers of blood cells are present (average of 10^7 per mL) in the blood of ascidians such as *Ciona*. Hemoblasts are considered to be undifferentiated cells, perhaps the equivalent of the prohemocytes of arthropods or the neoblasts of annelids. Blood cells in ascidians proliferate in the connective tissue next to the atrium. The pharyngeal hematopoietic nodule of this animal contains a large number of hyaline and granular cells called "leukocytes" with supposed intermediary forms of differentiation between blast and granular mature types. The granular form is likely to be involved in postphagocytic activity, like in earthworms.[49] Adoptive transfer of alloimmunity in the solitary tunicate *Styela* can be achieved via lymphocyte-like cells.

In *Amphioxus*, cells with phagocytic capacity have been identified in the coelom with a morphology resembling more the phagocytic echinoderm cells than urochordate blood cells, a fact that is consistent with the new systematic positions of amphioxus and echinoderms.[50] Both free cells and the lining of the perivisceral coelom are able to phagocytose bacteria. Cells with the morphologic appearance of lymphocytes and expression of lymphocyte-specific genes were detected in this species, the earliest identification of such cells in phylogeny.[51]

Hematopoiesis in the Invertebrates

The history of the hemocytes is associated with that of the mesoderm among triploblastic organisms. The bilaterian ancestor was most likely a small acoelomate or pseudocoelomate worm similar to extant platyhelminths (flatworms) (see Fig. 4.1). A specialized vascular system or respiratory system was probably lacking, although cells specialized for transport and excretions were likely present because they exist in most extant bilaterian phyla. One can further assume that groups of mesoderm cells in the bilaterian ancestor could have formed epithelial structures lining internal tubules or cavities (splanchnopleura). In coelomates, the mesoderm transforms into an epithelial sac, the walls of which attach to the ectoderm (somatopleura) and the inner organs (splanchnopleura). Blood vessels are formed by tubular clefts bounded by the splanchnopleura. Excretory nephrocytes are integrated into those vascular walls, which also gives rise to blood cells circulating within the blood vessels (the pronephros of anurans and head kidney of teleost fish are important hematopoietic organs in vertebrates). Thus, further evolutionary changes separated the three systems, but there was a close original connection between them.

The origin of hemocytes has been investigated mainly in arthropods. When examining principles that govern hematopoietic pathways, similarities have been observed with vertebrates, raising interesting evolutionary issues.[37,40] In jawed vertebrates, the yolk sac or its equivalent gives rise to blood precursors that are primarily erythroid in nature (but see the following: recent data suggest that B1 cells and macrophages are also derived from this embryonic tissue). In succession, definitive hematopoiesis occurs in the aorta/gonad/mesonephros (AGM) region of the embryo, encompassing all of the different cell types and multipotent progenitors (although this is controversial). Like in the vertebrates, hematopoiesis in insects is biphasic. One phase occurs in the embryo and the other during larval development. Additionally, these waves occur in distinct locations of the embryonic head mesoderm and the larval lymph gland. In the early embryo expression of the GATA factor, serpent (*Srp*) can be detected in the head mesoderm. This GATA family of zinc-finger transcription factors is conserved from yeast to vertebrates where they are involved in various aspects of hematopoiesis. Blood cell formation in the head

follows *Srp* expression, whereas in the lymph gland there is a long delay between *Srp* expression and the appearance of the lymph gland–derived hemocytes.[38] Hematopoiesis in the head mesoderm and yolk sac may be related evolutionarily. A further similarity occurs at the AGM/lymph gland level in *Drosophila*. The lymph gland develops from a part of lateral mesoderm that also gives rise to vascular and excretory cells, much like the vertebrate AGM. The conserved relationship between blood precursors and vascular and excretory systems is intriguing.

Hematopoiesis and Transcription Factors in the Vertebrates

As mentioned previously, transcription factors of the family PAX 2/5/8; GATA 1, 2, 3; ets/erg; and runt domain–containing factors have been cloned in several invertebrates. One plausible model to explain the genesis of true lymphocytes in vertebrates is that closely related members of transcription factor families are the result of a relatively late divergence in lineage pathways followed by specialization of duplicated genes.[52] These duplications could be those that apparently occurred during the history of chordates (see MHC and "Origins" section[2]). Within deuterostomes, the generation of true GATA 2 and 3 probably occurred after echinoderms diverged from the chordate branch and the GATA, ets, early B-cell factor, and Pax5-dependent pathways of T-/B-cell differentiation are thus specific to vertebrates. It is already known that lampreys express a member of the purine box 1/spleen focus–forming virus integration-B gene family that is critically and specifically involved in jawed vertebrate lymphocyte differentiation. Expression has been detected in the gut, which may be related to the fundamental nature of "gut-associated lymphoid tissue (GALT)" as a lymphoid cell–producing organ.

In vertebrates, the generation of T-, B-, and NK lymphocyte lineages from pluripotent hematopoietic stem cells depends on the early and tissue-specific expression of *Ikaros* (and related loci), which by means of alternative splicing produces a variety of zinc-finger DNA-binding transcription factors. The orthologs of *Ikaros*, *Aiolos*, *Helios*, and *Eos* have been identified in the skate *Raja eglanteria*, where two of the four *Ikaros* family members are expressed in their specialized hematopoietic tissues (epigonal and Leydig's organs; see subsequent discussion) like in mammals.[52] In lower deuterostomes, single genes that seem to be related to the ancestor of the Ikaros and Ets family of transcription factors exist, further suggesting that the division of labor between the family members in the jawed vertebrates was a result of en bloc duplications.[52,53] The conservation of *Ikaros* structure and expression reinforces its role as a master switch of hematopoiesis. We discuss this topic further in the lymphoid tissues section.

Responses of Hemocytes

In this section, we simply touch on classical and specific responses in the invertebrates, responses that are more universal are found in the innate immunity section. Proliferation of hemocytes upon stimulation is an unresolved issue in the invertebrates; clearly, clonal selection resulting in extensive proliferation is not the rule. The turnover of cell populations has been the object of numerous, often unconvincing experiments. Still, new data have emerged, and it is clear that in several invertebrates, proliferation occurs in certain cell types following encounters with pathogens. Very little cell proliferation occurs in the circulation of crayfish, but cells in the hematopoietic tissue divide after an injection of the PAMP β1-3-glucan. New cells in the circulation developed into functional synthetic germinal centers (GCs) and GCs expressing the proPO transcript. RUNT protein expression was upregulated prior to release of hemocytes. In contrast, proPO was expressed in these cells only after their release into the circulation.[54]

By contrast to the study of transcription factors that regulate hematopoiesis, relatively little is known about cytokines that drive hematopoiesis among invertebrates. It was reported that differentiation and growth of hematopoietic stem cells in vitro from crayfish required the factor astakine, which contains a prokineticin domain[55]; prokineticins are involved in vertebrate hematopoiesis, another case of conservation during the evolution of growth factors and blood cell development.

Parasitization of *Drosophila* by the wasp *Leptopilina boulardi* leads to an increase in the number of both lamellocytes and crystal cells in the *Drosophila* larval lymph gland. This is partially due to a limited burst of mitosis, suggesting that both cell division and differentiation of lymph gland hemocytes are required for encapsulation. In genetic backgrounds where ecdysone levels are low (*ecdysoneless*), the encapsulation response is compromised and mitotic amplification is absent. This ecdysone-dependent regulation of hematopoiesis is similar to the role of mammalian steroid hormones such as glucocorticoids that regulate transcription and influence proliferation and differentiation of hematopoietic cells.[56]

Phagocytosis

To obtain phagocytosis at the site of microorganism invasion implies recruitment of cells via chemoattraction. In vertebrates, this can be done by several categories of molecules such as proinflammatory chemokines/cytokines or the complement fragments C3a and C5a (as mentioned in the following section, C3a fragments as we know from mammals may be found in tunicates but not other nonvertebrates; yet, C3 may be cleaved in different ways in the invertebrates). C3b, mannose-binding lectin (MBL), and many other lectins can function as opsonins, and recent studies of the PGRPs, thioester-containing proteins (TEPs), DSCAMs, and eater have added to this repertoire.[36] Ingestion follows phagocytosis, and then killing occurs by an oxidative mechanism with the production of reactive oxygen radicals and nitric oxide. These mechanisms are conserved in phylogeny, and other basic mechanisms are being examined in more detail now in protozoan models.[57] Signaling pathways in common between vertebrates and the protozoon *Dictyostelium* include involvement of cyclic AMPs, integrins, and perhaps mitogen-activated protein (MAP) kinase cascades. Unique to all jawed vertebrates studied to date, the activation of

phagocytes also leads to upregulation of the antigen processing machinery, costimulatory molecules, and proinflammatory cytokines that can enhance adaptive immunity.

INNATE IMMUNE RESPONSES

Immune responses are often subdivided into recognition, signaling, and effector phases, which are subjected to different pressures, defined by whether orthology is maintained and the relative divergence rates of the genes responsible for the various phases. Recognition molecules are from evolutionarily conserved families, but as described previously, their genes are subjected to rapid duplication/deletion so that orthology is rarely preserved. By contrast, signaling pathways can be conserved (see Fig. 4.5), despite the fact that the genes are often divergent in sequence. Effector molecules can either be extremely conserved (eg, reactive oxygen intermediates) or extremely divergent to the point of being species-specific (eg, AMP). Here, we break the immune response down into these three phases, beginning with the recognition phase.

Initiation of an immune reaction can theoretically involve either the recognition of nonself, altered self, or the absence of self. Nonself-recognition can take place with receptors (pattern recognition receptors [PRRs]) that detect PAMPs, which were originally defined by Janeway and colleagues as evolutionarily conserved epitopes displayed by pathogens but not host cells.[58,59] The second mode, altered self, is typified by molecules that are induced in self-cells during infections and recognized by conserved defense molecules, similar to the SOS systems mentioned in the MHC section, or by peptide presentation on MHC molecules. A third mechanism, "am I still myself," depends upon recognition of self-tags and their changes in expression[60] (eg, NK recognition of self-MHC molecules through KIR and C-type lectins). These latter two mechanisms have not been described in the invertebrates for immune defense against pathogens, but it would not be surprising if they were revealed in the future, considering the new features of invertebrate immune systems that have been discovered recently and the usage of this mode of recognition in many invertebrate histocompatibility systems.

Whether the invader is related to its host (cells from individuals of the same species or cells from a parasitoid) or are very distant from the host (fungi and bacteria in metazoa), there are different principles of recognition. Yet PAMP determinants have been identified on very different organisms—sugars such as β1-3 glucan of fungi, lipopolysaccharide (LPS) and peptidoglycans of bacteria, phosphoglycan of some parasites, and especially nucleic acids of bacteria and viruses—and they can trigger similar cascades of events. The foreign ligand can be bound by a molecule in solution that initiates an effector proteolytic cascade (eg, clotting or the complement cascade). On the other hand, a proteolytic cascade can be initiated and result in the production of a self-ligand that interacts with a cell surface or endosomic or cytosolic receptor. In this way, there need not be a great diversity of cell surface receptors, especially in the absence of clonal selection.

Of the over 1 million described species of animals (see Fig. 4.1), approximately 95% are invertebrates representing 33 phyla, some with one species (*Placozoa*, *Cycliophora*) and others with over 1 million (*Arthropoda*). Because they have major differences in body plans, development, size, habitat, etc., wildly different types of immune systems in diverse species should be expected. Early studies of invertebrate immunology reached no consensus of how immunity should be examined, but because vertebrate cellular adaptive immunity was often defined (indeed, was discovered for T cells) through transplantation reactions, attempts to reveal specific memory by allograft rejection were often used. After many unsuccessful attempts to demonstrate memory of such responses (see the following) and after extensive molecular studies, a consensus was reached that an invertebrate adaptive immune system involving somatic generation of antigen receptors and their clonal expression was highly unlikely. However, the term "innate" is rigid and masks the possibility of other somatic alterations of invertebrate immune system molecules, as will be discussed.[61,62] We will categorize the molecules based on their location within the cell.

Intracellular Recognition

Nucleotide-Binding Domain Leucine-Rich Repeat (NLR)

One major group of intracellular sensors in animals and plants is the NLR family (see Figs. 4.2 and 4.4).[14,63] Each of the family members has a central NB/NACHT (nucleotide-binding domain) and C-terminal LRR used for recognition, and a unique N-terminal domain. The subfamilies are defined by their N-terminal domains, coiled-coil and toll-IL-1 receptor (TIR) in plants, and baculoviral inhibitory repeat, caspase-recruitment domain (CARD), pyrin domain (PYD), and activation domain in animals (see Fig. 4.4). Thus far, the NLRs have been found in deuterostomes but not protostomes (see Fig. 4.1), which is surprising considering that plants have intracellular defense proteins with a similar structure, and seem to have been derived via convergent evolution.[14]

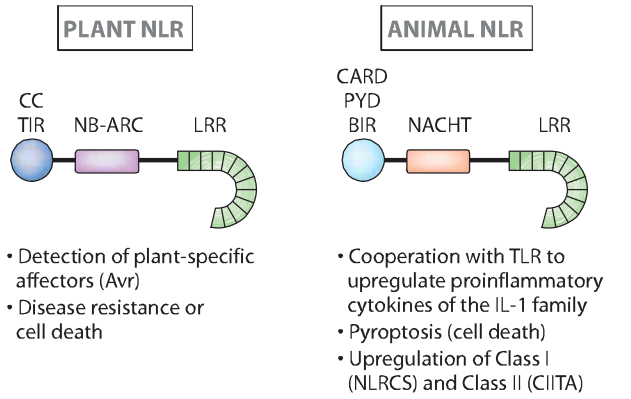

FIG. 4.4. Structure and Major Functions of Nucleotide-Binding Domain Leucine-Rich Repeats in Plants and Animals.[14] Although the structures are quite similar, and recognition can be analogous, these families seem to have arisen via convergent evolution (see text for more details).

The specificity of plant NLR depends principally on the LRRs, and these are targets for diversifying selection, as described previously for multigene families. Plant NLR can recognize pathogen effectors (pathogen-derived avirulence factors), or viral and fungal PAMPs directly via the LRR domains, or via modifications of a host target that interacts with the N-terminal domains, altered self if you will (see Fig. 4.4). This type of activation in plants is termed effector-triggered immunity, which is specific of the NLRs (see Fig. 4.4). A host of downstream effectors are generated, some involved in defense but others activating cell death pathways.[14,64]

Best described in mammals, the nucleotide oligomerization domain (NOD)/NLR recognizes PAMPs such as peptidoglycan and induces an autophagy-mediated destruction of intracellular pathogens as well as production of proinflammatory cytokines; however, it remains controversial whether there is direct or indirect recognition of the PAMPs (similar to some responses in plants). Polymorphisms in the NOD proteins are associated with inflammatory bowel diseases. The NLRP and NAIP NLRs are activated by in ways that are not well understood by various PAMPs or danger-associated molecular patterns and form inflammasomes, best known for the activation of caspase 1 and the processing of pro–IL-1beta (or mature IL-18) for release from cells.[14,65] The founding member of the family, CIITA, has long been known to upregulate class II genes (and associated genes, such as cathepsins and invariant chain), and its function is somewhat outside the norm. Another member, NLRC5, has been shown to upregulate MHC class I expression, but the mechanism is unknown.[66] While the shuttling of the vertebrate NLRs CIITA and NLRC5 to the nucleus seems to be a derived characteristic, movement of plant NLRs into the nucleus to activate transcription occurs, either directly or after recruitment of transcription factors like WRKY described in the following.

NLRs are expressed by echinoderm coelomocytes, again representing a highly diversified family[48] (> 200 members, similar to the TLRs and SRCRs). As mentioned, it is surprising that these genes do not seem to be represented in protostomes, and thus the emergence of the family in plants and deuterostomes occurred through convergent evolution.[67] On the contrary, in the vertebrates a search of the *Danio rerio* (and other teleosts) database have yielded a large number of NLR sequences, more similar to the situation in plants.[63] In humans, most NLR genes are encoded in clusters on chromosomes 11p15, 16p12, and 19q13, where six sequences are found in a single telomeric region.

Rig-I-Like Receptors (RLR)

The retinoic acid-inducible gene (RIG)-I is an intracellular defense molecule that is unrelated to the NOD proteins, with N-terminal CARD and C-terminal helicase domains.[68] With the helicase domain, RIG-1 binds to an uncapped 5′ phosphate group, which is diagnostic of viral RNAs. RIG-I also recognizes short double-stranded RNAs, while a second member of this family MDA5 recognizes long double-stranded RNAs. These molecules contain two CARD domains at the N-terminus, a DEXDc domain, a helicase domain, and a regulatory domain. Ligands bind to the regulatory domain, inducing a conformational change leading to interaction with the adaptor protein MAVS (or IPS-1) and ultimately to the induction of type I interferons (IFNs). A third member of the RLR family is LPG2, which lacks the CARD domain; this molecule was originally believed to be a negative regulator of RIG-I/MDA5-induced signaling, but that has been called into question.

This family is found in all of the vertebrates and in lower deuterostomes, such as amphioxus and echinoderms.[63] Somewhat surprisingly, the RLR family is only mildly expanded in sea urchins (12 members). While there is no report of bonafide RLR family members in protostomes but RLR activity is present,[69] the cnidarian sea anemone has been reported to have a RLR homologue,[70] again showing the importance of studying this taxon for the emergence of immune-related molecules.

Cytosolic Deoxyribonucelic Acid (DNA) Sensors

There are four mechanisms of cytosolic DNA sensing, three of them, the DNA-dependent activator of IFN-regulatory factor, IFI16, and RNA polymerase III (which converts viral DNA into RNA recognized by RIG-I), induce type I IFN production through the intermediate STING, a protein associated with the endoplasmic reticulum (ER).[71] In addition to being an intermediate in IFN upregulation (through IFN regulatory factor-3), STING is also a PRR in its own right, responding to the PAMP cyclic dinucleotides produced by intracellular bacteria like *Listeria*; this suggests that STING was originally a PRR, and then was co-opted by several other PRR sensors to induce effector functions.[72] IFI16 is part of the AIM2-like receptor family; the founding member, AIM2, like the inflammasome, activates caspase-1 to process pro–IL-1beta.

These new molecules/mechanisms have so far only been studied in mammals, but it would be surprising if they were not operative (at least) in other vertebrates as a way to combat DNA viruses. To date, they have not been found in the sea urchin or jawless fish databases.

Tripartite Motifs

Tripartite motif (TRIM) proteins belong to a family induced by type I and II IFNs, with 68 members in the human genome. TRIMs are involved in resistance against pathogens in mammals, especially lentivirus (eg, human Trim 5α is a retroviral restriction factor with activity against human immunodeficiency virus).[73,74] The activity of proteasomes, responsible for cytosolic protein degradation, has been implicated in the TRIM5α-dependent attenuation of retroviral reverse transcription. TRIMs contain an N-terminal moiety composed of three modules: RING (with an E3 ubiquitinase activity)-Bbox-coiled xoil motif followed by different C-terminal domains. TRIMs fit into two major categories by the function of their C-terminal domain: Category 1 with a PHD, MATH, ARF, FNIII, exoII, or NHL domains, and Category 2 with a B 30-2 domain shared with butyrophilins and other proteins and essential for ligand binding.[75] The tertiary structure of TRIM21 revealed two binding pockets in the B30.2 domain formed by six variable loops.[76]

Despite reports to the contrary, the TRIM family is ancient.[77] The family has been greatly diversified in vertebrates and in a taxon-specific manner, as observed for many multigenic immune families.[77] The zebrafish genome harbors a striking diversity of a subset of Category 2 TRIMs not encountered in mammals, called finTRIM, with 84 genes distributed in clusters on different chromosomes. This subset, specific of teleosts, is overexpressed after virus infection in the trout. In the B30.2 domain, residues under positive selection are concentrated within a viral recognition motif first recognized in mammalian Trim 5α.[78]

Finally, *trim* genes encoding Category 2 proteins are preferentially located in the vicinity of MHC or MHC gene paralogs both in fish and human, suggesting that they may have been part of the ancestral MHC.[79] The B30.2 domains most closely related to finTRIM are found among NLRs, indicating that the evolution of TRIMs and NLRs was intertwined by exon shuffling.[80] Exon shuffling was likely responsible for the presence of the B30.2 domain in butyrophilin and TRIM genes where it was perhaps favored by the proximity of gene in the MHC. It has been argued that during evolution the combination of SPRY and PRY motifs that build up the B30.2 domain were selected and maintained for immune defense.[81]

P47 GTPases

Among IFN-inducible immunity-related genes with an interesting evolutionary history, immunity-related GTPases (IRG/p47 in mouse) function as cell-autonomous resistance factors by disrupting the vacuolar membrane surrounding parasites (eg, toxoplasma).[82] The IRG system studied primarily in mice (absent in humans[83]) is present throughout mammals but the number, type, and diversity of genes differ greatly even between closely related species, one of the common themes in immunity described previously.

Concerning the evolutionary origin of the IRGs, the homologs of zebrafish and pufferfish seem to form two teleost-specific groups, another common theme in this chapter. Their putative promoter regions suggest an expression regulated by an IFN. Homology searches failed to find any convincing ancestral form to the vertebrate IRG proteins in the genomes of invertebrates, but in phylogenetic trees vertebrate IRGs clusters with some families of bacterial GTPases. Thus, IRGs may be derived from a prokaryotic GTPase acquired by a horizontal transfer subsequent to the appearance of eukaryotes.[82]

Integral Membrane (and Sometimes Secreted) Proteins

C-Type Lectins

Lectins were originally defined by their ability to bind carbohydrates in a calcium-dependent manner (how C-type lectins got their name[84]) and some have been described previously (and throughout the chapter). They are found in many phyla in both the deuterostome and protostome lineages in both membrane and/or secreted forms (eg, MBL described in the following). A large number of C-type lectins have been uncovered in the mosquito genome, and some are involved in bacterial defense through direct binding and others through the melanization reaction.[85] Some C-type lectins are encoded in the NKC, including the Ly49 and NKG2 families, as well as CD94 and several other members of the family are central to NK-cell function in mammals. A molecule resembling CD94 but unlikely to be an ortholog (see the following) has been detected on a subset of hemocytes in *Botryllus* and *Ciona*, the functions of which are unknown.[86] Another large gene family that is implicated in the response of the sea urchin to immune challenge includes 100 small C-type lectins,[48] consistent with the enormous expansion of several immune defense families in this animal. We describe other functions of C-type lectins in the NK cell sections.

Scavenger Receptors

The SRCR superfamily is an ancient (from sponges to chordates) and highly conserved group of cell surface and/or secreted proteins, some of which are involved in the development of the immune system as well as the regulation of both innate and adaptive immune responses; they are especially well known for their function in macrophages.[87] Group B SRCR domains usually contain eight regularly spaced cysteines that allow the formation of a well-defined intradomain disulfide-bond pattern. Scavenger receptors are best known for their housekeeping function of taking up lipids modified by oxidation or acetylation, but they have many other functions as well, such as uptake of apoptotic bodies (eg, croquemort in *Drosophila* of the CD36 subfamily[88]).

SRCRs have been studied mainly in the coelomocytes of echinoderms. Within a few hours after bacterial injection, sea urchin coelomocytes upregulate a variety of genes including an extremely diverse family of SRCRs.[48,89] A very large number of SRCR domains are present (approximately 1,200), but each individual may express different groups of SRCR genes at different levels (and even with differential splicing). To assume that they are all involved in defense is premature, as SRCR genes can be both up- and downregulated after infection with bacteria. As mentioned, this high level of gene duplication is a general rule in the echinoderms.

In mammals, the SRCR family as a whole is also poorly defined but is involved in endocytosis, phagocytosis, and adhesion, and some members acts as PRRs that bind to LPS or other bacterial components. SRCRs are widespread in the human genome and participate as domains in the structure of numerous receptors (eg, S4D-SRCRB, CD6, CD5-L, CD163), but without showing the high level of duplication seen in the echinoderm families.[87]

Down Syndrome Cell Adhesion Molecule

DSCAM in *Drosophila* and other arthropods was described originally by neurobiologists as an axon-guidance protein, dependent upon a large number of isoforms (> 30,000) generated by alternative splicing for the IgSF domains and the transmembrane segment. DSCAM is also involved in insect immunity, expressed in cells of the hematopoietic lineage, and clearly capable of binding to bacteria; like in the nervous system, a large number of splice variants are generated,

clearly different from the ones expressed in neurons.[24] In *Drosophila*, the DSCAM gene is composed of 115 exons, 95 of which encode alternative possibilities for splicing of exons 4, 6, and 9. The molecule consists of 10 IgSF domains and 6 FN domains, and present as either a membrane or soluble form, presumably generated by proteolysis of the membrane form. Each cell expresses only a fraction of the isoform repertoire.

Knock out (RNAi) and anti-DSCAM treatment significantly suppresses phagocytosis, at least in *Drosophila*. Soluble DSCAM constructs with different exon combinations were found to have differential pathogen-binding properties.[90] In addition, suppression of DSCAM in mosquitoes results in an impaired immunity to *Plasmodium*; exposure of hemocytes to different pathogens in culture gives rise to specific modifications and selection of alternative splicing patterns. A similar finding was made in crustaceans, in which particular DSCAM isoforms were induced in response to different pathogens in one species[91] and epitope II was under selection in a study in *Daphnia*.[92] The diversification of DSCAM seems to be specific of arthropods as neither flatworm nor sea urchin nor vertebrate DSCAM are diversified. The vertebrate DSCAM has only two forms, using two alternate TM exons. The cytoplasmic tail can also be modified by alternative splicing that could change its signaling properties by modulation of tyrosine-based motifs.[93] Human DSCAM is duplicated on chromosomes 21 and 11, but does not appear to be involved in immunity.

Peptodoglycan-Recognizing Protein and β1-3 Glucan Receptors

PGRPs are found in a wide range of organisms but have been best studied in insects, where they are classified into short (S) and long (L) forms. S forms are soluble and found in the hemolymph, cuticle, and fat-body cells.[94] L forms are mainly expressed in hemocytes as integral membrane proteins where their final structure depends on combinatorial association of different isoforms, modulated by alternative splicing. We provide a short description here, but delve more deeply in the discussion of the insect toll and immune deficiency (IMD) pathways subsequently (Fig. 4.5).[36] The expression of insect PGRPs is often upregulated by exposure to bacteria. PGRPs can activate the toll or IMD signal transduction pathways (see the following) or induce proteolytic cascades that generate AMPs, melanization, or induce phagocytosis. PGRPs directly kill bacteria by inducing a suicide mechanism, first demonstrated to be activated by a type of unfolded protein (stress) response in prokaryotes.[95] Besides their defense functions, insect PGRPs expressed in the gut are believed to promote homeostasis with commensal bacteria (also discussed briefly in the following). Both soluble and transmembrane forms are present in sea urchins, some with potential catalytic function.[48]

In vertebrates, PGRPs are all secreted and have direct microbicidal activity. Best studied in zebrafish, PGRPs are

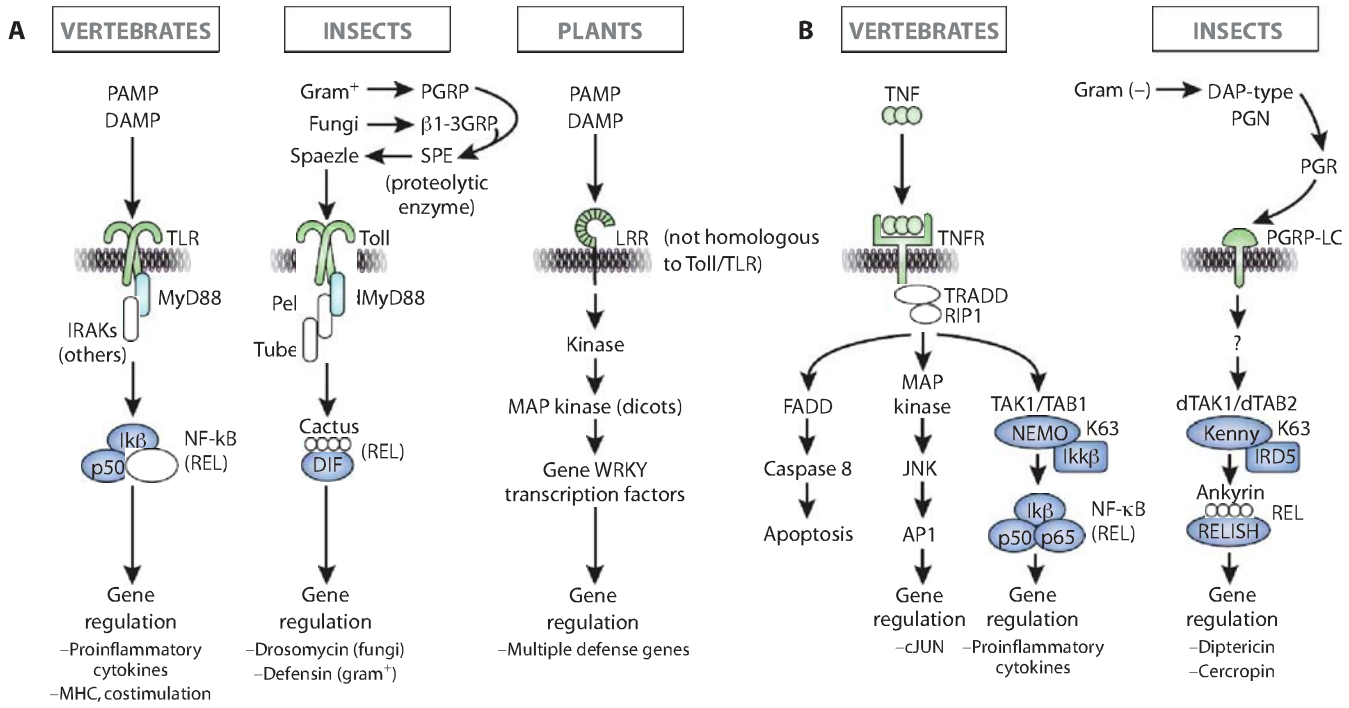

FIG. 4.5. Comparison of Immune Response Induction, Intracellular Pathways, and Immune Outcome in Insect (eg, *Drosophila*), Vertebrates (eg, human), and Plants (a Composite of Pathways in Monocots and Dicots). Note that the initiation of the response and the outcome(s) in insects and vertebrates are quite different for both the toll/toll-like receptor pathway (*left*, **A**) and the immune deficiency/tumor necrosis factor pathways (*right*, **B**), but the intracellular signaling pathways are well conserved evolutionarily in insect and vertebrate (details in the text). Note as well that plants use similar molecules for recognition (leucine-rich repeat–containing molecules) and have similar intracellular pathways with kinase cascades, but *all* of the molecules of recognition, signaling, and effector are derived by convergent evolution as compared to animals. This figure was modified from Beutler et al.[152]

expressed in many tissues such as gills, skin, and intestine, providing immune defense. They are expressed before the development of adaptive immunity and likely provide an important protective role.[96] The human PGRP genes are found on the MHC chromosomal paralogs 1q21 and 19q13/p13. All detected splice-variant isoforms bind to bacteria and peptidoglycan. Like the fish molecules, mammalian PGRPs are also positioned at epithelial surfaces and promote intestinal homeostasis by discriminating somewhat between commensal (eg, lactobacilli) and pathogenic bacteria. Knockout mice have increased pathogenic bacteria on mucosal surfaces that induce colitis after injury in the dextran sulphate sodium autoimmune assay.[97]

β1-3 glucan receptor proteins (β1-3GRPs, formerly known as gram-negative binding proteins (GNBPs) are related to bacterial β1-3 glucanases.[98] They are found in insects and other arthropods where they bind bacteria, fungal β-1, 3-glucans, LPS, and/or bacterial lipoteichoic acid (without necessarily showing glucanase activity) . An ortholog is present in the sea urchins, but not in vertebrates to date. *Drosophila* GNBP1 together with PGRP-SA are required to activate the toll pathway in response to infection.[36]

Toll and Toll-like Receptors

The toll receptors were originally described in *Drosophila* as genes involved in early development, specifically in dorsoventral patterning. Later, they were also shown to be essential sensors of infection, initiating antimicrobial responses.[36,99] This family was then revealed to be a major force in innate immunity in the vertebrates as well.[100,101] As mentioned previously, across the metazoa structurally closely related members of the toll family range from not being involved in immunity (in *C. elegans* and apparently in the horseshoe crab), to being the equivalent of a cytokine receptor (in *Drosophila*), to being PRR in the vertebrates and invertebrates.[102] Six spaetzle-like and eight toll-like molecules have been identified in *Drosophila*, but only one or two of them are clearly immunity-related.[36,102] In jawed vertebrates, they belong to a multigene family of PRR specific for diverse PAMPs and exhibiting different tissue distributions and subcellular locations.[27] In humans, many are on chromosome 4p and q (TLR 2, 1, 6, 10) but the others are distributed on chromosomes 9, 1, 3, and X.

Ectodomains of TLRs comprise 19 to 25 tandem repeats of LRR motifs made of 20 to 29 aa capped by characteristic N- and C-terminal sequences. All of the toll receptors are homologous and appear similar in domain constitution among all animals. They also share the TIR domain, which is the intracellular segment shared with the IL-1/-18/-33 receptors of vertebrates, as well as other molecules in plants. TIR domains associate with Myd88 to initiate signaling cascades culminating in the activation of NFκB/Rel (see the following) (see Fig. 4.5).

In *Drosophila*, the toll dimer is triggered by an interaction with the unique ligand spaetzle, which is the product of a series of proteolytic cascades, with the most critical enzyme identified (spaetzle-processing enzyme). Activation of the cascades triggers the production of antimicrobial peptides (see Fig. 4.5). The specificity of recognition is not achieved at this receptor level but rather in solution via other intermediates (see the following). *C. elegans* has only one toll receptor, and rather than being antimicrobial responses, it promotes avoidance of a flatworm pathogen upon engagement; the signaling mechanism for the *C. elegans* TOL (toll) is not evolutionarily conserved (it clearly does not induce the NF-κB pathway) and is under investigation.[67] A toll/TLR gene is present in the sea anemone, a cnidarian, but not in other cnidarians such as hydra or coral, which nevertheless have TIR domains associated with other molecules.[35] A TIR domain of the toll-receptor types was detected in sponges, but, like IL-1R in vertebrates, it is associated with a receptor with three IgSF domains.[103] Plants do not have toll/TLR per se, but do have LRR-containing transmembrane sensors that function in a similar fashion[102] (see Fig. 4.5). In summary, toll/TIR arose before the split of protostomes and deuterostomes, but has been lost in some invertebrate groups and has been recruited to perform multiple functions.

The arsenal of TLRs in vertebrates is endowed with specific and diverse capacities. Each vertebrate TLR has its range of specificities and, in addition, combinations of different TLR can create different binding specificities (eg, the association of TLR2 with TLR6 or TLR1 and 2[104] or even TLR2 homodimers in regulatory T cells[105]). This divergence in recognition function is well illustrated by the phylogenetic analysis of the toll and toll-related receptors in different phyla such as arthropods and vertebrates. Toll and related proteins from insects and mammals cluster separately in the analysis, indicating independent generation of the major families in protostomes and vertebrates.[102] Consistent with the expansion of SRCR and NLR genes in sea urchins, hundreds of TLRs also were found in this species.[48] TLRs of the protostome type are in small numbers (3 members) while the vertebrate type has been enormously amplified, all within a single family (222 members) and most without introns. Vertebrate TLRs do not diverge rapidly and evolve at about the same rate, and while there have been some duplications in amphibians and fish, they are not greatly expanded like in the echinoderms (no more than approximately 20 genes in any species).

Signaling Through Innate Surface Recognition Molecules

Four pathways of innate immunity triggering have conserved elements in eukaryotes: the toll/TLRs, the TNF-α/IMD receptors, the intracellular NOD, and the JAK/STAT. Although toll receptors have been found in almost all triploblastic coelomates, most of the work and the elucidation of pathways have been accomplished in *Drosophila* and *Anopheles*.[36] The diversity of AMPs that can be produced via the toll/IMD pathways is substantial, and as described previously is classified in several categories depending upon the type of pathogen recognized (eg, gram (+), drosocin, gram (−), diptericin; fungal, drosomycin) with different effector functions (see Fig. 4.5). Insect antimicrobial molecules were originally discovered by the late Hans G. Boman and colleagues in 1981, a seminal finding that

heralded the molecular analyses of innate immunity in the invertebrates.[106]

Toll and Immune Deficiency Pathways

As described, invertebrate toll receptors are homologous to the vertebrate TLR, in the sense that they are integral membrane LRR-containing proteins (see Fig. 4.5). *Drosophila* toll is activated after it binds spaetzle, the product of a proteolytic cascade activated in solution after the interaction of molecules produced by fungi or gram-positive bacteria with GNBP and PGRP.[107] The TIR cytoplasmic domain of the toll receptor then interacts with MyD88 (itself having a TIR domain) followed by Tube and Pelle, leading to activation of the homologous NF-κB system (Cactus or Diff) that then induces transcription of various defense peptides.[36,99,108] This is remarkably similar to the cascade of events following activation of mammalian TLRs where after their interaction with PAMPs at the cell surface, a cascade is induced through TLR including MyD88, IRAK, TRAF, TAK1, to NF-κB via the IKK signalosome. Thus, infection-induced toll activation in *Drosophila* and TLR-dependent activation in mammals reveal a common ancestry in primitive coelomates (or previous), in which defense genes under the control of a common signaling pathway lead to activation of Rel family transactivators.

The *Drosophila* IMD pathway is employed in responses to gram-negative bacteria[109] (see Fig. 4.5). After interaction with the cell surface receptor PGRP-LC mentioned previously, in a cascade similar to the mammalian TNF-αR signaling pathway, *Drosophila* tak1, an IKK signalosome, and a Relish-mediated (instead of Diff) NF-κB step, results in transcription of antibacterial peptides like diptericin. The *Drosophila* intracellular pathway is similar to the mammalian TNF-α receptor cascade, which also progresses via a death domain Mekk3, the signalosome, and NF-κB resulting in cytokine production. In both cases, a link to pathways leading to programmed cell death is possible; overexpression of *Drosophila* IMD leads to apoptosis. When the activation of either the fly toll or IMD pathway is considered, they are analogous to a mammalian cytokine/cytokine receptor system (eg, TNF-α) in which a soluble self-molecule activates cells via a surface receptor. Fitting with the paradigm put forward on recognition, signaling, and effector phases of the immune response, the diversity of external recognition systems is not matched by an equivalent diversity of intracellular signaling pathways.[22] There are conserved signaling cascades coupled to the receptors, giving the impression of conservation of the innate immunity pathways; yet, these pathways are also used in development, so which is primordial remains an open question.

Plants do not have toll/TLR, but do have transmembrane LRR-containing microbial sensors, of which FLS2 that binds to flagellin, is best characterized[27] (see Fig. 4.5). These molecules do not have an intracellular TIR domain (note that TIR domains exist in plants, but not associated with the TM sensors), but do recruit a kinase of a similar nature to the toll/TLR kinases (the so-called non-RD kinases) to activate downstream mitogen-activated protein kinase cascades. However, as mentioned previously, the NFκB transcriptional system arose early in the animal kingdom[110]; plants employ a different system of transcriptional activators, the WRKY molecules, which are activated directly by the mitogen-activated protein kinase cascades, similar to transcription factor found in animals, AP1.

Extracellular Soluble Receptor with Effector Cascade

Proteolytic cascades are initiated immediately following interaction of foreign material bound by preformed proteins in solution, and this principle is conserved throughout evolution. Indeed, the proteolytic cascade upstream of production of the toll ligand spaetzle resembles the complement or clotting cascades. The PPO cascade of arthropods leading to melanization and the genesis of antibacterial products described in the following is another example in which peptidoglycans on microbial surfaces initiate the cascade resulting in the degranulation of hemocytes.

The Complement System

The best-studied immune proteolytic cascade that is surprisingly well conserved in the animal kingdom is complement[111,112] (Fig. 4.6). In contrast to the other defense molecules that we have discussed, orthologous complement genes can be detected in all of the deuterostomes without a great deal of expansion/contractions of the gene family. The three major functions of complement in jawed vertebrates are 1) coating of pathogens to promote uptake by phagocytes (opsonization); 2) initiation of inflammatory responses by stimulating smooth muscle contraction, vasodilatation, and chemoattraction of leukocytes; and 3) lysis of pathogens via membrane disruption. Additionally, in the vertebrates, C′ is vital for the removal of immune complexes as well as elicitation of adaptive humoral immunity. The focal point of complement is C3, which lies at the intersection of the alternative, classical, and lectin pathways of complement activation. It is the only known immune recognition molecule (besides its homologue C4) that makes a covalent bond with biologic surfaces via a thioester linkage. C3 has a nonspecific recognition function, and it interacts with many other proteins, including proteases, opsonic receptors, complement activators, and inhibitors. In the alternative pathway, C3 exposes its thioester bond in solution, and in the presence of cell surfaces lacking regulatory proteins that block C3 activation (by cleaving it into iC3b), it associates with the protease factor B (B or Bf). After binding to C3, B becomes susceptible to cleavage by the spontaneously active factor D, resulting in formation of the active protease Bb that in combination with the covalently attached C3 cleaves many molecules of C3 in an amplification step. Another nonadaptive recognition system, the lectin pathway, starts with the MBL (or the lectin ficolin), which is a PRR of the collectin family that binds mannose residues on the surface of pathogens and can act as an opsonin. MBL is analogous to C1q with its high-avidity binding to surfaces by multiple interaction sites through globular C-terminal domains, but apparently it is not homologous to C1q. Like C1q, which associates with the serine proteases C1r and C1s, the MBL-associated serine proteases (MASPs) physically interact with MBL and not only activate

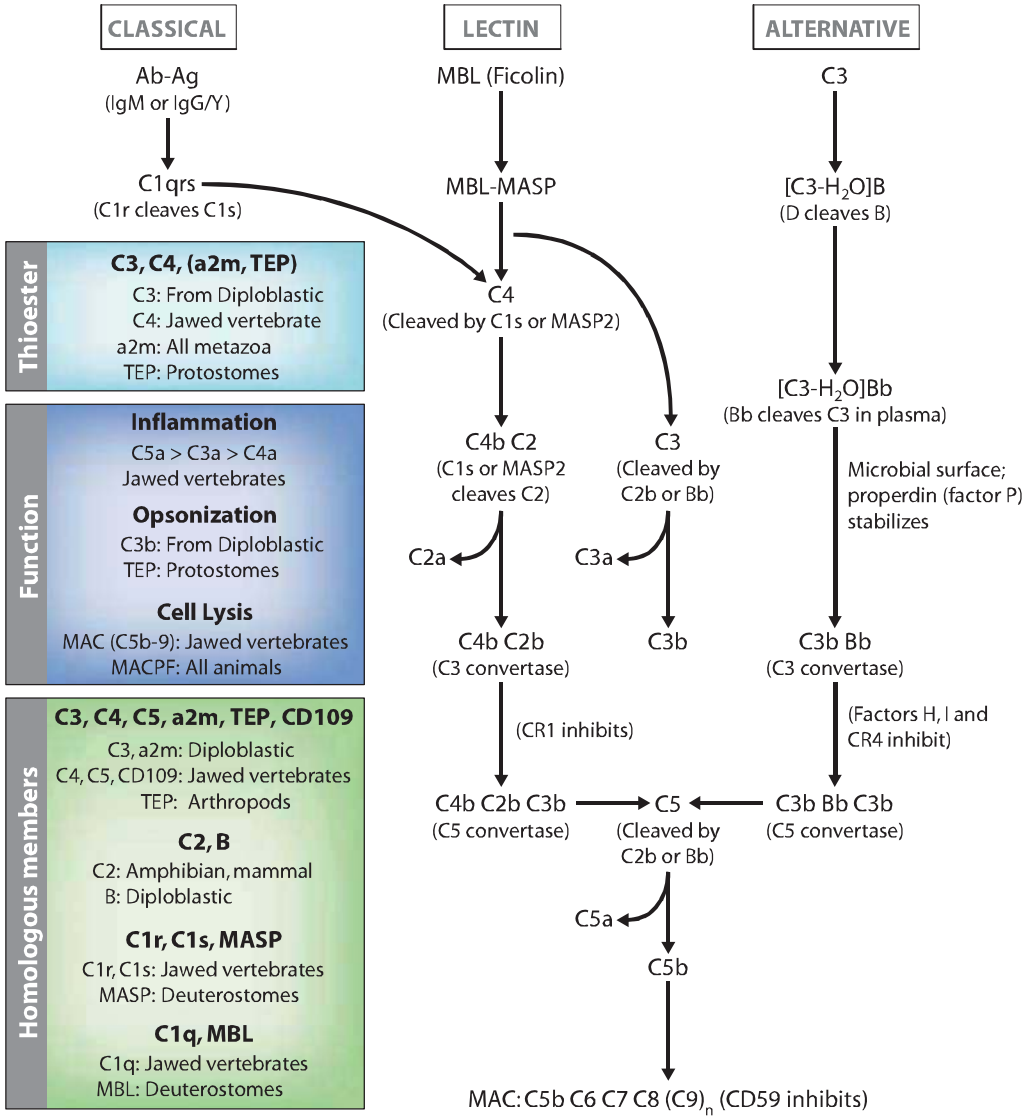

FIG. 4.6. Evolution of the Complement System. The general pathways and appearance of the various components in the phylogenetic tree are emphasized.

the classical pathway of complement by splitting of C4 and C2 (the same function as C1s; MASP2 appears to be the active protease), but also can activate the alternative pathway in ways that are not understood and thus completely bypass the classical pathway. Indeed, MASP-1 and -2 are homologs of C1r and C1s (see Fig. 4.6). Both C1q and MBL can promote the uptake of apoptotic bodies by phagocytes, via collectin receptors. Another lectin, ficolin, can also initiate the MASP pathway,[113] and it would not be surprising if other activators were discovered in the future (eg, the ancient molecule C-reactive protein is also capable of activating C′). Finally, the classical pathway, which is dependent upon antibody molecules bound to a surface, results in the same potential effector outcomes described previously for the alternative pathway. Novel molecules initiating this pathway are C1q, C1r, C1s, C4, and C2, as well as specific negative regulatory proteins such as C4-binding protein.

C3 and MBL (and ficolin) are vital players in the immediate innate immune response in vertebrates, and both have been described in nonvertebrate deuterostomes.[48,112] Thus far, the best-studied invertebrate systems for investigation of C3 evolution are the sea urchin and the ascidians *Halocynthia* and *Ciona*, in which C3 and B molecules and genes have been analyzed in some detail. In contrast to the very high levels of C3 found in the plasma of jawed vertebrates, sea urchin C3 is not expressed at high levels but is induced in response to infection in coelomocytes.[114] The C3 opsonic function clearly has been identified, but so far initiation of inflammatory or lytic responses (if they exist) has not been obvious. Receptors involved in the opsonization in echinoderms have not been identified, but in the ascidian gene fragments related to the C3 integrin receptor CR3 were identified, and antisera raised to one of the receptors inhibited C3-dependent opsonization.[115]

Hagfish and lamprey C3-like genes were thought to be ancestral C3/C4 genes because the sequence predicts two processing sites (leading to a three-chain molecule), like C4, but a C3-like properdin-binding site is clearly present.[116] However, like C3 in other animals the hagfish protein is composed of only two chains of 115 and 72 kDa, and sea urchin and ascidian C3 sequences predict only two chains (one proteolytic processing site). The lamprey, but not sea urchin C3, has a recognizable C3a fragment known from gnathostomes to be involved in inflammation, so the role of complement in inflammation may be a vertebrate invention (but see the following).

TEPs have been isolated from *Drosophila* and the mosquito *Anopheles*, as well as several other arthropods.[117,118] While the insect molecules function in a C3-like fashion (opsonization), phylogenetic analysis shows them to be more related to α2-macroglobulin (note that a few insects actually have molecules more related to C3). TEPs in insects function as opsonins, binding to parasites and promoting their phagocytosis or encapsulation. The evolution multimember TEP families in *Drosophila* and mosquito followed independent evolutionary paths, perhaps as a result of specific adaptation to distinct ecological environments as described in the introduction. The *Drosophila* genome encodes six TEPs (whereas there are 15 genes in *Anopheles*, again consistent with the major expansion of many immune gene families in the mosquito), three of which are upregulated after an immune challenge. Mosquito TEPs are involved in killing of parasites, and the reaction is regulated by LRR-containing molecules to avoid destruction of self-tissues; thus, full-blown complement-like systems complete with inhibitors have arisen independently in protostomes and deuterostomes.[119,120]

C3-like genes are present in cnidarians[121,122] and in the horseshoe crab *Limulus*.[123] Good phylogenetic support was obtained for their relationship to C3, as compared to other members of the thioester-containing family like the TEPs. Thus the emergence of C3 as a defense molecule predates the split between protostomes and deuterostomes. A gene resembling the proteolytic enzyme Bf was discovered in these protostomes as well (and in sea anemones), suggesting that the fundamental system was in place a billion years ago (see Fig. 4.1). The lack of C3 in many other protostomes suggests that the ancestral gene was lost and replaced by the TEPs.[117,118]

In jawed vertebrates and some lower deuterostomes, certain species express more than one C3 gene, suggesting that the innate system might compensate in animals that do not optimally make use of their adaptive immune system.[124] Changes in the amino acid composition of the C3-binding site are found that may somehow regulate the types of surfaces bound by the different isotypes.[125] Likewise, in lower chordates such as *Ciona*, C3 and other complement components can be duplicated.[115] Diversification of the carbohydrate recognition domains has been observed also in the *Ciona* MBP family (nine members).

Like Ig/TCR/MHC, the classical pathway and the terminal pathway membrane-attack complex (MAC) appears first in cartilaginous fish.[112] However, as MBL can activate the classical pathway in mammals, it is possible that some portion of this pathway exists in prejawed vertebrates. Nevertheless, C4 and C2 genes have not been detected to date in jawless fish or invertebrates. A bonafide C2 homologue has only been identified to the level of amphibians, although duplicate B genes were isolated from cartilaginous fish and teleost fish that may function both in the classical and alternative pathways. The lytic or MAC pathway, which is initiated by the cleavage of C5 into C5a and C5b, also has not been described in taxa older than cartilaginous fish. Thus, opsonization and perhaps the induction of inflammatory responses were the primordial functions of the lectin/complement pathways. However, a complementary DNA clone for CD59, a molecule that inhibits MAC formation in self-cells, was identified from a hagfish library, and some of the terminal components of the pathway have been detected in lower deuterostomes with no described functions.[116] Interestingly, proteins with the MAC/perforin domain have been detected throughout the animal kingdom,[48] and some are even involved in cytotoxic reactions; however, it seems that only vertebrates have bonafide terminal C′ components that are highly evolved for targeted destruction of cell membranes. The perforin gene itself also seems to have arisen in gnathostomes, from an ancient MAC/perforin domain-containing gene, macrophage-expressed gene 1 protein, which dates back to sponges; thus, cellular cytotoxic reactions in the invertebrates described in the following must use novel cytotoxic effector molecules.[126]

C3, C4, C5, and α2m (and TEP) are members of the same small family. A cell-surface–expressed (GPI-linked) member of this family, CD109, has been shown to associate with the transforming growth factor (TGF)-β receptor and modulate its expression.[127] The protease inhibitor, α2m, clearly present in invertebrates (protostomes and deuterostomes) and vertebrates, is thought to be the oldest, but obviously this must be viewed with caution considering the data in cnidarians. Along with its ability to bind to and inactivate proteases of all known specificities through a "bait region," it also has been shown to be opsonic in some situations. α2m, C3, C4, and CD109 (as well as the TEPs) have internal thioester sites, so this feature is primordial; C5 subsequently lost the site. The first divergence probably occurred between α2m and C3, with C5 and then C4 emerging later in the jawed vertebrates.[128] Consistent with Ohno's vertebrate polyploidization scheme is the fact that C3, C4, and C5 genes are located on three of the four previously described paralogous clusters in mammals, and this is also fits with the absence of classical (no antibody) and lytic (no MAC) pathways in phyla older than cartilaginous fish.[2] α2m is encoded at the border of the NKC in mice and human, and there are similarities between these regions and the other MHC paralogs (see Fig. 4.13). The C3a and C5a receptors that promote the inflammatory responses upon complement activation have been identified in several vertebrates and (perhaps) some lower deuterostomes; like the chemokine receptors they are G-protein coupled receptors whose genes may also be found on the ohnologs (C3aR, chr 12p13; C5aR, chr 19q13). If indeed such receptors are found in the prejawed vertebrates

as suggested by recent pioneering experiments in *Ciona* and *Styela*, it will be interesting to determine whether they are involved in some type of inflammation, thought to be the domain of the vertebrates.[129]

Melanization (Prophenoloxidase Cascade)

A major defense system in invertebrates is the melanization of pathogens and damaged tissues,[130] popularized by poor Gregor in Kafka's *Metamorphosis*, when the cockroach Gregor undergoes a melanization reaction from an apple thrown into his thorax by his father. The process is controlled by the circulating enzymes PPO and phenol oxidase. The system is activated by β1-3GRP, PGRP, LPS-binding proteins, and other proteins that can bind to various PAMPs (see Fig. 4.3). The complexes launch a cascade of serine protease activities resulting in cleavage of the pro-form of a prophenoloxidase-activating enzyme into the active form that in turn activates the PPO into phenol oxidase. This leads to the production of quinones and finally melanin. Melanization can completely inhibit parasite growth, whereas concomitant with PPO activation, many other immune reactions are initiated, such as the generation of factors with antimicrobial-, cytotoxic-, opsonic-, or encapsulation-promoting activities. The presence of specific proteinase inhibitors (of the serpin family) prevents unnecessary activation of the cascade and overproduction of toxic products. Phenoloxidase is the key enzyme responsible for the catalysis of melanization. It is a marker of the PPO activating system, and it can be an immune effector by itself as demonstrated in ascidians. It is therefore interesting to assess its conservation within all metazoa. A survey of the different organisms revealed the presence of phenoloxidase in many deuterostome and protostome phyla, and related molecules are also present in sponges. In arthropods, several PPO genes are present in the genome (nine in *Drosophila* and *Aedes*). Some may have different "immune" functions such as injury repair. Several components that would maintain the role of melanization in immunity may be lacking in different phyla even if many elements are conserved, and so far the best examples of melanization associated with immunity are still found almost exclusively among arthropods and to a lesser extent in annelids. Despite the presence of molecules involved the pathway, the PPO cascade per se does not exist in vertebrates.

Effector Molecules

Peroxidasin

Among molecules containing LRR motifs, peroxidasin occupies a special place because of its involvement in hemocyte biology in insects and because of its homology to the LRR motifs in the agnathan VLR and Ig domains similar to Ig itself. *Drosophila* peroxidasin is an assembly of a cysteine-rich motif, six LRR, and four IgSF domains.[131] The molecule is conserved in vertebrates, although a role in immunity has not been reported. Another molecule called peroxinectin, with similarity at the level of the peroxidase region, has been described in crustaceans and shown to be associated with immunity via the PPO cascade.[132] Its involvement in immunity is unlike any other effector so far described but illustrates the utility of LRR in many different types of molecules and processes. Pathogens bound by AMPs can be phagocytosed or walled off by a barrier of flattened hemocytes and ECM. The ECM forms a basement membrane that becomes stabilized partly through peroxidases that generate tyrosine-tyrosine bonds. The combination of LRR and Ig structures suggests that peroxidasin may precisely mediate adhesion of cells to the ECM.

As mentioned, a large number of LRR-Ig–containing proteins has been discovered, most of them playing roles in embryologic development.[32] Many LRR-Ig proteins are encoded in paralogous regions in the vicinity of immune genes, showing an ancient direct connection between the families (see the following).

Fibrinogen-Related Proteins

FREPs are proteins that were first discovered in the hemolymph of snails with an IgSF moiety (one or two V-like domains) and a fibrinogen domain. The fibrinogen domain is found in a large number of defense molecules throughout the animal kingdom (eg, the ficolins). FREP gene expression is upregulated following exposure to the mulluscan parasites such as schistosomes[133]; a snail strain resistant to schistosomes shows an upregulation of the FREP 2 and 4 genes of up to 50-fold. The original discovery of FREPs followed the recovery of snail proteins that bound to worm antigens, and thus this is one case in which the correlation between an invertebrate receptor and its ligand is clear. However, it is not known whether the IgSF or fibrinogen domain (or both) bind to the antigen or which effector functions are induced after FREP binding.

FREP diversity is remarkable in that there are many polymorphic genes as well as alternate messenger RNA splicing to generate the diversity. In addition, based on the number of genes and alleles in individual snails, there appears to be a somatic diversification mechanism that modifies FREP genes, either via mutation or gene conversion in the region that encodes the IgSF domains.[134] Over 300 unique sequences were found in 22 snails, consistent with a somatic diversification mechanism. Currently, there is no mechanism to account for the mutations, but data accumulate for somatic modifications and the use of FREPs in critical defense against pathogens.[135]

FREPs are also present in arthropods where they lack the Ig domains,[136] once again with an expansion of genes in the mosquito *Anopheles gambiae* as compared to *Drosophila*. RNAi studies have shown that subsets of FREP genes are vital for defense against malarial parasites, and different FREPs bind to bacteria with different affinities.[137] Homo- and heterodimers can form between different FREPs, and multimers can be fashioned that likely increase the avidity of binding. In summary, this ancient family of defense molecules has all of the attributes described in the introduction: rapidly evolving multigene family, conservation in divergent protostomic invertebrate phyla, somatic diversification via alternative splicing, and perhaps an unknown mutational/gene conversion mechanism, as well as heterodimeric (and multimeric) association.

185/333

There are a number of additional expanded gene families in the sea urchin genome that encode proteins with immune-related functions. The *185/333* genes were first noted because they are highly upregulated in coelomocytes after exposure to LPS, constituting up to 7% of the messenger RNAs in such cells.[138] Subsequently, transcripts were shown to be upregulated by many different types of PAMPs. The encoded proteins have no detectable similarity to any other gene family, but they are highly diversified and are produced (at least) by a subset of coelomocytes.

There are estimated to be at least 50 185/333 genes in the sea urchin genome.[139] The gene is composed of two exons, one encoding the leader and the other encoding the mature protein. The second exon is made up of so-called elements and repetitive sequences that are quite different from gene to gene, which can to a large extent explain the diversity of expressed loci. However, there are hints of RNA editing, a unique form of alternative splicing, somatic mutation (perhaps targeting cytosine residues), and other (perhaps) novel mechanisms of diversity to explain the incredible number of different isoforms; additionally, like many other defense molecules, there is evidence of protein multimerization. Whatever the mechanism of diversity generation, it would be surprising if this family were not vital for defense in echinoids. Furthermore, the presence of this unique multigene family in sea urchins is consistent with the great expansion of other immune gene families in this group, including TLRs, SRCRs, and NLRs.[7]

Variable Domain Chitin-Binding Proteins

VCBP, first discovered in *Amphioxus* but present in *Ciona* as well, consist of two Ig domains of the V type but with a different folding motif when compared to Ig or TCR V domains[140] followed by a chitin-binding domain. The chitin-binding domain resembles chitinases found throughout the animal kingdom, and like dedicated chitinases VCBP is usually expressed in the gut. Apparently, there are no cell-surface–expressed forms and thus all VCBPs are likely to be secreted, effector molecules. In *Amphioxus*, their diversity is enormous, apparently entirely because of polymorphism and polygeny, and not somatic alterations. Each individual can carry up to five genes per haplotype, and in limited studies (11 individuals), no identical haplotype has been encountered.[141,142] The general structure of the V domain is like that of the vertebrate rearranging antigen receptors, but with some unusual properties, including packing in a "head-to-tail" dimeric fashion, totally unlike Ig and TCR. VCBP diversity does not reside in the Ig/TCR CDR resides, but rather in the A, A′, and B strands, like in DSCAM.

By contrast to the *Amphioxus* VCBP, there are only a few nonpolymorphic *Ciona* VCBP genes, which are expressed by gut epithelium and amebocytes. Soluble VCBPs bind to bacteria and induce opsonization in amebocytes. It is hypothesized that these molecules may perform a function similar to mucosal IgA in vertebrates, which provides a "firewall" protecting from invasion of intestinal bacteria and promoting homeostasis.[143] If true, this would provide a link to the regulation of commensals in early deuterostomes. It should be noted here, however, that recent data suggest that "tolerance" of commensals occurs in the protostomic invertebrates as well.

Antimicrobial Peptides (Defensins)

Each metazoan taxon produces a variety of molecules with intrinsic antimicrobial activity,[144,145] the majority of which fall into three major categories: defensins, cateli-cidins, and histatins. Even in species with very small genomes (such as the tunicate *Oikopleura* [60 Mb genome]), selection pressures have been strong enough to lead to expansion of the Phospholipase a2 family, with 128 members.[146] Some families are evolutionarily conserved but generally they diverge rapidly and orthologous relationships are not apparent. The best studied group of AMPs is the *defensins*, which are amphipathic cationic proteins; their positively charged surface allows them to associate with negatively charged membranes (more common in pathogens), and a hydrophobic surface that allows them to disrupt the membranes, either by disordering lipids or actually forming pores. Most of the molecules are proteins, but an antimicrobial lipid called squalamine, which also is modeled to have hydrophobic and positively charged surfaces, is found at very high levels in dogfish and lamprey body fluids.[147] Defensins can either be constitutively expressed (eg, in respiratory epithelia in mammals) or inducible (eg, see the following for *Drosophila* and see Fig. 4.5). Certain responses that seem systemic, like the production of *Drosophila* defensins, can also take place locally in the damaged tissues themselves; otherwise, a systemic response is initiated in organs distant from the site of infection such as the fat body in *Drosophila* where induction of bactericidal peptide expression occurs.[36] Defensins are the focus of great attention in commercially bred species such as oysters, mussels, and crustaceans. Besides their direct defense functions, in mammals defensins play other roles, such as chemotaxis and immune regulation.

Penaedins. One set of diverse AMPs is the penaedins, present in crustaceans (shrimp). Penaedins are small antimicrobial peptides (5 to 7 kda) that bind to bacteria and fungi, and consist of a conserved leader peptide followed by an N-terminal proline-rich domain and a C-terminal cysteine-rich domain.[7,148] Most of the diversity is found in the proline-rich domain,[149] suggesting that it is most important for recognition, but both domains are required for recognition of bacteria and fungi. Four classes of penaedins, PEN2 to 5, are expressed by shrimp hemocytes. A great diversity of isoforms is generated, with substitutions and deletions within the proline-rich domain, suggesting that this domain recognizes ligand; nevertheless, both domains seem to be required for function. Like the VCBPs, each penaeidin class seems to be encoded by a unique gene and isoform diversity is generated by polymorphism. Multiple copies of penaedin genes are present in different species, and there is rapid expansion and contraction even within closely related organisms.

Responses to Viruses in the Invertebrates

Compared to immunity to extracellular pathogens in the invertebrates, the study of responses to intracellular pathogens like viruses is in its infancy.[150] First discovered in plants and in *C. elegans*, the RNAi pathway of defense against viruses is also operative in *Drosophila* and *Anopheles*.[151] Double-stranded RNAs (viral or otherwise) are recognized by the enzyme Dicer 2, generating small interfering RNAs that can associate with complementary RNAs and induce their degradation.

Viruses also induce an "IFN-like response" through a cytokine receptor (domeless) that is homologous to the IL-6 receptor and signals through the JAK/STAT pathway.[152] After viral infection, unknown signals (RNA, perhaps) induce the production of cytokines of the unpaired family that bind to domeless on neighboring cells and upregulate a large number of genes involved in defense (see Fig. 4.3). Nothing is known about the effector pathways of these responses, but mutants of one of the induced genes results in increased viral load. It should be noted that this pathway is not cell autonomous, inconsistent with the IFN pathway in vertebrates.

In a paradigm-changing paper, foreign antigen was shown to directly interact with *Drosophila* toll7, resulting in the induction of antiviral autophagy and inhibition of viral replication.[153] Toll-7 interacted with the glycoprotein from vesicular stomatitis virus at the cell surface to initiate the response. Thus, the dual paradigm of indirect and direct recognition by toll and TLR, respectively, must now be modified. There are several other tolls without functions in insects that might be involved in immunity, perhaps via such mechanisms.

In summary, arthropods (and presumably other invertebrates) use an RNAi pathway as well as a signaling pathway to combat viruses.[150,152] As opposed to the systemic plant RNAi response, the same pathway in protostomes is rather cell autonomous. This response was lost in the vertebrates, presumably because 1) viruses have been able to effectively counter this response and render it ineffective; 2) there have been remarkable evolutionary innovations in the vertebrate innate and adaptive immune systems to combat viruses; and 3) vertebrates use a Dicer pathway extensively to regulate expression of their own genes. The discovery of a viral immune response quite like a type I IFN response in vertebrates demonstrates that the three major signaling pathways of defense in *Drosophila*, toll, IMD, and JAK/STAT, are similar to the vertebrate TLR/IL-1R, TNF, and IL-6/IFN pathways, respectively (the first two homologous). Finally, other insect tolls may interact directly with PAMPs to promote antiviral defense such as autophagy or apoptosis. This is a field in which rapid progress will be made in the near future.

Natural Killing Activity Across Metazoa

The word "cytotoxicity" encompasses vastly different protocols of cell killing by of different cell types. It can be an effector function of cells of the adaptive (cytotoxic T-lymphocyte [CTL] or NKT cells) or of the innate arm (bonafide NK, see the following) of the jawed vertebrate immune system. Similarly the term "NK cells" covers different cell types and different functions. NK cells of vertebrates can "recognize" missing self-MHC class I, but also ligands induced on stressed cells following virus infection, transformation or stress. They can also have an immunoregulatory role by interactions with antigen-presenting cells (APCs). Many of these features obviously profit from a comparative approach.

In natural killing, the common denominator is a spontaneous reaction (ie, it does not require any [known] antigenic priming but only cell contact). Some form of natural killing can be observed from the earliest metazoans onwards. Some marine sponge and corals avoid fusion with one another by mechanism of cytotoxic cells or induce apoptosis at the level of the teguments.[154] Phenomena more similar to vertebrate NK killing are observed in sipunculid worms where allorecognition among populations was shown to result in killing of allogeneic erythrocytes by lymphocyte-like cells.[155] Similar cases can be also encountered in annelids and mollusks,[156] The role of IgSF and lectin receptors known to be involved as NK cell receptors in vertebrates has not been examined in these invertebrates, even though some candidate homologs have been identified. Note that the mechanism(s) cannot be perforin-mediated, based on the recent bioinformatics analysis on MAC/perforin domains described previously in the complement section.[126]

When comparative morphology or function is not informative, searching for conservation of transcription factors or cell surface markers may be useful. Survey of IgSF genes across databases have not yielded any promising candidates to date. Despite the presence of polymorphic IgSF members of the receptor tyrosine kinase family in sponges, their role in allorecognition or killing has not been demonstrated.[157] As mentioned, similarities were found among the lectin families, especially in prochordates where cytotoxicity has been reported and associated with a discrete population of hemocytes the granular amoebocytes. The urochordate genome (*Botryllus*, *Halocynthia*, *Ciona*) encodes many lectins with or without typical carbohydrate recognition signatures, among them a putative CD94 homolog has been cloned and its expression followed in *Botryllus*[158]; its predicted sequence does not match well to vertebrate CD94. This gene is differentially regulated during allorecognition in *Botryllus*, and a subpopulation of blood cells has the receptor on their surface in both *Botryllus* and *Ciona*. Phagocytosis is inhibited by an antiserum recognizing the *Ciona* homologue.[159]

Other C-type lection homologs of CD209 and CD69 are linked to the CD94/L gene on *Ciona* chromosome 1 (L.D.P., personal observation). Could they be part of a "pre-NK complex"? Interestingly, all the human homologs of those lectin genes are present either in the NK complex on 12 p13 (CD94, CD69) or on an MHC paralog 19p13 (CD209). Taken together with studies of the chicken MHC which encodes some C-type lectins (see the following), the data suggest that a conserved MHC-linked region containing several lectin genes was present before the emergence of MHC class I and II genes.[2]

In addition, a number of genes encoding membrane proteins with extracellular C-type lectin or immunoreceptor tyrosine-based inhibitory motifs (ITIMs) and immunoreceptor tyrosine-based activation motif s (ITAMs) (plus their associated signal transduction molecules) were identified in *Ciona*, which suggests that activating and inhibitory receptors have an MHC class I– and II–independent function and an early evolutionary origin.[160] The ligands of these *Ciona* molecules are of great interest to uncover.

Natural Killer and Natural Killer–Like Cells

NK cells express both activating and inhibitory cell surface receptors; in fact, the paradigm for positive and negative signaling via such receptors began with these cells[60,161]; however, activating and inhibitory receptors (often paired) are conserved throughout vertebrates and invertebrate deuterostomes, and are expressed in hematopoietic cells of all types. In NK cells, stimulation of the activating receptors, which associate with proteins having an intracellular ITAM (CD3ζ, DAP12, or DAP10 conserved at least to the level of bony fish[162]), results in killing of target cells. Inhibitory signaling receptors all possess cytoplasmic ITIMs, which recruit phosphatases and generally are dominant over the activating receptors. These receptors fall in two categories IgSF and C-type lectin group V(II). In general, NKRs recognize MHC class I molecules of either the classical or nonclassical type, the latter sometimes encoded by viruses.[163]

General Evolution of Natural Killer Receptor Families. As mentioned, NK cells in mammals can use different types of receptors, even encoded by different gene families, IgSF (KIR) in primates and C-type lectins (Ly49) in rodents. A few receptors are conserved but most others are highly variable. Very few families show conservation of domains throughout the jawed vertebrates.[163] When dealing with the origin of these genes in invertebrates, one has to imagine under what pressures they evolved. The question remains as to whether NK cells, or NK-like cells, preceded the emergence of T- and B-lymphocytes.

As described in the introduction, NKRs are the most rapidly evolving molecular component of the gnathostome immune system. Most ligands for these diverse NKRs are MHC class I molecules, or molecules of host or pathogen origin related to MHC class I. The KIR families are divergent, as very few genes are conserved even between chimpanzees and humans, and there are different numbers of genes in KIR haplotypes within a species. Humans have two KIR haplotypes A and B, one encoding a large number of activating receptors and the other very few.[23] It is speculated that the haplotypes are under balancing selection within the population both for defense against virus and for maternal/fetal interactions. By contrast, CD94 and NKG2 receptors are conserved throughout mammals. So whereas receptors for polymorphic class I molecules are divergent, those for nonpolymorphic or stress-induced class I molecules are relatively conserved (despite the fact that their ligands Qa1 and HLA-E are not orthologous). NK cells play other important roles in other innate immune responses, for example in antiviral immunity. NK cell recognition of virus-infected cells engages the activating KIR and Ly49 receptors and NKG2D in this process. Thus, viruses are hypothesized to supply the evolutionary pressure on diversification of NKRs. In fact, it has been shown in mice that inhibitory receptors can rapidly mutate into activating receptors when viral "decoy" class I molecules evolve to engage inhibitory receptors.[164] Generally speaking, inhibitory receptors are older and more conserved, whereas activating receptors evolve more rapidly and can be derived from inhibitory receptors via mutations that result in loss of the ITIM.[165]

Comparative Studies of Natural Killer Function. NK cells were detected in *Xenopus* by in vitro ^{51}Cr-release assays. Splenocyte effectors from early thymectomized frogs spontaneously lyse allogeneic thymus tumor cell lines that lack MHC antigen expression.[166] This activity is increased after the injection of tumor cells or after treating the splenocytes in vitro with mitogens, suggesting lymphokine activation of the killers. Splenocytes isolated with an anti-NK monoclonal antibody (mAb) revealed large lymphoid cells with distinct pseudopodia. Immunohistology indicated that each anti-NK mAb routinely labeled cells within the gut epithelium but NK cells were difficult to visualize in spleen sections.[167]

In amphibians, NK cell studies are especially interesting because of natural experiments done by nature (ie, the absence or low levels of MHC classical class I during larval life of some species like *Xenopus*).[168] They are bonafide NK cells, distinct from T cells, as they fail to express TCR Vβ transcripts. NK emerge in late larval life, 7 weeks postfertilization, which is about 2 weeks after the time when cell surface class I can be detected. The proportion of splenic NK cells remains very low until 3 to 4 months of age, but by 1 year there is a sizeable population. Therefore, NK cells fail to develop prior to MHC class I protein normal expression (at least NK cells of the type that can be measured with these assays and with NK cell–specific mAbs) and do not contribute to the larval immune system, whereas they do provide an important backup for T cells in the adult frog by contributing to antitumor immunity.

NK cells have also been described in a number of teleost fish with the most in-depth studies in catfish, in which there are clonal likes of cytotoxic cells,[167] some that clearly lack TCR expression.[169] A subset of the fish NK cell bears a high-affinity FcR that can be utilized for antibody-dependent cellular cytotoxicity.[170] Other subsets of NK cells spontaneously kill allogeneic targets. Further study of these cloned lines may provide much needed information on NK function in phylogeny.

Phylogeny of Natural Killer Lectins

Besides the well-described Ly49 family of receptors in rodents (and horse) and the CD94 and NKG2 families in all mammals, other mammalian NKRs are of interest. Studies in mammals have shown that some NKC-encoded lectin-like receptors in the Nkrp-l family can recognize other lectin-like molecules, termed Clr, also encoded in the NKC.[171] Having linked loci encoding receptor-ligand pairs suggests a genetic

strategy to preserve this interaction; perhaps the CD94 homologs of invertebrates are genetically linked to genes encoding their ligands. In addition, as described in the following, the close genetic linkage of receptor and ligand genes is a common theme in "histocompatibility reactions" throughout the animal and plant kingdoms described below.

In chickens, a single gene similar to CD94/NKG2 is encoded in a region syntenic with the mammalian NKC.[172] It is linked to CD69, another C-type lectin also encoded in the NKC of mouse/human. Chickens and quail MHC encode two C-type lectin NKR B-lec and B-NK, the latter being most similar to NKPR1.[173] Other C-type lectins are found in the RFP-y locus, one that is also most similar to NKRP1.[174] These linkages give credence to the idea that the NKC and MHC were syntenic in early jawed vertebrates (see the following). While C-type lectin genes with some similarity to mammalian NKR have been detected in ectothermic vertebrates, no convincing orthology or synteny to the NKC has been found to date. In mammals including marsupials, NKG2D is conserved, and CD94/NKG2 is found in mammals and birds (as well as NKPR1).[163] If such C-type lectin NKR are found in the future in cold-blooded vertebrates, they will have to be studied in functional assays.

Given the apparent lack of MHC class I and class II in agnathans and their convergently acquired adaptive immune system (see the following), it is difficult to envisage how NK cells with receptors of any type might function in these animals. It should be mentioned, however, that sequence similarity might be difficult to detect for an MHC peptide-binding region (PBR), given the rapid rate of evolution of this gene family. Furthermore, it would not be shocking if there were NK cells with ligands encoded by other gene families—in mammals, ligands for some activating NKRs have not been identified. It would be of interest to study the non-VLR–expressing lymphocytes in agnathans (if such cells exist) for their killing potential or gene expression.

Phylogeny of Immunoglobulin Superfamily Natural Killer Receptors

IgSF-activating receptors have been recognized from cartilaginous fish onwards with a convincing activating NKp44 homolog first found in carp (called NILT), but with no functional data.[175] Subsequently, this family was found in other bony and cartilaginous fish and definitive orthology to mammalian NKp44 was shown. The activating receptor NKp30 is also conserved, with orthologs found to the level of cartilaginous fish[176]; in addition, as described in the MHC section, there are V(J) genes within the frog MHC called XMIV that are ancient homologues of NKp30 and may be NKRs of both activating and inhibitory types.[177] The ligand for NKp30 has recently been uncovered, the stress-induced molecule B7H6.[178] Interestingly, there is a perfect correlation between the presence or absence of NKp30 and B7H6 in the vertebrate line, with both genes lacking in birds and bony fish but present in all other gnathostome classes.[176] Furthermore, phylogenetic trees suggest a close relationship between NKp30 and NKp44, consistent with their ancient origins within the vertebrate line.

There are other bony fish–specific IgSF NKR families. One family, the novel immune-type receptors (NITRs),[179] have one or two Ig domains with a charged residue in the TM and could therefore be associated (by analogy) to an ITAM DAP12 equivalent, and indeed was shown to interact with mammalian DAP12.[162] Like NKp30, the NITR N-terminal V domain is also of the VJ type. NITRs were originally believed to be part of the LRC, but this was shown to be unlikely upon further analyses. NITRs can be expressed by cells of the hematopoietic lineage, presumably lymphocytes. NITRs have been found in all bony fish, with rapid contraction and expansion of the gene family, with the majority of proteins predicted to have inhibitory ITIMs in their cytoplasmic tails. In zebrafish, there are many NITR genes that group into 12 distinct families.[180] An extreme level of allelic polymorphism is apparent, along with haplotype variation and family-specific isoform complexity. By contrast, only 11 related genes encoding distinct structural forms have been identified in the channel catfish, and the relatively small number of genes allowed functional studies to be performed. Additionally, taking advantage of the ability to grow clonal lines of catfish hematopoietic cells, one granular cell line lacking all markers of B/T cells was shown to express several NITRs. Expressed NITRs were fused to an ITAM-containing motif and transfected into a T-cell hybridomas line with a nuclear factor of activated T cells (NFAT) promoter, and the specificity for particular catfish MHC alleles was maintained.[181] Subsequent crystal structure analysis showed the NITR V domains to form dimers, much the same as Ig/TCR heterodimeric V regions. Thus, the sequence analysis (ITAM/ITIM), signaling properties, involvement in cytotoxicity, and recognition of MHC molecules identify the NITRs as excellent candidates for NKRs in bony fish. Furthermore, the work serves as a paradigm for study of potential NKRs when homology and/or conserved synteny are lacking or ambiguous.

IgSF inhibitory receptors usually form larger families of molecules in comparison to activating receptors. This function can be devoted to two distinct families of receptors, giving another example of the extremely rapid evolution of these molecules. There are many ITIM-carrying IgSF integral membrane receptors across the classes of vertebrates, and they seem to have had independent histories as it is difficult to convincingly detect orthologous genes between species. This is especially true of multigene families in fish, with members equipped with possible ITIMs, including the teleost NITR and LITR, and bird CHIR and CD300L.[163] Several members of these families can be expressed on NK cells, but expression studies are in their infancy in fish. It is sometimes difficult to distinguish FcR families from NK KIR-like domains, and both FcR and KIR seem to stem from a same lineage. Given the role of the FcR binding to bonafide antibodies and conferring specificities to cells of the innate arms of the immune system, it is likely that these molecules will be restricted to jawed vertebrates. The KIR activity that can incorporate pathogen and virus recognition may be more primitive, but the ancestry of KIR is not well understood, and the bonafide KIR family seems to be restricted to primates.[163]

Genes encoding the classical FcRs on the long arm of human chromosome 1 (1q21–23) are linked to other FcR-like genes. A large multigene family, which includes genes encoding the FcγR and the NK cell Ig-like receptors, is located in the LRC (human chromosome 19q13). This region could in fact be paralogous to 1q23 and may even have been originally associated with the MHC (see the following). These families belong to a larger class of activating or inhibitory receptors. Their phylogenetic conservation in birds, amphibians, and bony fish suggests a biologic importance even though the size of the families, their expression pattern, and the specific nature of the receptors vary greatly among species.[182] In several cases, a commitment to a task in the immune system may not be conserved among homologous members, and the evolutionary fate of the family will be probably affected. Comparison of key residues in the domains may suggest a possible common involvement in MHC recognition for the two families recently discovered in birds (CHIR)[183] and the teleosts (LITR).[184] Other families were generated within a single class or even within a single order of vertebrates (eg, the KIR described previously). The relationships of KIR with many other multigene families such as IpLITR or NITR remain to be explored. What was the scenario that led to the present mammalian situation? If the fish observation on potential MHC binding holds true, the IgSF type of receptor seems to be the most primitive NKR.[163]

Other Immunoglobulin Superfamily Families to Explore Further

In more primitive vertebrates, the physical or genetic linkage of relatively large IgSF families is well documented in the teleost NITR but not yet elucidated in the case of other interesting families in prochordates like the VCBP. In the sea urchin genome, many IgSF await a complete analysis and will certainly contribute to a better understanding of the evolution and origin of Ig/TCR.[48] Large families of LRR-IgSF in amphioxus[185] could perhaps represent interesting intermediaries in the genesis of either VLR in agnathans or Ig/TCR in gnathostomes (see Fig. 4.10). In hagfish, the discovery of leukocyte expressed receptors agnathan-paired receptors (APARs) revealed what might have been a precursor of Ig or TCR.[186] APARs resemble Ag receptors and are expressed in leukocytes and predicted to encode a group of membrane glycoproteins with organizations characteristic of paired Ig-like receptors. Based on their transmembrane regions, APAR-A molecules are likely to associate with an adaptor molecule with an ITAM and function as activating receptors. In contrast, APAR-B molecules with an ITIM are likely to function as inhibitory receptors Thus, the *APAR* gene family has features characteristic of paired Ig-like receptors. APAR V domains have a J region and are more closely related to those of TCR/B-cell receptor (BCR) than any other V-type domain identified to date outside of jawed vertebrates. Thus, the extracellular domain of APAR may be descended from a VJ-type domain postulated to have acquired recombination signal sequences (RSS) in a jawed vertebrate lineage (see Fig. 4.10).

In jawed vertebrates, three such receptor families with VJ-type domains have been identified: a small family of mammalian proteins known as signal-regulatory proteins, a large family of the previously described teleost NITR, and the MHC-linked XMIV in *Xenopus*. These molecules are examined in more detail in the conclusion. Many IgSF proteins expressed in the immune systems are also expressed in nervous systems where the signaling cacscades may be conserved. This selection of IgSF domains, in two different systems in which homologies are found in molecules with quite different functions, may reveal adaptation capacities and constraints exerted on surface receptors.[187]

VERTEBRATE ADAPTIVE IMMUNITY

Not long ago, it was believed that only jawed vertebrates had a true adaptive immune system. From the previous discussion of invertebrate immune responses, clearly mechanisms exist to generate high levels of immune diversity—even at the somatic level—one of the hallmarks of an adaptive response.[7] As described in the introduction, many in the field agree that the boundary between innate and adaptive immunity is artificial, and it may not be a useful dichotomy when studying immune responses in diverse organisms.[61,62] Despite this reluctance to exclusively classify systems as innate or adaptive, some features clearly fall into the latter category, such as clonal expansion of uncommitted lymphocytes and specific memory. These conditions are not fulfilled for the DSCAM, FREP, 185/333, or any other invertebrate systems described previously, but of course we must be open to new mechanisms besides the conventional outlook of adaptive immunity. Additionally, such adaptive immunity arose in concert with the emergence of lymphocytes in the lower chordates, as these cells clearly are the major players; we will discuss this in the conclusion.

Immunoglobulins

A typical Ig molecule is composed of four polypeptide chains (two heavy [H] and two light [L]) joined into a macromolecular complex via several disulfide bonds (Fig. 4.7). Each chain is composed of a linear combination of IgSF domains, and almost all molecules studied to date can be expressed in secreted or transmembrane forms.

Immunoglobulin Heavy Chain Isotypes

Like all other building blocks of the adaptive immune system, Ig is present in all jawed vertebrates (see Fig. 4.7). Consistent with studies of most molecules of the immune system, the sequences of IgH chain C region genes are not well conserved in evolution and insertions and deletions in loop segments occur more often in C than in V domains. As a consequence, relationships among non-μ isotypes (and even μ isotypes among divergent taxa) are difficult to establish.[188] Despite these obstacles, the field has developed a working evolutionary tree among all of the isotypes.

Immunoglobulin M

IgM is present in all jawed vertebrates and has been assumed to be the primordial Ig isotype. It is also the isotype expressed earliest in development in all tetrapods; until recently, it was believed to be the case in fish as well, but

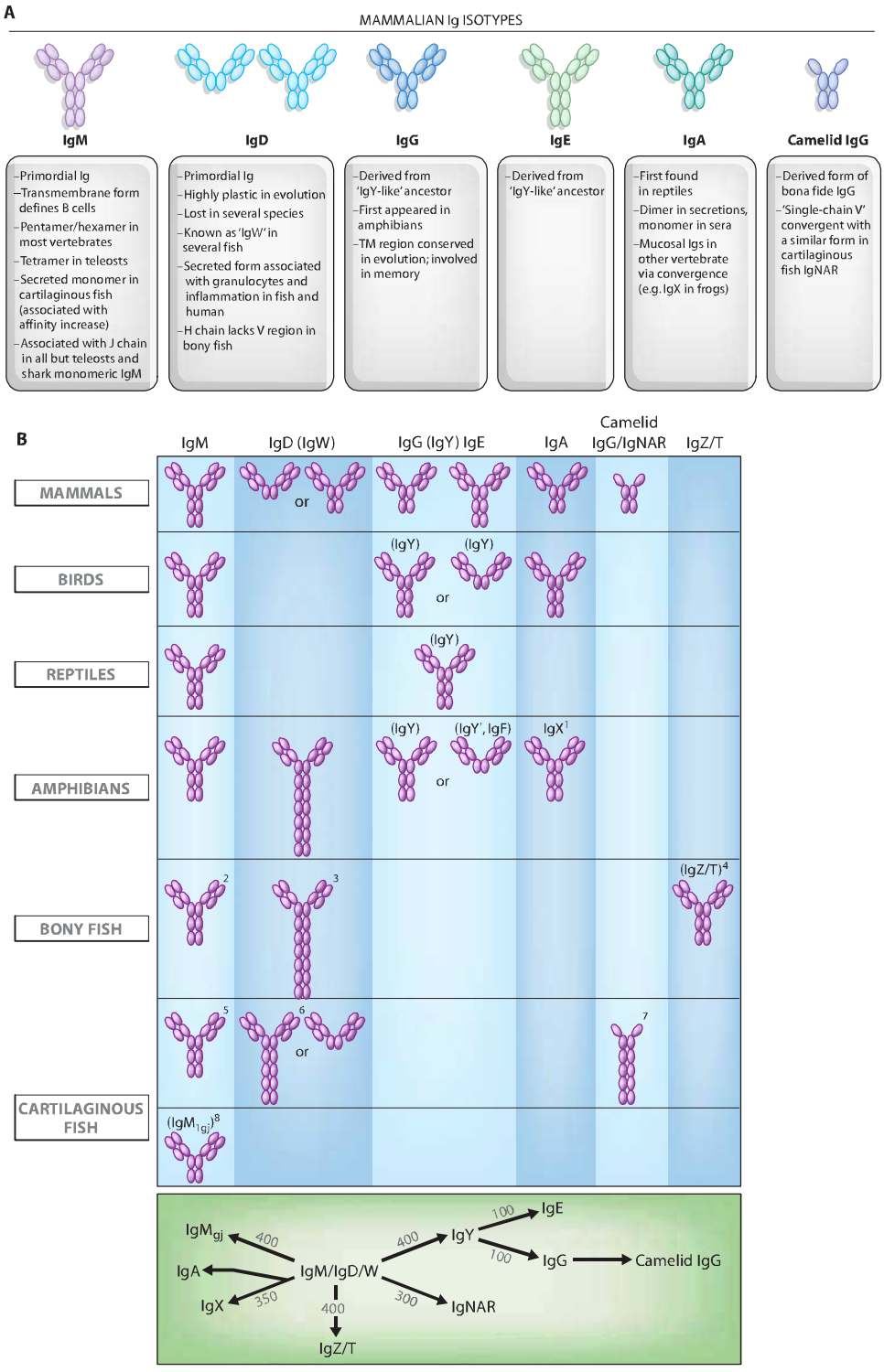

FIG. 4.7. Immunoglobulin (Ig) Isotypes in the Jawed Certebrates. A: Mammalian isotypes and their relationships to Igs in vertebrates from other classes. Each oval represents an Ig superfamily C1 domain. IgD is shown in two forms, mouse (*left*) and human (*right*).[2] **B**: Major Ig isotypes in all vertebrates. The *bottom panel* displays the approximate divergence times of all isotypes. IgM/D/W was found at the inception of adaptive immunity. 1, IgX is in the IgA column because it is preferentially expressed in the intestine, and IgA seems to have been derived from an IgX ancestor; IgX seems to have been derived from both IgM and IgY ancestors; 2, secreted IgM in teleost fish is a tetramer, and the transmembrane (TM) form only has three C domains; 3, the teleost fish IgD H chains incorporate the μC1 domain via alternative splicing in the TM form, and the secretory form does not have a V region and does not associate with L chains; 4, the new bony fish isotype, IgZ/T, may not be found in all fish species; 5, the secreted form of shark/skate IgM is present as a pentamer and monomer at approximately equal levels; 6, the TM form of IgW has four C domains; 7, a major TM form of IgNAR has three C domains, and IgNAR is related to camelid IgG by convergent evolution; 8, no TM form has been found (to date) for IgM_{1gj}. The *bottom panel* displays the approximate divergence times of all isotypes. IgM/D/W was found at the inception of adaptive immunity.

this view has changed (see the following). The secretory μ H chain is found in all vertebrates, usually consists of one V and four C1 domains, and is heavily glycosylated. H chains associate with each other and with L chains through disulfide bridges in most species, and IgM subunits form pentamers or hexamers in all vertebrate classes except teleost fish, which form tetramers.[189] The μ C_H4 domain is most evolutionarily conserved, especially in its C-terminal region, whereas the CH2 domain evolves at the fastest rate.[188] There are several μ-specific residues in each of the four C_H domains among vertebrates suggesting a continuous line of evolution, which is supported by phylogenetic analyses. Like TCR TM regions, μ TM regions are also well conserved among sharks, mammals, and amphibians, but the process by which the Ig TM messenger RNA is assembled varies in different species. In all vertebrate classes except teleosts, the μ TM region is encoded by separate exons that are spliced to a site on μ messenger RNA located approximately 30 basepairs from the end of the C_H4-encoding exon. By contrast, splicing of teleost fish μ messenger RNA takes place at the end of C_H3 exon.[190] In holostean fish (gar and sturgeon), cryptic splice donor sites are found in the C_H4 sequence that could lead to conventional splicing, but in the bowfin there is another cryptic splice donor site in C_H3.[191] The TM region itself is interesting as it is the only one that does not contain a residue capable of making an ionic bond with the ITAM-containing molecule (in this case, Ig-α and Ig-β.) As mentioned, some modifications apparently related to the particular environment were noticed in the Antarctic fish *Trematomus bernacchii*. There are two remarkable insertions, one at the V_H-C_H1 boundary and another at the C_H2-C_H3 boundary; the latter insertion results in a very long CH2-CH3 hinge region. Rates of nonsynonymous substitutions were high in the modified regions, suggesting strong selection for these modifications. These unusual features (also unique glycosylation sites) may permit flexibility of this IgM at very low temperatures.[192]

It has been known for a long time that in all elasmobranchs, IgM is present at very high amounts in the plasma of cartilaginous fish and that it is found in two forms: multimeric (19S) and monomeric (7S).[193] It is unlikely that the two forms are encoded by different gene clusters because 1) peptide maps are identical; 2) early work by Clem found the sequences of the cysteine-containing tail of 19S and 7S H chains to be identical; and 3) all identified germline VH families are represented for the 19S form.[194] Although most studies (but not all) reported that 19S and 7S are not differentially regulated during an immune response, in a recent study, the 19S response wanes over time and a stable 7S titer is maintained for periods of up to 2 years after immunization.[195] In addition, antigen-specific 7S antibodies observed late in the response have a higher binding strength than those found early, suggesting a maturation of the response, also generally at odds with the previous literature. Finally, when specific antibody titers were allowed to drop, a memory response was observed that was exclusively of the 7S class. This work has shown that a "switch" indeed occurs in the course of an immune response; whether the "switch" is due to an induction of the 19S-producing cells to become 7S producers or whether there are lineages of 19S- and 7S-producing B cells is an open question. One working hypothesis is that J chain expression is important for regulating whether a B cell makes 19S or 7S Ig, but of course that could be at the lineage level or the switch level (see the following).

Immunoglobulin M_{1gj}

Nurse shark *Ginglymostoma cirratum* expresses an IgM subclass in neonates.[196] The V_H gene underwent V-D-J rearrangement in germ cells ("germline-joined" or "gj," see the following). Expression of H_{1gj} is detected in primary and secondary lymphoid tissues early in life, but in adults only in the primary lymphoid tissue, the epigonal organ (see the following). H_{1gj} associates covalently with L chains and is most similar in sequence to IgM H chains, but like mammalian IgG it has three rather than the typical four IgM constant domains; deletion of the ancestral IgM second domain thus defines both IgG and IgM_{1gj}. Because sharks are in the oldest vertebrate class known to possess antibodies, unique or specialized antibodies expressed early in ontogeny in sharks and other vertebrates were likely present at the inception of the adaptive immune system. It is suggested that this isotype interacts either with a common determinant on pathogens or a self–waste product.

Immunoglobulin New Antigen Receptor and New Antigen Receptor-T-Cell Receptor

A dimer found in the serum of nurse sharks and so far restricted to elasmobranchs, IgNAR is composed of two H chains each containing a V domain generated by rearrangement and five constant C1 domains.[197] IgNAR was originally found in sera, but TM forms exist as ccomplementary DNA and cell-surface staining is detected with specific mAbs. The single V resembles a fraction of camel/llama (camelid) IgG that binds to antigen in a monovalent fashion with a single V region, but it clearly was derived by convergent evolution. In phylogenetic trees, NAR V domains cluster with TCR and L chain V domains rather with that V_H. A molecule with similar characteristics has also been reported in ratfish, although it was independently derived from an ancestral Ig like the camelid molecule emerged from bonafide IgG.[198] IgNAR V region genes accumulate a high frequency of somatic mutations (see the following).

The crystal structure of a Type I IgNAR V regions showed that, in contrast to typical V regions, they lacked CDR2 and had a connection between the two IgSF sheets much like an IgSF C domain.[199] The domain wraps around its antigen (hen egg lysozyme [HEL]), with the CDR3 penetrating into the active site of the enzyme. The structure of a Type II V region has a disulfide bond between CDR1 and CDR3 that forces the most diverse regions of the molecule to form raised loop, similar to what has been described for camelid V domains. In total, the differential placement of disulfide bonds forces major changes in the orientations of CDR1 and CDR3, and provides two major conformations for antigen binding.[200] The structure of a Type II NAR bound to HEL showed that it also interacted with the active site of the enzyme.

While analyzing the TCRVδ repertoire in nurse sharks, an entirely new form of this chain, which encodes three domains, V-V-C, was detected.[201] The C is encoded by the single-copy Cδ gene, and the membrane-proximal V is encoded by a Vδ gene that rearranges to the DJδ elements. The membrane distal V domain is encoded by a gene in the NAR family, found in a rearranging VDJ cluster typical of all cartilaginous fish Ig clusters. The NAR-TCR V genes, unlike IgNAR V genes which have three D segments, only have a single D region in each cluster. The particular Vδ loci linked to each NAR-TCR gene—called NAR-TCR–supporting Vδ—encode a cysteine in CDR1 that likely makes a disulfide bridge with the NAR-TCR V domain. The J segment of the rearranged NAR-TCR V gene splices at the RNA level directly to the supporting Vδ segment, which has lost its leader exon (Fig. 4.8). This organization likely arose from an IgNAR V cluster that translocated to the TCRδ locus upstream of a Vδ gene segment. After modifications of the supporting Vδ genes, this entire V-V gene set duplicated and diverged several times in different species of sharks. About 25% of the expressed nurse shark TCR δ repertoire is composed of this TCR (encoded by 15 to 20 V-V genes in this species), and have proposed that the typical γ/δ TCR acts as a scaffold upon which sits the single chain NAR V.

Our interpretation is that, true to the proposal that γ/δ TCRs interact with free antigen, the NAR V is providing a binding site that can interact with antigen in a different way than conventional heterodimeric Vs. Thus, this is the first case in which a particular V region family has been shown to be associated with a BCR and TCR; in the case of the BCR, the function likely resides within the Fc portion of IgNAR and for the TCR the function (cytokine secretion, killing) lies within the T cell itself. Interestingly, a second TCRδ chain locus has also been described in marsupials and monotremes with properties similar to NAR-TCR.[202] In this case, there are also two V domains, but in marsupials one (proximal to the membrane) is germline-joined, and only the membrane-distal domain undergoes rearrangement. In monotremes, both of the V regions undergo rearrangement, like for the NAR-TCR. This new TCRδ locus is preferentially expressed early in development. This type of TCR is described in more detail in the TCR section.

Immunoglobulin R/Immunoglobulin New Antigen Receptor/Immunoglobulin W/Immunoglobulin X/Immunoglobulin D

All elasmobranchs studied to date have another isotype called IgW. It was probably discovered long ago in skates as a non-IgM secreted isotype called IgR, but no protein sequence of this molecule has been obtained for confirmation.[203] Subsequently, an Ig gene was discovered in skates encoding a three-domain molecule with an unusual secretory tail that was named IgX (not to be confused with another isotype with that name in amphibians).[204,205] A high molecular weight (MW) species detected by northern blotting with an IgW probe suggested that there might be a longer form of this isotype, subsequently shown to be true in the sandbar shark (IgW), nurse shark (IgNARC—it was so-named because the C domains had highest similarity to IgNAR C domains), and skate.[206,207] It was originally believed that sharks only expressed the long (seven-domain) IgW form, but they were later shown to have both secretory forms; the reason for the discrepancy was shark-to-shark variation in expression of the short form, for unknown reasons. The major IgW TM form, like IgM, is composed of five domains, but variants with three domains—like the secretory forms—were also detected.[208] Very little is known about the function of this isotype, as IgW-specific mAbs have not been generated as they have for IgM and IgNAR.

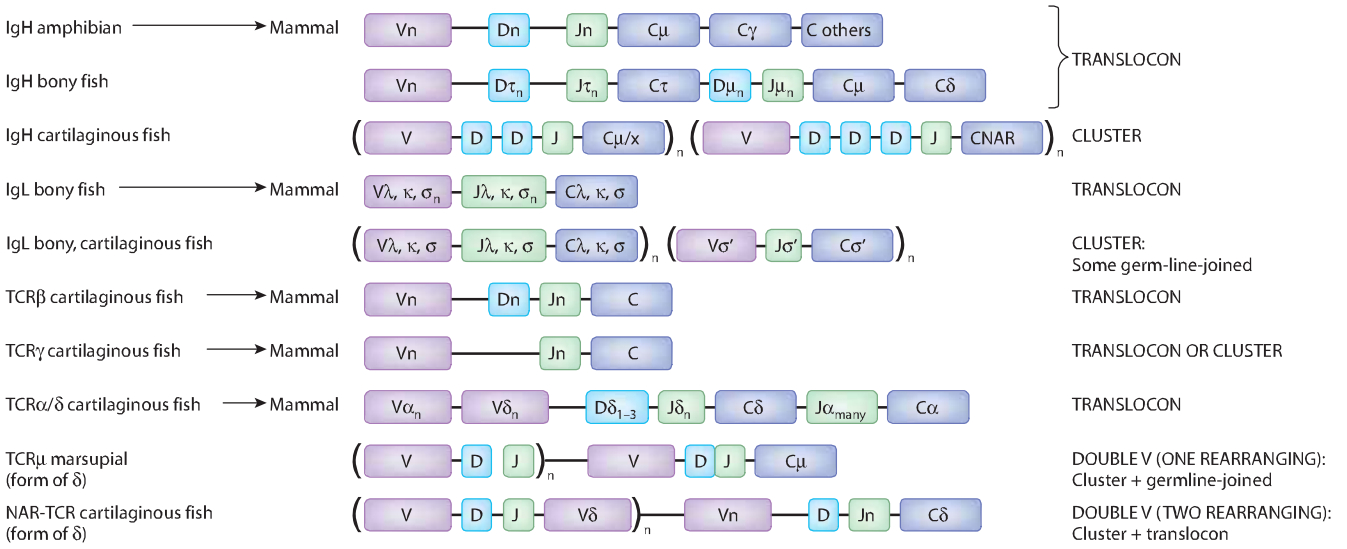

FIG. 4.8. Organization of Immunoglobulin (Ig) Heavy and Light (L) Chain and T-Cell Receptor (TCR) Genes in All Jawed Vertebrates. All cold-blooded gnathostomes except bony fish have three L chain isotypes, κ, λ, and σ; mammals have κ and λ; and birds have only λ. RSS are found at the 3′ end of V segments, 5′ end of J segments, and on both sides of D segments. Marsupial μ TCR is related to Ig in its V regions and TCRδ in its C region (and is not related to the μ of IgM).

IgW was thought to be a dead-end isotype in the cartilaginous fish, but a homologue was found in the lungfish.[209] It also is present in two secreted forms, one with eight domains and the other, like in the elasmobranchs, with three domains (unfortunately the secretory tail was not sequenced, and the TM form was not studied.) An Ig isotype was found in *Xenopus tropicalis* most related to the lungfish IgW.[210,211] Computer searches of databases for the *X. tropicalis* genome project uncovered a new isotype flanked by the IgM and IgX genes at the IgH locus. The deduced amino acid sequence obtained from the exons on the genomic scaffold suggests a nine-domain molecule. The N-terminal C domains and the TM regions are most similar to mouse and human IgD regions, and its genomic location also suggest that it is an IgD equivalent. Thus, these new data reveal that like IgM, IgW/D is an isotype that was present at the emergence of all extant vertebrate taxa.

Catfish IgD was the first member of this Ig class found outside of mammals,[212] but this was not well accepted until the isotype was found in all vertebrate classes. It is found attached to FcR on a subset of myeloid cells, and the secreted form does not contain a V region as the leader is spliced to the CH2 domain in plasma cell RNA.[213] This strongly suggests that the IgD is acting as a PRR, perhaps interacting with a conserved region of a pathogen leading to innate immunity. The work on catfish prompted a reappraisal on the role of secreted IgD in humans.[214] Like the fish IgD, the human IgD is bound to a subset of myeloid cells, which are activated upon IgD cross-linking. The receptor on human cells has not been identified, but this new work argues for strong evolutionary conservation of the function of secreted IgD.

An interesting feature of the IgD/W locus is its high plasticity in evolution, both in terms of the number of domains in different fish species and the plethora of splice variants found, at least in cartilaginous fish.[194,215] In sharks, two of the C domains were derived approximately 250 million years ago by a tandem duplication event, and there was a *Xenopus*-specific, two-domain tandem duplication event as well. Within teleost fish, the number of C exons for this isotype is different in various species and the secreted and TM forms are encoded by different loci in the catfish. In addition to the splice variants previously described in the cartilaginous fish and lungfish, in teleosts the IgM C1 domain exon is spliced into the IgD transmembrane transcript.[212] Even in mammals, there are different numbers of C domains in different species, and even exons that have emerged quite recently in evolution (see Fig. 4.7). It is our impression that this is the Ig locus that evolution "plays with," perhaps using it for different functions in vertebrate taxa. The beginning of understanding the function of the secreted form of IgD is a triumph for comparative immunology; likely the evolutionary approach will reveal the function of the TM form as well.

Immunoglobulin Z/T

A third, novel bony fish isotype was uncovered in screens of the EST and genomic databases called IgZ in zebrafish[216] and IgT in trout.[217] Its genomic organization parallels the TCR α/δ locus in that the IgZ/T D, J, and C elements are found between the VH and Cζ/Cμ exons (see Fig. 4.8). IgZ/T is a five-domain H chain that associates with L chains (see Fig. 4.7). The authors of the zebrafish paper proposed that lymphocytes bearing IgZ may be B1-cell equivalents, but a preliminary VH repertoire analysis did not suggest that unique sets of V regions are expressed on IgZ compared to IgM. IgT is not preferentially expressed over IgM early in trout development.

Recent work has shown that IgT is the mucosal Ig of trout, with preferential expression in the intestine and skin.[218] Mucosal immunization with the parasite *Toxoplasma gondii* showed a preferential induction of specific IgT. While all teleosts have lost the J chain in evolution,[219] biochemical analysis of IgT showed that it contained a secretory piece derived from the poly-Ig receptor (pIgR). Furthermore, like mammalian dimeric IgA, IgT coats trout commensal bacteria, presumably acting as a "firewall" preventing commensals from breeching the blood–brain barrier. In sum, IgT, while displaying unique evolutionary features, has attained almost all of the characteristics of IgA via convergent evolution.

Other Isotypes Related to Immunoglobulin G, Immunoglobulin E, Immunoglobulin A, and the Class Switch

Other isotypes consist of four C domains in nonmammalian vertebrates including *Xenopus* IgY and IgX, non-μ isotypes of *Rana*, IgY of axolotl, and IgA and IgY of birds.[194,220] In *Xenopus*, IgY is thymus-dependent; IgM and IgX are not, although thymectomy impacts specific IgM antibody production (ie, antigen-specific IgM can be produced, but there is neither an increase in affinity after immunization nor elicitation of plaque-forming cells). IgM and IgX plasma cells are abundant in the gut,[221] whereas IgY is expressed primarily in spleen. Axolotl IgM is present in the serum early during development and represents the bulk of specific antibody synthesis after antigenic challenge.[222] In contrast to *Xenopus* IgY, the axolotl ortholog appears late in development and is relatively insensitive to immunization. From 1 to 7 months posthatching, axolotl IgY is present in the gut epithelium associated with a secretory component.[223] IgY progressively disappears from the gut and is undetectable in the serum of 9-month-old animals. Thus, axolotl IgY, like *Xenopus* IgX and trout IgT, may be analogous to mammalian IgA. *Xenopus* IgX seemed to be most similar to IgM, but as more sequences have become available it does seem to be orthologous to IgA, obviously consistent with its assumed function (Criscitiello, personal communication). In addition, IgX was formerly believed to be derived from IgM, but recent data suggest that the N-terminal C-domains were derived from an IgY ancestor and the C-terminal domains from IgM.[224]

The TM and cytoplasmic domains of *Xenopus* TM IgY share residues with avian IgY and mammalian IgG and IgE, suggesting that mammalian/avian isotypes share a common ancestry with amphibian IgY.[225] This homology is especially interesting because studies in mice suggested that the IgG cytoplasmic tail is the central molecular element promoting rapid memory responses of plasmablasts.[226,227] The motif in

the IgY/G tail is also found in other receptors and seems to recruit PI3kinase, which amplifies the signal derived from Ig-α and -β. Finally, another *Xenopus* isotype was discovered from the databases, called IgF or IgY′ (because it is closely related to IgY, probably duplicating within the *Xenopus* lineage). Like other isotypes described in this section, it only has two C domains, but rather than alternative splicing, the gene is organized in such a manner.[211] There has been no biochemical identification of IgF.

CSR results from fusing switch regions upstream of the μ gene and another 3′ isotype gene, accompanied by the deletion of the intervening sequences. Among vertebrates, mammalian and bird class switch regions are GC-rich and contain tandem repeats in which certain motifs, such as TGGGG, GGGGT, AGCT, and GGCT, are abundant. Because of the GC richness of these regions, transcription generates stable R loops, which provide single-stranded substrates for activation-induced deaminase (AID) activity. The first comparative studies on switch were done in the amphibian *Xenopus* where the switch regions are not GC-rich but adenine-thymine (AT)-rich and cannot form R loops.[228] Replacement in mouse of the switch region with a *Xenopus* switch region showed that it mediated efficient CSR and that the junctions were associated with the short palindromic AGCT motifs, already recognized as the main component in *Xenopus* switch.[229] As predicted from the absence of R loops, the *Xenopus* switch region supported recombination in both orientations. The breakpoints were located in the AGCT palindrome-rich region of the switch box. Other motifs have been identified in the other switch regions, such as in IgX and chicken isotypes; all of these correspond to the DGYW hotspot consensus. AID-mediated deamination in the context of these motifs may be the conserved major event in the initiation of CSR. As described in the following, until recently it was believed that CSR first appeared in amphibians, but recent data have uncovered a precursor to switch in cartilaginous fish (but not teleosts). Thus, we should add another conserved feature of adaptive immunity that appears to have arisen in the early gnathostomes.[230]

As mentioned, a single gene can encode different Ig forms, like for duck IgY, cartilaginous fish IgW, and camelid IgG loci. It has been suggested that the two avian IgY short and long forms could be the functional equivalents of both IgE and IgG, respectively; the same may be true of the cartilaginous fish IgW short and long forms with two and six domains, respectively. Also as mentioned, IgF or IgY′ also falls into this category but not through alternative splicing.[194,211]

Immunoglobulin Light Chains

L chains can be classified phylogenetically not only by their sequence similarity, but also by the orientation of their V and J RSS, which differ for mammalian λ and κ. There has been much debate regarding the affiliations of L chains in various vertebrates, but as sequences have accumulated, we have a grasp on their phylogenetic emergence. Contrary to what was believed, κ and λ L chains emerged early in vertebrate evolution, in the cartilaginous fish or placoderms. In addition, a third L chain, σ, originally described in *Xenopus* as a dead-end isotype, is in the ancestral clade and is present in all cold-blooded vertebrates.[231]

Elasmobranchs have four L chain isotypes (Type I, II, III, and IV), and the combined data suggest that they are present in all chodricthyans.[231] Type IV is the ortholog of the unusual *Xenopus* σ L chain, and Type I is a dead-end variant of this isotype called σ′ or σ-cart (for cartilaginous fish). Type II L chains are of the λ isotype and have been cloned from all groups of cartilaginous fish; all genes of this isotype are "germline-joined." The type III is clearly κ-like, at least in the V region and RSS orientation (κ is the only antigen receptor gene in which the V is associated with a 12mer RSS). These relationships are more noticeable in the V sequences, which have defining characteristics such as CDR length; although, the C sequences also fall into the same clusters, they do so with much less phylogenetic support. Different elasmobranch species express the L chain isotypes preferentially (eg, κ in nurse sharks, σ in horned sharks, and λ in sandbar sharks); this pattern of expression may be due to expansions/contractions of the different isotype genes in various elasmobranch lineages.

Almost all of the L chains in bony fish can be categorized as either κ or σ, despite the large number of gene expansions and contractions in this group.[232] As expected, recent data have shown that λ exists in some species, consistent with the work in sharks.[233] Genes in different species can be found either in the cluster type organization, translocon, or some intermediate type, and this organization is especially well suited to allow for receptor editing, either to generate the repertoire or as a consequence of binding to self-ligand.[234,235]

mAb studies suggested the existence of three *Xenopus* L chain isotypes of 25, 27, and 29 kD with heterogeneous two-dimensional gel patterns and preferential association of some L chain isotypes with IgY H chains.[236] Indeed, three *Xenopus* L chains genes have been isolated: ρ (now κ), σ, and λ. Only one C gene is present in the ρ locus, and it encodes the most abundant L chain. The V and J RSS are of the κ-type and the five identified J segments are nearly identical. The locus is deleted, like mammalian κ, when the other isotype genes are rearranged. Southern hybridizations with genomic DNA from different animals showed V_L sequences to be both diverse and polymorphic. The λ *Xenopus laevis* L chain isotype predicted from the biochemical studies consists of six distinct V_L families. In the σ locus, the J segment has an unusual replacement of the diglycine bulge by two serines. The *Rana* major L chain type has an unusual intrachain disulfide bridge that is seemingly precludes covalent association of its H and L chains.[237]

Two L chain types were identified in reptiles. Chickens and turkeys only express one L chain (λ) with a single functional V and J gene, and the manner by which diversity is generated is likely responsible for this unusual evolutionarily derived arrangement (see the following). Nonproductive rearrangements are not detected on the unexpressed L chain allele, and thus there is a strong pressure to generate functional joints (see the following). Such a system probably rendered a second (or third) L chain locus

superfluous.[236] Within mammals (marsupial *Monodelphis domestica*), the Vλ repertoire is comprised of at least three diverse families related to distinct placental families, suggesting the divergence of these genes before the separation of metatherians and eutherians more than 100 million years ago. Opossum λJC sequences are phylogenetically clustered, as if these gene duplications were recent and the complexity of the λ locus seems greater than that found at the H chain locus.[238]

In summary, all vertebrate groups except the birds have two to four L chain isotypes, all of which can be categorized as κ, λ, or σ (and in cartilaginous fish σ-cart).[231] However, we are still at a loss to understand the significance of possessing multiple isotypes as there is scant evidence that L chains have any effector functions. It has been suggested that different isotypes may provide distinct CDR conformations in association with H chains, or there may be L chain/H chain preferences that provide some advantage that is not obvious.[239,240] At least the differential CDR sizes for σ as compared to κ/λ suggest that the former rationale is plausible. The preferential association of certain L chains with particular IgH isotypes is suggestive of different functions as well, but there is no evidence for such functions to date.

V_H Evolution

Diversity of the immune repertoire depends on the variety of V segments inherited in the germline and upon the further diversification by rearrangement (CDR3 only) and SHM (all CDR). Early in life, the repertoire depends chiefly on the inheritable genes as one finds little N-region diversity and somatic mutation (with exceptions; see the following). A central question is how antibody germline V genes diversify CDR during evolution while they are subject to homogenizing forces operating in most multigene families. Perhaps environmental antigens have played a major role in shaping the germline repertoire and have selected some V_H/V_L germline sequences used by neonates. V_H families arose in a bony fish lineage and have been conserved for hundreds of millions of years.[241] Conserved regions defining families are found on solvent-exposed faces of the V_H, at some distance from the antibody-combining site. Phylogenetic analyses show clustering of V_H into groups A, B, C, D, and E. All cartilaginous fish V_H belong to the monophyletic group E; bony fish V_H genes to cluster into all Groups (one group [D] unique only to them). By contrast, group C includes bony fish sequences as well as V_H from all other classes except cartilaginous fish. Another phylogenetic analysis classifies mammalian V_H genes in three "clans" (I, II, and III), which have coexisted in the genome for > 400 million years. Only in cartilaginous fish does it appear that V_H gene families have been subjected to concerted evolution that homogenized member genes (except for the IgM_{lgj} V region described previously). It has been debated whether Ig V genes could be under direct positive selection or not because these genes hypermutate somatically. However, several features (eg, codon bias) and discovery of high replacement/silent ratios in germline gene CDR codons indeed argue for positive selection during evolution.[242,243]

In summary, much of the V_H germline repertoire has been conserved over extremely long periods of vertebrate evolution. The birds and some mammalian species that rely on GALT to generate Ig diversity (see the following) are exceptions with a reduced germline repertoire (at least expressed repertoire), but as will be seen in the following, gene conversion and SHM compensate for this situation in formation of the primary repertoire. Even in the cartilaginous fish where there is a single V_H family, there is nevertheless great heterogeneity in CDR1 and CDR2 sequences (as well as hypermutation) that must boost diversity in the expressed repertoire.

The J Chain

The joining (J) chain is a small polypeptide, expressed by plasma cells, that regulates polymer formation of IgA and IgM.[244] J-chain incorporation into dimeric IgA and pentameric IgM endows these antibodies with the ability to be transported across epithelial cell barriers. J chain facilitates creation of the binding site for pIgR (secretory component in the Ig polymers), not only by regulating the polymeric structure but apparently also by interacting directly with the receptor. Therefore, both the J chain and the pIgR/secretory component are key proteins in secretory immunity. J chain complementary DNAs have been reported in all jawed vertebrates except the teleost fish, which have lost it.[245] The existence of *Xenopus* J chain suggests that, unlike mouse IgM, *Xenopus* IgM forms hexamers with J chain; alternatively, the previous electron microscopy studies identified IgX as the hexameric isotype (the ξ chain has a stop codon before the Cys of C_{H4} domain and thus cannot make a covalent attachment to J chain). The highest level of J chain expression was detected in frog and bird intestine, correlating well with a role for J chain in mucosal immunity (although obviously not for IgX secretion). Elasmobranch J chain shows high similarity to the N-terminal half of J chains from other vertebrates, but is divergent or even absent in the other regions. This result suggests that the function of J chain may be solely for IgM polymerization in elasmobranchs, and the transporting function arose later in evolution; consistent with this idea, *Xenopus*, but not shark, J chain is capable if interacting with human IgA and pIgR.[245] As mentioned previously, the loss of J chain in bony fish does not preclude the interaction of IgT with pIgR and transport across epithelia, implying a strong pressure to maintain a mucosal-specific Ig.[218] There was a claim for the presence of J chain in many protostomes because a homologue was cloned in earthworms, but no J chain sequences have appeared in the numerous protostomes or deuterostome invertebrate databases.[246]

T-Cell Receptors

Ig, TCRs, and MHC class I and class II are all composed of IgSF domains. The membrane-proximal domains of each Ig/TCR/MHC chain are IgSF C1-set domains, while the N-terminal domains of Ig and TCR proteins are V-set domains encoded by genes generated via rearrangement of two or three gene segments during ontogeny (the membrane-distal domains of

MHC are a special case; see the following). In all vertebrates studied to date, TCRs are membrane-bound and never secreted, while almost all Ig proteins have transmembrane and secreted forms.[247]

α/β Constant Domains

Genes encoding the two types of TCR, α/β (which accounts for all known MHC-restricted regulatory and effector functions) and γ/δ (which to the best of our knowledge recognizes antigens in an Ig-like manner and may play immunoregulatory or homeostatic roles during certain infections), existed in the earliest jawed vertebrates. The α/β TCR is considered rather "boring" evolutionarily, as all gnathostomes from the basal cartilaginous fish appear to have this MHC restricted form.[248] As mentioned, although many IgSF members exist in the invertebrates, thus far no bonafide Ig/TCR sequences (ie, IgSF genes generated by somatic rearrangements) have been isolated from jawless vertebrates or invertebrates, although a renewed search in jawless vertebrates may be fruitful (see the following).

While TCR genes have been cloned from representatives of most vertebrate classes, few biochemical data are available, except in birds where α/β and γ/δ TCR have been identified with mAbs.[249] In amphibians, the *Xenopus* α/β TCR was coimmunoprecipitated with cross-reactive antibodies raised against human CD3ε chains[250] (note that this same antibody indentifies CD3 epsilon from many divergent species, which is quite unusual,[251] in turtles). α chains from diverse vertebrates are poorly conserved and the structure of the Cα IgSF domain itself is unusual: only strands A, B, C, E, and F can be identified, although strands E and F are shorter than those of mammals and strand D is absent; this modification has an important role in TCR dimerization and subsequent signaling.[252] The lack of conservation in this extracellular domain as well as deletions found in bird and teleost fish TCR (especially in the connecting peptide) suggest that the coreceptor may be structurally distinct from mammalian CD3 complex components.

Pre-Tα, which associates with TCR β chains during thymocyte development, has been identified only in warm-blooded vertebrates.[253] The pre-Tα protein has no V domain, and its interaction with the TCRβ chain is unique, based on recent structural studies.[254] Unlike all of the other TCR chains, pre-Tα has a long cytoplasmic tail, which seems to be important for T-cell differentiation. Interestingly, the pre-Tα gene is linked to the MHC in mammals, and more phylogenetic studies should be performed to determine whether this linkage group is ancient (see the following).

The TM region and cytoplasmic tail of Cα are the most conserved parts of the molecule. Cα and Cβ TM segments in all species have the so-called conserved antigen receptor transmembrane motif (CART) motif, in which conserved amino acids form an interacting surface with the CD3 complex.[255] Besides CART, the opposite TM face with conserved residues Ile-Lys-Leu interacts with other CD3 components. The cytoplasmic region is remarkably conserved among all vertebrates. TCR β genes have been sequenced from several species of cartilaginous and bony fish and two species of amphibians (axolotl and *Xenopus*). In addition to the typical IgSF domain features, there are several conserved regions among vertebrate TCRβ chains, especially at positions 81 to 86, probably involved in TCR dimerization. There are also remarkable differences: the solvent-exposed segment 98 to 120 in mammals is absent in all nonmammalian vertebrates. This loop has been shown in mouse TCR to be important for negative selection events in the thymus; perhaps the absence of this region in nonmammalian vertebrates results in subtle differences in tolerance induction as compared to mouse/human.[256] The number of Cβ genes varies in different species.

Like Cα, Cβ sequences are not well conserved in evolution (eg, the *X. laevis* Cβ gene does not cross-hybridize with *X. tropicalis* genomic DNA, and catfish Cβ has only 41% to 42% identity with other teleost Cβ and 26% identity with horned shark Cβ). Two different catfish Cβ complementary DNA sequences were identified, suggesting the existence of either two loci or allotypes, as is found in mammals.[257] Indeed, the damselfish Cβ was shown to be encoded by two polymorphic genes, and this feature seems to extend to other teleosts. As the polymorphic sites are believed to interact with the associated CD3-signaling molecules, the authors suggested that signals might be transduced to T cells in different ways depending on the particular expressed Cβ allele. The damselfish Cα gene seems to be encoded by polymorphic alleles as well.[258]

α/β T-Cell Receptor Variable Domains

Because T-cell recognition is MHC-restricted, TCR V regions have been evolutionarily selected for different properties as compared to Ig; indeed, TCR V regions are much less similar to each other than are Ig V regions from other antigen receptor loci.[257] Furthermore, TCR Vs, unlike IgV_H, have conserved CDR3 lengths, suggesting that there is a restricted size for recognition of MHC-peptide complexes.[259] α loci in all vertebrates examined have many J segments, and consistent with the mammalian paradigm, the absence of D and the large numbers of J segments favors the potential for receptor editing during thymic positive selection.[260] A number of Vβ gene families are another evolutionarily conserved feature. There are 12 families in nurse sharks and 19 in *Xenopus*. In axolotls Vβs are classified into nine categories each with 75% or more nucleotide identity; as only 35 genes were cloned, there are probably more families, and several are related to mammalian Vβ genes (human Vβ13 and Vβ20[257]).

There is evidence that the TCR has coevolved with MHC, displayed by the canonical types of interactions seen in crystal structures.[261] Vβ sequences from many different vertebrates share residues in CDR2 that interact with class II and CD1 MHC molecules (Y-46 and Y-48, especially).[262] It will be of interest to search for other conserved interaction sites as crystal structures become available.

Studies of several teleost α/δ loci suggests an organization similar to mouse/human, but with more rearrangement by inversion and more gene segments than are found in mammals.[263] Pulsed field gel analysis suggested that the horned shark α and δ TCR loci are closely linked.[264] There

has been a recent, major modification in our thinking of the α/δ locus that will be described in the following.

The γ/δ TCR

Complementary DNA sequences from the skate have significant identity with prototypic mammalian γ and δ TCR genes with extensive V region diversity, putative D segments in δ, and varying degrees of junctional diversity.[248] In the nurse shark, there is a great diversity of the δ V regions, the highest among the TCR chains; consistent with this diversity is the presence of the NAR-TCRδ, which is found in approximately 25% of the δ Vs.[257] The NAR-TCR has been found in all cartilaginous fish to date, including holocephalins, whereas NAR and IgW have only been detected in elasmobranchs. Thus, it is possible that NAR (especially the V region) arose as a TCR and was transferred to an Ig cluster sometime in evolution.

In axolotls, Vδ diversity was diminished in thymectomized animals and TCR δ chains are expressed by cells in lymphoid organs, skin, and intestine.[265] Chicken γ/δ T cells were identified long ago. Expression is found in thymus, spleen, and a γ/δ T-cell line, but not in B cells or α/β T-cell lines. Three V subfamilies, three J gene segments, and one C gene were identified at the TCR γ locus. All Vγ subfamilies participate in rearrangement during the first wave of thymocyte development, and the γ repertoire diversifies from embryonic day 10 onwards with random V-J recombination, nuclease activity, and P- and N-nucleotide addition.[249]

In ruminants and chickens (so-called GALT species; see the following), the γ/δ repertoires are quite diverse, and there seems to be ligand-mediated selection of γ/δ cells during ontogeny. In sheep, where γ/δ TCR diversity is thymus-dependent and follows a developmentally regulated progression, no invariant γ/δ TCRs are found.[266] The degree of γ/δ expression is correlated with the evolution of the TCR V families in warm-blooded vertebrates. Indeed, mammals can be classified into "γ/δ low" (humans and mice, in which γ/δ T cells constitute limited portion of the T-cell population) and "γ/δ high" (chicken, sheep, cattle, and rabbits, in which such γ/δ cells comprise up to 60% of T cells). TCR V genes form subgroups in phylogenetic analyses, and humans and mice have representative loci in most subgroups whereas the other species appear to have lost some.[267] Thus, γ/δ-low species have a high degree of TCR-V gene diversity, while γ/δ-high species have limited diversity. Interestingly, this pattern is similar to that found for IgV_H genes.

Recent work in *Xenopus* has shown that the presence of prototypic V_H at the TCRδ locus, in addition to typical α and δ V domains.[268] Furthermore, consistent with the presence of VH, the αδ locus is closely linked to IgH and IgL λ in the frog genome. These data suggest that these heterodimeric loci arose via a *cis* duplication early in vertebrate history.[263] The TCRμ locus in monotremes and marsupials[202,269] can now be extended to the chicken as well,[270] although like *Xenopus* there are chicken V_H with a three-domain molecule as is found in monotremes, marsupials, and sharks. In total, all of the nonplacental mammals except bony fish show some sort of chimeric Ig/TCR locus, with TCR δ V domains that are more Ig-like than TCR-like. Generally speaking, comparative studies have revealed that the γ/δ TCR can either be used for innate recognition (eg, the cells in the skin of mice or the blood of humans) or adaptively with an enormous repertoire in ways we do not understand—this will be discussed in more detail in the conclusion. Consistent with the potential of γδ TCR to interact with nominal antigen, SHM has been detected in the γ genes of the sandbar shark, although it is not known whether the mutations generate the repertoire or appear in mature T cells after immunization.[271,272]

Immunoglobulin Gene Organization

V_H Regions

A rearranged V_H gene consists of a leader segment, encoded by a canonical split exon, followed by four framework regions and three CDRs (see Fig. 4.8). Canonical V_H CDR1 nucleotide sequences are conserved in all jawed vertebrates and serve as targets for SHM.[242,243] A major germline difference is the lack of conserved octamers and TATA box in the 5′ region of shark Vs. In all species, functional V genes are assembled by rearrangement and joining of germline V, D, and J elements. Cartilaginous fish H chains are encoded by large numbers of clusters (> 100 in horned shark and dogfish; approximately 15 in the nurse shark). For IgNAR, there are only four V genes/haploid genome and only a few IgW V genes are detected in nurse sharks, but a large number in skates and dogfish.[273] There are widely varying numbers of V genes in different species; importantly, the V_H complexity does not seem to limit diversity of the antibody repertoire in any ectothermic vertebrate studied to date. There are actually fewer functional human V_H (44 functional, 79 pseudogenes that fall into seven families) than in many ectotherms. Dynamic reorganization of the H chain V regions seems to have occurred at least eight times between 133 and 10 million years ago.[241] Perhaps species that utilize somatic mutation/selection "optimally" rely less on germline diversity and therefore fewer functional genes are required. Only approximately 10% of *Xenopus* V_H are pseudogenes in the three families ($V_{H1–3}$) that have been exhaustively studied; thus, *Xenopus* with fewer lymphocytes has a greater number of functional V_H genes than humans.

Chondrichthyan Germline-Joined Genes

In all vertebrate species, functional Ig genes are assembled by rearranging DNA segments scattered on the chromosome. However, in cartilaginous fish some V genes are the products of V(D)J rearrangement in eggs/sperm.[196,274,275] Type I L chain (σ) genes are all germline-joined in skates but split in horned sharks, and the piecemeal germline joins (eg, VD, VDD, VDDJ) found in many horned shark H chain gene clusters and in nurse shark L chain Type I (σ) clusters strongly suggest that the germline-joining is a derived feature. Definitive proof came from a study of a germline-joined nurse shark Type III (κ) L chain gene, shown by phylogenetic analysis to have been joined within the last 10 million years[236]; this was followed by the identification of the nurse shark germline-joined IgM_{1gj} described previously.[196] When there is a *mixture* of joined and conventional genes, the split genes are expressed in adults, while the joined genes are

expressed at significant levels only early in ontogeny. When *all* of the genes in a particular family are joined (eg, skate Type I L chain genes and Type II [λ] L chains in all elasmobranchs), they continue to be expressed into adult life at high levels. In mammals, what may appear like germline-rearranged V genes are in most cases processed pseudogenes (eg, pseudo Vκ on chromosome 22 in human or in mouse). However, it is possible that the surrogate L chain gene VpreB is the product of a germline-joining event in the line leading to mammals.[276] Additionally, as described previously, there is a marsupial germline-joined V that serves as the membrane-proximal domain of TCRμ.[202]

Organization of Rearranging Genes

As mentioned, shark IgH-chain genes are structured into tens to hundreds of clusters, each consisting of V, D, J, and C elements[277]; all evidence from studies in horned shark, nurse shark, skate, and sandbar shark (and holocephalin ratfish) suggest that V, D, and J genes rearrange only within one cluster (but, as detailed in the following, switch may occur between clusters, most likely as a consequence of antigen stimulation). While there is extensive N-region diversity and sometimes usage of two D segments (three Ds in IgNAR), and there are V_H subfamilies having substantial CDR1/2 heterogeneity, diversity of the primary repertoire is lower than in other vertebrates as there is no (or infrequent) rearrangement between clusters. The special constitution of the shark H and L chain loci suggests an exclusion mechanism similar to that of mouse TCR γ loci, also found in clusters. It appears that only one V_H transcript is expressed in each lymphocyte, consistent with isotypic exclusion, despite the many clusters (see the following).[278,279] Bony fish (teleosts and chondrosteans like the sturgeon), frogs, reptiles, and mammals have very similar architectures of their H chain locus—the so-called translocon configuration. As described previously, multiple families of V_H genes, each consisting of many apparently functional elements (1 to 30 per family), are separated from a smaller number of genomic D and J elements. The possibility of combinatorial rearrangement enables more diversification than is possible with the cartilaginous fish clusters for a given number of segments. In birds, the organization is similar but all V genes except those most 3′ to the D elements are pseudogenes (see the following). One exception is the coelacanth (*Latimeria*), in which V genes are immediately followed by D segments, and then multiple J segments as are found in all tetrapods.[280]

L chain gene organization is variable. In elasmobranchs, the organization is the same (ie, in clusters) as the H-chain locus without the D segments. The prototypic horned shark Type I (σ) L chain has a cluster organization in which V, J, and C segments are closely linked. As mentioned previously, bony fish L-chain genes have the shark cluster-type organization, but some species have multiple V genes in some clusters, demonstrating that there is rapid evolution of not only sequence but gene organization as well in this taxon.[232] In *Xenopus*, there are multiple Vκ(ρ) presumably derived from one family: five J and a single C gene segment. In cartilaginous fish and birds there has been coevolution of Ig gene architecture for H and L loci, but the teleosts have shown that this is not a rule. As mentioned previously, two groups have suggested that the teleost gene organization seems to promote receptor editing.[234,281]

D Segments

D segments are always present in one of the two loci encoding an Ig/TCR heterodimer (IgH and TCR β,δ), and the pressure maintaining this asymmetry is unknown. Cartilaginous fish usually have two D genes/H chain cluster, and there are only minor variations among the clusters; one RSS follows the 12-12 paradigm seen in tetrapods and the other is like the TCR 12-23. In teleosts, amphibians, and reptiles where the organization of the H chain locus is similar to humans, the number of Ds deduced from complementary DNAs ranges from 10 to 16. Two germline D segments have been identified in *Xenopus*, and their RSS follow the rules defined in mammals. In birds, there are 15 very similar D_H.

There are several reasons why D segments may have been preserved throughout evolution. Incorporation of D segments augments CDR3 diversity and size, obviously directly influencing the combining site.[259] Three different Ds contribute to IgNAR CDR3, and besides generating great diversity, CDR3 length and amino acid composition fulfills special tertiary structure requirements: D-encoded cysteine residues bond with cysteine(s) in the body of the V domain, thereby stabilizing a loop involved in the antigen-binding of this unusual monomeric receptor.[197,200] A similar situation has been reached by convergence in the monomeric variant of camel IgG.[198] Finally, rearrangement of one locus "locks it in" and allows the second locus to undergo receptor editing, as is the case for negative selection of the B-cell repertoire and positive selection of the T-cell repertoire. Finally, in mice, one of the TCRδ D segments encodes a section that interacts with the ligand, the nonclassical class I molecule T lymphocyte antigen (TLA).[282]

Agnathan Variable Lymphocyte Receptor Structure and Function

In the 1960s, jawless vertebrates (hagfish and lampreys; see Fig. 4.1) were reported to mount humoral responses to foreign antigens. However, for anti–group A streptococcal antigens, the hagfish "antibodies" (at least a proportion of them) were actually the complement component C3[283]! Lamprey and hagfish were long known, however, to possess cells resembling lymphocytes and plasma cells,[284] with expression of lymphocyte- or at least leukocyte-specific genes,[285] but the quest for RAG or bonafide Ig/TCR/MHC genes was a complete failure. Reports of specific memory in allograft rejection and other specific humoral responses were difficult to reconcile with absence of the RAG-based rearranging machinery and the possibility of generating specific lymphocyte clones.

Our view of the jawless fish immune system was radically transformed when Pancer et al. prepared complementary DNA libraries of naïve lymphocyte RNA subtracted from lymphocyte RNA derived from lamprey larvae (ammocoetes) that had been immunized to a bacterial/PAMP mixture, and found a highly diverse set of LRR sequences enriched in the

immunized animals.[286] The clones were not only diverse in sequence, but also in the number of LRR "cassettes" found between invariant 5′ (LRR NT) and 3′ (LRR CT) cassettes. Unlike what has been discussed previously for immune genes many species (especially sea urchins), there were not a great number of the germline NT and CT cassettes, suggesting that a somatic recombination process, convergent with Ig/TCR rearrangement, occurred in the developing lamprey lymphocytes (Figs. 4.9 and 4.10). The genomic organization of germline VLR (the original gene discovered was VLRB) have 5′ and 3′ LRR cassettes separated by an intron. Upstream and downstream of the invariant exons are a large number of LRRs, which become inserted between the 5′ and 3′ cassettes during lymphocyte differentiation. Individual lymphocytes apparently express a uniquely rearranged VLR gene in monoallelic fashion. The potential VLR repertoire can be as great as 10^{14}, vastly outnumbering the lymphocytes within an individual.[287,288]

Fragments of homology between the LRR cassettes allow for joining and then priming of synthesis of a copy of the particular transferred cassette.[289] Because these small regions of homology are found throughout the cassettes, "hybrid" LRR can also be formed, further enhancing the diversity over a simple insertion of cassettes. The insertion of LRRs can occur at either end of the somatic recombinant, but it is always 5′-to-3′ on the growing strand. This type of genomic modification resembles the initial stage of gene conversion, but because there is not a complete transfer of genetic material between two homologous gene segments, but actually an addition of sequence, the assembly of the mature VLR is more similar to a recombinatiorial mechanism described in yeast called "copy choice." The enzymology of "copy choice" has not been examined; because a gene conversion-like process occurs, it is likely that an APOBEC family member is involved. Indeed, APOBEC family members were detected in the lamprey genome project and are expressed in a lymphocyte-specific fashion[288]; in fact, the two members that have been discovered, CDA1 and CDA2, are differentially expressed in agnathan lymphocyte subsets (see the following). Because APOBEC family members are involved in repertoire building in jawless and jawed vertebrates, mutation/gene conversion may have predated RAG-mediated repertoire building of the repertoire (see the following).

VLR homologs were found in two additional lamprey species and in hagfish, the only other order of living jawless vertebrates reviewed in Boehm et al.[13] As in the sea lamprey, the incomplete hagfish germline VLR generate somatically highly diverse repertoires. Interestingly, the *Amphioxus* genome harbors a large number of intronless VLR-like sequences that could represent an alternative germline VLR diversity akin to the echinoderm gene families (TLR, SRCR) described previously; one could even speculate that they are related to the ancestral VLR before invasion of its analogous "RAG transposon" (see the following).

There are three VLR types: A, B, and C. VLR-B cell-surface receptors and secreted molecules are analogous to the jawed vertebrate membrane and secreted BCR, and it was proposed that cell activation was similar to T-independent pathways in jawed vertebrates (ie, either direct stimulation through the surface VLR or surface VLR stimulation in combination with a PRR/PAMP interaction). It was suggested that the jawless vertebrate adaptive system might be dedicated exclusively to humoral immunity, with the PAMPs providing the second signal to activate lymphocytes.[290] Thus, it was proposed that the lamprey system was "B cell-centric" and therefore focused on humoral immunity.

This model has been disproved as subsequent work demonstrated that the VLRA and VLRB were differentially expressed (see Fig. 4.10). The former receptor could not be found in plasma and was expressed exclusively as

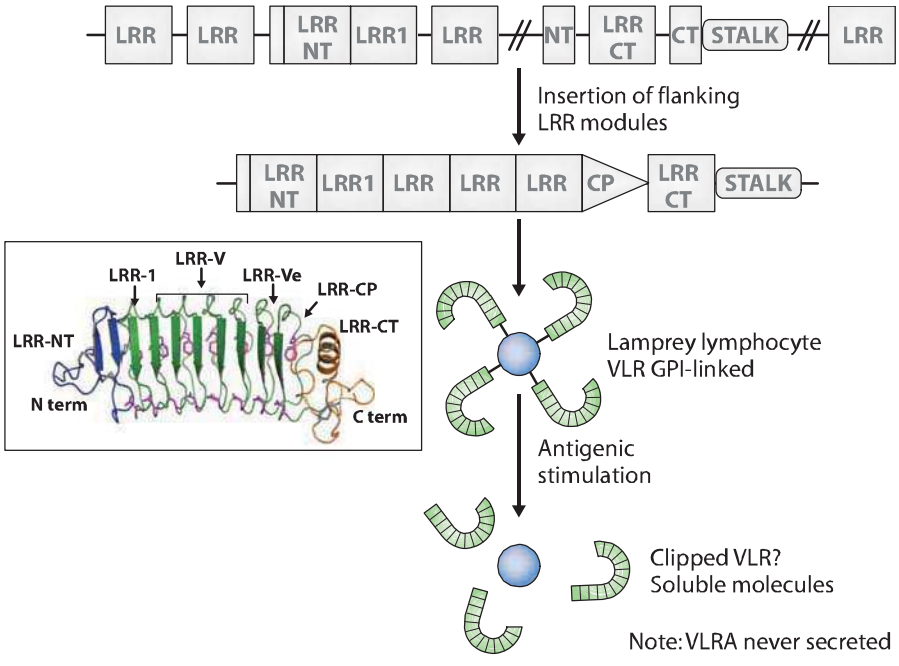

FIG. 4.9 Genetics, Generation of Diversity, and Speculative Cell Biology of the Hagfish Variable Lymphocyte Receptor (VLR) System.[13] The *top line* shows an incomplete VLR gene (NT and CT, N-terminal and C-terminal cassettes, respectively). During lymphocyte ontogeny, upstream and downstream LRR cassettes are inserted between the NT and CT gene segments, resulting in an intronless, mature VLR gene (*second line*). VLR proteins are attached to the lymphocyte surface via a glycophosphatidylinositol linkage, and may be released into the blood upon antigenic stimulation (also see Fig. 4.10). *Boxed* is a hagfish VLR protein, the first structure to be elucidated by Kasahara and colleagues.[499] Note the loop at the C-terminal leucine-rich repeat, which is mentioned in the text as a prominent region inserting into antigens, like single-domain antibodies.[293,294]

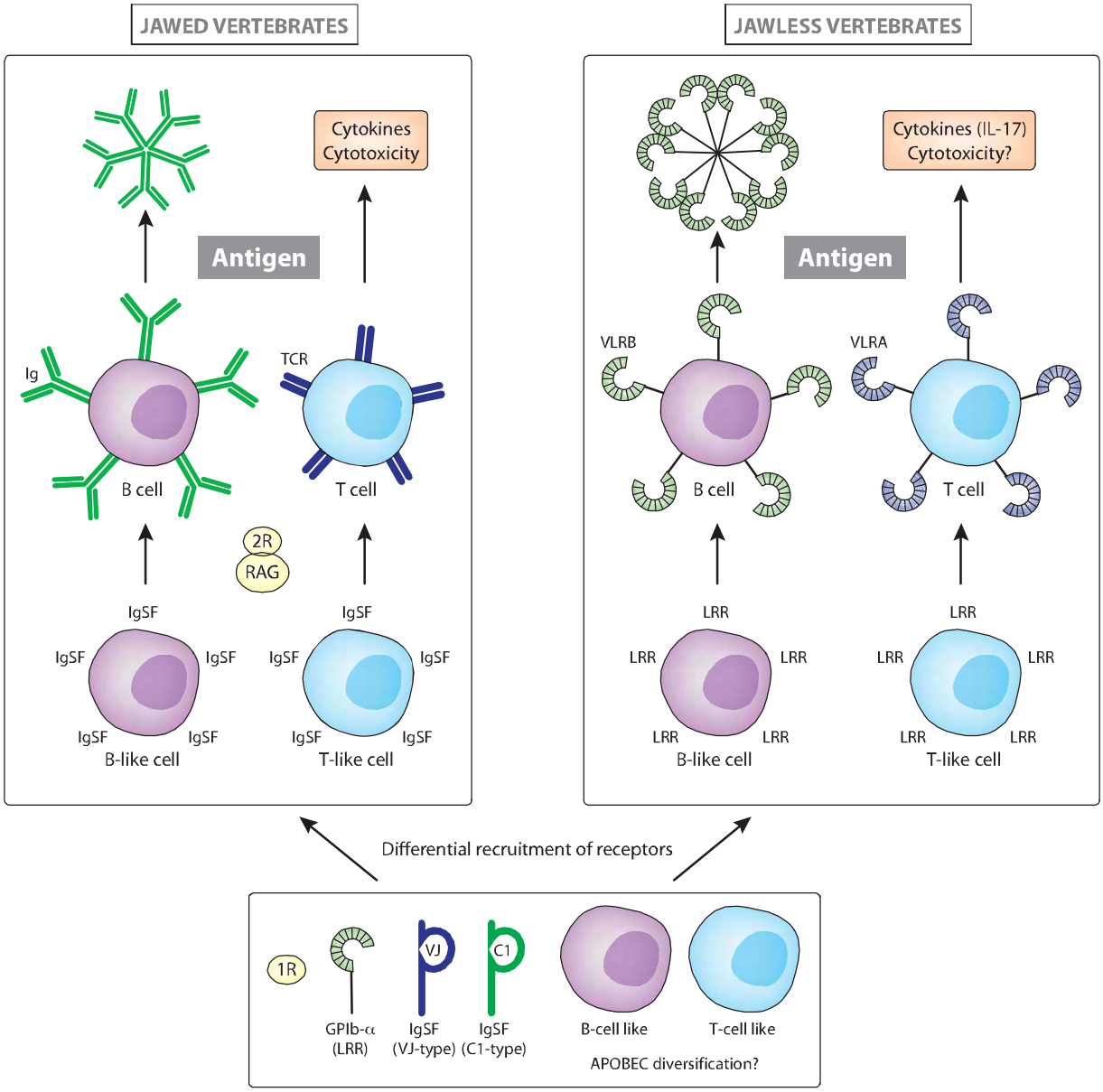

FIG. 4.10. Emergence of Variable Lymphocyte Receptor (VLR) and Immunoglobulin (Ig) Early in Vertebrate Evolution. T and B cells likely preceded the divergence of the leucine-rich repeat and Ig superfamily antigen receptors. The VLR is most similar to a molecule called GPIb-α[288] present in *Amphioxus,* and Ig superfamily antigen receptors are derived from molecules with so-called VJ and C1 domains, probably in the proto–major histocompatibility complex (see text for candidates). Both precursor genes were present in basal chordates. The RAG transposon and 2R likely ignited the appearance of the Ig superfamily receptors (Ig/T-cell receptor) (see text for full explanation). This figure also shows B and T cells today in jawless and jawed vertebrates, with secretion of IgM from gnathostome B cells and a VLR tetramer of dimers from VLRB-secreting lamprey B cells. Figure modified from Flajnik and Kasahara.[2]

a cell-surface receptor, while, as described, the latter was present as both a lymphocyte receptor and a secreted molecule.[291] When microarray expression analysis was performed on VLRA- and VLRB-positive cells, the patterns were consistent with expression profiles in gnathostome T cells and B cells, respectively. The lamprey adaptive scheme, therefore, parallels the situation in jawed vertebrates where Ig is found both as a cell-surface receptor on B cells and as a secreted effector molecule in the serum, whereas the TCR is present only as a cell-surface receptor on T cells; effector functions in T cells, such as cytokine secretion or production of cytotoxic mediators, are properties of the T cells themselves. It is not clear how VLRC fits into this scheme at the moment.

As expected for members of this family, the framework, or backbone, of the LRR is very similar between the cassettes. Diversity between cassettes is concentrated in the concave surface, presumably the region coming in contact with antigen. The crystal structures of two VLR-B molecules have been determined, one specific for HEL[292] and the other for H-trisaccharide.[293] In both cases, the concave surface makes contacts as well as a loop in the LRR-CT, which in the case of HEL inserts into the active site, similar to

what has been found for single-domain camelid and shark Vs.[198] One VLR-A crystal structure was determined, also specific for HEL.[294] This VLRA was derived from a HEL-hyperimmunized adult lamprey, which apparently underwent affinity maturation with induced molecules having affinities in the picomolar range. The antigen is bound in the region of the molecule that is most diverse among all VLRAs; surprisingly, the LLRNT among all VLRA are not diverse, suggesting that this region might bind to a self–"restricting element" and the rest of the binding site to the true antigen.

These are early days, but the comparisons to date suggest that VLRA and VLRB recognize antigen in different ways. Like gnathostome BCR, VLRB is expressed at the cell surface and is secreted as a pentamer of dimers, much like IgM (see Fig. 4.10). The affinities detected to date from immunized animals are in the micromolar range, again similar to low-affinity pentameric IgM in gnathostomes. By contrast, VLRA has never been detected in the sera or secreted in culture, fitting with its similarity to TCR. The affinity of VLRA for soluble antigen was high (in the nanomolar range) for the HEL-immunized animal, which appeared to undergo affinity maturation.[294] Combining the information with the conserved and diverse regions of the VLRA bidding site, perhaps VLRA recognizes antigen (budding virus?) associated with a self-protein (MHC analogue?) at the cell surface. It remains to be seen whether VLRA clones will be found from other immunized animals, and the HEL experiment was an artifact of the immunization schedule and not physiologic.

The independent development of two different strategies for receptor somatic diversification at the dawn of vertebrate evolution approximately 500 million years ago reveals the magnitude of the selective pressure applied on to the immune system and the emergence of individualized adaptive responses (see Fig. 4.10). Are VLR responsible for the graft rejection results seen in the old experiments? If so, this is most likely a "convergent MHC" coopted for the VLR system. Skin grafting experiments (or other tests of alloreactivity) should be repeated to study potential VLR involvement. The VLR system is discussed below as well in the lymphoid tissues section and the conclusion.

Adaptive Immune Responses in Gnathostomes

The quality of T-cell and B-cell responses depends on the heterogeneity and the diversity of the antigen receptor repertoires, and the ability to select cells in secondary lymphoid tissues. Because of the indefinite number of potential Ig/TCR V region sequences in most taxa, potential diversity exceeds the number of available lymphocytes; furthermore, all jawed vertebrates are capable of AID-dependent SHM or gene conversion after antigen stimulation. Yet, while potential repertoires are diverse in all vertebrate classes, and polymorphic MHC class I and II and TCR genes have been isolated from all classes, antibody diversity in nonmammalian vertebrates is relatively low.[295–297] The expressed repertoire has been studied via structural studies, affinity measurements during the maturation of the immune response, enumeration of antigen-binding Igs by isoelectrofocusing (IEF), and idiotypic analysis. Sequences of Ig and TCR genes expressed over the course of a response help to estimate diversity at another level, allowing studies of V genes diversified by gene conversion and/or somatic mutation during a response in a precise way. In the following survey, we describe studies of specific antibody synthesis, T-cell responsiveness (T-B collaboration, MHC restriction).

Cartilaginous Fish

Natural antibodies binding many antigens have been detected at surprisingly high levels in chondrichthyans and in some teleosts. In older experiments, after immunization the horned shark mounted a low-affinity 19S (pentameric) IgM antibody response, which varied little among individuals and did not increase in affinity after prolonged immunization.[298]

The relative homogeneity and large number of V genes hindered SHM studies until a single unique reference horned shark IgM V_H gene was found. Mutations in this gene were slightly more frequent than those in *Xenopus* (see the following). This first study proved that SHM preceded diversity obtained by combinatorial association of gene segments in evolution.[299] In contrast to mutations in the horned shark IgM V_H genes, unusual patterns of somatic mutation were detected in nurse shark IgNAR (see the following) and Type II (lambda) germline-joined L chains. Half of the mutations (338/631) occur in tandem without the GC bias seen in *Xenopus* or horned shark H chain V genes. Tandem mutations and point mutations that take place simultaneously were not generated by gene conversion as there are no repeated patterns or potential donor genes.[300,301] The germline-joined L chain genes can only diversify through SHM, perhaps like the hypothetical prototypic V region gene prior to RAG-mediated rearrangement (ie, SHM may have preceded gene rearrangement as the primordial somatic diversification mechanism)[300] (see the following). Lastly, a reappraisal of mutation at H chain and other L chain loci in the nurse shark showed that the mutations were not so different from the L chains; the differences from the previous work were the different shark species and the analysis of all H chain loci in the species rather than only one unusual locus.[302]

As mentioned, the small number of IgNAR genes also made it possible to analyze SHM, and in the first experiments, random complementary DNAs were examined.[197,296] The mutation frequency was about 10 times that of *Xenopus* and horned shark IgM, and even higher than in most studies in mammals. lt was difficult to establish a pattern for the mutations due to their high frequency and because they are often contiguous, like in the L chain gene study described previously. Mutations even in randomly isolated clones appeared to be under positive selection in IgNAR secretory but not TM clones, strongly suggesting that mutations do not generate the primary repertoire like in sheep but arise only after antigenic stimulation.[303,304] In total, the shark mutations seem quite mammalian-like but with unusual features. Analysis of the mutations in noncoding DNA suggests

an AID-dependent process coupled with an error-prone polymerase.[305]

Affinity maturation and memory generation can be detected in sharks.[195] Soon after immunization with HEL, an IgM response can be detected, primarily of the pentameric class. Over time, 7S IgM and IgNAR responses develop, and the 7S antibodies have a higher binding strength than those of the 19S class. When titers were permitted to drop to baseline (or close to baseline), a memory response was induced by immunization of antigen without adjuvant. However, unlike responses in higher vertebrates, the titers do not increase over those in the primary response, suggesting a unique type of regulation of antigen-specific IgM and IgNAR. Nevertheless, these data strongly suggest that the hallmarks of an adaptive response occur in sharks. This type of response is reminiscent of IgA responses of intestinal lymphocytes in mice.[306] In another study, a family of HEL-specific IgNAR clones was followed over time after immunization, and a 10-fold increase in the affinity of an already high-affinity germline clone (10^{-9} M) was observed.[307] These results suggest that affinity maturation, memory, and "switch" to the monomeric IgM isotype occurs, but it takes much longer to attain these adaptive hallmarks compared to mammals, perhaps a paradigm for ectotherms (see the following).

Recent work showed that isotype switch can occur in sharks, despite many reports to the contrary.[230] Previously, studies of nurse and horned sharks complementary DNAs showed that the V and C regions were derived from the same gene clusters. By contrast, in the new work, immunized animals showed switching between IgM clusters and even between IgM and IgW clusters. It is not known whether there is any functional significance to the switch, but one might predict the switch could dictate a change from 19S to 7S antibodies (see the following), or a modification of effector class in IgM and IgW. None of the classic hotspots (RGYW) upstream of constant region genes in tetrapods vital to switch appear to be targeted to initiate the switch in sharks, but other repetitive elements were detected that might play a role.

The role of T cells in shark immune responses has not been studied in detail. No thymectomy experiments have been performed, and T cells have not been monitored during an immune response. Shark mixed lymphocyte responses (MLR) and graft rejection have been attempted, MLR with little success (probably for technical reasons) and grafts with the demonstration of a chronic type of rejection for which the genetics has not been analyzed. However, from the MHC and TCR studies, it is clear that all of the molecular components are available for proper antigen presentation in sharks and skates, and studies of splenic architecture suggestive class II+ dendritic cells in the white pulps argue for a prominent T-cell regulatory role in adaptive immunity.[308] Furthermore, an increase in binding strength and memory response, as well as mutation and now switch, also strongly suggests a T-cell involvement in humoral immunity. Finally, recent studies of the thymus have shown that the architecture and expression of well-known markers are wholly consistent with a typical T-cell regulation of adaptive immunity.[257] In summary, it appears that all of the components of adaptive immunity in mammals occur in the cartilaginous fish.

Bony Fish

There are high levels of low-affinity natural antibody (up to 11% of total Ig) to nitrophenylacetate in some bony fish. Natural antibodies in catfish have been correlated with resistance to virus infection or furonculosis. As a rule, and similar to cartilaginous fish, little affinity maturation has been detected in fish, although some changes in fine specificities were noticed in the trout with a sensitive enzyme-linked immunosorbant assay–based test.[309] The mild increase in trout antibody affinity (similar to that found in *Xenopus* and shark IgNAR) is attributed to selection of either minor preexisting B-cell populations or somatic mutants. In partially-inbred self-fertilized or gynogenetic trout, variability of specific responses is even more restricted. Affinity measured by equilibrium dialysis was of the order of 2.0×10^{-6} M for trinitrophenol (TNP)-specific antibodies. A large literature deals with vaccination attempts in teleost fish, due to their economic importance. The availability of catfish B-cell, macrophage, and T-cell lines have been instrumental in analyses of antibody production.[310] There are puzzling differences in responses from different teleost groups, much like differences between urodeles and anurans (amphibians). Cod, for example, do not respond well to specific antigen and have very high levels of "natural antibodies"; recent studies showing that cod have lost class II genes provides an explanation for the poor humoral responses (see the following).

Like the sharks, isolation of TCR genes and the existence of a polymorphic class I and class II molecules suggest that antigen presentation is operative teleosts, but unlike sharks, functional experiments examining mammalian-like T-APC interactions have been performed. TCR messenger RNAs are selectively expressed, and specific TCR rearrangements have been detected in catfish clonal cell lines, which produce factor(s) with leukocyte growth–promoting activity reviewed in Miller et al.[310] Modifications of the trout T-cell repertoire during an acute viral infection (rhabdovirus) have also been followed.[311,312] In nonintentionally immunized trout, adaptation of the spectratyping technique for TCRβ CDR3 length revealed a polyclonal naïve T-cell repertoire. After an acute infection with viral hemorrhagic septicemia virus, CDR3 size profiles were skewed for several Vβ/Jβ combinations, corresponding to T-cell clonal expansions. Both "public" and "private" T-cell expansions were detected in the infected genetically identical individuals. The "public" response resulted in expansion of Vβ4/Jβ1-positive T cells that appeared first in the primary response and were boosted during the secondary response. Further work examined fine specificity of the viral T-cell response, which is a model for studies in cold-blooded animals.

Recent results suggest that, despite the fact that IgM is the major Ig expressed in an immune response, high- and low-affinity IgM appears to be a function of the degree of polymerization of the tetramer.[309] It is suggested that high-affinity interactions of TM IgM on B cells modifies the enzymes

involved in disulfide-bond formation, resulting in modifications of secreted IgM.[313] In addition, expression of transcription factors in different subpopulations of trout B cells suggests that the typical populations of naïve, memory, and long- and short-lived plasma cells exist in bony fish; these populations have been followed in blood, spleen, and head kidney by measuring proliferation and B cell–specific transcription factors.[314–316] Such studies should prove very useful for vaccination of large populations of teleosts.

Studies of SHM in teleost fish have been hampered by the lack of good reference genes, but it has been clear that AID-targeting motifs are found in Ig V genes.[317,318] Furthermore, AID is expressed in the spleen during immune responses, again consistent with its role in SHM.[319] A recent study examined mutations in a reference zebrafish L chain gene and detected the typical mutational pattern described previously (without the tandems seen in sharks).[320] AID from several bony fish species is capable of inducing CSR in mammalian B cells, despite the fact that teleosts do not undergo CSR[318]; perhaps the new results that demonstrate switching in sharks may shed light on this conundrum.[230]

As mentioned, species living in extreme cold develop adaptive structural differences in their Igs.[192] At the level of global immune response, temperature exerts a great influence in ectothermic vertebrates in general, low temperature generally being immunosuppressive. Lowering the water temperature from 23° to 11°C over a 24-hour period suppresses both B- and T-cell functions of catfish for 3 to 5 weeks as assessed by in vitro responses.[321] Virgin T cells are most sensitive to this cold-induced suppression, a property shared with mammals when tested appropriately. Fish have developed ways to adapt to the lack of fluidity of their B-cell membranes by altering the composition of fatty acid by using more oleic acid at low temperatures. After appropriate in vivo acclimation, catfish T cells are better able to cap cell surface molecules at low assay temperatures than are B cells, suggesting that capping is not the low temperature-sensitive step involved in T-cell immunosuppression in catfish.

In the NK section, we briefly discussed fish cytotoxic cells.[169] In vitro studies have now shown that leukocytes from immunized fish specifically kill a variety of target cells (allogeneic erythrocytes and lymphocytes, hapten-coupled autologous cells); fish CTL of the αβ (and perhaps γδ) lineages as well as NK cells were found (see previous discussion). Naïve catfish leukocytes spontaneously kill allogeneic cells and virally infected autologous cells without sensitization, and allogeneic cytotoxic responses were greatly enhanced *by in vitro* alloantigen stimulation.[322] Cloned cytotoxic cells contain granules and likely induce apoptosis in sensitive targets via a putative perforin/granzyme or Fas/FasL-like interactions. All catfish cytotoxic cell lines express a signal-transduction molecule with homology to the Fcγ chain of mammals; this chain with an ITAM is an accessory molecule for several activating receptors on mammalian NK cells.[161] Importantly, these cytotoxic cells do not express a marker for catfish nonspecific cytotoxic cells. As described previously, nonspecific cytotoxic cells have been found in other fish species, including trout, carp, damselfish, and tilapia, and they spontaneously kill a variety of xenogeneic targets, including certain fish parasites and traditional mammalian NK cell targets.

Amphibians

Differences in immune system features between urodele (axolotl) and anuran (*Xenopus*) amphibians, already discussed for MHC and Ig complexity, are also seen in immune responses. Rarely is such divergence seen within one vertebrate class (although the two groups diverged over 250 million years ago!). Urodeles express a very restricted antibody repertoire in response to specific antigen that peaks at 40 days postimmunization, and is entirely of the IgM class, even though the serum also contains IgY.[323] They do not respond well to thymus-dependent antigens, which may be due to lack of T-cell help, yet their expressed TCR diversity looks normal. A population of axolotl B cells proliferates specifically in response to LPS and also secretes both IgM and IgY. Moreover, a distinct lymphocyte subpopulation proliferates significantly in response to the T cell mitogens Con A. T cells from young axolotls (before 10 months) do not have this functional ability. Axolotl T cells also can be stimulated with to SEA/SEB, known from mammalian studies to be superantigens.[324]

Anuran larvae can respond specifically (with only 10^6 lymphocytes) to many antigens, with a modest affinity maturation of the IgM anti-dinitrophenol (DNP) response.[325] In adults, the number of different anti-DNP antibodies does not exceed 40, versus 500 in mammals. In secondary responses, the peak of the response is about 10-fold higher and is reached in 2 weeks; there are no major changes in affinity over this initial rise. Isogeneic *Xenopus* produce homogenous antibodies to DNP, xenogeneic red blood cells, or phosphorylcholine with identical or similar IEF spectrotypes and idiotypes, while outbred individuals differ.[326] Both IEF spectrotypes and idiotypes are inheritable, suggesting that diversity is a reflection of the germline repertoire without a major contribution from somatic mutations. Thus, somatic mutations were followed during the course of an antigen-specific immune response at the peak of the modest affinity maturation.[327] The V_H genes, like their mammalian homologues, contain the sequence motifs that target hypermutation, as described previously. Of the 32 members of the V_H1 family involved in the anti-DNP response, expression of only 5 was detected, indicating that immunization was being monitored. Few mutations were detected (average: 1.6 mutations per gene; range: 1 to 5), and there was not a strong preference for mutations in CDR1 and 2 and virtually none in CDR3. Like in the horn shark IgM study noted previously (but not IgNAR or Type II L chains), the mutations were targeted to GC bases, and such a pattern has been suggested to be the first phase of the SHM phase in mouse/human; perhaps *Xenopus* has lost the second phase of the process that results in an evening of mutation frequency for all bases. While the mutation frequency was lower than in mammalian B cells, the rates were only four- to sevenfold less in *Xenopus*. Thus, there is no shortage of variants, and the reasons for the low heterogeneity and poor affinity mat-

uration may be due to less than optimal selection of the mutants. Indeed, because of a relatively low ratio of replacement to silent mutations in the CDRs, it was argued that there is no effective mechanism for selecting mutants, which in turn might be related to the absence of GC in *Xenopus*. In summary, the data from hypermutation, complementary DNA heterogeneity, and spectratype dominance suggests that in the absence of refined modes of selection in late-developing clones, B cells producing somatic mutants may be outcompeted by antibodies generated earlier in the response.

Essential T-cell functions in anurans have been demonstrated with in vitro assays for T-B collaboration and MHC restriction, demonstrating the similarity of the role of MHC in *Xenopus* and mammals.[328] Regulatory T cells have been shown indirectly in hematopoeitic/thymic chimaeras for control of CTL generation and in antibody responses. Ig synthesis can be enhanced following late thymectomy in axolotl or *Xenopus*, again implying a role for thymic-dependent regulatory cells. Thymectomy early in life totally prevents CSR from IgM to IgY, but not IgX synthesis; thus, T cells are absolutely required for the switch to the "IgG-like isotype" and for high-affinity IgM responses, but switch to the mucosal Ig can be T-independent. Switching can also be induced in tadpoles, although one must hyperimmunize animals for this response, due to a paucity of T cells in larvae. The switch is also temperature-dependent, and as described previously for channel catfish, ectotherm T cells are quite temperature-sensitive. AID is expressed in lymphocytes in the spleen as well as in secretory cells.[329] However, consistent with many previous studies, there is no evidence for a typical GC response to date in amphibians or any other ectothermic species. The continued expression of AID in plasma cells (and, presumably, early in embryogenesis) is of interest and deserves further study.

Similar to studies in mammals, the chaperone gp96 has been shown to shuttle peptides into cells making them targets for MHC-restricted CTL lysis.[330] Immunization of frogs with gp96 from a thymic tumor results in the elicitation of CTL that display antitumor activity. Elegant experiments with gp96 vaccination have also shown that CTL activity against minor histocompatibility antigens is MHC-restricted. As mentioned previously, NK cells have been characterized in *Xenopus* with mAbs that recognize non-B/T cells. Those cells kill MHC class I–negative target tumor cells but not class I–positive lymphocytes, and after thymectomy these cells are enriched in the spleen.[331] CD8+ cells expressing TCR were isolated with the same mAb, suggesting the existence of amphibian NKT cells; expression of the mAb epitope on cells is induced by phorbol 12-myristate 13-acetate (PMA)/ionomycin, and is also detected in CTL when MHC-dependent cytotoxicity is reduced.[332] Robert and colleagues have developed one of the best-defined model of innate and adaptive immunity to viral infection in cold-blooded vertebrates, the ranavirus FV3 in *Xenopus*.[70,333] Involvement of CD8 cytotoxic cells and humoral responses have been studied over the course of primary and secondary infections with this virus.[334,335] The system is now primed for the study of other cell type involvement, ontogeny, repertoire, etc.

Reptiles

Lack of an increase in affinity and homogeneity of IEF spectrotypes suggest low-antibody heterogeneity in reptiles. In the turtle *Pseudemys scripta*, a number of genomic VH sequences, representing possibly four families, were isolated, as was a genomic Cμ, all shown to be encoded at a single locus. In northern hybridizations, the Cμ4 probe detected two transcripts; of the four VH groups, only one was expressed, and multiple bands indicated the presence of at least two non-μ transcripts. Among 32 unique VDJ rearrangements from one animal, there were 22 sequence variants in FR4, suggesting either a large number of J segments or somatic modification.[336] The latter interpretation is supported by point mutations found in FR3 and CDR3. For T cells, there are no data on T effector function, but there are studies on the behavior of T-cell population changes due to seasonal and hormonal variations. Thymocytes from the turtle *Mauremys caspica* proliferate in response to phytohemagglutinin (PHA) and ConA, and can kill tumor target cells by both antibody-dependent cellular cytotoxicity–mediated and NK-mediated cytotoxicity. Proliferative responses to PHA and Con A were higher for both sexes in spring and for females in winter than in the other seasons.[337]

Birds and Mammals

The poor increase in affinity of chicken anti-DNP and anti-fluorescein antibodies again indicates lower heterogeneity in chickens. Few changes occur after immunization, even if one waits 1 year after several injections. Perhaps similar to the trout study described previously, a restricted population of high-affinity antibodies was found only after immunization in complete Freund's adjuvant (CFA).[338] Hyperconversion and somatic mutation in Ig genes have been found in splenic GC B cells after immunization.[339] The relatively poor affinity maturation of the chicken response may be due to a balance between gene conversion and somatic mutation. Indeed, modification of V genes with segments of DNA is not an optimal strategy for fine-tuning antibody responses.[340] In the rabbit, there is also conversion/mutation by B cells in GCs after immunization. Within mammals, large variations are found from marsupials with no obvious secondary response, to mouse with 1,000-fold increases in affinity, but the basis for the relatively poor responses has not been established.

In conclusion, although all vertebrates have a very large potential for generating diverse antibodies after immunization, only some mammals studied to date make the most of this potential. Perhaps pressures on the immune system of cold-blooded vertebrates have been less intense due to a stronger innate immunity, and architecture of their lymphoid system is not optimal for selecting somatic mutants, or the great rises in affinity detected in antihapten responses are not physiologically relevant.[297] An immune system using somatic diversification at its "best" is well adapted to species where the value of single individuals is important (ie, species with small progenies); has that been the condition for the creation and selection of somatic rearrangement and of the optimal usage of somatic mutations? If this explanation provides a rationale for the utilization of somatic mechanisms

in generating a repertoire and improving it, it does not tell us why it works so well in certain species and not in others. Perhaps one key is the organization of secondary lymphoid organs. Likely a combination of factors (eg, quantitative and qualitative effects such as endothermy, secondary lymphoid tissues, mutation versus conversion, the hypermutation mechanism itself, rates of proliferation thogen and lymphocyte], etc.) are at work in the regulation of antibody responses.[296,297] Finally, significant differences have been detected in the mutational mechanism and targeting in ectotherms (eg, GC-richness in amphibians, tandem mutations in sharks, TCR loci mutation in sharks, etc.) that may enrich our understanding of the general mutational mechanism, including the enzymes involved and the targeting to V genes.[318]

Lymphoid Tissues

In addition to the molecules and functions characteristic of adaptive immunity, primary (lymphocyte-generating) and secondary (immune response–generating) lymphoid tissues also define the specific immune system[341] (Fig. 4.11). The thymus is present in all jawed vertebrates, and recent evidence suggests its origin at the dawn of vertebrate emergence.[342] *All* animals have hematopoietic cell–generating tissues, and outside of the so-called GALT species, B cells develop in such bone marrow equivalents in all jawed vertebrates. With the advent of clonal selection, the accumulation and segregation of T and B cells in specialized organs for antigen presentation became necessary and indeed the spleen as such an organ is found in all jawed vertebrates, but not in agnathans or invertebrates.

All jawed vertebrate species rearrange their antigen receptor genes somatically (except the case mentioned previously for some shark germline-joined genes[276]). Besides rearrangement, with combinatorial joining of gene segments and imprecision of the joins, there are two other sources of diversity to generate the repertoires: the terminal deoxynucleotidyl transferase (TdT) enzyme that modifies boundaries of rearranging gene segments, and somatic mutations, found exclusively in B cells usually introduced during immune responses. However, progression of rearrangement during B- and T-cell development and diversification follow different rules in different vertebrates.[220] It is conceivable that species hatching early with just a few lymphocytes are under pressures to develop a rapid response and may not use the same mechanisms as species protected by the mother's uterine environment. It is also possible that immune systems of species with few offspring are under stronger pressures than species that have many offspring, and this could be reflected in the manner diversity is generated. Studies of B- and T-cell differentiation have been performed in many vertebrates. RAG and TdT genes have been cloned in representatives of all vertebrate classes, probes that allow the monitoring of lymphocyte development (see the following). Reagents have become available permitting a monitoring of T-cell appearance in the lymphoid organs of ectotherms (crossreactive anti-CD3 sera mentioned previously or TCR probes), as well as mAbs and gene probes specific for Ig H/L chains that allow examination of B cells. As a rule, the thymus is the first organ to become lymphoid during development. Another emerging rule is that development of the thymus-dependent MHC-restricted T-cell repertoire is similar in all species, and this is reflected in the evolution of TCR gene organization described previously as well as a core set of molecules that are required for thymocyte migration and differentiation[343]; in contrast, B-cell repertoire generation differs dramatically among different species, at times even within the same class of vertebrates.[220]

Cartilaginous Fish

Like all other major adaptive immune system components, cartilaginous fish are the first in evolution to possess a prototypic thymus originating from pharyngeal pouches. As

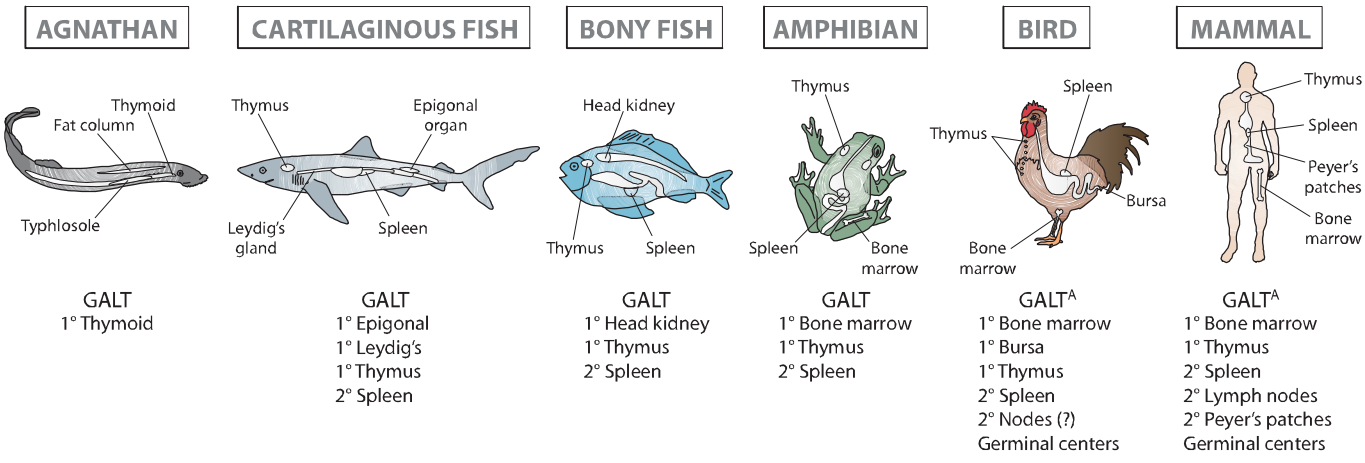

FIG. 4.11. Evolution of Lymphoid Tissues in the Vertebrates. All jawed vertebrates have a thymus and a spleen with demarcated T- and B-cell zones. Fish have different bone marrow equivalents (epigonal, Leydig's gland, head kidney), and amphibians are the oldest group with lymphopoietic bone marrow (also the first to have a typical immunoglobulin [Ig]H chain class switch, although sharks seem to have switch as well, despite the IgH cluster organization). Germinal centers are found only in warm-blooded vertebrates. A thymus equivalent (thymoid) has recently been discovered in agnathans.[375]

in mammals, it has a distinct cortex/medulla structure, and TdT expression was detected in thymocytes with cross-reactive antisera and more recently by northern blotting and in situ hybridization, where it is found throughout the cortex.[257] Interestingly, unlike most other vertebrates, age is not an indicator as to the size of the cartilaginous fish thymus; it can be small or large at any stage of development. GALT is also found in elasmobranchs, but lymphoid tissue in the spiral valve (intestine) clearly does not have typical secondary lymphoid tissue structure; the spleen is the only tissue with compartmentalization of cells into discrete T-cell and B-cell zones.[308] The Leydig's and epigonal organs (associated with the gonads) are lymphopoietic and erythropoietic, producing mainly granulocytes and lymphocytes, and there is high RAG expression in these tissues (see the following). Lymphocytes form nodules in the epigonal organ, probably indicative of differentiative events. In addition, many plasma cells are found peppered throughout the epigonal, fitting with the bone marrow connection.

At hatching, when dogfish embryos are exposed to waterborne antigens, structural development of the lymphomyeloid tissues is well advanced.[344] In the nurse shark, neonatal spleen white pulp consists entirely of class II–negative B cells; by 5 months after birth, T-cell zones appear adjacent to the B-cell zones. Both the B-cell and T-cell zones are vascularized, and no detectable marginal zone separates red pulp from white pulp. Class II–positive dendritic-like cells are found throughout the white pulp.[345]

In the skate *Raja eglanteria*, Ig and TCR expression is sharply upregulated relatively late in development (8 weeks) by quantitative polymerase chain reaction. IgM expression is first detected in the spleen of young skates but IgW is expressed first in gonad, liver, Leydig's organ, and thymus.[346] In adults, Leydig's organ and spleen are sites of the highest IgM and IgW expression. In nurse sharks, IgM1gj and 19S IgM appears in the serum before 7S IgM and IgNAR, and this profile is reflected in the lack of IgNAR+ cells in the spleen until 2 months after birth. RAG and TdT expression in the thymus and epigonal organ of the nurse shark suggests that lymphopoiesis is ongoing in adult life. In contrast to most other vertebrates, N-region diversity is detected (albeit reduced by approximately 50%) in skate and nurse shark IgM and IgNAR CDR3 from the earliest stages analyzed, suggesting that a diverse repertoire is important for young elasmobranchs.[308] As mentioned previously, a subset of Ig genes is prerearranged in the germline of chondrichthyans, and many of those germline-joined genes are transcribed in the embryo and hatchling, but not in the adult. This pattern fits with the expression of the nurse shark IgM_{1gj} with its germline-joined V region, and suggests that some germline-joined genes "take advantage" of their early transcriptional edge and thus some clusters can be selected for specialized tasks in early development. With many gene clusters, it is not known how "clusteric exclusion" is achieved at the molecular level (and why the germline-joined gene expression is extinguished in adult life), but as mentioned previously, studies in two cartilaginous fish species suggest that only one H chain cluster is expressed in each lymphocyte.[278,347]

It was suggested, based on the paucity of rearranged gene segments at the nonexpressed IgH loci, that there are limiting factors regulating accessibility to RAG proteins and a short time window for rearrangement of accessible loci in B cells.[347]

The architecture of cartilaginous fish Ig loci allows greatest diversity only in CDR3 because the CDR2 and CDR1 are always encoded in the germline and V segments do not combine with (D)J segments from other clusters. Yet the number of possible CDR3 is essentially limitless, and the number of germline clusters is also high (at least 15 genes in each species and as many as 100 genes; and usually three rearrangement events take place because two D segments are in each cluster). Thus, the potential diversity is greater than the number of lymphocytes, the general rule for generation of diversity in the vertebrates.

Bony Fish

In all teleosts examined, the thymus is the primary organ for T-lymphocyte generation and head kidney the primary organ for B-cell development. The teleost thymus gland originates from the pharyngeal pouches and can be uni-, bi-, or trilobed, depending on the species,[348] and it is the first organ to become lymphoid. The cortex/medulla architecture is not as precise in other vertebrate species, but the duality of the compartment is apparent and varies from species to species.[349] The spleen contains the basic elements seen in other vertebrates—blood vessels, red pulp, and white pulp—but the distinction between red and white pulp is less obvious (the white pulp being poorly developed). In spleen, the ellipsoids, which are actually terminal capillaries, have a thin endothelial layer surrounded by fibrous reticulum and an accumulation of cells, mainly macrophages. Lymphocyte accumulations are often seen in their vicinity, especially during immune responses, which have been suggested to be primitive GCs, but they are not homologous; as mentioned, AID expression is found in cells[319] during immune responses. Red pulp is rich in melanomacrophage centers, groups of pigment-containing cells at bifurcations of large blood vessels, which may regulate immune responses. The other main lymphoid organ is head kidney, believed to function as mammalian bone marrow. The transparent zebrafish is being developed as a new model to study T-cell differentiation.[343]

In the sea bass *Dicentrarchus labrax*, a mAb detects differentiating T cells (perhaps pre-T cells) as well as mature T cells as evidenced by the presence of TCR messenger RNA in the sorted populations. Cells seem to migrate from surrounding mesenchyme and subsequently mature in the thymus like in all vertebrates studied so far. T cells appear earlier in ontogeny (between 5 to 12 days after hatching) than cytoplasmic Ig+ pre-B cells, which are detected only at 52 days posthatching. Adult levels of T and B cells are reached between 137 to 145 days after hatching, which is quite a long time compared to young amphibians.[350,351]

Teleost RAG1 differs from mammalian RAG1 genes by the presence of an intron of 666 base pairs (an intron is also found in the sea urchin RAG1 gene in a similar position[352]).

Compared with other RAG1 sequences, trout RAG1 has a minimum of 78% similarity for the complete sequence and 89% similarity in the conserved region (amino acid 417-1042). RAG1 transcripts are detected starting at day 20 after fertilization. Trout TdT is highly expressed within the thymus and to a lesser extent in the pronephros beginning at 20 days postfertilization, which correlates with the appearance of lymphocytes in these two tissues.[353] Because the H chain cluster is in the translocon configuration and there are many V_H families, it is assumed that diversity is generated in the mouse/human mode.

As described previously, studies of mature B-cell activation and homing have been done in trout. Immunization of animals results in the production of short-term Ig-secreting cells in the blood and spleen and long-lived plasma cells in the head kidney. Further analysis with B cell–specific transcription factors like PAX5 and BLIMP1 reinforced the functional studies and showed that the blood contains primarily "resting" B cells and the head kidney both plasma cells and B-cell precursors.[314,354] These last findings appear to be true of the cartilaginous fish as well, with the epigonal organ as the head kidney primary lymphoid tissue equivalent. Interestingly, recent studies have shown trout B cells to be quite efficient at phagocytosis, raising questions about myeloid/lymphoid lineage commitment in the vertebrates.[355] It is predicted that B cells from all ectothermic vertebrates are capable of phagocytosis (shown for *Xenopus*), and the work was recently extended to B1 cells in mice.[356] It certainly will be of interest to study these innate characteristics of B-cell subsets in the future, considering the early appearance of B1 cells and macrophages differentiating from the yolk sac of mouse embryos.[328,357]

Amphibians

In anurans, the thymus develops from the dorsal epithelium of the visceral pouches (the number of pouches varies with species) and is the first tissue to become lymphopoietic. It is colonized from days 6/7 onward by precursors derived from lateral plate and ventral mesoderm through the head mesenchyme. Precursors proliferate in situ as the epithelium begins to express MHC class II molecules but not classical class I molecules. By day 8, thymic cortex/medulla architecture resembles that of other vertebrates.[328] Amphibians possess a spleen with red and white pulp, GALT with no organized secondary lymphoid tissue, and many nodules (but no lymph nodes), with lymphopoietic activity in the kidney, liver, mesentery, and gills. The general morphology of lymphoid organs varies greatly according to species and changes with the season. In *Xenopus*, splenic white pulp is delineated by a boundary layer, and the central arteriole of the white pulp follicle terminates in the red pulp perifollicular area, a T-dependent zone. Anurans, like all ectothermic vertebrates, lack GCs. In *Bufo calamita*, colloidal carbon particles injected via the lymph sac are trapped by red-pulp macrophages, which then move through the marginal zone to the white pulp.[358] Giant, ramified, nonphagocytic cells found in both white and red pulp have been proposed to be dendritic cells. *Xenopus* bone marrow does not appear to be a major lymphoid organ from histologic observation, but high RAG expression in this tissue suggests lymphopoietic activity.[359] The maintenance of RAG expression throughout adult life suggests that lymphocytes are continually produced.

Thymectomy decreases or abolishes allograft rejection capacity, MLR and PHA responsiveness, IgY antibody synthesis, and all antibody responses that increase in affinity to classic thymus-dependent antigens.[331,360] MLR reactivity matures before the ability to mount IgY responses in primary responses. Thymectomy at 7 days of age delays allograft rejection and abrogates specific IgY responses, whereas later in life it only abrogates antibody responses. Thymectomy performed later greatly affects the pool of peripheral T cells, as monitored with mAbs specific for molecules such as CD8. Early thymectomy results in the complete absence of T cells, but lymphocytes with T-cell markers, perhaps corresponding to NKT cells, can still be detected. In *Xenopus*, thymocytes induce weak graft-versus-host reactions, whereas splenic T cells are good helpers and strong graft-versus-host inducers. The thymus contains some IgM-producing B cells and memory cells poised to switch to IgY synthesis, and in vitro responses are downregulated by naïve thymus cells. *Xenopus* B cells respond in vitro to low doses of LPS not by proliferation, but rather by Ig synthesis, and also respond to PMA. Old reports of B-cell proliferation can be attributed to contaminants in LPS preparations.[328]

Urodele embryos initially produce five pairs of thymic buds, the first two of which disappear.[284] This results in a three-lobe thymus in *Ambystoma*, but in *Pleurodeles* and *Triturus* it forms one lobe. No cortex-medulla boundary is present, and the thymus generally resembles a canonical cortex. There are at least three types of stromal epithelial cells. There is no lymphopoietic activity in axolotl bone marrow, and hematopoiesis takes place in the spleen and in the peripheral layer of the liver. The spleen is not clearly divided into white and red pulp.

About 40% of TCR-β VDJ junctions in 2.5-month-old *Ambystoma* larvae have N-additions, compared to about 73% in 10- to 25-month-old animals.[247] These VDJ junctions had approximately 30% defective rearrangements at all stages of development, which could be due to the slow rate of cell division in the axolotl lymphoid organs and the large genome in this urodele. As mentioned previously, many axolotl CDRβ3 sequences, deduced from in-frame VDJ rearrangements, are the same in animals of different origins. In contrast, in *Xenopus*, rearrangement starts on day 5 after fertilization for the V_H locus, and within 9 days all V_H families are used. V_H1 rearranges first followed by V_H3, and by day 9/10 V_H 2, 6, 9, and 10 begin being rearranged and then V_H 5, 7, 8, 11 on day 13. For VL, the κ locus is the first to rearrange on day 7 (2 days after V_H), a situation similar to that found in mammals. During this early phase, B cells are present in the liver, where their number increases to approximately 500 cells.[325] Later in larval life, rearrangement resumes at metamorphosis, as suggested by the low incidence of pre-B cells and by the reexpression of RAG during the second histogenesis of the lymphoid system. T cells show a similar type of RAG expression/cell renewal during

ontogeny as the B cells, and the larval and adult Vβ T-cell repertoires differ significantly. Even early in development, tadpoles express a highly variable TCR-β repertoire despite the small number of lymphocytes (8,000 to 10,000 splenic T cells); little redundancy in TCR complementary DNA recovered from young larvae implies that clone sizes must be extremely small, unlike in axolotls.

In *Xenopus*, no lymphoid organ apart from the thymus is detectable until day 12 when the spleen appears and with it the ability to respond to antigen. For B cells, until this time no selection occurs as suggested by the random ratio of productive/non-productive VDJ rearrangements (2:1). After day 12, this ratio becomes 1:1 (ie, the rearrangements have been selected). Complementary DNA sequences on days 10 to 12 (when the number of B cells increases from 80 to 500) are not redundant as if each sequence was represented by one cell.[325] RAG expression together with the detection of DNA rearrangement circles in the bone marrow suggests that rearrangement is ongoing throughout life and is not restricted to an early period, like in birds and rabbits. Tadpole rearrangements are characterized by a lack of N-region diversity, like in mammals but not axolotls or shark/skate (see previous discussion), and thus very short CDR3.[361] During ontogeny, TdT appears in significant amounts in thymus of tadpoles at metamorphic climax, but little expression is detected at earlier stages, which correlates well with the paucity of N-region addition in larval IgH chain sequences.[362] Studies of the ontogeny of the *Xenopus* immune system have revealed a less efficient tadpole immune response (skin graft rejection and Ig heterogeneity and affinity); the absence of TdT expression during tadpole life fits well with the findings of lower larval Ig (and perhaps TCR) diversity.

Reptiles

In all reptiles studied, the thymic cortex and medulla are clearly separated. The spleen has well-defined white and red pulp regions, but T- and B-cell zones have not been delineated with precision.[348] In *Chrysemys scripta*, white pulp is composed of two lymphoid compartments: lymphoid tissue surrounds both central arterioles and thick layers of reticular tissue called ellipsoids. Even after paratyphoid vaccine injection, splenic GCs are not formed, as in fish and amphibians. Splenic red pulp is composed of a system of venous sinuses and cords. In *Python reticulatus*, dendritic cells involved in immune complex trapping have been identified and may be related to mammalian follicular dendritic cells. GALT develops later than spleen during development, and it appears to be a secondary lymphoid organ (but does not seem to contain the equivalent of the bursa of Fabricius). Lymph node–like structures, especially in snakes (*Elaphe*) and lizards (*Gehyra*), have been reported.

Reptiles, the evolutionary precursors of both birds and mammals, are a pivotal group, but unfortunately the functional heterogeneity of reptile lymphocytes is poorly documented. There seems to be T-/B-cell heterogeneity because an antithymocyte antiserum altered some T cell–dependent functions in the viviparous lizard *Chalcides ocellatus*. Embryonic thymocytes responded in MLR at all stages, but ConA responsiveness increased gradually during successive stages and declined at birth. In the alligator (*Alligator mississippiensis*), like in mammals after glass-wool filtration, nonadherent peripheral blood leukocytes (PBL) responded to PHA and not to LPS, whereas adherent cells were stimulated by LPS.

Birds

The thymus, which develops in chickens from the third and fourth pharyngeal pouches, consists of two sets of seven lobes each with definitive cortex/medulla. The thymus becomes lymphoid around day 11 of incubation. Splenic architecture is less differentiated than in mammals. It is not lymphopoietic during embryogenesis as RAG-positive cells are found mainly in yolk sac and blood. Birds are the first vertebrate group where follicular GCs and T-dependent areas comprising the periarteriolar lymphatic sheath are encountered. Plasma cells are located in the red pulp. γ/δ TCR+ T-lymphocytes are chiefly concentrated in sinusoids, whereas α/β T cells fill the periarteriolar lymphatic sheath.[249] Lymph nodes seem to be present in water and shore birds but not in chickens and related fowl.

The bursa of Fabricius is a primary lymphoid organ unique to birds in which B cells are produced.[363] It arises at day 5 of development and involutes 4 weeks later. T-B heterogeneity is obviously well defined in birds (indeed, the "B" in B cell stands for bursa.) The effects of thymectomy—T- and B-cell collaboration and generation of MHC-restricted helper and killer cells—are very similar to mammals, the other class of warm-blooded vertebrates.

During the embryonic period, chicken stem cells found in yolk sac and blood rearrange their IgH and L V genes simultaneously over a very restricted period of time, and very few cells colonize each bursal follicle (about 10^4 follicles).[364] Three weeks after hatching, these cells have differentiated in the bursa and then seed the secondary lymphoid tissues, after which time B cells are no longer be generated from multipotent stem cells; thus, only approximately 2×10^4 productive Ig rearrangements occur in the life of the chicken. When an antiserum to chicken IgM was administered in ovo to block this early bursal immigration, there were no stem cells arising later in development that can colonize the bursa, and these chickens lacked B cells for their entire lives.[365] Although the general Ig locus architecture is similar to that of frogs and mammals, only one rearrangement is possible as there is only one functional V_L or V_H on each allele.[366,367] Diversity is created during bursal ontogeny by a hyperconversion mechanism in which a pool of pseudogenes (25 ψL and approximately 80 ψH) act as donors and the unique rearranged gene acts as an acceptor during a proliferative phase in bursal follicles. For H chains, the situation is more complex as there are multiple D elements. During ontogeny, selection of productive rearrangements parallels the selection of a single D reading frame, suggesting that the many D segments favor D-D joins to provide junctions that are diversified by gene conversion; the hyperconversion mechanism can also modify Ds because most donor pseudogenes are fused VD segments. The gene conversion process requires AID, which is also required for SHM and CSR.[368]

Because diversification by gene conversion occurs after Ig rearrangement and cellular entry into bursal follicles, and there is only a single germline V_H and V_L expressed on all developing B cells, it was tempting to implicate a bursal ligand binding to cell surface IgM to initiate and sustain cellular proliferation and gene conversion. However, surface expression of IgM devoid of V regions permitted the typical B-cell developmental progression, demonstrating that such receptor/ligand interactions are not required.[369] Thus, currently we know little of how cells enter the bursa, which signals induce them to proliferate/convert, and how cells arrest their development and seed the periphery.[364]

Generation of Diversity in Mammals

Mechanisms leading to the generation of repertoire diversity vary among mammalian species.[220] Categories can be made depending upon the mode of B-cell development: rabbits, cattle, swine, and chickens, unlike fish, amphibians, reptiles, and primates/rodents, use a single V_H family, of which only a few members (sometimes only one) are functional. To diversify their antibody repertoire, this group uses gene conversion or hypermutation in hindgut follicles of GALT early in life (rather than bone marrow throughout life) and is therefore known as the "GALT group." At the rabbit H locus, as in the chicken, a single V_H is expressed in most peripheral B cells. During development, B cells that have rearranged this particular V in the bone marrow (and other sites) migrate to the appendix where this rearranged gene is diversified by gene conversion using upstream donor V segments.[370,371] This development of B cells in rabbits is dependent on the intestinal microflora, and efforts are being made to define the potential bacterial superantigens involved, as well as binding sites on IgM necessary for the differentiation.[372] In ruminants, the ileal Peyer patches are the bursa-like primary B cell–generating tissues.[373] Although bursa, appendix, and sheep ileal Peyer patches show morphologic similarities, the mechanisms generating diversity are different: conversion in the chicken and hypermutation in sheep, and both in the rabbit. As described previously, most of the "GALT group" also appears to lack IgD; thus, IgD might serve some purpose in repertoire development in some groups of mammals and not others.

In summary, the organization of the lymphoid tissues is perhaps the only element of the immune system that shows increasing complexity that can be superimposed on the vertebrate phylogenetic tree. The absence of primary and secondary lymphoid tissues (thymus and spleen) is correlated with the absence of a rearranging and/or hypermutating receptor family in other animals, with the exception of the agnathan VLR in which the relevant lymphoid tissues are being defined (see the following). While all jawed vertebrates have a true secondary lymphoid tissue (spleen), ectotherms lack lymph nodes and organized GALT. In addition, while ectotherms clearly have B-cell zones resembling follicles, and despite the clear ability for ectothermic B cells to undergo SMH and at least some degree of affinity maturation, GCs with follicular dendritic cells are not formed after immunization; clearly, this was a major advance in the evolution of the vertebrate immune system.

The potential repertoire of Ig and TCR as well as VLR-combining sites is enormous in all vertebrates. The potential antigen receptor repertoire in all species for both T and B cells is far greater than could ever be expressed in an animal because of cell number limitations. Not all species or all gene families use combinatorial joining for repertoire building, but all species assemble V, (D), and J gene segments to generate their functional Ig genes during B-cell ontogeny, and the imprecision of this assembly creates great somatic diversity. Thus, from this survey in various species, one could not predict that there would be major differences in immune responses in representatives of different vertebrate classes, and yet as mentioned previously the mouse/human antibody responses are superior to those in many taxa.

More on Evolution of the Thymus

The discovery of a second (cervical) functional thymus in mice has raised ontogenetic and immunologic questions.[342,374] Is this second thymus the result of an atavism, or does it correspond to what is seen in human when a cervical thymus can form under certain pathogenic conditions during the migration of the thymus to its final mediastinal location? There are examples of cervical thymi in primitive mammals such as marsupials but also in some prosimians. An "extra" thymus also is reminiscent of the multiple thymi encountered quite frequently in cold-blooded vertebrates.

As described, the thymus is promiscuous with regard to its precise developmental origin.[284] The thymus arises from pouches two to six in cartilaginous fish, from the second pouch in frogs, the second and third in reptiles, and from the third and/or fourth in bony fish, birds, and mammals. The final number of thymi can also be variable. It ranges from five pairs of organs in sharks, to four in caecilian amphibians, to three in urodeles, and finally to one in many teleost fish species, anurans, and many mammals. The thymus of reptiles varies in term of location and number of lobes, reflecting variation in embryonic origin. The adult thymus may be found anywhere from the base of the heart to the neck. For example, in lizards and snakes, there are two lobes on each side of the neck with no subdivision in lobules. The crocodilian thymus is an elongated chain-like structure, not unlike that of birds. In turtles, the thymus is a pair of lobes divided in lobules at the bifurcation of the common carotid associated with the parathyroids. The multilobed thymus in birds is not the equivalent of the multiple thymi found in sharks because the subdivision is secondary to primary organogenesis. Regardless of the underlying ontogenetic mechanism leading to the development the cervical thymus in mice, the result is suggestive of the secondary "thymus spreading" in birds, but it differs with regard to an uneven final size distribution. All marsupials, except koalas and some species of wombats that only have a cervical thymus, have a thoracic thymus similar to that of placentals. Some marsupials (kangaroos and possums) also have a cervical thymus; their thoracic thymus derives from the third and fourth pharyngeal pouches, whereas the cervical thymus arises mainly from the ectoderm of the cervical sinus with some participation from the second and third pouch (like in reptiles).

The second mouse thymus seems to show primitive characteristics such as the existence of a single lobe as is found in amphibians, a superficial location, and a position compatible with an origin involving another pharyngeal pouch (presumably the second) that would be a marsupial and therefore perhaps a reptilian character. Embryology helps in determining the possible scenarios: During mouse ontogeny, the canonical thymus anlage can be recognized, beginning on day 11.5 in development, as a group of Foxn1-expressing cells located ventrally in the third pharyngeal pouch. If the cervical thymus were derived from an independent anlage, one would rather expect to detect Foxn1+ cells in the endoderm outside the third pouch. This argues for a common origin of thoracic and cervical thymi and against a second thymus anlage outside the third pouch, and, hence, against the above hypothesis that the cervical thymus represents an atavistic organ.

A Thymus in Jawless Fish?. Until recently, it was believed that the thymus appeared in evolution with the emergence of adaptive immunity in the extinct placoderm lineage approximately 500 million years ago. There has never been any controversy concerning the presence of a thymus in all living jawed vertebrates, and as described, its requirement for T-cell differentiation is universal. The lack of a thymus in the jawless fish was consistent with the non-Ig/TCR adaptive immunity in these animals, but this has changed in a new study.[375]

The finding that lampreys have two types of lymphocytes heralded a renewed search for a thymus equivalent in these animals.[291] Indeed, there was an extensive literature on this topic over the past century identifying accumulations of cells in various cranial regions, suggesting that lymphocytes could be differentiating in these areas. However, it has always been clear that there is no specialized tissue with a defined cortex and medulla in lampreys or hagfish, as is seen in all gnathostomes. This conclusion was not well supported because there were no molecular markers for either lymphocytes or thymic epithelium in jawless fish. Indeed, consistent with almost all other accumulated data, a recent study concluded that there was "no evidence for a thymus in lampreys."[343(p189)] In that study, the major transcription factor involved in the development of T cells described previously, *foxn1*, was expressed by the pharyngeal epithelium, but the lack of expression of any known lymphocyte markers made it unlikely that this region was truly the thymus equivalent; a similar transcription profile was seen in the gill epithelium of the model basal chordate *Amphioxus*, which truly seems to lack an adaptive immune system.

The discovery of "T cells" in lampreys opened a new panorama with the expression of T cell–specific genes besides the antigen receptors. As mentioned, Pancer and colleagues discovered that lamprey lymphocytes express two APOBEC family member genes, CDA1 and CDA2, and suggested that they were involved in the rearrangement (and perhaps mutational) events in the VLR genes.[288] In the T/B split paper described previously, these APOBEC family members seemed to be expressed specifically in either VLRA (CDA1) or VLRB (CDA2) cells. In the new study, CDA1 was shown to be expressed by lymphocytes in close proximity to the *foxn1*-positive pharyngeal epithelial cells.[375] Furthermore, only in these "developing" lymphocytes, but not in mature VLRA-positive cells, could a high percentage (approximately 25%) of out-of-frame VLRA genes be detected, implying that cells were differentiating in this region. In summary, this tissue in lampreys, which was christened the "thymoid," 1) is derived from the pharyngeal epithelium, 2) expresses classical thymic epithelial markers such as *foxn1* and notch ligands, and 3) is associated with developing VLRA cells, based on expression of the APOBEC family member CDA1, out-of-frame VLRA gene sequences, and failure to respond to activation signals (such as the T cell mitogen PHA) that stimulate mature lymphocytes. In addition, consistent with the high percentage of cells with a nonfunctional receptor, many lymphocytes undergo apoptosis in the thymoid, which is also comparable to the situation in jawed vertebrates. Much more work is necessary to understand this system, but the basic finding is extraordinary.

Because the thymoid is expressed at the tips of all of the gill filaments,[375] thymectomy will not be possible in jawless fish. Perhaps procedures will be developed to block the interactions between the VLRA cells and the pharyngeal epithelium, or the development of the thymoid itself can be disrupted. Assuming that this tissue indeed is the thymic equivalent in lampreys, what is the significance of having the VLRA cells develop in a unique organ? In gnathostomes, T cells recognize antigen in the form of peptides in association with MHC class I or class II molecules. Because of the high levels of MHC polymorphism, as mentioned previously, T cells are positively selected in the thymus for cells that recognize antigen in association with the thymic MHC. Despite major effort, neither MHC molecules nor the specialized proteins associated with antigen processing have been detected in the jawless fish, and thus if there is positive selection it must be orchestrated by a convergent system of antigen processing/presentation. In the same vein, perhaps there is an AIRE equivalent expressed by the thymoid that ensures deletion of self-reactive clones[376]; if so, it would also imply that a convergent antigen presentation system will be discovered in lampreys. It will be of interest to reexamine differentiation of lymphocytes in the pharyngeal epithelium of basal chordates; perhaps this will lead us to an understanding of the origins of adaptive immunity in the vertebrates. Finally, what is the significance of T cells developing in association with the pharyngeal epithelium? Is it because this area in the gill region is evolutionarily plastic or is there some relevance to exposure of the thymoid to the external environment?

Discovery of a New Proteasome Element Expressed in Thymus. Recently, a new form of the proteasome only expressed in the thymus was discovered in mammals, called β5t. The β5t gene was generated via a cis duplication from LMPX (β5), and in its absence cytotoxic T cells are impaired.[377] Like many other components of adaptive immunity we have discussed, β5t is found in all gnathostomes, beginning with the cartilaginous fish. As we will see in the following, the LMPX equivalent of the immunoproteasome, β5i or LMP7, is polymorphic in amphibians and bony fish,

suggesting that it is a lynchpin in formation of the immunoproteasome.[378] It is interesting as well that both β5t and β5i (and all of the immunoproteasome elements) have been lost in birds (see the following).

The Major Histocompatibility Complex

T cells distinguish self from nonself through the presentation of small peptides bound to MHC class I and class II molecules (ie, MHC restriction). The genetic restriction of T cell–APC collaboration, processing of antigen by professional APCs, and T-cell education in the thymus described in mice hold true (or is assumed) for most jawed vertebrate classes. For technical reasons, no MHC-regulated T-cell responses have been documented in cartilaginous fish, but the identification of polymorphic class I and II and rearranging TCRα/β genes and segregated T- and B-cell zones in spleens (see previous discussion) strongly suggest that functional analyses will reveal MHC restriction of adaptive responses.[257] By contrast, urodele amphibians and teleost cod are notorious for their poor immune responses (see the following), but biochemical and molecular evidence suggests that class II polymorphism is low in the axolotl and cod have lost the entire class II–based immune system. Recent results in agnathans and other vertebrates have opened our eyes to new functions in MHC.

Class I/II Structure Through Evolution

The three-dimensional organizations of class I and class II are remarkably similar: the two membrane-distal domains of both molecules form a PBR composed of two antiparallel α helices resting on a floor of eight β strands, and the two membrane-proximal domains are IgSF C1. Although sequence identity among class I and class II genes in vertebrates is low (like most other immune genes), the four extracellular domain organization and other conserved features are likely to be found in the ancestral class I/II gene.[379] An intrachain disulfide bridge exists within the class I PBR α2 and class IIβ1 domains, but not the class I/II PBR α1 domains, and phylogenetic trees show that these respective domains are most similar. Bony fish class II α1 domains, like class II DMα molecules, do have a disulfide bridge. The exon/intron structure of class I and class II extracellular domains is also well conserved, but some teleosts have acquired an intron in the exon encoding the IgSF β2 domain. Other conserved features of class I genes include a glycosylation site on the loop between the α1 and α2 domains important in biosynthesis (shared with class II β chains), a Tyr and one to three Ser in the cytoplasmic regions that can be phosphorylated in mammals, as well as several stabilizing ionic bonds. Class II with its two TM regions differs from class I with only one; conserved residues in the class II α and β TM/cytoplasmic regions facilitate dimerization. In summary, because sequence similarity is very low among MHC genes in different taxa, these conserved features are important for function, biosynthesis, and maintenance of structure.

β2m was the second IgSF molecule (C1 type) ever to be identified, originally found at high levels in the urine of patients with kidney disease. It associates with most class I molecules (see the following). Besides mammals, β2m genes have been cloned from representatives of all jawed vertebrate classes. The β2m gene is outside the MHC in all tetrapods and bony fish tested, and is a single copy gene in all species except cod and trout, in which it has undergone multiple duplications. Based on the levels of similarity between the various domains of class I and class II, it was predicted that β2m was originally encoded in the MHC; indeed, recent work has shown that it is linked to the MHC in the nurse shark, adjacent to the ring3 gene.[380] Like TCR/Ig/class I/II, β2m has not been found in jawless vertebrates, so its origin remains mysterious.

Classical and Neoclassical Class I and Class II

Class Ia (classical) and class Ib (nonclassical) genes are found in all of the major groups of jawed vertebrates. Class Ia genes are defined by their ubiquitous expression, their presence in the MHC proper, and by high polymorphism (in most species). In addition, class Ia proteins almost always have eight conserved residues at both ends of the PBR that interact with "mainchain" atoms of bound peptides and constrain their size to eight or nine residues; this feature often distinguishes class Ia from class Ib (see the following). Thus, tight binding of peptides, a likely source of conformational changes in class I allowing transport through the endoplasmic reticulum and cell surface expression, is an evolutionarily conserved trait.[381] In nonmammalian vertebrates, one of these residues at the C-terminus is lysine rather than tyrosine, the functional significance of which is unclear.[379]

The class Ia/Ib distinction holds in most taxa: one to three polymorphic class Ia genes are expressed ubiquitously in most species, whereas other minimally polymorphic or monomorphic class Ib genes are expressed in a tissue-specific fashion. The class Ib genes can be split into two major groups: one set that is most related to the class Ia genes within a taxon and thus recently derived, and one group that is ancient and emerged early in evolution.[381] In mouse and human, the set most closely related to class Ia genes are closely linked within the MHC. In nonmammalian vertebrates, however, this first set is found in gene clusters on the same chromosome as the MHC proper but far enough away to segregate independently from MHC (eg, chicken [*Rfpy*] and *Xenopus* [*XNC*]). One *Xenopus* class Ib gene is expressed specifically in the lung and thus likely has a specialized function, and another gene (XNC10) is expressed by T cells and may serve as a ligand for unconventional subsets of T cells.[382,383] Chicken Rfpy is associated with resistance to pathogens, and the recent structure of one of these class Ib genes has shown an unusual hydrophobic structure in the groove.[384] Class Ib genes related to but unlinked to the classical class I have also been found in bony and cartilaginous fish, and a lineage of class II–linked class I genes was discovered in bony fish.[385] Thus, class Ib genes that arise in each taxon seem to have true class I–like functions, but perhaps have become specialized (sometimes the distinction between class Ia and class Ib is blurry; see the following). The second set of older class Ib genes that predates divergence of taxa can have very different functions.[381] For example, the neonatal Fc receptor is involved in binding

and transport of IgG molecules across epithelia as well as protecting them from degradation (the Brambell receptor). Furthermore, molecules only described so far in mammals are composed only of a PBR without IgSF domains; these unusual class I molecules do not bind peptides but rather are important for the regulation of NK- and T-cell function during infection. The paradigm for these SOS responses is the MHC class I-related protein (MIC) and UL16-binding protein (ULBP) class Ib molecules, which clearly do not bind peptides. Some teleost class Ib genes that fall outside the major cluster of fish class I genes may fit into this category.[385,386] Finally, molecules like CD1 bind nonpeptidic antigens for presentation to innate-like NKT cells. The phylogenetic analysis predicts that CD1 and FcRN are old class I genes (see the following on CD1); the age revealed by the phylogenetic tree also correlates well with the hypothesis that ancient duplication events predating the emergence of jawed vertebrates resulted in the appearance of CD1, FcRN, and MHC-linked class I genes (see the following). Was the original function of class I linked to antigen presentation (peptidic or otherwise), induction of an SOS response, or to housekeeping functions? We do not have the answer because class Ia and class Ib molecules are present in the oldest living gnathostomes. The discovery of class I–like genes in animals derived from ancestors predating adaptive immunity or in the jawless fish would help resolve this question, but to date no recognizable MHC molecules or their kin have been detected in prejawed vertebrates; this topic is especially of interest since the dichotomy of lymphocytes as well as a thymus candidate in jawless fish were discovered.[291,375]

Class II molecules also have nearly invariant and evolutionarily conserved residues that bind to main-chain atoms of peptides, but these are in the center of the groove.[379] Thus, tight binding to main-chain peptide atoms occurs in the center of the class II PBR, and peptides are free to protrude from both ends. The only nonclassical class II molecules so far identified are the previously mentioned DM molecules that lack these residues and DO proteins. DM molecules so far have been cloned only from tetrapods[177] and have not been detected in any fish species despite the large genomic and EST databases for the bony fish. Thus, they either had not emerged at the time fish arose or were lost in the bony fish lineage; phylogenetic trees suggest the latter, and would be consistent with the rapid rate of genome evolution in the teleosts. DO class II molecules, believed to modulate DM function, have only been detected in placental mammals.[387] The invariant chain is another protein found only in gnathostomes[388]; its recent role in class I cross-presentation suggests that it may have been important for biosynthesis of both class I and class II.[389]

Class I/II Expression

In *Xenopus* species, immunocompetent larvae express high levels of class II on APCs such as B cells, but express only very low levels of class Ia molecules on all hematopoietic cells until metamorphosis.[328] Expression of the immunoproteasome element *lmp7* and all identified class Ib isotypes is also very low.[390] Larval skin and gut, organs with epithelia in contact with the environment, appear to coexpress class I (transcripts) and class II. Such expression may provide immune protection during larval life; perhaps expression of class Ia is limited to organs that undergo massive destruction and remodeling at metamorphosis. Class II molecules also change their distribution after metamorphosis and are highly expressed by unstimulated T cells.[391] Axolotl class II molecules are also regulated differentially during ontogeny, expressed in young animals on B cells, and then expanded to all hematopoietic cells, including erythrocytes, later in life.[392] Changes in MHC expression are not correlated with cryptic metamorphosis in axolotls, but class II expression by erythrocytes is correlated to the switch from larval to adult globins. Unlike *Xenopus*, class I transcripts isolated so far are expressed early in ontogeny, from hatching onwards.

Carp class I and class II transcripts are detected in embryos 1 day after fertilization and reach a plateau at day 14. However, the suspected class Ia protein does not appear until week 13, whereas β2m can be detected several weeks earlier.[393] It was suggested that another class I molecule is expressed during early development of the carp hematopoietic system, perhaps one of the unusual nonclassical molecules that groups outside the teleost cluster. Interestingly, class I is expressed in the brain of young, but not adult fish, suggesting that class I molecules may play a role in neurogenesis.[394]

As mentioned previously, the cod was recently shown to have lost class II genes, as well as the invariant chain and CD4 genes.[395] The apparent lack of the entire class II system of antigen recognition correlates well with the inability of cod to make antigen-specific antibody responses.[396] The authors speculate that the large numbers of class I molecules in cod may somehow compensate for the loss of a class II system. Axolotls, which have very low class II polymorphism,[397] also have a highly expanded class I system.[398] It is possible that in both cases there has been a "use it or lose it" scenario, in which a major arm of the immune system was lost due to a low pathogen load.

Major Histocompatibility Complex Gene Organization

As class I and class II proteins are structurally similar, it is no surprise that their genes are linked, a primordial trait subsequently lost only in bony fish (Fig. 4.12).[2] But why are structurally unrelated class I processing genes, including the immune proteasome components *lmp2* and *lmp7* and the *TAP* and *TAPASIN* genes, also found in the MHC? There are two possible scenarios: primordial linkage of ancestral processing and presenting genes in the MHC, or later recruitment of either the processing/presenting genes into a primordial MHC. Based on the presence of similar clusters of MHC genes on paralogous chromosomal regions in humans and mice, Kasahara et al.[17,399] proposed that ancestors of class I, class II, proteasome, transporter, and class III genes were already linked before the emergence of the adaptive immune system. Genomewide duplications around the time of the origin of vertebrates (the so-called 2-Round or 2R hypothesis), as proposed by Ohno et al.,[16] may have provided the raw material from which the immune system genes was assembled (see the following; Fig. 4.13). As for all other adaptive immune genes described so far, neither class I/II nor immunoproteasome/transporter associated with antigen processing (TAP) have been isolated from hagfish nor

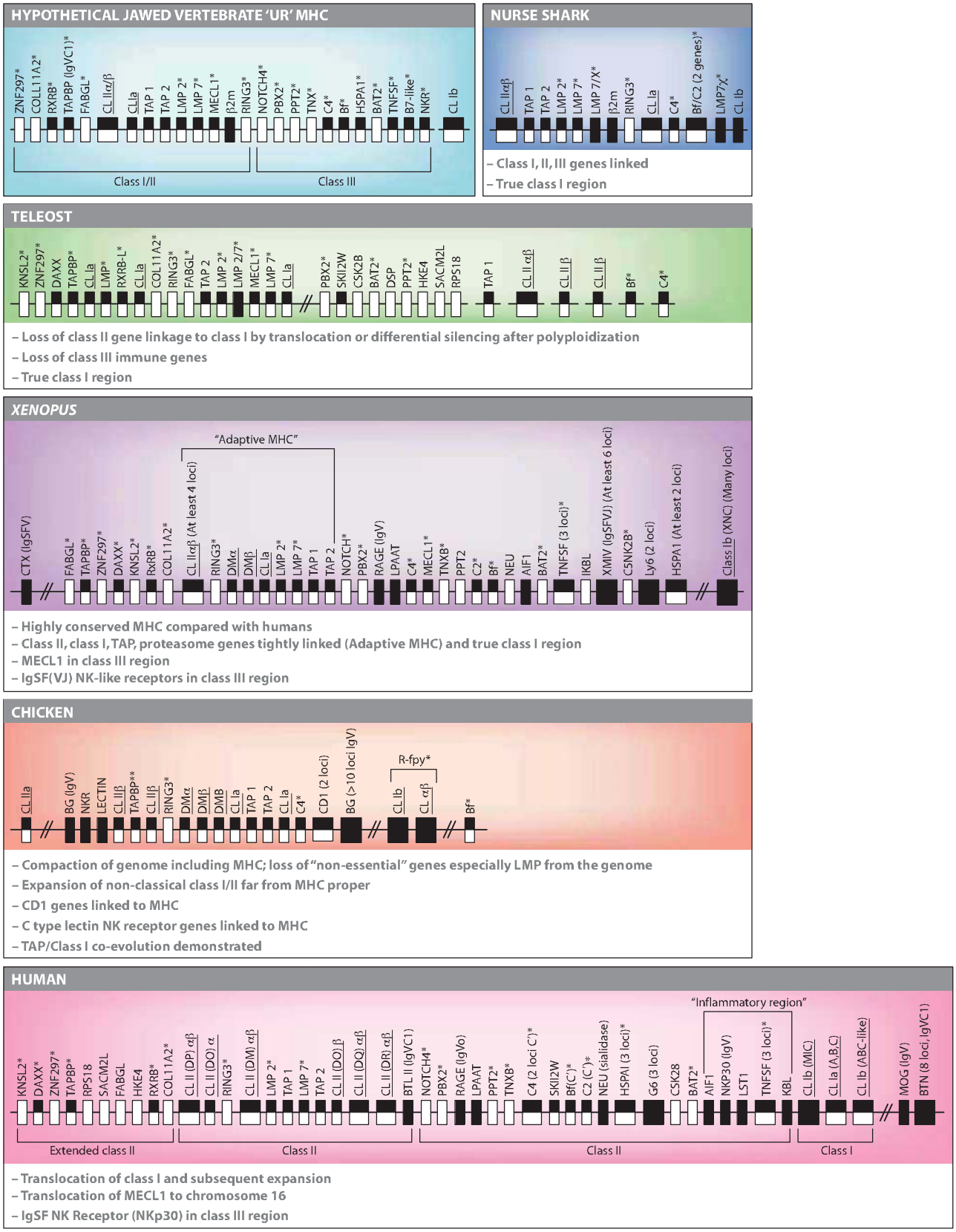

FIG. 4.12. Major Histocompatibility Complex (MHC) Evolution. *White* indicates that the gene is found in at least one other species besides human; *black* designates a gene with known or inferred immune function; *asterisk* indicates an ancestral gene found on at least two paralogous MHC regions in mammals; *large box* indicates at least two genes in the particular region; *double slash* indicates linkage far away on the same chromosome; *space between lines* indicates that the genes are on different linkage groups. The teleost MHC is a composite of the zebrafish, trout, *Fugu*, and medakafish maps. Extensively modified from Flajnik and Kasahara.[498]

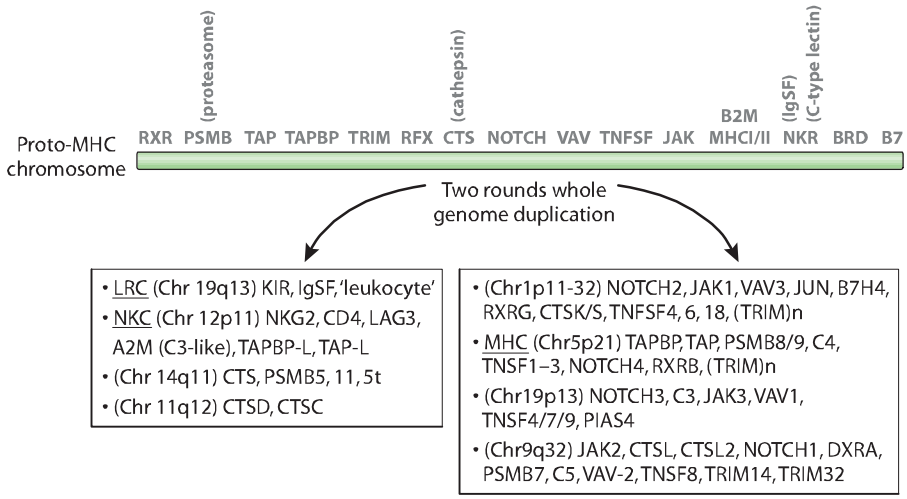

FIG. 4.13. Genomewide Duplication (2R) Model of Major Histocompatibility Complex (MHC) Evolution. Displayed are the MHC paralogous regions and their human chromosomal designations, first identified by Kasahara and modified over the years.[2,17] Note that such paralogous regions have also been studied for chemokine receptors,[477] tumor necrosis factor and tumor necrosis factor receptors,[459] immunoglobulin superfamily molecules involved in cell-cell interactions,[442] and others. Note that the natural killer cell complex and leukocyte receptor complex, respectively, are likely to have been in the Ur MHC based upon linkages seen today in various taxa. The *box on the right* indicates the original MHC paralogous regions identified by Kasahara on chromosomes 1, 6, 9, and 19.[17] The *box on the left* shows paralogous regions uncovered in further analyses.[500] Modified from Flajnik and Kasahara.[2]

lampreys, and all of these genes as well as other genes involved in immunity could have emerged as a consequence of the duplications. Because class I genes are found on two or three of the clusters, class I–like molecules may have predated class II in evolution. Indeed, NK-like recognition of a class I or class I–like molecule encoded in an ancestral linkage group may have been at the origin of the adaptive immune system (see the following).

TAP is an interesting case in that the MHC-linked TAP 1/2 genes were clearly not part of the Ur MHC, but rather they were "recruited" to the MHC early in evolution. TAPs are members of the very large ABC transporter family, and are most closely related to bacterial ABC transporters; this suggests that TAP1/2 were derived from a bacterial, or more likely a mitochondrial gene, via horizontal transfer.[400] Furthermore, a close homologue of TAP1/2, TAP-L (TAP-like), unlike all of the other Ig/TCR/MHC genes we have discussed, is present in jawless fish.[401] The function of TAP-L is not known, but it may be involved in cross-presentation[402] or "typical" presentation in jawless fish. In summary, the TAP genes are not following any of the "rules" that seem to hold true for the other genes involved in adaptive immunity, and this story will be exciting to monitor, especially in the context of the agnathan VLR system.

In all nonmammalian vertebrates, and even in marsupials, the immunoproteasome and TAP genes are closely linked to class I genes, not to class II, in a true "class I region."[2] This result is most striking in bony fish (*Fugu*, zebrafish, medaka, trout) because class I/lmp/TAP/TAPBP and class II are found on different chromosomes.[403,404] The class III region, historically defined by the innate immune genes such as Bf/C2, and C4 are also present in the *Xenopus* and elasmobranch MHC, showing that the class III association of class I/II with such genes is ancient.[177] If Kasahara's interpretation is correct (ie, MHC syntenic groups found on different mammalian chromosomes resulted from ancient block duplications), it is expected that the physical association of ancestral class I, II, and III genes predated the emergence of jawed vertebrates, and such syntenies in ectothermic vertebrates are not surprising. Indeed, linkage studies in nonvertebrates *Amphioxus* and *Ciona* do support an ancient linkage of class I, II, and III genes.[405] Taken together, the data reveal that lack of synteny of class I, class II, and class III genes in teleosts is a derived character. Independent assortment of class I and class II may allow these genes to evolve at different rates: in some teleosts, class Ia alleles form ancient, slowly evolving lineages, whereas class II genes evolve at similar rates as mammalian MHC alleles.[406,407]

The chicken MHC, the B complex, is on a microchromosome, and intron sizes and intergenic distances are both quite small so that the entire complex is only a few hundred kb as compared to over 4,000 kb in humans and *Xenopus*.[408] Class Ia (BF), class IIβ (BL and DM), and TAP genes are in the MHC, but there is no evidence for immunoproteasome genes, and almost all class III genes have been deleted except for C4. The quail MHC is similar, although is somewhat expanded, especially in genes related to the C-type lectin NK receptors.[409] Although most class III genes are found on other chromosomes, *lmp2/7* and *MECL1* genes are actually absent from the genome; indeed, peptides bound to chicken class I molecules sometimes have C-terminal glutamic acid or aspartic acid, which are rare after proteolysis by mammalian proteasomes containing lmp2 and lmp7. Indeed, the recent crystal structure of a chicken class I molecule shows that the alleles can interact with a broader array of peptides as compared to mammalian class I alleles; the PBR is "broader" and one of the conserved peptide-binding residues is noncanonical.[410] To explain the correlation of

diseases with particular haplotypes, Kaufman proposed that the chicken has a minimal essential MHC composed of only those genes absolutely required to remain in the complex. This concept has been reinforced recently by an analysis of resistance to viral infection (Rous sarcoma virus) governed by classical class I molecules.[411,412] Additionally, the presence of polymorphic TAP alleles closely linked to particular class I alleles has demonstrated coevolution of the transporters and peptide-binding molecules.[413]

Surprisingly, CD1 genes are closely linked to the chicken MHC.[414,415] As mentioned previously, CD1 genes in mammals seem to be on one of the MHC paralogous regions and it was suggested that it arose as a consequence of the en bloc duplication. While this is still possible (ie, there was differential silencing of CD1 and other class I genes on the two paralogous chromosomes), the more likely scenario is that CD1 arose by gene duplication within the MHC itself in an ancestor of warm-blooded vertebrates; no bonafide CD1 genes have been detected to date in any fish or amphibians, so the entire NKT system may be specific of warm-blooded vertebrates.

In summary, in all animals except placental mammals, classical class I genes map closely to the *TAP*, *lmp*, and tapasin genes, suggesting that the processing, transport, and presenting genes were in an original "class I region."[416] The tight linkage of the functionally, but not structurally, related genes strongly suggests that such genes coevolve within particular MHC haplotypes. Indeed, in *Xenopus* there are biallelic lineages of class Ia, LMP7, and TAP, which are always found as a set in wild-caught animals.[417] Although teleosts underwent an explosive adaptive radiation 100 million years ago and primordial syntenies have been lost in many cases, there are deep lineages of class Ia genes in many species, also found for *Xenopus* and cartilaginous fish class Ia genes. A study in medaka suggests that divergent noncoding regions between the class I–processing and –presenting genes do not permit recombination between lineages, hence preserving the linkage disequilibrium.[418] Nonaka et al. proposed that these deep proteasome/class I lineages emerged at the dawn of vertebrate immunity and may even have been maintained perhaps by convergent evolution in certain groups.[419] In (most) eutherian mammals, the class I region is not closely linked to lmp/TAP and is very unstable, with rapid duplications/deletions expected in a multigene complex (see Fig. 4.12); the same class I instability extends to the non–MHC-linked class Ib genes in *Xenopus* species.[420]

EVOLUTION OF ALLORECOGNITION

Histocompatibility Reactions in Invertebrates

Scrutinizing graft rejection within a species (allograft) across the animal kingdom demonstrated that allorecognition was almost universal among metazoa. Scrutinizing specific memory in the same systems was *the* major tool to establish the universality or not of adaptive immunity. Moreover, it fueled speculations the origins of vertebrate MHC. Most of these studies led to inconclusive results because of the poor immunogenetic aspects of the reactions. However, the genetic regions and mechanisms responsible and molecular underpinnings of such alloreactions are now becoming known, and no (or little) resemblance to the vertebrate MHC has been found even though regions homologous of the extended vertebrate MHC have been identified in some organisms but without functional correlation.[405,421]

Colonies of *Porifera*, *Cnidaria*, *Bryozoa*, and *Tunicata* (see Fig. 4.1) often compete for space and may develop histocompatibility reactions in the zone of contact. In addition, cell-lineage parasitism, in which the somatic and/or germ cell lineage of one partner replaces that of the other, may ensue if colonies fuse into a chimera. Thus, effector functions following allorecognition also protect the genetic integrity of the individual. In some species, fusion is apparently restricted to tissues of the same individual (complete matching), in other species such as bryozoans, fusion also occurs between genetically distinct individuals if they share kinship (partial matching). Two divergent invertebrate phyla have been studied in detail: *Cnidaria* and *Tunicata*.

Porifera

Sponges (*Porifera*) and placoza are the phylogenetically oldest extant metazoan phyla. Sponges, whether marine or freshwater species, possess a sophisticated histocompatibility system.[422] Elements of the sponge immune system involved in these reactions have been analyzed at the molecular level. Sponge cells associate in a species-specific process through multivalent calcium-dependent interactions of carbohydrate structures on a 200 kd extracellular membrane-bound proteoglycan called "aggregation factor," well studied in *Microciona* and *Geodia*.[422] The glycan moiety is involved in cell adhesion and exhibits differences in size and epitope content among individuals, suggesting the existence of allelic variants. Therefore, strong carbohydrate-based cell adhesion evolved at the very start of Metazoan history. Other genes involved in these reactions include one that is similar to the vertebrate MHC-linked allograft inflammatory factor and another to the T-cell transcription factor. AIF-1 and T-cell transcription factor genes are upregulated in vivo after tissue transplantation, and in vitro in mixed sponge cell reaction.[423] Polymorphic IgSF molecules are found on the surface of sponge cells, but their relationship to allorecognition events (if one exists) is not clear.[424]

Allogeneic recognition in vitro led to apoptotic cell death in one partner and survival in the other.[425] The process is controlled by a differential expression of the proapoptotic and prosurvival proteins that are characteristic for the initiation of apoptosis (caspase, MA3, ALG-2 protein) and the prevention of programmed cell death (2 Bcl-2 homology proteins, FAIM-related polypeptide, and DAD-1–related protein). In an apoptotic mixed cell combination, characteristic apoptotic genes were expressed, while in the nonapoptotic aggregates the cell-survival genes are upregulated.[423] In another species, *Microciona*, allogeneic interactions also

induce cellular reactions involving gray cells (sponge immunocytes) and finally apoptosis. Analogous (but most likely not homologous) to T-cell responses, the response is inhibited by cyclosporin A.[426]

From observation of 50 pairs of larval grafts within one F1 progeny of the marine sponge *Crambe*, 75% could fuse, a proportion suggesting that the genetic control depends on one locus and sharing of one haplotype results in fusion; a 100% fusion between mother and offspring is consistent with this interpretation. Unfortunately, the issue is complicated as individuals from a given mother may not have the same father. Still, the few data available are consistent with a single or at least major histocompatibility locus (or region), the "rule" in other invertebrate phyla described in the following.

Cnidaria

The existence of highly polymorphic histocompatibility loci was demonstrated long ago in various corals where apoptosis induction was responsible for the death of partners in incompatible combinations,[154] whereas in sea anemone nematocyte discharge was induced between incompatible individuals.[427] The colonial cnidarian *Hydractinia* was the only species to provide a model to analyze genetic control of such reactions. In 1950, Hauenschild noticed that allorecognition seemed to be under the control of a single genetic region with multiple alleles.[428] Colonies of *Hydractinia* encrust the shells of hermit crabs, where they grow by elongation and branching of stolons. Embryos and larvae fuse indiscriminately. However, when two or more larvae are recruited to the same substratum, colonial forms may come into contact through their stolons. If the two colonies are histocompatible, stolon tips adhere and fuse, establishing gastrovascular connections and a permanent genetic chimera. If tips of incompatible colonies fail to adhere, they swell (hyperplastic stolons) with the migration of nematocysts, which discharge and damage the tissues. In addition, transitory fusions can also occur in a few cases. Similar to the genetics of the sponge alloreactions previously mentioned (and in other invertebrates and plants), colonies fuse if they share one or two haplotypes, reject if they share no haplotypes, and display transitory fusion if they share only one allele at one haplotype and no alleles at the other. Examination of the polymorphic locus governing this reaction (*alr*) revealed the involvement of two closely-linked polymorphic loci 1.7 cm apart (likely encoding receptor and ligand), *alr1* and *alr2*.[429] *Alr1* encodes a three IgSF domain surface molecule, not unlike other metazoan nectin-like molecules, with a cytoplasmic tail equipped with multiple signaling motifs (including a ITAM-like motif). In the first domain, the molecule shows a high level of variability at particular residues, suggesting positive selection. Alr1 is embedded in a genetic region consisting of multiple IgSF members. Alr2 encodes another highly polymorphic gene with three IgSF domain IgSF with the distal V-like domain being the most variable, and an ITIM-like motif in the cytoplasmic tail adjacent to various phosphorylation motifs. Like the corals studied by Theodor,[430] a high level of allele diversity was found at the alr2 level as nearly all sampled alleles encoded unique gene products.[431]

Urochordates

Compared to *Cnidaria*, tunicates shared a recent common ancestor with the vertebrates (see Fig. 4.1), and thus might be expected to have a histocompatibility system more related to MHC. Allorecognition has been studied in both colonial and solitary ascidians. In *Botryllus*, a colonial ascidian, extra attention was afforded this system because the locus controlling histocompatibility was linked to (or was the same locus as) the locus controlling fertilization by preventing self-fertilization. For fusion, at least one histocompatibility locus must be shared between the colonies and for fertilization the sperm must be mismatched from the egg.[432]

Metamorphosis in *Botryllus* is followed by budding that eventually gives rise to a large colony of asexually derived genetically identical individuals (zooids), united through a vascular network. At the periphery of the colony, the vasculature ends in ampullae, which are the sites of interaction when two colonies meet during their expansion. The interaction results in either fusion of the two ampullae to form a single chimeric colony sharing a common blood supply or a rejection reaction during which the interacting ampullae are destroyed, thus preventing vascular fusion. Hemocytes (morula cells) are involved in the reaction.[433] Fusion or rejection is governed by a single highly polymorphic (tens to hundreds of alleles) locus called the FuHC (for fusion/histocompatibility; 10 wild-type individuals collected from around the Monterey Bay area yielded 18 cFuHC alleles). As mentioned, when two colonies share one or both FuHC alleles, they will fuse; rejection occurs if no alleles are in common. However, like in *Hydractinia*, intermediary pathways have been reported in the past and have not been entirely elucidated.

The C-terminal region of the FuHC molecule consists of a nectin-like segment with three Ig domains, showing most similarity to chicken Igsf4 and related members conserved in all vertebrates as well as to one of the *Ciona* nectins.[434] Those members are found in a tetrad of paralogs in vertebrates but not linked to the tetrad of MHC paralogs (see the following). The N-terminal region contains an epidermal growth factor (EGF) domain and some other unrecognizable regions not conserved among ascidians. Any two FuHC alleles differ by an average of 4% at the nucleotide level. Unlike MHC class I and class II, polymorphic residues in FuHC alleles are not concentrated in particular regions, and alternative splicing can generate a fragment devoid of the IgSF moiety.

Some 200 kb away from FuHC is a second polymorphic (and polygenic) locus, *fester*, which is inherited in distinct haplotypes.[435] Diversified through extensive alternative splicing, with each individual expressing a unique repertoire of splice forms (each individual expressing up to three splice variants in addition to the regular full-length product), it potentially exists as both membrane-bound and secreted forms, all expressed in tissues intimately associated with histocompatibility. After *fester* knockdown, the histocompatibility reaction is blocked at the stage of initiation as

if the colonies ignored each other. By contrast, when Fester was blocked with specific mAbs, fusion reactions were unaffected, but rejection reactions were turned into fusions in an allele-dependent manner. These data combined with its genetic location suggest that Fester encodes the FuHC ligand. Fester contains a short consensus repeat (or sushi domain) often found in vertebrate complement receptors. Beside these two polymorphic products, another *Botryllus* cell surface–expressed nonpolymorphic molecule related to fester, uncle fester, is required for incompatibility reactions, while fester seems to be required for allele discrimination and inhibition of killing.[436] So, several independent pathways seem to control the final outcome of the interaction between individuals.[437] It should be mentioned that a recent study, based essentially on the incongruence between histocompatibiity profiles and FuHC polymorphism, questions the validity of these reports.[438]

Solitary Ascidians

In *Halocynthia roretzi*, a "polymorphism of color" has been observed and histocompatibility reset by a mixed hemocyte technique in vitro resulting in a melanization reaction likely to involve the PPO cascade.[439] Depending on the strains, the percentage of positive reaction varied from approximately 55% to 70%, indicative of polymorphism. Grafting experiments had already shown the existence of allorecognition in solitary ascidians, and investigation at the cellular level had demonstrated the occurrence of cytotoxic cells in such organisms.[440] In order to shed light on allorecognition in urochordates and on the molecules involved in preventing self-fertilization, gonadal complementary DNAs of three genetically unrelated *Ciona intestinalis* individuals were compared by suppression subtractive hybridization. This led to the discovery of the highly polymorphic variable complement receptor–like 1 gene coding for a transmembrane protein with several short consensus repeat domains (short consensus repeat/complement control protein [CCP]), a motif shared with the variable fester receptor of *Botryllus* described previously.[441] Genes encoding variable complement receptor–like are in the same linkage group as a set of IgSF domains with homology to nectin (CD155/poliovirus receptor [PVR], cortical thymocyte protein [CTX]/junctional-adhesion molecule [JAM] family members, etc.) and other adhesion molecules of which related members can be found also on one genetic region in vertebrate, the 19q 34 human chromosome segment with the extended LRC (see the following).[442]

The Meaning of Histocompatibility Reactions in the Invertebrates

The association between allorecognition in *Botryllus* and fertilization led to the proposal that histocompatibility systems were selected during evolution to avoid inbreeding. The hypothesis made sense in the case of sessile colonial invertebrates that might have difficulty dispersing their gametes and therefore are susceptible to inbreeding depression. Indeed, the partial matching mentioned previously is a general characteristic of fusion compatibility in colonial invertebrates, perhaps driven by "selection operating on an error-prone genetic system for self-recognition that is perhaps constrained by derivation from a gametic function selected to reduce inbreeding.[443] Furthermore, even in mammals there is a large literature suggesting a selection both at the mate-choice and pregnancy levels for preserving heterozygocity at the MHC.[443a] However, the possibility of a common genetic system, or linked systems, governing fusion and gametic compatibility awaits confirmation. Because animals that are neither sessile nor unable to disperse their gametes can possess alloimmune responses is inconsistent with the general hypothesis. Moreover, inbreeding avoidance can only explain the selection of histocompatibility alleles if the histocompatibility loci are genetically linked to a large fraction of its genome. This is inconsistent with the tight linkage of histocompatibility genes to a single major locus, especially in invertebrates. So inbreeding avoidance is unlikely to contribute significantly to the selection of histocompatibility alleles, although in jawed vertebrates, selection for heterozygocity at the MHC itself has obvious advantages.

Another hypothesis is that alloimmunity was selected because it avoided intraspecific parasitism and/or competition for attachment sites.[444] Indeed, after fusion of compatible colonies, bloodborne germline or totipotent stem cells are transferred between colonies, and can expand and differentiate in the newly arising, asexually derived individuals of the vascular partner.[445] This can result in a situation where only one genotype is represented in the gametic output of the fused individuals. The FuHC polymorphism in *Botryllus* could function to restrict to compatible individuals the vascular fusion and the germline parasitism. The high allelic polymorphism characteristic of all invertebrate recognition systems may have evolved in response to selection for fusion with self rather than kin. Fusion with self will allow the development of a colony that benefits from fusion while eliminating the possible cost of somatic cell parasitism, and thus would be the raison d'etre of the allorecognition mechanisms.[446]

However, most animals are not sessile and thus are neither prone to intraspecific competition nor to compete for limited substrate attachment sites. Tunicates besides *Botryllus* have evolved differently (eg, in *Diplosoma*, large numbers of chimaeras are encountered in nature). In addition, the intraspecific competition hypothesis demands that individuals maintain expression of histocompatibility alleles, even when the expression of these alleles enables their own destruction during intraspecific competition. These considerations suggest that intraspecific competition might affect histocompatibility allele frequencies in some organisms under certain conditions.

Both previous hypotheses are essentially based on observation made in colonial tunicates, but colonial living has evolved several times independently. Urochordates are most related to the vertebrates, but the data argue against any of the genes involved in their histocompatibility reactions being ancestral to MHC class I and class II. It is clear in all of these studies that a strong pressure for polymorphism is the rule, but the evidence from all of these fascinating

reactions suggests that such "pressures" select for similar systems via convergence, at least at the level of the receptor. The selecting molecular environment can perhaps show more conservation (see the folllowing). On the other hand, as described previously, genetic regions with homology to the vertebrate MHC have been detected in the invertebrates, yet are (most likely) unrelated to the reactions detailed here (see conclusion).

As described, in all of these allorecognition systems, convergent mechanisms have been encountered to reach an analogous end, and thus it is unlikely that a unique "cause" arose for selecting them in diverse organisms. However, in an attempt to find an ultimate and general explanation to the selection during evolution of highly polymorphic allorecognition systems, it has been suggested that pathogen and retroviruses are the force behind the selection of allopolymorphism.[447] This does not imply intimate phylogenetic relationships between the systems observed but rather emphasizes analogous solutions when facing similar pressures. We discuss "connections" between vertebrate immunity and these histocompatibility reactions in the conclusion.

CYTOKINES AND CHEMOKINES

Many cytokines/chemokines and their receptors, like most molecules of the immune system, evolve rapidly. However, consistent with the "Big Bang" theory, it is an emerging picture that the majority of cytokines and chemokines found in mouse and human are also found in the genome and EST projects of nonmammalian jawed vertebrates, best studied in chickens and certain bony fish, but now extending to the cartilaginous fish as well. This suggests that whatever the initiating pressures in the evolution of the Ig/TCR adaptive immune system, the network of cytokines and chemokines emerged (practically) full blown early in evolution. A picture starts to emerge, but the gestalt is far from clear; this is, in part, because the situation in mouse human is clouded by the plasticity of T helper cell lineages, as well as an entirely new subset of immune cells, the innate lymphoid cells (ILCs), that produce cytokines previously thought to be within the domain of adaptive lymphocytes.

The Proinflammatory Cytokines, Interleukin-1 Family Members, Interleukin-6, Interleukin-8, and Tumor Necrosis Factor α

IL-1 and related family members IL-18 and IL-33, IL-6, TNF-α, and IL-8 (CXCL8) are the prototypic cytokines associated with inflammatory responses, which are defined by induction of vasodilatation and vascular permeability, and upregulation of innate immune system–specific molecules that have direct functions or that costimulate/attract T and B cells. Classically, many of these activities can be assayed in supernatants from PAMP (eg, LPS)-stimulated phagocytes by determining whether thymocytes are induced to proliferate when one also adds suboptimal concentrations of T-cell mitogens. It was reasonable to hypothesize that such cytokines, which act both at a distance as well as in a cognate fashion, might be found in the invertebrates. Indeed, IL*-1–like *activities* have been described for echinoderm coelomocytes (either IL-1–like production by such cells, or the ability of the cells to respond to mammalian IL-1), but unfortunately no molecular data revealing the structures of the active invertebrate cytokine/cytokine receptor have been reported. In fact, no ortholog has been detected in the genome projects from protostomic invertebrates (see Fig. 4.1), and only IL-17 and TNF homologues have been detected in nonvertebrate deuterostomes and protostomes,[48,448,449] and IL-8 in agnathans; thus, we may consider these as primordial cytokines related to the vertebrate versions. A molecule from earthworms capable of activating the prophenoloxidase defense pathway cross-reacted with a mAb directed to mammalian TNF-α.[450] However, this molecule had no homology to TNF-α upon sequencing.

IL-1 activity as measured by costimulation assays or as a consequence of PAMP stimulation has been detected in all nonmammalian vertebrates. IL-1β upregulation has been detected after treatment of macrophages with LPS, consistent with its inflammatory function in mammals. In addition, injection of gram-negative bacteria into trout induced IL-1β expression in many tissues.[451] Identity with the mammalian IL-1β gene in all other species ranges from 28% to 40% (identity between mammalian IL-1α and IL-1β is about 25%). In nonmammalian species, IL-1β lacks the so-called ICE cleavage site, important for function in mouse/human.[452] IL-18 is an IL-1–related cytokine, and in contrast to IL-1 seems more focused in its function of potentiating TH1 responses. IL-18 has been detected in birds and fish, but the tissue distribution in fish seems to be expanded as compared to mammals. Additionally, IL-18 in nonmammalian vertebrates contains the ICE cleavage site, unlike its cousin IL-1β. Other IL-1–related cytokines, including IL-33 and the IL-1F series, have also been found in several jawed vertebrates, but with little study of functional activity. This will be of great interest to uncover, considering the IL-1 family members' roles in regulating Th1, Th2, and Th17 (and others?) differentiation.[453]

Both chicken IL-1β and the IL-1R were identified and have been expressed as recombinant proteins.[454] The IL-1R homology to mammalian orthologs is quite high (61% identity), but the highest similarity is found in the cytoplasmic domains. In addition, there are four blocks of high similarity to the cytoplasmic tail of toll/TLR proteins, and IL-1R and TLR use similar signal transduction cascades (see previous discussion).

As mentioned, IL-8 (actually, the CXC chemokine CXCL8) has been identified in the jawless lamprey and seems to be expressed specifically in B cells, whereas the IL-8 receptor is found in T cells; this has been one of the mechanisms proposed to permit the cells to interact in a cognate fashion.[13,291] IL-8 has also been found in various gnathostomes such as trout, flounder, and perhaps chicken; a chicken CXC chemokine called K60 clusters with IL-8 in phylogenetic trees and is upregulated in macrophages stimulated with LPS, IL-1β, and IFN. Interestingly, Marek disease virus expresses an IL-8 homologue (v-IL-8), which may be

involved in inducing immune deviation.[455] A molecule related to the IL-R was detected in the sea urchin genome, but the ligand has not been found[48]; becuase there are so many members of the IL-1 family, the search should continue.

The TNF family in mammals includes the canonical TNF-α, lymphotoxin (LT)α, and LTβ, all encoded at the distal end of the MHC class III region,[403] as well as a large number of other members with diverse immune functions. TNF-α is the best studied of these cytokines, and it is one of the key regulators of innate and adaptive immunity. The other two cytokines have a more limited tissue distribution and function, and are noted in their roles in lymphoid tissue development, especially in the formation of splenic white pulp and segregation of T and B cells in lymph nodes. In contrast to IL-1, TNF homologues have been detected in cnidarians, protostomes (see previous discussion), sea urchin,[48] *Ciona*,[448] and *Amphioxus*,[456] consistent with the idea that such a multifunctional cytokine would predate the jawed vertebrate adaptive immune system. Homologues have also been cloned from several teleost species, and TNF-α expression in leukocytes is upregulated within 4 hours after treatment with LPS, IL-1β, and PMA. While there is good phylogenetic support for orthology of fish TNF-α to that of mammals, the other TNF genes seem to be teleost-specific duplicates rather than the LT genes.[457] Conversely, in *Xenopus*, the three TNF family members described previously in mammals are closely linked in the class III region of the MHC.[177] This is a bit of a surprising result as all of these family members seem to be lacking in chickens, consistent (according to the authors) with a lack of lymph nodes in these animals; however, the unusual nature of the MHC in birds (eg, the immunoproteasome genes are missing) rather is consistent with a loss of TNF genes in these animals, which is indeed surprising, especially for TNF-α.[458]

An excellent review detailing the evolution of all TNF and TNF receptor family members demonstrated that all of the genes are found in paralogs on four chromosomes, consistent with the 2R hypothesis. This work demonstrated that the earliest member was linked to the proto MHC, and thus it is not surprising that TNF is an ancient gene family.[459]

IL-6 and its related members IL-11 and IL-31 are also found in most gnathostomes examined, with few functional data.

Interleukin-2 and the γC Family of Cytokines

The family of cytokines that signals through the γC receptor includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Similar to what was described for most molecules involved in adaptive immunity, all of these cytokines (except perhaps IL-9) are present in all gnathostomes studied.[460] Costimulation assays of thymocytes, as described previously for IL-1, and perpetuation of T-cell lines with stimulated T-cell supernatants are performed to detect IL-2 or "T-cell growth factor" activities. Unlike IL-1, IL-2–like factors generally stimulate cells only from the same species, and it is a "cognate" cytokine, meant for release only between closely opposed cells, or as an autocrine factor. From teleost fish to mammals, stimulated T-cell supernatants costimulate thymocyte proliferation or can maintain the growth of T-cell blasts, and the ortholog has been detected in bony fish and birds. The chicken IL-2 protein is only 24% identical to human IL-2 and only 70% identical to a near cousin, the turkey.[454] IL-15, a relative of IL-2, has also been cloned in the chicken. A candidate IL-2R in chicken was identified by a mAb recognizing a 50-kDa molecule only on stimulated T-cells (thus an IL-2Rα homologue). This mAb blocks costimulation by IL-2–like molecules in chicken T-cell supernatants and also reduces the capacity of T-cell blasts to absorb IL-2–like activity from supernatants. IL-2 has been studied in the bony fish *Fugu*. In both chicken and the deduced *Fugu* IL-2 protein, there is a second set of cysteine residues, which are found in IL-15 and thus is a primordial feature.[461]

As mentioned, γC is the signaling subunit of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors; absence of this chain in mammals leads to major defects in lymphocyte development ("boy in the bubble"). In fish, a γC homologue was cloned in rainbow trout with unusually high identity (44% to 46%) to mouse/human genes.[462] IL-1β, but not LPS, upregulated the trout gene in macrophage cultures and a fibroblast cell line. Since then, many other orthologs have been uncovered in fish, often duplicated in various species.[460]

IL-21, which is encoded adjacent to IL-2 in mammals, has been found in ectothermic vertebrates.[460] Because one of its major functions is the differentiation of T-follicular cells and the GC response, it will be of interest to study it role in those animals.

IL-7 is involved in lymphocyte differentiation as well as homeostasis of mature lymphocytes. Recently, mutations in Il-7R and downstream signaling molecules in the zebrafish have shown a block in T-cell development.[463] These mutants will permit future study of lymphocyte production in this crucial developmental model.

TH2 Cytokines: Interleukin-4, Interleukin-5, Interleukin-9, and Interleukin-13

Reponses to extracellular pathogens, especially filarial worms, are largely regulated by TH2 cytokines in mammals. IL-4 is the most pleiotropic cytokine in this regard, stimulating the production of neutralizing antibodies, stimulation of eosinophils and mast cells, and an antagonism of TH1 responses. IL-4, IL-5, and IL-13 (and granulocyte macrophage colony-stimulating factor) are encoded in the so-called TH2 cytokine complex in mammals, and all of the genes are coordinately upregulated after stimulation of a nearby locus-control region. This same "TH2 complex" is found in chickens but with a psuedogene for IL-5, but in *Xenopus* only the IL-4 gene has been identified to date. Fish have genes in which the ancestor of IL-4 and IL-13 seems to be in a preduplicated state, syntenic with genes in the TH2 complex.[464] Again, duplicates of IL-4/-13 and their receptor have been found in various fish.[460,465]

At this stage of study, it may be that lower vertebrates do not have "full-blown" TH2 responses, consistent with the lack of canonical allergic responses in ectothermic vertebrates. On the other hand, we must be careful because cytokine genes evolve rapidly and relying on synteny is not always

dependable, especially in the teleost fish. It will be of interest to see whether IL-4 in nonmammalian vertebrates will promote switching to isotypes that are incapable of promoting inflammatory responses. In addition, further studies of this family and its receptors in teleost and cartilaginous fish will reveal whether this facet of the adaptive immune response is indeed a relative newcomer.

Interferons

IFNs, classically known for their antiviral properties, are divided into three groups: type I, type II, and type III. Perhaps surprisingly, this entire group has only been uncovered in jawed vertebrates to date. However, as described previously, viral immunity in insects is partially regulated via a JAK/STAT response.[152] Types I (α and β) and III (λ) IFN are widely expressed in cells of many types and induces inhibition of viral replication in neighboring cells, as well as molecules of the innate immune system such as inducible nitric oxide synthase (iNOS) and IFN regulatory factor-1.[466] In contrast, type II IFN (IFNγ or immune IFN) is synthesized by activated T cells, activates macrophages, and upregulates class I, class II, immune proteasome subunits, and TAP, and a large number of other genes.

Antiviral activity is detected in supernatants from virally infected fish fibroblasts, epithelial cell lines, and leukocytes. All of the biochemical properties of mammalian type I IFN (eg, acid-stable, temperature-resistant) are present in these fish supernatants, and the putative IFN reduces viral cytopathic effects in homologous cell lines infected with virus.[466] In vivo, passive transfer of serum from virally infected fish protects naïve fish from acute viral pathogenesis. There appears to be two lineages of type I IFNs in fish that are specific to this group. In chickens there are up to 10 closely related, intronless type I IFN genes.[455] Sequence identity to human type I IFN ranges from 25% to 80%, with the apparent functional gene having highest similarity. Interestingly, bats have greatly expanded their type I and III IFN genes and receptors (as well as many other genes associated with viral immunity).[467] These animals are highly infected with (and susceptible to) virus, and some IFN forms are constitutively expressed at low levels.

While type I/III IFNs can be highly duplicated in some vertebrates, this is not true of IFN receptors, and in some cases many cytokines will used two to four receptors within a species. How can such a system work and result in different readouts? It appears that even single amino acid changes in the cytokine can induce differential conformational changes in the receptor that can modify downstream signaling.[468] This paradigm will be exciting to follow in a phylogenetic context.

Type II or IFNγ has been cloned in most jawed vertebrates. The chicken gene is 35% identical to human type II IFN and only 15% identical to chicken type I IFN.[454,469] Recombinant chicken IFN stimulates nitric oxide production and class II expression by macrophages. The gene has been cloned in many fish, and has been studied mostly in the trout where it has been shown to upregulate CXCL10 and activate protein kinase C. Thus, at least in chickens and teleost fish, type II IFN seems to have the same function as in mammals, suggesting that the TH1-type responses emerged early in vertebrate evolution.[466]

Heterodimeric Cytokines

The IL-12 family are composed of heterodimers that share chains (p19, p40, p35, and EB13) and include IL-12, IL-23, IL-27, and IL-35. IL-12 is generally considered to be a proinflammatory cytokine produced by APCs that promotes a TH1 response after exposure to intracellular pathogens. Consistent with the ancient derivation of TH1 responses, the two subunits of IL-12 p70 (p35 and p40) have been found in chickens and several teleost species.[454,470] The p40 subunit of IL-12 can also associate with p19 to form the cytokine IL23, which is involved in the perpetuation of TH17 cells. IL-27 generally inhibits all TH subsets but expands regulatory T cells and is composed of p28 and EB13; IL-35 is a relatively new regulatory T–produced cytokine composed of p35 and EB13. All of these chains have been detected in gnathostomes and several studies have been done to examine their expression at the RNA level; however, to date no reagents have been prepared to the heterodimers for functional analysis.

Transforming Growth Factor-β and Interleukin-10

TGF forms a large family with pleiotropic effects in many developmental systems. For the immune system, TGF-β isoforms are best known for their capacity to suppress adaptive immune responses (even across species barriers), although they can also stimulate lymphocytes under certain conditions, especially in mucosal surfaces. TGF-β inhibits macrophage activation in trout and growth of T-cell lines in *Xenopus* species. Recombinant *Xenopus* TGF-β, like the mammalian form, also can inhibit IL-2–like dependent growth of splenic lymphoblasts.[471] Four TGF-β isoforms were isolated from chickens, as opposed to three major forms in mammals.[455] The three major forms of the cytokine have been isolated in several teleost species.[470]

IL-10 is often considered along with TGF-β because it is mostly an immunosuppressive cytokine with multiple effects; both cytokines are expressed in subsets of "regulatory T" cells in mammals. This cytokine was originally discovered by its ability to suppress TH1 responses, but now is known to have a much expanded role in immunity. IL-10 has been found in chickens and a large number of teleosts.[455,470] As usual, functional experiments have lagged behind the molecular work, but recombinant chicken IL-10 can block IFNγ production by splenocytes. IL-10 is a class II cytokine, related to IFNγ, IL-19, IL-20, IL-24, and IL-26. IL-22, a cytokine released by TH17 cells and ILCs in mammals, is upregulated in fish vaccinated with pathogens and is correlated with immune protection.[472]

Note that there have been very few "regulatory T" experiments reported in the comparative literature, but many older experiments that suggest that such cells exist. Over 25 years ago, experiments in *Xenopus* showed that graft rejection could be delayed when lymphocytes from metamorphosing animals were adoptively transferred into adult frogs.[473] This

result suggested that a "wave" of suppressor cells emerged near the time of metamorphosis that evolved to protect animals from autoimmunity when adult-specific molecules were expressed. It is time to reexamine such experiments with modern tools. In a recent experiment in zebrafish, immunization of animals with central nervous system (CNS) antigens induced an autoimmune reaction. Overproduction of foxp3 inhibited the production of IL-17 and IFNγ in these animals, ameliorating the disease.[474]

Interleukin-17

TH17 cells in mammals are important for inflammatory responses to extracellular bacteria via indirect stimulation of neutrophils. Despite its late appearance in the immunologic literature, IL-17 appears to be one of the most ancient cytokines, with homologues in protostomes and lower deuterostomes, and extensively amplified in sea urchins. Even the different isoforms seem to be conserved among the jawed vertebrates, which should initiate many new avenues of study.[470] The family is composed of IL-17A/F, with the A and F forms being the best studied to date. IL-17A/F genes have been found in all mammals studied, as well as IL-17C/E. The most ancient IL-17 form is IL-17D, present in both oysters and agnathans.[291,449] This form is expressed in lamprey T cells but not B cells, and has been proposed as a helper factor for cognate interactions.[13] Finally, like many other immune genes, as many as 20 IL-17 genes have been found in sea urchins, again suggesting expanded innate immunity in this species.[475]

Cytokine Summary

The isolation of nonmammalian cytokines and cytokine receptor genes has lagged behind molecular characterization of antigen receptors and MHC. However, with the advent of the genome and EST projects, we are rapidly acquiring a comparative view of this field, at least at the genetic and molecular level. Teleost fish and chickens have paved the way in this field, but cartilaginous fish and agnathan databases will soon be complete and provide the big picture. Already, it seems that cytokines like TNF and IL-17 seem to be most primordial, with homologues in *Ciona*, *Amphioxus*, and sea urchins, as well as deeper homologues in certain protostomes. Conversely, *none* of the other so-called adaptive cytokines (or even type I/III IFNs) seem to be present in the lower deuterostomes, consistent with the Big Bang theory of adaptive immunity.[1,2] If it is true that the jawless fish lack these genes as well, despite their convergent adaptive immune system, one can point to the evolution of lymphoid organs and the segregation of lymphocyte subsets into discrete areas to help to explain the explosion and recruitment of these genes. We discuss this concept in more detail in the conclusion section, especially regarding the relationship of adaptive lymphocytes with ILCs.

Despite our "big picture" knowledge of the emergence of cytokines, obviously functional experiments have lagged behind. Furthermore, is it true that TH2 responses are evolutionary latecomers, or are our early attempts at finding the genes in ectotherms because of some "missing pieces" in the databases (or our ability to find some of the genes)? Some genes may have been lost, as described previously for the chicken—again, further studies of amphibians and cartilaginous fish should help in our understanding. In mammals, the LT TNF family members are important in the development of lymph nodes and splenic white pulp, and yet the three genes are present in lymph node–less *Xenopus*—what are their functions in ectotherms? If we can uncover these other roles in nonmammalian vertebrates, it could be quite informative to reinvestigate such functions in mammals. Finally, chickens and fish have evolved their own paralogs of some of the well-known cytokine genes; their study should reveal selection pressures on particular species that could also be quite informative in our understanding of the gestalt of the cytokine network.

General Evolution of Chemokines and Their Receptors

Chemokines and chemokine receptors are essential for many aspects of the immune system, including inflammation, the differentiation of lymphoid tissues, trafficking of hematopoietic cells during ontogeny and immune responses, and even stimulation of cells under various conditions.[476] With the advent of the genome and EST projects, a seminal paper showed that, consistent with the Big Bang theory of adaptive immune system evolution, all of the major classes of chemokines and their receptors were present in the bony fish lineage, and probably will be found in cartilaginous fish as well when the genomes are completed.[477] Recently, the analysis was updated to include all of the vertebrates in the databases.[476] Similar to most of the immune-related genes discussed throughout the chapter, bony fish have more chemokines/receptors than any other vertebrate, including amphibians and mammals.

Interestingly, no CC (so-called homeostatic) or CXC (so-called inflammatory) chemokines/receptors were detected in lower deuterostomes like *Ciona* and sea urchin. The chemokine receptors are member so the G protein–coupled receptor family, and of course such molecules are found in all animals. However, no members of the specialized family (G protein–coupled receptor γ) to which chemokine receptors belong were found in lower deuterostomes. The chemokine receptor genes are found on four chromosomes in mammals, four of which contain the HOX genes, one of the gene families that provided evidence for the 2R hypothesis early in the reawakening of this theory. The distribution of chemokine receptor genes on these chromosomes correlates very well with 2R, and helps to account for the large-scale gene expansion of this family in the jawed vertebrates. Thus far, very few chemokines/receptors (eg, IL-8 [CXCL8] and CXCR4) have been detected in agnathans, but as mentioned previously, agnathan T cells express IL-8R and B cells IL-8, believed to be a factor in promoting the cognate interactions between the cells.[291] CXCL12, a chemokine vital for thymic differentiation, is also present in lamprey, consistent with new work on the agnathan thymus discussed previously.[375] In summary, it appears that a few chemokines/receptors arose at the dawn of vertebrate adaptive immunity, and they

were greatly expanded by both en bloc and *cis* duplications and became "full-blown" shortly after the 2R Big Bang.[476] Currently, this is our most complete story of the evolution of a family of immune genes in deuterostomes.

ORIGINS OF ADAPTIVE IMMUNITY

The immune system of vertebrates is unique because the antigen-specific receptor expressed by lymphocytes, which initiates cascades leading to activation of the adaptive immune system, is not the product of a complete germline-inherited gene. Rather, receptors are generated somatically during lymphocyte ontogeny from gene segments scattered at a particular locus. As described previously, the receptors in gnathostomes are IgSF members composed of V and C domains, with the C domains being of the rare "C1" type, which is shared by MHC class II and class I molecules. There are many specific questions: 1) Did MHC class I or class II come first?; 2) What is the origin of the MHC PBR?; 3) Was the MHC involved with innate immunity before the emergence of adaptive immunity?; 4) Did somatic rearrangement or somatic mutation come first to diversify antigen receptors?; 5) Which of the extant antigen receptors in gnathostomes, α/β TCR, γ/δ TCR, or IgH/L (if any) resembles the primordial receptor?; and 6) How did two different antigen receptor gene families emerge in evolution, *after* the appearance of separate lymphocyte populations? The answers to these questions are speculative, but deductions can be made based upon the wealth of molecular and emerging functional data. We base many of our arguments on the large-scale duplications that were revealed for MHC by Kasahara[17] in 1996. The remarkable syntenies of paucicopy genes on the paralogous regions, and the recent finding that an animal that predated the duplications (*Amphioxus* and *Ciona*) has only single copy genes in the same syntenic group orthologous to the four mammalian copies, make it clear the en bloc duplications were involved.[405,421] Further analysis of this region in model lower deuterostomes, as described in the histocompatibility section, will continue to be vital in our understanding of early immunity. Genetic analyses in protostome lineages have not been very informative, but we must reevaluate once we piece together data from more deuterostome species.

Major Histocompatibility Complex Origins

Class I and class II molecules have been found only on the jawed vertebrates—so far, painstaking screenings of the lower deuterostome or lamprey/hagfish databases have yielded no indication of these proteins. Based upon phylogenetic analyses and thermodynamic arguments, most investigators believe that class II preceded class I in evolution.[478] However, as stated previously, class I is much more plastic than class II as there are many different types of class I molecules, some that do not even bind to peptides. Because class I genes may be on two or three MHC paralogs, and they can have functions outside the immune system; this is evidence that the primordial "PBR" may not have even bound to anything. If this is true, and because class I and class II do bind peptides, it would suggest class I arose first. Again, genome scans of jawless fish and lower deuterostomes should be informative on this point, but to date no luck! As described previously, we should be diligent because with the discovery of T cells and a thymus-like structure in lamprey, one expects some sort of "MHC" in the agnathans[291,375]; to date, the only homologue we have uncovered to date is TAP-L, which may hold the key to antigen presentation in this group.[401]

From the paralog data, genes encoding the complement components C3 and Bf, TNF superfamily members, the signaling molecule Vav, B7 family members, and proteasome subunits among other genes (such as tripartite motif-containing) should have been present in the proto-MHC, before emergence of the adaptive immune system (see Fig. 4.13). Some of these genes were found in the *Amphioxus* and *Ciona* "MHC" linkage groups, and C3 and Bf genes are linked in the sea urchin. A fifth paralogous region on human chr 12p13 contains the α2-macroglobulin gene (recall the C3/4/5 homologue), a tapasin homologue, the C3a-receptor, and this "complex" is linked to the NKC. Taken together, the data suggest that the proto-MHC included vital nonhomologous genes of the innate immune system, which perhaps were linked to allow coordinate regulation of expression. After the en bloc duplications, Pontarotti et al. has suggested that "functional restraints upon the complex were relaxed" and hence the duplicated members could evolve new functions, including features indispensable to the adaptive system.[405] If indeed innate immunity genes were already linked to allow upregulation at times of infection, it is no surprise that the adaptive immune system piggybacked on such a gene complex. A final point: why did the duplicate genes survive rather well over hundreds of millions of years; *cis* duplicates have been shown to degenerate rapidly over evolutionary time. Evidence suggests that duplicates arising from polyploidy (and by inference large en bloc duplications) survive better than *cis* duplicates, most likely because they cannot be inactivated by unequal crossovers; the ability of the genes to survive over very long periods, perhaps combined with strong selection pressures, would allow for subspecialization.[479]

Origins of Rearranging Receptors

The Rearranging Machinery

Most models propose that the generation of somatically rearranging receptors occurred abruptly in evolution via the generation of the RAG machinery made of two lymphocyte-specific proteins, RAG1 and RAG2. *RAG* genes have so far been isolated in all classes of jawed vertebrates and have been quite conserved. In every case examined, *RAG1* and *RAG2* genes are closely linked and in opposite transcriptional orientation. Some regions of RAG1 and RAG2 are similar to bacterial recombinases or to molecules involved in DNA repair (eg, RAD16) or the regulation of gene expression (such as rpt-1r). Similarities to prokaryotic proteins and the gene structure suggest that vertebrates acquired the RAG machinery by horizontal transfer and transposition from bacteria.[19,480] Indeed, *RAG* genetic organization has

some transposon characteristics: the RSS are reminiscent of sequences involved in targeting excision of transposons. A class of transposons detected in several protostome and deuterostome invertebrate species that shows similarity to the catalytic domain of RAG1.[481] It is believed that RSS were derived from the terminal inverted repeats of this transposon, called *transib*. In invertebrate genomes there are many of such "RAG1 core regions," but only one has an open reading frame throughout the core and shows similarity to vertebrate RAG1 in other regions as well. Furthermore, a *RAG2* homologue is adjacent to the urchin *RAG1* gene, in a similar orientation as is found in gnathostomes.[352] This finding was a big surprise and suggests that both *RAG* genes were in place approximaltey 100 million years before the origin of the Ig/TCR system. Their tissue distribution and expression during ontogeny are not known; nor have any candidate genes been recognized to date with RSS that might be recognized by the echinoderm homologues. It must be admitted that it is unclear how this new result fits into the puzzle of the origins of adaptive immunity. Because transib is so often found in the animal kingdom, it is certainly part of the transposon. By contrast, RAG2 is now believed to have been present in the genome, and recent data have shown it to interact with active chromatin.[482,483] Thus, the new idea is that RAG2 recruits RAG1 to open chromatin, and rag1 is the active enzyme in the major rearrangement events, which is wholly consistent with the transposon hypothesis with RAG1 (ie, transib) being the genome "invader."

Another source of somatic antigen receptor diversity shared by all gnathostomes characterized to date is a unique DNA polymerase, TdT, which diversifies CDR3 during Ig and TCR gene rearrangement through the addition of nucleotides in a template-independent fashion.[484] Furthermore, as detailed previously, its expression serves as an unambiguous developmental marker for the sites of lymphopoiesis. TdT has been highly conserved in both sequence (> 70% amino acid similarity, > 50 amino acid identity) and overall structure during vertebrate evolution. An amino acid alignment of all known TdT sequences reveals that some, but not all, structural motifs believed to be critical for TdT activity are particularly well conserved in all vertebrates studied. TdT protein alignments, and the crystal structure for rat β-polymerase, support the hypothesis that both evolved from a common ancestral DNA repair gene. In addition, four protein kinase C phosphorylation sites are conserved, and hence may be involved in TdT regulation. Homologues related to the ancestor of polymerase β and TdT have been found in sea urchin and other lower deuterostomes. Thus, unlike RAG, TdT has evolved by gradual evolution from a polymerase family and was recruited for immune system function.

Rearrangement or Somatic Hypermutation First?

Because all antigen receptor genes use somatic rearrangement of V genes to generate diversity in CDR3 regions as well as to promote combinatorial diversity, there is no doubt that this mechanism is at the heart of adaptive immunity. Indeed, as described, most investigators believe that the introduction of the tranposable element into a V gene was *the* driving force in the abrupt appearance of vertebrate adaptive immunity; the finding of the RAG genes in sea urchins challenges this notion at some level. However, it cannot be overemphasized that SHM is also at the origins of the immune system; furthermore, all evidence to date suggests a gradual evolution of the SHM machinery (the AID/APOBEC family and associated polymerases/mismatch repair proteins) rather than the "hopeful monster" generated by the famous RAG transposon. Thus, diversity generated via SHM or gene conversion may have existed in an adaptive immune system prior to rearrangement, and V gene rearrangement was superimposed onto this already existing system.[296,300] Indeed, the presence of APOBEC family members in the jawless fish (CDA1 and CDA2, as described previously) suggest that diversity generated via mutation/conversion preceded rearrangement. The RAG-induced rearrangement break and subsequent repair provided something new and novel in gnathostomes, not only diversity in sequence but heterogeneity in size[300]; this was a remarkable innovation and likely indeed heralded the sophistication of jawed vertebrate adaptive immunity.

Which Antigen Receptor First? Phylogenetic analyses have suggested that among IgSF receptors γ/δ TCR-like ancestor may have predated α/β TCR and Ig H/L.[485] This would suggest that direct antigen recognition, perhaps by a cell surface receptor, arose first in evolution followed by a secreted molecule and an MHC-restricted one. Hood and colleagues argue that phylogenetic analyses over such large evolutionary distances obscure true relationships among the antigen receptor genes (eg, the relationships of the molecules in the phylogenetic trees has to impose multiple loss/gain of D segments in the different antigen receptor families) and suggest a model based upon genomic organization, not so different from the Kasahara model.[263] They propose an alternative phylogeny in which an ancestral chromosomal region with linked genes encoding both chains of an ancestral antigen receptor heterodimer, one having D segments the other not (eg, IgH/IgL). The α and δ TCR loci are still closely linked in all vertebrates analyzed (human chromosome 14), and a pericentric inversion is suggested to have separated the TCR β and γ loci (linked on human chromosome 7). This model predicts that D segments only emerged once, and also explains the existence of inverted V elements in both the TCR β and δ loci. This model does not predict which antigen receptor is oldest, but does provide a "simple view" of receptor evolution, consistent with the Kasahara/Ohno model. Recent data have added to this scenario. First, as mentioned previously, VH elements have been found at the TCR-δ locus in many vertebrates, including sharks, amphibians, birds, and marsupial mammals.[269] In part, this is due to the close proximity of the IgH/L (lambda) and TCR alpha/delta loci in many living vertebrates (especially *Xenopus*[268]), consistent with the Hood hypothesis of ancient en bloc duplications of antigen receptor loci.[268] Additionally, the idea that most TCR-δ receptors do not recognize MHC-restricted antigen suggests that they can continue to "borrow" elements from Ig loci over evolutionary time.

Thus, an alternative scenario is that MHC-restricted antigen recognition arose first, perhaps derived from a NKR with a "VJ" IgSF domain that recognized SOS proteins, like the ULBPs or MIC today. The rearrangement break induced by

RAG occurs in CDR3, which is in the center of the antigen receptor–combining site, in perfect position to interact with peptide bound to MHC, which could have been another innovation to detect foreign antigens within cells.[486] Thus, such cells would be most like extant αβ T cells with an MHC-restricted antigen receptor encoded by linked genes. Once rearrangement was introduced, the combining site may have been "relaxed" so that it was no longer forced to be MHC-restricted (see the section on TCR above). The genomewide duplication then would provide two types of TCR, one MHC-restricted and the other not, like γδ TCR today. One locus, according to the Hood model, then underwent an en bloc cis duplication to give rise to the IgH/L loci. Ig, then, developed a new differentiation pathway with an alternative splicing mechanism for the transmembrane and secreted forms of BCRs.

For the origin of the rearranging receptors, IgSF lineages have to be traced back through phylogeny as such receptors generated by somatic rearrangement do not exist outside the jawed vertebrates. In a quest for molecules related to elusive ancestors, without focusing on genes expressed in the immune systems of various phyla (ie, structure is more important than function in this case), the most homologous sequences and gene architectures in the various metazoan phyla must be scrutinized.

V(J) and C1 Domains

C1 domains are found in the antigen receptors, MHC class I and class II, and very few other molecules (see Fig. 4.10). This IgSF domain is so far most prevalent in gnathostomes, as if C1 domains arose concurrently with the adaptive immune system and coevolved with it. What was the value of the C1 domain, and why is it found almost exclusively in adaptive immune system–related molecules? All of these molecules interact with coreceptors such as CD3 (TCR), Igα and β chains (Ig on B cells), CD4, and CD8 (with MHC on opposing cells), and it is conceivable that in sections of IgSF domain in which C1 differs from the C2 there is a specific region favoring interaction with other molecules.

The G strand of Ig/TCR V domains is encoded by the J gene segment, separated from the V region-encoded A-F strands, and rearrangement is necessary in order to assemble a complete V gene. The primary structure of each Ig/TCR chain bears hallmarks of the dimeric nature of the receptor in which they participate. A diglycine bulge (Gly-X-Gly), present in all V domains, is thought either to be a beneficial adaptation, or to promote dimer formation by inducing a twist in the G strand that results in V domain pairing that appropriately orients the CDR. Monitoring this feature, therefore, might reveal genes that had the ability to form dimers similar to that of modern antigen-specific receptors. In V genes that do not somatically rearrange, the G stand is an integral part of the V exon. In other remotely related IgSF genes, introns have invaded the V domain exon creating a variety of V gene families. Many examples of such events can be found in the history of the Ig superfamily, for example in the genes encoding CD8 and CTX.[33,487]

As described, no Ig/TCR genes have been isolated from hagfish or lampreys, although there are some tantalizing molecules potentially related to their ancestors (eg, APAR, see previous discussion). Were Ig and TCR "invented" in a class of vertebrates now extinct (eg, the placoderms, which are more primitive than cartilaginous fish but more advanced than agnathans)? The discovery of the three tapasin paralogs all with C1 domains suggests an origin prior to the full establishment of the vertebrate genome.[33]

V domains, either alone (eg, IgNAR) or in association with another V domain (eg, Ig H/L), recognize the antigenic epitope and are therefore the most important elements for recognition. For this reason, they will be the first to be traced back in metazoan evolution by asking whether V domains exist in invertebrates. Domains with the typical V fold, whether belonging to the true V-set or the I-set, have been found from sponges to insects (although not necessarily involved in immune reactions; the first ones were discovered by nonimmunologists among molecules involved in nervous system differentiation in invertebrates [eg, amalgam, lachesin, and fascicilin]). Invertebrates also use IgSF members in immunity, but so far they are not V domains, but more I- or C2-set (eg, mollusk defense molecule, hemolin, DSCAM). However, the mollusk FREPs have a V domain at their distal end, associated with a fibrinogen-like domain. As described, they are involved in antiparasitic reactions and form a multigenic family with polymorphism.[135] Similarly, as mentioned previously, prochordate VCBP has a VJ domain, but it mediates innate immunity.[143]

Besides searching for VC1-encoding genes in nonvertebrates, surveying the human genome for such genes has been fruitful. Indeed, nonrearranging V-containing molecules, either VJ alone, VC1, or C1 alone, have been found in the human genome. Interestingly, many of them are present in the MHC class III region (human chromosome 6p21) or its paralogs (see Fig. 4.12 and 4.13). Two MHC-linked gene segments stand out: a single VJ, NKP30, and a gene containing a VJC1 core, tapasin (TAP-binding protein), involved in antigen processing. NKP30, made of a single Ig domain of the VJ type, is an NK cell–activating receptor, and it may offer a link to cell types in invertebrates. It could be a relative of an ancient receptor whose history is linked to the emergence of MHC class II and class I; unlike most NKRs, NKp30 is evolutionarily conserved, present from sharks to mammals.[176] In order to resemble an ancestor, the NKP30 V domain need only be associated with a C1 domain. In fact, a C1 single-domain gene, pre–TCR-α, is also encoded in the MHC.[253] Besides Ig and TCR, tapasin is one of the rare cases, if not the only other case, of a gene segment with a VJC1 structure existing on several paralogous linkage groups (6, 9q33, 19q13). In other words, while this gene is related to the rearranging receptor structure, it is undoubtedly very old, and probably predated the ancient block (genomewide) duplications. It could have acted as a donor of C1 to a V domain–containing gene in the MHC class III region (like the XMIV), which then could have been the first substrate of the rearrangement. Another set of molecules with distal VJC1 segments, the signal-regulatory proteins (VJ C1 C1), and the poliovirus receptor (VJ C1 C2) could represent another group linked to the history of the Ig and TCR (see the following).

TREM1 and TREM2, receptors on monocytes/neutrophils involved in inflammatory responses, are composed of single VJ domains whose genes are MHC-linked. Myelin oligodendrocyte protein (MOG) and P0, two single V domains

involved in the synthesis of myelin sheath, are encoded in the MHC paralogous region on chromosome 1. Chicken BG, which is related to MOG but probably has a different function, is encoded in the chicken MHC. Butyrophilin, CD83, and tapasin all have VJ domains, and butyrophilin also has a C1-type domain. More distant relatives with VJC2-based architectures are also found in MHC (receptor for advanced glycosylation end products, CTX, lectin-related genes; see Fig. 4.12), and some of these genes related to the rearranging receptor ancestors are found on several paralogs (whether the MHC paralogs or other), suggesting that the V-C1 core was generated early in vertebrate evolution subsequent to the emergence of the chordate superphylum (see Fig. 4.13). Among all these molecules, butyrophilin is perhaps not on the direct track to antigen-specific receptors. Its C domain, although proven to be C1 through its crystal structure, is more like a C2 at the primary sequence level, and belongs to the CD80/86 family, rather than the TCR. Finally, tracing the VJ NITR gene family previously described in evolution may lead us to an understanding of the original NKC/LRC/MHC, as well as identifying more candidate genes related to the ancestral gene invaded by the RAG transposon.

Many invertebrate molecules not involved in immune responses are present as a distal V domain associated with one or more C2-type domains. In the vertebrates many molecules have retained this feature such as CD2 and CTX. Some members resemble "primitive" antigen receptors and several of them map, for instance in humans, to at least two tetrads of paralogous genes, the MHC on chromosomes 1, 6. 9 (12), 19 and the LRC on chromosomes 1, 3, 11, 19 (21) (see Fig. 4.13).[160,442] Many form dimers and are expressed in lymphocytes, where they form a family of adhesion molecules. The crystal structure analysis of a CTX-related molecule (JAM) revealed a unique form of dimerization, suggesting that the diversity of ligand binding and domain-interactions used by different IgSF domain is extensive. Two JAM molecules form a U-shaped dimer with highly complementary interactions between the N-terminal domains. Two salt bridges are formed in a complementary manner by a novel dimerization motif, R (V, I, L) E. The receptor for advanced glycosylation end products gene has a rather "generic" receptor function as it recognizes aged cells exposing particular carbohydrate motifs. CD47, another conserved IgSF member with a CTX-like V domain, suggests an ancient function.[488] Perhaps such nonrearranging VJC1 genes that regulated cytotoxicity/phagocytosis were predecessors of the antigen receptors. Some molecules with VJ/C1 cores involved in cell-cell interactions often serve as ports of entry for viruses. In a move from "property to function," an arms race consequence could result in a virus receptor developing into an immune receptor. The best examples of such molecules are in the CTX JAM family, in which receptor interactions with viruses may trigger an apoptosis, a primitive form of antiviral immunity.[33,489]

Emergence of T and B Cells

As described previously, agnathans have now been shown to have cells in the T and B lineages, similar to what is found in gnathostomes.[291] Because the jawless and jawed vertebrates have antigen receptors derived from different gene families, at first glance it seemed that the T-B split occurred twice in evolution. However, the gene expression profiles for the lamprey T and B cells are too similar to the patterns in gnathostome lymphocyte populations to be derived by convergent evolution; furthermore, the development of lamprey T cells in a thymus-like structure also suggests evolution from a common ancestor. Thus, it seems most likely that both the LRR and IgSF receptor families were present in the common ancestor,[2,13] with the VLR system predominating early in vertebrate evolution (see Fig. 4.10). Such a scenario exists today with NKRs in mammals, with different gene families (C-type lectin or IgSF) predominating in different species.[12]

Because APOBEC family members are most likely involved in VLR assembly and perhaps in mutation, there may have been a primordial IgSF receptor that was also modified somatically by an AID-like molecule, which functioned side-by-side with VLRs. The rearrangement break caused by the RAG transposon in a primordial IgSF gene offered a great advantage, as was detailed previously, and the IgSF receptors then could have supplanted VLRs in gnathostomes, while maintaining the AID-dependent machinery to diversify it further. It is difficult to imagine how a rearrangement break in an antigen receptor gene generated by LRR insertion could be advantageous; conversely, as mentioned previously, a break in the CDR3 loop in the center of the binding site is in perfect position to interact with peptide bound to MHC. While lampreys have a thymoid for development of their "T cells," as described previously, there was a great leap in complexity of thymus structure in the gnathostomes, consistent with a new TCR/MHC system in which positive and negative selection became a requirement for a functioning adaptive immune system. In addition, the general transcriptional and cytokine/chemokine networks for T-cell (and B-cell) development would have also been in place in the jawless vertebrates; in gnathostomes, this network became more complex in the Big Bang (eg, see the chemokine section in which the leap in sophistication can be examined with precision).

In summary, a working hypothesis proposes that the divergence of lymphocytes into T and B cells occurred early in vertebrate evolution (see Fig. 4.10). LRR- and IgSF-based antigen receptors existed simultaneously as well at this time (in the same organism or organisms in the same epoch), with VLR perhaps predominating in early vertebrates. The RAG transposon insertion into an IgSF receptor (perhaps one already diversifying by mutation or conversion), along with a genomewide duplication, resulted in the Ig/TCR/MHC-based system of gnathostomes, with all of its accompanying sophistication in all aspects of adaptive immunity, including development of the thymus and spleen, complexity of cytokine and chemokine networks, and intimate interactions of cells with primary and secondary lymphoid tissues.

CONCLUSION

Since the last edition of *Fundamental Immunology* was published 5 years ago, there have been several astonishing findings, as well as other interesting discoveries and integrations, regarding the evolution of the structure and function of the

immune system. Furthermore, there have been many genome and EST projects that have allowed us to determine whether or not particular genes or gene families are present in different taxa. Here, we briefly describe these findings and discuss their broad relevance and relationship to future studies.

A new system of defense in bacteria and archaebacteria against bacteriophage, CRISPR, has been elucidated.[490] Short phage DNA sequences have been acquired by prokaryotic genomes to direct sequence-specific protection against phages. These elements are transcribed into small RNAs that recruit endonucleases to cleave the phage nucleic acid. This system is widespread and its study is in its infancy. The discovery of this system fits well with studies of plants and animals over the last decade uncovering the plethora of mechanisms used to detect foreign nucleic acids (eg, TLR, NLR, RIG, APOBEC, etc.), as described previously. It should be clear to all immunologists by now that *all* living things must distinguish self from nonself, and have developed intricate mechanisms to achieve this end.

Cytokines such as IFNγ, IL-2, and IL-4, and other adaptive cytokines (and type I/III IFNs) and their receptors are not present outside of jawed vertebrates, nor is the vast array of chemokines seen in gnathostomes. However, at the moment it appears that (almost) all of the cytokines/chemokines discovered in mammals are also present in basal jawed vertebrates: Big Bang indeed! IL-17, a cytokine garnering great attention in basic immunology, and TNF, a central cytokine in both innate and adaptive immunity, seem to be the first cytokines to have emerged in evolution based on studies of lower deuterostomes. If, as suggested from studies in mammals, IL-17 is important for responses to extracellular bacteria, nonvertebrate deuterostomes may hold the key to understanding the physiology of the entire system (ie, in the absence of the competing TH1, TH2, and regulatory T cells), and one may be able to study the dynamics of IL-17 development and function in lower deuterostomes and even protostomes.

Additionally, over the past few years a new type of cell, the ILC, has come to prominence.[491] These cells are predominantly found in epithelia and mucosa and are involved in homeostasis and a first line of defense. As they do not bear antigen receptors, ILCs respond via cytokine receptors or PRRs. ILCs express cytokines consistent with T-cell subsets, and it is tempting to propose that they preceded adaptive T cells in evolution, and the cytokine-production profiles were coopted by the antigen receptor–bearing cells. However, as mentioned, it appears that "adaptive" cytokines arose late in evolution, coincident with adaptive immunity. Thus, it seems more like that ILCs arose simultaneously with the adaptive cells in the immunologic Big Bang, and there has been adaptation over time. However, as IL-17 and TGF-β are old cytokines, it has been proposed that their opposing functions arose before adaptive immunity.[492] TGF-β belongs to an ancient family of cytokines, and at least one of its functions in mammals is to antagonize proinflammatory responses (regulatory T differentiation, regulation of IgA synthesis, etc.). One model suggests that sophisticated innate immunity arose in mucosal tissues, regulated by the pro- and anti-inflammatory properties of IL-17 and TGF-β, respectively, by their expression in gut epithelia or by infiltrating hematopoietic cells; later, this was superimposed onto adaptive lymphocytes in the vertebrate lineage. Consistent with this idea, sea urchins have an expanded family of IL-17 genes,[48] suggestive of a very important function for this cytokine. So, such a system may have been the forerunner of both the ILCs and adaptive lymphocytes, perhaps found in tissues directly exposed to the environment.

Additionally, we are poised for more seminal studies of mucosal immunity. Mammals have dedicated secondary lymphoid tissues in their mucosa, and thus it is often difficult to tease apart the innate from adaptive responses in these tissues, except in contrived knockout or transgenic models. By contrast, ectothermic vertebrates have no Peyer patches or mesenteric lymph nodes, and thus are excellent models to examine responses in the lamina propria, which are predominantly T-independent.[493]

Lastly, studies of how the microbiota interact with the immune system have filled journals to the gills over the past 5 years. McFall-Ngai suggested that the driving force for adaptive immunity was not so much to fight off invaders, but rather to tolerate commensals,[494] perhaps not so different from Weaver's proposal.[492] This framework of interactions between leukocytes, and between leukocytes and commensals/pathogens, may be the key to understanding the genesis of adaptive immunity.

A new defense system in *Drosophila*, important for viral defense, has been uncovered. Previously, the recognition of pathogens by TLRs in the invertebrates was believed to be indirect, interacting with self-molecules similar to a cytokine interaction. However, *Drosophila* Tol7 has now been shown to interact directly with viruses, similar to TLRs in vertebrates,[153] but, as expected, the effector phase is different from the canonical (historical) toll pathway. There are several other tolls in the protostomes without a described function that can be studied in a new light.

One of the great triumphs in the field of comparative immunology was the discovery that similar mechanisms are used to recognize extracellular pathogens and initiate immune responses. It has been approximately 15 years since the *Drosophila* toll/IMD systems and their relationship to vertebrate TLRs and TNF receptors has been uncovered. Over the past few years, the intracellular defense mechanism such as NOD/NLR, RIG, helicase, etc., have been elucidated in vertebrates, and we are beginning to piece together their evolutionary histories. The amazing similarities between the deuterostome and plant NOD/NLR systems will challenge us to understand their recognition events for years to come.[14]

The VLR system continues to amaze us.[13] Most comparative immunologists never expected a unique antigen receptor system to be uncovered in jawless fish. To then realize that the divergence of lymphocytes into two lineages occurred in the ancestor of jawed and jawless fish was astonishing. Now to have uncovered a primitive type of thymus candidate in jawless fish is a total shock. All of these discoveries force us to acknowledge that we should not be surprised by future discoveries in the evolution of immunity, such as a "convergent MHC," or an antigen presentation system not based on peptide presentation, or even a primordial type of adaptive immune system in lower deuterostomes or protostomes.

Furthermore, from comparative studies, Boehm and colleagues have proposed a simple model for the most fundamental elements required for thymocyte differentiation, which has been superimposed onto the lamprey thymus-like structures.[375,495] Further study of this minimal network of transcription factors, chemokines, and cytokines could uncover the origins of thymus differentiation in lower deuterostomes that clearly have no T/B cells.

Certain groups of organisms are interesting to study for different reasons. The genome projects in cnidarians demonstrated that certain gene families were more ancient than previously believed (from studies of the classical models *Drosophila* and *C. elegans*).[35] Thus, this taxonomic group has become the new rock stars of comparative immunology, not only for their immune complexity in the absence of a mesodermal germ layer, but also for elucidation of a histocompatibility reaction that has fascinated us for decades.[496]

Teleost fish have been shown again and again to have rapidly evolving immune systems, often expanding gene families in unprecedented ways (eg, NLR, tripartite motif-containing), generating specific paralogs and losing others (eg, cytokines, chemokines, etc.), losing canonical syntenies found in all other vertebrates (eg, MHC class I and class II), and having unique forms of ancient molecules (eg, tetrameric IgM with no J chain). The third genomewide duplication (3R) is believed to have been a major force in this instability and rapid evolution.[20] This group provides an opportunity to study the plasticity of immune systems in related organisms over a short period of evolutionary time. With the cod story, they even offer a natural class II knockout system[395]!

Amphibians with metamorphosis offer models to study two modalities of self-tolerance, metamorphosis, and polyploidy. The larval immune system must somehow be suppressed with the appearance of adult self-determinants during metamorphosis and the refurbishing of the immune system. The amphibian *Xenopus* speciates by polyploidy, allowing an experimental system to examine the effects of whole-genome duplications on immune system loci (silencing, deletion, gene conversion, conservation), a key issue given the importance attributed to the genomewide duplications in the evolution of immune systems in vertebrates.[328,399]

Birds offer another case of rapid evolution, probably because of attack by viruses. For the MHC, birds have lost immunoproteasomes and the thymic-specific proteasome, and have modified their class I groove to be more promiscuous in peptide binding. Chickens (but not ducks) have lost the RIG-1 gene, apparently making them susceptible to influenza.[497] Clearly, birds have something fundamental and unique to teach us about how pathogens (most likely viruses in this case) can force a remodeling of innate and adaptive immunity.

The genomewide duplications early in the evolution of the vertebrates (the 2R hypothesis) have been confirmed in a variety of studies, and they were indeed important in the Big Bang emergence of the adaptive immune system. The 2R paradigm is useful for studying *any* gene family found on multiple chromosomes in the vertebrates; good examples of how it has aided in our understanding of immunity were the study of IgSF members involved in cell-cell interaction/costimulation, and the evolution/emergence of chemokines and TNF family members. An emerging paradigm is the possibility that genes encoding NKC, LRC, MHC, as well as the antigen receptors and costimulatory molecules (and other immune genes) were all linked at an early point in evolution.[2,498] In addition, while the agnathan adaptive system is similar to the Ig/TCR/MHC system of gnathostomes, the great sophistication that we see in the latter is not present in lampreys and hagfish. Our best guess at the moment is that the RAG transposon, in combination with the genomewide duplications, both superimposed onto an already existing adaptive framework, resulted in the Big Bang of adaptive immunity (see Fig. 4.10).

With the genome projects and advances in molecular biology, we have made strides in understanding old problems in vertebrate adaptive immunity, such as 1) when the L chains emerged and what the significance of more than one isotype is; 2) which antigen receptor (if any of the extant ones) came first, and all of them came to evolve to their present state; 3) when IgD emerged and what its function is in different vertebrate phyla, especially the transmembrane form; and 4) how γ/δ T cells recognize antigen. We propose that are two arms of the γ/δ T-cell lineage, one innate and the other adaptive, similar to B cells and α/β T cells.

The long-awaited molecular mechanisms of histocompatibility in nonvertebrates have been at least partially uncovered in plants, *Botryllus*, and *Hydractinia*. The common themes thus far are that the genes encoding the polymorphic receptors/ligands are closely linked and that the genes all appear to be unrelated to the vertebrate MHC. However, there are tantalizing links to immune gene clusters found throughout the animal kingdom that suggest there may be an underlying fundamental similarity in recognition, perhaps related to 1) the paucity of surface receptors available because of multiple constraints resulting in sharing of receptor/ligands, and 2) the signaling cascades.

Despite the great variation in mechanisms of allorecognition across phyla, are there commonalities? Whether in cnidarians or in tunicates, the polymorphic genetic regions involved contains from one to many IgSF members resembling members of the nectin PVR/CD155 family. In *Ciona*, these genes are not only closely linked in a single region but also are homologs found in the LRC. Could this region be the conserved and link aspects of immunity between vertebrates and invertebrates? The frequent presence of ITIM and ITAM indeed suggest the conservation of receptors interacting among these molecules.[160] It is clearly of ancient origin. With paralogs on four different chromosome regions in gnathostomes as well as an ancient connection to MHC and NKC, this region was present before 2R. It is not surprising now to detect very different molecular families involved in immune recognition events: LRR, lectins, IgSF. The comparison of allorecogniton in invertebrates has perhaps helped to focus our attention on genetic regions that may have provided the "context" for leukocyte interactions.

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SECTION III

Immunoglobulins and B Lymphocytes

CHAPTER 5

Immunoglobulins: Structure and Function

Harry W. Schroeder Jr • David Wald • Neil S. Greenspan

INTRODUCTION

Immunoglobulins (Igs) are marked by a duality of structure and function.[1] In common with other members of the Ig superfamily,[2] they provide the immune system with a conserved set of effector molecules. These effectors can activate and fix complement and they can bind to Fc receptors on the surfaces of granulocytes, monocytes, platelets, and other components of the immune response. Both activation of complement and binding to Fc receptors can contribute to the induction or maintenance of inflammation. They also provide the immune system with a polyclonal set of diverse ligand binding sites, which allow Igs, as a population, to recognize an almost unlimited array of self- and non–self-antigens, which may range from compounds as fundamental to life as deoxyribonucleic acid to manmade molecules that could not have played a role in the evolution of the immune system.

Differential splicing allows individual Ig molecules to serve as either membrane-bound receptors for the B cell that allow antigen-specific activation or as soluble effectors, which act at a distance. In vivo, proper effector function requires more than just antigen-specific binding; it requires successful neutralization of the offending antigen while avoiding potentially pathogenic self-reactivity.

The receptor and effector functions of each individual Ig can be localized to a separate region or domain of the molecule. Each variable (V) or constant (C) domain consists of approximately 110 to 130 amino acids, averaging 12,000 to 13,000 kD. A typical light (L) chain will thus mass approximately 25 kD, and a three C domain Cγ H chain with its hinge will mass approximately 55 kD.

Immunoglobulins are Heterodimers

Igs are heterodimeric proteins, consisting of two H and two L chains (Figs. 5.1 and 5.2). The eponymous Ig domain serves as the basic building block for both chains. Each of the chains contains a single amino-terminal V Ig domain and one, three, or four carboxy-terminal C Ig domains. H chains contain three or four C domains, whereas L chains contain only one. H chains with three C domains tend to include a spacer hinge region between the first (C_H1) and second (C_H2).[3]

At the primary sequence level, Igs are marked by the interspersion of regions of impressive sequence variability with regions of equally impressive sequence conservation. The V domains demonstrate the greatest molecular heterogeneity, with some regions including non-germline–encoded variability and others exhibiting extensive germline conservation across 500 million years of evolution.[4] The molecular heterogeneity of the V domains permits the creation of binding sites, or *paratopes*, which can discriminate between antigens that may differ by as little as one atom. Thus it is the V domains that encode the receptor function and define the monovalent specificity of the antibody. The H chain C_H1 domain, which is immediately adjacent to the V, associates with the single L chain C domain. Together, the C_H1 and C_L domains provide a stable platform for the paired set of V_H and V_L domains, which create the antigen binding site. The distal C_H2 and C_H3 domains, for those antibodies with a hinge, or the C_H3 and C_H4 domains, for those with an extra (C_H2) domain, typically encode the effector functions of soluble antibody. Each of these Ig C_H domains is encoded by a separate exon. Although the sequences of the individual C_H domains are constant within the individual (and nearly constant within a species), they can vary greatly across species boundaries. The carboxy-terminal C_H domain encodes a secretory tail, which permits the antibody to exit the cell.

Also encoded within the germline sequence of each C_H gene are two membrane/cytoplasmic tail domain exons, termed M1 and M2. Alternative splicing removes the secretory sequence typically encoded by the terminal C_H3 or C_H4 domain and replaces it with the peptides encoded by the M1 and M2 exons, converting a secretory antibody to a

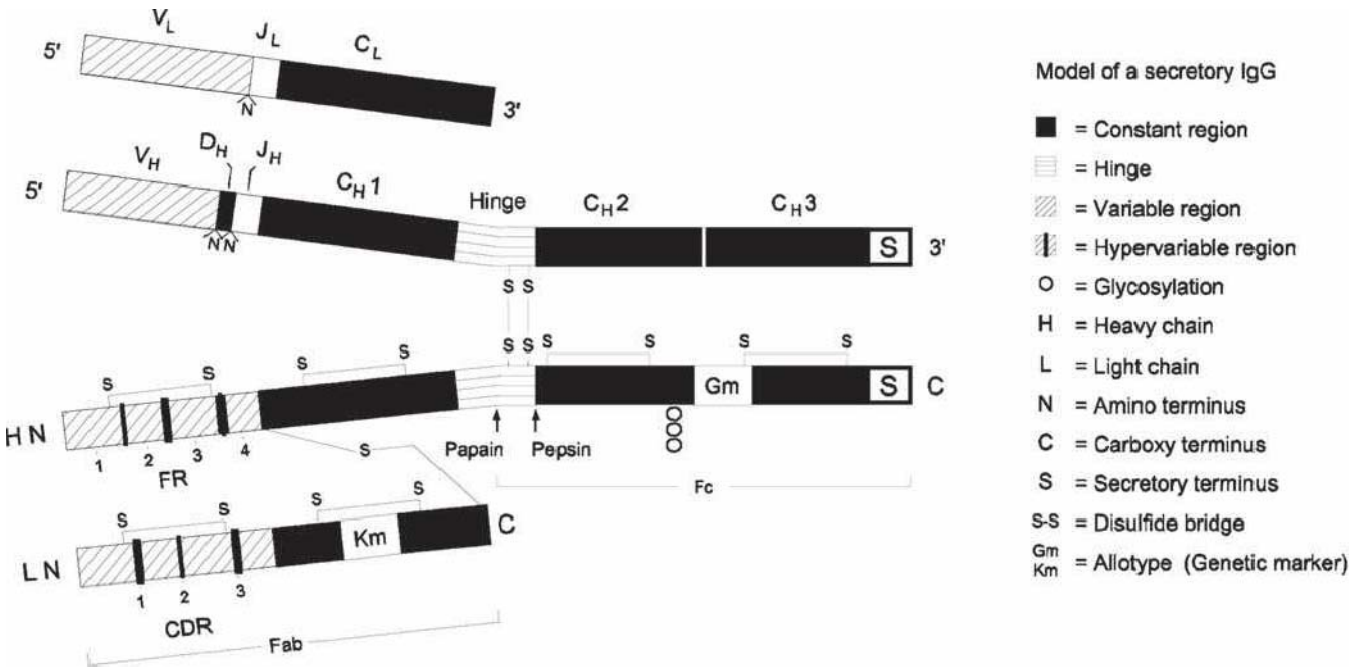

FIG. 5.1. Model of a Secreted Immunoglobulin (Ig)G. The germline exonic derivation of the sequence is shown at the *top*, and the protein structure is shown at the *bottom*. The location of the various cysteine residues that help hold both the individual domains and the various Ig subunits together are illustrated. Papain digests IgG molecules above the cysteine residues in the hinge that holds the two H chains together yielding two Fab molecules and an Fc, whereas pepsin digests below releasing an $(Fab)'_2$ fragment and two individual Fcs (which are typically degraded to smaller peptide fragments). The location of some allotypic variants is illustrated. C_H2 domains can be variably glycosylated, which can also affect Ig structure and effector function.

membrane-embedded receptor.[5] Between species, the membrane/cytoplasmic tail region is the most highly conserved portion of the C_H domains, which befits its role as a link to the intracellular signal transduction pathways that ultimately regulate B-cell function.

Paratopes and Epitopes

The immunoglobulin-antigen interaction takes place between the paratope, the site on the Ig at which the antigen binds, and the *epitope*, which is the site on the antigen that is bound. It is important to appreciate that antibodies do not recognize antigens; they recognize epitopes borne on antigens.[6] This makes it possible for Igs to discriminate between two closely related antigens, each of which can be viewed as a collection of epitopes. It also is one scenario that permits the same antibody to bind divergent antigens that share equivalent epitopes, a phenomenon referred to as *cross-reactivity*.

It has been estimated that triggering of effector functions in solution typically requires aggregation of three or more effector domains, and thus tends to involve the binding of three or more epitopes.[6] For antigens encoding repeating epitope structures, such as polysaccharides or antigen aggregates, binding of a single polymeric Ig molecule carrying multiple effector domains, such as pentameric IgM, can be sufficient to induce effector function. For antigens encoding diverse epitopes, which is more typical of monodisperse single-domain molecules in solution, triggering of inflammatory effector functions may require the binding of a diverse set of Ig molecules, all binding the same antigen, but at different epitopes.[7]

Membrane and Secretory Immunoglobulin

Alternative splicing allows Igs to serve either as soluble antibodies or as membrane-bound antigen receptors. In their role as antibodies, Igs are released into the circulation from where they may traffic into the tissues and across mucosal surfaces. In their role as the B-cell antigen receptor, they are anchored to the membrane by means of their M1:M2 transmembrane domain. Soluble antibodies can also be pressed into service as heterologous cell surface antigen receptors by means of their attachment to membrane-bound Fc receptors.[8] This permits the power of antibody recognition to be extended to nonlymphoid cells, such as Fc-expressing granulocytes, macrophages, and mast cells. The major difference between these two forms of cell surface receptors is that Igs as B-cell antigen receptors provide a monoclonal receptor for each B cell, whereas antibodies bound to Fc receptors endow the cell with a polyclonal set of antigen recognition molecules. This gives greater flexibility and increases the power of the effector cells to recognize antigens with multiple non-self–epitopes.

Isotypes and Idiotypes

Igs can also serve as antigens for other Igs. Immunization of heterologous species with monoclonal antibodies (or a restricted set of Igs) has shown that Igs contain both common

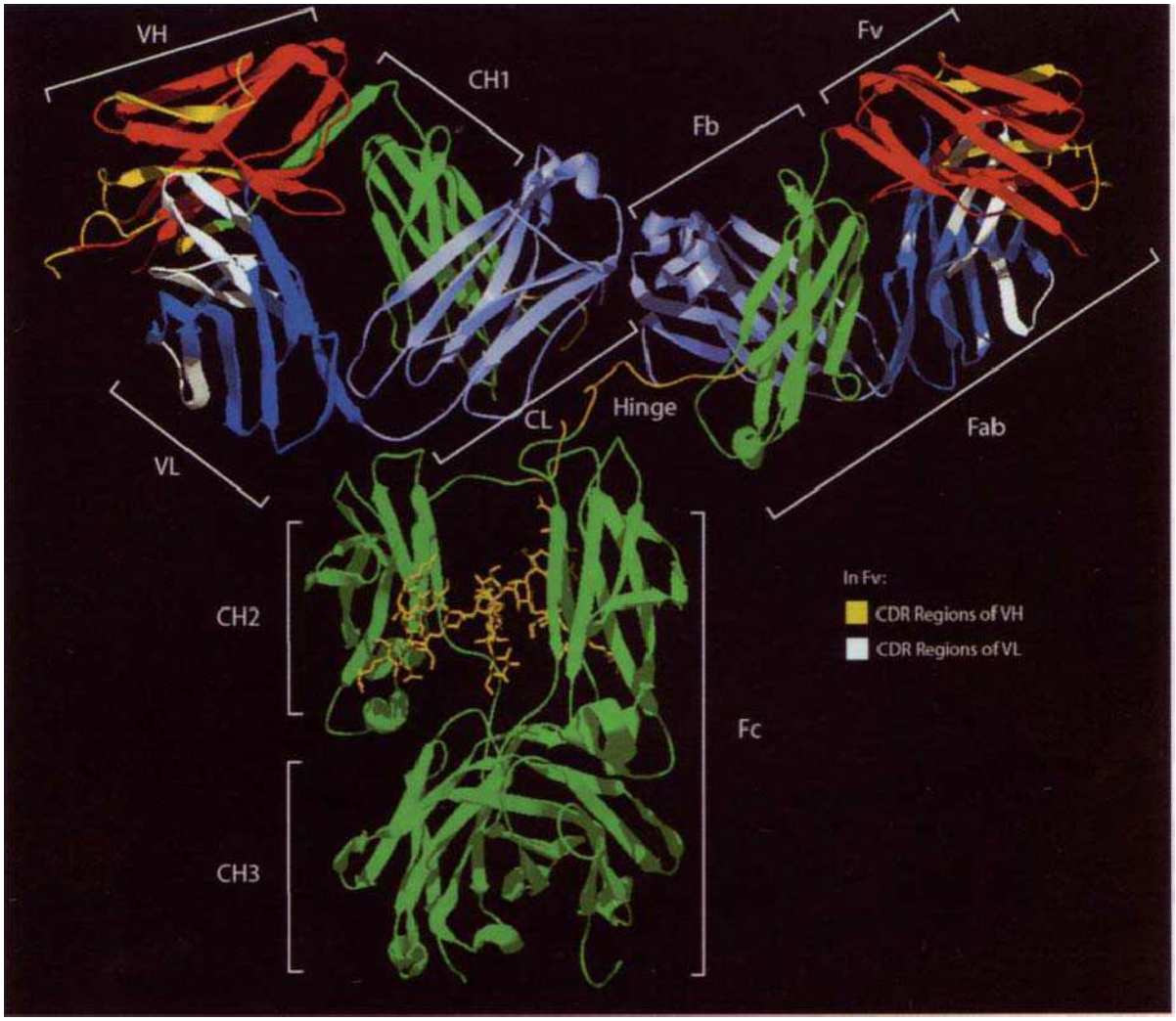

FIG. 5.2. Ribbon Diagram of a Complete Immunoglobulin (Ig)G1 Crystal (1 hzh in the protein data bank [PDB] from Data of Harris et al.[202]). The major regions of the Ig are illustrated. The heavy-chain constant regions (*green*) also include the hinge (*yellow*) between the first two domains. Cγ2 is glycosylated (also seen in *yellow*). The heavy- and light-chain variable regions (*red* and *dark blue*, respectively) are N terminal to the heavy- (*green*) and light-chain (*light blue*) constant regions. Complementarity determining region loops in the heavy- and light-chain variable regions (*yellow* and *white*) are illustrated as well.

and individual antigenic determinants. Epitopes recognized within the V portion of the antibodies used for immunization that identify individual determinants are termed *idiotypes* (Fig. 5.3), whereas epitopes specific for the constant portion are termed *isotypes*. Recognition of these isotypes first allowed grouping of Igs into recognized classes. Each class of Ig defines an individual set of C domains that corresponds to a single H chain constant region gene. For example, IgM utilizes μ H chain C domains and IgE utilizes ε C domains.

Some V domain epitopes derive from the germline sequence of V gene exons. These shared epitopes, commonly referred to as *public idiotopes* or *cross-reactive idiotypes* (see Fig. 5.3), are, from a genetic perspective, isotypic because they can be found on many Igs of different antigen-binding specificities that derive from the same germline V region. Examples include the cross-reactive idiotypes found on monoclonal IgM rheumatoid factors derived from individuals with mixed cryoglobulinemia, each of which can be linked to the use of individual V gene segments.[9]

Classes and Allotypes

Each of the various classes and subclasses of Igs has its own unique role to play in the immunologic defense of the individual. For example, IgA is the major class of Ig present in all external secretions. It is primarily responsible for protecting mucosal surfaces. IgG subclasses bind Fc receptors differently, and thus vary in effector function.[10] Determinants common to subsets of individuals within a species, yet differing between other members of that species, are termed *allotypes* and define inherited polymorphisms that result from allelic forms of immunoglobulin C (less commonly, V) genes.[11]

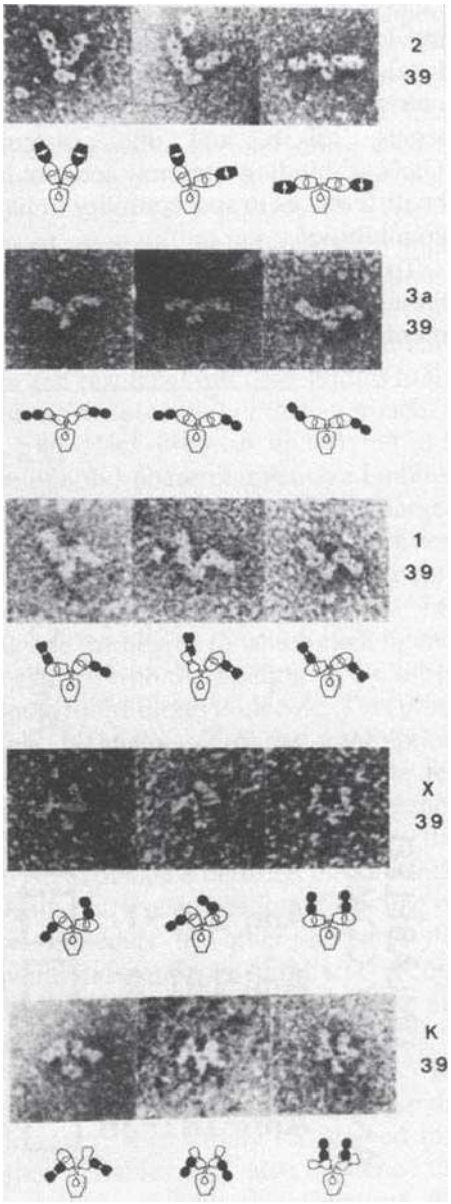

FIG. 5.3. Electron micrographs (*top*; ×350,000) and interpretive diagrams (*below*) of murine mAb hybridoma group A carbohydrate (HGAC) 39 (specific for the cell wall polysaccharide of *Streptococcus pyogenes*) in complex with anti-idiotypic mAb Fab fragments. HGAC 39 is represented in the diagrams as an open figure, and the Fab anti-Id probes are represented as solid figures. The Fab arms of the antibody targets and probes are drawn to indicate their rotational orientation as planar (oval with open center), intermediate (bone shape with or without central opening), or perpendicular ("dumbbell shaped"). Different complexes illustrate the range of Fab-Fab angles made possible by segmental flexibility. 1, anti-Idl-1 Fab; 2, anti-Idl-2 Fab; 39, HGAC 39; 3a, anti-Idl-3a Fab; K, anti-Cκ Fab; X, anti-IdX Fab. Idl designates an individual idiotope, and IdX, a crossreactive idiotope. Antibody complexes were stained with 2% uranyl formate as described by Roux et al.[108] Reproduced from Proceedings of the National Academy of Sciences (from Roux et al.[108] with permission).

Glycosylation

N-linked carbohydrates can be found in all constant domains as well as in some variable domains.[12] The structure of the attached N-linked carbohydrate can vary greatly, depending on the degree of processing. These carbohydrates can play a major role in Ig function.[13] For example, human IgG molecules contain a conserved glycosylation site at Asn 297, which is buried between the C_H2 domains.[14] This oligosaccharide structure is almost as large as the C_H2 domain itself. O-linked sugars are also present in some Igs.[12] Human IgA1, but not IgA2, possesses a 13 amino acid hinge region that contains three to five O-linked carbohydrate moieties.[15] A deficiency in proper processing of these O-glycans can contribute to IgA nephropathy, which is a disease that is characterized by the presence of IgA1-containing immune complexes in the glomerular mesangium.[16]

A HISTORICAL PERSPECTIVE

The identification of Ig as a key component of the immune response began in the 19th century. This section describes the history of the identification of Ig and introduces fundamental terminology.

Antibodies and Antigens

Aristotle and his contemporaries attributed disease to an imbalance of the four vital humors: the blood, the phlegm, and the yellow and black biles.[17] In 1890, Behring (later, von Behring) and Kitasato reported the existence of an activity in the blood that could neutralize diphtheria toxin.[18] They showed that sera containing this humoral antitoxin activity would protect other animals exposed to the same toxin. Ehrlich, who was the first to describe how diphtheria toxin and antitoxin interact,[19] made glancing reference to "Antikörper" in a 1891 paper describing discrimination between two immune bodies, or substances.[20] The term antigen was first introduced by Deutsch in 1899. He later explained that antigen is a contraction of "Antisomatogen+-Immunkörperbildner," or that which induces the production of immune bodies (antibodies). Thus, the operational definition of antibody and antigen is a classic tautology.

Gamma Globulins

In 1939, Tiselius and Kabat immunized rabbits with ovalbumin and fractionated the immune serum by electrophoresis into albumin, alpha-goblulin, beta-globulin, and gamma-globulin fractions.[21] Absorption of the serum against ovalbumin depleted the gamma-globulin fraction, hence the terms immunoglobulin and IgG. "Sizing" columns were used to separate Igs into those that were "heavy" (IgM), "regular" (IgA, IgE, IgD, IgG), and "light" (light chain dimers). Immunoelectrophoresis subsequently permitted identification of the various Ig classes and subclasses.

Fab and Fc

In 1949, Porter first used papain to digest IgG molecules into two types of fragments, termed Fab and Fc (Table 5.1).[22]

TABLE 5.1 Definitions of Key Immunoglobulin Structure Nomenclature

Fc	A constant region dimer lacking C_H1
Fab	A light chain dimerized to V_H-C_H1 resulting from papain cleavage; this is monomeric because papain cuts above the hinge disulfide bond(s)
$F(ab)'_2$	A dimer of Fab' resulting from pepsin cleavage below the hinge disulfides; this is bivalent and can precipitate antigen
Fab'	A monomer resulting from mild reduction of $F(ab)'_2$: an Fab with part of the hinge
Fd	The heavy chain portion of Fab (V_H-C_H1) obtained following reductive denaturation of Fab
Fv	The variable part of Fab: a V_H-V_L dimer
Fb	The constant part of Fab: a C_H1-C_L dimer
pFc'	A C_H3 dimer

From Carayannopoulos and Capra[208] with permission.

Papain digested IgG into two Fab fragments, each of which could bind antigen, and a single Fc fragment. Nisonoff developed the use of pepsin to split IgG into an Fc fragment and a single dimeric $F(ab)_2$ that could cross-link antigens.[23] Edelman broke disulfide bonds in IgG and was the first to show that IgG consisted of two H and two L chains.[24]

Two Genes, One Polypeptide

The portion of the constant domain encoded by the Fc fragment was the first to be sequenced and then analyzed at the structural level. It could be readily crystallized when chilled. The heterogeneity of the V domain precluded sequence and crystallographic analysis of an intact Ig chain until Bence-Jones myeloma proteins were identified as clonal, isolated Ig light chains. These intact chains could be purified and obtained in large quantities, which finally permitted rational analysis of antibody structure and function.[25] Recognition of the unique nature of a molecule consisting of one extremely variable V domain and one highly conserved C domain led to the then-heretical Dreyer-Bennett proposal of "two genes, one polypeptide,"[26] which was subsequently and spectacularly confirmed by Tonegawa.[27]

THE IMMUNOGLOBULIN DOMAIN

The Ig domain is the core unit that defines members of the Ig superfamily (reviewed in Williams and Barclay[2] and Harpaz and Chothia[28]). This section describes the Ig domain in detail.

The Immunoglobulin Superfamily

Each Ig domain consists of two sandwiched β-pleated sheets "pinned" together by a disulfide bridge between two conserved cysteine residues (Fig. 5.4). The structure of the β-pleated sheets in an Ig domain varies depending on the number and conformation of strands in each sheet. Two such structures, V and C, are typically found in Igs. C-type domains, which are the most compact, have seven antiparallel strands distributed as three strands in the first sheet and four strands in the second. Each of these strands has been given an alphabetical designation ranging from amino terminal A to carboxy-terminal G. Side chains positioned to lie sandwiched between the two strands tend to be nonpolar in nature. This hydrophobic core helps maintain the stability of the structure to the point that V domains engineered to replace the conserved cysteines with serine residues retain their ability to bind antigen. The residues that populate the external surface of the Ig domain and the residues that form the loops that link strands can vary greatly in sequence. These solvent exposed residues offer multiple targets for docking with other molecules.

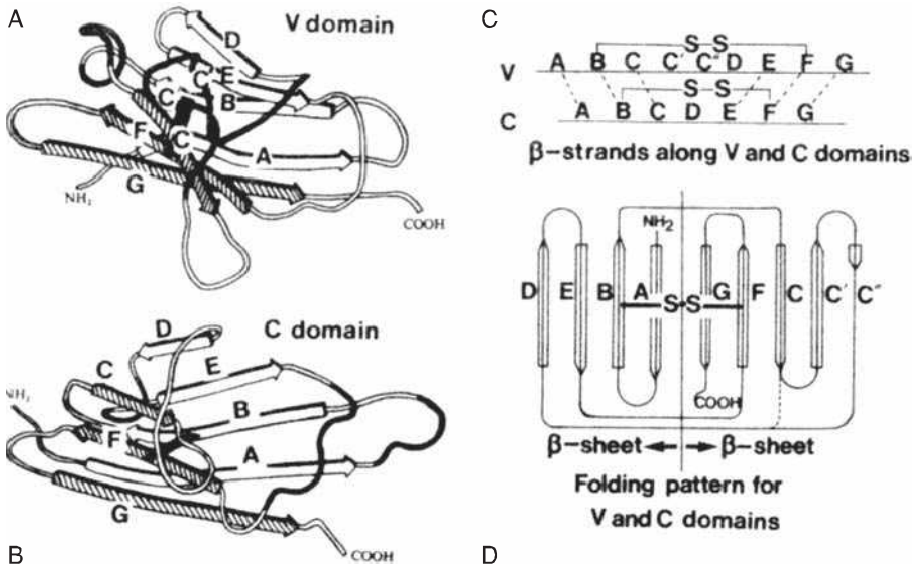

FIG. 5.4. The Immunoglobulin Domain. A: A typical variable domain structure. Note the projection of the C-C′ strands and loop away from the core. **B:** A typical compact constant domain structure. **C:** The cysteines used to pin the two b-sheets together are found in the B and F strands. **D:** The folding pattern for variable and constant domains.[2]

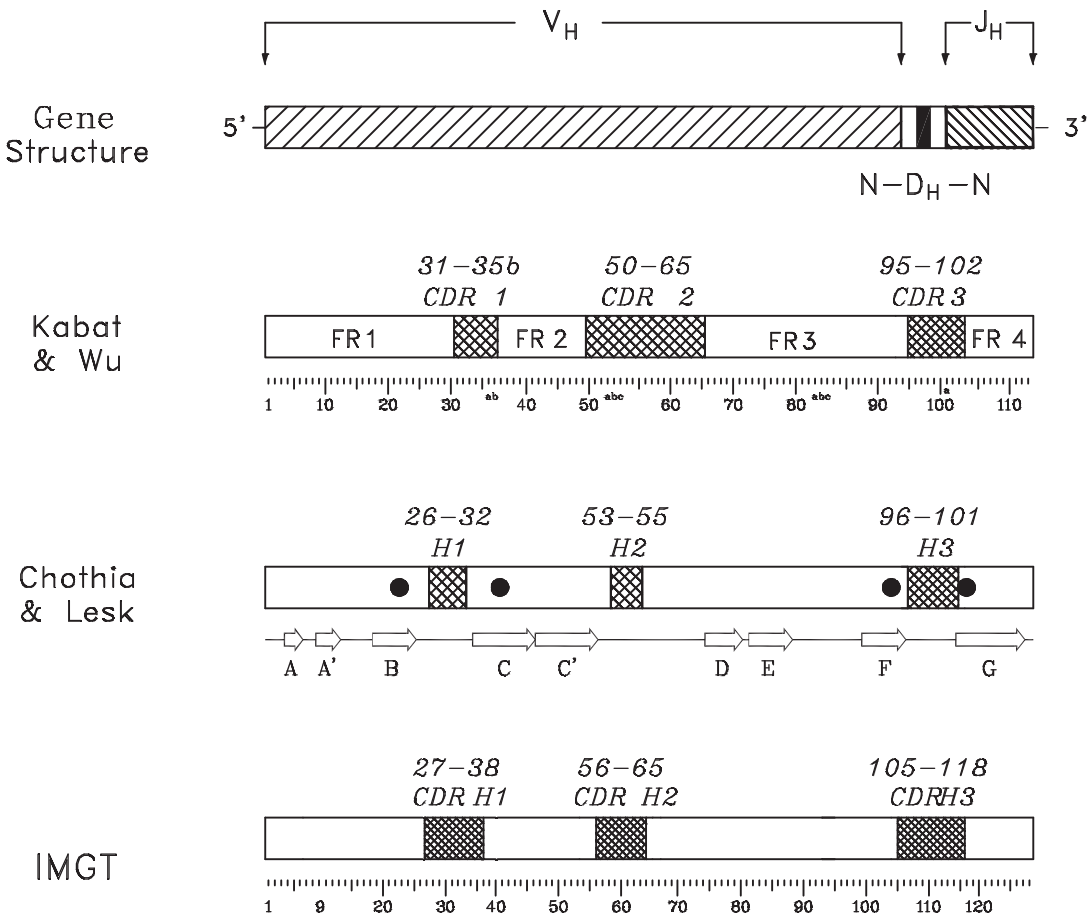

FIG. 5.5. Sequence Conservation and Hypervariability within a Heavy Chain Variable (V) Domain. The primary sequence the V domain can be divided into four regions of sequence conservation, termed framework regions (FRs), and three regions of hypervariability, termed complementarity determining regions (CDRs). A schematic of the genomic origin of the variable domain is shown at the top of the figure. The classic separation of the sequence into FR and CDR by Kabat et al.[31] is shown below the gene structure. The letter designation for individual β strands is given beneath the Chothia and Lesk nomenclature,[32] which focused more on structure. The positions of each of the four invariant residues of the V_H chain (FR1 Cys22, FR2 Trp36, FR3 Cys92, and FR4 Trp103) are shown as *darkened circles* on the Chothia and Lesk model. The ImMunoGeneTics information system® designation has attempted to rationalize sequence variability with structure and is the current nomenclature of choice.[33]

The V Domain

V-type domains add two additional antiparallel strands to the first sheet, creating a five strand–four strand distribution. Domain stability results from the tight packing of alternately inward-pointing residue side chains enriched for the presence of hydrophobic moieties to create a hydrophobic domain core. The H and L variable domains are held together primarily through noncovalent interaction between the inner faces of the β sheets.[29,30]

Early comparisons of the primary sequences of the V domains of different antibodies identified four intervals of relative sequence stability, termed framework regions (FRs), which were separated by three hypervariable intervals, termed complementarity determining regions (CDRs) (Figs. 5.5 and 5.6).[31] The exact location of these intervals has been adjusted over the years, first by a focus on the primary sequence,[31] then by a focus on the three-dimensional structure,[32] and, more recently, by a consensus integration of the two approaches by the international ImMunoGeneTics information system® (IMGT)[33] (see Fig. 5.5). (For students of the Ig repertoire, IMGT maintains an extremely useful website, http://www.imgt.org, which contains a large database of sequences as well as a multiplicity of software tools.)

The C and C' strands that define a V domain form FR2. These strands project away from the core of the molecule (see Figs. 5.4 and 5.6) where they take on a conserved structure that is parallel and opposite to the FR2 of the companion V and adjacent to the FR4 of the complementary chain. Approximately 50% of the interdomain contacts in the hydrophobic core of the V domain are formed by contacts between the FR2 of one chain and the FR4 of the complementary chain.[30] Another 30% to 45% is contributed by contacts between the CDR3 and the FR2 or CDR3 of the complementary domain. The overall interdomain contact includes between 12 and 21 residues from the L chain V domain and 16 to 22 residues from H chain V domain, most of which are contributed by the FR2, CDR3, and FR4 regions.

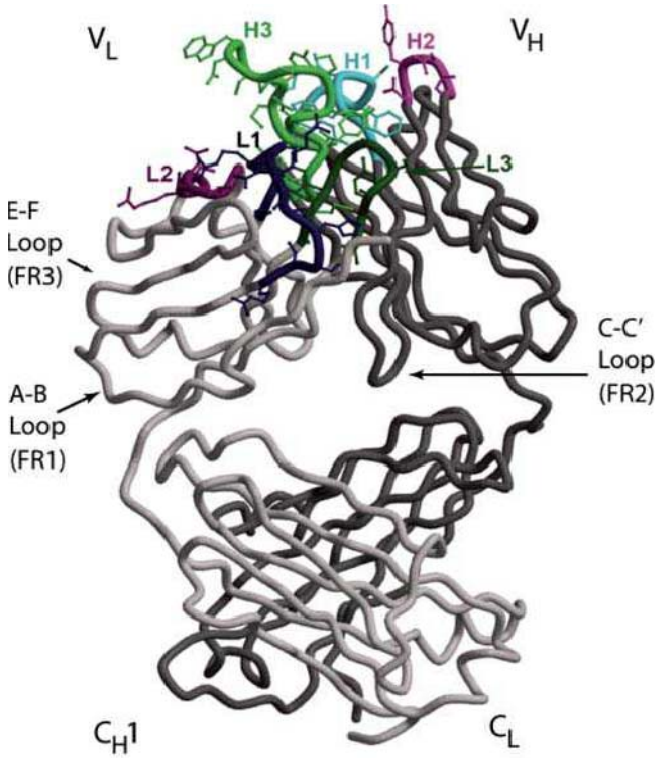

FIG. 5.6. The Structure of an Fab. The antigen-binding site is formed by the heavy (H) and light (L) chain B-C, C′-C″, and F-G loops. Each loop encodes a separate complementarity determining region (CDR). The location of CDRs H1, H2, H3, L1, L2, and L3 are shown. The opposing H and L chain C-C′ strands and loop help stabilize the interaction between VH and VL. This C-C′ structure is encoded by the second V framework region, FR2. The inclusion of this structure permits the variable (V) domains to interact in a head-to-head fashion. The E-F strands and loop are encoded by the FR3 region and lie directly below the antigen-binding site. The A-B strands and loop encode FR1 and lie between the CH1 and CL domains and the rest of the Vs. The beta sheet strands of the CH1 and CL domains rest crosswise to each other. The illustration is modified.[203]

There are approximately 40 crucial sequence sites that influence variable domain inter- and intradomain interactions.[32,34] Four of these sites are relatively invariant: the two cysteines that form the disulphide bridge between the beta sheets and two tryptophan (phenylalanine in Jκ) residues, one near the beginning of the C strand and the second near the beginning of the G strand, that pack against the bridge to add stability. Beyond these and other common core residues, Ig domains can vary widely in their primary amino acid sequences. However, a common secondary and tertiary structure characteristic of the core Ig V domain tends to be preserved.

FAB STRUCTURE AND FUNCTION

Introduction

It is the Fab domain that allows Ig to discriminate between antigens. The Fab is individually manufactured to precise specifications by individual developing B cells. It shows an amazing array of binding capabilities while maintaining a highly homologous scaffold. This section describes the characteristics of the Fab domain, its component V domains, and the *paratope*, which is the part of the Fab that actually binds antigen.

Fab, Fv, and Fb

The antigen-binding fragment (Fab) is a heterodimer that contains an L chain in its entirety and the V and C_H1 portions of the H chain (see Figs. 5.1 and 5.6). The Fab in turn can be divided into a variable fragment (Fv) composed of the V_H and V_L domains, and a constant fragment (Fb) composed of the C_L and C_H1 domains (see Table 5.1). Single Fv fragments can be produced in the laboratory through genetic engineering techniques.[35] They recapitulate the monovalent antigen binding characteristics of the original, parent antibody. Other than minor allotypic differences, the sequences of the constant domains do not vary for a given H chain or L chain isotype. The eponymous V domain, however, is quite variable.

Generation of Immunoglobulin Variable Domains by Recombination

Ig V domain genes are assembled in an ordered fashion by a series of recombination events.[27,36–39] The elegant mechanisms used for the assembly of these genes and the Fvs they create are fully discussed in Chapter 6. However, in order to understand the relationship between antibody structure and function, a brief review is in order.

In BALB/c mice, Ig V assembly begins with the joining of one of 13 diversity (D_H) gene segments to one of four joining (J_H) gene segments. This is followed by the joining of one of 110 functional variable (V_H) gene segments.[40–42] Each D_H gene segment has the potential to rearrange in any one of six reading frames (RFs), three by deletion and three by inversion. Thus, these 127 gene segments can come together in 3.4×10^4 combinations. The numbers of gene segments can vary quite widely between different mouse strains, but the process of assembly and the multiplicative effect of combinatorial diversity is the same.

In the final assembled V domain, the V_H gene segment encodes FRs H1 to H3 and CDRs H1 and H2 in their entirety (see Figs. 5.1 and 5.5), and the J_H encodes FR-H4. CDR-H3 is created de novo in developing B cells by the joining process. CDR-H3 contains the D_H gene segment in its entirety, as well as portions of the V_H and J_H gene segments.

After a functional H chain has been created, L chain assembly begins. Mice contain two L chain loci, κ and λ. In C57BL/6 mice, the κ locus includes five Jκ gene segments and 140 Vκ gene segments, of which 4 and 73 have been shown to be functional, respectively.[43–45] This provides 292 combinations. There is only one Cκ.[46] The BALB/c λ locus contains three Vλ, three functional Jλ, and two or three functional Cλ chains.[47,48] The λ constant domains are functionally indistinguishable from each other. Due to gene organization, the λ repertoire provides at most seven combinations. Each V_L encodes FRs L1 to L3, CDR L1 and L2, and two thirds of CDR-L3 (see Fig. 5.1). Each J_L encodes one-third of CDR-L3 and FR4 in its entirety. Any one

H chain can combine with any one L chain, thus 211 V, D, and J gene segments can provide approximately 1×10^7 different H:L combinations.

At the V→D and D→J junctions, the potential for CDR-H3 diversity is amplified by imprecision in the site of joining, allowing exonucleolytic loss as well as palindromic (P junction) gain of terminal V_H, D_H, or J_H germline sequence. B cells that develop after birth express the enzyme terminal deoxynucleotidyl transferase (TdT) during the H chain rearrangement process.[27,36] TdT catalyzes the relatively random incorporation of non-germline–encoded nucleotides between V_H and D_H, and between D_H and J_H. Each three nucleotides of N addition increase the potential diversity of CDR-H3 20-fold. Thus sequences with nine nucleotides of N addition each between the V→D and D→J junctions would enhance the potential for diversity by $(20)^6$, or by 6×10^7; six-fold greater than the potential diversity provided by VDJ gene segment combinations. These genomic gymnastics permit the length of CDR-H3 to vary from 5 to 20 amino acids among developing B cells in BALB/c bone marrow.[49] Together, imprecision in the site of VDJ joining and N addition provides the opportunity to create nearly random CDR-H3 sequence, potentially freeing the CDR-H3 repertoire from germline sequence constraints. Although a limited amount of N addition is observed between V_L and J_L in human,[50,51] N addition in murine L chains is distinctly uncommon. Moreover, the length of CDR-L3 appears to be under relatively strict control, greatly limiting the potential for somatic L chain junctional diversity.[48,50] Thus, CDR-H3 represents the greatest focus for the initial somatic diversification of the antibody repertoire.

Segmental Conservation and Diversity within the V Domain

Although the large numbers of V gene segments might give the impression of a smooth incremental range of available diversity, multigene families are thought to evolve in concert through mechanisms of gene conversion, and V gene segments are no exception. Sequence relationships allow grouping them into families and clans of sequences that share nucleotide homology,[52] as well as structural features. Close inspection of the V_H gene repertoire has shown that these family relationships reflect segmental gene conversion coupled with selection for function.[4,53,54]

Due to the need to maintain a common secondary and tertiary core Ig V domain structure capable of associating randomly with a complementary V chain to form a stable Fv, the core sequence of FR2, which is encoded by the V_H gene segment, and the core sequence of FR4, which is encoded by the J_H gene segment, are highly conserved among all Ig V domains. Conversely, the need to generate a diverse repertoire of antigen-binding sites has led to extensive diversity in the CDR-1 and CDR-2 intervals. One might presume that the FR1 and FR3 intervals, which form the external surface of the antibody, would not be under any particular constraints, but sequence comparisons suggest otherwise.

Given the need to diversify the CDRs and the need to preserve FR2, it is not surprising that family identity, which might reflect ancestral relationships, can be assigned by the extent of FR1 and FR3 similarity.[55] Of these, FR1 appears to be under the greatest constraints, with V_H gene segments belonging to different families both within and across species barriers exhibiting extensive similarities in FR1 sequence (Fig. 5.7). Sequence similarities in FR1 and, to a lesser extent, FR3 allow grouping of human and murine V_H families into three clans of related sequences, presumably reflecting an early divergence in sequence from a primordial V_H gene sequence (Fig. 5.8).

Constraints on the Sequence and Structure of Variable-Encoded Complemenatarity Determining Regions

The antigen-binding site of an Ig is formed by the juxtaposition of the six hypervariable H and L chain V domain

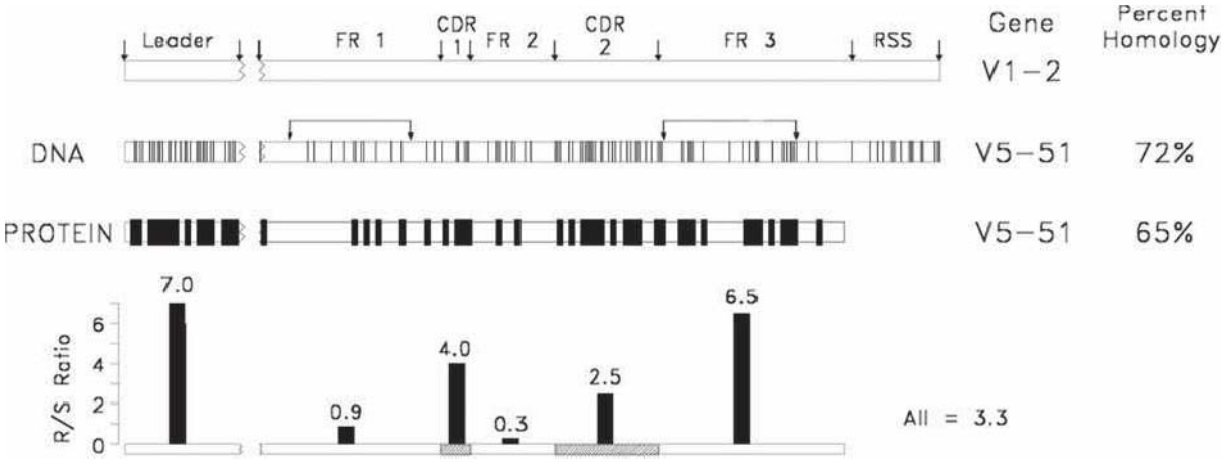

FIG. 5.7. A comparison of two human Clan I V_H sequences that belong to different V_H families (modified[4]). Shown is a comparison of the deoxyribonucleic acid and amino acid sequences of the V5-51 and V1-2 gene segments. Each line depicts a divergence in a nucleotide or amino acid at that position. Shown at the bottom is a replacement/silent site substitution analysis by interval. Random mutation tends to exhibit an R/S ratio of 2.9. The smaller the ratio, the greater the preservation of sequence. The intervals identified by the arrows predict the family and clan of origin.

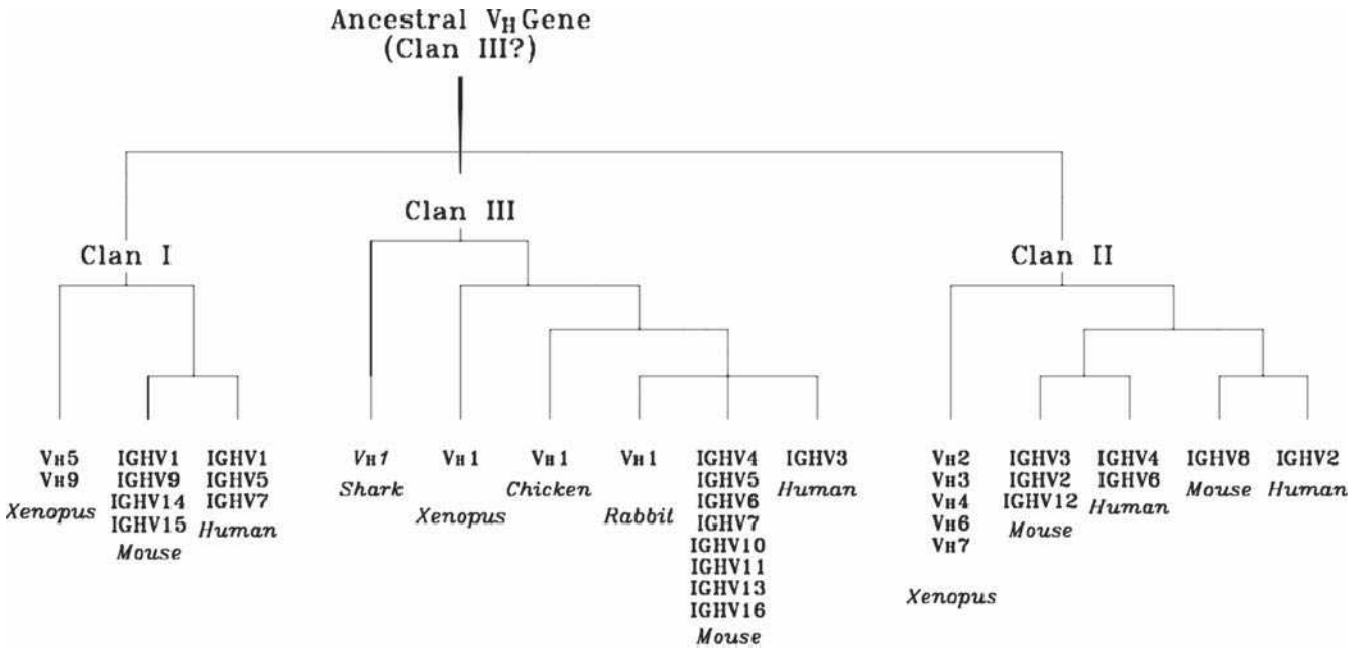

FIG. 5.8. Evolutionary Relationships among Vertebrate V_H Families. The sizes or relative placements of the evolutionary connecting lines are not to scale. V_H sequences from all mammalian species analyzed to date can be placed into one of these three clans (modified[4]).

intervals: CDRs-H1, -H2, and -H3; and CDRs-L1, -L2, and -L3.[31] The CDR sequences of V gene segments tend to be enriched for codons where mutations maximize replacement substitutions.[56] This includes the RGYW motif that facilitates somatic hypermutation.[57,58] While evolution appears to favor CDR1 and CDR2 sequences that facilitate codon diversity, it also appears to preserve specific loop structures.

Although there is great variation in the sequence and size of these CDRs, it has been shown that five of them, CDR-H3 being the notable exception, possess one of a small set of main-chain conformations termed canonical structures.[32,34,59–62] Each canonical structure is determined by the loop size and by the presence of certain residues at key positions in both the loop and framework regions. It can be calculated that the total number of possible combinations of canonical structures, or structure classes, is 300.[63] However, only 10 of these combinations, or classes, are sufficient to describe the majority of human and mouse Fab sequences. Among specific classes, the lengths of CDR-H3 and -L1 appear to correlate with the type of recognized antigen. Antibodies with short loops in -H2 and -L1 appear to be preferentially specific for large antigens (proteins), whereas antibodies with long loops in -H2 and -L1 appear to be preferentially specific for small molecules (haptens).[63]

Given that the sequence and structure of the framework regions, which define families, influences the canonical structure of V_H-encoded CDRs, it is not surprising that the structure repertoire of canonical structures is strongly associated with family and clan identity.[64] This implies restrictions to the random diversification of the hypervariable loop structures (canonical structures) and their combinations within the same V_H gene segment (canonical structure classes). It further suggests evolutionarily and structurally imposed restrictions operating to counteract the random diversification of these CDRs.

Diversity and Constraints on the Sequence and Structure of CDR-H3

The combination of VDJ assortment, variation in the site of gene segment rearrangement, and N nucleotide addition makes CDR-H3 the most variable of the six hypervariable regions. In some cases, the sequence of CDR-H3 appears designed to provide optimal flexibility.[65] Correspondingly, it has been more difficult to assign canonical structures to the CDR-H3 loops similar to those observed for the V-encoded CDRs. However, insight into a gradient of possible structures has been gained.

CDR-H3 can be separated into a base, which is adjacent to the frameworks, and a loop. The base tends to be stabilized by two common residues, an arginine at Kabat position 94 (IMGT 106) and an aspartic acid at Kabat 101 (IMGT 116).[32,33] These form a salt bridge which, together with the adjacent residues, tends to create one of three backbone conformations, termed kinked, extrakinked, and extended.[66] In some sequences with kinked or extrakinked bases, it is possible to predict whether an intact hydrogen-bond ladder may be formed within the loop of the CDR-H3 region, or whether the hydrogen-bond ladder is likely to be broken.[67,68] However, for many CDR-H3 sequences, especially those that are longer, current tools provide less than optimal predictions for the structures of individual CDR-H3s.

Despite the potential for totally random sequence provided by the introduction of N nucleotides, close inspection has shown that the distribution of amino acids in the

CDR-H3 loop is enriched for tyrosine and glycine,[31,69] and relatively depleted of highly polar (charged) or nonpolar (hydrophobic) amino acids, although the precise pattern depends on the species of origin.[70] This pattern of amino acid utilization is established early in B-cell development, prior to the expression of Ig on the surface of the cell (Fig. 5.9)[49,71–73] and reflects evolutionary conservation of J_H and D_H gene segment sequences. In particular, although the absolute sequence of the D_H is not the same, the pattern of amino acid usage by RF is highly conserved. Of the six potential RFs, RF1 by deletion is enriched for tyrosine and glycine. RF2 and RF3 by deletion are enriched for hydrophobic amino acids, as they are by inversion. RF1 by inversion tends to encode highly polar, often positively charged, amino acids.[69] Various species use different mechanisms to bias for use of RF1 by deletion, to limit use of hydrophobic RFs, and to restrict or prevent use of RFs enriched for charged amino acids. Forced rearrangement into RFs with charged amino acids yields an altered repertoire enriched for charge and depleted of tyrosine and glycine.[71]

The distribution of CDR-H3 lengths can also be regulated both as a function of differentiation and as a function of ontogeny.[49,74] In association with long V-encoded CDRs, short CDR-H3s create an antigen-binding cavity at the center of the antigen-binding site, and CDRs of intermediate length can create an antigen-binding groove. Each species appears to prefer a specific range of CDR-H3 lengths.[75] Long CDR-H3s, which can create "knobs" at the center of the antigen-binding site, are unevenly distributed between species and reflect both divergence in germline sequence and somatic selection.

The Antigen-Binding Site is the Product of a Nested Gradient of Regulated Diversity

The tension between the need to conserve essential structure and the need to emphasize diversity in an environment subject to unpredictable antigen challenge appears to create a gradient of regulated diversity in the Fv. The most highly conserved components of the Fv are FR2 and FR4, which form the hydrophobic core of the V_H:V_L dimer (see Figs. 5.4 and 5.5). FR1, which in the H chain presents with three conserved structures, helps form the ball and socket joint between the V_H and C_H1. FR3, which in the H chain defines the family and provides 7 different structures in human versus 16 different structures in mouse, frames the antigen-binding site (Fig. 5.10). The V-encoded CDRs, -H1, -H2, -L1, -L2, and most of -L3, are programmed for diversity. However, conserved residues within these CDRs, which interact with V family–associated FR3 residues, constrain diversity within a

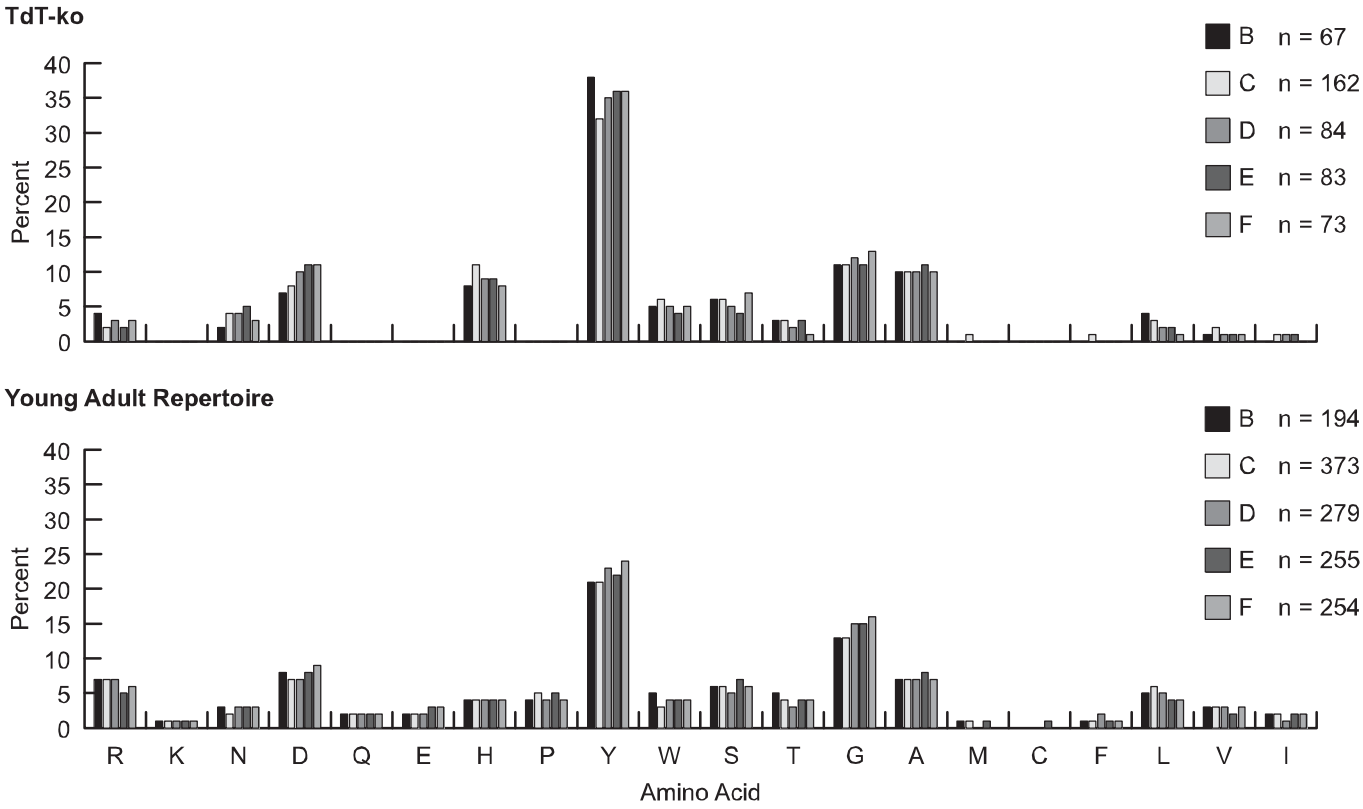

FIG. 5.9. Both in the absence or presence of N-addition, the preference for tyrosine and glycine in complementarity determining region-H3 begins early and intensifies with B-cell development. V_H7183DJCμ transcripts were cloned and isolated from fractions B (pro-B cells) through F (mature B cells) from the bone marrow of 8- to 10-week-old terminal deoxynucleotidyl transferase-sufficient and terminal deoxynucleotidyl transferase-knockout BALB/c mice.[73] The amino acids are arranged by relative hydrophobicity, as assessed by a normalized Kyte-Doolittle scale.[204,205] Use is reported as the percent of the sequenced population. The number of unique sequences per fraction is shown.

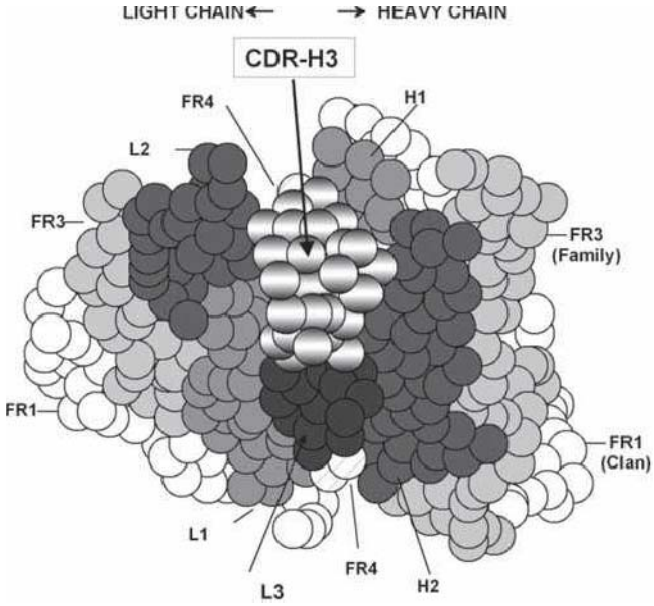

FIG. 5.10. Location and Generation of Complementarity Determining Region-H3. A: A cartoon of the classic antigen-binding site (modified[4]). Due to its central location, most antigens bound to the antibody will interact with complementarity determining region-H3.

preferred range of canonical structures. CDR-H3, the focus of junctional diversity, lies at the center of the antigen-binding site. The conformation of its base tends to fit within three basic structures. The loop varies greatly in sequence, yet still maintains a bias for the use of tyrosine and glycine. Thus, diversity increases with proximity to the tip of the antigen-binding site but appears to be held within regulated limits.

The extent and pattern of diversity in CDR-H3 can have a critical effect in the biologic function of Ig as a soluble effector molecule. Absence of N addition, with its enhancement of tyrosine and glycine usage (see Fig. 5.9) facilitates B-cell development, whereas enrichment for charged amino acids impairs it.[71,76,77] However, both the absence of N addition and enrichment for charged amino acids impair immune responses and protection in vivo.[71,76,78]

Somatic Hypermutation and Affinity Maturation

Following exposure to antigen and T-cell help, the V domain genes of germinal center lymphocytes can undergo mutation at a rate of up to 10^{-3} changes per base pair per cell cycle,[79] a process termed *somatic hypermutation*. Somatic hypermutation allows *affinity maturation* of the antibody repertoire in response to repeated immunization or exposure to antigen. Although affinity maturation often preserves the canonical structure of the CDR loops, the distribution of diversity appears to differ between the primary and antigen-selected repertoire.[80] In the primary repertoire, diversity is focused at the center of the binding site in CDR-H3. With hypermutation, somatic diversity appears to spread to the V-encoded CDRs in the next ring of the binding site (see Fig. 5.10), enabling a more custom-tailored fit.

Binding of "Superantigens" to Nonclassic Variable Domain Antigen-Binding Sites

Not all antigens bind to the paratope created by the classic antigen-binding site. Antigens that can bind to public idiotopes on V domain frameworks[81] and recognize large portions of the available repertoire are termed superantigens. There are indications that B cell superantigens influence the pathogenesis of some common infections, such as those caused by *Staphylococcus aureus*.

ANTIGEN-ANTIBODY INTERACTIONS

Technological advances in biomolecular structure determination, analysis of molecular dynamics, protein expression and mutagenesis, and biophysical investigation of receptor-ligand complex formation have facilitated significant advances in the understanding of antigen-antibody interactions. Particularly valuable has been the integration of high-resolution structural data with thermodynamic and kinetic analyses on a number of antigen-monoclonal antibody complexes. In this section, some of the key insights arising from these studies will be reviewed.

Molecular Flexibility

Like many other protein domains, V domains exhibit varying degrees and modes of molecular flexibility. Evidence suggests that some V modules (ie, V_L-V_H pairs) can adopt two or more conformations with meaningful frequencies in the unbound state. Because these different conformational states can exhibit distinguishable binding proclivities, molecular flexibility provides monoclonal antibodies with a mechanism for polyspecificity.[82] Molecular flexibility can also play a role in binding a single ligand, which, in such instances, may be better understood as a process of binding rather than as a simple event.[83,84] The extent of conformational adjustment by antibody or antigen required for complex formation can influence both the thermodynamics and kinetics of that process.

Role of Water

A significant role for water molecules has become clear from the study of high-resolution structures and thermodynamic analyses of antigen-antibody complexes. Water molecules exhibit a broad range of association times with protein surfaces. Thus some of the more tightly protein-associated of these solvent molecules effectively behave as parts of the protein.[85] Water molecules found in the antigen-antibody interface, whether constitutively bound or newly recruited, can make important contributions to both the intrinsic (ie, monovalent) affinity of the complex and to the differential affinities for different ligands (ie, specificity).[86] Amino acid residues of antigen and antibody can interact, indirectly, through hydrogen bonds to one or more water molecules.[86]

Thermodynamics and Antigen-Antibody Interactions

Specific residues in the antibody V domains or the antigen can contribute to complex formation in different ways, and some residues can contribute in multiple ways.[87] In addition

to making van der Waals contact, residues can be important due to contributions to the free energy of complex formation or to the differential free energy of complex formation for two or more different ligands. Some residues may contribute primarily to modulation of the association rate, the "relaxation" of the forming complex, or the dissociation rate.[83,88] Other contact residues contribute minimally to the energetics of complex formation, and yet other noncontact residues can be significant thermodynamic contributors.[89,90]

Structural and thermodynamic/kinetic comparisons of antibodies with germline V domain sequences and with somatically mutated V domain sequences have provided new insights into the structural and energetic bases for affinity maturation.[89,90] Mutations in both contact and noncontact residues can have major consequences, positive or negative, for the affinity with which an antibody binds an antigen. They can also favor tighter binding by enhancing V domain rigidity, thereby reducing the entropic penalty associated with complex formation.[90] Antibodies derived from secondary or later responses that have incorporated somatic mutations have been shown to exhibit less than absolute specificity. Even these "mature" antibodies can bind multiple ligands when screened on libraries of peptides or proteins,[91,92] a lesson likely to be relevant to most biomolecules.

It has been routine to distinguish between interactions in which antibodies bind to protein antigens versus those in which antibodies bind to carbohydrate antigens. Recent studies of antibodies that bind to human immunodeficiency virus-1 gp120 with high affinity and that exhibit potent and broad neutralizing activity suggest that antibodies can bind to epitopes composed of both peptide and glycan elements.[93,94]

IMMUNOGLOBULIN "ELBOW JOINTS" AND "HINGES"

The structure of the constant domains can affect antibody-antigen interactions by influencing the range of molecular flexibility permitted between the two Fabs. In this section, the role of the Ig hinge will be discussed.

Elbow Joints

Individual Ig V and C domains tend to create rather rigid dimers. However, the antibody molecule as a whole, which consists of four or more such dimeric modules linked like beads on a string, can be viewed as a paradigm of molecular flexibility.[95] Flexibility begins between the Fv and the Fb of the Fab at what is termed the elbow bend or elbow angle.[96] This reflects both a ball and socket interaction between the FR1 of the H chain and the C_H1 domain,[97] and the identity of the L chain.[96] Five residues (three in V_H and two in C_H1) that are highly conserved in both antibodies and T-cell receptors make the key contacts that constitute this "joint." Elbow angles, assessed from crystal structures of homogeneous Fab fragments, range from 130 degrees to 180 degrees. λ light chains appear to permit an Fv to adopt a wider range of elbow angles than their κ chain counterparts.

Hinges

Some Ig isotypes contain a structural element that does not strictly correspond to the canonical structural motifs of Ig superfamily V and C domains, which is termed the hinge. Where it occurs, it is located between the C-terminus of the C_H1 and the N-terminus of the C_H2 domains. In isotypes such as IgG and IgA, the hinge is encoded by one (or more) separate exon(s). In isotypes with four C_H domains (ie, IgM and IgE), the C_H2 domain serves in place of a classical hinge.

The hinge, or the C_H2 domain in Igs that lack a hinge, permits an Fab arm to engage in an angular motion relative both to the other Fab arm and to its Fc stem (Figs. 5.3 and 5.11). This permits the two Fab arms to cover a range from maximal extension to an almost parallel alignment. The range of motion of the Fab arms reflects the nature of the hinge region, which in some C genes is rigid and in others, such as human IgA1, functions more as a tether for each individual Fab than as a support. This flexibility has major implications for antibody function, because it enables a bivalent antibody molecule to bind epitopes in a variety of relative spatial arrangements.

Among human IgG subclasses, the most unusual hinge region is that of IgG3. Unlike other human IgG hinge regions, the IgG3 hinge is encoded by a quadruplicated hinge exon, making it the longest hinge (62 amino acids) by far (Table 5.2). The primary structure of this hinge has been divided into upper, core (or middle), and lower hinge regions with somewhat different functional associations. The upper hinge in particular has been associated with the magnitude of segmental flexibility as assessed by fluorescence emission anisotropy kinetics[98]

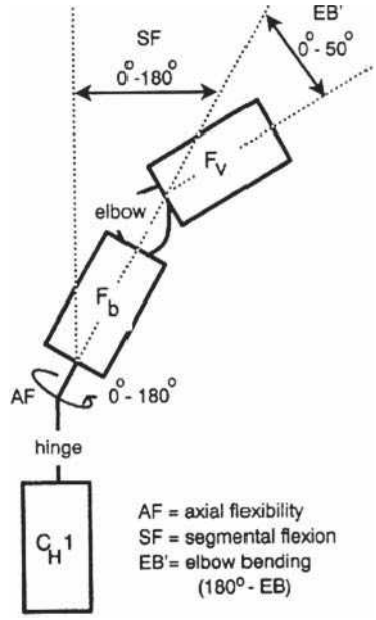

FIG. 5.11. Illustration of the Motions and Flexibility of the Immunoglobulin. Axial and segmental flexibility are determined by the hinge. The switch peptide (elbow) also contributes flexibility to the Fab. The measure of the elbow angle is defined with respect to the Fv and Fb axes of two-fold symmetry. From Carayannopoulos and Capra[206] with permission.

TABLE 5.2 Properties of Hinges in Immunoglobulin G, A, and D

Ig Type	Upper Hinge Length	Middle Hinge Length	Lower Hinge Length	Genetic Hinge Configuration (Amino Acids/Exon)	Susceptibility to Proteolysis	Special Features
IgG1	4	10	6	15		
IgG2	3	8	6	12		
IgG3	12	49	6	17-15-15-15		
IgG4	7	4	6	12		
IgA1	1	23	2	19	High	Heavy O-linked glycosylation
IgD				34-24	High	Extensive charged amino acids; heavy O-linked glycosylation at N terminus

and with the magnitude of Fab-Fab flexibility by immunoelectron microscopy.[99] The core or middle hinge appears to serve, at least in part, a spacer function. The lower hinge functions primarily to facilitate C_H2-C_H2 interactions.

Segmental flexibility of the Ig molecule, conferred mainly by the hinge, permits or facilitates simultaneous binding through two or more Fab arms. Such monogamous bivalency or multivalency, which enhances overall binding,[100,101] is a crucial factor permitting biosynthetically feasible antibody concentrations to offer adequate immunity against replicating pathogens. While it is more speculative why the Fab-Fc geometry needs to vary, it may have to do with optimizing effector function activation when antigen is bound, such as maintaining antigen binding when Fc receptors are simultaneously engaged.

The attribution of flexibility control to the hinge is supported by protein engineering studies in which V domain–identical IgGs of different subclasses were analyzed.[98,102] This basic conclusion is also supported by studies in which hinge regions have been selectively mutated or swapped among human or mouse IgG subclasses.[103–105] One of these studies indicated that structural variation among subclasses in the C_H1 domain also influenced segmental flexibility as assessed by nanosecond fluorescence polarization measurements.[103] Early suggestions that IgG subclass–related differences in activating the classical pathway of complement were explained by differences in segmental flexibility[98] were not confirmed by the studies in which mutant hinge regions were created.[103–105]

There are several types of molecular motion attributable to the hinge region that contribute to overall segmental flexibility (see Fig. 5.11). These include flexing between Fab arms (motion toward or away from one another in the same plane), Fab arms moving in and out of the same plane, Fab arms rotating along their long axes, and Fab arms moving in or out of the same plane as the Fc region.[106,107] The inter-Fab angles observed by electron microscopy range from 0 degrees to 180 degrees.[108,109] Similarly, Fab arm long-axis rotations can extend up to 180 degrees.[110]

Another key role of the hinge is the maintenance of the C_H2-C_H2 interaction (ie, effectively constraining molecular mobility within the Fc region itself). The lower hinge stabilizes C_H2-C_H2 contacts by providing the key cysteine residues involved in inter-H chain disulfide bonds. Experiments in which IgG molecules were modified to eliminate the hinge region demonstrate that covalent linkage between the hinge regions just "upstream" of the two C_H2 domains is critical for the preservation of IgG effector function.[111]

HEAVY CHAIN STRUCTURE AND FUNCTION

What might be termed the **fundamental strategy of humoral immunity** is a two-step process that begins with the identification by antibodies (of appropriate binding specificity) of the molecules or molecular complexes that should be eliminated. Following such identification (ie, noncovalent complex formation), antibodies can then trigger other molecular systems (eg, complement) or cells (eg, phagocytes) to destroy or remove the antigenic material—guilt by association at the molecular level. Thus, antigen specificity, determined primarily by the V domains in the Fab arms, is physically and functionally linked to effector function, the activation of which is primarily attributable to the C domains of the Fc region. The effector functions associated with the humoral immune response primarily involve either complement or Fc receptor–bearing cells, such as neutrophils, macrophages, and mast cells. As might be expected, therefore, the Fc contains sites for noncovalently interacting with complement components, such as C1q, and with Fc receptors. This section focuses on the structure and function of the Fc regions of the various Ig classes and subclasses.

Structure and Function of the Fc

The necessity for interacting effectively with relatively conserved molecules such as C1q and Fc receptors provides a selective basis for maintaining the primary structures of the Fc region, at least where changes would undermine such intermolecular contacts. The degeneracy possible in noncovalent molecular recognition events permits selected primary sequence variations without catastrophic alterations in function. However, there are allotypic differences in the H chain constant domains among both human and mouse Igs. In some cases, these allotypic differences are associated with variation in function, at least in vitro. For example, two recombinant V domain–identical IgG3 antibodies, of

different allotypes, exhibited differential abilities to bind C1q or initiate antibody-dependent cell-mediated cytotoxicity.[112] Nevertheless, the H chains of the human IgG subclasses are particularly well conserved, with > 90% identity of amino acid sequences. In other mammalian species that have two or more IgG subclasses, they tend to exhibit less amino acid sequence identity than the human IgG subclasses.

By convention, the H chain constant domains are numbered from N-terminal to C-terminal, with C_H1 residing in the Fab arm and the remaining two (IgG, IgA, IgD) or three (IgM, IgE) C domains (C_H2, C_H3, and, if relevant, C_H4) residing in the Fc. In IgM and IgE, the C_H2 domain largely plays the role of the hinge region. The C domains of different isotypes and from different species share several key structural features. In distinction from V domains, which consist of four- and five-stranded β-pleated sheets linked through an intrachain disulfide bond, C domains consist of three- and four-stranded β-pleated sheets linked through an intrachain disulfide bond (see Fig. 5.4). Across isotypes, amino acid sequence identity for C_H domains is approximately 30%, while for subclasses (within an isotype) the amino acid sequence identity for C_H domains is in the range of 60% to 90%. Important physical and biological properties of the human Ig isotypes are summarized in Table 5.3.

As noted previously, the traditional and accepted functional anatomy of Igs attributes antigen binding (both specificity and affinity) to the V modules in the Fab arms and effector function activation to the Fc region. While this scheme is both well supported and appealing, there is considerable (perhaps not widely appreciated) evidence that in some cases structural variations in H chain domains (ie, C_H domains and hinge) can influence both the affinity of the antibody for antigen and the discrimination among antigens.[113–118] Although these instances of C domain influence on ligand binding (through the V domains) primarily involve multivalent antigens, there are also reports that suggest C domain influence in instances of monovalent recognition.[119–121] Mechanisms for these effects in the context of binding multivalent antigens include isotype-related differences in segmental flexibility as well as the tendency for self-association. Results from a study comparing resistance to pneumococcal infection for IgG3-deficient and IgG3-producing mice are consistent with the notion that the cooperative binding permitted by murine IgG3 antibodies contributes to the effectiveness of humoral immunity.[122]

Fc Glycosylation

All Igs contain N-linked oligosaccharides, and it is becoming increasingly clear that this glycosylation plays significant roles in Ig structure and function. Though the type and extent of glycosylation varies among isotypes, an N-linked oligosaccharide on Asn 297 in the C_H2 domain is conserved on all mammalian IgGs and homologous portions of IgM, IgD, and IgE. As the average serum IgG contains 2.8 oligosaccharides, there is often glycosylation present in the V domain as well.[123] The consensus sequence for the V domain N-linked oligosaccharides is not present in the germline, but it can be created during somatic hypermutation.[124] Glycosylation in the Fc region has been shown to be important for antibody half-life and effector functions.[125–127] Glycosylation of the Fc domain influences complement activation as Ig hypoglycosylation influences affinity for C1q as well as Ig binding to the FcR, possibly due to its effects on Ig structure.[128] Differential sialylation of the core Fc polysaccharide has recently been shown to have dramatic effects on the proinflammatory versus anti-inflammatory activity of IgG.[129,130] Furthermore, IgD N-linked glycans are necessary for IgD to bind to the IgD receptor on T cells.[131]

V domain glycosylation potentially affects the affinity for antigen, antibody half-life, antibody secretion, and organ targeting. Interestingly, glycosylation has been shown to be capable of both positively and negatively affecting antigen binding.[132–135] The biologic significance of Ig glycosylation can be seen from studies demonstrating that IgG from patients with rheumatoid arthritis is galactosylated to a lesser extent (termed IgG G0) than IgG from normal controls. In some cases, hypogalactosylation correlates with disease activity.[136] Hypogalactosylation of IgG has also been found to occur in other chronic inflammatory diseases such as Crohn's disease and systemic lupus erythematosus.[137]

Immunoglobulin M

IgM is an isotype of firsts. It is ontogenetically primary, being expressed first on developing B lineage cells. IgM is also the isotype that initially dominates the primary humoral immune response. It is probably, along with IgD, a phylogenetically primitive isotype in jawed vertebrates (an almost first) and may be the most phylogenetically stable isotype.[138]

IgM serves important immunological functions both on the surfaces of B-lymphocytes and in the fluid phase in the blood and in the mucosal secretions. On the cell surface, IgM consists of two identical μ H chains and two identical L chains (μ_2L_2). It is initially expressed on B lineage cells in noncovalent association with surrogate L chains, and subsequently, following successful L chain gene rearrangement, with κ or λ light chains. On the mature B-cell surface, IgM is noncovalently associated with two other polypeptide chains, Ig-α (CD79a) and Ig-β (CD79b).[139–141] These integral membrane proteins serve to transduce signals when surface IgM binds to and is cross-linked by cognate antigen.

In the secreted form, IgM can consist of either pentamers $(\mu_2L_2)_5$ or, less often, hexamers $(\mu_2L_2)_6$.[142] The μ_2L_2 monomers of the pentameric form are linked one to another by disulfide bonds in the C_H4 domains. Two of these monomers are, on one side, disulfide bonded not to another μ chain but to a 15,000 Da-polypeptide, called J chain. J chain is also found in polymeric IgA. There may be multiple patterns of such disulfide bonding, such that different cysteines participate in different monomeric units.[143]

Application of electron microscopy to polymeric IgM molecules has suggested that IgM can adopt two different quaternary arrangements: star and staple.[144,145] All of the antigen-binding sites are arrayed in radial fashion, in the same plane as one another and the Fc regions, in the star

TABLE 5.3 Properties of Immunoglobulin Isotypes

Class or subclass properties	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgE
Molecular weight of secreted form (kDa)[a]	950(p)	175	150	150	160	150	160(m), 300(d)	160(m), 350(d)	190
Sedimentation coefficient	19S	7S		6.6S			7S	11S	8S
Functional valency	5 or 10	2	2	2	2	2	2 or 4	2 or 4	2
Interheavy disulphide bonds per monomer	1	1	2	4	11	2	2	2	1
Membrane Ig cytoplasmic region	3	3	28	28	28	28	14	14	28
Secreted Ig tailpiece	20	9	2	2	2	2	20	20	2
Other chain	J chain (16 kDa)	9	—	—	—	—	J chain (16 kDa) secretory component (70 kDa)		—
N-glycosylation sites	5	3	1	1	2	1	2	4	7
O-glycosylation sites	0	7	0	0	0	0	8	0	0
Carbohydrate average (%)	10–12	9–14	2–3	2–3	2–3	2–3	7–11	7–11	12–13
Adult level range (age 16–60) in serum (mg/ml)[b]	0.25–3.1	0.03–0.4	5–12	2–6	0.5–1	0.2–1	1.4–4.2	0.2–0.5	0.0001–0.0002
Approximate % total Ig in adult serum	10	0.2	45–53	11–15	3–6	1–4	11–14	1–4	0.004
Synthetic rate (mg/kg weight/day)	3.3	0.2	33	33	33	33	19–29	3.3–5.3	0.002
Biological half-life (days)	5–10	2–8	21–24	21–24	7–8	21–24	5–7	4–6	1–5
Transplacental transfer	0	0	++	+	++	++	0	0	0
Complement activation classical pathway (Clq)	++++	0	+++	+	++++	0	0	0	0
Complement activation alternative pathway	0	0	0	0	0	0	+	0	0
Reactivity with protein A via Fc	0	0	++	++	+/–	++	0	0	0
Allotypes	—	—	G1m	G2m	G3m	—	—	A2m	Em
Biological properties	Primary antibody response, some binding to pIgR, some binding to phagocytes	Mature B-cell marker	Placental transfer, secondary antibody for most responses to pathogen, binds macrophages and other phagocytic cells by FcγR				Secretory Ig, binds pIgR		Allergy and parasite reactivity, binds FcεR

Ig, immunoglobulin; d, dimer; m, monomer; p, pentamer.
[a] Light chain molecular weight is 25 kDa.
[b] Total = 9.5-21.7 mg/ml.
Compiled from Carayannopoulos and Capra,[208] Lefranc and Lefranc,[209] Kuby,[210] and Janeway et al.[211]

arrangement. In the staple form, the Fab arms bend out of the plane of the Fc regions. It has been conjectured that the staple form is utilized in binding simultaneously to two or more epitopes on multivalent antigens, such as bacterial or viral surfaces.

A major pathway through which soluble IgM mediates immunity or immunopathology is the activation of the classical pathway of complement. On a per molecule basis, relative to other isotypes, IgM is highly active in activating the classical pathway and can thereby effectively opsonize bacterial pathogens. In select cases (eg, *Neisseria meningitidis*), binding of IgM to bacterial surfaces, followed by complement activation, can cause direct lysis of the bacteria through the insertion of the membrane attack complex into the bacterial membrane.[146] IgM, like polymeric IgA, can reduce the effective number of colony- or plaque-forming units for, respectively, bacteria and viruses, through agglutination. Significant physical and biological properties of IgM and the other Ig isotypes are shown in Table 5.3.

Immunoglobulin D

IgD is primarily of interest in its membrane form, as the soluble form of IgD is found in relatively modest concentrations in the blood and other body fluids. The cell surface form of IgD is found along with IgM on all mature, naïve B cells, where it appears capable of transducing activating and tolerizing signals.[147] As is true for IgM, the membrane form of IgD associates noncovalently with Ig-α (CD79a) and Ig-β (CD79b). Simultaneous cell surface expression of two H chain isotypes expressing the same V_H domains and the same L chains occurs via differential ribonucleic acid splicing.[148]

IgD exhibits greater sensitivity to proteolytic cleavage than IgM, which is consistent with a relatively short serum half-life of only 2.8 days. The relatively long hinge region is a primary target for proteolysis. Relatively modest efforts have historically been devoted to investigating of the functional roles of IgD antibodies, especially in the secreted form, in comparison to the other isotypes. However, new information suggests that IgD antibodies are produced in the upper respiratory mucosa by an unanticipated mechanism of class switch recombination and that these antibodies participate in host defense against pathogens relevant to this anatomical environment. In addition, secreted IgD antibodies can bind to basophils, through a receptor that is yet to be identified, and when these antibodies are cross-linked by cognate antigen, the basophils release potent mediators that influence immune reactivity, inflammation, and pathogen viability.[149]

Immunoglobulin G

IgG is the predominant isotype (approximately 70% to 75% of the total Ig) in the blood and extravascular compartments. The four human IgG subclasses (IgG1, IgG2, IgG3, and IgG4) are named in order of their relative serum concentrations, with IgG1 the most prevalent and IgG4 the least. There are differences in effector functions (eg, complement activation and Fc receptor binding) and other biological properties (such as serum half-life) among these subclasses. However, there are also crucial functional commonalities, such as placental passage (see Table 5.3).

IgG antibodies are the hallmark of immunological memory in the humoral immune response. In addition to the isotype switch from IgM to IgG in a secondary antibody response, somatic hypermutation can lead to affinity maturation, a process by which the average affinity of antibody for the antigen eliciting the immune response can increase.

IgG antibodies contribute to immunity directly and through the activation of complement or FcR-bearing cells. Important examples of immunity mediated directly through antibody binding include neutralization of toxins (eg, diphtheria toxin) and viruses (eg, poliovirus). Medically important examples of IgG-induced complement activation include immunity to encapsulated bacterial pathogens leading either to opsonization and destruction within phagocytes (eg., *Streptococcus pneumoniae*) or to direct complement-mediated lysis (eg, *Neisseria meningitidis*). Activation of FcR-bearing cells by IgG antibodies has also been implicated in immunity to pathogens (eg, *Cryptococcus neoformans*).[150] The consensus view is that human IgG1 and IgG3 isotypes are effective activators of the classical complement pathway. While some older sources state that IgG2 and IgG4 are weak or nonactivators of the classical complement pathway, more recent evidence suggests that when epitope density is high, IgG2 is effective in activating complement.[151,152] One possible source for the isotype-related variation in complement-activating ability is variation in affinity for C1q (IgG3 > IgG1 > IgG2 > IgG4), the portion of the first component in the classical pathway that physically contacts the C_H2 domains of antibodies. However, isotype-associated differences in complement activation have also been found to occur at steps of the cascade subsequent to the binding of C1q to antibody.[112,153] For example, in one study of chimeric monoclonal antibodies engineered to express identical V domains and representing all four human IgG subclasses, the IgG3 antibody fixed C1q better than the IgG1 antibody, but the IgG1 molecule was more effective in mediating complement-dependent cell lysis than the IgG3 molecule.[153] Thus, it is probably not possible to rank the relative abilities of the IgG subclasses to activate complement in a single absolute hierarchy.

The affinities of IgG subclasses for Fc receptors vary from about 5×10^5 M^{-1} to about 10^8 M^{-1}. Recent studies in the mouse suggest that the relative contributions of IgG subclasses to various immunopathological processes depend on their relative affinities for the activating versus inhibiting isoforms of FcR.[154]

A remarkable attribute of IgG (for three of the four subclasses) is its serum half-life of about 23 days. This property, attributable to the Fc region and its interaction with the neonatal Fc receptor (FcγRn), has been exploited for therapeutics through the genetic fusion of solubilized receptors, (eg, cytotoxic T-lymphocyte antigen 4) to IgG Fc regions.[155]

A recently derived insight into the function of IgG4 antibodies relates to their apparently unique ability to spontaneously dissociate into half-molecules and to form antibodies composed of different half-molecules with distinct antigen

specificities (ie, bispecific antibodies that cannot cross-link antigen and thus have anti-inflammatory activity).[156] This phenomenon may be of particular relevance to specific immunotherapy in the setting of allergy.[157]

Immunoglobulin A

While IgG is the clearly predominant isotype in the blood, IgA is the dominant Ig isotype in the mucosal secretions as well as in breast milk and colostrum.[158]

In the blood, 10% to 15% of the Ig is IgA (vs. 65% to 75% IgG). Moreover, IgA has a shorter half-life than IgG in serum. The predominant form of IgA in human serum or plasma is monomeric (ie, α_2L_2), but there are small quantities of dimers $[(\alpha_2L_2)_2]$ and fewer still trimers and tetramers. Secretory IgA consists of dimers and lesser amounts of trimers and tetramers associated with one joining (J) chain (distinct from the J region in the heavy chain V domain) and one SC chain (see following discussion). The latter is the extracellular portion of the polymeric Ig receptor (pIgR), which is expressed by mucosal epithelial cells and transfers polymeric IgA or IgM from basolateral to apical surfaces, thereby providing most of the Ig content of the mucosal secretions. The J chain is disulfide bonded to the tail pieces, short C-terminal extensions of the C_H3 domains, of the two IgA monomers of a dimer, while SC forms a disulfide bond to a cysteine in one C_H2 domain of one monomer.

The two α H chain C region genes correspond to two IgA subclasses, IgA1 and IgA2. IgA1 is the predominant (> 80%) IgA subclass in the serum. While IgA2 is the major form in some human mucosal secretions, such as those in the large intestine and the female genital tract, there is variation in the relative proportion of IgA1 and IgA2 in different secretions. The shorter hinge region of IgA2 confers increased resistance to bacterial proteases that might be encountered in the mucosal environment. The extended hinge region of IgA1 is believed to permit molecules of this isotype to accommodate variable epitope spacings on multivalent antigens. While the long hinge region of IgA1 molecules might be expected to confer relatively high susceptibility to proteolysis, relative protection against the activity of bacterial proteases is provided by heavy O-linked glycosylation in the hinge (Fig. 5.12). Nevertheless, the IgA1 hinge is uniquely susceptible to IgA proteases produced by certain pathogenic bacteria.[159]

Secretory IgA (S-IgA) has been shown to participate in immunity against a range of viral, bacterial, and parasitic pathogens at mucosal surfaces. The relative absence of functional complement and phagocytes in mucosal secretions is consistent with a different mix of mechanisms for mediating immune effects associated with S-IgA versus, for example, serum IgG. Mechanisms associated with IgA are less dependent on inflammation-producing molecules or cells, such as inhibition of microbial adherence through V module-mediated specific binding to microbial adhesins, agglutination of microbes, blocking of microbial receptors for cell surface carbohydrates with IgA-associated glycans, and mucus trapping (in which binding of S-IgA to bacteria makes them more adherent to host-generated mucus). There is also evidence that polymeric IgA can neutralize viruses

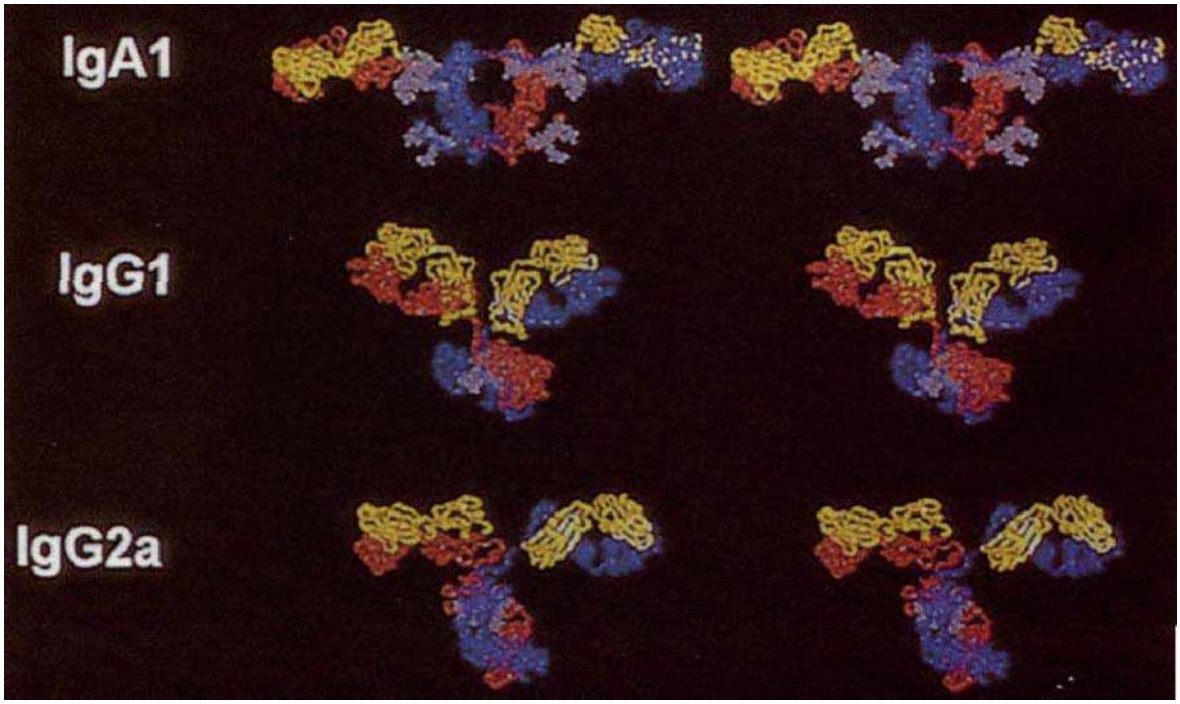

FIG. 5.12. A comparison of an x-ray and neutron-solution–scattering theoretical model (human immunoglobulin (Ig)A1) and x-ray crystal (murine IgG1 and IgG2a) structures. Light chains (*yellow*), heavy chains (*red* and *dark blue*), and glycosylation (*light blue*) are illustrated. The extended length of IgA1 over that of IgG can be seen along with extensive glycosylation that characterizes this isotype. From Boehm et al.[207] with permission.

in some circumstances by interfering with steps postattachment, such as internalization. In some cases, posttranslational modifications of viral surface molecules, related to proteolytic events associated with epithelial cell transit, lead to IgA antibodies expressing protection-related antigen specificities that have no parallel in the IgG pool.[160,161] Some of the contributions of IgA to immunity are mediated through binding to FcαRI (CD89) on human neutrophils, monocytes/macrophages, and eosinophils. For example, cells bearing FcαRI on their plasma membranes can phagocytose IgA-antigen complexes. FcαRI binds to the IgA Fc region between the Cα2 and the Cα3 domains.[162] Amino acid residues in the IgA Fc region critically involved in the interaction with CD89 are indicated in Figure 5.13.

The ability of IgA to activate complement is controversial. At present, the preponderance of evidence suggests that IgA does not activate the classical complement pathway and only weakly, and under some pathophysiological circumstances, activates the alternative complement pathway. However, there is evidence suggesting that, in vitro at least, polymeric, but not monomeric, IgA can activate the complement pathway dependent on mannose-binding lectin.[163]

Additional properties of the polymeric forms of IgM and IgA are considered in the following section.

Immunoglobulin E

IgE is best known for its association with hypersensitivity reactions and allergy, but this isotype is also of interest in the context of immunity to parasites. In the blood, IgE is present at the lowest concentration of any of the Ig isotypes (with roughly five orders of magnitude less IgE than IgG) and has the shortest half-life. The unimpressive quantitative representation of IgE in the blood is related to the high affinity of IgE antibodies for FcεRI, often referred to as the high-affinity Fc receptor for IgE. FcεRI is expressed on mast cells, basophils, Langerhans cells, and eosinophils. Due to the high affinity of FcεRI for its IgE ligand, mast cells and basophils are covered with relatively long-lived FcεRI-IgE complexes.

The interaction between IgE and FcεRI has an affinity of ~10^{10} L/M. It primarily involves contacts between FcεRI and amino acids in the C_H3 domains, with some contributions from amino acids in the C_H2 domains. Although each IgE potentially has two sites for interacting with the FcεRI, the stoichiometry has been shown to be 1:1. Furthermore, it has been suggested that IgE binds to FcεRI in a kinked conformation.[164]

Upon ligation with bivalent or multivalent antigens specifically recognized by the bound IgE molecules, the FcεRI molecules transduce signals that activate the mast cells or basophils to secrete potent mediators of inflammation, such as histamine. These mediators are responsible for the symptoms associated with asthma, allergic rhinitis, and anaphylaxis.

There is a second receptor for IgE. FcεRII (CD23), a type II membrane protein, is expressed on monocytes/macrophages, B-lymphocytes, natural killer cells, follicular dendritic cells, Langerhans cells, eosinophils, activated epithelial cells, and platelets. It binds monomeric IgE with an affinity of ~10^7 L/M, roughly three orders of magnitude lower than the affinity of FcεRI for IgE. Functional consequences of FcεRII-IgE interaction on macrophages include secretion of mediators of immediate hypersensitivity as well as cytokines and chemokines. There is also evidence suggesting that the FcεRII-IgE interaction can contribute to antigen capture and presentation to both B and T cells.

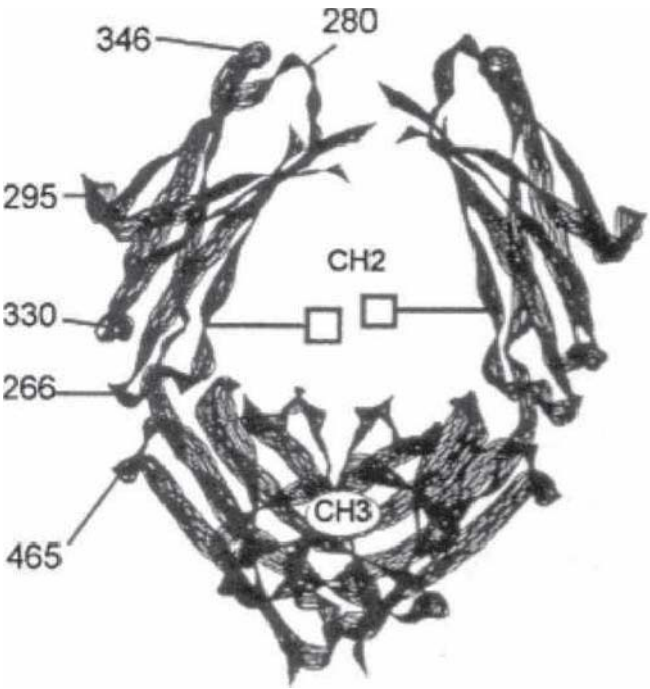

FIG. 5.13. Illustration of the Residues Essential for the Binding of immunoglobulin (Ig)A to FcαR (CD89). Residues represent mutations made in IgA C_H regions as mapped on an Fcγ fragment. Of the residues mutated, L465 and L266 were found to be important for binding to CD89. From Carayannopoulos et al.[162] with permission.

Fc Receptor Immunoglobulin Interactions

There are four receptors that bind the Fc regions of IgG molecules in humans and five such receptors in mice. Three of the human receptors are expressed primarily on hematopoietic cells directly involved in immune responses: FcγRI, FcγRII, and FcγRIII. Among the FcγR involved in antibody effector functions, there is important variation in affinity for IgG molecules. FcγRI binds IgG with relatively high affinity, permitting the binding of monomeric IgG. In contrast, FcγRII and FcγRIII bind to IgG with relatively low affinity. Consequently, these latter two receptors do not bind significant quantities of monomeric IgG but preferentially interact with IgG that has been effectively aggregated through interaction with bivalent or multivalent antigens (ie, immune complexes). Key amino acid residues involved in the binding FcγRs and FcεR1 with their corresponding Ig can be seen in Figure 5.14. Allotypic variations in FcγRIIA can also influence affinity for IgG ligands and subsequent effector function.[165]

There are different isoforms of FcγRII and FcγRIII. Of particular functional relevance, FcγRIIA is activating,

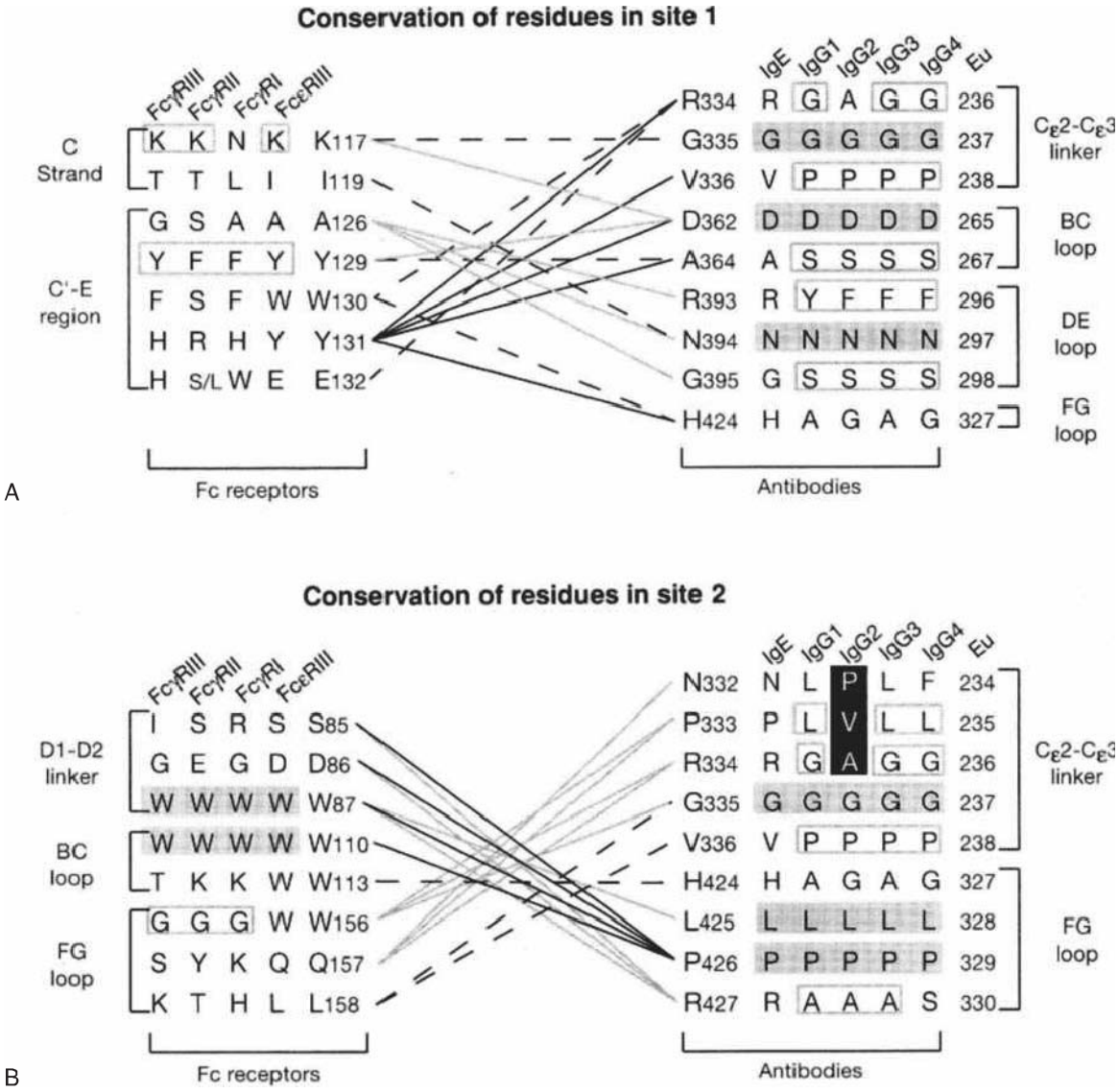

FIG. 5.14. Certain residues are conserved between Fcγ Rs and FcεRI as well as between immunoglobulin (Ig)G and IgE that facilitate binding. Two sites participate: site 1 in **(A)** and site 2 in **(B)**. *Heavy lines* indicate the highest number of contacts and *dashed lines* indicate the least. Of considerable note are residues W87 and W110 in site 2 of the receptors and P426 in the Ig that form a core "praline sandwich" in the interaction between Ig and receptor. From Garman et al.[208] with permission.

whereas FcγRIIB is inhibiting. In mouse models, deficiency of FcγRIIB can be associated with an autoimmune syndrome similar to human lupus.[166] Studies in mice have also suggested that the effectiveness of antibodies of the various murine IgG subclasses in mediating FcγR-dependent effector functions are correlated with the ratio of affinities of antibodies of those subclasses for activating versus inhibiting FcγR.[154]

FcγRIIIA is expressed on natural killer cells, whereas FcγRIIIB is expressed on neutrophils. The former receptor is attached to the membrane by a standard transmembrane polypeptide, whereas the latter is attached via a glycophospholipid tail.

The fourth human receptor, the neonatal FcγR, or FcγRn (sometimes referred to as the Brambell receptor), transports IgG across the placenta. It also plays a crucial role in protecting IgG from proteolytic degradation, thereby prolonging serum half-life. While FcγRI, FcγRII, and FcγRIII are members of the immunoglobulin superfamily, FcγRn is structurally similar to major histocompatibility complex class I molecules, including noncovalent association with β_2-microglobulin. The interaction between FcγRn and IgG involves amino acid residues in the C_H2-C_H3 interface[167] and is pH-sensitive. This latter property is consistent with the ability of FcγRn to bind IgG in acidic vesicular compartments and then release it into the neutral-pH environment of the blood. A crystallographic structure of the rat FcγRn in a 1:1 complex with a heterodimeric Fc (containing only one FcγRn-binding site) reveals that there are conformational changes in the Fc on binding to FcγRn.[168] The investigators also identified three titratable

salt bridges that confer pH-dependent binding of the IgG Fc to the FcγRn.

There are other FcR that interact with the non-IgG isotypes: IgM, IgD, IgA, and IgE. The FcR for IgA and IgE are covered in the respective sections devoted to the corresponding isotypes. In mice, an additional Fcα/μR binds both IgA and IgM, whereas in human these receptors are distinct.[169,170] Functional attributes of these receptors and the FcδR are continuing to be studied.

There are functional as well as structural parallels among FcR for IgG and non-IgG isotypes. Some of the features of Fc-FcR interaction that are conserved across isotypes are illustrated in Figure 5.14.

Transmembrane and Cytoplasmic Domains

Igs are expressed in both membrane and secreted forms. In contrast to the secreted form, the membrane Ig contains a transmembrane and a cytoplasmic domain. The transmembrane domain is a typical single-pass polypeptide segment consisting of 26 hydrophobic residues, extending from the C-terminus end of the C-terminal C_H domain and which forms an alpha-helix followed by a variable number of basic amino acids. The cytoplasmic portion of the Ig H chain ranges in length from 3 amino acids for IgG to 30 amino acids for IgE.

Both membrane expression of Ig and the integrity of the cytoplasmic domain are important in antibody function. As the cytoplasmic domain is rather short, membrane-bound IgM is not thought to "signal" directly, but through the associated Igα and Igβ molecules.[171] However, disruption of either membrane expression of IgG1 in mice or the cytoplasmic tail results in the failure to generate an effective IgG1 response and IgG1 memory.[172] Mice lacking IgE membrane expression exhibit significant impairment in IgE responses and have extremely low levels of secreted IgE.[173] For those Igs with longer membrane:cytoplasmic tails, membrane Ig affects signaling beyond its association with Igα and Igβ. Specific residues in the transmembrane domain have been identified that are crucial for signal transduction while having no effect on the association with Igα and Igβ.[174]

HIGHER ORDER STRUCTURE

Many of the biological functions of IgA and IgM are dependent on their ability to form multimeric structures. This section will discuss the role of multimeric Ig in immune function.

Dimers, Pentamers, and Hexamers

The majority of multimeric IgA exists as dimers and, less commonly, trimers and tetramers, while IgM forms pentamers and occasionally hexamers. The polymeric structures of these antibodies enhances their functional affinity (avidity) for antigen, is essential for their active transport (both IgA and IgM) across epithelial cells to mucosal secretions, and in the case of IgM, enhances the activation of the classical pathway of complement. Once multimerized, IgA or IgM in a complex with J chain can bind to pIgR and cross mucosal epithelial cells.[175,176] Though IgM can undergo transcytosis to the mucosal secretions, its principal action is in the serum.

The ability of IgA and IgM to multimerize is due to a tailpiece, an additional C-terminal segment of 18 amino acids in the secreted forms of the μ and α heavy chains. Tailpieces of both IgM and IgA contain a penultimate cysteine (residue 575 in IgM and 495 in IgA) that forms two different disulfide bonds important for multimer formation. In an Ig monomeric unit containing two identical H chains, one cysteine residue forms intermonomeric subunit bonds, whereas the remaining cysteine residue on the other heavy chain bonds to a cysteine on the J chain.[177–180]

Another cysteine residue, Cys414, also forms intermonomeric subunit disulfide bonds in IgM, and this bond is important in hexamer formation.[181] Besides disulfide bonds, the highly conserved glycan linked to Asn563 in IgM (and the homologous region in IgA) is also important for multimerization.[182]

Domain-swapping experiments demonstrate that the tailpiece regulates multimerization in the context of the specific H chain. While addition of the α tailpiece to IgM has little effect on IgM polymerization, the introduction of the μ tailpiece to IgA leads to higher-order IgA polymers.[183] Based on this finding, it has been proposed that IgM polymerization is more efficient than IgA polymerization.

The J Chain

The J chain, an evolutionarily conserved 137 amino acid polypeptide produced by B-lymphocytes, functions to regulate multimer formation and to promote linkage of multimeric Ig to pIgR on epithelial cells. The J chain consists of a single domain in a beta barrel conformation and does not show sequence similarity to Ig domains.[184] It contains eight cysteine residues that participate in disulfide bonds with two tailpiece cysteines, as described previously, as well as function to stabilize its own structure through intramolecular bonds.[183,185] The J chain influences the polymerization of the multimers, as in the absence of J chain, IgA forms fewer dimers and IgM forms fewer pentamers.[186]

The J chain exists in all polymeric forms of IgA and is important in IgA polymerization and secretion across the mucosa. J chain is not required for IgM polymers but is required for external secretion. While IgM pentamers contain J chain, hexamers almost always lack it. The makeup of IgM is biologically significant because IgM hexamers have about 20-fold greater complement-activating activity than IgM pentamers. The presence of increased levels of hexameric IgM has been postulated to play a role in the pathogenesis of Waldenström macroglobulinemia and cold agglutinin disease.[187]

Immunoglobulin Transport

Transport of dimeric IgA and pentameric IgM to the mucosal secretions occurs after binding to pIgR that is present on the basolateral surface of the lining epithelial cells. The J chain is essential for the secretion of IgA and IgM and,

as described previously, influences the polymeric structure of the Ig.[175] pIgR is a transmembrane receptor synthesized by mucosal epithelial cells that contains seven domains including five extracellular V-like domains, a transmembrane domain, and a cytoplasmic domain.[188] Once bound to pIgR, polymeric Ig is endocytosed and transported to the apical surface of the cell. pIgR is then proteolytically cleaved between the fifth and sixth domains to release a complex (termed secretory Ig) containing the H, L, and J chains, and the SC, which represents the cleaved extracellular portion of pIgR.[189,190] As pIgR undergoes constitutive transcytosis in the absence of polymeric Ig, free SC is also released into the mucosal secretions. SC has several biological functions, including protecting the Ig from degradation by proteases and binding bacterial antigens such as the *Clostridium difficile* toxin A.[191,192] SC also functions to localize sIgA to the mucus layer to help protect against invasion by pathogens.[193] Results from pIgR-null mice demonstrate that alternate pathways exist to transport polymeric Ig to the mucosal secretions as some secretory Ig still crosses the epithelial cells in the absence of pIgR.[194] Results from pIgR-null mice also demonstrate the importance of high levels of secretory antibodies as these mice are more susceptible to mucosal infections with pathogens such as *Salmonella typhimurium and Streptococcus pneumoniae*.[195,196]

The epithelial transcytosis of polymeric Ig has several biological implications. First by delivering the Ig to the mucosal surface, it enables antibodies to bind to pathogenic agents and prevent them from penetrating the mucosa, a process termed immune exclusion. Second, transcytosing antibody can neutralize viruses intracellularly.[197,198] Finally, polymeric Ig can bind to antigens in the mucosal lamina propria and excrete them to the mucosal lumen (where they can be removed from the body) by the same pIgR-mediated transcytosis process.[199,200] Some pathogens can exploit the pIgR-mediated transcytosis process in reverse to penetrate the mucosa. For example, the pneumococcal adhesin, CbpA, can bind pIgR at the epithelial apical surface, leading to bacterial penetration of the mucosa.[201]

CONCLUSION

Igs are extremely versatile molecules that can carry out many biological activities at the same time. The need to be able to recognize unique antigen structures prior to any previous exposure coupled with the need to maintain host cell receptor or complement recognition properties presents a truly unique challenge for the system. As has been described, the system incorporates diversity within specific constraints. The precise biological niches may differ, but the overall design for these molecules is the same.

The flexibility and biologic properties of Igs have made them a major focus of molecular engineering. Igs are being used as therapeutic agents, as well as for biotechnology applications. These opportunities have led to a resurgence of interest in the structure–function aspects of antibodies as we approach "designer antibodies." Both the variable and constant portions of these molecules are current substrates for engineering purposes, offering the potential for altering both receptor and effector function. The study of antibodies began with the need to understand how sera could neutralize toxins. It is likely that antibodies will continue to be a major focus for those who seek to take fundamental principles of protein chemistry to the bedside.

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CHAPTER 6

Immunoglobulins: Molecular Genetics

Edward E. Max • Sebastian Fugmann

INTRODUCTION

To respond to a foreign molecule (antigen) on an invading pathogen, the "humoral" immune system generates antibodies, or immunoglobulins (Igs), that can bind specifically to the offending antigen. Each antibody molecule is composed of two identical light (L) chains and two identical heavy (H) chains, all linked by disulfide bonds to form a symmetric Y-shaped tetramer. The ability of the immune system to generate an antigen-specific antibody depends on the fact that, before exposure to antigen, millions of naïve resting B cells circulate in the individual, each cell displaying on its membrane several thousand identical copies of a single unique species of antibody; these serve as B-cell receptors (BCRs) for that lymphocyte. Only a tiny fraction of the B cells express a BCR capable of binding to any particular antigen. When these B cells bind their antigen, they become activated to proliferate and mature into antibody-secreting plasma cells, which manufacture large amounts of antibody specific for the activating antigen. To be able to generate antibodies against a universe of diverse pathogens, this "clonal selection" mechanism for specific antibody secretion requires an enormous diversity of Ig species expressed on naïve B cells prior to antigen exposure. Indeed, in the 1960s the number of different antibody sequences in the repertoire of typical mouse was estimated in the millions. To encode this many sequences seemed to require an unreasonably high percentage of the mammalian genome (now estimated to contain only about 30,000 genes). Understanding the genetic source of Ig diversity—Ig gene assembly—was the first major challenge and achievement of the molecular biologic investigations of antibody genes, and this will be discussed first in this chapter.

A week or so after antigen administration, the B-cell response changes in two ways that generally improve the protective functions of antibodies. B cells initially express antibodies of the IgM isotype, but cells that migrate into germinal centers receive T-cell-derived stimuli that can induce them to switch to production of IgG, IgA, or IgE without changing their antigen specificity; this switch results from a deoxyribonucleic acid (DNA) recombination event known as class switch recombination (CSR). In addition, over the course of an immune response, the affinity of antibody for antigen gradually improves as a result of somatic hypermutation (SHM) of antibody genes, coupled to selection for B cells expressing high-affinity antibodies. CSR and SHM are discussed later in this chapter.

In this chapter, well-established facts about Ig genes are summarized concisely, while areas currently under investigation are considered in more detail, with particular attention to topics expected to interest immunologists.

OVERVIEW OF IMMUNOGLOBULIN GENE ASSEMBLY

In the 1960s, investigators determined the amino acid sequences of Igs secreted by several mouse myelomas (clonal tumors of B-lymphocytes that secrete a single pure species of Ig). The N-terminal domains of the L and H chains—each roughly 100 amino acids—were highly diverse between different myeloma proteins and were designated variable (V) regions. In contrast, sequences of the remaining domains of the proteins were essentially identical for every myeloma Ig of a given class (and so they were designated constant [C] region domains). The advent of recombinant DNA technology allowed comparisons of V region genes expressed in different myelomas with the corresponding sequences in nonlymphoid DNA (commonly referred to as "germline" DNA). It was found that each myeloma V gene is composed of several segments that are separated in germline DNA; these germline segments must undergo one or more DNA recombination events to assemble a complete V region.[1] For example, each complete Vκ gene from a myeloma or B-lymphocyte encodes roughly 108 amino acids and is assembled by linking one of about 40 germline Vκ segments (encoding amino acids 1 through 95) to one (of five) "joining" or Jκ segments encoding residues 96 to 108. Similarly, a complete Vλ gene is assembled from one germline Vλ segment and one Jλ segment. H chains are assembled from three segments; a diversity (D) segment is interposed between V_H and J_H. In developing B cells, the germline gene segments are assembled into functional V exons by a process named V(D)J recombination (Fig. 6.1).

V(D)J recombination is a "cut and paste" process in which the DNA between two recombining V, D, or J gene segments is excised from the chromosome, and the two remaining DNA segments are joined together to reseal the DNA break. The two principal proteins executing the "cut" phase of this process are encoded by the recombination activating genes (RAG)1 and 2. These proteins recognize unique sequences, known as recombination signal sequences (RSSs), that flank and mark each eligible V, D, and J gene segment (RSSs are described further in the following). After the RAG proteins cut the DNA, the subsequent "joining" of the gene segments relies largely on ubiquitous DNA repair factors.

How Recombination Contributes to Diversity

V(D)J recombination contributes in several distinct ways to the diversity of antigen-binding specificities. First, there is

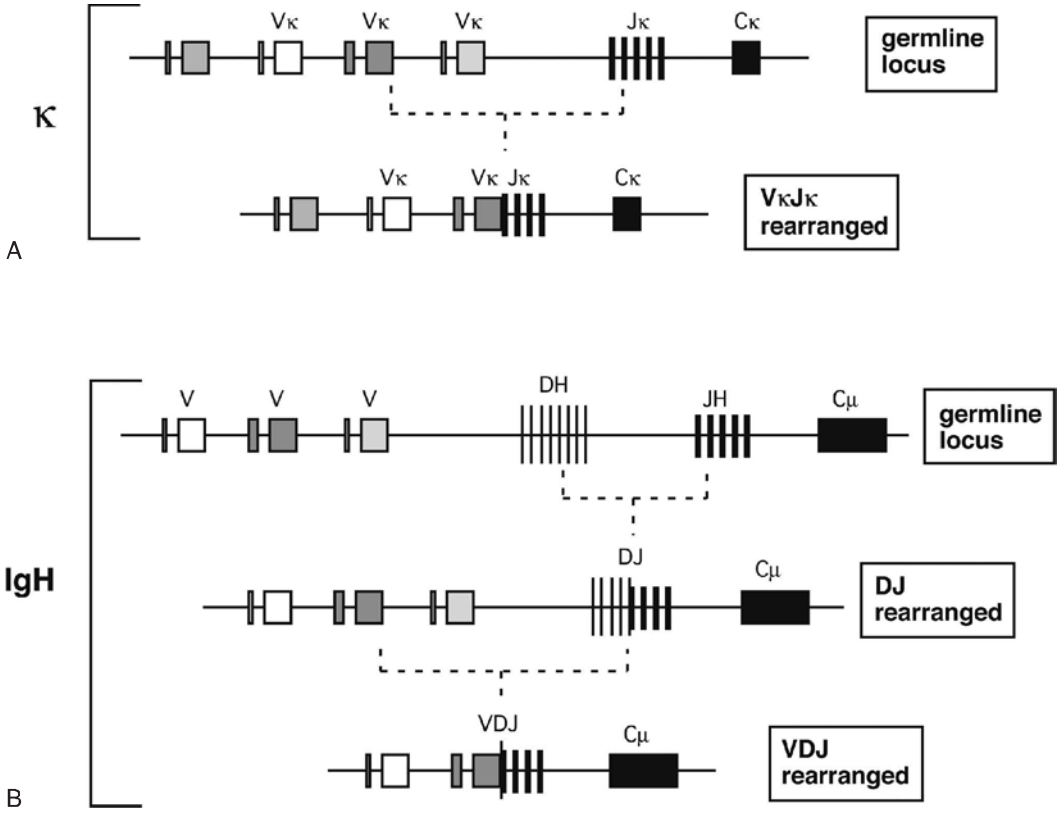

FIG. 6.1. Variable Assembly Recombination. **A:** In the κ locus, a single recombination event joins a germline Vκ region with one of the Jκ segments. **B:** In the immunoglobulin H locus, an initial recombination joins a diversity segment to a J_H segment. A second recombination completes the variable assembly by joining a V_H to DJ_H.

combinatorial diversity, as each Ig locus contains multiple V, D (in case of the IgH locus), and J segments that can be combined in many ways. The total number of theoretically possible combinations of V_H, D_H, J_H, V_L, and J_L, is the multiplication product of the numbers of possible H chains—about 40 (V_H) × 27 (D_H) × 6 (J_H) or 6480 combinations in humans—times the number of possible L chain combinations (about 290), or almost 2 million. This repertoire is vastly larger than could be achieved by devoting the same total lengths of DNA sequence to preassembled variable region exons. Second, there is junctional diversity generated by flexibility in the position of joining between gene segments. This was initially recognized by comparisons of nucleotide sequences of various myeloma Vκ genes to their germline Vκ and Jκ precursors. As shown in Figure 6.2A, these comparisons revealed that the crossover point between sequence derived from a germline Vκ region and a Jκ region could vary in different myelomas, increasing the diversity of amino acids around codons 95 and 96. H chain VDJ exons exhibit this flexibility at both V-D and D-J junctions, yielding striking variation in the lengths D region–derived segments, from zero to about 14 amino acids. And additional junctional diversity is produced by the addition of nucleotides not present in any germline elements: "N" and "P" nucleotides, discussed below. Importantly, the three-dimensional structures of Igs established by X-ray crystallography reveal that the V_L-J_L junction and the V_H-D_H-J_H junction each encode one of the three "complementarity determining region" loops of L or H chain that can contact antigen; thus, this junctional diversity is directly functionally relevant for diversifying antigen binding.

The imprecision of V(D)J recombination increases Ig diversity, but at a cost. Because the precise boundaries between V, D, and J result from independent stochastic events, only about one-third of all recombination events maintain the correct reading frame through the J segments. Gene rearrangements leading to functional Ig genes are often referred to as "productive," while out-of-frame rearrangements are labeled "nonproductive."

Function of Recombination Signal Sequences

Analysis of DNA sequences flanking the germline V, D, and J gene segments revealed highly similar sequence motifs that have subsequently been shown to define targets for V(D)J recombination: the RSSs, which serve as the recognition sequences for the V(D)J recombinase proteins RAG1 and RAG2, as mentioned previously. Notably, RSSs lie adjacent to L- and H-chain Ig gene segments and to T-cell–receptor (TCR) gene elements throughout phylogeny. RSSs consist of a conserved seven base pairs (bps) long "heptamer" (consensus: CACAGTG) and a nine bp long "nonamer" sequences

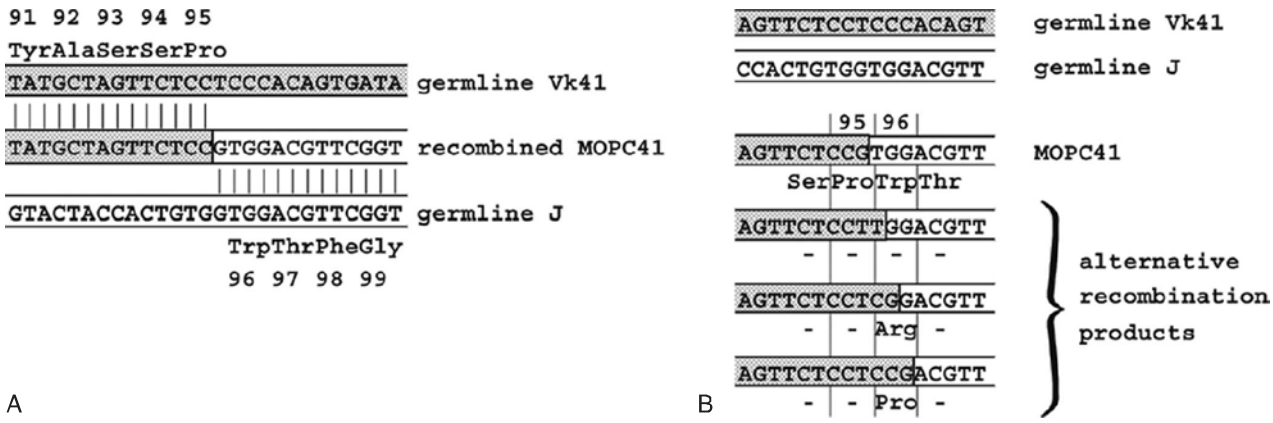

FIG. 6.2. Vκ-Jκ Recombination at Single Base Resolution. A: The sequence of the recombined MOPC41 κ gene around the VJ junction is shown (*center*) with the sequences of the two germline precursors (Vκ41 and Jκ1) shown above and below. The germline origins of the recombined gene are indicated by the vertical lines and the shading of the V-derived sequence. **B:** The consequences of joining the same germline sequences (from part *A*) at four different positions are shown. Of the four alternative recombination products illustrated, the top one is that actually found in MOPC41. The second example has a single nucleotide difference but no change in encoded amino acid sequence. The third and fourth alternatives yield Arg or Pro at position 96; both of these amino acids have been found at this position in sequenced mouse κ chains.

(consensus: ACAAAAACC) that are separated by less well-conserved spacers of either approximately 12 or 23 bp in length (Fig. 6.3). Based on the spacer lengths, the two classes of RSSs are referred to as 12-RSSs and 23-RSSs, respectively. (Note that some laboratories use the term recombination signal instead of RSS in their publications.)

Recombination occurs almost exclusively between coding sequences associated with RSSs of different spacer lengths, a requirement referred to as the "12/23-rule" (i.e., the recombination between two 12-RSSs [or two 23-RSSs] is "forbidden" and does not occur *in vivo*). Within each gene locus, all gene segments of one class (e.g., all Vs in the Igκ locus) carry RSSs with the same spacer length. Thus the 12/23 rule drives appropriate recombination events leading to functional VJ and VDJ products, and prevents futile recombination events, such as between two V or two J gene segments. While the heptamer and nonamer are the major determinants of RSS function necessary for V(D)J recombination, increasing evidence suggests that spacer sequences can modulate recombination efficiencies of compatible gene segments (e.g., they affect the non-random usage of human Vκ elements[2]).

THE THREE IMMUNOGLOBULIN GENE LOCI

To understand the contribution of the germline V, D, J element repertoire to Ig diversity, several laboratories undertook cloning and sequence analysis of individual V region genes from the IgH, Igκ, and Igλ loci of human and mouse. More recently, the complete sequences of all human and mouse Ig loci have been determined as part of the genome sequencing projects for these two species (available online at www.ncbi.nlm.nih.gov, though annotation that describes

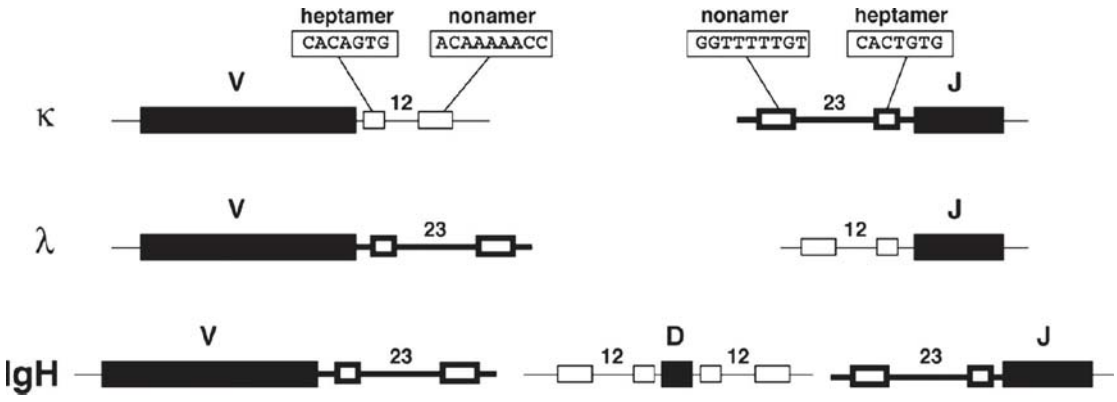

FIG. 6.3. Conserved Elements Flank Germline Variable (V), Diversity (D), and Joining (J) Region Genes. Conserved heptamer and nonamer recombination signal sequences (RSSs) lie adjacent to V, D, and J coding sequences and are important for targeting V(D)J recombination. The heptamer and nonamer elements are separated by spacer regions of about 12 basepairs (bp) (*thin lines*) or 23 bp (*thick lines*). Depending on the locus, V regions may be flanked by 12 bp or 23 bp RSS; and similarly for J regions. But one of each type of element must be present for recombination to occur, a requirement that prevents futile recombination events (e.g., J to J).

TABLE 6.1 Overview of the Number of Variable, Diversity, and Joining Segments in Each of the Three Immunoglobulin Loci in Humans and Mice

Locus	Species	V		D		J	
		Functional	*Pseudogenes*	*Functional*	*Pseudogenes*	*Functional*	*Pseudogenes*
IgH	Mouse	110	85	10		4	
	Human	40	83	24	3	6	3
Igκ	Mouse	95	45			4	1
	Human	46	87			5	
Igλ	Mouse	3				3	1
	Human	36	56			4–5	2–3

D, diversity; Ig, immunoglobulin; J, joining; V, variability.
Pseudogenes are recognized based on sequence defects that would preclude function (premature stop codons, defective recombination signal sequences, defective splice sites). The numbers in this table are approximate, owing to variation between mouse strains and between individual humans.

function and refers to earlier literature is incomplete). It is important to point out that Ig gene loci are not identical between individuals (humans) or between individual strains of inbred mice. Several Internet resources are devoted to providing convenient updated access to Ig germline gene sequences. The international ImMunoGeneTics database (http://imgt.org) includes a database for Ig and TCR genes from a variety of species, and includes maps, sequences, lists of chromosomal translocations, and multiple helpful links. IgBLAST (www.ncbi.nlm.nih.gov/igblast/) is a service of the National Center for Biotechnology Information and allows a submitted sequence to be searched against known annotated germline V, D, and J sequences.

The Murine Immunoglobulin H Germline Variable, Diversity, and Joining Gene Segments

V_H Segments

The murine V_H region extends over about 2.5 megabases on chromosome 12 and includes roughly 100 functional segments (depending on mouse strain) plus additional V_H pseudogene segments (Table 6.1). All V_H elements are in the same transcriptional orientation as the D, J_H, and C_H regions.[3] The V_H segments are classified into 16 distinct families based on sequence similarity; V_H elements within a family show more than 80% nucleotide sequence identity. Elements of individual V_H families are largely clustered together, though some interdigitation occurs (Fig. 6.4). The V_H families can be grouped into three "clans" based on sequence conservation primarily of their framework regions (framework region 1, codons 6 to 24, and framework region 3, codons 67 to 85, respectively), which form the more conserved structural backbone of the Ig variable region. Importantly, these clans are conserved between man, mouse, and frog, suggesting that their emergence in the repertoire preceded the amphibian-reptile divergence.[4]

Diversity and J_H

About 50 kb downstream of the most 3′ V_H element resides the murine D cluster spanning about 80 kb, depending on

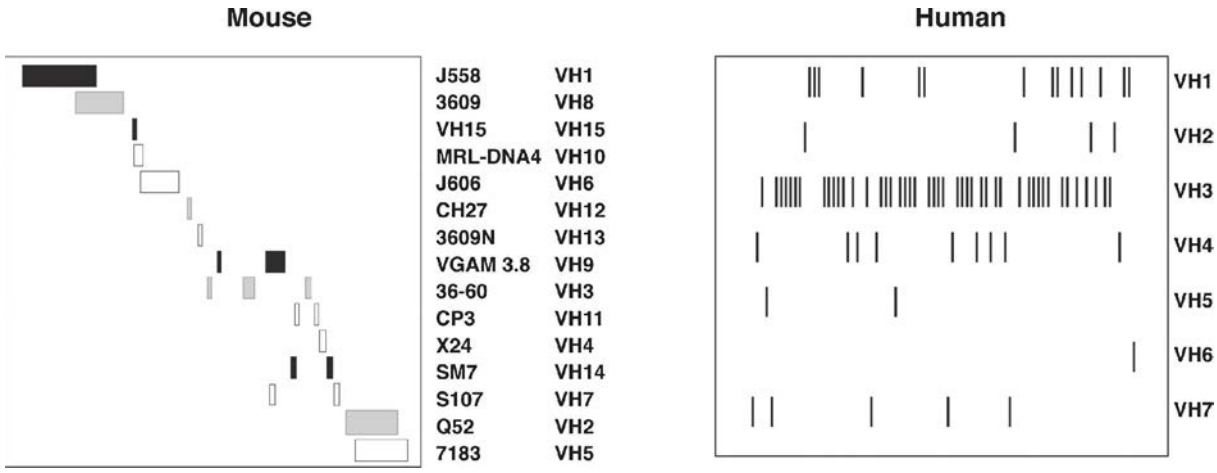

FIG. 6.4. Maps of the Murine and Human V_H Loci. The 15 known murine V_H gene families are shown in their approximate map positions. Each *rectangle* represents a cluster of V_H genes of the indicated family; the clan identification of the V_H families is indicated by the color of the rectangle: *black* for clan I, *gray* for clan II, and *white* for clan III. Although some interdigitation is shown by overlapping families (e.g., the Q52 and 7183 families), the murine V_H families are largely clustered. In contrast, all human V_H genes (*vertical lines*) of a prototypic haplotype are shown in the right panel; extensive interdigitation of families is apparent.

the mouse strain (see Table 6.1). Each D segment is flanked by 12 RSSs on both sides, so that the 12/23 rule ensures that all assembled V genes carry a D element between their V and J segments (which are both flanked by 23 RSSs that prevent direct V to J rearrangements).

The murine D elements are classified into four families: DSP2, DFL16, DST4, and DQ52. Although D regions could theoretically contribute to Ig diversity by being read in all three frames, the mouse has evolved mechanisms that strongly favor one of them.[5] Four functional germline J_H sequences reside about 0.7 kb downstream of the most 3′ D region, DQ52.

The Human Immunoglobulin H Germline Variable, Diversity, and Joining Gene Segments

V_H Segments

The human V_H locus spans 1.1 Mb at the telomeric end of chromosome 14 (14q32.33) (see Table 6.1). The human germline V_H segments—numbering roughly 40 to 45—fall into seven families that, in contrast to the family clusters characteristic of the murine locus, are extensively interdigitated (see Fig. 6.4). Some human V_H sequences are polymorphic owing to V_H insertions or deletions in different allelic chromosomes. Twenty-four additional germline V_H sequences have been mapped to chromosome 15 and 16 and represent nonfunctional "orphans" that were apparently duplicated from the IgH locus on chromosome 14.[6]

Diversity and J_H Regions

Twenty-six human D elements are located in an ~40 kb region about 20 kb downstream of VH6, the most 3′ of the V_H genes.[7] This D cluster is comprised of four tandem duplications of a 9.5 kb segment containing a representative of each of six D families. The twenty-seventh D element—DHQ52—is the only one showing sequence similarity to a mouse segment (DQ52) and shares a homologous location just 5′ to J_H1. In contrast to mice, humans use all reading frames of D elements.[7] One reading frame encodes primarily hydrophilic residues, one encodes hydrophobic residues, and one includes frequent stop codons. Some D elements contain stop codons that can be removed by nuclease trimming during VDJ assembly. As in mice, the human J_H cluster is immediately downstream of DHQ52.

Heavy Chain Constant Regions

Murine and human genomic clones containing C region H-chain (C_H) genes include separate exons encoding the ~100 to 110 amino acid Ig domains. These domains were independently identified by internal homologies of amino acid sequences and by three-dimensional structural analysis (X-ray crystallography). The exons are separated from each other by introns of roughly 0.1 to 0.3 kb. Thus, for example, the mouse γ2b protein has three major domains (CH1, CH2, and CH3) with a small hinge domain between CH1 and CH2. The gene structure may be summarized as follows:

CH1	intron	**hinge**	intron	**CH2**	intron	**CH3**
(292)	(314)	(64)	(106)	(328)	(119)	(322)

where the numbers in parentheses represent the number of nucleotides in each segment. As an interesting contrast, the hinge region of the α gene is encoded contiguously with the CH2 domain with no intervening intron, while the unusually long human γ3 hinge is encoded by three or four hinge exons.

Genomic Organization of the C_H Region

Each B-lymphocyte initially produces IgM by expressing an assembled variable region linked to Cμ, but may use CSR (discussed later in this chapter) to replace Cμ with one of the several C_H regions lying downstream, thereby allowing expression of IgG, IgA, or IgE (Fig. 6.5A). Eight murine C_H genes span about 200 kb of DNA on chromosome 12; these genes were linked by contiguous clones in 1982.[8] Several γ pseudogenes lie within the clustered γ functional genes[9] (Fig. 6.5B). The coding sequences of all C_H genes are oriented in the same direction.

The human C_H genes were similarly cloned, and then eventually completely linked by the Human Genome Project. The human IgH locus contains a large duplication, with two copies of a γ–γ–ε–α unit separated by a γ pseudogene (see Fig. 6.5B). One of the duplicated ε sequences is also a pseudogene, and a third closely homologous ε-related sequence—a "processed" pseudogene—is present on chromosome 9.

The IgH locus has also been examined in several other species besides mouse and human, and several notable differences have been observed. Rabbits, for example, have 13 Cα sequences and only a single Cγ gene[10]; this unusual expansion of genes contributing to mucosal immunity may be related to the peculiar habit of coprophagy in these animals. In contrast to the multiplicity of rabbit Cα genes, pigs have only one Cα gene and eight Cγ genes. Camels are unusual in having H chains that function in the absence of L chains.[11]

Membrane versus Secreted Immunoglobulin

Igs are found either as secreted molecules in the serum or as membrane-bound receptors. The membrane-bound μ chains contain a C-terminal hydrophobic transmembrane domain consisting of 26 uncharged hydrophobic amino acids encoded by additional membrane exons, and these residues anchor the protein in the cell membrane lipid bilayer. The membrane (μ_m) and secreted (μ_s) forms are derived from the same gene by alternative splicing (Fig. 6.6). The same general gene structure has been found for other C_H genes, suggesting that differential splicing accounts for the two forms of all Ig isotypes.

Early B cells make roughly similar quantities of both μ_m and μ_s, whereas maturation to the plasma cell stage is associated with strong predominance of μ_s production, facilitating high-level secretion of circulating Ig. The balance between the two ribonucleic acid (RNA) splice forms of μ has been interpreted as a competition between splicing of the CH4 and M1 exons versus the cleavage/polyadenylation at the upstream μ_s poly(A) site. These processes are mutually exclusive because CH4-M1 splice removes the μ_s poly(A) site, while cleavage at the μ_s poly(A) site removes the membrane exons.

Cis-regulatory elements (and corresponding transacting RNA binding proteins) control the balance between these

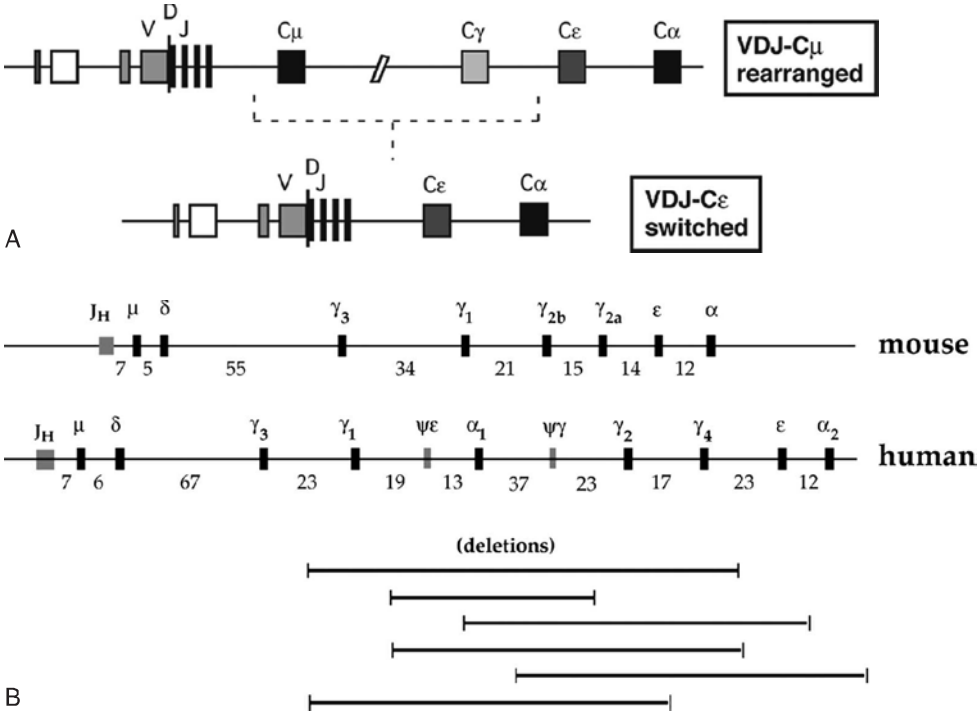

FIG. 6.5. Deletional Isotype Switch Recombination. A: The expression of "downstream" heavy chain genes is accomplished by a recombination event that replaces the Cμ gene with the appropriate heavy chain constant gene (Cε is shown as an example), deleting the deoxyribonucleic acid between the recombination breakpoints. **B:** The murine and human heavy chain constant region genes are diagrammed with the approximate intergene distance indicated below (in kb); various literature values for these distances differ somewhat, possibly due to allelic polymorphisms. The human locus shows a large duplication of γγεα sequences.

processes. They include a GU-rich element downstream of the μs poly(A) site,[12] the polyadenylation factor cleavage stimulator factor 64,[13] and the U1A protein.[14] These factors likely function downstream of B-lymphocyte–induced maturation protein-1 (BLIMP-1) whose expression in Ig-secreting plasma cells was also found to be critical for μ_s poly(A) site utilization.[15] *Cis*-acting sequences affecting the ratio of alternative splice forms have been described for other isotypes besides Cμ, particularly Cα.[16]

Membrane Ig serves as the antigen-recognition component of the BCR that is critical for initiating the signal for lymphocyte activation following contact with antigen. Transduction is mediated by an associated protein dimer composed of the BCR components Igα and Igβ (CD79a and CD79b) whose cytoplasmic domains contain immunoreceptor tyrosine-based activation motifs similar to those found in the CD3 chains mediating TCR signaling. Additional signaling is mediated by conserved tyrosines in the cytoplasmic tails of the IgG and IgE H chains, which serve as a phosphorylation-dependent docking sites for the signaling adapter Grb2.[17] Binding of Grb2 enhances BCR signaling and subsequent B-cell proliferation.

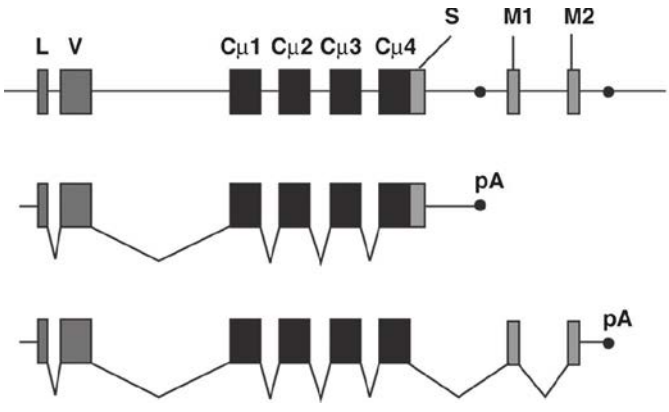

FIG. 6.6. Two Ribonucleic Acids (RNAs) Generated from the μ Gene by Alternative Processing. The *top line* illustrates the exons of the μ gene (*black rectangles*) in an expressed, rearranged μ gene. A primary transcript including all the exons present in the deoxyribonucleic acid can be processed as shown to yield either μ_s RNA (containing a C-terminal "secreted" sequence) or μ_m RNA (containing the two membrane exons).

Kappa Light Chain Genes

Murine Germline Vκ Locus

The murine Vκ locus spans about 3.2 mb on chromosome 6[18] and contains 20 Vκ families, some of which are shared by human and mouse (see Table 6.1). Vκ sequences within a single family are largely clustered together. Some Vκ elements lie in the opposite orientation to that of the Jκ and Cκ elements, and these Vκ segments undergo VJ recombination by an inversion rather than deletion (Fig. 6.7). A few Vκ sequences have been localized to chromosome 16 and 19 and are considered orphan genes.

Human Germline Vκ Locus

The human Vκ locus (see Table 6.1) lies on the short arm of chromosome 2 (2p11-2) spanning ~2 mb of DNA.[19] The locus includes a large inverted duplication, so that most Vκ sequences exist in pairs with one copy lying in the cluster proximal to Jκ (and in the same orientation) and a second copy (inverted) in the distal cluster. The average sequence similarity between duplicates is 98.9%, suggesting the duplication occurred less than 5 million years ago. This is consistent with the absence of such duplication in chimpanzees, which diverged from the human lineage approximately 6 million years ago. Interestingly, about 5% of human alleles also lack the distal duplication.

Outside the Igκ locus, at least 25 orphan Vκ segments have been identified in clusters on chromosome 1, 2, and 22. The orphan cluster located in the long arm of chromosome 2 was probably separated from the major locus—on the short arm of this chromosome—by a pericentric inversion (which must have occurred rather recently in evolution as it is absent from chimpanzee and gorilla).

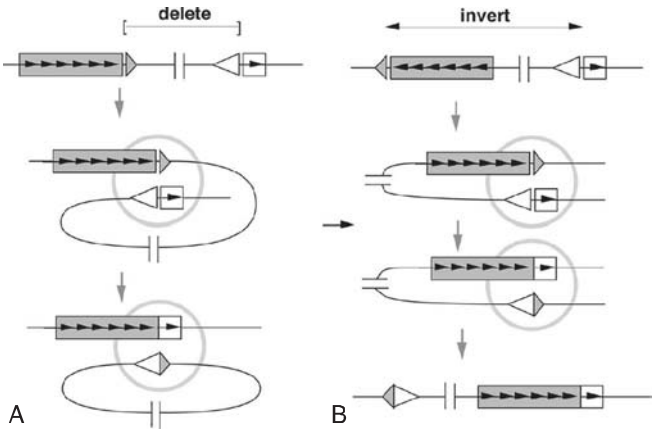

FIG. 6.7. The Same "Micro" Mechanism of Recombination can Join Vκ and Jκ by Deletion or Inversion, Depending on the Relative Orientation of the Two Precursors in Germline DNA. **A:** When V coding sequence (*shaded rectangle*) and J coding sequence (*white rectangle*) are oriented in the same 5′ → 3′ direction in germline DNA (as indicated by the *internal arrowheads*), the recombination yields a VJ coding joint plus a DNA circle containing the signal joint (*apposed triangles*). **B:** If V is oriented in the opposite direction in germline DNA, an identical recombination reaction at the "micro" level (*inside shaded circle*) leaves the signal joint linked to the recombined VJ coding joint.

Jκ and Cκ Elements

In comparison to the H chain genes, the organization of the C region segments in the κ locus is relatively simple (see Table 6.1). A single Cκ gene with a single exon and with no reported alternative splice products is found in both mouse and human. While all five Jκ elements are functional in humans, the third J element in mice has not been observed in functional κ L chains.

Apart from the typical Vκ-Jκ rearrangements, an additional recombination event occurs uniquely in the κ locus. The event is mediated by V(D)J recombination utilizing a 23-RSS element—designated Recombining Sequence in the mouse[20] and Kappa Deleting Element in the human[21]—that is positioned in an intergenic region downstream of Cκ; the recombination results in the deletion of the Cκ exon. Hence, Cκ fragments are undetectable on Southern blots of DNA from λ-expressing human lymphoid cells,[22] as in most B cells the Cκ genes are apparently deleted from both chromosomes before Igλ gene rearrangement begins.

Lambda Light Chain Genes

Murine λ Locus

In laboratory mouse strains, only about 5% of the B-lymphocytes utilize Igλ L chain, and the diversity of these L chains is meager due to the very small number of V region genes (Fig. 6.8). Complete sequence analysis[23] of the murine locus revealed two V-J-C clusters (Vλ2-Vλx-Jλ2Cλ2-Jλ4Cλ4 and Vλ1-Jλ3Cλ3-Jλ1Cλ1) separated by about 110 kb. Each Jλ is linked to its own Cλ region gene, but Jλ4 is nonfunctional. Recombination occurs largely within each cluster, although Vλ2Cλ1 products are occasionally observed. The ancestry of the Vλx element is uncertain, as it is rather dissimilar to the other Vλ segments; indeed, it resembles Vκ as much as Vλ. In contrast to the Igκ locus, the Vλ segments are flanked by 23-RSS and the Jλ gene segments by 12-RSS (see Fig 6.3).

Human λ Locus

The human Vλ region was characterized by intensive cloning, sequencing, and mapping of Vλ elements and ultimately by the complete sequence analysis of 1 Mb covering the entire locus[24] (see Table 6.1). Within the Vλ cluster lies the human VpreB gene (discussed below), as well as several genes and pseudogenes unrelated to the Igλ system.

λ L chains are much more abundant in man than in mouse (about 40% of human L chains are λ versus about 5% in mouse). Four forms of human λ chains have been classified serologically, with differences residing in a small number of amino acids in the C region. The serologic classification of Kern+ corresponds to a glycine at position 152 versus a serine in Kern–. The Oz+ designation corresponds to a lysine at position 190 versus an arginine in the Oz– variant. Similarly, Mcg+ λ chains (versus Mcg–) contain asparagine 112 (versus alanine), threonine 114 (versus serine) and lysine 163 (versus threonine).

Four functional human Jλ-Cλ segments and three pseudogenes are clustered within an approximately 33 kb region of DNA (see Fig. 6.8) and the four major expressed human λ isotypes correspond to the functional JCλ1, JCλ2, JCλ3, and

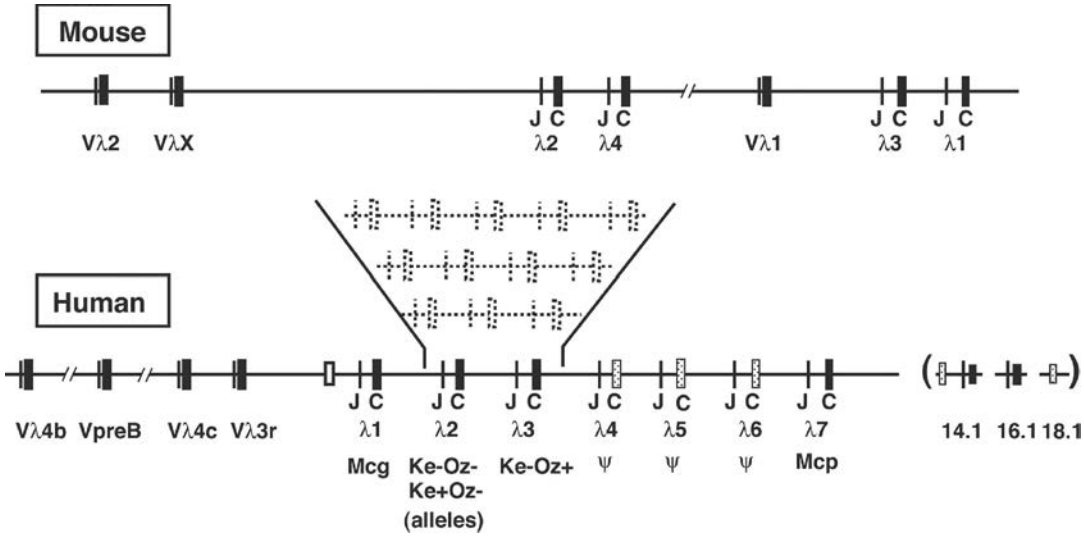

FIG. 6.8. Germline λ Genes. The maps in this figure are schematic (i.e., not to scale). **A:** The murine λ gene system includes four JC complexes and three V genes, as shown. **B:** The human λ locus includes multiple V genes, of which only three are shown. The human VpreB "surrogate" light chain gene is located within the Vλ cluster. The Cλ locus includes a segment of seven JC complexes plus three additional unlinked sequences. The hatched JC complexes diagrammed above the seven linked λ sequences represent polymorphic variants with additional duplications of the JC unit. The 14.1 sequence—the human λ5 "surrogate" light chain homolog—lies downstream of the JC cluster. Exon 1 of the 14.1 gene is homologous to an exon upstream of Jλ1 (as indicated by the *unlabeled white rectangle*).

JCλ7, with the latter encoding an isotype provisionally designated Mcp.[25] JCλ6 may be functional in some individuals, and the common allele—which has a 4 bp insertion leading to a deletion of the C-terminal third of the Cλ region—can nevertheless undergo Vλ-Jλ recombination, encoding a truncated protein that can associate with H chains. A variety of polymorphic variants of the human λ locus have been detected, apparently the result of gene duplication, as shown in Figure 6.8.[26] Lastly, three Cλ-related sequences have been discovered near the major Jλ-Cλ cluster. One of these, designated λ14.1, represents the human homolog of the murine "surrogate" L chain λ5 (see following discussion).

λ-Related "Surrogate" Light Chains

Ig H chains cannot reach the cell surface without pairing with Ig L chains. However, Ig μ H chains can be detected on the surface of pre–B cells whose Igκ and Igλ loci are still in their germline configuration and thus do not produce L chains. In these cells, a "surrogate L chain" (SLC) composed of two smaller proteins, VpreB and λ5, facilitates the surface expression of the μ H chain protein. The first component (λ5) was identified as the product of a gene expressed exclusively in pre–B cells that showed high sequence similarity to the J and C regions of the λ locus,[27] As four murine Cλ genes were already known, it was designated λ5. The second component of the SLC was identified as a gene residing about 4.7 kb upstream of λ5 in the mouse genome. Based on its similarities to both Vλ and Vκ (and its expression in pre–B cells), it was called VpreB1. A second, nearly identical sequence in the mouse genome is named VpreB2 and appears to be functional,[28] and a less similar VpreB3 has also been described. Neither λ5 nor VpreB genes show evidence of gene rearrangement in B or pre–B cells, and homologs have been found in every mammalian species examined.

The two SLC proteins form a L chain–like heterodimer that is able to fulfill some functions of a true L chain, including association with μ H chains to permit surface μ expression prior to the availability of κ or λ L chains. Thus, when a μ H chain gene was transfected into an Ig-negative myeloma line, no surface μ expression was observed unless λ5 and VpreB genes were also transfected.[29] Surface μ chains are covalently linked to the λ5 protein, while the VpreB1 protein is noncovalently associated. The expression of μ-SLC on the surface of pre–B cells triggers the onset of Vκ-Jκ rearrangement, as discussed below.

In humans, three λ5-like sequences are located downstream of the human Cλ cluster on chromosome 22 (see Fig. 6.8), but only one—designated 14.1—appears to be functional. The human VpreB homolog lies within the Vλ cluster[30] in contrast to murine VpreB, which lies close upstream of λ5.

V(D)J RECOMBINATION

The mechanism by which germline variable region segments (V_L and J_L, or V_H, D, and J_H) are assembled in the DNA to form a complete active V region has been pursued ever since Ig gene recombination was first discovered. In this section we will address 1) the molecular mechanism of the reaction, 2) the topology of the recombination events, 3) the components of the recombinase machinery, and 4) the regulation of that machinery during B-cell development.

Molecular Mechanism of V(D)J Recombination

Recombination Model Overview

A model for the detailed mechanism of the V(D)J recombination event must account for the observed features of the recombination *products* and of their germline *precursors*. In the germline *precursors*, the RSSs with their heptamer, nonamer, and appropriate 12 or 23 bp spacers are necessary and sufficient to create efficient recombination targets; model substrates in which RSSs flank DNA sequences completely unrelated to Ig genes are competent to undergo recombination. The model shown in Figure 6.9 will serve as a framework for discussion of the recombination mechanism. The recombination is thought to begin with binding of the RAG1-RAG2 complex to the RSSs that flank the two gene segments to be recombined. Simultaneous DNA cleavage occurs precisely between the RSSs and the gene segments. The two ends of the RSSs (frequently named "signal ends") are joined directly, forming "signal joints." In contrast, the ends of the gene segments (also referred to as "coding ends") are processed prior to joining and are ultimately ligated together, giving rise to "coding joints" and completing the recombination event.

Recombination Products: Coding Joints and Signal Joints

In the recombination *products*, signal joints are typically direct ligation products of the signal ends: the RSSs are joined directly at the heptamers ("back-to-back"), and nucleotide additions or deletions at these junctions are quite rare. The properties of the coding joints, however, are more complex, as the joining reaction at these DNA ends is "imprecise." The following features are frequently present:

1. Deletions: variable number of bases are deleted from the ends of the coding regions (in comparison to the "complete" sequence in the germline precursor)
2. Nongermline ("N") nucleotides: random nucleotides (with a bias toward G and C) are added by a template-independent DNA polymerase (discussed below). The sequence of these N nucleotides has no relationship to the germline V, D, or J sequences.
3. Palindromic ("P") nucleotides: the ends of the coding gene segments are sealed by a DNA hairpin structure (see Fig. 6.9, and discussed in the following). "Opening" of these hairpins frequently occurs by nicking at some distance away from the hairpin tip leading to single-stranded overhangs. Filling in of such overhangs by DNA polymerases generates DNA palindromes that mirror the nucleotides at the end of the V, D, or J segment.[31] P nucleotides are generally only one or two bps, but they can be longer, especially in mice with the severe combined immunodeficiency defect (SCID) disorder in which the opening of the hairpins occurs in an aberrant manner.

Recombination Intermediates: Blunt Signal Ends and Hairpin Coding Ends

To study broken DNA ends as intermediates in V(D)J recombination, several laboratories employed ligation-mediated–polymerase chain reaction (LM-PCR) to detect signal ends. This technique involves ligating blunt double-stranded oligonucleotide linkers to blunt genomic DNA breaks, and then amplifying the ligation junctions between a primer in the ligated oligonucleotide and a primer based on known sequence from the ligated genomic DNA; amplification products can

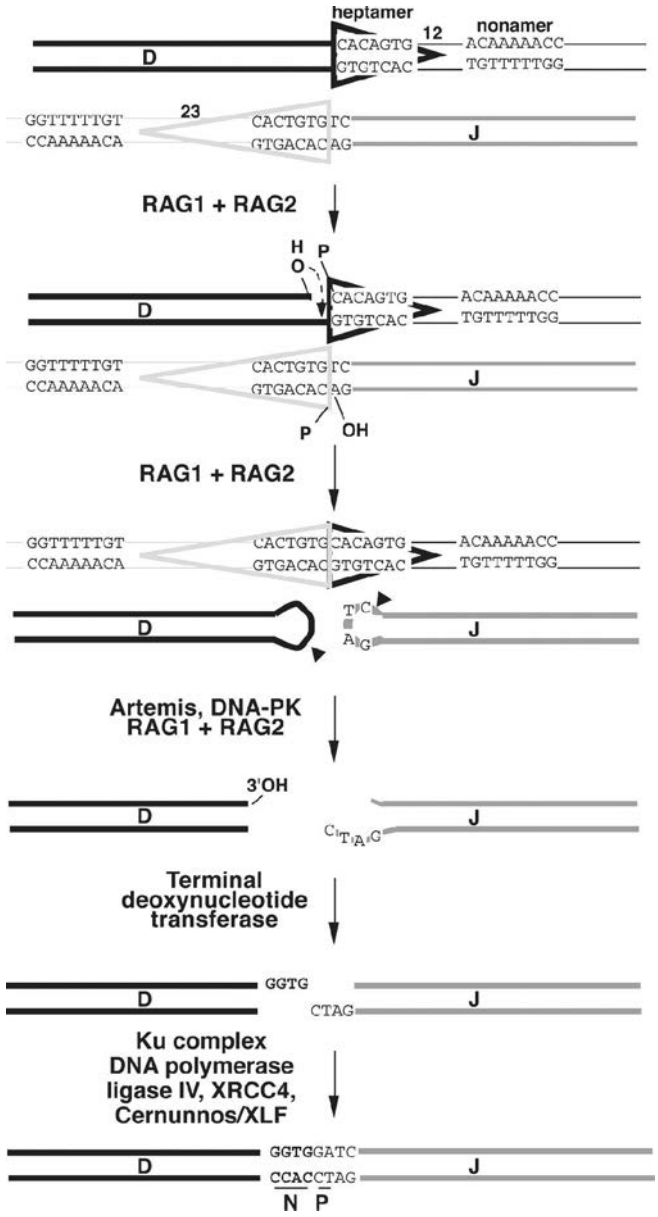

FIG. 6.9. Model for V Assembly Recombinations. All V assembly recombination reactions (in immunoglobulin and T-cell receptor genes) may proceed by a common mechanism, illustrated here by D-J recombination. The recombination signal sequences (RSSs) are included in *triangles*, which is the conventionally used RSS graphic. Hairpin loops are created on coding ends dependent on the action of the two Recombination Activating Genes: RAG1 and RAG2. After the opening of the hairpin loops, the pictured diversity coding sequence shows the effects of "nibbling" by exonuclease, but the joining coding sequence is spared and shows P nucleotide generation; N region addition is pictured in this example as occurring only on the diversity region end. In reality, exonuclease digestion and N nucleotide addition can occur on either (or both) ends. The steps in the proposed mechanism are discussed in the text.

then be cloned and sequenced. LM-PCR analyses of both TCR and Ig genes undergoing V(D)J recombination showed the signal ends to be blunt double strand breaks (dsbs), usually exactly at the heptamer border.[32] Similar LM-PCR experiments failed to detect the coding ends unless they were pretreated with mung bean nuclease, a single-strand-specific endonuclease that recognizes the distortion of DNA at a hairpin structure. Sequences of these LM-PCR products from coding ends suggested that the hairpins are precisely at the end of the coding elements, usually without loss or gain of a single nucleotide.[33] By Southern blot analyses, coding ends were found to have two properties suggestive of a hairpin-like structure: 1) resistance to exonuclease treatment, and 2) doubling of the apparent length of restriction fragments under denaturing electrophoresis conditions.[34]

Hairpin ends represent V(D)J recombination intermediates that, in wild-type cells, are opened at the hairpin tip (or a few nucleotides away from it) by the Artemis nuclease (discussed below). P nucleotides result from opening the loop at an asymmetric position (see Fig. 6.9); this model would explain why P nucleotides are never observed at coding ends that have been "nibbled" after opening of the hairpin. P nucleotide segments in the rare coding joints observed in SCID mice are unusually long and likely result from resolution of hairpins by nicking enzymes that, unlike Artemis, do not focus on the area near the tip of hairpin loops but instead nick in variable positions in the double-stranded hairpin "stem."[34]

Topology of V(D)J Recombination

Deletion versus Inversion

If a V segment and a J segment are both oriented in the same direction, they can recombine by excising the DNA between the coding sequences and ligating the two coding ends. Ligation of the two signal ends produces a DNA circle that generally lacks replication origins and therefore fails to replicate as cells divide after V(D)J recombination. Such excision circles are therefore generally absent in mature B-lymphocytes that have already undergone several rounds of proliferation after completing the Ig gene assembly. By isolating circular DNA from cells actively undergoing Vκ-Jκ rearrangement, it is possible to isolate and characterize the circular molecules bearing signal joints.[35]

As mentioned previously, some germline Vκ genes are oriented in the opposite direction from the Jκ-Cκ region. In these cases, VJ recombination occurs by an inversion of the DNA between the recombining V and J segments, leaving both the VκJκ coding joint and the signal joint (formed by ligating the RSSs) retained in the chromosome (see Fig. 6.7). This demonstrates that the enzymatic machinery "sees" only the DNA in the immediate vicinity of the recombination site and is insensitive to the topology of the DNA strands far from this site.

Nonstandard Joints

In addition to the canonical coding and signal joints, several "nonstandard" recombination joints have been documented, that, though not contributing to physiologic Ig gene assembly, represent tell-tale signs of a recombination event.[36]

In the first phase of V(D)J recombination, the DNA is cut at both gene segment–RSS boundaries that participate in the reaction, thereby generating four DNA ends. In principle, there are three possible topologies in which these DNA ends can be rejoined:

1. "Signal and coding joints": the standard reaction product in which the two coding ends get joined generating the assembled VJ gene and the 12-RSS/23-RSS signal joint.
2. "Open and shut joints": the RSSs get ligated back to the gene segments from which they were released. These joints are topologically identical to the starting DNAs, but can be distinguished from them if nucleotides have been added or deleted at the junctions.
3. "Hybrid joints": joints in which the RSSs have traded places so that the 23-RSS that was flanking the Vκ segment is now linked to the Jκ segment, and vice versa.

"Hybrid" and "open and shut" joints have been observed in transfected plasmids bearing artificial recombination substrates[36] as well as in endogenous Ig loci *in vivo*.[37]

Secondary V(D)J Recombination

As discussed previously, imprecise joining of gene segments causes about two-thirds of all recombination products to be out-of-frame. Thus, a B-lymphocyte could end up with nonproductively rearranged Igκ genes on both alleles. However, germline Vκ segments lying upstream of an initial Vκ-Jκ recombination junction can recombine with Jκ segments lying downstream of the junction, producing a "secondary" recombination event, as shown in Figure 6.10A.

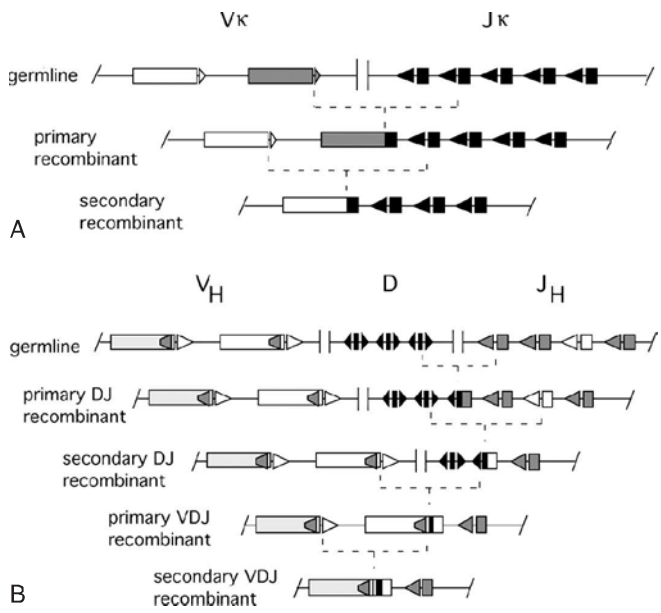

FIG. 6.10. Secondary Recombinations. A: In the κ light chain system, a primary recombination can be followed by recombination between an upstream V and a downstream J. **B:** Analogous secondary recombinations can occur in the heavy chain system between upstream D and downstream J segments. After V(D)J recombination eliminates all 12-RSS signal elements from the chromosome, secondary recombination can still occur between V_H (23-RSS) and an internal heptamer within the V_H coding sequence of the VDJ unit.

Such secondary recombination also occurs in cells that have assembled a productive Vκ-Jκ joint if the encoded antigen binding domain recognizes an autoantigen. This type of secondary recombination, known as "receptor editing," is considered in more detail later in this chapter.

In the IgH locus, secondary D-J rearrangements sometimes occur, but only until V_H-DJ_H recombination removes all unused upstream D_H segments (Fig. 6.10B). VDJ rearrangement eliminates all the 12-RSSs from the IgH locus that could pair with the 23-RSSs flanking the upstream V_H elements. Sometimes, these V_H segments do, however, recombine with an established VDJ unit, displacing most of the originally assembled V_H element,[38] a process sometimes called V_H replacement. Such events are mediated by cryptic RSSs (mainly a heptamer sequence) that is present near the 3′ end of about 70% of all V_H genes (see Fig. 6.10B). Such internal cryptic RSSs are not generally found in L chain genes. As discussed previously for the L chain, secondary recombination represents a rescue mechanism for cells with nonproductive rearrangements on both H chain chromosomes, and for cells whose encoded antibody recognizes an autoantigen.

The V(D)J Machinery

Since the discovery of V(D)J recombination as the process that assembles the germline antigen receptor gene segments into functional genes, one major question was the identity of the enzymatic machinery catalyzing this complex set of reactions. Genetic and biochemical work by a large number of laboratories led to identification of a total of 13 different proteins that have been shown to be directly involved in V(D)J recombination: RAG1, RAG2, HMG1, Ku70, Ku80, DNA-PKcs, Artemis, pol μ, pol λ, TdT, XRCC4, Cernunnos/XRCC4-like factor (XLF), and DNA ligase IV. The only lymphoid-specific factors are RAG1, RAG2, and TdT; all others are ubiquitously expressed in all cell types, and this feature allows investigators to study aspects of V(D)J recombination by ectopically expressing the RAG proteins in nonlymphoid cells. A recent biochemical tour de force study showed that coding joint formation could be recapitulated *in vitro* using artificial recombination substrates and highly purified preparations of all 13 proteins.[39] The respective coding joints showed all of the features typically observed *in vivo* (nucleotide deletion, N nucleotide, and P nucleotide addition), suggesting that most, if not all, of the factors involved in the coding end processing steps of V(D)J recombination have been identified. In contrast, signal joint formation was not observed. This step seems to require the removal of the RAG proteins after the cleavage reaction and is likely to require additional factors as yet unidentified.

Recombination Activating Gene Proteins: Mediators of Early Steps in V(D)J Recombination

A major advance in the investigation of V(D)J recombination was the identification of two genes whose products are critical for this process in the B and T cell lineages. In the pioneering experiments, Schatz and Baltimore[40] stably transfected fibroblasts with a construct containing a selectable marker whose expression was dependent on V(D)J recombination; as expected, no measurable recombination occurred in this nonlymphoid cell. However, when either human or murine genomic DNA was transfected into these fibroblasts, a small fraction of recipient cells stably expressed recombinase activity, activating the selectable marker. This suggested that a single transfected genomic DNA fragment was able confer recombinase activity in a fibroblast. (Presumably the fibroblast contained endogenous copies of the same genes, but their expression was repressed by mechanisms that could not repress the transfected genes.) This active fragment was cloned and turned out to contain two closely linked genes, designated RAG1 and RAG2, respectively. Both RAG1 and RAG2 are essential for recombination; therefore, these genes would not have been discovered by this transfection technique if they had not been closely linked in the genome. The genes are notable for having no introns splitting up their open reading frame in most species, and for their opposite transcriptional orientation in all species examined.

A crucial role for the RAG genes in V(D)J recombination was supported by the conservation of these genes in all jawed vertebrate species analyzed thus far, from shark through man. RAG1 and RAG2 are expressed together in developing B and T cells, specifically at the stages at which V(D)J recombinase activity is required for the assembly of Ig and TCR genes. Moreover, mouse strains in which either gene has been eliminated by homologous recombination (gene "knockouts") have no mature B or T cells, as the result of their inability to initiate V(D)J recombination.[41,42] Similarly, a subset of human patients with SCID syndrome characterized by the complete absence of T- or B-lymphocytes have been found to have null mutations in RAG genes.[43] Patients with hypomorphic alleles often have a complex set of features (oligoclonal T cells, hepatosplenomegaly, eosinophilia, decreased serum Ig but elevated IgE) known as the Omenn syndrome, which can also be caused by defects in other genes involved in V(D)J recombination. Interestingly, the same RAG mutation in different patients can cause either Omenn syndrome or SCID, depending on unknown factors.[44]

RAG1 shows intrinsic binding affinity for the RSS nonamer sequence via its nonamer binding domain even in the absence of RAG2. Exhaustive mutational analysis has revealed that RAG1 contains the catalytic center of the RAG complex, composed of three amino acids critical for all enzymatic activity: D600, D708, and E962.[45,46] RAG2, on the other hand, serves as a regulatory cofactor; it has no intrinsic binding affinity for RSSs, but once bound to RAG1 improves the strength and specificity of RAG1 RSS contacts.[47,48] It is also enhances RAG activity on chromosomal substrates and it restricts V(D)J recombination to the G0/G1 stage of the cell cycle (both features are discussed below).

Attempts to determine the molecular role of the RAG proteins in cell-free recombination assays were initially hampered by poor solubility of the proteins, but functional analyses of truncated RAG genes (using RAG expression vectors cotransfected into fibroblasts along with recombination substrate plasmids) revealed that surprisingly large segments of both proteins could be deleted without eliminating recombinase activity, and some of the remaining core regions were

soluble and could be handled relatively easily in experiments. This work allowed the demonstration that in a cell-free *in vitro* system, core regions of the two RAG proteins together are capable of carrying out cleavage of substrate DNAs as well as hairpin formation on the coding end.[49]

The RAG-mediated cleavage occurs in two steps: first a nick is introduced on the top strand between a gene segment and the adjacent heptamer (see Fig. 6.9), then the 3′-hydroxyl group participates as the nucleophile in a direct transesterification reaction to attack the phosphodiester bond adjacent to the heptamer on the bottom strand (see Fig. 6.9), yielding a DNA hairpin structure on the coding end and a new 3′-hydroxyl group on the 3′ end of the bottom heptamer strand.[50] After DNA cleavage, the RAG proteins remain in a complex with the DNA ends and facilitate aspects of the joining phase. Mutant forms of RAG1 or RAG2 have been reported that are competent for cleavage but show impairment in coding or signal joint formation.[51]

While nicking can occur asynchronously at the 12-RSS and 23-RSS, hairpin formation is "coupled" and occurs synchronously at both RSSs. *In vitro*, coupled cleavage requires only the RAG proteins, HMG1/2 (discussed below) and Mg^{2+} as the divalent metal ion in the reaction buffer. *In vivo*, DNA dsb formation at an individual RSS is dangerous as it could give rise to translocations, and it is thought that Mg^{2+} promotes an optimal molecular "architecture" for controlled V(D)J recombination. *In vivo* experiments indeed suggest that RAG proteins may bind to and introduce a nick at a single 12-RSS, but do not complete DNA cleavage until a matching 23-RSS is captured into the RAG-RSS complex.[52]

In addition to the "classical" activities of RAG proteins on DNA segments containing RSSs, these proteins can also catalyze DNA strand cleavage on "nonstandard" substrates.

1. Transposition. *In vitro*, purified recombinant core RAG proteins can catalyze the excision and insertion of a DNA fragment with signal ends into foreign DNA, acting as a transposase.[53,54] This property provides additional support for the early speculation that the V(D)J recombination system may have originated by insertion of transposon-like DNA fragment encoding RAG genes (and bearing RSSs at its ends) into a primordial antigen receptor gene, thereby generating a pair of separated V and J gene segments. This model of the origin of V(D)J recombination is consistent with the many mechanistic similarities at the molecular level between Ig gene rearrangements and transposition,[55] and the recent identification of the Transib transposase family that shows striking sequence similarity to RAG1 and is widespread in insect, echinoderm, helminth, coelenterate, and fungal genomes.[56] The recent finding of an apparent homolog of the entire RAG1 and RAG2 gene locus in a sea urchin genome suggests that the two RAG genes may have entered the genome of a common ancestor of all deuterostomes far earlier than the Ig-/TCR-based adaptive immune system developed.[57] It remains unclear whether the primordial RAG transposon encoded solely RAG1 (which would then have integrated next to the primordial RAG2 gene) or both RAG1 and RAG2. The transposase activity of RAGs, however, seems to be almost completely suppressed *in vivo*, and the C-terminus of RAG2 may have evolved to control this potentially deleterious activity.[51,58–60]
2. V_H replacement. As mentioned previously, recombination events can occur between a V_H 23-RSS and cryptic RSS within rearranged V_H coding sequences. An *in vitro* model suggests that in V_H replacement, the RAG proteins nick both DNA strands without forming a hairpin coding end.[61] Whether this is indeed a completely different activity is unclear.
3. Translocations at non-RSS sequences. The RAG complex also generates two nicks to cleave within the major breakpoint region of the *Bcl2* gene. This 150-bp segment is the target of a common RAG-catalyzed translocation between the IgH locus and the *Bcl2* gene occurring in most follicular lymphomas. In this segment, there are no RSSs, and the RAG proteins recognize an unusual sequence-dependent DNA conformation different from the normal B-form double helix.[62]

Although the "core" RAG proteins have been useful for elucidating the molecular mechanism of the cleavage step of V(D)J recombination in biochemical studies, it is clear that the "noncore" portions of each protein confer important functions, as expected from their sequence conservation across species. Broadly speaking, the "noncore" regions ensure regulated and efficient recombination on the physiological substrates (i.e., imperfect RSSs deviating from the perfect consensus heptamer and nonamer) in the context of chromatin. The functions of the "noncore" regions have largely been inferred by comparing V(D)J recombination products from cells expressing core RAG proteins versus full-length versions, and more recently by *in vitro* studies using full-length RAG proteins that are now available for such analyses.

The C-terminal region of RAG2 has multiple functions and is important for achieving normal numbers of B- and T-lymphocytes *in vivo*,[63] for the formation of precise signal joints during IgH recombination,[64] and for protecting against RAG-mediated DNA transposition.[51,65] These functions are thought to be conferred at least in part, by a plant homeo domain (PHD) zinc finger fold that is formed by amino acids 414 to 487 in murine RAG2. This PHD domain binds specifically to the tails of histone H3 that are trimethylated at lysine 4 (H3K4Me3),[66–68] a histone modification that is associated with "open" chromatin and that is uniquely present on "accessible" RSSs in Ig loci (discussed below). *In vitro* studies suggest that the binding of the RAG2 PHD domain to histone tails causes a conformational change that increases the catalytic activity of the RAG complex.[69]

Furthermore, the RAG2 C terminus regulates RAG2 protein levels—and hence V(D)J recombinase activity—across the cell cycle to prevent dsbs during DNA synthesis or mitosis, when such breaks could lead to chromosomal deletions.[32] RAG1 protein and messenger RNA (mRNA) transcript levels of both RAG genes vary little across the cell cycle, but phosphorylation of RAG2 at Thr490 by the cyclin-dependent

kinase cdk2 mediates its destruction via ubiquitination and proteasomal degradation during S phase.[70] Mice expressing RAG2 with a T490A mutation (which cannot be phosphorylated) showed RAG2 protein and dsbs throughout the cell cycle, demonstrating the importance of the RAG2 degradation signal in cell-cycle control of V(D)J recombination.[71,72]

The N-terminal noncore region of RAG1 is required *in vivo* for optimal RAG1 activity and for the formation of precise signal joints in D-J recombination.[64] This region of RAG1 contains a RING finger domain that seems to be required for ubiquitination of several proteins, including histone H3.[73]

Apart from the obvious importance of the RAG proteins in understanding the initial steps of V(D)J recombination, knowledge of these proteins and their genes has allowed two major technical advances that have opened the way to many additional experiments. First, various nonlymphoid cell lines with known defects in various DNA repair genes have been transfected with the RAG genes to identify genes involved V(D)J recombination (these factors are described below). Second, availability of the RAG1 and RAG2 knockout mice has been instrumental in a large number of immunology studies. These mice completely lack functional B cells or T cells, and are not "leaky" like SCID mice, which develop some functional B and T cells, especially as the animals age. Thus the RAG-deficient mice can be used to study the importance of the "innate" immune system (i.e., responses that occur in the absence of antigen-specific lymphocytes) in particular immune responses. They can also be used as recipients for various lymphocyte populations to explore the roles of different cell types. They can also be used as recipients for various lymphocyte populations to explore the roles of different cell types. They can be transfected with transgenes encoding specific Ig genes to study the roles of specific antibodies in B cell development and in immune responses. Finally, they can be used in "RAG complementation" experiments designed to assess the phenotype—in lymphocytes—of various other gene knockouts.[74] In RAG complementation, embryonic stem cells in which the gene of interest has been knocked out by homologous recombination are injected into homozygous RAG2 knockout (RAG2−/−) blastocysts. This procedure yields chimeric mice in which all B and T cells derive from the embryonic stem cells deleted for the gene of interest, as these are the only source of intact RAG genes to support lymphocyte development. Such animals can be made more easily than a knockout mouse line, and can be used to study the effect of gene deletion in lymphocytes independent of effects the deletion may have in other cells. In particular, for cases where the gene knockout causes embryonic lethality due to effects on nonlymphoid cells, RAG complementation allows the selective knockout in lymphocytes to be studied in the background normal gene expression in nonlymphoid cells.

High Mobility Group Proteins

The search for RAG cofactors that stimulate cleavage activity in biochemical assays led to the identification of HMG1.[75] HMG1 (and the closely related HMG2) are abundant and ubiquitous proteins that bind DNA in a non–sequence-specific manner and to cause a local bend in DNA. The two RAG proteins can form a stable signal complex with a 12-RSS, but efficient complex formation with a 23-RSS requires the addition of either HMG1 or HMG2.[76] HMG1/2 apparently stabilizes the bending of the 23-RSS that is induced by the RAG proteins themselves.[77]

Nonhomologous End Joining Components

The RAG proteins are the essential lymphocyte-specific factors in the DNA cleavage phase of V(D)J recombination, but DNA repair factors that are part of a DNA repair pathway known as nonhomologous end joining (NHEJ) are essential for the joining phase. NHEJ is the major pathway for repair of dsbs (such as those induced by ionizing radiation or reactive oxygen species) during the G0-G1 phases of the cell cycle. (In the S and G2 phases, the additional chromatid genome copy enables breaks to be repaired by homologous recombination.) The six classical core components of NHEJ are Ku70, Ku80, DNA-PKcs, XRCC4, DNA Ligase IV, Artemis, and Cernnunos/XLF, but additional proteins play a role in some models of NHEJ.

The DNA-PK Complex. The first gene for an NHEJ component to be recognized as participating in V(D)J recombination was the SCID gene. This gene was originally identified as being mutated in the *scid* mouse strain that is immunodeficient due to a marked impairment in V(D)J recombination of both Ig and TCR genes. Lymphocytes from *scid* mice are able to perform the RAG-mediated cleavage reaction, and can also form signal joints, but are markedly defective in coding joint formation. Subsequently, it was found that the *scid* mutation also impairs NHEJ, causing radiosensitivity.

The gene mutated in the *scid* mouse strain encodes DNA-PKcs, a large protein (460 kD) with a kinase domain near its C terminus that is related to phosophoinositide-3-kinase (PI3K). This kinase is DNA-dependent and represents the catalytic subunit (hence "cs") of a heterotrimer known as the DNA-PK complex. The other components are Ku70 and Ku80 (also referred to as Ku86), which were originally identified as the autoantigens recognized by a patient antiserum (Ku was the coded name of the patient, and the numbers refer to the approximate size of the proteins, 70 kD and 80 to 86 kD, respectively). Together, these two very abundant proteins form a heterodimer that binds to the ends of double-stranded DNA independent of the nucleotide sequence of the DNA. The DNA-Ku complex can then recruit DNA-PKcs and activate autophosphorylation of this protein.[78] *In vitro* activation of DNA-PKcs was found to be efficient when DNA ends either were at high concentration or, if at low concentration, were on DNA fragments long enough to circularize readily. In contrast, when the DNA-PKcs was located on the ends of DNA fragments too short to circularize (and too dilute for efficient intermolecular interactions with other DNA ends), the DNA-PKcs activation was much reduced. These observations suggest that kinase activation can occur only after two DNA ends are brought together by DNA-PKcs in "synapsis."[79,80] Further phosphorylation of

DNA-PKcs inactivates the protein and may prepare it for removal once DNA ends have been sealed.

Ku genes are highly conserved through evolution, and homologs are even found encoded in the genome of some bacteria, consistent with a function in general NHEJ not restricted to V(D)J recombination. While mice with a targeted deletion of DNA-PKcs resemble the original *scid* mutation (i.e., defective coding but functional signal joint formation[81,82]), Ku70 and Ku80 mutant cell lines are defective in both signal and coding joint formation, and Ku70- and Ku80-deficient mice exhibit a complete block in B- and T-cell development due to their inability to undergo V(D)J recombination.[83–85]

DNA Ligase IV and XRCC4. An important role of activated Ku-DNA-PKcs complex is to recruit the additional components of NHEJ. One such component is DNA ligase IV, which is recruited to the Ku complex and activated by the protein XRCC4.[86,87] The evidence suggests that DNA ligase IV is the essential ligase that joins DNA ends in V(D)J recombination and NHEJ. Human patients with ligase IV deficiency (characterized by hypomorphic alleles) have a severe phenotype including chromosomal instability, developmental and growth retardation, radiosensitivity, and immunodeficiency with a T–B–NK+ phenotype.[88] The rare D_H-J_H junctions detected show extensive nucleotide deletion consistent with delayed ligation and prolonged exonuclease digestion.[89] In mice, disruption of either the XRCC4 or the DNA ligase IV gene causes embryonic lethality associated with neuronal apoptosis. Crossing these mice with p53 mutants does not improve V(D)J recombination, but rescues the mice from embryonic lethality, suggesting that neuronal cells may be unusually susceptible to p53-triggered apoptosis induced by normal low-level DNA damage during brain development; a similar mechanism may explain the severe human phenotype.[90] DNA ligase IV is the only NHEJ component absolutely required to join compatible sticky DNA ends *in vitro*, though XRCC4 can stimulate this activity significantly.[87]

Cernunnos/XRCC4-like Factor. The next NHEJ component was independently discovered by two laboratories. One group used yeast two-hybrid screening to search for proteins interacting with XRCC4.[91] The other group searched for the gene causing a syndrome of T+ B lymphocytopenia, increased radiosensitivity, and microcephaly in a Turkish family; these investigators used functional cDNA rescue of a patient's cell line from a radiomimetic drug to identify the gene.[92] The protein identified by both groups is a 299 amino acid nuclear protein, which was named Cernunnos or XLF. The protein has a predicted secondary structure similar to that of XRCC4, to which it binds in cells[93] as expected from its isolation via two-hybrid screen. When Cernunnos/XLF-deficient fibroblasts were transfected with RAG genes and a recombination substrate, imprecise signal joining was observed, similar to the defect in patients with hypomorphic DNA ligase IV mutations. These experiments all suggest a role for Cernunnos/XLF linked to the function of XRCC4 and ligase IV.

Artemis. The coding ends generated by RAG cleavage cannot be directly ligated because of their hairpin structure, and therefore V(D)J recombination requires a single-strand endonuclease activity to cleave the hairpins. This activity is conferred by the protein named Artemis, which was discovered through positional cloning of the genetic defect in a group of human SCID patients with defects in V(D)J recombination and increased radiation sensitivity.[94] Patients with homozygous null mutations of Artemis survive (no embryonic lethality) and show sensitivity to γ irradiation as well as defects in coding joints, while signal joint formation is normal. Hypomorphic Artemis mutations can cause features of the Omenn syndrome similar to those observed with hypomorphic RAG gene mutations.[95] Purified recombinant Artemis protein has an intrinsic exonuclease activity *in vitro*; however, when complexed with DNA-PKcs in the presence of DNA ends, it gains a single-strand endonuclease activity and, in an ATP-dependent step, becomes phosphorylated at multiple sites in the C-terminal region of the protein.[96,97] The Artemis endonuclease can cleave synthetic and RAG-generated hairpin ends as well as other single-stranded DNA near a transition to double-strand DNA.[98]

DNA Polymerase X Family Members. If a hairpin opening leaves blunt ends or complementary sticky ends (like the ends generated by many restriction enzymes), *in vitro* joining experiments suggest that these ends can be joined by ligase IV without any additional processing.[99] However, as Artemis probably opens most hairpins noncomplementary DNA overhangs, further processing of DNA ends generally occurs before ligation completes the recombination. This processing may include further nuclease digestion (by Artemis or exonucleases) and apparently also involves variable DNA extension by three DNA polymerases—polymerase λ, polymerase μ, and terminal deoxynucleotidyl transferase (TdT)—all of which are members of the polymerase X family. Interestingly, all three proteins contain a Brca1-C-terminus domain, which is thought to confer binding to Ku.[100]

Terminal Deoxynucleotidyl Transferase and N Regions. TdT, the primary source of untemplated "N region" additions in VDJ junctions, is an enzyme uniquely expressed in the thymus and bone marrow; in the B lineage, it is expressed almost exclusively in pro-B cells. It catalyzes the nontemplated addition of nucleotides to the 3′ end of DNA strands. Though no template determines the nucleotides added, the enzyme adds dG residues preferentially, consistent with N region sequences observed in VDJ joints. Both TdT expression and N nucleotide addition are characteristically absent from fetal lymphocytes.[101] N region addition is common in H chain genes (recombined in pro-B cells) but rare in murine L chain genes (recombined in pre-B cells), though perhaps somewhat less rare in human.[102] This is consistent with the observation that in mice the expression of a μ H chain may downregulate TdT expression,[103] contributing to the reduced level during the stage of L chain recombination.

Lymphocytes with engineered defects in their TdT genes produced rearranged Ig V regions with almost no N additions. Conversely, when TdT expression was engineered in cells undergoing κ or λ L chain rearrangement, the level of

N nucleotide addition to these coding joints was dramatically increased. Furthermore, mice engineered to undergo premature Vκ-Jκ joining in pro-B cells show an increased frequency of N region nucleotides in their recombined Vκ genes.[104] These results suggest that the low frequency of N region sequences in normal κ or λ recombinations is caused by the reduced levels of TdT at this stage of B-cell development (see following discussion).

The absence of N region addition in TdT mutant mice, as well as in normal fetal lymphocytes, is associated with an increase in the frequency of recombination junctions with microhomologies. These are short stretches of nucleotides that are present close to the end of both germline gene segments involved in the recombination event. These junctions suggest a joining intermediate in which the complementary single-stranded regions from the two coding ends hybridize to each other, much as "sticky ends" generated by restriction endonucleases can facilitate ligation of DNA fragments. This alternative joining pathway may restrict the diversity of neonatal antibodies; the resulting antibodies are possibly enriched in specificities for commonly encountered pathogens, or have broadened specificity, as has been reported for TCRs lacking N regions.[105] Decreased N region nucleotides and a high incidence of homology-mediated recombination have also been found in the rare coding joints formed in Ku80−/− mice, consistent with a role for Ku in recruiting TdT or supporting its action.[106]

Polymerase μ and Polymerase λ. Polymerase μ and polymerase λ are ubiquitously expressed polymerases. Both readily fill in single-strand gaps in DNA and apparently participate in V(D)J recombination by filling in single-strand 3′ overhangs generated by asymmetric hairpin opening. Without this filling in, such overhangs might be resected by nucleases. Indeed, when *in vitro* NHEJ reconstitution experiments are performed using purified proteins and DNA fragments with overhanging ends, the omission of polymerase μ or polymerase λ increases the deletional trimming at junctions.[100] Similar excessive deletions at VDJ junctions are observed in mice lacking polymerase μ or polymerase λ. Remarkably, however, polymerase μ knockout mice show abnormalities only in their L chains,[107] whereas the deletions in polymerase λ knockouts are restricted to their H chains.[108] This selectivity may be explained by corresponding changes in the relative mRNA levels for these two polymerases at different stages of B-cell development.

Other Participants in V(D)J Recombination

DNA Damage Response Factors. In eukaryotic cells, DNA breaks initiate signals that halt cell division, induce DNA repair, and in some cases trigger apoptosis. Several proteins apart from NHEJ components can be detected at DNA breaks induced by V(D)J recombination or irradiation, including γ-H2AX, a phosphorylated form of the histone H2AX; ATM, the product of the gene mutated in the disease ataxia telangiectasia; Nbs1 (or nibrin), the product of the gene mutated in Nijmegen breakage syndrome; and 53BP1, p53 binding protein 1. The importance of these proteins in V(D)J recombination is not clear because defects in all three are compatible with near normal V(D)J recombination. Possibly, they participate in backup mechanisms to prevent aberrant V(D)J recombination and thus translocations.

Pax5/B-Cell–Specific Activator Protein. Pax5 (also known as B-cell–specific activator protein; BSAP) is a transcription factor required for normal B-cell development. Pax5-deficient mice are able to complete DJ_H recombination, but V_H to DJ_H recombination is impaired except for certain V_H genes located proximal to the D regions. Interestingly, 94% of human and mouse V_H coding genes were found to have potential Pax5 binding sites. Surprisingly, Pax5 was found to coimmunoprecipitate with RAG proteins, to potentiate *in vitro* cleavage of a V_H gene RSS, and to enhance V_H to DJ_H recombination in RAG-transfected fibroblasts; the latter enhancement required intact Pax5 binding sites in the V_H sequence.[109]

REGULATION OF V(D)J RECOMBINATION IN B-CELL DEVELOPMENT

The expression of only one antigen binding specificity by each B-lymphocyte is a crucial requirement of the clonal selection model of the humoral immune response. Thus, the recombination events that occur between Ig gene segments are carefully regulated so that most B cells express only one L chain isotype, either Igκ or Igλ (isotype exclusion), and use only one of the two alleles of H and L chain genes (allelic exclusion). These constraints ensure that each B cell expresses a single H_2L_2 combination. Current evidence suggests that V(D)J recombination is controlled largely at two levels: regulation of the RAG protein activity and regulation of accessibility of the germline V, D, and J elements to the recombinase machinery. Both of these are controlled by the stage of B-cell development; conversely, the expression of Ig provides a signal critical for regulating maturation of B cells. A brief scheme of B-cell development is presented in the following as background.

B- and T-lymphocytes differentiate from pluripotent hematopoietic stem cells in the fetal liver and bone marrow (Fig. 6.11). The primordial lymphoid progenitor has the potential to differentiate into B- or T-lymphocytes or natural killer cells. Among the earliest markers that indicate B-lineage specificity are the non-Ig components of the pre-BCR: Igα, Igβ, and λ5. CD19, which functions as a coreceptor in signal transduction, first appears in large proliferating "pro-B" cells, which also express several other distinguishing surface markers including c-kit, B220, TdT, and CD43. RAG gene expression in pro-B cells initiates D to J rearrangements on both alleles. Subsequently, recombination with germline V_H elements occurs; if the recombination is "productive" (i.e., yielding an "in-frame" VDJ junction), a μ H chain protein can be produced. This protein appears on the B-cell surface along with SLC in a pre-BCR (also named μ-SLC) complex that also includes Igα and Igβ. As the resulting large pre-B cells proliferate, RAG gene expression declines. After several rounds of division, the cells become smaller, stop dividing, turn up RAG gene expression once more, undergo L chain recombination, and express surface

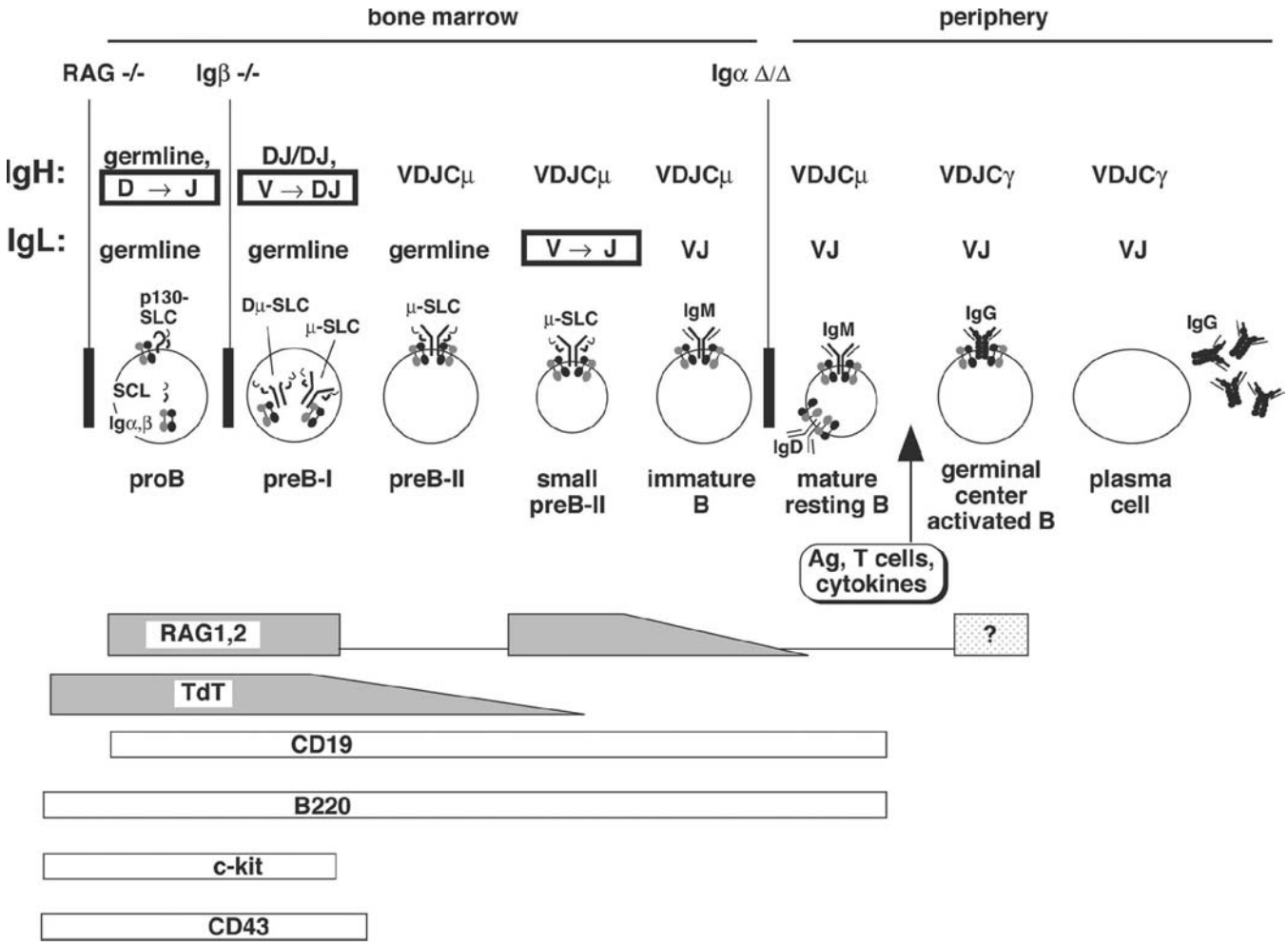

FIG. 6.11. Immunoglobulin (Ig) Gene Recombination in B-Cell Development. A simplified scheme of B-cell development is presented as a background for discussion of Ig gene recombination. The stages occurring in the bone marrow versus in the periphery (e.g., lymph nodes, spleen) are shown, along with the status of IgH and IgL genes at each stage. A graphic depicting the Ig-related proteins displayed on the surface at each stage is presented; at the bottom, the stage-dependent expression of recombination activating genes and terminal deoxynucleotidyl transferase—both important in V(D)J recombination—is schematically depicted.

IgM. These "immature B cells" again turn down RAG expression. In these IgM+IgD– immature B cells, contact with autoantigens may upregulate RAG expression again to facilitate receptor editing (discussed in more detail below). When immature B cells eventually also express surface IgD, they become "mature B cells" and migrate into the periphery, ready to be triggered by antigen exposure.

Allelic Exclusion and Regulated V(D)J Recombination

The previous description of B-cell development serves as a background to understand an explanation of allelic exclusion that was first proposed by Alt and colleagues[110] and has been supported by subsequent experiments. According to this model the functional rearrangement of an L (or H) chain gene in a particular B cell would inhibit further L (or H) chain gene rearrangement in the same cell. If the inhibition occurred promptly after the first functional rearrangement, then two functional Igs could never be produced in the same cell. An initial nonproductive rearrangement would have no inhibitory effect, so recombination could continue until a functional product resulted or until the cell used up all its germline precursors.

In pro-B cells, the first Ig gene rearrangements join D to J_H segments (commonly on both chromosomes), and this is followed by V_H to DJ_H recombination. If the first V_H to DJ_H recombination in a pro-B cell produces a functional VDJ gene, a functional μ H chain will be expressed on the cell surface paired with the SLCs. The expression of this pre-BCR complex has been shown to have two consequences. First, it blocks further H chain recombination by decreasing RAG gene expression[111] and by reducing target accessibility, as reflected in decreased V_H gene transcription.[112] The latter is important for rendering the IgH locus inaccessible during subsequent rearrangement of the Igκ and Igλ loci. If the initial V_H to DJ_H rearrangement is nonfunctional (e.g., out of frame), subsequent V_H to DJ_H recombination occurs on the other allele. If the VDJ recombination product on the

second chromosome is also nonproductive, then the cell has reached a dead end and is eliminated by apoptosis.[113]

The second consequence of pre-BCR expression is the initiation of Ig L chain recombination. This effect was originally deduced from the rarity of κ-expressing cells without H chain gene rearrangement, suggesting that H chain expression is required for κ recombination. As additional evidence, a functional μ gene introduced into early B-lineage cells can cause RAG gene expression and turn on transcription of unrearranged Vκ genes. These are designated "sterile" transcripts because they cannot encode a κ protein, but they are required for Vκ-Jκ recombination. When this recombination ensues, the possibilities for functional and nonproductive Vκ-Jκ rearrangements resemble those discussed previously for the H chain. Expression of a functional κ chain that can associate with μ to form a surface-expressed IgM molecule results in the downregulation of RAG gene expression and suppression of further κ rearrangements. By this mechanism, functional rearranged VκJ-Cκ transgenes can suppress rearrangement of endogenous κ genes.[114]

Most B cells show isotypic exclusion (i.e., they express either κ or λ but not both). Furthermore, κ rearrangement seems to occur before λ. Thus in normal and malignant human B-lymphoid cells, κ-expressing cells generally have their λ genes in germline configuration, while in λ-expressing cells, κ genes are either rearranged (rarely) or deleted (most commonly) by recombination signal recombination events discussed previously in this chapter.[22] The mechanisms that dictate the order of L chain recombination remain unknown. Plausible models include either the selective suppression of λ recombination until all options on the Igκ locus are exhausted or differences in the timing of the developmental programs controlling κ and λ accessibility.

Regulation of RAG Expression

A complete explanation of RAG gene expression would explain its lymphoid specificity, the two waves of RAG expression (during IgH and IgL rearrangements) and the autoantigen-induced upregulation associated with receptor editing. Although our current knowledge is still incomplete, several *cis*-regulatory elements that regulate RAG expression have been characterized. Surprisingly, the elements and mechanism for regulating expression during B- and T-cell development are distinct. RAG1 and RAG2 are transcribed toward each other in opposite directions, driven by promoters near the respective transcription start sites. Three B-cell–specific enhancers—designated Erag, D3, and Ep—have been reported, lying about 23 kb, 8 kb, and 1.6 kb, respectively, upstream of RAG2.[115–117] The B-cell–specific function of these regulatory regions is likely explained by the intersecting specificities of transcription factors that interact with them, including Pax5, E2A, FoxP1, FoxO1, NFATc1, and Ikaros. NFκB, which binds at several locations in the RAG enhancers, and FoxO1 (binding to Erag) were found to be important mediators of the upregulation of RAG expression in cells undergoing receptor editing.[118,119] Regulation of RAG2 protein across the cell cycle has been discussed previously in this chapter.

Parameters Affecting Recombinational Accessibility and Transcription

V(D)J recombination is triggered by RAG expression in the development of both B and T cells, yet Ig gene recombination is largely confined to B cells (exception: early T cells typically show D-J_H recombination); TCR gene recombination is exclusive to T cells. A widely accepted explanation for this locus specificity is provided by the "accessibility" model.[120] This model proposes that only those gene segments programmed for recombination at a given stage of B- and T-cell development are "accessible" to the RAG recombinase. One clue suggesting this model was that susceptibility to recombination and transcription of germline gene elements seem to be tightly correlated.[120] For example, many germline V_H genes are transcribed at the pre-B cell stage, just at the time when these genes are targets for recombination; these transcripts—designated "sterile" like the Vκ transcripts mentioned previously—are not seen in more mature B cells in which H chain recombination has been terminated. In support of the accessibility model, recombinant RAG proteins incubated with nuclei purified from pro-B cells (which generate sterile transcripts in the IgH locus) were found to cleave DNA at Ig J_H RSSs, but not at TCRδ RSSs; conversely, in pro-T nuclei the TCRδ RSS was cleaved, but not an Ig gene RSS.[121]

One molecular correlate of accessibility is the epigenetic state of DNA in the nuclear chromatin. The minimal repeat unit of chromatin is the nucleosome, which consists of eight core histones (two copies each of H2A, H2B, H3, and H4) with 146 bp DNA wrapped around it. *In vitro*, RAG proteins are unable to bind to and cut DNA wrapped around nucleosomes,[122,123] and hence nucleosomes have to be shifted or removed (a process called chromatin remodeling) to allow access. An alternative but not mutually exclusive approach to gain access is post-translational modification of the histone tails, which regulates the tightness of DNA-nucleosome contacts. The following section provides an overview of how accessibility of the Ig gene loci for RAG activity is regulated by several distinct but interconnected epigenetic mechanisms. We discuss a few important examples for each mechanism and refer to comprehensive review articles for an in-depth discussion.

Subnuclear Localization

In general, inactive genes tend to be located in the periphery of nuclei, while active genes are recruited to a more central nuclear location.[124] It is unclear whether the location per se dictates the chromatin state of a locus or whether the movement is a consequence of a locus being "opened." Fluorescence in situ hybridization (FISH) with large (~100 kb) probes specific for Ig loci is routinely used to reveal the position of Ig gene loci and control genes in the nucleus. The IgH and Igκ loci are located at the nuclear periphery in hematopoietic progenitors and pro-T cells, but move to central areas of the nucleus in pro-B cells.[125] As only the IgH locus gets rearranged at this stage, the correlation of position with accessibility is not perfect.

Transcription

Transcription typically occurs in "open" chromatin, and an emerging theme suggests that while some locus "opening" has

to precede transcription, transcription per se also positively reinforces this chromatin state. As mentioned previously, transcription of individual elements within Ig gene loci correlates well with their availability for V(D)J recombination at that stage. In early pro-B cells when D to J_H recombination takes places a "μo" transcript starts upstream of DQ52 and proceeds all the way through the J_H elements[126,127]; and only after DJ_H rearrangement do V_H sterile transcripts appear.[120] It is currently unclear whether transcription per se is directly linked to recombination or whether the correlation is largely mediated by similar requirements for gaining access to chromatinized DNA.

Histone Modifications

Posttranslational modifications of histone tails are important epigenetic marks of the chromatin state (also referred to as the "histone code"). Distinct marks correlate well with actively transcribed and inactive (or repressed) gene loci. As these patterns hold true for Ig loci as well, active marks (e.g., histone acetylation) and inactive marks (e.g., the methylation of lysine 9 on histone H3, H3K9Me2) correlate well with both transcription and recombination accessibility.[128] Importantly, one particular mark for open chromatin, H3K4Me3, is directly linked to the RAG recombinase. As discussed previously, histone tails with this modification are recognized by the PHD domain of RAG2; strikingly, the distribution of RAG2 in Ig loci matches exactly the pattern of H3K4Me3 (which mark accessible gene segments).[129] Beyond facilitating the recruitment of the recombinase, this histone modification also increases cleavage activity *in vitro*.[69]

Methylation

Most cytosine residues within CpG dinucleotides are methylated in mammalian DNA, but genes that are actively expressed in a particular cell are generally relatively hypomethylated in that cell type, implying that DNA methylation inhibits transcription. DNA methylation also seems to inhibit V(D)J recombination. The developmental maturation from pro-B to pre-B cells is associated with progression from a κ locus that is largely methylated, nontranscribed, and nonrearranging to one that is hypomethylated, transcribed, and rearranging.[130,131] Furthermore, methylation of artificial recombination substrates blocked V(D)J recombination when transfected into a recombination competent B-cell line[132]; V(D)J recombination of a transgenic construct occurred only when it was unmethylated.[133] Methylation and histone acetylation are interrelated; for example, the methyl-CpG-binding protein MeCP2 recruits histone deacetylases, which reduce acetylation of histones.

Gene Localization in the Nucleus

While accessibility allows the RAG proteins to selectively bind to appropriate sets of RSSs at each developmental stage, V(D)J recombination also requires that a pair of compatible gene segments (and their RSSs) are in close physical proximity. This becomes a particularly daunting requirement for gene segments > 1 mb apart in linear DNA sequence. Such distant segments are apparently brought close together in the nucleus, a process of "locus compaction" that loops out large regions of intervening DNA. Evidence for this model derives from FISH experiments showing greater compaction of the IgH locus in pro-B cells poised to undergo V(D)J recombination than in their earlier hematopoietic progenitors.[125] B cells deficient in Pax5 are impaired in recombination of the most distal V_H regions and show less movement of these regions towards the J_H-C locus than normal cells.[134] Data from a recently developed high-resolution FISH method provide a detailed model of the three-dimensional structure of the IgH locus, revealing rosette-like structures with central hubs from which several loops extend.[135]

cis Mediators of Accessibility and Looping

All previously described properties are dependent on *cis*-regulatory elements within the Ig loci, including classic promoters and enhancers. Individual promoters are present upstream of all V elements in all Ig loci, while the downstream D and J elements share a smaller number of promoters. Enhancers are present in each Ig locus, and they are thought to confer the transcriptional activation of each locus at the appropriate stage of B-cell development. Murine κ and IgH loci have intronic enhancers in the intron between J and C (iEκ and Eμ, respectively), and all three loci have enhancers downstream of C coding regions. (For example, downstream of the murine IgH locus is a complex of four enhancers, collectively known as the 3′regulatory region.) Promoters and enhancers were originally defined based on their role in regulating transcription, but these and other recently reported elements appear to play additional roles in Ig gene recombination. Transcriptional activation and the correlated locus "opening" is mediated by the recruitment of transcription factors that in turn recruit histone modifying and chromatin-remodeling enzymes. In addition, promoters and enhancers regulate transcription through the formation of DNA loops, some of which are critical determinants of the three-dimensional structure of Ig loci, thereby affecting V(D)J recombination as well as CSR (as discussed below).

An enhancer may activate transcription of several genes within a given gene locus, but its effects may be deleterious if it can activate other nearby genes requiring different patterns of expression. To prevent enhancer function beyond appropriate domains, boundary elements known as insulators establish borders between gene loci that are differentially regulated. The protein CCCTC binding factor (CTCF) is commonly found at insulators and also functions by creating DNA loops. One such insulator apparently lies downstream of a complex of enhancers at the 3′ end of the IgH locus, the 3′-regulatory region, where it may protect genes further downstream from being regulated by the Ig enhancer elements.[136] A recently discovered regulatory element with insulator properties is the intergenic control region-1 (IGCR1), which participates in CTCF–dependent looping between the Eμ and 3′ regulatory region enhancers.[137–139] Based on results of deletion of this region, the IGCR1 element suppresses recombination of V_H to D segments not already joined to J_H, prevents V_H to DJ_H recombination in T cells, and mediates the BCR-induced signal that terminates sterile V_H transcription and recombinational accessibility of V_H segments after a productive VDJ recombination leads to expression of a μ protein.

Late RAG Expression: Receptor Editing and Receptor Revision

Although the RAG genes are generally downregulated by a signal mediated by the appearance of membrane IgM at the end of the pre–B-cell stage, RAG gene expression and V(D)J recombination can recur later during "receptor editing" of autoreactive B cells in the bone marrow. One estimate suggests that about 25% of Igs are products of receptor editing.[140] After production of an initial Igκ. protein, receptor editing by L chain rearrangement can occur three ways: an initial VκJκ junction could be deleted by recombination between an upstream V and downstream J on the same chromosome ("secondary recombination," as discussed previously), VκJκ recombination could occur on the other (allelic) copy of the κ locus, or Vλ-Jλ recombination could be activated.

Most replacement of productively rearranged L or H chain genes likely serves to extinguish an antibody that was autoreactive, thus complementing two other mechanisms to silence autoantibodies: anergization and cell deletion by apoptosis. Early studies with transgenic autoantibodies suggested that anergy or deletion were the main fates of self-reactive B cells, but these conclusions may have depended on the nonphysiologic inability of the cells to silence the transgenic autoantibody by receptor editing. More recent studies involving autoantibody "knockin" genes—i.e., productive V[D]J recombined genes swapped into the physiologic positions within the IgH or Igκ gene loci using homologous recombination —have shown that receptor editing is the major mechanism for B cell tolerance.[141,142] This conclusion was also supported by a study of mice expressing a transgenic antibody against the murine Cκ constant region, a model of a self-superantigen; these mice provided evidence of receptor editing leading to virtually 100% λ L chain expression.[143]

IMMUNOGLOBULIN GENE ALTERATIONS IN GERMINAL CENTERS

Several days after exposure to an antigen, B cells accumulate in local lymph nodes, gut-associated lymphoid tissue, and spleen, and begin additional maturation steps in germinal centers (GCs). During the GC response, antigen-driven B cells undergo cycles of proliferation and their Ig genes undergo two unique alterations. 1) Lymphocytes switch from making IgM to making a new H chain isotype by the process of CSR. This process introduces dsbs upstream of Cμ in a specific repetitive noncoding DNA segment—the "switch region"—and in a similar switch region upstream of the new target Cx region; the DNA between the breaks is then deleted, and the ends of the remaining chromosomal DNA are rejoined so that the assembled VDJ region now lies upstream of the new Cx gene (Fig. 6.12). 2) In the other GC-associated gene alteration, the affinity of the antibody for its antigen increases by a process that introduces random mutations in V_H and V_L—somatic hypermutation (SHM)—and then selects for B cells producing higher-affinity antibodies. For many years, CSR and SHM were considered to be unrelated processes, but several lines of evidence provided hints that they might share some mechanistic features. First, while dsbs are expected intermediates for the DNA recombination underlying class switching, DNA breaks in V regions were also detected accompanying SHM. Second (and conversely), in addition to the mutations occurring in V regions associated with SHM, mutations were also observed surrounding the recombination junctions of CSR. Third, RNA transcription was found to be required for both processes.

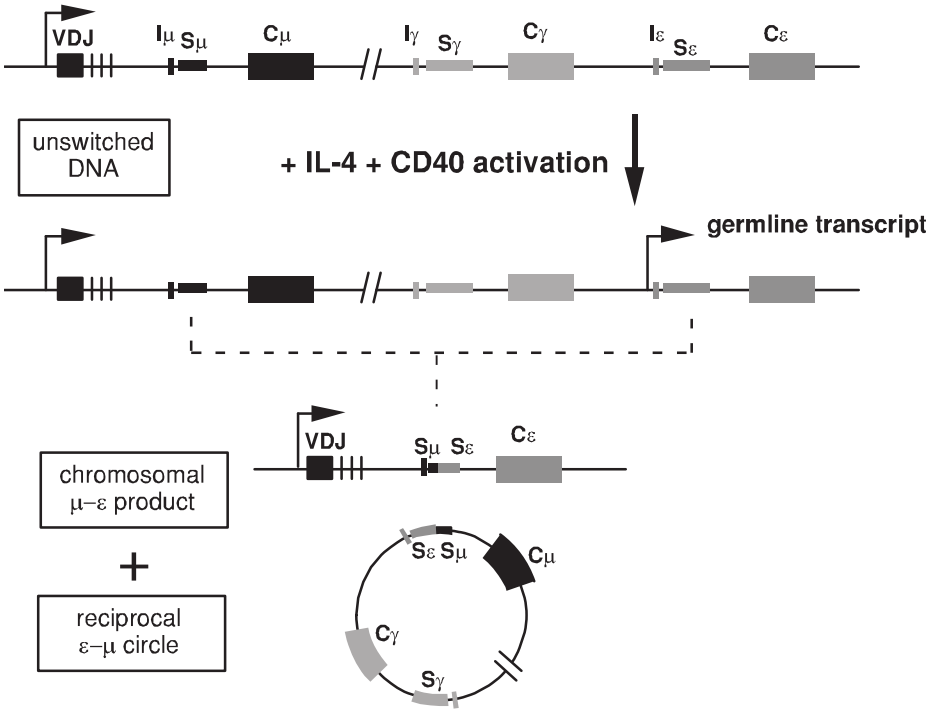

FIG. 6.12. Switch Regions and Composite Switch Junctions. The recombination breakpoints in isotype switch recombination fall within repetitive "switch" (S) regions. Stimuli that activate switch recombination (IL-4 and CD40 activation in the example shown) generally promote transcription across the target S region, initiating just upstream at the "I" exon. Recombination between Sμ and Sε produces two composite switch junctions: an Sμ-Sε junction retained in chromosomal DNA, and a reciprocal Sε-Sμ junction found in fractions of circular DNA. Polymerase chain reaction amplification across either composite junction can be used to study switch recombination.

Activation-Induced Deaminase

A fourth and dramatic link between CSR and SHM was the discovery that both processes require the protein known as activation-induced deaminase (AID). The gene encoding AID (known as *Aicda*, for *a*ctivation *i*nduced *c*ytosine *dea*minase) was discovered[144] by a subtractive strategy designed to screen for transcripts that were expressed in a murine B-cell line when induced to undergo CSR, but that were not expressed in uninduced cells. AID is expressed almost exclusively in GC B cells and in B cells activated *in vitro*, though exceptions to this generalization will be discussed later. Mice engineered with a targeted defect in the *Aicda* gene are completely deficient in CSR and SHM. The same defects are seen in patients with a homozygous defect in the human *Aicda* gene, a condition known as the hyper-IgM syndrome-2.[145] These patients have elevated serum levels of IgM because their B cells cannot undergo efficient CSR. AID is not only necessary for CSR and SHM, but apparently sufficient (in a mammalian cell at least), as overexpression of AID in fibroblasts can confer transcription-dependent CSR of an artificial switch construct[146] and transcription-dependent SHM of a transfected model mutation target DNA.[147] These experiments suggest that AID is the only B-cell–specific protein required for SHM and CSR. AID is also required for somatic Ig gene conversion in those species (e.g., rabbit and chicken) that use that process to somatically diversify Ig genes.

As translated from the cDNA, AID is a ~24 kD 198 amino acid protein that forms homodimers. AID shows 34% amino acid identity with the RNA editing enzyme APOBEC1, which catalyzes the deamination of a cytosine residue to uracil in a specific position in the mRNA encoding apolipoprotein B. The human *Apobec1* and *Aicda* genes are genetically linked, both lying at chromosome 12p13. Three other APOBEC1-related genes on other chromosomes have also been identified, but are not thought to participate in SHM or CSR.

Like APOBEC1, recombinant AID protein has a cytidine deaminase activity *in vitro*; it was initially proposed that, by analogy with APOBEC1, AID functions by deaminating cytidines in specific RNAs to produce novel edited transcripts encoding one or more proteins required for CSR and SHM. However, no evidence of AID-dependent edited RNAs has been reported, nor does AID deaminate RNA cytidines *in vitro*. Instead, current evidence indicates that AID acts on DNA, deaminating cytidines to uracil—in V regions for SHM, and in switch regions for CSR. The resulting uracils would be then recognized as both Watson-Crick mismatches and abnormal DNA bases by the cell's genetic surveillance machinery, triggering error-prone repair of V regions to produce SHM, or DNA cleavage to mediate CSR.

This DNA-deamination model for AID is consistent with various properties reported for this protein *in vitro* or in cells.

1. In transfected *Escherichia coli* cells, AID mutates cytidine to uracil in DNA, a result that would be unexpected by the RNA-deamination model, as bacteria presumably lack the specific mammalian RNA targets predicted by that model.[148,149]
2. Defective SHM and CSR are observed with inhibition or genetic inactivation of uracil-N-glycosylase (UNG), an enzyme that removes uracil residues from DNA,[150,151] consistent with the idea that CSR and SHM involve a step in which AID-produced uracil in DNA is removed by UNG. Moreover, B cells from *Ung*–/– mice accumulate uracils in V and in switch regions in an AID-dependent manner.[152]
3. AID isolated from B-lymphocytes can deaminate cytidine to uracil in single-strand DNA *in vitro*[153–155] or in double-strand DNA that is being transcribed *in vitro*, presumably because transcription causes localized regions of single-strandedness.[155–157]
4. AID is found to be associated with IgH genes *in vivo* in B cells undergoing CSR,[158–160] as assessed by Chromatin ImmunoPrecipitation (ChIP) using an anti-AID antibody.
5. A DNA sequence motif WRC—where W is A or T (*w*eak Watson-Crick pairs) and R (pu*r*ine) is A or G—that has been recognized as a hotspot target of SHM is also a preferred target for AID deamination *in vitro*,[154,157] and the hotspot preference can be altered by engineering amino acid changes in the segment of the AID protein thought to recognize the hotspot target in DNA.[161–163]

In light of these and other observations, the DNA deamination model for AID action is now almost universally accepted. The AID protein is highly conserved from fish to human, with all species having approximately 200 amino acids and sharing sequence similarities throughout the protein. In all species, AID contains a motif common to the active site of all cytidine deaminases: H[A/V]E – $X_{(24–36)}$ – PCXXC. This motif is also found in other members of the AID-APOBEC gene family, including APOBEC2, APOBEC3 (with several distinct paralogs in human), and APOBEC4.[164] AID is encoded in the five exons of the *Aicda* gene.

Structure-function relationships of AID have been probed by examining cross-species sequence comparisons and the effects of natural and engineered mutations in the protein. Remarkably, mutations in the N-terminus of AID impaired SHM but not CSR, whereas mutations or deletion in the C-terminus selectively impaired CSR,[165–167] suggesting the possibility that the N- and C-terminal regions of the protein contact specific cofactors required (respectively) for SHM and CSR function. Other functional features of the protein include a dimerization domain, several phosphorylatable residues, segments affecting nuclear localization, and target sequences for association with other proteins.

Although AID was initially recognized for its participation in SHM and CSR in GC cells, more recent reports have reported several other roles for this protein, both beneficial and deleterious.

1. *Epigenetic methylation.* Selective DNA methylation of cytidines is an epigenetic mechanism that participates in the regulation of transcription, and the methylation pattern of DNA is normally replicated when DNA is replicated prior to cell division. In primordial germ cells of

early embryogenesis, a global erasure of methylation occurs as part of the reprogramming to pluripotency, and AID apparently participates in this process.[168,169]

2. *Germline mutation.* AID can deaminate methylcytosine to thymine, which unlike uracil is a natural DNA base. This reaction in germ cells may contribute to the most frequent germline point mutation observed in mammals: the transition from CpG to TpG.[170]
3. *Genomic protection.* AID and other APOBEC family members protect cells against retroviral infections and against the spread of endogenous retroviruses.[171–173]
4. *Tolerance, apoptosis.* AID appears to be important for the establishment of B-cell tolerance in humans and mice, in that the low levels of AID expression observed in immature and transitional B cells appear to be necessary for suppressing the appearance of autoantibodies.[174–176] This effect may be related to the apparent requirement of AID for normal levels of apoptosis, which contribute to the elimination of B cells expressing autoantibodies.
5. *Oncogenic mutations and translocations.* Soon after AID was discovered, it was observed that overexpression of the protein caused tumors in transgenic mice,[177] and even normal expression of AID in B cells contributes to tumorigenic translocations and oncogenic mutations.[178] A notable example of AID-stimulated translocation is the recurrent *c-myc/Ig* translocation seen in Burkitt lymphoma and murine myelomas. Other cancers where AID plays a role through mutation include intestinal and lung cancer.

Based on studies of AID+/− heterozygous mice expressing roughly 50% of normal AID, the level of AID activity seems to be the limiting factor for both SHM and CSR, but is also limiting for oncogenic translocations and mutations.[179,180] Therefore, AID activity must be regulated robustly to balance between its multiple beneficial and deleterious actions. The regulation of AID activity is extremely complex, involving multiple levels of control, and is still incompletely understood.

Transcriptional and Posttranscriptional Regulation of AID Expression

Early studies showed that AID expression in B cells is upregulated by IL-4 and CD40 engagement. In more recent investigations, four DNA regions that regulate *Aicda* gene expression have been identified. They include an upstream enhancer responsive to T-cell signals, a promoter, a regulatory region just downstream of exon 1, and an additional enhancer downstream of the *Aicda* gene.[181–186]

The microRNA miR-155 has been shown to suppress AID expression.[187,188] MicroRNAs are short (21 to 23 nt) RNAs that hybridize to complementary sites in numerous target RNAs, triggering their degradation or inhibiting their translation. A target for miR-155 lies in the 3′ untranslated region of the AID transcript. AID expression was increased in miR-155–deficient mice and in mice whose *Aicda* gene was mutated to disrupt the 3′UTR target sequence. In both cases, consequences of the increase in AID expression included increased off-target mutations and increased *c-myc-IgH* translocations. Perhaps to protect against such effects, miR-155 is itself upregulated by the same signals that induce *Aicda* transcription in B cells.

Posttranslational Regulation

The level of AID activity in the nucleus is affected by its distribution between cytoplasm and nucleus, by the protein half-life, and by phosphorylation at various positions in the protein.

AID was found to shuttle between nucleus and cytoplasm.[189] Most of the protein is cytoplasmic and only a small fraction is in the nucleus, where it can act on DNA to cause CSR and SHM as well as "off-target" deleterious effects. Movement between cytoplasm and nucleus is controlled by at least three independent mechanisms: active nuclear import mediated by an N-terminal nuclear localization signal,[190] active nuclear export mediated by a C-terminal nuclear export signal,[189,191] and a cytoplasmic retention mechanism apparent when active import and export mechanisms are both blocked. Two proteins that may facilitate AID import independently of the nuclear localization signal are GANP[192] and CTNNBL1.[193] Nuclear-cytoplasmic distribution affects AID stability, because in the nucleus AID is targeted for ubiquitination and proteasomal degradation, shortening its half-life.[194] Conversely, in the cytoplasm, the chaperone protein Hsp90 protects AID from proteasomal degradation.[195]

In addition to ubiquitination, AID is also subject to posttranslational phosphorylation at several distinct residues in the protein, including Ser3, Thr27, Ser38, Thr140, and Tyr184. Of these, the functional relevance of the Ser38 site is best understood. Phosphorylation of AID Ser38 is essential to recruit replication protein A (RPA), a trimeric single-strand DNA binding protein[159,196] that may be required to stabilize a single-strand DNA target for AID. Preparations of AID purified from transfected *E. coli* or from nonlymphoid eukaryotic cells lack Ser38 phosphorylation; they can deaminate single-strand DNA *in vitro*, but are inactive in an assay for deamination of double-strand DNA transcribed *in vitro* by T7 polymerase. Activity in the latter assay requires AID that is phosphorylated at Ser38 and associated with RPA. In the AID protein, Ser38 lies within a conserved phosphorylation site [RRXX(T/S)] for the cyclic-AMP dependent protein kinase A (PKA), and multiple experiments have confirmed that PKA phosphorylates AID at Ser38 *in vitro* and physiologically *in vivo*.[196,197] The physiologic importance of Ser38 phosphorylation is strongly supported by observations of the effect of mutating this serine to alanine. The AID^{S38A} protein has essentially normal activity in deaminating single-stranded DNA *in vitro*, but severely reduced activity in deaminating transcribed double-strand DNA *in vitro*.

Less is known about the other sites of AID phosphorylation. At Thr140, alanine replacement causes more modest inhibition of AID activity than Ser38Ala, preferentially affecting SHM *in vivo*, but without significant effect on *in vitro* catalytic activity.[198] In contrast to Ser38 and Thr140, phosphorylation of Ser3 apparently suppresses *in vivo* AID activity; alanine replacement at this position increases SHM,

CSR, and oncogenic translocation, also without affecting *in vitro* catalytic activity.[199]

Clearly, multiple mechanisms regulate the level of activity of AID in B-cell nuclei, but these mechanisms cannot explain how—within nuclei—AID acts on Ig V genes and switch regions at a much higher frequency than on the rest of the genome. Targeting mechanisms differ somewhat between SHM and CSR, and will be discussed in the context of those processes.

Somatic Hypermutation

The hypothesis that antibody genes inherited in the germline might be subject to somatic mutation in lymphocytes during the life of an individual was suggested as an explanation for the diversity of antibodies several years before recombinant DNA technology became available to clarify the role of V(D)J recombination. Persuasive evidence for somatic mutation was reported in the 1970s: analyses of Vλ1 amino acid sequences of murine myeloma antibodies showed many instances of a particular prototype sequence, plus several variants containing unique single amino acid substitutions that could be explained by single nucleotide changes. The prototype was interpreted as reflecting the germline sequence, with the variants arising by somatic mutation.[200] Subsequent investigations at the DNA level revealed myeloma V region sequences that deviated from their germline counterparts, verifying the principle of somatic mutation.

Somatic mutations are much rarer in IgM than in antibodies with "switched" isotypes (IgG, IgA, and IgE) made by B cells that have been exposed to AID in GCs, but they do occur; antibodies with "switched" isotypes without mutations are also found. These observations suggest that though both SHM and CSR normally occur in GC B cells, the two processes are unlinked.

Role of Hypermutation in Immune Responses

To understand the role of SHM in the antibody response, several groups have studied the extent of Ig gene mutation at different times after the immunization of mice. Studies of the responses to p-azophenylarsonate (Ars), phosphorylcholine, influenza hemagglutinin, oxazalone, and several other antigens have all indicated that the initial response after primary immunization is established by antibodies showing no somatic mutation. About 1 week after immunization, mutated sequences begin to be observed, increasing during the next week or so. Booster immunizations yield sequences showing additional mutations.

Many hybridomas made late in the immune response produce mutated antibodies with a higher antigen affinity than the unmutated (sometimes loosely called "germline") antibodies made early after immunization. The shift to higher affinity is a phenomenon long recognized at the level of (polyclonal) antisera and has been termed "affinity maturation." This phenomenon can now be explained as the result of an "evolutionary" mechanism selecting antibodies of progressively higher affinity from the pool of randomly mutated V sequences.

According to this model, at the time of initial antigen exposure an animal has a set of naïve B-lymphocytes expressing IgM with germline (unmutated) versions of Ig variable regions resulting from gene rearrangements that occurred prior to immunization. Because of the diversity of available V_H, D, J_H, V_L, and J_L sequences as well as the impressive recombinational potential described previously, some B cells will express Ig molecules capable of binding the antigen with modest affinity. Antigen binding stimulates these B cells to proliferate and to move to lymphoid follicles, where they form GCs. In the GC environment, AID is expressed and SHM machinery is activated, generating random mutations in the Ig genes of stimulated GC B cells. Many of these mutations reduce the affinity of the encoded antibody for antigen,[201] and some may induce autoantibody specificities (i.e., the ability to bind to self-molecules[202]). As clearance of the antigen lowers the antigen concentrations, only the cells displaying high affinity for antigen will be stimulated effectively; cells displaying lower-affinity antibodies or antibodies with affinity for self-antigens are subjected to programmed cell death ("apoptosis").[203,204] The preferential proliferation of the high-affinity cells and their maturation to secreting plasma cells causes an increase in the average affinity of the antibodies in the serum. Some high-affinity cells become memory cells, persisting long after the initial antigen exposure, ready to respond to a subsequent antigen exposure with rapid production of high-affinity antibody. In this model, the driving force for affinity maturation—analogous to natural selection in the evolution of species—is selection for high-antibody affinity in the face of falling antigen concentration. The importance of this selective force is suggested by the observation that affinity maturation can be inhibited by repeated injection of antigen (which removes the selective pressure for high affinity)[205] or by overexpression of the antiapoptotic protein Bcl-XL (which allows survival of B cells expressing low affinity antibody).[206]

Cellular Context of Somatic Mutation

Each GC appears to be populated by a small number of antigen-specific founder B cells[207] and an unusual Thy-1 negative T-cell population, also antigen-specific,[208] The GC environment promotes contact between the B cell and follicular dendritic cells— which store, process, and present antigen—and T-lymphocytes, which activate somatic mutation in part via CD40-CD40Ligand interaction.[209] In a widely accepted model of GC function, SHM occurs in a subpopulation of B cells known as centroblasts. These cells proliferate in the "dark zone" of the GC and bear characteristic surface markers including IgD, CD38, and the receptor for peanut agglutinin. Proliferating GC centroblasts give rise to nondividing centrocytes in the "light zone" of the GC; there centrocytes are programmed for apoptosis unless they are rescued by follicular dendritic cell–presented antigen and T-cell activation via CD40 engagement. Selection for high-affinity antibodies occurs because cells expressing high-affinity antibodies are most efficiently rescued from apoptosis. Surviving centrocytes may return to the dark zone to undergo several successive cycles of mutation and proliferation followed by selection. This model is supported by direct observation (by two-photon microscopy) of B cells moving between light zone and dark zone.[210] Migration of

B cells to follicles or GC zones is thought to be controlled by a complex system of chemotactic receptor /ligand pairs including CXCR5/CXCL13, CXCR4/SDF1, CCR7/CCL9 (or CCL21), S1P1/sphingosine-1-phosphate, and EBI2/its lipid ligand.[211] Although certain features of this classical model have been challenged, the broad outlines have received strong support from studies in which cells in the dark zone or light zone of individual GCs can be marked by a photoactivatable green fluorescent protein (GFP) and then followed for several hours using two-photon imaging as the cells migrate through a mounted lymph node.[212]

The notion that GC B cells compete to avoid apoptosis on the basis of antigen affinity of their BCR has been has been supported by a study that directly compared caspase activation (an apoptosis marker) in B cells expressing transgenic antibodies with higher versus lower affinity for the same antigen; the lower-affinity cells were found to undergo a significantly higher rate of apoptosis.[213] The mechanism by which high-affinity antigen binding selects for survival is not fully understood. One possibility is that a higher-affinity BCR directly delivers a stronger activation signal to the B cell, inhibiting apoptosis. However, a second possibility is that a higher-affinity BCR could capture antigen more efficiently, enabling stronger antigen presentation to GC T cells, which could then deliver stronger survival signals to the B cell via secreted or surface protein interactions. Consistent with the second model, experiments using two-photon microscopy have documented that that B cells from mice immunized with a fluorescent antigen can capture that antigen from follicular dendritic cells in the GC.[214] To test whether B-cell antigen capture might confer survival independently of the BCR, investigators engineered B cells in ovalbumin-primed mice to deliver ovalbumin via a surface lectin instead of via the BCR; they observed that the internalized ovalbumin antigen was capable of conferring a B-cell survival advantage in the GC in the absence of BCR engagement.[212] However, effective BCR signaling can enhance antigen processing and presentation,[215] so internalization/presentation and BCR signaling may work together to promote GC B-cell survival and mediate selection for high affinity.

Most experiments on SHM have focused on GC cells, as does the discussion in this chapter. However, T-cell–independent SHM may also occur in a population of less mature cells, which may populate the splenic marginal zone and which may increase the repertoire of circulating lymphocytes prior to antigen exposure, especially in young individuals,[216,217] or perhaps function in tolerance induction, as discussed previously. Also, mice lacking histologically detectable GCs as a result of lymphotoxin-α deficiency are capable of SHM and affinity maturation.[218] T-cell–independent antigens can induce a low level of SHM in B cells.[219]

Molecular Mechanism of Hypermutation

AID deaminates cytidine to uracil, an analog of thymine. Thus, if replicated before repair, an original C:G base pair would, in the daughter cell receiving the uracil-bearing DNA strand, mutate to a T:A. But it was initially not clear how cytidine deamination could affect A:T base pairs, which are targeted in 50% to 60% of mutations observed in SHM. By analyzing abnormalities in SHM that are observed in various mutant B cells, Di Noia and Neuberger[150] formulated a model for SHM that has explained this question and has gained wide acceptance (Fig. 6.13). The model proposes that after AID-catalyzed deamination creates a uracil residue in the target DNA, the possible outcomes depend on how the resulting mismatch is resolved. 1) The U:G mismatch may be replicated as described above, resulting in what are known as phase 1A mutations. 2) The uracil base may, before replication, be excised by UNG, creating an abasic site. Normally, the creation of such abasic sites is the first step of the base excision repair pathway, in which subsequent steps remove the sugar-phosphate backbone, leaving a single nucleotide gap that is then restored to a C:G bp by DNA polymerase β and DNA ligase. If the DNA replicates before the abasic site is repaired, the strand with the abasic site may directly engage translesional polymerases (which are error-prone, as discussed below) to insert an unpaired nucleotide (i.e., any nucleotide) opposite the abasic lesion, leading to phase 1B mutations (see Fig. 6.13). 3) The original U:G mismatch—or possibly the abasic site created by UNG action—may be recognized by the mismatch repair (MMR) system of the cell. MMR triggers excision of the DNA strand for several nucleotides surrounding the mismatch, and this strand is then resynthesized by polymerase β, in the case of faithful repair, or by error-prone polymerases, in the case of SHM. Error-prone repair inserts mispaired bases, which may become fixed on one strand by replication, creating mutations (designated phase 2) from both A:T and G:C bps at some distance from the position of the original U:G mismatch. If a mispaired base is recognized by MMR before replication, a new cycle of MMR may be initiated, extending mutation even farther from the initial deamination event.

Role of Uracil-N-Glycosylase in Somatic Hypermutation. The UNG gene encodes two proteins that differ in their N termini as a result of alternative promoters that generate different initial coding exons. UNG1 is expressed in mitochondria, whereas UNG2 is nuclear. Hydrolytic deamination of cytosines to uracil occurs at a significant rate in all eukaryotic and prokaryotic cells, and misincorporation of dUTP during replication further contributes to the load of uracil in DNA. UNG2 plays a major role in mitigating this load by initiating faithful base excision repair.

In SHM, the faithful repair of uracil is somehow subverted to introduce mutations. *Ung*–/– mice and human patients with a rare form of HyperIgM immunodeficiency due to *UNG* mutations have similar immunologic phenotypes. First, as discussed in the following, *Ung*–/– individuals are profoundly defective in CSR, as expected if this process requires AID-catalyzed deamination of cytidine followed by *UNG*-catalyzed removal of uracil. Although the frequency of mutation is roughly normal in *UNG*-deficient individuals, mutations at C are almost exclusively transitions of C to T (phase IA). As suggested by Figure 6.13, *Ung*–/– individuals would not create the abasic sites that lead to C → G and C → A mutations by replication, though some mutations of these kinds could be produced by MMR. Indeed, the frequencies of mutations

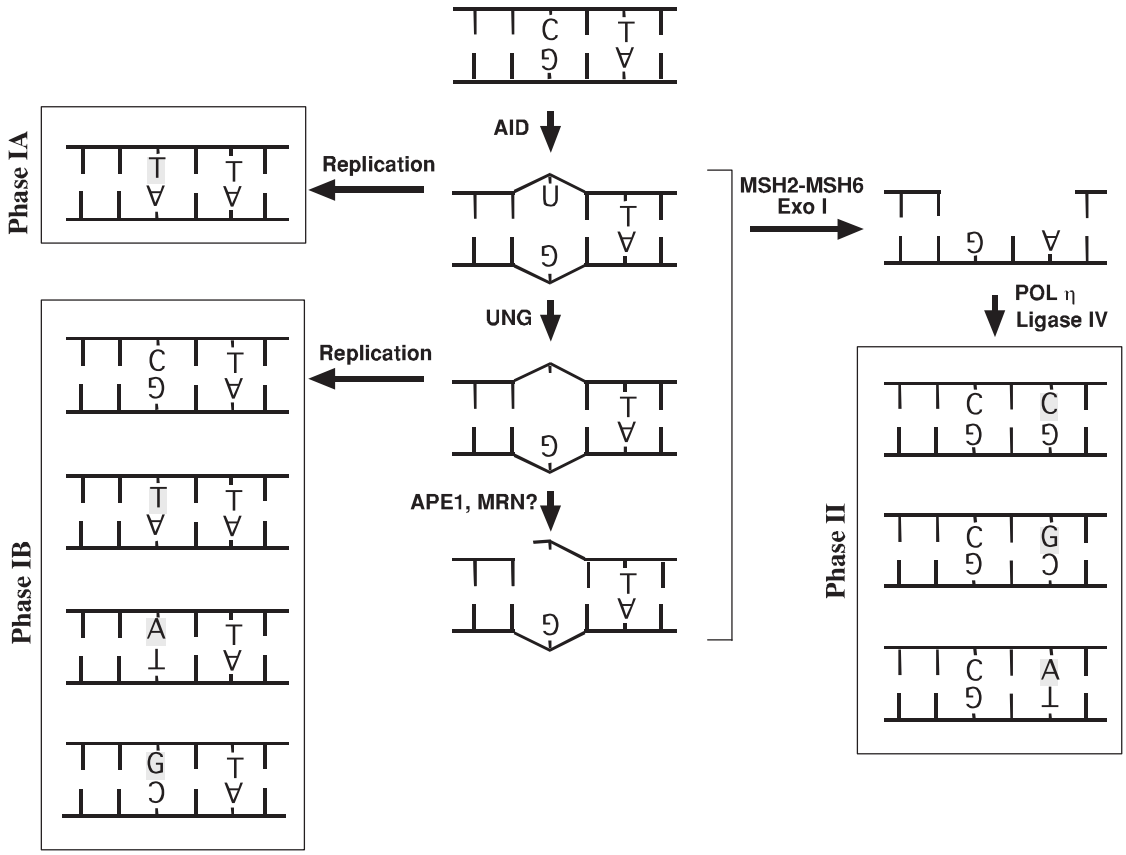

FIG. 6.13. Mechanistic Model of Reactions Triggered by Activation-Induced Deaminase (AID) in Somatic Hypermutation and Class Switch Recombination (CSR). The graphic at the top depicts a small region in a VH gene showing a targeted C:G basepair and a nearby T:A pair. After AID-catalyzed deamination of a C residue, the DNA may be subjected to various modifications as shown, leading to mutations indicated by nucleotides in gray rectangles. The model is described in the text. The Phase II mutations occur when mismatched bases incorporated by an error-prone polymerase are replicated before they can be corrected by MMR. The same mechanism explains two switch-region–associated events occurring during CSR: DNA strand breaks (see center panel at bottom) and mutations..

from A, T, and G in *Ung*−/− individuals are normal, apparently resulting from MMR activated by the U:G mismatch in many cells.

Mismatch Repair in Somatic Hypermutation. MMR is a highly conserved mechanism that detects abnormalities in DNA, including mispaired nucleotides and abnormal bases, and repairs them. The eukaryotic MMR system has two main components. MutS binds tightly and specifically to DNA defects and recruits MutL. The Mut complex then activates a latent nuclease activity to remove a segment of the DNA strand including the mismatched base; this gapped strand is then resynthesized by a DNA polymerase. Mammals have several MutS homologs, including three reported in somatic cells—MSH2, MSH3, and MSH6—which exist in the cell as heterodimers. MSH2-MSH6 (also known as MutSα) is the most abundant form and is specialized for recognizing single base gaps or mismatches, while MSH2-MSH3 (MutSβ) recognizes larger gaps and insertion/deletion loops. The mammalian MutL proteins are heterodimers consisting of MLH1 paired with PMS1, PMS2, or MLH3. Apart from their effects on SHM and CSR, mutations in MMR genes, especially MSH2 and MLH1, underly hereditary nonpolyposis colorectal cancer. Recent efforts in several laboratories have led to *in vitro* reconstitution of mammalian MMR with purified components, enabling powerful analysis of this complex mechanism.[220] In addition to the MutS and MutL proteins, the system requires the following components: Exo1 to excise the gap; replication protein A, which binds to the single strand DNA in the gapped region (and is known to bind to phosphorylated AID, as discussed previously); proliferating cell nuclear antigen (PCNA), which promotes processivity by encircling DNA in a sliding ring clamp and by recruiting other components; DNA polymerase δ; DNA ligase I; and several other proteins.

Knockouts of MSH2, MSH6, and Exo1 have been studied by several laboratories and show consistent decrease in B-cell SHM from A:T bps (with a corresponding increase in the percentage of mutations from G:C bps).[221,222] These results, in the context of the model of Figure 6.13, suggest that MMR triggered by the MSH2-MSH6 heterodimer primarily introduces mutation at A:T bps. The predilection for

mutating A:T bps matches the activity of polymerase η, as discussed in the next section, implying that this polymerase may be the one most frequently engaged by the MMR machinery in repairing AID-generated lesions. Indeed, MSH2-MSH6 is capable of binding to a U:G mismatch, MSH2 can bind to polymerase η in cell extracts, and MSH2-MSH6 can stimulate the activity of polymerase η *in vitro*.[223]

Error-Prone Polymerases. Although avoidance of error is a high priority for most DNA replication, error-prone DNA synthesis served an important function long before SHM evolved to mutate Ig genes; indeed, error-prone polymerases have been most thoroughly studied in *E. coli* and yeast. These polymerases are useful in all cells for replication of DNA containing focal lesions that would block replication by stringent high-fidelity polymerases. However, the tolerance of these error-prone polymerases to abnormalities in DNA structure is accompanied by tolerance of non–Watson-Crick basepairing in the replication of normal DNA and by absence of DNA proofreading activity. More than a dozen of these polymerases have evolved in eukaryotes, each specializing in different aspects of "translesion DNA synthesis" (TLS) and showing differing spectra of infidelity in replication of normal DNA. Clear evidence for participation of several TLS polymerases in SHM has come from comparing the known *in vitro* activities of a particular polymerase with the SHM abnormalities seen in mice or humans with mutations in the corresponding polymerase gene.

Polymerase η characteristically inserts mismatched bases opposite T nucleotides, and individuals lacking this polymerase show the predicted abnormality: decreased mutations at A:T base pairs. This was first demonstrated in patients with xeroderma pigmentosum variant disease, whose defects in polymerase η subject them to sunlight-induced skin cancers in addition to their abnormal SHM; a similar defect in mutations from A:T bps was subsequently shown for polymerase η knockout mice.[224,225] Polymerase η binds to MSH2-MSH6, as mentioned previously, and is upregulated in cells undergoing SHM.

In addition to polymerase η, the TLS polymerase REV1 has been shown to participate in SHM. REV1 is known to preferentially insert cytosine residues opposite uracil or abasic sites, and *Rev1* knockout B cells were found to be impaired in C to G mutations, especially in the nontranscribed (coding) strand.[226] Polymerase ζ,[227,228] polymerase κ,[229] and polymerase θ[230–232] knockouts have also been reported to show altered SHM patterns, but these effects are smaller or not seen in all circumstances.

Error-prone repair apparently operates in competition with faithful repair carried out by DNA polymerase β. The human B-cell line BL2 is known to undergo SHM, but in subclones of this line with higher SHM proficiency, polymerase β levels were very low; overexpression of polymerase β in a proficient subclone suppressed SHM activity.[233] Compared to wild-type B cells, polymerase β–deficient B cells (developing in a wild-type recipient) showed increased switch region mutation after induction of CSR *in vitro*.[234]

A fundamental question about the action of error-prone polymerases is how they are specifically engaged for SHM of Ig genes, given that most spontaneous cytosine deamination in nonlymphoid cells is accurately repaired. One apparent clue is that some TLS polymerases are upregulated in B cells undergoing SHM. In addition, when the sliding clamp protein PCNA is monoubiquitinated at lysine residue K164, it is known to bind and activate TLS polymerases including polymerase η. When this ubiquitination is prevented, either by knockout of the specific E3 ubiquitin ligase of PCNA or by a K164R mutation at the ubiquitination site in PCNA, SHM is abnormal. $PCNA^{K164P}$ mice were found to have a dramatically decreased ability to mutate A:T sites during SHM, similar to the phenotype of the polymerase η knockout, as though polymerase η requires monoubiquinated PCNA in order to participate in SHM.[235] However, these clues do not fully explain why Ig genes in GC B cells are less faithfully repaired than other genes in the same cells. This question is part of the larger issue of mutational targeting, as discussed below.

Targeting and Distribution of Mutations

The mutation rate of Ig genes in GC B cells undergoing SHM may reach as high as 10^{-3} mutations/bp/generation, or about 10^6 times higher than the normal genomic mutation rate;[236] this elevated rate could be lethal to B cells if mutations were not carefully targeted specifically to Ig genes. Several other genes highly expressed in GC B cells were also found to be mutated at lower levels (e.g., *Bcl6* and *Cd95*[237]), though several other genes expressed in GC B cells at comparable levels are not mutated. Apparently, some of the features that target SHM to Ig genes may be shared by other genes.

Recently, a genome-wide analysis has made it clear that AID binds much more widely to the genome than was previously appreciated. When chromatin from mouse B cells incubated with lipopolysaccharide (LPS) and IL-4 was analyzed by ChIP with an anti-AID antibody, 5910 genes were found by deep sequencing of the immunoprecipitated DNA (ChIP-seq),[160] including many previously described AID targets. Although this is a large number, it is still a small minority of the genome. As judged by the number of sequence tags recovered, AID binding to the IgH locus was substantially higher than binding to any other region. A sample of genes identified as AID-binding by ChIP-seq showed significant mutation frequency, whereas control genes did not. The AID-binding genes were associated with high levels of mRNA transcription (by RNA-seq), by a chromatin mark commonly associated with transcription—histone H3 trimethylated on Lys4 (H3K4me3)—and by density of Pol II binding. However, only the IgH locus, with its uniquely high levels of AID occupancy and mutations, bound to RPA; this binding was AID-dependent and was diminished in the S38A and T140A mutants of AID described previously.

The difference between AID-targeting and SHM frequency has been reinforced by analysis of SHM in panels of non-Ig genes isolated from murine Peyer patch B cells.[238] All the non-Ig genes were mutated at levels much lower than the 2.2×10^{-2} mutations per bp detected near J_H4, but substantial numbers of mutations were found at *Bcl6*, *Pim1*, *CD79B*, and several other previously reported targets of SHM. The mutations were almost completely AID-dependent, as in cells from *Aicda*–/– mice, all genes showed very low mu-

tation frequencies that were barely above the sequencing error rate. However, different genes varied widely in the way their mutation rates were affected by the double knockout *Ung–/– Msh2–/–*, in which only phase 1A mutations should be possible. At one extreme, *c-myc*, which is known to mutate at a very low rate in GC B cells, showed a mutation rate in the *Ung–/– Msh2–/–* cells almost 17-fold higher than in the wild-type, as though almost all AID-induced deamination in the wild-type B cells had been faithfully repaired by UNG- and Msh2-dependent mechanisms. In contrast, *Bcl6* mutations were only 1.3-fold higher in the double knockout, suggesting that faithful repair of this gene was dramatically less active.

Thus SHM susceptibility depends not only on differential AID targeting across the genome but also on differential ratios of error-prone versus faithful repair. The distinction between these two variables (deamination and repair) has only recently been recognized, so it was not taken into account in many earlier studies described in the following.

RNA Transcription. A relationship between SHM and RNA transcription is suggested by the observation that unrearranged V_H and Vκ genes are generally neither transcribed nor mutated, but become susceptible to both processes when V(D)J recombination moves them close to their (respective) intronic enhancers, Eμ and iEκ. In contrast, the λ locus lacks an enhancer between J and C; unrearranged Vλ regions are transcribed in B cells[239] and can be mutated.[240] The Ig coding sequences are apparently not specifically required to target SHM, as transgenic V coding sequence can be replaced by a human β-globin gene or prokaryotic *neo* or *gpt* gene without affecting the hypermutation rate downstream of the promoter.

Mutations around a given V region are distributed in a domain that begins roughly 100 to 200 bps downstream of the promoter, extends for ~1.5 to 2 kb downstream, and then tapers off long before the RNA transcriptional termination. This distribution led to the hypothesis that after transcriptional initiation, a "mutator factor" attaches to the transcriptional machinery, attacks DNA as the transcription complex moves downstream, and eventually falls off, so that further transcription proceeds without mutations.[241] Consistent with this model, a VκJκ-Cκ transgene bearing a second Vκ promoter engineered upstream of the Cκ region was found to incur mutations over a second domain extending into the Cκ region, in addition to the usual V region mutations.[242] Conversely, the insertion of an irrelevant 2 kb DNA fragment between a Vκ promoter and the leader (signal peptide) exon prevented mutation within the Vκ transgene, which now apparently lay downstream of the mutational domain.[243] (Mutations also occur in domains surrounding repetitive switch regions upstream of C_H genes. The frequency of these mutations is similar to that in V regions, but the domains are larger, correlating to some extent with the length of the switch region, as discussed in the following.) The key mutator factor is apparently AID, which can deaminate double-strand DNA *in vitro* only when the DNA is transcribed. One hypothesis suggests that DNA near the advancing RNA polymerase complex becomes negatively supercoiled or partially single-stranded, which might facilitate access of the DNA to AID.[244] Another explanation, involving R loops, is described below. Evidence for patches of single-stranded DNA in transcribed V regions undergoing SHM has been obtained using a bisulfite technique.[245]

In vivo dependence of SHM frequency on transcriptional activity has been confirmed by several findings. In one study, SHM was studied in knockin mice with a prerecombined V_HDJ_H region driven by either of two V region promoters; SHM rates in these knockin strains were highly correlated with transcription driven by the different promoters.[246] In another study, a tetracycline-inducible GFP reporter gene engineered with a stop codon was stably transfected into a B-cell line. The rate of reversion of the stop codon by SHM (allowing GFP expression) was found to be directly related to the transcription rate, as regulated by a tetracycline analog.[247] Other studies examining SHM in models where transcription was altered by mutating enhancers have supported the relationship between SHM and enhancer-induced transcription. Some non-Ig enhancers could support SHM in a stably transfected cell line,[248] but others could not, leading to the suggestion that specific elements in enhancers might confer susceptibility to SHM. One candidate element is the sequence CAGGTG, which is a target for E-box transcription factors and which is found in several Ig enhancers. When a murine κ enhancer containing CAGGTG was linked to a GFP gene inactivated by a premature stop codon, stable transfectants of chicken B-cell line DT40 were found to produce mutants that expressed GFP, but a single mutation in the CAGGTG motif prevented SHM without changing transcription.[249] Inactivation of the E-box transcription factor E2A in DT40 cells was found to reduce SHM of endogenous Ig genes without significant effects on Ig gene (or AID) transcription.[250]

However, some conflicting conclusions have been drawn about enhancer-dependence of SHM depending on whether experiments investigated transgenes versus constructs created in the endogenous context using homologous recombination (i.e., knockouts or knockins). For example, the downstream IgH enhancers HS3b and HS4 were found important for SHM in transgenes[251] but dispensable in the context of endogenous genes.[252]

In one case, transcriptional enhancer activity has been clearly separated from an associated SHM stimulating activity. Just downstream of the chicken Ig λ 3′ enhancer lies a DNA segment whose deletion in the DT40 B-cell line severely impaired SHM without dramatically affecting transcription; this segment could confer SHM when inserted into a non-Ig locus.[253,254]

To summarize, it appears that transcription is necessary but not sufficient for targeting hypermutation, but the additional elements required for SHM have not been defined as of this writing (though E2A function may be among them).

Chromatin Marks. A parameter related to transcription of Ig genes (i.e., their context in chromatin) has also been studied in relation to SHM targeting. Culture of a B-cell line under conditions that upregulate SHM (in the presence of T cells and anti-IgM) led to increased acetylation of histone H3 and H4 at V region but not C region DNA, as assessed by ChIP analysis[255] paralleling the distribution of mutations. The increased histone acetylation was not a consequence of AID action as it occurred when AID expression (and SHM) was inhibited by antisense treatment; moreover, when cells were treated with the deacetylase inhibitor trichostatin A, the C region was both acetylated and subjected to SHM. Other histone modifications that may be correlated with SHM are phosphorylation or monoubiquitination of histone H2B.[256,257]

Hotspot Focusing. Mutations occurring within V region genes expressed *in vivo* in B cells may be highly selected for antigen-binding function of the expressed antibody. To analyze the spectrum of mutations produced by SHM unbiased by selection, investigators have studied nonproductively rearranged VDJ alleles or "passenger" transgenes engineered with stop codons to prevent expression as a protein. In these genes, mutational "hot spots" as well as "cold spots" have been recognized, apparently due to local sequence features that may promote or suppress somatic mutation. The consensus sequence WRC (i.e., [A/T][G/A][C]) is the most consistent hotspot for mutation, presumably reflecting the predilection of AID for *in vitro* deamination of this sequence. It is possible that evolution has concentrated mutational hot spot frequencies in CDR regions to enhance the potential for diversity generation in the parts of the protein critical for antigen contact.[258]

The previous discussion has identified several factors influencing the targeting of SHM, a process in which a unique triggering event—AID-dependent deamination of DNA—is followed by a cascade of other events that depend on mechanisms common to most cells. All of these steps, including the AID-dependent trigger, can be affected by biologic parameters common to all cells, such as transcription, enhancers, epigenetic state, DNA repair mechanisms, etc. Many of these same mechanisms affect CSR as well as SHM. Recently, three additional biologic parameters have been found to influence AID-triggered events in CSR: RNA polymerase stalling, the RNA splicing in spliceosomes, and RNA degradation by exosomes. It is possible that these three processes impinge on both SHM and CSR, but because they were discovered in the context of CSR, they are discussed in the following section.

Heavy Chain Switch

Switch Regions and Switch Junctions

As briefly mentioned previously in this chapter, isotype switching involves removal of Cμ from downstream of the rearranged H chain VDJ gene and its replacement by a new downstream C_H region. This occurs by a deletional recombination—CSR—in which the recombinational breakpoints generally occur within G-rich repetitive DNA sequences known as switch (or S) regions lying 5′ of each C_H region (except Cδ). While most switch breakpoints fall in the S regions, some are in nearby nonrepetitive DNA. The S region of the mouse μ gene, Sμ, is located about 1 to 2 kb 5′ to the Cμ coding sequence and is composed of numerous tandem repeats of sequences of the form (GAGCT)n(GGGGT), where n is usually 2 to 5 but can range as high as 17. All of the S regions of downstream isotypes include pentamers similar to GAGCT and GGGGT embedded in larger repeat units rather than precisely tandemly repeated as in Sμ. In support of the critical role of S regions for CSR, knockout of Sγ1 by homologous recombination essentially abolished expression of IgG1 from that allele,[259] and mice with a deletion of Sμ were also dramatically impaired in CSR.[260,261]

A switch recombination between, for example, μ and ε genes produces a composite Sμ-Sε sequence (see Fig. 6.12). From a comparison between the sequence of an Sμ-Sε composite switch region and the sequences of the germline Sμ and Sε, one can localize the exact recombination sites between Sμ and Sε that occurred in each allele. Such comparisons have indicated that there is no specific site, either in Sμ or in any other S region, where the recombination always occurs, although clusters of recombination sites have been reported at two specific regions within the tandem repeats of murine Sγ3 region.[262] Thus, unlike the enzymatic machinery of V-J recombination, the switch machinery can break and join sequences in a broad target region, and as the recombination targets are in intronic DNA, there are no reading frame complications. Often both IgH alleles in a single cell undergo switching to the same downstream isotype. Some alleles undergo sequential switching events; for example, a common pathway to IgE expression is an initial μ → γ1 switch, followed by a CSR between the composite Sμ-Sγ1 switch region and the Sε region.[263] Sγ1- Sε switching may even occur independently of Sμ.[264] Although most CSR occurs as a deletion within a single IgH allele, switching between two allelic chromosomes was detected at a frequency of roughly 7% to 10% in mouse and rabbit.[265,266]

DNA fragments excised by switch recombination have been cloned from fractions of circular DNA isolated from cells actively undergoing isotype switch recombination. Thus, at least some of the excised DNA segments ligate their ends to form "switch circles"; these contain composite switch junctions that are in theory reciprocal to the composite switch junction retained on chromosomal DNA (see Fig. 6.12). Because switch circles are not linked to centromeres and do not apparently contain origins of replication, they are not efficiently replicated. Therefore, they are not found in cells that have divided many times after switching (e.g., in myelomas or hybridomas).

Many composite switch junction sequences show mutations near the recombination breakpoint when compared to the corresponding germline switch sequences. Indeed, many features of CSR are shared by SHM (as indicated previously in this chapter), including the requirements for transcription, AID, UNG2, and MMR components for normal CSR and SHM. However, CSR is more complex in that it involves simultaneous targeting to two DNA regions (i.e., switch

regions of Cμ and the target Cx), it requires dsbs, and it consequently requires mechanisms to repair the breaks. AID can theoretically trigger single-strand breaks on both DNA strands by the mechanisms discussed for SHM, including cleavage by APE-1. If these breaks are close enough together, they can effectively form a dsb with staggered ends, and if dsbs occur in two switch regions, the DNA repair mechanisms common to all cells may rejoin the broken ends to complete a CSR event. Indeed, B cells in which Sμ and Sγ1 were deleted and replaced by recognition sites for the yeast homing endonuclease I-SceI were capable of mediating some μ → γ1 CSR in the absence of AID if I-SceI was expressed,[267] highlighting the participation of ubiquitous AID-independent repair mechanisms for CSR. These mechanisms depend on the NHEJ components described previously in this chapter plus backup participation by alternative end-joining mechanisms discussed below.

Regulation of Isotype Switching: Proliferation and AID Expression

Isotype switching occurs physiologically in animals about 1 week after immunization with T-dependent antigens, at about the same time that somatic mutation of Ig genes begins. Both processes normally occur in GCs of lymphoid organs, a location that facilitates interactions between B cells, T cells, and follicular dendritic cells presenting antigen. As demonstrated by *in vitro* switching experiments, T cells promote switching by secretion of cytokines (especially IL-4 and transforming growth factor β) as well as by cell-to-cell contact.

A major component of the cell contact signal is mediated by an interaction between the B-cell surface marker CD40 and its ligand—designated CD40L, CD154, or gp39—which is expressed on activated T cells (primarily CD4+). CD40 is a member of the TNF–receptor family, while CD40L belongs to the TNF–ligand family. The dependence of switching on the CD40-CD40L interaction is highlighted by the genetic disease known as the X-linked hyper-IgM syndrome-1, which was found to be caused by a defect in the human gene encoding the CD40L/gp39.[268] Like AID-deficient patients with hyper-IgM syndrome-2 described previously, patients with X-linked hyper-IgM syndrome-1 have elevated concentrations of IgM in their serum and almost no Igs of other isotypes. In addition, their antibodies fail to show affinity maturation or evidence of B-cell memory responses. Similar defects are seen in humans with mutations in their CD40 gene, an autosomal recessive disease designated hyper-IgM 3.[269] CSR impairment may also be caused by abnormal function of CD40 signaling pathway components including IKKγ (also known as NEMO), NFκB proteins, and C-Jun N-terminal kinase. Mouse strains with engineered defects in CD40 are defective in SHM and T-dependent CSR, but respond with normal isotype switching to T-independent antigens. The T-independent switching pathway may be especially important for gut-associated switching to IgA.[270] One role of the CD40 engagement is to induce B-cell proliferation. Indeed, other proliferative stimuli (e.g., LPS or IgM or IgD crosslinking) can support cytokine-induced isotype switching *in vitro* in the absence of T cells and CD40 activation. The relationship of CSR to cell division is supported by evidence that switching is linked to the cell cycle[271] and to the number of cell divisions after stimulation,[272] a phenomenon which may reflect cell division-related regulation of AID expression.[273] However, apart from activating proliferation, CD40 has additional effects that may facilitate switching, including upregulation of IL-4 responsiveness and IL-4 receptor number,[274] upregulation of sterile "switch transcripts" (discussed below), and upregulation of AID expression. Activated B cells also express CD40L, which can not only trigger CD40 signaling but also transduce a "reverse" signal affecting B-cell function.[275] Independent of CD40, B-cell activation can independently be stimulated by TLR ligands and cytokines such as APRIL, another member of the TNF family.[276]

Regulation of Class Switch Recombination to Specific Isotypes: Promoters, Enhancers, and Chromatin

Different isotypes are known to predominate in different immune responses depending on the antigen, route of antigen administration, and several other parameters. These different parameters act in part by influencing the cytokine milieu of the B cells. IL-4, for example, promotes the expression of IgE and IgG1, whereas TNF-β promotes switching to IgA. These lymphokines have been proposed to act by making the target isotype "accessible" to switch recombinase machinery that may be largely non–isotype-specific. The accessibility is associated with expression of a "sterile" or "germline" RNA transcript that initiates upstream of a target S region (see Fig. 6.12) and extends through the target C region. The germline transcript is spliced so that a noncoding upstream exon known as an I ("intron") region is joined to the first coding exon of the C region. (This contrasts with the "productive" transcript containing VDJ spliced to the C region.) The same experimental conditions—particularly the same cytokines—that favor the accumulation of germline transcripts from a particular isotype generally also stimulate switch recombination to the same isotype. In many cases, the signals transduced by the cytokine receptor have been elucidated. For example, IL-4 stimulates germline transcription by activating the transcription factor STAT6, which binds to one of several IL-4 response motifs in the promoter region upstream of Iε and Iγ1. CD40 engagement also acts in part through NFκB-mediated binding to I region promoters.[277]

Gene targeting experiments have shown that mouse strains lacking the I region (and its promoter) of a particular isotype do not switch to that isotype, reinforcing the idea that sterile transcription is necessary for CSR.[278] The low extent of sequence conservation of the I exons and the lack of consistent open reading frames suggest that these transcripts do not encode a functional protein. Indeed, the exact sequence of the I region may be irrelevant as an I region can be replaced by an unrelated sequence and still support CSR.[279] However, the transcribed exon upstream of the S region apparently needs a splice donor site allowing splicing to the downstream C region, as a targeted construct lacking such a splice donor site was reportedly unable to support CSR even though transcription through the S region occurred.[280]

Apart from I region promoters, germline transcription and isotype switching are also regulated by IgH enhancers. A combined deletion of the murine 3′-regulatory region enhancers HS3b and HS4 was found to cause a significant impairment in switching to most isotypes, although switching to IgG1 was unaffected (and IgA only moderately decreased).[281] The diminished switching was associated with diminished germline transcription of the same isotypes, suggesting that an important function of the enhancers in CSR is to increase germline transcription. The relative independence of γ1 from regulation by enhancers may be related to the putative locus control region associated with that gene.[282]

Enhancers are believed to function via physical interactions between enhancer-bound proteins and promoter-bound proteins, creating a DNA loop that brings enhancers into close proximity to promoters. Such looping may have special significance in promoting DNA recombination between segments of DNA lying great linear distances apart in the chromosome, as has been discussed in the context of V(D)J recombination. Chromosome conformation capture experiments have shown that Eμ and 3′-regulatory region enhancers are in close proximity in mature resting B cells, and that when B cells are stimulated to switch to γ1 by LPS + IL4 (or to γ3 by LPS alone), the corresponding I region promoter moves close to the two enhancers.[283,284] This looping would bring Sμ, which lies just downstream of Eμ, close to the Iγ1 (or Iγ3) promoter, presumably facilitating recombination between Sμ and the Sγ region.

Another parameter correlated with regional transcriptional regulation is the chromatin context of the genes, including specific modifications of histone proteins, as discussed previously in this chapter. For example, acetylation of histone H3 (H3Ac) is associated with DNA regions of increased "accessibility" to transcription (as well as to experimental digestion by restriction enzymes), while transcriptional promoters tend to be marked with trimethylation of H3 at lysine4 (H3K4me3). In B cells stimulated to switch to γ1 by LPS + IL4 (or to γ3 by LPS alone), the corresponding I region and switch regions show increased H3Ac and H3K4me3 marks.[285,286] The importance of the H3K4me3 mark for transcription and CSR is highlighted by evidence that preventing this mark—by B cell–specific knockout of PTIP, a component of the machinery that catalyzes this modification—leads to impaired germline γ transcription and defective CSR to γ isotypes.[287] PTIP knockout B cells show decreased DNA looping between the 3′ enhancer region and Iγ region promoters, suggesting that PTIP contributes to this looping.[288] Knockdown of other factors that are necessary to maintain H3K4me3 also reduce CSR efficiency, including the components of the complex known as FACT (*fac*ilitates *c*hromatin *t*ranscription).[289]

Surprisingly, a histone modification generally associated with gene silencing—trimethylation of H3 lysine 9 (H3K9me3)—has also been reported to mark switch regions targeted for CSR in both mouse and human B cells.[290,291] Recently, a screen for nuclear proteins associating with AID *in vitro* identified KAP1 (KRAB domain–associated protein 1),[292] a transcriptional repressor that associates with heterochromatin protein 1, as binding to H3K9me-modified chromatin. By coimmunoprecipitation, KAP1 was confirmed to bind to AID *in vivo*, and B-cell–specific conditional knockout of KAP1 was found to diminish AID binding to Sμ and to impair CSR efficiency by about 50%.

Switch Region Targeting of AID in Class Switch Recombination: R Loops, Paused Polymerase II, and AGCT

A model in which AID loads onto an RNA polymerase complex and acts on DNA as the transcription complex travels downstream was discussed previously in the context of AID function in SHM, and a similar model apparently applies in the context of CSR. The domain of AID susceptibility in switch regions can be deduced from the distribution of C:G → T:A mutations in B cells that are defective for both UNG and MMR, because (as shown in Fig 6.13) in these cells the only AID-dependent mutations would be U:G mismatches resolved by replication to T:A. A study of the distribution of such mutations in clones from *Ung–/–*, *Msh2–/–* B cells found that a domain of mutations began about 150 bp 3′ of the Iμ transcription start site and extended 4 to 5 kb downstream, with diminishing mutation frequency near Cμ.[293] The location of AID binding in B-cell DNA can also be directly determined by ChIP-seq analysis, as discussed previously. AID was found to bind to many loci that are transcribed in B cells; indeed, the patterns of AID and RNA polymerase II binding detected by ChIP are very similar.[160] RPA, however, was efficiently bound only at IgH switch regions, and this binding was inhibited by the Ser38Ala mutation that blocks the critical Ser38 phosphorylation discussed previously.

One likely consequence of germline transcription in facilitating CSR involves the formation of a stable RNA:DNA complex known as an R-loop. In this structure, RNA transcribed from the template strand of a DNA molecule binds tightly to that strand with Watson-Crick basepairing, displacing the other DNA strand, which forms a single-stranded loop. In support of the R-loop model, cell-free transcription across G-rich switch regions was found to generate a stable association of the transcript RNA with the template DNA[294]; significantly, no substantial association occurred when the switch region was transcribed in reverse orientation, leading to a C-rich transcript, or when the transcribed template was a DNA fragment other than a switch region. The displaced DNA strand in S region R loops was susceptible to deamination by AID.[155] Evidence that such R-loops form *in vivo* over S regions has been obtained from bisulfite analysis of single-strand DNA regions in B cells.[295,296]

The tightly bound RNA-DNA complex on the template strand of an R-loop may explain both the high level of polymerase II accumulation and the AID-induced mutations in the vicinity of switch region DNA.[297] Progression of the polymerase II complex transcribing through the switch region might be impeded by the RNA bound to the template strand, leading to an accumulation of "stalled" polymerase II molecules; the "stalling" of AID molecules associated with the transcription complex might prolong exposure of the DNA to deamination by AID. Some support for this scheme was reported from an *in vitro* model in which AID-triggered mutations in a transcribed DNA substrate were

increased when transcription was slowed by reducing the nucleotide triphosphate concentration.[298] The importance of stalled polymerase II for CSR was reinforced by the results of a screen for factors whose knockdown by specific shRNAs would inhibit CSR[299]: one protein recovered in this screen was the murine homolog of suppressor of Ty 5 (Spt5), a transcription elongation factor known to be associated with stalled polymerase II. Spt5 knockdown decreased both CSR and switch region hypermutation, as well as AID occupancy of the Sμ region, without affecting cellular levels of AID protein or germline transcripts. By coimmunoprecipitation experiments, AID and Spt5 were found to associate. And genes with high Spt5 occupancy by ChIP analysis were found to be most susceptible to mutations induced by AID overexpression. Polymerase II stalling has been extensively studied in yeast and drosophila, and is found in many genes that require more rapid changes in expression than can be achieved by modulating transcriptional initiation.[300] Apparently, the complex of proteins mediating polymerase II stalling has been adapted in B cells for the special function of regulating CSR of IgH genes.

The R-loop model explains how the "upper" nontranscribed DNA strand would become single-stranded and accessible to AID, but it raises the question of how AID might gain access to the "lower" template DNA strand, held in a tight RNA-DNA hybrid. This strand is known to be accessible to AID because it undergoes C → T mutations in *Ung*−/− *Msh2*−/− double knockout B cells.[293] A likely answer to this question has come from an analysis of proteins bound to AID when mixed with a B cell extract plus *in vitro*–transcribed switch region DNA.[301] The complex of AID and transcribed DNA was found to bind to components of the multisubunit RNA exosome. The exosome is an evolutionarily conserved structure containing nine core proteins that can associate with RNA nucleases, leading to degradation of RNA from template DNA; the exosome could thus potentially expose the template DNA strand to AID. Indeed, shRNA knockdown of one exosome component in the murine B cell line CH12F3 inhibited CSR. Moreover, exosome components were found to associate with AID *in vivo* by immunoprecipitation experiments and were detected (by ChIP) bound to switch region DNA in cells activated for CSR. Finally, in a deamination assay of a model switch region DNA transcribed *in vitro* by T7 polymerase, the addition of AID + RPA + PKA led to deamination of only the nontemplate strand, but the further addition of exosome components led to deamination on the template strand as well. These experiments highlight the exosome as a likely candidate for explaining AID action on the template strand of R-loops, though an unhybridized "lower strand" may also be produced as a result of transcription-dependent supercoiling or antisense transcription.

However, the propensity for R-loop formation is not the only property of switch regions that facilitates CSR. When the Sγ1 region was inverted, it retained about 25% of the wild-type CSR activity.[302] As this inverted, and now C-rich, DNA segment could not form an R-loop, this result suggests that while the R-loop contributes to CSR, other features of the S region that are preserved in the inverted sequence also play a role. Replacement of a natural murine Sμ sequence with the AT-rich frog Sμ sequence, which cannot form an R-loop, supported somewhat reduced but still substantial frequencies of CSR, and it functioned equally well in either orientation. Significantly, in either orientation, the recombination junctions were clustered in a portion of the S region that is rich in repeats of the sequence AGCT, which is a special case of the WRC consensus sequence for AID targeting, being present on both strands as a self-complementary palindrome. Indeed, the AGCT-rich region was a good substrate for *in vitro* deamination by AID when transcribed in association with RPA. The AGCT motif is enriched in all mammalian S regions and may be particularly effective as a target for CSR because its presence in clusters on both strands promotes closely spaced nicks on opposite strands, or even a dsb with a single base overhang if the cytosines in both strands of the same AGCT motif are targeted.[303] The density of AGCT in S regions correlates with the location of switch junctions better than the density of WRC or the boundaries of G-richness or R-loops.[296] These results all suggest that clusters of AGCT may represent a target for CSR that evolved in amphibians, with R-loop formation evolving later in mammals to further enhance AID accessibility to S region DNA.

DNA Breaks as Intermediates in Class Switch Recombination

The recombination event that underlies isotype switching includes DNA breaks and rejoining events that must involve both strands of DNA. Although the RAG-induced DNA breaks that initiate V(D)J recombination occur at the corresponding position on the two strands (yielding a blunt end and a hairpin), the nature of the ends in the initial CSR cleavage is not so clear. An early compilation of switch junctions[304] found only infrequent instances of microhomology at the junction (i.e., short sequence segments that are identical in the unrearranged S sequences near the recombination breakpoint). As these microhomology examples would be consistent with invasion of one DNA strand from Sμ targeting a short homologous region in a downstream S region (or vice versa), the rarity of such junctions has been interpreted as an indication that CSR only rarely occurs by strand invasion and instead usually proceeds by ligation of blunt DNA ends. However, dsbs with staggered ends would generally be the result of the widely accepted mechanism shown in Figure 6.14 (bottom left): a DNA break on one strand might result from AID-catalyzed cytosine deamination, removal of the resulting uracil by UNG, and single-strand cleavage 5′ to the abasic site by an endonuclease, probably apurinic-apyridinic endonuclease 1 (APE1). The initial staggered ends could be converted to blunt ends through exonuclease trimming of a 5′ or 3′ single strand overhang, through filling in of a shorter 3′ end by a DNA polymerase, or through a combination of both processes. Filling in by error-prone polymerases could explain the mutations commonly observed around the switch junction, as mentioned previously.

Evidence supporting initially staggered DNA breaks in CSR was reported from occasional switch junctions observed in a model CSR substrate designed with two oppositely

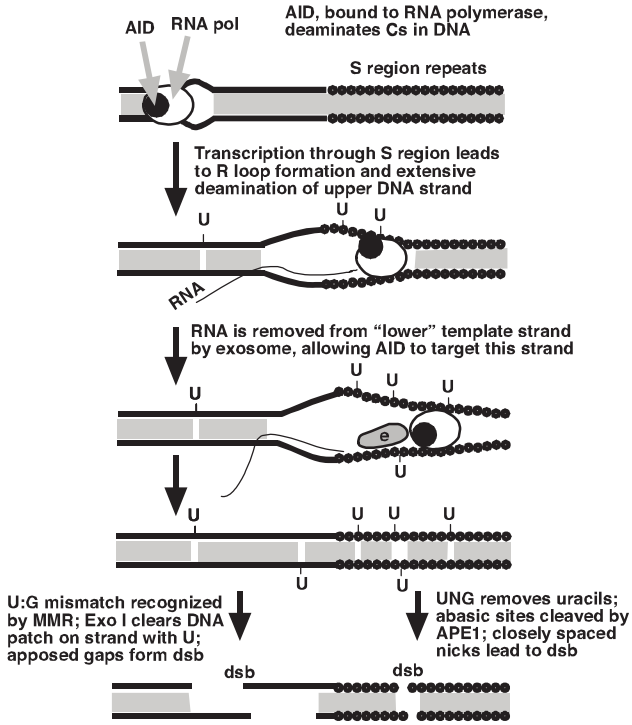

FIG 6.14. Mechanistic Model of Class Switch Recombination. This figure incorporates features of the most widely accepted models, but some aspects are uncertain at present. Two strands of deoxyribonucleic acid (DNA) near the 5′ boundary of a switch region are shown in *black*, with the S region repeats indicated by *dots*. DNA with normal Watson-Crick basepairing is indicated by the *gray shading* between the DNA strands. Ribonucleic acid (RNA) polymerase moves to the right, transcribing the sequence into an RNA strand; multiple other proteins accompany the RNA polymerase, but only activation-induced deaminase (AID) (*black circle*) is shown. AID can deaminate C residues to U in single-stranded R-looped DNA displaced by the RNA-DNA complex. The exosome complex (*e* in the drawing) degrades RNA in the RNA-DNA hybrid, leaving an unpaired lower strand where AID can deaminate C residues. DNA cleavage triggered by processing of uracils can lead to double-strand breaks in pathways dependent or independent of mismatch repair components as shown.

oriented S regions such that CSR would occur by inversion, preserving both recombination junctions on the same chromosome. Several duplications at the ends of inverted DNA after CSR suggested that complementary overhangs at dsbs had been filled in before joining.[305] Additional evidence for staggered breaks has come from several investigators using ligation-mediated–polymerase chain reaction (LM-PCR) to detect double-strand DNA ends at switch regions in cells undergoing CSR.[306–308] LM-PCR protocols involving ligation of a blunt linker directly to blunt ends from genomic DNA were successful in amplifying blunt ends from DNA of B cells activated for CSR, but when the DNA was pretreated with T4 polymerase, which would convert staggered end cuts to blunt, the yield of amplified LM-PCR products was significantly increased, suggesting that most of the ends in the isolated genomic DNA (before T4 polymerase treatment) were staggered.

When the CSR-associated dsbs in Sμ were examined by LM-PCR in B cells from normal and AID−/− individuals, the dsbs were significantly fewer in AID−/− B cells, though not completely absent. Furthermore, microscopic foci of the modified histone γH2AX (which rapidly accumulates at dsbs) were found at IgH genes (localized by FISH) in B cells undergoing CSR; these foci were strikingly diminished in AID−/− B cells, consistent with AID-dependence of dsbs in CSR.[309] These foci, as well as switch region dsbs, occur predominantly in the G1 phase of the cell cycle.[310] Like the off-target deamination by AID discussed previously in the context of SHM, off-target DNA breaks are another potential consequence of AID. B cells from mice overexpressing AID showed a high incidence of chromosomal translocations and DNA breaks compared with *Aicda*−/− mice; in the context of homozygous p53 deficiency (which allows cell growth despite DNA damage), many of the mice developed B-cell lymphomas.[311] In a study designed to systematically examine the locations of dsbs in mouse B cells stimulated to undergo CSR, ChIP was used to identify DNA associated with Nbs1, a protein that rapidly binds to dsbs *in vivo*, as discussed below.[312] This analysis detected hundreds of reproducible AID-dependent DNA break locations, some of which were syntenic with DNA rearrangements found in human B-cell lymphomas. Many of the AID-dependent dsbs occurred in nontranscribed regions, unlike the CSR-related dsbs in IgH switch regions. Evidence indicates that AID-dependent off-target dsbs are normally repaired largely by homologous recombination: B cells defective for the homologous recombination component XRCC2 were found to harbor many more γ-H2AX foci than were found in *Xrcc2*+/+ cells or in *Aicda*−/− *Xrcc2*−/− cells.[313] B cells apparently protect against these breaks by upregulating *Xrcc2* transcription when activated for CSR.

Ubiquitously Expressed Components of Class Switch Recombination Machinery

UNG and Mismatch Repair. The model of Figure 6.13 suggests that after AID-catalyzed deamination of a cytidine residue, the resulting uracil is removed by *UNG*, creating an abasic site that is converted by APE1 to a single-strand DNA break (nick), which can become double-stranded if there is a nearby nick on the opposite strand. This model predicts that both CSR and the creation of dsbs would be severely impaired in the absence of *UNG*. Indeed, B cells from *Ung*−/− mice showed almost complete inability to switch *in vitro* to IgG1 or IgG3 secretion, and significant impairment in IgA secretion.[151] dsbs in *Ung*−/− B cells, as detected by LM-PCR, were also significantly reduced, but not abolished.[308] Human patients with homozygous *UNG* deficiency due to mutations in both *UNG* alleles showed a hyper-IgM phenotype, with a more profound defect than in *Ung*−/− mice: the patients showed essentially no IgG, IgE, or IgA secretion by stimulated B cells, and no dsbs (by LM-PCR) in Sμ.[314]

A role for APE1 or APE2 in CSR is supported by the observation that in mice with engineered deficiencies these genes, B cells induced for CSR show decreased switching and decreased induction of dsbs.[315]

The complementary pathways of UNG and MMR action in SHM were discussed previously (see Fig. 6.13); current evidence suggests a similar participation of MMR in CSR. Although

CSR was dramatically impaired in *Ung*–/– B cells (e.g., *in vitro* switching to IgG1 was reduced to about 6% of wild-type), the double knockout *Ung*–/– *Msh2*–/– caused significant further impairment (to 1.5% of wild-type IgG1 switching).[316] Evidence suggests that the C-terminal 10 amino acids of AID that are required for CSR but not SHM may function by stabilizing the interaction of UNG2 and the MSH2-MSH6 dimer to DNA.[317]

If a single-strand break created on one strand by UNG and APE1 is too far away from the the closest single-strand break on the opposite strand to create a dsb, then Exo1 engaged by MMR can chew from a nick on one strand toward a nick on the opposite strand, creating a dsb.[310,318] Consistent with this idea, mice engineered with a knockout of the MMR component Exo1 show a significantly decreased efficiency of CSR, to roughly 15% to 30% of normal.[319]

Thus, many of the same AID-triggered mechanisms that induce mutations in V_H regions operate in switch regions to induce dsbs. Once created, these dsbs are resolved by mechanisms distinct from the mechanisms resolving mismatches in SHM; however, as discussed in the following, some of the components that resolve dsbs generated in V(D)J recombination play a similar role in CSR.

End Joining Proteins in Class Switch Recombination. If AID triggers DNA cleavage at switch regions, ubiquitous DNA repair and ligation enzymes could participate in the subsequent DNA repair steps of CSR, as in V(D)J recombination. This possibility has been tested by engineered gene knockouts. However, as NHEJ knockouts would impair V(D)J recombination, thus blocking B-cell development prior to the stage of CSR, investigators have studied NHEJ in CSR using mouse strains with knockins of productive recombined H and L chain genes, which can undergo CSR. IgH/IgL knockin mice with intact Ku genes were able to switch to downstream isotypes, but the corresponding Ku-70– or Ku-80–deficient mice were reported to be dramatically impaired in CSR, although decreased cell proliferation could have contributed to this effect as more recent studies using different conditions report 30% to 50% residual CSR activity.[320]

The other "core" factors of NHEJ—XLF/Cernunnos, Ligase4, and XRCC4—catalyze the DNA ligation in NHEJ and have also been investigated for participation in CSR.[320–324] B cells deficient in any one of these genes show variably decreased CSR efficiency, with increased junctional microhomologies compared with normal B cells. This observation suggests that one or more fairly robust backup pathways—commonly called alternative NHEJ—can repair dsbs using microhomology-based ligation when "classic" NHEJ is inoperative. This conclusion is consistent with studies of alternative NHEJ repair of dsbs unrelated to CSR.[78] The proteins CtIP,[325] PARP1,[326,327] and XRCC1[328] apparently contribute to alternative NHEJ during CSR, as experimentally reduced expression of each protein decreases microhomology at CSR junctions.

Apart from these core NHEJ components, Artemis and DNA-PKcs are required for joining ends that require processing, like the hairpin coding ends produced in V(D)J recombination discussed previously in this chapter. Artemis-deficient murine B cells have no obvious impairment in CSR efficiency[329] but show several-fold increases (compared to wild-type) in the number of chromosome aberrations in the IgH locus detected by FISH assays[330]; these aberrations are AID-dependent and only observed after activation of B cells for CSR. In humans, Artemis deficiency results in a SCID syndrome with severe defects in B- and T-cell development, but rare Sμ-Sα recombination junctions amplified from patient B cells were found to show a high index of microhomologies,[331] suggesting that Artemis is required for normal classic-NHEJ resolution of dsbs in human CSR.

Studies on the role of DNA-PKcs in CSR have yielded somewhat conflicting conclusions on whether this protein is required for maximally efficient CSR, with the results of individual studies perhaps depending on the knocked-in Ig genes used, the specific mutations of DNA-PKcs, or the genetic background of mice studied.[332,333] However, *DNA-PKcs*–/– B cells showed evidence of AID-dependent chromosome aberrations in the IgH locus, similar to those seen in *Artemis*–/– cells but more numerous.[330] Thus it appears that both Artemis and DNA-PKcs are required for efficient repair of at least a subset of the dsbs associated with CSR.

DNA Damage Response Proteins in Class Switch Recombination. NHEJ is one component of a larger mechanism for detecting and repairing dsbs, collectively known as the DNA damage response (DDR). Because DNA dsbs are potentially damaging to the cell, and occur in all cells through accidents of DNA metabolism, toxic chemicals, and radiation, DDR appeared early in evolution, and many components are conserved from yeast to mammals. Defects in DDR can cause developmental abnormalities, cancer predisposition, and sensitivity to radiation, as well as immunodeficiency resulting from impaired V(D)J recombination or CSR. DDR factors participate in a baroque cascade of interactions to cluster at dsbs and initiate repair. The complexities of the DDR are beyond the scope of this chapter, but are described in several recent reviews.[78,334]

Several DDR components have been documented to participate in CSR and are listed below; mutations in some of these cause specific genetic syndromes in humans, often associated with immunodeficiency.

1. Nbs1, product of the gene that is defective in the human disease Nijmegen breakage syndrome.[309,335,336] Nbs1 functions as part of a complex that also includes Mre11 and Rad50 (MRN complex). Mre11 has a nuclease activity, and may contribute to DNA cleavage in CSR; mice and humans with defective Mre11 also show impaired CSR.[337,338]
2. ATM, product of the gene *m*utated in the human disease *a*taxia *t*elengectasia and a member of the phosphatidylinositol-3′-kinase family.[339,340]
3. γ-H2AX—the phosphorylated form of histone H2AX—which rapidly accumulates in foci at dsbs, and helps to assemble other proteins at dsbs to prevent the breaks from progressing to chromosome translocations.[341]
4. The E3 ubiquitin ligase RNF8 (Ring Finger 8), which is known to monoubiquitinate histones at dsbs.[342]
5. The E3 ubiquitin ligase RNF168, found to be mutated in the RIDDLE (*r*adiosensitivity, *i*mmuno*d*eficiency, *dy*smorphic features, *le*arning difficulties) syndrome.[343]

6. 53BP1 (p53 binding protein 1) originally discovered as a protein binding to the tumor suppressor p53, but subsequently found to function in checkpoint control and to localize rapidly to DNA breaks *in vivo*. *53BP1*−/− murine B cells show dramatic impairment in CSR (to 55 to 25% of wild-type), but increases in the frequency of AID-triggered deletions within Sμ,[344] suggesting that formation of dsbs and repair of closely-spaced breaks does not require 53BP1, but joining of more distant dsbs depends on this protein.

Although the we currently lack a detailed model explaining the functions of all the DDR factors in dsb repair and specifically in CSR, the emerging evidence suggests that these proteins have mutual interactions and distinct but related roles, so that the elimination of any one protein reduces CSR efficiency but permits residual CSR to occur by pathways that remain intact.

Other Proteins that may Target AID for Class Switch Recombination. As discussed in the context of SHM, accurate targeting of AID is important since off-target activities of this protein can be deleterious, and off-target dsbs triggered by AID could be particularly dangerous. Several searches for proteins that might contribute to targeting of AID to switch regions have uncovered candidates for this function. In one study,[345] biotinylated AID was used as bait to fish for *in vitro* binding proteins, which were identified by mass spectrometry. One protein identified by this screen was PTBP2 (polypyrimidine tract-binding protein 2) which is considered to be a regulator of RNA splicing. Knockdown of PTBP2 by shRNA in CH12 B cells caused a substantial decrease in binding of AID to Sμ (determined by ChIP) and significant impairment of CSR. The possible role of a splicing regulator in CSR could be related to the puzzling observation that germline transcripts must be spliced in order for efficient CSR,[280] as mentioned previously.

A screen for proteins that could bind selectively *in vitro* to AGCT sequences—which are found clustered in many switch regions—identified another protein that might target AID in CSR: the 14-3-3 proteins, a family of seven members widely expressed and known to bind to many signaling proteins.[346] ChIP assays on B cells stimulated for CSR showed that 14-3-3 proteins bind to switch regions in an isotype-specific way (depending on stimulus conditions) in both normal and *Aicda*−/− cells, and reduction of 14-3-3 activity by either a peptide inhibitor or by genetic engineering decreased both CSR efficiency and AID binding to switch region DNA. Finally, a bimolecular fluorescence complementation assay revealed that in B-cell nuclei AID and 14-3-3 molecules form a complex that is dependent on the AID C-terminal amino acids required for CSR. These experiments suggest that 14-3-3 proteins are additional candidates for AID targeting molecules.

PKA, the cAMP-regulated protein kinase responsible for phosphorylation of AID Ser38 discussed previously, has also been suggested as a candidate that targets AID-dependent DNA cleavage to switch regions.[347] PKA was found by ChIP to bind to switch regions of stimulated B cells from normal and *Aicda*−/−mice, whereas the binding of RPA depended on Ser38-phosphorylated AID. Cellular concentrations of cAMP were found to rise rapidly after stimulation to CSR, and a genetic inactivation of PKA activity that prevented its activation by cAMP reduced both RPA binding and CSR. These data suggest a model in which CSR stimuli recruit both PKA and nonphosphorylated AID to switch regions, and AID gains the ability to recruit RPA and trigger dsbs efficiently only when cAMP-activated PKA phosphorylates Ser38.

Additional components of CSR may be discovered by analysis of patients with a hyper-IgM phenotype unexplained by defects in known components.[314]

CONCLUSION

Recombinant DNA technology has revolutionized the study of the antibody response. Initial investigations used powerful cloning and sequencing methods to define the structure of the Ig genes as they exist in the germline and in actively secreting B-lymphocytes. Subsequent experiments have begun to shed light on the mechanisms of the processes unique to these genes: V(D)J recombination, CSR, and SHM.

The knowledge of Ig genes gained so far has answered some of the most puzzling mysteries about antibody diversity, as discussed previously, and has also led to many practical ramifications involving these genes that are beyond the scope of this chapter. As one example, cloned Ig genes have allowed the production of recombinant monoclonal antibodies and the bioengineering of Ig-fusion proteins that exploit the exquisite specificity of antibody V region binding (e.g., antibody-toxin fusions) or the ability of Ig C region domains to extend serum half-life or engage Fc receptors. Other engineered derivatives utilizing Ig genes include single-chain antibodies, bispecific antibodies, and "intrabodies" designed not to be secreted from a cell but rather to bind to intracellular targets.[348–350] Ig V gene fragments cloned into bacteriophage so as to express single-chain V regions on the phage surface (phage display libraries) can be used to obtain specific monoclonal antibodies without immunization or use of mammalian cells, and *in vitro* mutation and selection protocols can mimic affinity maturation to yield high-affinity antibodies.[351] Even Ig gene regulatory regions have been exploited to achieve B-cell–specific expression of oncogenes[352] and of intracellular toxins that could be used to target B lymphomas.[353] Apart from these biotechnology applications, Ig gene probes have led to the identification of numerous proto-oncogenes that become activated by translocation into Ig gene loci.[354] For instance, Bcl2 was initially discovered as the target of Ig H chain translocation in follicular lymphoma, and provided an entry into an entire family of apoptosis-related genes. A final example of medical benefit from Ig gene technology has been the use of patient-specific Ig gene rearrangements of leukemias or lymphomas to monitor disease status by PCR.[355,356]

Further practical applications of Ig genes can be anticipated in the future, as well as a deeper scientific understanding of their molecular biology and their contribution to the immune system.

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CHAPTER 7

Antigen–Antibody Interactions and Monoclonal Antibodies

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INTRODUCTION

The basic principles of antigen–antibody interaction are those of any bimolecular reaction. Moreover, the binding of antigen by antibody can, in general, be described by the same theories and studied by the same experimental approaches as the binding of a hormone by its receptor, of a substrate by enzyme, or of oxygen by hemoglobin. There are several major differences, however, between antigen–antibody interactions and these other situations. First, unlike most enzymes and many hormone-binding systems, antibodies do not irreversibly alter the antigen they bind. Thus, the reactions are, at least in principle, always reversible. Second, antibodies can be raised, by design of the investigator, with specificity for almost any substance known. In each case, one can find antibodies with affinities as high as and specificities as great as those of enzymes for their substrates and receptors for their hormones. The interaction of antibody with antigen can thus be taken as a prototype for interactions of macromolecules with ligands in general. In addition, these same features of reversibility and availability of a wide variety of specificities have made antibodies invaluable reagents for identifying, quantitating, and purifying a growing number of substances of biologic and medical importance.

One other feature of antibodies that in the past proved to be a difficulty in studying and using them, compared to, say, enzymes, is their enormous heterogeneity. Even "purified" antibodies from an immune antiserum, all specific for the same substance and sharing the same overall immunoglobulin (Ig) structure, will be a heterogeneous mixture of molecules of different subclass, different affinity, and different fine specificity and ability to discriminate among cross-reacting antigens. The advent of hybridoma monoclonal antibodies[1–3] has made available a source of homogeneous antibodies to almost anything to which antisera can be raised. Nevertheless, heterogeneous antisera are still in widespread use and even have advantages for certain purposes, such as precipitation reactions. Therefore, it is critical to keep in mind throughout this chapter, and indeed much of the volume, that the principles derived for the interaction of one antibody with one antigen must be modified and extended to cover the case of heterogeneous components in the reaction.

In this chapter, we examine the theoretical principles necessary for analyzing, in a quantitative manner, the interaction of antibody with antigen and the experimental techniques that have been developed to study these interactions as well as to make use of antibodies as quantitative reagents. Furthermore, we discuss the derivation, use, and properties of monoclonal antibodies.

THERMODYNAMICS AND KINETICS

The Thermodynamics of Affinity

The basic thermodynamic principles of antigen–antibody interactions, as we indicated previously, are the same as those for any reversible bimolecular binding reaction. We review these as they apply to this particular immunologic reaction.

Chemical Equilibrium in Solution

For this purpose, let S = antibody binding sites, L = ligand (antigen) sites, and SL = the complex of the two. Then for the reaction

$$S + L \rightleftharpoons SL \tag{1}$$

the mass action law states

$$K_A = \frac{[SL]}{[S][L]} \tag{2}$$

where K_A = association constant (or affinity) and square brackets = molar concentration of the reactants enclosed. The import of this equation is that for any given set of conditions such as temperature, pH, and salt concentration, the ratio of the concentration of the complex to the product of the concentrations of the reactants at equilibrium is always constant. Thus, changing the concentration of either antibody or ligand will invariably change the concentration of the complex, provided neither reactant is limiting, that is, neither has already been saturated, and provided sufficient time is allowed to reach a new state of equilibrium. Moreover, because the concentrations of antibody and ligand appear in this equation in a completely symmetrical fashion, doubling either the antibody concentration or the antigen concentration results in a doubling of the concentration of the antigen–antibody complex, provided the other reactant is in sufficient excess. This proviso, an echo of the one just discussed, is inherent in the fact that [S] and [L] refer to the concentrations of free S and free L, respectively, in solution, not the total concentration, which would include that of the complex. Thus, if L is not in great excess, doubling [S] results in a decrease in [L] as some of it is consumed in the complex, so the net result is less than a doubling of [SL]. Similarly, halving the volume results in a doubling of the total concentration of both antibody and

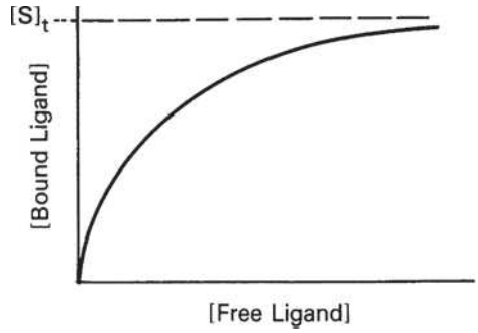

FIG. 7.1. Schematic Plot of Bound Ligand Concentration as a Function of Free Ligand Concentration at a Constant Total Concentration of Antibody-Combining Sites, $[S]_t$. The curve asymptotically approaches a plateau at which [bound ligand] = $[S]_t$.

ligand. If the fraction of both reactants tied up in the complex is negligibly small (as might be the case for low-affinity binding), the concentration of the complex quadruples. However, in most practical cases, the concentration of complex is a significant fraction of the total concentration of antigen or antibody or both, so the net result is an increase in the concentration of complex but by a factor of <4. The other important, perhaps obvious, but often forgotten, principle to be gleaned from this example is that because it is concentration, not amount, of each reactant that enters into the mass action law (Equation 2), putting the same amount of antigen and antibody in a smaller volume will increase the amount of complex formed, and diluting them in a larger volume will greatly decrease the amount of complex formed. Moreover, these changes go approximately as the square of the volume, so volumes are critical in the design of an experiment.

The effect of increasing free ligand concentration [L], at constant total antibody concentration, on the concentration of complex, [SL], is illustrated in Figure 7.1. The mass action law (Equation 2) can be rewritten as

$$[SL] = K_A[S][L] = K_A([S]_t - [SL])[L] \qquad (3)$$

or

$$[SL] = \frac{K_A[S]_t[L]}{(1 + K_A[L])} \qquad (3')$$

where $[S]_t$ = total antibody site concentration; that is, [S] + [SL]. Initially, when the complex [SL] is a negligible fraction of the total antibody $[S]_t$, the concentration of complex increases nearly linearly with increasing ligand. However, as a larger fraction of antibody is consumed, the slope tapers off and the concentration of complex, [SL], asymptotically approaches a plateau value of $[S]_t$ as all the antibody becomes saturated. Thus, the concentration of antibody-binding sites can be determined from such a saturation binding curve (see Fig. 7.1), taking the concentration of (radioactively or otherwise labeled) ligand bound at saturation as a measure of the concentration of antibody sites.* This measurement is sometimes referred to as antigen-binding capacity.

*This point is strictly true only for univalent ligands, but most multivalent ligands behave as effectively univalent at large antigen excess, where this plateau is measured.

The total concentration of ligand at which the antibody begins to saturate is a function not only of the antibody concentration but also of the association constant, K_A, also called the affinity. This constant has units of molar (M^{-1}) or L/mol, if all the concentrations in Equation 2 are molar. Thus the product $K_A[L]$ is unitless. It is the value of this product relative to 1 that determines how saturated the antibody is, as can be seen from Equation 3′. For example, an antibody with an affinity of 10^7 M^{-1} will not be saturated if the ligand concentration is 10^{-8} M (product $K_A[L] = 0.1$) even if the total amount of ligand is in great excess over the total amount of antibody. From Equation 3′ the fraction of antibody occupied would be only 0.1/1.1, or about 9%, in this example. These aspects of affinity and the methods for measuring affinity are analyzed in greater detail in the next section.

Free Energy

Regarding thermodynamics, the affinity, K_A, is also the central quantity because it is directly related to the free energy, ΔF, of the reaction by the equations

$$\Delta F° = -RT \ln K_A \qquad (4)$$

$$K_A = e^{-\Delta F°/RT} \qquad (4')$$

where R = so-called gas constant (1.98717 cal/°K·mol), T = absolute temperature (in degrees Kelvin), ln = natural logarithm, and e = base of the natural logarithms. The minus sign is introduced because of the convention that a negative change in free energy corresponds to positive binding. The ΔF° is the standard free-energy change defined as the ΔF for 1 mol antigen + 1 mol antibody sites combining to form 1 mol of complex at unit concentration.

It is also instructive to note an apparent discrepancy in Equations 4 and 4′. As defined in Equation 2, K_A has dimensions of M^{-1} (ie, L/mol), whereas in Equation 4′, it is dimensionless. The reason is that for Equation 4′ to hold strictly, K_A must be expressed in terms of mole fractions rather than concentrations. The mole fraction of a solute is the ratio of moles of that solute to the total number of moles of all components in the solution. Because water (55 M) is by far the predominant component of most aqueous solutions, for practical purposes, one can convert K_A into a unitless ratio of mole fractions by dividing all concentrations in Equation 2 by 55 M. This transformation makes Equation 4′ strictly correct, but it introduces an additional term, −RT ln 55 (corresponding to the entropy of dilution), into Equation 4. This constant term cancels out when one is subtracting ΔF values but not when one discusses ratios of ΔF values.

An important rule of thumb can be extracted from these equations. Because ln 10 = 2.303, a 10-fold increase in affinity of binding corresponds to a free-energy change ΔF of only 1.42 kcal/mol at 37°C (310.15°K). (The corresponding values for 25 and 4°C are 1.36 and 1.27 kcal/mol, respectively.) This is less than one-third the energy of a single hydrogen bond (about 4.5 kcal/mol). Looked at another way, a very high affinity of 10^{10} M^{-1} corresponds to a ΔF of only 14.2 kcal/mol, approximately the bonding energy of three hydrogen bonds. (Of course, because hydrogen bonds with water are broken during the formation of hydrogen bonds between

antigen and antibody, the net energy per hydrogen bond is closer to 1 kcal/mol.) It is apparent from this example that of the many interactions (hydrophobic and ionic as well as hydrogen bonding) that occur between the contact residues in an antibody-combining site and the contacting residues of an antigen (such as a protein), almost as many are repulsive as attractive. It is this small difference of a few kilocalories between much larger numbers corresponding to the total of attractive and the total of repulsive interactions that leads to net "high-affinity" binding. If ΔF were any larger, binding reactions would be of such high affinity as to be essentially irreversible. Viewed in this way, it is not surprising that a small modification of the antigen can result in an enormous change in affinity. A single hydrogen bond can change the affinity manyfold, and similar arguments apply to hydrophobic interactions and other forms of bonding. This concept is important when we discuss specificity and antigen structure later.

Effects of Temperature, pH, Salt Concentration, and Conformational Flexibility

It was mentioned previously that K_A is constant for any given set of conditions such as temperature, pH, and salt concentration. However, it varies with each of these conditions. We have already seen that the conversion of free energy to affinity depends on temperature. However, the free energy itself is also a function of temperature

$$\Delta F^\circ = \Delta H^\circ - T\,\Delta S^\circ \qquad (5)$$

where ΔH = change in enthalpy (the heat of the reaction),[†] ΔS = entropy (a term related to the change in disorder produced by the reaction),[2] and T = absolute temperature (in degrees Kelvin).

It can be shown that the association constant K_A will thus vary with temperature as follows:

$$\frac{d \ln K_A}{dT} = \frac{\Delta H^\circ}{RT^2} \qquad (6)$$

or equivalently,

$$\frac{d \ln K_A}{d(1/T)} = \frac{-\Delta H^\circ}{R} \qquad (6')$$

The derivation of these equations is beyond the scope of this book.[4] However, the practical implications are as follows. First, one can determine the standard ΔH° of the reaction from the slope of a plot of ln K_A versus 1/T. Second, for an interaction that is primarily exothermic (ie, driven by a large negative ΔH, such as the formation of hydrogen bonds and polar bonds), the affinity decreases with increasing temperature. Thus many antigen–antibody interactions have a higher affinity at 4°C than at 25°C or 37°C, so maximum binding for a given set of concentrations can be achieved in the cold. In contrast, apolar or hydrophobic interactions are driven largely by the entropy term T ΔS, and ΔH° is near zero. In this case, there is little effect of temperature on the affinity.

As for the effects of pH and salt concentration (or ionic strength) on the affinity, these vary depending on the nature of the interacting groups. Most antigen–antibody reactions are studied near neutral pH and at physiologic salt concentrations (0.15 M NaCl). If the interaction is dominated by ionic interactions, high salt concentration lowers the affinity.

Conformational flexibility of an antigen can also affect the affinity by affecting the entropy term in Equation 5. An outstanding example comes from the thermodynamics of binding of a series of broadly neutralizing monoclonal antibodies to the human immunodeficiency virus (HIV) envelope protein gp120. The energetics of binding between HIV gp120 and its cellular receptor cluster of differentiation (CD)4[5] or to a panel of monoclonal antibodies[6] has been studied in detail. The results provide insight into the ways protein flexibility can help the virus to evade antibody immunity. They may also explain part of the difficulty in eliciting antibodies of this type when immunizing with the native protein.

GP120 is a glycoprotein that exhibits at least two conformational states: one exists on the virus, and it changes to the other when it binds the CD4 receptor on the cell surface. Monoclonal antibodies F105 and b12 bind residues within the CD4 binding site on gp120 while monoclonal 2G12 binds the opposite surface. The CD4 binding site defines a neutralizing surface that is conserved among a broad range of HIV isolates.

As shown in Table 7.1, CD4 and each antibody demonstrated a strongly negative ΔF, corresponding to high affinity binding. However, as shown by microcalorimetry, they arrived at the affinity in different ways. The CD4 and F105 have a strong negative ΔH of binding, but they also have a large negative entropy due to the conformational change required, as shown by strongly positive values for –TΔS. This entropy effect greatly reduces the overall ΔF of binding, so they depend on a very large negative ΔH to bind. In contrast, monoclonal b12 has moderate levels of both ΔH and –TΔS, resulting in a nearly identical ΔF. Monoclonal 2G12 binds gp120 with a less favorable ΔH but no entropy cost.

The two discrete conformations of gp120 may be called the open and closed forms. The excursion between these two conformations affects the entropy of binding. CD4 and F105 only bind the open form, which reduces randomness and creates an entropy barrier. Monoclonal b12 is less dependent on one conformation, so it binds with less entropy cost, and 2G12 is indifferent to the two forms, so it has no entropy effect at all.

The strong entropy effect observed for these monoclonals may illustrate one way for the virus to evade antibody binding, through a mechanism called conformational masking.

TABLE 7.1 Energetics of Antibody Binding to gp120 Core Structure

Ligand	ΔF	ΔH	–TΔS
CD4	–10.4	–48.4	38.2
F105	–11.4	–30.0	18.6
b12	–12.3	–18.0	5.7
2G12	–7.8	–6.2	–1.6

CD, cluster of differentiation. From Kwong, et al.[8]

[†]For a more complete description of these concepts, see a physical chemistry text such as Moore.[4]

The randomness of gp120, as found on the virus, means that it is rarely in the open conformation, making it difficult for most antibodies such as F105 to bind. Only the rare antibody that is b12-like can bind multiple forms and eventually pull gp120 into its most favorable conformation.

The same effect may explain the difficulty in eliciting antibodies to this site using native gp120. If it is rarely in the open form, it will be unable to trigger B cells to make antibodies that require this form, and it will deliver only a weak antigenic stimulus to those that bind partially to different conformations, such as b12. These considerations suggest that a more favorable vaccine antigen could be made if gp120 could be anchored in the open conformation, so it could stimulate B cells to make antibodies that require this form. The hallmark of this structure would be its ability to bind F105 and b12 with a good ΔH and reduced values of $-T\Delta S$.

Kinetics of Antigen–Antibody Reactions

A fundamental connection between the thermodynamics and kinetics of antigen–antibody binding is expressed by the relationship

$$K_A = \frac{k_1}{k_{-1}} \tag{7}$$

where k_1 and k_{-1} are the rate constants for the forward (association) and backward (dissociation) reactions.

The forward reaction is determined largely by diffusion rates (theoretical upper limit 10^9 L/mol/sec) and by the probability that a collision will result in binding, that is, largely the probability that both the antigen and the antibody will be oriented in the right way to produce a good fit as well as the activation energy for binding. The diffusive rate constant can be shown[7] to be approximated by the Smoluchowski equation

$$k_{dl} = 4\pi a D(6 \times 10^{20}) \tag{7a}$$

where a = sum of the radii in centimeters of the two reactants, D = sum of the diffusion constants in cm^2/sec for the individual reactants, and the constant 6×10^{20} is necessary to convert the units to $M^{-1} \cdot sec^{-1}$. For example, if $a = 10^{-6}$ cm and $D = 10^{-7}$ cm^2/sec, then $k_{dl} \approx 7.5 \times 10^8$ $M^{-1} \cdot sec^{-1}$. Association rates will generally be slower for large protein antigens than for small haptens. This observation may be due to the smaller value of D, to the orientational effects in the collision, and to other nondiffusional aspects of protein–protein interactions. Therefore, association rates for protein antigens are more frequently on the order of 10^5 to 10^6 $M^{-1} \cdot sec^{-1}$ (see following discussion). However, this observation can also be partly understood from diffusion-limited rates alone. If the radii of hypothetically spherical reactants are r_1 and r_2, then in Equation 7a, $a = r_1 + r_2$, whereas D is proportional to $1/r_1 + 1/r_2$. The diffusive rate constant is therefore proportional to

$$(r_1 + r_2)\left(\frac{1}{r_1} + \frac{1}{r_2}\right) = \frac{(r_1 + r_2)^2}{r_1 r_2} \tag{7b}$$

From this result, it can be seen that if $r_1 = r_2 = r$, then r cancels out and the whole term in Equation 7b is simply equal to 4. Thus, for the interaction between two molecules of equal size, the diffusive rate constant is the same regardless of whether those molecules are large or small.[8] However, if one molecule is large and the other small, the rate is greater than if both molecules are large. This difference occurs because reducing the radius r_1 while keeping r_2 constant (and larger than r_1) has a greater effect on increasing the diffusion constant term D, proportional to $(1/r_1 + 1/r_2)$, in which the smaller radius produces the larger term than it has on the term a, which is still dominated by the larger radius r_2. For example, if $r_2 = r$ as shown, but $r_1 = 0.1r$, then the numerator in Equation 7b is only reduced from $4r^2$ to $1.21r^2$, whereas the denominator is reduced from $1r^2$ to $0.1r^2$. Thus, the ratio is increased from 4 to 12.1. Viewed another way, the greater diffusive mobility of the small hapten outweighs its diminished target area relative to a large protein antigen because the larger target area of the antibody is available to both.

The dissociation rate (or "off rate") k_{-1} is determined by the strength of the bonds (as it affects the activation energy barriers for dissociation) and the thermal energy kT (where k is Boltzmann constant), which provides the energy to surmount this barrier. The activation energy for dissociation is the difference in energy between the starting state and the transition state of highest energy to which the system must be raised before dissociation can occur.

As pointed out by Eisen,[9] if one compares a series of related antigens, of similar size and other physical properties, for binding to an antibody, the association rates are all very similar. The differences in affinity largely correspond to the differences in dissociation rates.

A good example is that of antibodies to the protein antigen staphylococcal nuclease.[10] Antibodies to native nuclease were fractionated on affinity columns of peptide fragments to isolate a fraction specific for residues 99 through 126. The antibodies had an affinity of 8.3×10^8 M^{-1} for the native antigen and an association rate constant, k_{on}, of 4.1×10^5 $M^{-1} \cdot sec^{-1}$. This k_{on} was several orders of magnitude lower than had been observed for small haptens,[11] as discussed previously. A value of k_{off} of 4.9×10^{-4} sec^{-1} was calculated using these results in Equation 7. This is a first-order rate constant from which one can calculate a halftime for dissociation (based on $t_{1/2} = \ln 2/k_{off}$) of 23 minutes. These rates are probably typical for high-affinity ($K_A \approx 10^9$ M^{-1}) antibodies to small protein antigens such as nuclease (molecular weight [MW] ≈ 17,000). The dissociation rate is important to know in designing experiments to measure binding because if the act of measurement perturbs the equilibrium, the time one has to make the measurement (eg, to separate bound and free) is determined by this halftime for dissociation. For instance, a 2-minute procedure that involves dilution of the antigen–antibody mixture can be completed before significant dissociation has occurred if the dissociation halftime is 23 minute. However, if the on rate is the same, but the affinity 10-fold lower, still a respectable 8×10^7 M^{-1}, then the complex could be 50% dissociated in the time required to complete the procedure. This caution is relevant when we discuss methods of measuring binding and affinity in the following.

Because knowledge of the dissociation rate can be so important in the design of experiments, a word should be said about techniques to measure it. Perhaps the most widely applicable one is the use of radiolabeled antigen. After equilibrium is reached and the equilibrium concentration of bound radioactivity determined, a large excess of unlabeled antigen is added. Because any radioactive antigen molecule that dissociates is quickly replaced by an unlabeled one, the probability of a radioactive molecule associating again is very small. Therefore, one can measure the decrease in radioactivity bound to antibody with time to determine the dissociation rate.‡

AFFINITY

It is apparent from the previous discussion that a lot of information about an antigen–antibody reaction is packed into a single value, its affinity. In this section, we examine affinity more closely, including methods for measuring affinity and the heterogeneity thereof, the effects of multivalency of antibody and/or of antigen, and the special effects seen when the antigen–antibody interaction occurs on a solid surface (two-phase systems).

Interaction in Solution with Monovalent Ligand

The simplest case is that of the interaction of antibody with monovalent ligand. We may include in this category both antihapten antibodies reacting with truly monovalent haptens and antimacromolecule antibodies, which have been fractionated to obtain a population that reacts only with a single, nonrepeating site on the antigen.** In the latter case, the antigen behaves as if monovalent in its interaction with the particular antibody population under study. The proviso that the site recognized (antigenic determinant) be nonrepeating, that is, occur only once per antigen molecule, of course, is critical.

If the combining sites on the antibody are independent (ie, display no positive or negative cooperativity for antigen binding), then for many purposes one can treat these combining sites, reacting with monovalent ligands, as if they were separate molecules. Thus, many, but not all, of the properties we discuss can be analyzed in terms of the concentration of antibody-combining sites, independent of the number of such sites per antibody molecule (2 for IgG and IgA, 10 for IgM).

To determine the affinity of an antibody, one generally determines the equilibrium concentrations of bound and free ligand, at increasing total ligand concentrations, but at constant antibody concentration. Alternatively, one can vary the antibody concentration, but then, the analysis is slightly more complicated. Perhaps the theoretically most elegant experimental method to determine these quantities is equilibrium dialysis,[12,13] depicted and explained in Figure 7.2, in which ligand (antigen) is allowed to equilibrate between two chambers, only one of which contains antibody, separated by a semipermeable membrane impermeable to antibody. The important feature of this method, as opposed to most others, is that the concentrations of ligand in each chamber can be determined without perturbing the equilibrium. The disadvantage of this method is that it is applicable only to antigens small enough to permeate freely a membrane that will exclude antibody. Another technical disadvantage is that bound antigen, determined as the difference between bound plus free antigen in one chamber and free antigen in the other, is not measured independently of free antigen.

Another category of method uses radiolabeled ligand in equilibrium with antibody and then physically separates free antigen bound to antibody and quantitates each separately. The methods used to separate bound and free antigen are discussed in the section on radioimmunoassay (RIA). These methods generally allow independent measurement of bound and free antigen but may perturb the equilibrium.

Scatchard Analysis

Once data are obtained, there are a number of methods of computing the affinity, of which we shall discuss two.

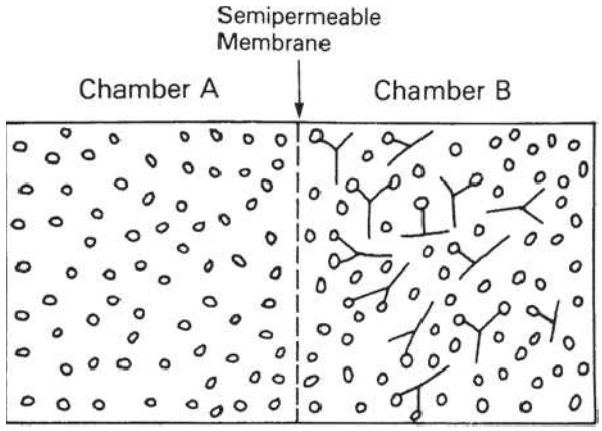

FIG. 7.2. Equilibrium Dialysis. Two chambers are separated by a semipermeable membrane that is freely permeable to ligand but not at all to antibody. Antibody is placed in one chamber (B) and ligand in one or both chambers. Regardless of how the ligand is distributed initially, after sufficient time to reach equilibrium, it will be distributed as follows. The concentration of free ligand will be identical in both chambers, but chamber B will have additional ligand bound to antibody. The concentration of bound ligand is thus the difference between the ligand concentration in the two chambers, whereas the free concentration is the concentration in chamber A. Because these concentrations must obey the mass action law, Equation 2, they can be used to determine the affinity K_A, from Equation 3 or 3′, by any of several graphical procedures, such as Scatchard analysis (described in the text).

‡This method assumes that all binding sites are independent, as is generally true for antibodies and monovalent ligands. If there were either negative or positive cooperativity in binding, then the change in receptor occupancy that occurs when a large excess of unlabeled antigen is added would probably perturb the dissociation rate of radiolabeled antigen molecules already bound to other sites.

**Such fractionated antibodies may contain mixtures of antibodies to overlapping sites within a domain on the antigen, but as long as no two antibody molecules (or combining sites) can bind to the same antigen molecule simultaneously, the antigen still behaves as effectively monovalent.

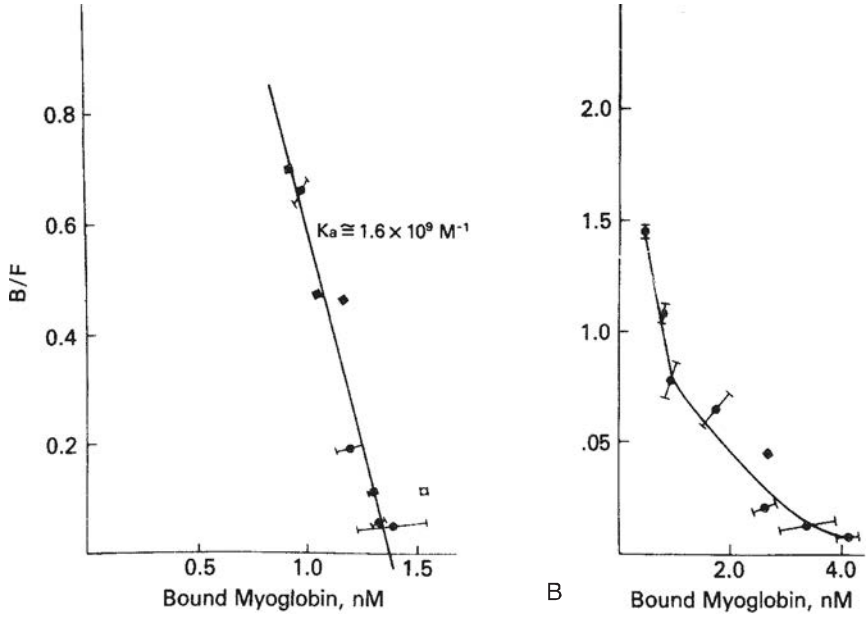

FIG. 7.3. Scatchard analysis of the binding of [^{3}H]-sperm whale myoglobin by a monoclonal antibody to myoglobin (A) and by the serum antibodies from the same mouse whose spleen cells were fused to prepare the hybridoma (B). The monoclonal antibody (clone HAL 43-201E11, clone 5) produces a linear Scatchard plot, whose slope, -1.6×10^9 M^{-1}, equals $-K_A$, and whose intercept on the abscissa gives the concentration of antibody-binding sites. In contrast, the serum antibodies produce a curved (concave up) Scatchard plot, indicative of heterogeneity of affinity. From Berzofsky et al.,[15] with permission.

Perhaps the most widely used is that described by Scatchard[14] (Fig. 7.3[15]). The mass action equilibrium law is plotted in the form of Equation 3

$$[SL] = K_A([S]_t - [SL])[L] \quad (3)$$

and B is substituted for [SL] and F for [L], referring to bound and free ligand, respectively. Then the Scatchard equation is

$$\frac{B}{F} = K_A([S]_t - B) \quad (8)$$

Note that a very critical implicit assumption was made in this seemingly very simple conversion. The [SL] within the parentheses in Equation 3 was intended to be the concentration of bound antibody sites so that ($[S]_t - [SL]$) = free [S]. However, in Equation 8, we have substituted B, the concentration of bound ligand. If the ligand behaves as monovalent, then this substitution is legitimate, as every bound ligand molecule corresponds to an occupied antibody site. However, if the ligand is multivalent and can bind more than one antibody site, then Equation 8 is valid only in ligand excess where the frequency of ligands with more than one antibody bound is very low. In this section, we are discussing only monovalent ligands, but this proviso must be kept in mind when the Scatchard analysis is applied in other circumstances.

From Equation 8, we see that a plot of B/F versus B should yield a straight line (for a single affinity), with a slope of $-K_A$ and an intercept on the abscissa corresponding to antibody-binding site concentration (see Fig. 7.3). This is the so-called Scatchard plot. An alternative version that is normalized for antibody concentration is especially useful if the data were obtained at different values of total antibody concentration, $[A]_t$, instead of constant $[A]_t$. However, for this version, one requires an independent measure of total antibody concentration, other than the intercept of the plot. Then one divides Equation 8 by the total concentration of antibody molecules (making no assumptions about the number of sites per molecule) to obtain

$$\frac{R}{c} = K_A(n - r) \quad (9)$$

where r = the number of occupied sites per antibody molecule, n = the total number of sites per antibody molecule, and c = free ligand concentration, that is, c = F. Thus,

$$r = \frac{B}{[\text{total antibody}]} = \frac{B}{[A]_t}$$
$$n = \frac{[\text{total sites}]}{[\text{total antibody}]} = \frac{[S]_t}{[A]_t}$$

where $[A]_t$ = total molar antibody concentration. In this form of the Scatchard plot, r/c versus r, the slope is still $-K_A$ and the intercept on the r axis is n. Thus one can determine the number of sites per molecule. Of course, if one determines $[S]_t$ from the intercept of Equation 8, one can also calculate the number of sites per molecule by dividing $[S]_t$ by any independent measure of antibody concentration. Thus, the only advantage of normalizing all the data points first to plot the r/c form arises when the data were obtained at varying antibody concentrations. If the antibody concentration is unknown but held constant, then the B/F form is more convenient and actually provides one measure of antibody (site) concentration. Because today we know the value of n for each class of antibody (2 for IgG and serum IgA, 10 for IgM), the concentration of sites and that of antibody are easily converted in many cases.

Heterogeneity of Affinity

The next level of complexity arises when one is dealing with a mixture of antibodies of varying affinity for the ligand. This is the rule, rather than the exception, when one deals with antibodies from immune serum, even if they are fractionated to be monospecific, that is, all specific for the same site

on the antigen. Contrast, for example, the linear Scatchard plot for a homogeneous monoclonal antibody to myoglobin (see Fig. 7.3A), with the curved Scatchard plot for the serum antibodies from the same mouse used to prepare the hybridoma monoclonal antibody (see Fig. 7.3B). This concave up Scatchard plot is typical for heterogeneous antibodies. In a system such as hormone receptor–hormone interaction, in which negative cooperativity can occur between receptor sites (ie, occupation of one site lowers the affinity of its neighbor), a concave up Scatchard plot can be produced by negative cooperativity in the absence of any intrinsic heterogeneity in affinity. However, in the case of antibodies, where no such allosteric effect has been demonstrated, a concave up Scatchard plot indicates heterogeneity of affinity.

Ideally, one would like to imagine that the tangents all along the curve correspond (in slope) to the affinities of the many subpopulations of antibodies. Mathematically, this is not strictly correct, but it is true that the steeper part of the curve corresponds to the higher affinity antibodies and the shallower part of the curve to the lower affinity antibodies. Graphical methods have been developed to analyze more quantitatively the components of such curves,[16,17] and a very general and versatile computer program (LIGAND) has been developed by Munson and Rodbard[18] that can fit such curves using any number of subpopulations of different affinity. For purposes of this chapter, we discuss only the case of two affinities and then examine the types of average affinities that have been proposed when one is dealing with much greater heterogeneity. We also examine mathematical estimates of the degree of heterogeneity (analogous to a variance).

When an antibody population consists of only two subpopulations of different affinities, K_1 and K_2, then we can add the component Equations 3′ to obtain

$$r = r_1 + r_2 = \frac{n_1 K_1 c}{(1 + K_1 c)} + \frac{n_2 K_2 c}{(1 + K_2 c)} \qquad (10)$$

so that

$$\frac{r}{c} = \frac{n_1 K_1}{(1 + K_1 c)} + \frac{n_2 K_2}{(1 + K_2 c)} \qquad (10')$$

where the subscripts correspond to the two populations. Then the graph of r/c versus r can be shown to be a hyperbola whose asymptotes are, in fact, the linear Scatchard plots of the two components (Fig. 7.4). This situation has been analyzed graphically by Bright.[19] Taking the limits as $c \to 0$ and as $c \to \infty$, it can easily be shown that the intercept on the abscissa is just $n_1 + n_2$ (or, in the form B/F versus B, the intercept is the total concentration of binding sites $[S]_t$), and the intercept on the ordinate is $n_1K_1 + n_2K_2$. Thus, one can still obtain the total value of n or $[S]_t$ from the intercept on the abscissa. The problem is in obtaining the two affinities, K_1 and K_2, and the concentrations of the individual antibody subpopulations (corresonding to n_1 and n_2). If K_1 is greater than K_2, one can approximate the affinities from the slopes of the tangents at the two intercepts (see Fig. 7.4), but these will not, in general, be exactly parallel to the two asymptotes, which give the true affinities, so some error is always introduced, depending on the relative values of n_1 and n_2 and K_1 and K_2. A graphical method for solving for these exactly has been worked out by Bright[19] and computer methods by Munson and Rodbard.[18]

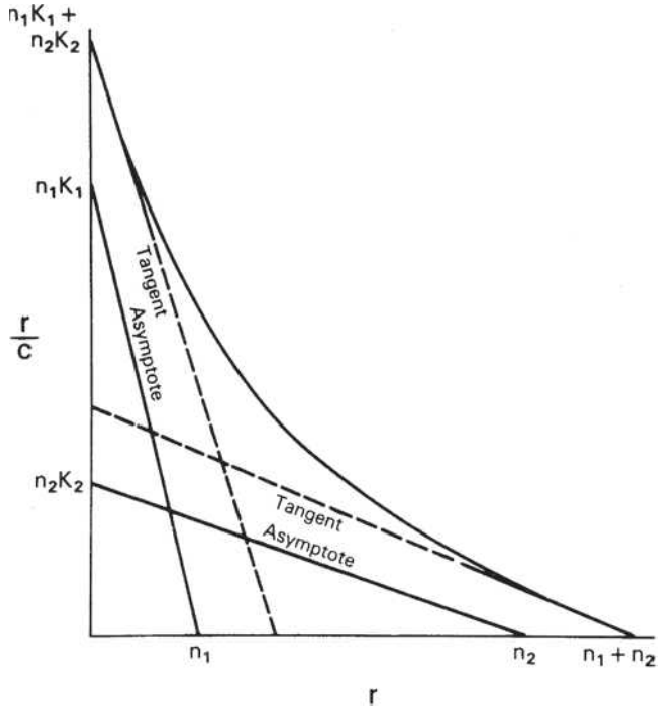

FIG. 7.4. Analysis of a Curved Scatchard Plot Produced by a Mixture of Two Antibodies with Different Affinities. The antibodies have affinities K_1 and K_2 and have n_1 and n_2 binding sites per molecule, respectively. The r is the concentration of bound antigen divided by the total antibody concentration (ie, bound sites per molecule), and c is the free antigen concentration. The curve is a hyperbola that can be decomposed into its two asymptotes, which correspond to the linear Scatchard plots of the two components in the antibody mixture. The tangents to the curve at its intercepts only approximate these asymptotes so that the slopes of the tangents estimate but do not accurately correspond to the affinities of the two antibodies. However, the intercept on the r axis corresponds to $n_1 + n_2$. Note that in this case n_1 and n_2 must be defined in terms of the total antibody concentration, not that of each component.

Average Affinities

In practice, of course, one rarely knows that one is dealing with exactly two subpopulations, and most antisera are significantly more heterogeneous than that. Therefore, the previously mentioned case is more illustrative of principles than of practical value. When faced with a curved Scatchard plot, one usually asks what the average affinity is, and perhaps some measure of the variance of the affinities, without being able to define exactly how many different affinity populations exist.

Suppose one has m populations each with site concentration $[S_i]$ and affinity K_i, so that at free ligand concentration [L], the fraction of each antibody that has ligand bound will be given by an equation of the form of Equation 3′:

$$B_i = \frac{K_i [S_i]_t [L]}{(1 + K_i [L])} \qquad (11)$$

Then the bound concentrations sum to give

$$B = \sum_{i=1}^{m} B_i = \sum_{i=1}^{m} \frac{K_i [S_i]_t [L]}{(1 + K_i [L])} \qquad (11')$$

Substituting F for [L] and dividing through by this quantity, one obtains

$$\frac{B}{F} = \sum_{i=1}^{m} \frac{K_i[S_i]_t}{(1 + K_iF)} \tag{12}$$

or equivalently,

$$\frac{r}{c} = \sum_{i=1}^{m} \frac{K_i n_i}{(1 + K_ic)} \tag{12'}$$

These can be seen to be generalizations of Equations 10 and 10′. Taking the limits as $F \rightarrow 0$ and $F \rightarrow \infty$, one again sees that the

$$\text{intercept on ordinate} = \sum_{i=1}^{m} K_i[S_i]_t \tag{13}$$

and the

$$\text{intercept on abscissa} = \sum_{i=1}^{m} [S_i]_t = [S]_t \tag{14}$$

Therefore, one can still obtain the total antibody site concentration from the intercept on the abscissa (Fig. 7.5).[20]

Two types of average affinity can be obtained graphically from the Scatchard plot.[20] Perhaps the more widely used K_0 is actually more accurately a median affinity rather than a mean affinity. It is defined as the slope of the tangent at the point on the curve where half the sites are bound, that is, where $B = [S]_t/2$ (see Fig. 7.5). A second type of average affinity, which we call K_{av}, is a weighted mean of the affinities, each affinity weighted by its proportional representation in the antibody population. Thus, we take the ratio

$$K_{av} = \sum_{i=1}^{m} \frac{K_i[S_i]_t}{[S]_t} \tag{15}$$

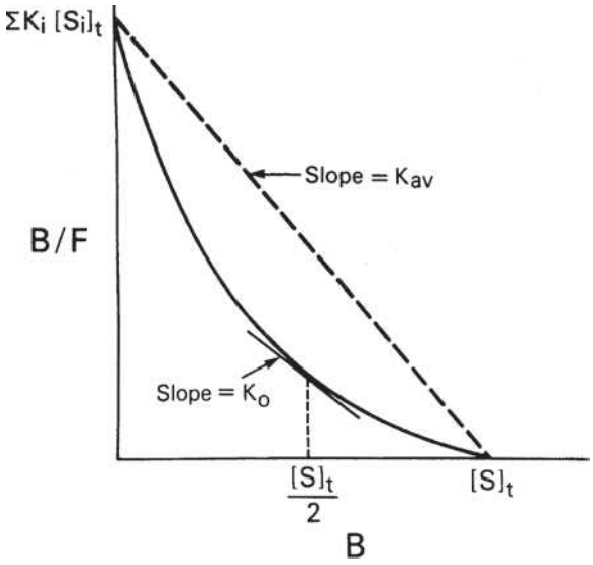

FIG. 7.5. Types of Average Affinities for a Heterogeneous Population of Antibodies, as Defined on a Scatchard Plot. K_0 is the slope of the tangent to the curve at a point where $B = [S]_t/2$, that is, where half the antibody sites are bound. Thus, K_0 corresponds to a median affinity. The K_{av} is the slope of the chord between the intercepts and corresponds to a weighted average of the affinities weighted by the concentrations of the antibodies with each affinity. Adapted from Berzofsky.[20]

From Equations 13 and 14, it is apparent that K_{av} is simply the ratio of the two intercepts on the B/F and B axes, that is, the slope of the chord (see Fig. 7.5). This type of weighted mean affinity, K_{av}, is therefore actually easier to obtain graphically in some cases than K_0, and we shall see that it is useful in other types of plots as well.

Indices of Heterogeneity: The Sips Plot

For a heterogeneous antiserum, one would also like to have some idea of the extent of heterogeneity of affinity. For instance, if the affinities were distributed according to a normal (Gaussian) distribution, one would like to know the variance.[21,22] More complex analyses have been developed that do not require as many assumptions about the shape of the distribution,[23–25] but the first and most widely used index of heterogeneity arbitrarily assumes that the affinities fit a distribution, first described by Sips,[26] which is similar in shape to a normal distribution. This was applied to the case of antibody heterogeneity by Nisonoff and Pressman,[27] and is summarized by Karush and Karush.[28] One fits the data to the assumed binding function

$$r = \frac{n(K_0c)^a}{(1 + (K_0c)^a)} \tag{16}$$

which is analogous to Equations 3′ and 11 (the Langmuir adsorption isotherm) except for the exponent a, which is the index of heterogeneity. This index, a, is allowed to range from 0 to 1. For $a = 1$, Equation 16 is equivalent to Equation 3, and there is no heterogeneity. As a decreases toward 0, the heterogeneity increases. To obtain a value for a graphically, one plots the algebraic rearrangement of Equation 16:

$$\log\left(\frac{r}{n - r}\right) = a \log c + a \log K_0 \tag{17}$$

so that the slope of $\log [r/(n - r)]$ versus $\log c$ is the heterogeneity index a.

C. DeLisi (personal communication) has derived the variance (second moment) of the Sips distribution in terms of the free energy RT ln K_0, about the mean of free energy. The result (normalized to RT) gives the dispersion or width of the distribution as a function of a:

$$\frac{\sigma^2_{Sips}}{R^2T^2} = \frac{\pi^2(1 - a^2)}{3a^2} \tag{18}$$

This is useful for determining a quantity, σ_{Sips}, which can be thought of as analogous to a standard deviation, if one keeps in mind that this is not a true Gaussian distribution. In addition, as noted previously, the use of the Sips distribution requires the assumption that the affinities (really the free energies) are continuously distributed symmetrically about a mean, approximating a Gaussian distribution. This assumption frequently is not valid.

The Plot of B/F versus F or T

Another graphical method that is useful for estimating affinities is the plot of bound/free versus free or total ligand concentration, denoted F and T, respectively[20] (Fig. 7.6). To simplify the discussion, let us define the bound/free ratio, B/F, as R, and define R_0 as the intercept, or limit, as free

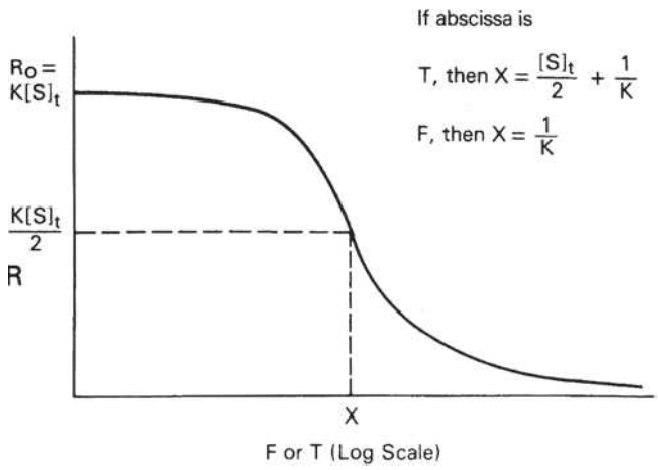

FIG. 7.6. Schematic Plot of R, the Bound/Free Ratio, as a Function of Free (F) or Total (T) Antigen Concentration. The curves have a similar sigmoidal shape, but the midpoint (where $R = R_0/2$) of the plot of R versus T has a term dependent on antibody site concentration ($[S]_t$), whereas the midpoint of the plot of R versus F is exactly 1/K, independent of antibody concentration. Adapted from Berzofsky.[20]

ligand $F \rightarrow 0$. First, for the case of a homogeneous antibody, from Equation 3′,

$$R = \frac{B}{F} = \frac{K[S]_t}{(1 + KF)} \tag{19}$$

and

$$R_0 = \lim_{F \to 0} \frac{B}{F} = K[S]_t \tag{20}$$

Let us define the midpoint of the plot (see Fig. 7.6) as the point at which R decreases to half its initial value, R_0, that is, at which $R = K[S]_t/2$. For the case of homogeneous antibody (ie, a single affinity), simple algebraic manipulation,[20] substituting $K[S]_t/2$ (ie, $R_0/2$) for B/F in Equation 8, will show that at this midpoint††

$$F = \frac{1}{K} \tag{21}$$

and

$$B = \frac{[S]_t}{2} \tag{22}$$

so that the total concentration, T, is

$$T = B + F = \frac{[S]_t}{2} + \frac{1}{K} \tag{23}$$

Thus, if one plots B/F versus F, the midpoint directly yields 1/K. However, it is frequently more convenient experimentally to plot B/F versus T. In this case, the midpoint is no longer simply the reciprocal of the affinity. As seen from Equation 23, the assumption that the midpoint is 1/K will result in an error equal to half the antibody-binding site concentration. Thus, in plots of B/F versus T, the midpoint will be a good estimate of the affinity only if $[S]_t/2 \ll 1/K$, that is, if the antibody concentration is low compared to the dissociation constant. In fact, if the affinity is so high that $1/K \ll [S]_t/2$, then one will merely be measuring the antibody concentration, not the affinity at all[20] (see Fig. 7.6).

In the case of a heterogeneous antiserum, we have already seen that

$$R_0 = \sum_i K_i[S_i]_t \tag{13}$$

Therefore, at the midpoint, when $B/F = R_0/2$, it is easy to see that

$$K_{av} = \left(\frac{B}{F}\right)\left(\frac{2}{[S]_t}\right) = \frac{R_0}{[S]_t} \tag{24}$$

Thus, one can still obtain the average affinity, as defined previously.[20]

Regardless of average affinities, the effect of affinity heterogeneity is to broaden the curve or to make the slope shallower. This can be seen by visualizing the curve of B/F versus F as a step function. Each antibody subpopulation of a given affinity, K_i, will be titrated to 50% of its microscopic B/F at a free ligand concentration $F = 1/K_i$. The high-affinity antibodies will be titrated at low F, but the low-affinity antibodies will require much higher F to be titrated. The resulting step function is analogous to the successive transitions corresponding to different pK values in a pH titration.

Intrinsic Affinity

The affinity, K_A, that we have been discussing so far is what has been termed the intrinsic affinity, that is, the affinity of each antibody-combining site treated in isolation. We have been able to do this, regardless of the valence of the antibodies, by using the concentration of combining sites, [S], in our equations rather than the concentration of antibody molecules, [A], which may have more than one site. Even without any cooperativity between combining sites, there is a statistical effect that makes the actual affinity different from the intrinsic affinity if the antibody is multivalent and one uses whole antibody concentration rather than site concentration. The way this difference arises can best be seen by examining the case of a bivalent antibody, such as IgG. We assume that the two sites are equivalent and neither is affected by events at the other. The ligand, as in this whole section, is monovalent. Then there are two binding steps

$$A + L \overset{K_1}{\rightleftharpoons} AL, \qquad AL + L \overset{K_2}{\rightleftharpoons} AL_2 \tag{25}$$

and the corresponding actual affinities are

$$K_1 = \frac{[AL]}{[A][L]}, \qquad K_2 = \frac{[AL_2]}{[AL][L]} \tag{26}$$

If the intrinsic affinity of both equivalent sites is K, then K_1 will actually be twice K because the concentration of available sites [S] will be twice the antibody concentration when the first ligand is about to bind in step 1. However,

††It is important to note that R_0 must be the limit of B/F as F truly approaches zero. In an RIA in which the concentration of tracer is significant compared to 1/K, reducing the unlabeled ligand concentration all the way to zero will still not yield the true limit R_0. The tracer concentration must also be negligible. If not, R_0 will be estimated falsely low, and the affinity will also be underestimated.

once one site is bound, the reverse (dissociation) reaction of step 1 can occur from only one site, namely, that which is occupied. Conversely, for the second step, the forward reaction has only one remaining available site; however, in the reverse reaction, $AL_2 \rightarrow AL + L$, either site can dissociate to go back to the AL state. The second site bound need not be the first to dissociate, and because the sites are identical, one cannot tell the difference. Thus, for step 2, the apparent concentration of sites for the reverse reaction is twice that available for the forward reaction, so the affinity K_2 for the second step will be only half the intrinsic affinity, K.

It is easy to see how this statistical effect can be extrapolated to an antibody with n sites[29]:

$$K_1 = nK \quad \text{and} \quad K_n = \left(\frac{1}{n}\right) K \tag{27}$$

For the steps in between, two derivations are available,[9,29] which yield

$$K_i = \frac{(n - i + 1)}{i} K \tag{28}$$

The actual affinity, rather than the intrinsic affinity, becomes important with monovalent ligands when one is interested in the effective affinity (based on a molar antibody concentration) under conditions where [L] is so low that only one site can bind antigen. Then for IgG or IgM (with 2 or 10 sites per molecule, respectively), the apparent affinity will be theoretically 2 or 10 times the intrinsic affinity. For most purposes, it is easier to use site concentrations and intrinsic affinities. The analyses given previously, such as B/F versus F or the Scatchard plot, whether B/F versus B or r/c versus r, will all yield intrinsic affinities. It is the intrinsic affinity that tells us something about the nature of the antibody–ligand interaction.

Once one enters the realm of multivalent ligands, the actual affinity or effective affinity involving multipoint binding between multivalent antibody molecule and multivalent ligand molecule can be much greater than the intrinsic affinity for binding at each site. This case is the subject of the next section.

Interaction with Multivalent Ligands

So far, we have discussed only situations in which the ligand is monovalent or effectively monovalent with respect to the particular antibody under study. However, in many situations, the ligand molecule has multiple repeating identical determinants, each of which can bind independently to the several identical combining sites on a divalent or multivalent antibody.[‡‡] Although the intrinsic affinity for the interaction of any single antibody–combining site with any single antigenic determinant may be the same as that discussed in the preceding section, the apparent or effective affinity may be much higher due to the ability of a single antibody molecule to bind more than one identical determinant of a multivalent antigen molecule. Karush[30] has termed this phenomenon "monogamous bivalency." Such monogamous binding can occur between two molecules in solution, or between a molecule in solution and one on a solid surface, such as a cell membrane or microtiter plate. We first discuss the situation in solution and then discuss the additional considerations that apply when one of the reactants is bound to a solid surface.

Monogamous Bivalency

Suppose a divalent antibody molecule reacts with antigen that has two identical determinants. This situation has been treated in detail by Crothers and Metzger,[31] and by Karush.[30] Let us call the two antibody sites S and S′, and the antigenic determinants D and D′, with the understanding that, in actuality, we cannot distinguish S from S′ or D from D′. The interaction can be broken up into two steps, a bimolecular reaction

$$\begin{array}{ccc} S & D & \\ | & + & | \\ S' & & D' \end{array} \overset{K_1}{\rightleftharpoons} \begin{array}{cc} S & D \\ | & | \\ S' & D' \end{array} \tag{29}$$

followed by an intramolecular reaction

$$\begin{array}{cc} S & D \\ | & | \\ S' & D' \end{array} \overset{K_2}{\rightleftharpoons} \begin{array}{cc} S & D \\ | & | \\ S' & D' \end{array} \tag{30}$$

The association constant for the first step, K_1, is related to the intrinsic affinity, K, simply by a statistical factor of 4 due to the degeneracy (equivalence) between S and S′ and between D and D′. This is a typical second-order reaction between antigen and antibody. However, the second step (Equation 30) is a first-order reaction because it is effectively an interconversion between two states of a single molecular complex, the reactants S′ and D′ being linked chemically (albeit noncovalently) through the S–D bond formed in the first step. Thus, the first-order equilibrium constant, K_2, is not a function of the concentrations of S–S and D–D in solution, as K_1 would be. Rather, the forward reaction depends on the geometry of the complex and the flexibility of the arms; in other words, the probability that S′ and D′ will encounter each other and be in the right orientation to react if they do come in contact depends on the distances and freedom of motion along the chain S′–S–D–D′ rather than on the density of molecules in solution (ie, concentration).

The reverse reaction for step 2, on the other hand, will have a rate constant similar to that for the simple monovalent S–D → S + D reaction, as the dissociation reaction depends on the strength of the S′–D′ (or S–D) bond and is not influenced by the other S–D interaction unless there is strain introduced by the angles required for simultaneous bonds between S and D and S′ and D′. Note that K_2 will inherently have a statistical factor of 1/2 compared to the intrinsic K'_2 for the analogous reaction if the S′–S–D–D′ link were all covalent because in the forward reaction of Equation 30 only one pair can react, whereas in the reverse reaction either S′–D′ or S–D could dissociate to produce the equivalent result.

[‡‡] If only the antigen is multivalent, and the antibody monovalent, such as an Fab fragment, the situation can be analyzed using the same statistical considerations discussed previously.

We would like to know the apparent or observed affinity for the overall reaction

$$\begin{array}{cc} S & D \\ | & | \\ S & D \end{array} + \begin{array}{cc} | & | \\ S & D \end{array} \overset{K_{obs}}{\rightleftharpoons} \begin{array}{cc} S & D \\ | & | \\ S & D \end{array} \qquad (31)$$

Because the free energies, ΔF_1 and ΔF_2, for the two steps are additive, the observed affinity will be the product of K_1 and K_2

$$K_{obs} = K_1 K_2 \qquad (32)$$

where we have defined K_1 and K_2 to include the statistical degeneracy factors.*** The equilibrium constants K_1 and K_2 are each the ratios of forward and reverse rate constants, as in Equation 7. Of these four rate constants, all are directly related to the corresponding terms for the intrinsic affinity between S and D except for the intramolecular forward reaction of step 2, as noted previously. Thus, the difficulty in predicting K_{obs} is largely a problem of analyzing the geometric (steric) aspects of K_2, assuming one already knows the intrinsic affinity, Crothers and Metzger[31] have analyzed this problem for particular situations. Qualitatively, we can say that whether K_2 will be larger or smaller than K will depend on factors such as the enforced proximity of S′ and D′ in step 2 and the distance between D and D′ compared to the possible distances accessible between S and S′, which in turn depends on the length of the antibody arms and the flexibility of the hinge between them. Thus, because K_1 can be approximated by K, except for statistical factors, the apparent affinity for this "monogamous bivalent" binding interaction, K_{obs}, may range from significantly less than to significantly greater than K^2. If K_2 is of the same order of magnitude as K, then K_{obs} will be of the order of K^2, which can be huge (eg, if $K \approx 10^9\ M^{-1}$, K_{obs} could be $\approx 10^{18}\ M^{-1}$). The halftime for dissociation would be thousands of years. It is easy to see how such monogamous bivalent interactions can appear to be irreversible, even though in practice the observed affinity is rarely more than a few orders of magnitude larger than the K for a single site, possibly due to structural constraints.[32]

If apparent affinities this high can be reached by monogamous bivalency, even greater ones should be possible for the multipoint binding of an IgM molecule to a multivalent ligand. Although IgM is decavalent for small monovalent ligands, steric restrictions often make it behave as if pentavalent for binding to large multivalent ligands. However, even five-point binding can lead to enormously tight interactions. Therefore, even though the intrinsic affinity of IgM molecules tends to be lower than that of IgG molecules for the same antigen,[30] the apparent affinity of IgM can be quite high.

Two-Phase Systems

The same enhanced affinity seen for multipoint binding applies to two-phase systems. Examples include the reaction of multivalent antibodies with antigen attached to a cell surface or an artificial surface (such as Sepharose or the plastic walls of a microtiter plate), the reaction of a multivalent ligand with antibodies on the surface of a B cell, a Sepharose bead, or a plastic plate, and the reaction of either component with an antigen–antibody precipitate. For the reasons outlined previously, "monogamous" binding can make the apparent affinity of a multivalent antibody or antigen for multiple sites on a solid surface be quite large to the point of effective irreversibility.

However, another effect also increases the effective affinity in a two-phase system. This effect applies even for monovalent antibodies (Fab fragments) or monovalent ligands. The effect arises from the enormously high effective local concentration of binding sites at the surface, compared to the concentration if the same number of sites were distributed in bulk solution.[33] Looked at another way, the effect is due to the violation, at the liquid–solid interface, of the basic assumption in the association constants, K_A, discussed previously, that the reactants are all distributed randomly in the solution. (To some extent, the latter is involved in the enhanced affinity of multivalency as well.) This situation has been analyzed by DeLisi[34] and DeLisi and Wiegel,[35] who break the reaction down into two steps: the diffusive process necessary to bring the antigen and antibody into the right proximity and orientation to react, and the reactive process itself. The complex between antigen and antibody, when positioned but not yet reacted, is called the encounter complex. The reaction can then be written

$$S + D \underset{k_-}{\overset{k_+}{\rightleftharpoons}} S \cdots D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SD \qquad (33)$$

where S = antibody site, D = antigenic determinant, k+ and k_- = forward and reverse diffusive rate constants, and k_1 and k_{-1} = forward and reverse reactive rate constants once the encounter complex is formed. If the encounter complex is in a steady state, the overall rate constants will be given by

$$k_f = \frac{k_1 k_+}{(k_1 + k_-)} \qquad (34)$$

$$k_r = \frac{k_{-1} k_-}{(k_1 + k_-)} \qquad (35)$$

where subscripts f and r = forward and reverse.[34] The association constant, according to Equation 7, is the ratio of these two, or

$$K_A = \frac{k_1 k_+}{k_{-1} k_-} \qquad (36)$$

The relative magnitudes of k_1 and k_- determine the probable fate of the encounter complex. Is it more likely to react to form SD or to break up as the reactants diffuse apart?

Now suppose that k is slow compared to k_1. Then the SD bound complex and the encounter complex, S ··· D, may interconvert many times before the encounter complex breaks up and one of the reactants diffuses off into bulk solution. If the surface has multiple antigenic sites, D, then even a monovalent antibody (Fab) may be much

***In some treatments where these statistical factors are not included in K_1 and K_2, the equivalent equation may be given as $K_{obs} = 2K_1K_2$.

more likely, when SD dissociates to S ··· D, to rereact with the same or nearby sites than to diffuse away into bulk solution, again depending on the relative magnitudes of these rate constants. This greater probability to rereact with the surface rather than diffuse away is the essence of the effect we are describing. A more extensive mathematical treatment of reactions with cells is given in DeLisi[34] and DeLisi and Wiegel.[35]

A somewhat different, and very useful, analysis of the same or a very similar effect was given by Silhavy et al.[36] These authors studied the case of a ligand diffusing out of a dialysis bag containing a protein for which the ligand had a significant affinity. Once the ligand concentration became low enough that there was an excess of free protein sites, then the rate of exit of ligand from the dialysis bag was no longer simply its diffusion rate nor was it simply the rate of dissociation of protein–ligand complex. These authors showed that under these conditions the exit of ligand followed quasi–first-order kinetics, but with a half-life longer than the half-life in the absence of protein by a factor of $(1 + [P]K_A)$

$$t_+ = t_-(1 + [P]K_A) \quad (37)$$

where [P] = protein site concentration, K_A = affinity, and t_+ and t_- = half-lives in the presence and absence of protein in the bag.

In this case, the protein was in solution, so the authors could use the actual protein concentration and the actual intrinsic affinity, K_A. In the case of protein on a two-dimensional surface, it is harder to know what to use as the effective concentration. However, the high local concentration of protein compartmentalized in the dialysis bag can be seen to be analogous to the high local concentration attached to the solid surface. The underlying mechanism of the two effects is essentially the same and so are the implications. For instance, in the case of dialysis, a modest 10 μM concentration of antibody sites with an affinity of 10^8 M^{-1} can reduce the rate of exit of a ligand 1000-fold. A dialysis that would otherwise take 3 hours would take 4 months. It is easy to see how this "retention effect" can make even modest affinities appear infinite (ie, the reactions appear irreversible). This retention effect applies not only to immunologic systems but also to other interactions at a cell surface or between cell compartments where the local concentration of a protein may be high. In particular, these principles of two-phase systems should also govern the interaction between antigen specific receptors on the surface of T cells and antigen–major histocompatibility complex (MHC) molecule complexes on the surface of antigen-presenting cells, B cells, or target cells.

One final point is useful to note. Because these retention effects depend on a localized abundance of unoccupied sites, addition of a large excess of unlabeled ligand to saturate these sites will diminish or abolish the retention effect and greatly accelerate the dissociation or exit of labeled ligand. This effect of unlabeled ligand can be used as a test for the retention effect, although one must be aware that in certain cases the same result can be an indication of negative cooperativity among receptor sites.

RADIOIMMUNOASSAY AND RELATED METHODS

Since it was first suggested in 1960 by Yalow and Berson,[37] RIA has rapidly become one of the most widespread, widely applicable, and most sensitive techniques for assessing the concentration of a whole host of biologic molecules. Most of the basic principles necessary to understand and apply RIA have been covered previously in this chapter. In this section, we examine the concepts and methodologic approaches used in RIA. For a detailed methods book, we refer the reader to Chard,[38] Rodbard,[39] and Yalow.[40]

The central concept of RIA is that the binding of an infinitesimal concentration of highly radioactive tracer antigen to low concentrations of a high-affinity–specific antibody is very sensitive to competition by unlabeled antigen and is also very specific for that antigen. Thus, concentrations of antigen in unknown samples can be determined by their ability to compete with tracer for binding to antibody. The method can be used to measure very low concentrations of a molecule, even in the presence of the many impurities in biologic fluids. Accomplishment of this requires an appropriate high-affinity antibody and radiolabeled antigen, a method to distinguish bound from free-labeled antigen, optimization of concentrations of antibody and tracer-labeled antigen to maximize sensitivity, and generation of a standard curve, using known concentrations of competing unlabeled antigen, from which to read off the concentrations in unknown samples as well as the best method for representing the data. We review all these steps and pitfalls in this procedure except the preparation of antibodies and labeled antigens.

Separation of Bound and Free Antigen

Whatever parameter one uses to assess the amount of competition by the unlabeled antigen in the unknown sample to be tested, it will always be a function of bound versus free, radiolabeled antigen. Therefore, one of the most critical technical requirements is the ability to distinguish clearly between antibody-bound radioactive tracer and free radioactive tracer. This distinction usually requires physical separation of bound and free ligand. If the bound fraction is contaminated by free ligand, or vice versa, enormous errors can result, depending on the part of the binding curve on which the data fall.

Solution Methods

Solution RIA methods have the advantage that binding can be related to the intrinsic affinity of the antibody. However, bound and free antigen must be separated by a method that does not perturb the equilibrium. Three basic types of approaches have been used: precipitate the antibody with bound antigen, leaving free antigen in solution; precipitate the free antigen, leaving antibody and bound antigen in solution; or separate free from antibody-bound antigen molecules in solution on the basis of size by gel filtration. This last method is too cumbersome to use for large numbers of samples and is too slow, in general, to be sure the equilibrium is not perturbed in the process. Therefore, gel filtration columns are not widely used for RIA.

Methods that precipitate antibody are perhaps the most widely used. If the antigen is sufficiently smaller (<30,000 MW) than the antibody that it will remain in solution at

concentrations of either ammonium sulfate[41] or polyethylene glycol, 6000 MW (10% W:W),[42] which will precipitate essentially all the antibody, then these two reagents are frequently the most useful. Precipitation with polyethylene glycol and centrifugation can be accomplished before any significant dissociation has occurred due to dilutional effects.[43] However, if the antigen is much larger than about 30,000 to 40,000 MW, these methods will produce unacceptably high background control values in the absence of specific antibody. If the antibody is primarily of a subclass of IgG that binds to staphylococcal protein A or G, one can take advantage of the high affinity of protein A or G for IgG by using either protein A (or G)-Sepharose or formalin-killed staphylococcal organisms (Cowan I strain) to precipitate the antibody.[44] Finally, one can precipitate the antibody using a specific second antibody, an anti-Ig raised in another species. Maximal precipitation occurs not at antibody excess but at the "point of equivalence" in the middle of the titration curve where antigen (in this case, the first antibody) and the (second) antibody are approximately equal in concentration. Thus, one must add carrier Ig to keep the Ig concentration constant and determine the point of equivalence by titrating with the second antibody. Even worse, the precipitin reaction is much slower than the antigen–antibody reaction itself, allowing reequilibration of the antigen–antibody interaction after dilution by the second antibody. Some of these problems can be reduced by enhancing precipitation with low concentrations of polyethylene glycol.

The other type of separation method is adsorption of free antigen to an agent, such as activated charcoal or talc, which leaves antigen bound to antibody in solution. Binding of antigen by these agents depends on size and hydrophobicity. Although these methods are inexpensive and rapid, they require careful adjustment and monitoring of pH, ionic strength, and temperature to obtain reproducible results and to avoid adsorption of the antigen–antibody complex. Furthermore, because these agents have a high affinity for antigen, they can compete with a low-affinity antibody and alter the equilibrium. Also, as charcoal quenches beta scintillation counting, it can be used only with gamma-emitting isotopes such as ^{125}I.

Solid-Phase Methods

Solid-phase RIA methods have the advantages of high throughput and increased apparent affinity due to the effects at the solid–liquid interface noted previously. However, they have the concomitant disadvantage that one is not measuring the true intrinsic affinity because of these same effects. The method itself is fairly simple. One binds the antibody in advance to a solid surface such as a Sepharose bead or the walls of a microtiter plate well. To avoid competition from other serum proteins for the solid phase, one must use purified antibody in this coating step. Once the wells (or Sepharose beads) are coated, one can incubate them with labeled tracer antigen with or without unlabeled competitor, wash and count directly the radioactivity bound to the plastic wells or to the Sepharose. The microtiter plate method is particularly useful for processing large numbers of samples. However, because the concentration, or even the amount, of antibody coating the surface is unknown and because the affinity is not the intrinsic affinity, one cannot use these methods for studying the chemistry of the antigen–antibody reaction itself. A detailed analysis of the optimum parameters in this method is given by Zollinger et al.[43]

A variation that does allow determination of affinity, based on the enzyme-linked immunosorbant assay (ELISA) described in the following, but equally applicable to RIA, was described by Friguet et al.[45] This uses antigen-coated microtiter wells and free antibody but measures competition by free antigen to prevent the antibody in solution from binding to the antibody on the plate (see Fig. 7.9B). Thus, the antibody bound to the plastic is antibody that was free in the solution equilibrium. The affinity measured is that between the antibody and antigen in solution, not that on the plastic, so it is not directly influenced by the multivalency of the surface. However, as pointed out by Stevens,[46] the determination of affinity is strictly accurate only for monovalent Fab fragments because a bivalent antibody with only one arm bound to the plastic and one bound by antigen in solution will still be counted as free. Therefore, there will be an underestimate of the ligand occupancy of the antibody combining sites and thus an underestimate of affinity. Stevens also points out a method to correct for this error based on binomial analysis. Subsequently, Seligman[47] showed that the nature and density of the antigen on the solid surface can also influence the estimate of affinity.

Optimization of Antibody and Tracer Concentrations for Sensitivity

The primary limitations on the sensitivity of the assay are the antibody affinity and concentration, the tracer concentration, and the precision (reproducibility) of the data. In general, the higher the affinity of the antibody, the more sensitive the assay can be made. Once one prepares the highest affinity antibody available, this parameter limits the extent to which the other parameters can be manipulated. For instance, because the unlabeled antigen in the unknown sample is going to compete against labeled tracer antigen, the lower the tracer concentration, the lower the concentration of the unknown sample, which can be measured up to a point. That point is determined by the affinity, K_A, as can be seen from the theoretical considerations discussed previously.[38] The steepest part of the titration curve will occur in the range of concentrations around $1/K_A$. Concentrations of ligand much below $1/K_A$ will leave most of the antibody sites unoccupied so that competition will be less effective. Thus, there is no value in reducing the tracer concentration more than a few-fold lower than $1/K_A$. Therefore, although it is generally useful to increase the specific radioactivity of the tracer and reduce its concentration, it is important to be aware of this limit of $1/K_A$. Increasing the specific activity more than necessary can result in denaturation of antigen.

Similarly, lowering the antibody concentration will also increase sensitivity, up to a point. This limit also depends on $1/K_A$ and on the background "nonspecific binding." Decreasing the antibody concentration to the point that binding of tracer is too close to background will result in

loss of sensitivity due to loss of precision. In general, the fraction of tracer bound in the absence of competitor should be kept greater than 0.2, and in general closer to 0.5.[48]

A convenient procedure to follow to optimize tracer and antibody concentrations is first to choose the lowest tracer concentration that results in convenient counting times and counting precision for bound values of only one-half to one-tenth the total tracer. Then, keeping this tracer concentration constant, one dilutes out the antibody until the bound/free antigen ratio is close to 1.0 (bound/total = 0.5) in the absence of competitor. This antibody concentration in conjunction with this tracer concentration will generally give near-optimal sensitivities, within the limits noted previously. It is important to be aware that changing the tracer concentration will require readjusting the antibody concentration to optimize sensitivity.

Analysis of Data: Graphic and Numerical Representation

We have already examined the Scatchard plot (bound/free versus bound) and the plot of bound/free versus free or total antigen concentration as methods of determining affinity. The latter lends itself particularly to the type of competition curves that constitute an RIA. In fact, the independent variable must always be antigen concentration, as that is the known quantity one varies to generate the standard curve. Let us use B, F, and T to represent the concentrations of bound, free, and total antigen, respectively. We have seen that the plot of B/F versus F is more useful for determining the affinity, K_A, than the plot of B/F versus T. However, in RIA, the quantity one wants to determine is T, and correspondingly, the known independent variable in generating the standard curve is T. Another difference between the situation in RIA and that discussed previously is that, in RIA, one has both labeled and unlabeled antigen. The dependent variable, such as B/F, is the ratio of bound tracer over free tracer, as only radioactive antigen is counted. The B/F for the unlabeled antigen will be the same at equilibrium, assuming that labeled and unlabeled antigen bind the antibody equivalently, that is, with the same K_A. This assumption is not always valid and requires experimental testing.

The sigmoidal shape of B/F versus F or T, when F or T (the "dose") is plotted on a log scale, has been seen in Figure 7.6. The shape for B/T versus F or T would be similar. Note that because B + F = T,

$$\frac{B}{F} = \frac{B}{(T-B)} = \frac{B/T}{(1-B/T)} \tag{38}$$

and

$$\frac{B}{T} = \frac{B/F}{(1+B/F)} \tag{39}$$

These transformations can be useful. If one plots B/F or B/T versus F or T on a linear scale, then the shape is approximately hyperbolic, as in Figure 7.7. The plot of B/F versus T (log scale) was one of the first methods used to plot RIA data and is still among the most useful. The most sensitive part of the curve is the part with the steepest slope.

It has been shown by probability analysis that if the antigen has multiple determinants, each capable of binding

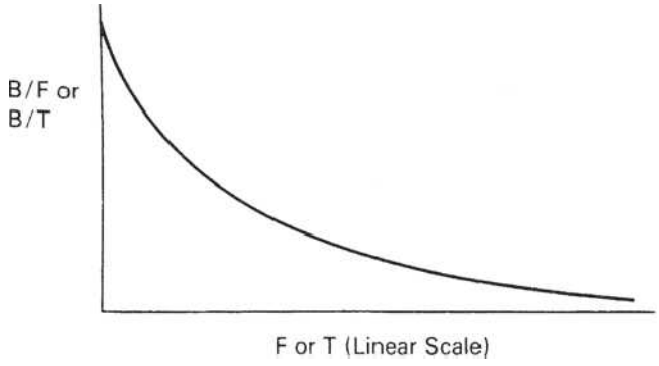

FIG. 7.7. Schematic Plot of B/F or B/T (the Bound Over Free or Total Antigen Concentration) as a Function of Free (F) or Total (T) Antigen Concentration, when Plotted on a Linear Scale. Contrast with similar plot on a log scale in Figure 7.6.

antibody molecules simultaneously and independently of one another, then the more such determinants capable of being recognized by the antibodies in use, the steeper will be the slope.[49] This effect of multideterminant binding on steepness arises because, in RIA, an antigen molecule is scored as bound whether it has one antibody molecule attached or several. It is scored as free only if no antibody molecules are attached. Thus, the probability that an antigen molecule is scored as free is the product of the probabilities that each of its determinants is free. The effect can lead to quite steep slopes and has been confirmed experimentally.[49]

A transform that allows linearization of the data in most cases is the logit transform.[50,51] To use this, one first expresses the data as B/B_0, where B_0 is the concentration of bound tracer in the absence of competitor. One then takes the logit transform of this ratio, defined as

$$\text{logit}\,(Y) = \ln\left[\frac{Y}{(1-Y)}\right] \tag{40}$$

where ln = the natural log (log to the base e). The plot of logit (B/B_0) versus ln T is usually a straight line (Fig. 7.8).

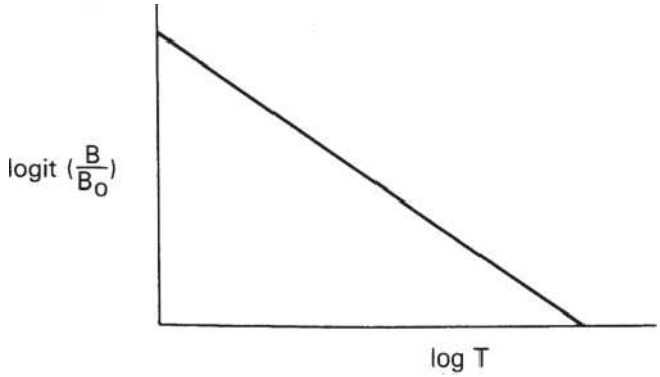

FIG. 7.8. Schematic Logit. Log plot used to linearize radioimmunoassay data. B and T are bound tracer and total antigen concentration, respectively, and B_0 is the value of B when no unlabeled antigen is added to tracer. The logit function is defined by Equation 40, logit (Y) = ln [Y/(1 – Y)].

The slope is usually −1 for the simplest case of a monoclonal antibody binding a monovalent antigen. The linearity of this plot obviously makes it very useful for graphical interpolation, which one would like to do to read antigen concentration off a standard curve. One additional advantage is that linearity facilitates tests of parallelism. If the unknown understudy is identical to the antigen used to generate the standard curve, then a dilution curve of the unknown should be parallel to the standard curve in this logit–log coordinate system. If not, the assay is not valid.

These and other methods of analyzing the data are discussed further by Feldman and Rodbard[52] and Rodbard,[39] including statistical treatment of data. Although a number of computer programs have become available for rapid analysis of RIA data without using manual plots of standard curves, they are all based on these and similar methods, and their accurate interpretation depends on an understanding of these concepts.

Corrections for B, F, and T

Before we leave this section on analysis of RIA data, we must point out a few controls and corrections to the data without which the results may be fallacious.

First, in any method that precipitates antibody and bound antigen (or uses a solid-phase antibody), there may always be a fraction of antigen that precipitates or binds nonspecifically in the absence of specific antibody. Thus, one must always run controls with normal serum or Ig to determine this background. The nonspecific binding usually increases linearly with antigen dose, that is, it does not saturate. This control value should be subtracted from B but does not affect F when measured independently, only F determined as T minus B. The total antigen that is meaningful is the sum of that which is specifically bound and that which is free. Nonspecifically bound antigen should be deleted from any term in which it appears.

A second correction is that for immunologically inactive radiolabel, that is, either free radioisotope, or isotope coupled to an impurity or to denatured antigen. The fraction of radioactive material that is immunologically reactive with the antibodies in the assay can be determined by using a constant, low concentration of labeled antigen and adding increasing concentrations of antibody. If there is no contamination with inactive material, all the radioactivity should be able to be bound by sufficient antibody. If the fraction of tracer bound reaches a plateau at <100% bound, then only this fraction is active in the assay. The importance of this correction can be seen from the example in which the tracer is only 80% active. Then, when the true B/F is 3 (B/T = 0.75), applying only to the active 80% of the tracer, the remaining 20%, which can never be bound, will mistakenly be included in the free tracer, doubling the amount that is measured as free. Thus, the measured B/F will be only 1.5 (ie, 0.6/0.4) instead of the true value of 3 (ie, 0.6/0.2). This factor of 2 will make a serious difference in the calculation of affinity, for instance, from a Scatchard plot. Also, it will result in a plateau in the Scatchard plot at high values of B/F, as with 20% of the tracer obligatorily free, B/F can never exceed 4 (ie, 0.8/0.2). To correct for this potentially serious problem, the inactive fraction must always be determined and subtracted from both F and T.

Nonequilibrium Radioimmunoassay

So far, we have assumed that tracer and unlabeled competitor are added simultaneously, and sufficient incubation time is allowed to achieve equilibrium. To measure the affinity, of course, equilibrium must be assured. However, suppose one's sole purpose is to measure the concentration of competitor by RIA. Then one can actually increase the sensitivity of the assay by adding the competitor first, allowing it to react with the antibody, and then intentionally adding the tracer for too short a time to reach a new equilibrium. One is essentially giving the competitor a competitive advantage. It can be shown that the slope of the dose-response curve, B/T versus total antigen added, is increased in the low-dose range—a mathematical measure of increased sensitivity. A detailed mathematical analysis of this procedure may be found in Rodbard et al.[53] Note, however, that use of such nonequilibrium conditions requires very careful control of time and temperature.

Enzyme-Linked Immunosorbent Assay

An alternative solid-phase readout system for the detection of antigen–antibody reactions is the ELISA.[54] In principle, the only difference from RIAs is that antibodies or antigen are covalently coupled to an enzyme instead of a radioisotope so that bound enzyme activity is measured instead of bound cpm. In practice, the safety and convenience of nonradioactive materials and the commercial availability of plate readers that can measure the absorbance of 96 wells in a few seconds account for ELISA's widespread use. Because both ELISA and RIA are governed by the same thermodynamic constraints, and the enzyme can be detected in the same concentration range as commonly used radioisotopes, the sensitivity and specificity are comparable. We consider three basic strategies for using ELISA assays to detect specific antibody or antigen.

As shown in Figure 7.9A, the indirect antibody method is the simplest way to detect and measure specific antibody in an unknown antiserum. Antigen is noncovalently attached to each well of a plastic microtiter dish. For this purpose, it is fortunate that most proteins bind nonspecifically to plastic. Excess free antigen is washed off, and the wells are incubated with an albumin solution to block the remaining nonspecific protein binding sites. The test antiserum is then added, and any specific antibody binds to the solid-phase antigen. Washing removes unbound antibodies. Enzyme-labeled anti-Ig is added. This binds to specific antibody already bound to antigen on the solid phase, bringing along covalently attached enzyme. Unbound antiglobulin-enzyme conjugate is washed off; substrate is then added. The action of bound enzyme on substrate produces a colored product, which is detected as increased absorbance in a spectrophotometer.

Although this method is quick and very sensitive, it is often difficult to quantitate. Within a defined range, the increase in optical density is proportional to the amount of specific antibody added in the first step. However, the amount of antibody bound is not measured directly. Instead, the antibody concentration of the sample is estimated by comparing it with a standard curve for a known amount of antibody. It is also difficult to determine affinity by this method because the solid-phase antigen tends to

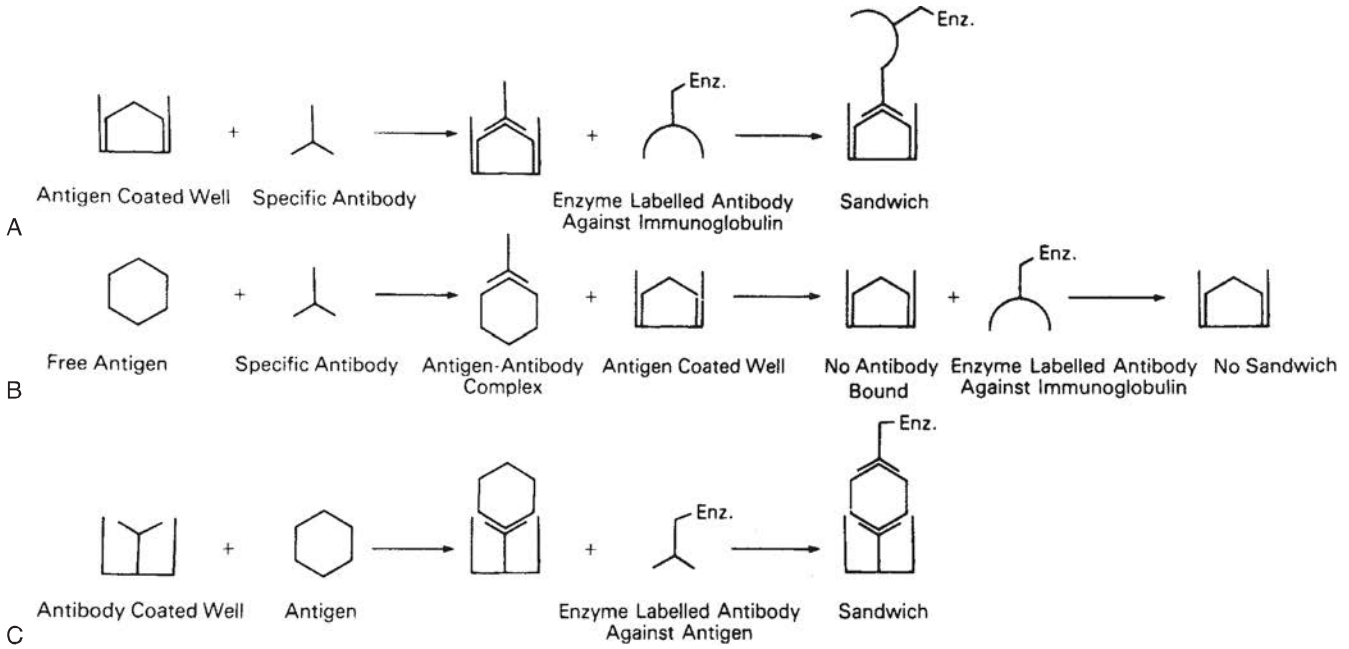

FIG. 7.9. Three Strategies for the Detection of Specific Antibody–Antigen Reactions Using the Enzyme-Linked Immunosorbant Assay Technique. A: Direct binding. B: Hapten inhibition. C: Antigen sandwich.

increase the apparent affinity. The sensitivity of this assay for detecting minute amounts of antibody is quite good, especially when affinity-purified antiglobulins are used as the enzyme-linked reagent. A single preparation of enzyme-linked antiglobulin can be used to detect antibodies to many different antigens. Alternatively, class-specific antiglobulins can be used to detect how much of a specific antibody response is due to each Ig class. Obviously, reproducibility of the assay depends on uniform antigen coating of each well, and the specificity depends on using purified antigen to coat the wells.

Figure 7.9B shows the competition technique for detecting antigen. Soluble antigen is mixed with limiting amounts of specific antibody in the first step. Then the mixture is added to antigen-coated wells and treated as described in Figure 7.9A. Any antigen–antibody complexes formed in the first step will reduce the amount of antibody bound to the plate, and hence will reduce the absorbance measured in the final step. This method permits the estimate of affinity for free antigen, which is related to the half-inhibitory concentration of antigen. Mathematical analysis of affinity by this approach was described by Friguet et al.[45] with modification by Stevens,[46] as discussed under RIA solid-phase methods. In addition, some estimate of cross-reactivity between the antigen in solution and that on the plate can be obtained.

Figure 7.9C shows the sandwich technique for detecting antigen. Microtiter plates are coated with specific antibody. Antigen is then captured by the solid-phase antibody. A second antibody specific for the antigen, and coupled to enzyme, is added. This binds to the solid-phase antigen–antibody complex, carrying enzyme along with it. Excess second antibody is washed off, and substrate is added. The absorbance produced is a function of the antigen concentration of the test solution, which can be determined from a standard curve. Specificity of the assay depends on the specificity of the antibodies used to coat the plate and detect antigen. Sensitivity depends on the affinity as well as amount of the first antibody coating the well, which can be increased by using affinity-purified antibodies or monoclonal antibodies in this step. The sandwich method depends on divalency of the antigen or else the two antibodies must be specific for different antigenic determinants on the same antigen molecule. When comparing two monoclonal antibodies to the same antigen, this technique can be used to ascertain whether they can bind simultaneously to the same molecule or whether they compete for the same site or sites close enough to cause steric hindrance.[55]

When antibodies are serially diluted across a plate, the last colored well indicates the titer. Specificity of binding can be demonstrated by coating wells with albumin and measuring antibody binding in parallel with the antigen-coated wells. Because it can be used to test many samples in a short time, ELISA is often used to screen culture supernatants in the production of hybridoma antibodies. The sensitivity of the method allows detection of clones producing specific antibodies at an early stage in cell growth.

An important caution when using native protein antigens to coat solid-phase surfaces (see Fig. 7.9A) is that binding to a surface can alter the conformation of the protein. For instance, using conformation specific monoclonal antibodies to myoglobin, Darst et al.[56] found that binding of myoglobin to a surface altered the apparent affinity of some antibodies more than others. This problem may be avoided by using the solution phase methods of Figure 7.9B or 7.9C.

ELIspot Assay

The normal ELISA assay can be modified to measure antibody production at the single cell level. In this method, tissue culture plates are coated with antigen, and various cell populations are cultured on the plate for 4 hours. During that time, B cells settle to the bottom and secrete antibodies, which bind antigen nearby and produce a footprint of the antibody-secreting cell. The cells are then washed off, and a second antibody, such as enzyme-labeled goat antihuman IgG, is added. Finally, unbound antibody is washed off, and enzyme substrate is added in soft agar. Over the next 10 minutes, each footprint of enzyme activity converts the substrate to a dark spot of insoluble dye, corresponding to the localized zone where the B cell originally secreted its antibody.

Using this method, it is possible to detect as few as 10 to 20 antibody-producing B cells in the presence of 10^6 spleen cells, and typical results for immunized mice range from 200 to 500 spot-forming cells per 10^6 spleen cells.[57,58] Clearly, to work at all, this assay must be capable of detecting the amount of antibody secreted by a single immune B cell and specific enough to exclude nonspecific antibodies produced by most nonimmune B cells. Sensitivity depends on the affinity and amount of antibodies secreted and may be optimized by titering the amount of antigen on the plate.

This type of assay is useful in analyzing the cellular requirements for antibody production in vitro, as the number of responding B cells is measured directly. It can also be used to detect antibodies made in the presence of excess antigen. For example, during acute infections[59] and in autoimmunity,[60] when antigen may be in excess over antibody, this assay makes it possible to measure antibody-producing B cells, even though free antibody may not be detectable in circulation. It can also be used to measure local production of self-reactive antibodies in a specific tissue, such as synovium. By using two detecting antibodies, each specific for a different Ig class and coupled to a different enzyme, and two substrates producing different colored dyes, cells secreting IgA and IgG simultaneously can be detected.[61] Recently, ELIspot was used to show that bacterial deoxyribonucleic acid (DNA)-containing CpG sequences is a polyclonal B-cell mitogen.[62]

ELIspot can also detect secreted cytokines, as opposed to antibodies, by coating the plate with a capture antibody and detecting antigen with an enzyme-coupled second antibody (as in a sandwich ELISA; see Fig. 7.9C). For example, using plates coated with monoclonal antibody to interleukin (IL)-4, T cells secreting IL-4 could be detected,[63] providing one measure of T helper 2 cells.

SPECIFICITY AND CROSS-REACTIVITY

The specificity of an antibody or antiserum is defined by its ability to discriminate between the antigen against which it was made (called the homologous antigen or immunogen) and any other antigen one might test. In practice, one cannot test the whole universe of antigens but only selected antigens. In this sense, specificity can only be defined experimentally within that set of antigens one chooses to compare. Karush[30] has defined a related term, selectivity, as the ability of an antibody to discriminate, in an all-or-none fashion, between two related ligands. Thus, selectivity depends not only on the relative affinity of the antibody for the two ligands but also on the experimental lower limit for detection of reactivity. For instance, an anticarbohydrate antibody with an affinity of 10^5 M^{-1} for the immunogen may appear to be highly selective, as reaction with a related carbohydrate with a 100-fold lower affinity, 10^3 M^{-1}, may be undetectable. On the other hand, an antibody with an affinity of 10^9 M^{-1} for the homologous ligand may appear to be less selective because any reaction with a related ligand with a 100-fold lower affinity would still be quite easily detectable.

Conversely, cross-reactivity is defined as the ability to react with related ligands other than the immunogen. More usually, this is examined from the point of view of the ligand. Thus, one might say that antigen Y cross-reacts with antigen X because it binds to anti-X antibodies. Note that in this sense, it is the two antigens that are cross-reactive, not the antibody. However, the cross-reactivity of two antigens, X and Y, can be defined only with respect to a particular antibody or antiserum. For instance, a different group of anti-X antibodies may not react at all with Y so that with respect to these antibodies, Y would not be cross-reactive with X. One can also use the term in a different sense, saying that some anti-X antibodies cross-react with antigen Y.

In most cases, cross-reactive ligands have lower affinity than the immunogen for a particular antibody. However, exceptions can occur in which a cross-reactive antigen binds with a higher affinity than the homologous antigen itself. This phenomenon is called heterocliticity, and the antigen that has a higher affinity for the antibody than does the immunogen is said to be heteroclitic. Antibodies that manifest this behavior are also described as heteroclitic antibodies. A good example is the case of antibodies raised in C57BL/10 mice against the hapten nitrophenyl acetyl. These antibodies have been shown by Mäkelä and Karjalainen[64] to bind with higher affinity to the cross-reactive hapten, nitroiodophenyl acetyl, than to the immunogen itself. Another example is the case of retro-inverso or retro-D peptides.[65–69] By reversing the chirality from L to D amino acids, and simultaneously reversing the sequence of amino acids, one can produce a peptide that is resistant to proteolysis and has its side chains approximately in the same position as the original L amino acid peptide, with the exception of some amino acids with secondary chiral centers such as Thr and Ile. However, the backbone NH_2 and COOH moieties are reversed. Antibodies that interact with only the side chains might not distinguish these peptides, whereas antibodies that interact with the main chain as well as side chains might distinguish them and have potentially higher or lower affinity. In a study of monoclonal antibodies to a hexapeptide from histone H3, some bound the retro-D form with higher affinity than the native sequence and some did not.[67,68] The former are examples of heterocliticity. In addition to greater binding affinity, the retro-D peptides may have even greater activity in vivo because of their resistance to proteolysis.[65–69] This stability makes them more useful as drugs as well.[65,66,70]

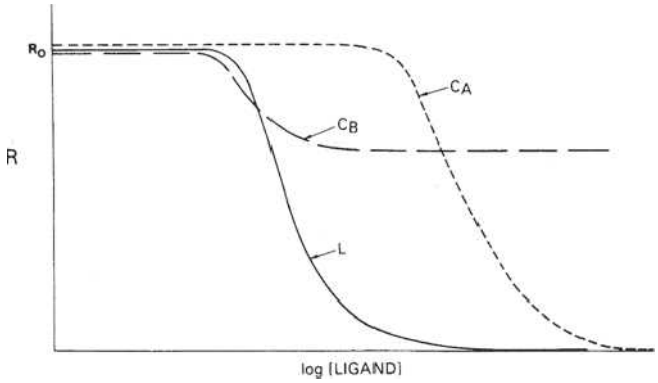

FIG. 7.10. Schematic Radioimmunoassay Binding Curves for Homologous Ligand L and Cross-Reacting Ligands. Cross-reacting ligand C_A manifests type 1 or true cross-reactivity demonstrated by complete inhibition of tracer ligand binding and a lower affinity. Ligand C_B displays type 2 cross-reactivity or determinant sharing, as recognized from the plateau at less than 100% inhibition, but not necessarily a lower affinity. The ordinate *R* is the ratio of bound/free radiolabeled tracer ligand, and R_0 is the limit of R as the concentration of all ligands, including tracer, approaches zero. From Berzofsky and Schechter,[71] with permission.

Cross-reactivity has often been detected by methods such as the Ouchterlony test, or hemagglutination (see the following for descriptions of both of these) or similar methods, which have in common the fact that they do not distinguish well between differences in affinity and differences in concentration. This practical aspect, coupled with the heterogeneity of immune antisera, has led to ambiguities in the usage of the terms "cross-reactivity" and "specificity." With the advent of RIA and ELISA techniques, this ambiguity in the terminology, as well as in the interpretation of data, has become apparent.

For these reasons, Berzofsky and Schechter[71] have defined two forms of cross-reactivity and, correspondingly, two forms of specificity. These two forms of cross-reactivity are illustrated by the two prototype competition RIA curves in Figure 7.10. In reality, most antisera display both phenomena simultaneously.

Type 1 cross-reactivity, or true cross-reactivity, is defined as the ability of two ligands to react with the same site on the same antibody molecule, possibly with different affinities. For example, the related haptens dinitrophenyl and trinitrophenyl may react with different affinity for antibodies raised to dinitrophenyl hapten. In protein antigens, such differences could occur with small changes in primary sequence (eg, the conservative substitution of threonine for serine), or with changes in conformation, such as the cleavage of the protein into fragments (Fig. 7.11).[71–75] If a peptide fragment contained all the contact residues in an antigenic determinant (ie, those that contact the antibody-combining site), it might cross-react with the native determinant for antibodies against the native form but with lower affinity because the peptide would not retain the native conformation. This type of affinity difference is illustrated by competitor C_A in Figure 7.10, in which complete displacement of tracer can be achieved at high enough concentrations of

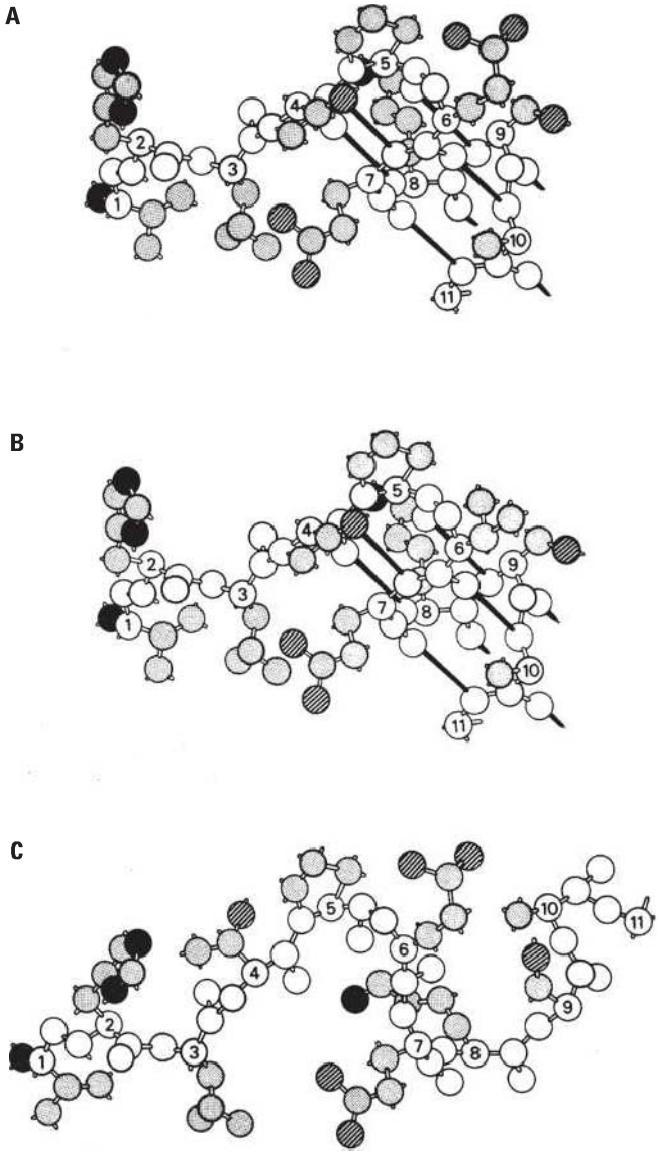

FIG. 7.11. An Artist's Drawing of the Amino Terminal Region of the β Chain of Hemoglobin. **A:** The first 11 residues of the β^A chain. **B:** The comparable regions of the β^S chain. The substitution of valine for the normal glutamic acid at position 6 makes a distinct antigenic determinant to which a subpopulation of antibodies may be isolated.[72,73] **C:** A schematic diagram of the sequence in (**A**) unfolded as occurs when the protein is denatured. This region may be cleaved from the protein, or the peptide synthesized,[74] resulting in changed antigenic reactivity. An antiserum prepared to hemoglobin (or the β chain thereof) might exhibit cross-reactivity with the structures shown in (**B**) and (**C**), but the molecular mechanisms would be different. Polypeptide backbone atoms are in *white* in the side chains, oxygen atoms are *hatched*, nitrogen atoms are *black*, and carbon atoms are *lightly stippled*. Adapted from Berxofsky and Schechter[71] and Dean and Schecter.[75]

C_A, but higher concentrations of C_A than of the homologous ligand, L, are required to produce any given degree of inhibition.

A separate issue from affinity differences is the issue of whether the cross-reactive ligand reacts with all or only a subpopulation of the antibodies in a heterogeneous serum.

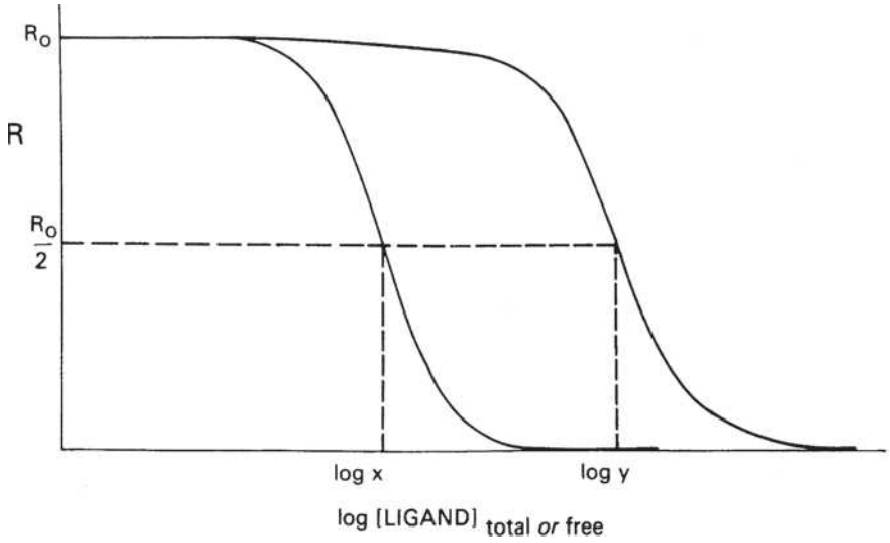

FIG. 7.12. Schematic Radioimmunoassay Binding Curves Showing the Effect of Affinity on the Midpoint and the Slope at the Midpoint and the Value of Using Free (Ligand) Rather than Total (Ligand). Ordinate R is the ratio of bound/free radiolabeled tracer ligand, and R_0 the limit of R as all ligand concentrations approach zero. If x and y are the concentrations of ligands X and Y that reduce R to exactly $R_0/2$, then if the abscissa is total ligand concentration, $x = 1/K_X + [S]_t/2$ and $y = 1/K_Y + [S]_t/2$, where $[S]_t$ is the concentration of antibody binding sites and K_X and K_Y the affinities of the antibody for the respective ligands. However, if the abscissa is free ligand concentration, $x = 1/K_X$ and $y = 1/K_Y$ so that the ratio x/y (or the difference log x – log y on a log plot) corresponds to the ratio of affinities K_Y/K_X. Note that the slopes at the midpoints are the same on a log scale, but that for Y would be only K_Y/K_X that for X on a linear scale. From Berxofsky and Schechter,[71] with permission.

This second type of cross-reactivity, which we call type 2 cross-reactivity or shared reactivity, therefore can occur only when the antibody population is heterogeneous, as in most conventional antisera. In this case, the affinity of the cross-reactive ligand may be greater than, less than, or equal to that of the homologous ligand for those antibodies with which it interacts. Therefore, the competition curve is not necessarily displaced to the right, but the inhibition will reach a plateau at less than complete inhibition, as illustrated by competitor C_B in Figure 7.10. As an example, let us consider the case of a protein with determinants X and Y, and an antiserum against this protein containing both anti-X and anti-Y antibodies. Then a mutant protein in which determinant Y was so altered as to be unrecognizable by anti-Y, but determinant X was intact, would manifest type 2 cross-reactivity. It would compete with the wild-type protein only for anti-X antibodies (possibly even with equal affinity), but not for anti-Y antibodies.

Of course, both types of cross-reactivity could occur simultaneously. A classic example would be the peptide fragment previously discussed in the case of type I cross-reactivity. Suppose the fragment contained the residues of determinant X, albeit not in the native conformation, but did not contain the residues of a second determinant, Y, which was also expressed on the native protein. If the antiserum to the native protein consisted of anti-X and anti-Y, the peptide would compete only for anti-X antibodies (type 2 cross-reactivity) but would have a lower affinity than the native protein even for these antibodies. Thus, the competition curve would be shifted to the right and would plateau before reaching complete inhibition.[†††]

In the case of a homogeneous (eg, monoclonal) antibody in which only type 1 or true cross-reactivity can occur, one can quantitate the differences in affinity for different cross-reactive ligands by a method analogous to the B/F versus F method described previously. Suppose that ligands X and Y cross-react with homologous ligand L for a monoclonal antibody. If one plots the bound/free (B/F = R) ratio for radiolabeled tracer ligand L as a function of the log of the concentration of competitors X and Y, one obtains two parallel competition curves (Fig. 7.12),[71] under the appropriate conditions (see subsequent discussion). The first condition is that the concentration of free tracer be less than $1/K_L$, the affinity for tracer. In this case, it can be shown[71] that

$$K_X \approx \frac{1}{[X]_{free}} \tag{41}$$

at the midpoint where $R = R_0/2$, where K_X = affinity for X. This is analogous to Equation 21 for the case in which unlabeled homologous ligand is the competitor. Also, in

[†††]An ambiguous case could occur experimentally in which the distinction between the two types of cross-reactivity would be blurred. For example, in the case of antibodies that all react with determinant X but have a very wide range of affinities for X, some such antibodies may have such a low affinity for cross-reactive determinant X′ that they would appear not to bind X′ at all. Then a competition curve using X′ might appear to reach a plateau at incomplete inhibition, even though all the antibodies were specific for X, and the only difference between X and X′ was affinity.

analogy with Equation 23, it can be shown that if the total concentration of competitor, $[X]_t$, is used instead of the free concentration, $[X]_{free}$, an error term will arise, giving

$$[X]_t \text{ (at } R = R_0/2) = \frac{1}{K_X} + \frac{[S]_t}{2} \quad (42)$$

Thus, with competitor on a linear scale, the difference in midpoint for competitors X and Y will correspond to the difference $1/K_X - 1/K_Y$ regardless of whether free or total competitor is plotted, but the ratio of midpoint concentrations will equal K_X/K_Y only if the free concentrations are used. This last point is important if one plots the log of competitor concentration, as is usually done, as the horizontal displacement between the two curves on a log scale corresponds to the ratio $[X]/[Y]$, not the difference.[71]

If a second condition also holds, namely, that the concentration of bound tracer is small compared to the antibody site concentration $[S]_t$, then the slopes (on a linear scale) of the curves at their respective midpoints (where $R = R_0/2$) will be proportional to the affinity for that competitor, K_X or K_Y (71). (Both conditions can be met by keeping tracer L small relative to both K_L and $[S]_t$.) When $[X]_{free}$ and $[Y]_{free}$ are plotted on a log scale, the slopes will appear to be equal (ie, the curves will appear parallel) because a parallel line shifted m-fold to the right on a log scale will actually be 1/m as steep, at any point, in terms of the antilog as abscissa.

When the antibodies are heterogeneous in affinity, the curves will be broadened and in general will not be parallel. When heterogeneity of specificity is present, and type 2 cross-reactivity occurs, it should be pointed out that the fractional inhibition achieved at the plateau in a B/F versus free competitor plot will not be proportional to the fraction of antibodies reacting with that competitor but will be proportional to a weighted fraction, where the antibody concentrations are weighted by their affinity for the tracer.[71]

These two types of cross-reactivity lead naturally to two definitions of specificity.[71] The overall specificity of a heterogeneous antiserum is a composite of both of these facets of specificity. Type 1 specificity is based on the relative affinities of the antibody for the homologous ligand and any cross-reactive ligands. If the affinity is much higher for the homologous ligand than for any cross-reactive ligand tested, then the antibody is said to be highly specific for the homologous ligand (ie, it discriminates very well between this ligand and the others). If the affinity for cross-reactive ligands is below the threshold for detection in an experimental situation, then type 1 specificity gives rise to selectivity as was discussed previously.[30] The specificity can even be quantitated in terms of the ratio of affinities for the homologous ligand and a cross-reactive ligand.[76] It is this type 1 specificity that most immunochemists would call true specificity, just as we have called type 1 cross-reactivity true cross-reactivity.

The common use of the term "cross-reactivity" to include type 2 or partial reactivity leads to a second definition of specificity, which applies only to heterogeneous populations of antibodies such as antisera. We call this type 2 specificity. If all the antibodies in the mixture react with the immunogen, but only a small proportion react with any single cross-reactive antigen, then the antiserum would be said to be relatively specific for the immunogen. Note that it does not matter whether the affinity of a subpopulation that reacts with a cross-reactive antigen is high or low (type 1 cross-reactivity). As long as that subpopulation is a small fraction of the antibodies, the mixture is specific. Thus, type 2 specificity depends on the relative concentrations of antibodies in the heterogeneous antiserum, not just on their affinities. Also note that one can use these relative concentrations of antibody subpopulations to compare the specificity of a single antiserum for two cross-reactive ligands. However, it would not be meaningful to compare the specificity of two different antisera for the same ligand by comparing the fraction of antibodies in each serum which reacted with that ligand. Although type 2 specificity may appear to some a less classic concept of specificity than type 1, it is type 2 specificity that one primarily measures in such assays as the Ouchterlony double immunodiffusion test, and it carries equal weight with type 1 specificity in such assays as hemagglutination, discussed in the following section. Type 2 specificity also leads naturally to the concept of "multispecificity," described in the following section.

Multispecificity

The theory of multispecificity, introduced and analyzed by Talmadge[77] and Inman,[78,79] and discussed on a structural level by Richards et al.,[80] suggests a mechanism by which the diversity and specificity of antisera can be expanded and also understood. The idea is that each antibody may actually bind, with high affinity, a variety of diverse antigens. When one immunizes with immunogen A, one selects for many distinct antibodies, which have in common only that they all react with A. In fact, each antibody may react with other compounds, but if fewer than 1% of the antibodies bind B, and fewer than 1% bind C, and so on, then by type 2 specificity, the whole antiserum will appear to be highly specific for A. Note that the subpopulation that binds B may react with an affinity for B as high as or higher than that for A so that the population would not be type 1 specific for A. This same population would presumably be selected if one immunized with B. The net result would be that the diversity of highly (type 2) specific antisera an organism could generate would be even greater than the diversity of B-cell clones (or antibody structures). This principle can explain how polyclonal antisera can sometimes appear paradoxically more specific than a monoclonal antibody.

OTHER METHODS

We mention only a few of the other methods for measuring antigen–antibody interactions. Other useful techniques include quenching of the tryptophan fluorescence of the antibody by certain antigens on binding[81] (a sensitive method useful for such experiments as fast kinetic studies), antibody-dependent cellular cytotoxicity, immunofluorescence including flow cytometry, immunohistochemistry, and inhibition by antibody of plaque formation by antigen-conjugated bacteriophage[82] (a method as sensitive as RIA as inhibition of even a few phage virions can be detected).

Quantitative Precipitin

Among the earliest known properties of antibodies were their ability to neutralize pathogenic bacteria and their ability to form precipitates with bacterial culture supernatants. Both activities of each antiserum were highly specific for the bacterial strain against which the antiserum was made. The precipitates contained antibody protein and bacterial products. The supernatants contained decreased amounts of antibody protein and, under the right conditions, had lost the ability to neutralize bacteria. However, quantitation of the antibody precipitated was difficult because the precipitate contained antigen protein as well as antibody protein. Heidelberger and Kendall[83,84] solved this problem when they found that purified pneumococcal cell wall polysaccharide could precipitate with antipneumococcal antibodies. In this case, the amount of protein nitrogen measured in the precipitate was entirely due to antibody protein, and the amount of reducing sugar was mostly due to the antigen. Plotting the amount of antibody protein precipitated from a constant volume of antiserum by increasing amounts of carbohydrate antigen gives the curve shown in Figure 7.13.

As shown in Figure 7.13A, the amount of antibody precipitated rises initially, reaches a plateau, and then declines. The point of maximum precipitation was found to coincide with the point of complete depletion of neutralizing antibodies and is called the equivalence point. The amount of antibody protein in the precipitate at equivalence is considered to equal the total amount of specific antibody in that volume of antiserum. The rising part of the curve is called the antibody excess zone (antigen limiting), and the part of the curve beyond the equivalence point is called the antigen excess zone.

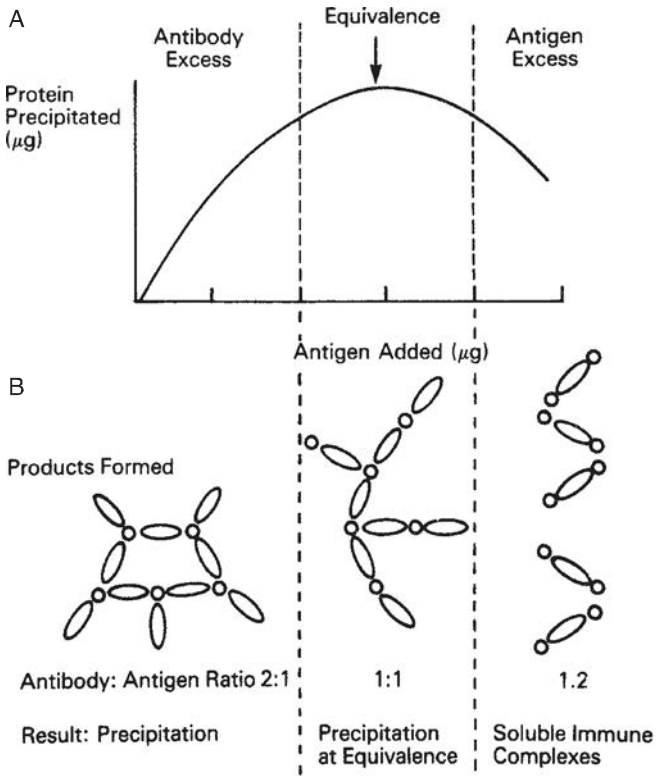

FIG. 7.13. Quantitative Immunoprecipitation. To a fixed amount of specific antibody are added increasing amounts of nonprotein antigen. The figure shows the amount of antibody protein **(A)** and the ratio of antibody to antigen **(B)** found in the precipitate. At antigen excess, soluble immune complexes are found in the supernatant, and the precipitate is decreased.

Supernatants and precipitates were carefully analyzed for each zone of antibody or antigen excess, as shown in Figure 7.13B. When antigen was limiting, the precipitate contained high ratios of antibody to antigen. The supernatant in this zone contained free antibody with no detectable antigen. As more antigen was added, the amount of antibody in the precipitate rose, but the ratio of antibody to antigen fell. At equivalence, no free antibody or antigen could be detected in the supernatant. As more antigen was added, the precipitate contained less antibody, but the ratio of antibody to antigen remained constant. The supernatant now contained antigen–antibody complexes because the complexes at antigen excess were small enough to remain in solution. No unbound antibody was detected.

The lattice theory[83,84] is a model of the precipitation reaction that explains these observations. It assumes that antibodies are multivalent and antigens are bivalent or polyvalent. Thus, long chains can form consisting of alternating antibody linked to antigen linked to antibody, and so on. The larger the size of the aggregate, the less soluble the product. In the antibody excess zone, branch points can form whenever three antibodies bind to a single antigen, resulting in a three-dimensional lattice structure, which precipitates. In the equivalence zone, when equimolar amounts of antibody and antigen are mixed, the likelihood of more than two antibodies binding the same antigen molecule decreases. With fewer branch points, the product is more likely to consist of long chains of alternating antibody and antigen molecules in the molar ratio of 1:1. At even higher antigen ratios, each antigen molecule will have 0 or 1 antibody bound, so shorter chain lengths are found, until the product is small enough to remain in solution. Such soluble antigen–antibody "immune complexes" are detectable in the antigen excess zone, where no free antibody is found.

Besides explaining the observed precipitation phenomena on a statistical basis, the lattice theory made the important prediction that antibodies are bivalent or multivalent. The subsequent structural characterization of antibodies revealed their molecular weight and valency. Antibodies are indeed bivalent, except for IgM, which is functionally pentavalent and forms precipitates even more efficiently.

Antigens can be polyvalent either by having multiple copies of the same determinant or by having many different determinants, each of which reacts with different antibodies in a polyclonal antiserum. The predominant antigenic determinants of polysaccharides are often the nonreducing end of the chain. Branched chain polysaccharides have more than one end and are polyvalent. Nonbranched chains such as dextran (polymer of glucose) are monovalent for end specific antidextran antibodies and do not precipitate them.[85] However, a second group of antidextran antibodies is specific for internal glucose moieties. Because each dextran polymer consists of many internal units, it is polyvalent for

internal α(1 → 6)-linked glucose-specific antibodies. Thus, unbranched dextran polymer can be used to distinguish between end-specific and internal-specific antibodies, as it will precipitate with the latter antibodies but not the former.[85,86] Monomeric protein antigens, such as myoglobin or lysozyme, behave as if they were polyvalent for heterogeneous antisera but monovalent for monoclonal antibodies. This results from the fact that each antigen molecule has multiple antigenic determinants but only one copy of each determinant. Thus, a polyspecific antiserum can bind more than one antibody to different determinants on the same molecule and form a lattice. However, using antibodies directed against a single determinant (such as a monoclonal antibody), no precipitate will form. In this case, antigen–antibody reactions must be measured by some other binding assay, such as RIA or ELISA.

Immunodiffusion and the Ouchterlony Method

One of the most useful applications of immunoprecipitation is in combination with a diffusion system.[87] Diffusion could be observed by gently adding a drop of protein solution to a dish of water without disturbing the liquid. The rate of migration of protein into the liquid is proportional to the concentration gradient times the diffusion coefficient of the protein according to Fick's law,

$$\frac{dQ}{dt} = -DA\frac{dc}{dx} \tag{43}$$

where Q = amount of substance that diffuses across an area A per unit time t; D = diffusion coefficient, which depends on the size of the molecule; and dc/dx = concentration gradient. Because antibody molecules are so large, their diffusion coefficients are quite low, and diffusion often takes 1 day or more to cover the 5 to 20 mm required in most systems. In order to stabilize the liquid phase for such long periods of time, a gel matrix is added to provide support without hindering protein migration. In practice, 0.3% to 1.5% agar or agarose will permit migration of proteins up to the size of antibodies while preventing mechanical and thermal currents. By carefully adjusting the concentration of antibody and antigen, these systems can provide a simple analysis of the number of antigenic components and the concentration of a given component. By adjusting the geometry of the reactants entering the gel, immunodiffusion can provide useful information concerning antigenic identity or difference, or partial cross-reaction, as well as the purity of antigens and the specificity of antibodies.

In single diffusion methods,[88–91] antibody is incorporated in the gel, and antigen is allowed to diffuse from one end of a tube gel in one dimension or from a hole in a gel in a petri dish in two dimensions. Over time, the antigen concentration reaches equivalence with the antibody in the gel, and a precipitin band forms. As more antigen diffuses, antigen excess is achieved at this position, so the precipitate dissolves and the boundary of equivalence moves farther. By integrating Fick's law, we find that the distance moved is proportional to the square root of time. If two species of antigen a and b are diffusing, and the antiserum contains antibodies to both, two independent bands will form. These will move at independent rates, depending on antigen concentration in the sample, diffusion coefficient (size), and antibody concentration in the agar. Similarly, in the two-dimensional method, at a given radius of diffusion, antigen concentration will be equivalent to the antibody in the gel, and a precipitin ring will form. The higher the initial antigen concentration, the farther the antigen will diffuse before precipitating and the wider the area of the ring will be. The area of the ring is directly proportional to the initial antigen concentration. This method provides a convenient quantitative assay that can be used to measure Ig classes by placing test serum in the well and antiserum to each class of human Ig in the agar. Sensitivity can be increased by lowering the concentration of antiserum in the gel, giving wider rings, as the antigen must reach a lower concentration to be at equivalence. However, the antiserum cannot be diluted too far, or no precipitate will form.

The double diffusion methods use the same principles, except that instead of having one reactant incorporated in the gel at a constant concentration, both antigen and antibody are loaded some distance apart in a gel of pure agarose alone and are allowed to diffuse toward each other. At some point in the gel, antigen diffusion and antibody diffusion will provide sufficient concentrations of both reactants for immunoprecipitation to occur. The line of precipitation becomes a barrier for the further diffusion of the reactants, so the precipitin band is stable. If the antigen preparation is heterogeneous, and the antiserum is a heterogeneous mixture of antibodies, different bands will form for each pair of antigen and antibody reacting at positions dependent on concentration and molecular weight of each. The number of lines indicates the number of antigen–antibody systems reacting in the gel. The ability of immunodiffusion to separate different antigen–antibody systems gives a convenient estimate of antigen purity or antibody specificity.

In the most widely used Ouchterlony method of double diffusion in two dimensions,[87] three or more wells are cut in an agarose gel in a dish in the pattern shown in Figure 7.14. Antigen a or b is placed in the upper wells, whereas antiserum containing anti-a or anti-b is placed in the lower well. Each antigen–antibody reaction system will form its own precipitin line between the wells. As shown in Figure 7.14A, this should extend an equal distance to the left and right of the wells. When different antigens are present in different wells (see Fig. 7.14C), the precipitating systems do not interact immunochemically, so the precipitin lines cross. However, when the same antigen is present in both wells (see Fig. 7.14B), each line of precipitation becomes a barrier preventing the other antigen and antibody from diffusing past the precipitin line. This shortens the precipitin line on that side of the well. In addition, antigen diffusion from the neighboring wells shifts the zone of antigen excess, causing the equivalence line to deviate downward and meet between the two wells. Complete fusion of precipitin lines with no spurs is called a line of identity, indicating that the antigen in each well reacts with all the antibody capable of reacting with antigen in the other well.

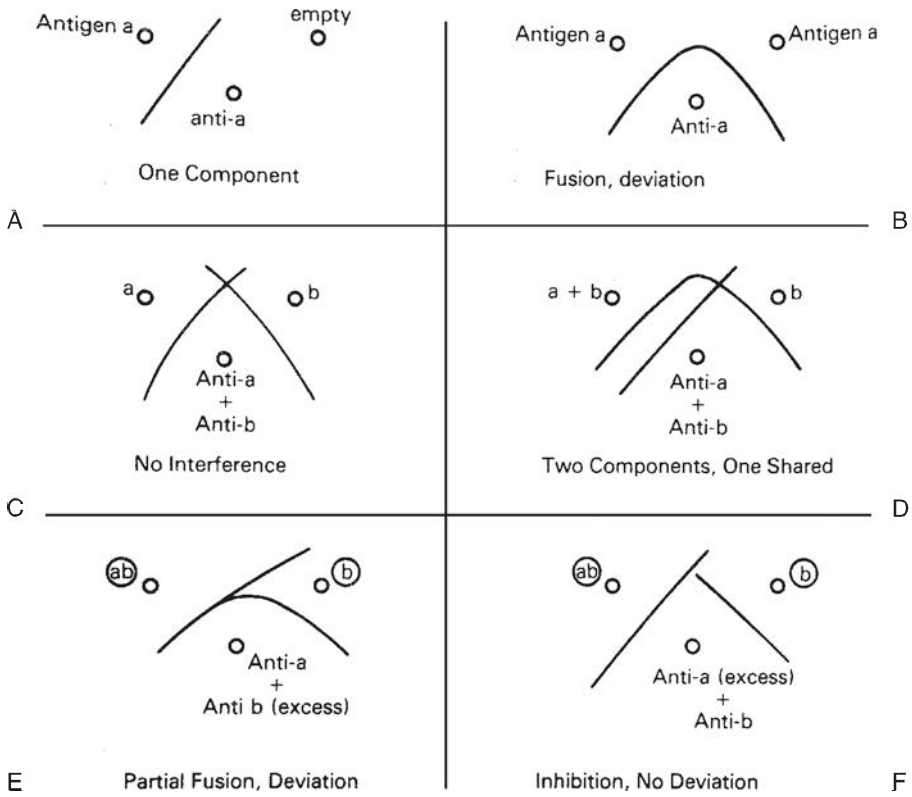

FIG. 7.14. Immunodiffusion of Two Components in Two Dimensions. Cross-reactions produce inhibition *(shortened bands)* or deviation *(curved bands)*. Lines of identity are shown in (B) and (D).[87]

The analytical power of this method is shown in Figure 7.14D. When a mixed antigen sample is placed in one well and pure antigen b is placed in the other well, antiserum to a plus b gives the pattern shown. Two precipitin lines form with the left well and one precipitin line with the right well. The line of complete fusion allows us to identify the second band as antigen b; the first band is antigen a. From their relative distance of migration, we can conclude that antigen a is in excess over antigen b, assuming their diffusion coefficients are comparable and both antibodies are present in equal amounts. Finally, because the precipitin line of antigen a-anti-a is not shortened at all, there is no contamination of the right sample with antigen a, and the two antigens do not cross-react.

The type of cross-reactivity detected by this Ouchterlony double immunodiffusion in agar is what we have defined previously as type 2 cross-reactivity. The method is not suitable for measuring affinity differences required for quantitating type 1 cross-reactivity. Sensitivity can be increased by use of radioactive antigen and detection of the precipitate by autoradiography.

Immunoelectrophoresis

Some antigen–antibody systems are too complex for double immunodiffusion analysis, either because there are too many bands or they are too close together. Immunoelectrophoresis combines electrophoresis in one dimension (Fig. 7.15) with immunodiffusion in the perpendicular direction. In the first step, electrophoresis separates the test antigens according to charge and size, in effect, separating the origin of diffusion of different antigens. This is equivalent to having each antigen start in a different well, as shown in the right-hand panel. A horizontal trough is then cut into the agar and filled with antiserum to all the components. Immunodiffusion occurs between the separated antigens and the linear source of antibody. The results for a mixture of three antigens approximate those shown for three antigens in separate wells.[87] Fusion, deviation, and inhibition between precipitin lines can be analyzed as described previously. The resolution of each band is somewhat decreased due to widening of the origin of diffusion during electrophoresis. However, the immunodiffusion of unseparated human serum proteins, for example, is greatly facilitated by prior electrophoresis. Starting from a single well, only the heavier bands would be visible. However, prior electrophoresis makes it possible for each electrophoretic species to make its own precipitin line. Monospecific antiserum can be placed in a parallel horizontal trough so that each band of precipitation can be identified. Immunoelectrophoresis is commonly used to diagnose myeloma proteins in human serum. The unknown serum is electrophoresed, followed by immunodiffusion against antibodies to human Ig heavy or light chains. A widening arc of IgG suggests the presence of an abnormal Ig species. At this same electrophoretic mobility, a precipitin line with anti-κ, but not anti-λ, reactivity strongly suggests the diagnosis of myeloma or monoclonal gammopathy, as these proteins arise from a single clone that synthesizes only one light chain. All normal electrophoretic species of human Igs contain both light chain isotypes, although κ exceeds λ by the ratio of 2:1 in humans. As shown in Figure 7.15C, the abnormal arc with γ mobility reacts

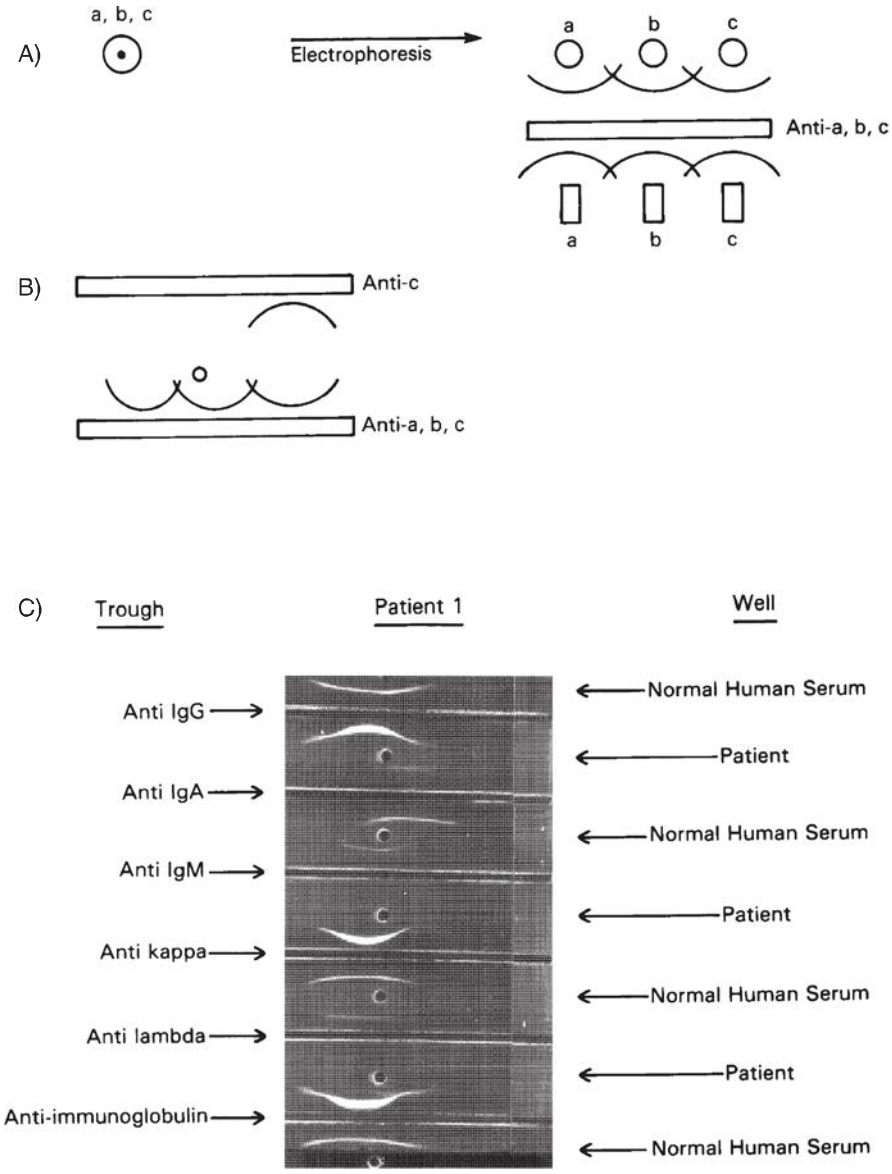

FIG. 7.15. Immunoelectrophoresis. A sample containing multiple components is electrophoresed in an agarose gel, separating the antigens in the horizontal dimension. Then a horizontal trough is cut into the gel and antiserum is added. Immunodiffusion between the separated antigens and the trough is equivalent to having separate wells, each with a different antigen.[87] This technique is used to identify a myeloma protein in human serum. Sera from the patient or normal individual were placed in the circular wells and electrophoresed. Antisera were then placed in the rectangular troughs and immunodiffusion proceeded perpendicular to the direction of electrophoresis. The abnormally strong reaction with anti-immunoglobulin (Ig) G and anti-κ, but no reaction with anti-λ antibodies, indicate a monoclonal protein (IgG,κ), as polyclonal Ig should react with both anti–light-chain antisera. Failure to form a band with anti-IgM and a reduced band with anti-IgA show typical reduction of normal Igs in this disease. (Photographs courtesy of Theresa Wilson, NIH Clinical Chemistry Section.)

with anti-IgG and anti-κ but not anti-λ antiserum. Thus, it is identified as an IgG-κ monoclonal protein.

Hemagglutination and Hemagglutination Inhibition

Hemagglutination

A highly sensitive technique yielding semiquantitative values for the interaction of antibody with antigen involves the agglutination by antibodies of red blood cells coated with the antigen.[92] For exogenous antigens that are adsorbed to the red blood cell surface, the reaction is called passive hemagglutination. Untreated red blood cells are negatively charged, and electrostatic forces keep them apart. Following treatment with tannic acid (0.02 mg/mL for 10 minutes at 37°C), however, they clump readily.

Untreated red blood cells are easily coated with polysaccharide antigens, which they adsorb readily. After tanning, the uptake of some protein antigens is good, giving a sensitive reagent, whereas for others it tends to be quite variable; coating red blood cells has been the limiting factor in the usefulness of this method for certain antigens. Apparently, slightly aggregated or partially denatured protein antigens are adsorbed preferentially.[92]

The test for specific antibodies is done by serially diluting the antiserum in the U-shaped wells of a microtiter plate and adding antigen-coated red cells. When cross-linked by specific antibodies, agglutinated cells settle into an even carpet spread over the round bottom of the well. Unagglutinated red cells slide down the sides and form a much smaller button at the very bottom of the well. The titer of a sample is the highest dilution at which definite agglutination occurs. With hyperimmune antisera, inhibition of agglutination is often observed at high doses of antibody, termed a *prozone effect*. Two interpretations have been offered: One is that, at great antibody excess, each cell is coated with antibody, so cross-linking by the same antibody molecule becomes improbable. The second interpretation is the existence of some species of inefficient or "blocking" antibodies that occupy

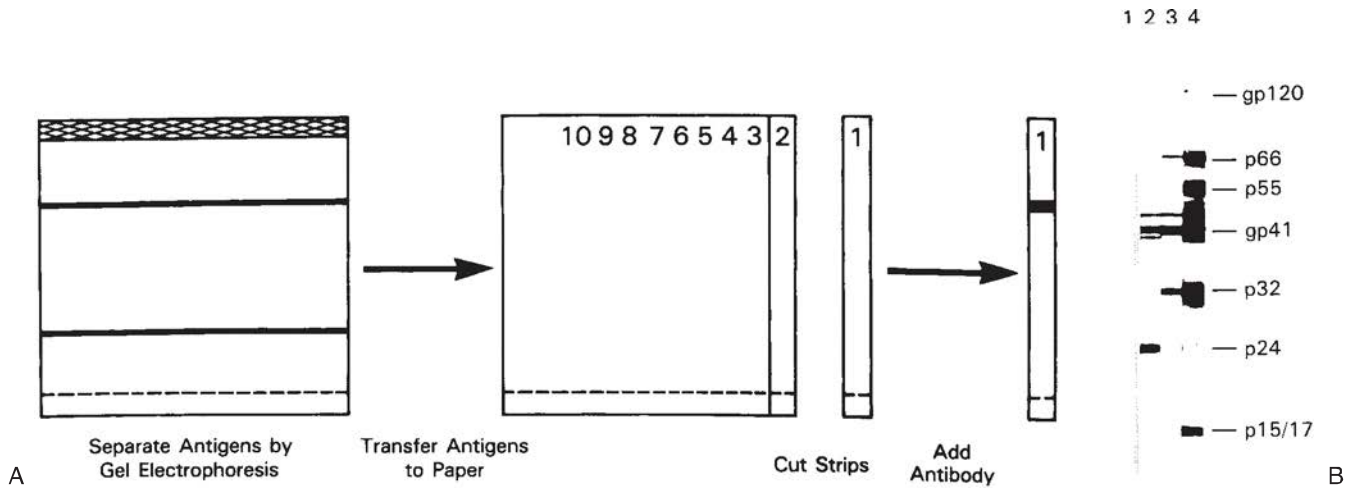

FIG. 7.16. A: Western blot technique. The antigen preparation is run through a polyacrylamide gel, which separates its components into different bands. These bands are then transferred to paper by electrophoresis in the horizontal dimension. The paper is cut into strips. Each strip is incubated with test antibodies, followed by further incubation with enzyme-labeled second antibodies. If the test antibodies bind to the component antigens, they will produce discrete dark bands at the corresponding positions on the strip. **B:** Clinical specimens from patients with acquired immunodeficiency syndrome tested on strips bearing human immunodeficiency virus-1 viral antigens, showing antibodies to viral gag (p15/17, p24, and p55 precursor), pol (p66 and possibly p32), and env (gp41 and gp120) proteins. Lane 1 is the negative control, and lanes 2 to 4 are sera from three different patients.

antigen sites without causing aggregation of cells.[9] To assure antigen specificity, the antiserum should be absorbed against uncoated red cells prior to the assay, and an uncoated red cell control should be included with each assay. IgM is up to 750 times more efficient than IgG at causing agglutination, which may affect interpretation of data based on titration. The titer may vary by a factor of 2 simply due to subjective estimates of the endpoint.

Once the hemagglutination titer of an antiserum is determined, its interaction with antigen-coated red blood cells can be used as a sensitive assay for antigen. To constant amounts of antibody (diluted to a concentration twofold higher than the limiting concentration producing agglutination) are added varying amounts of free antigen. Agglutination will be inhibited when half or more of the antibody sites are occupied by free antigen. In a similar fashion, the assay can be used for the detection and quantitation of anti-idiotype antibodies that react with the variable region of antibodies and sterically block antigen binding.

Immunoblot (Western Blot)

A most useful technique in the analysis of proteins is polyacrylamide gel electrophoresis, in which charged proteins migrate through a gel in response to an electric field. When ionic detergents such as sodium dodecyl sulfate are used, the distance traveled is inversely proportional to the logarithm of molecular weight. The protein components of complex structures, such as viruses, appear as distinct bands, each at its characteristic molecular weight. Because antibodies may be unable to diffuse into the gel, it is necessary to transfer the protein bands onto a nitrocellulose membrane support first, where they are exposed for antibody binding.[93]

The immunoblot is often used to detect viral proteins with specific antibodies that bind these proteins on the nitrocellulose blot. Then a second antibody, which is either enzyme conjugated or radiolabeled, is used to detect the antigen–antibody band. Crude viral antigen preparations can be used, as only those bands that correspond to viral antigens will be detected.

Typical results are shown in Figure 7.16. HIV-1 was cultured in susceptible H9 cells. The viral proteins were separated by polyacrylamide gel electrophoresis and detected by immunoblot, using the serum of infected patients. Each antigen band recognized by the antiserum has been identified as a viral component or precursor protein. The gp160 precursor is processed to mature gp120 and gp41 envelope proteins, whereas a p66 precursor is processed to the p51 mature form of reverse transcriptase, and a p55 precursor becomes the p24 and p17 gag and matrix proteins of the virus.[94] The practical uses of the HIV western blot include diagnosing infection, screening blood units to prevent HIV transmission, and testing new vaccines.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) uses the electromagnetic properties of light to measure the binding affinity of a variety of biologic molecules, including antigen–antibody pairs. In this method, polarized light passes through a glass plate coated on the back surface with a thin metal film, usually gold. Biologic materials binding to the metal film behind the plate can alter its refractive index in ways that affect the angle and intensity of reflected light.

At angles close to perpendicular, light will pass through the glass, although it will bend at the interface due to

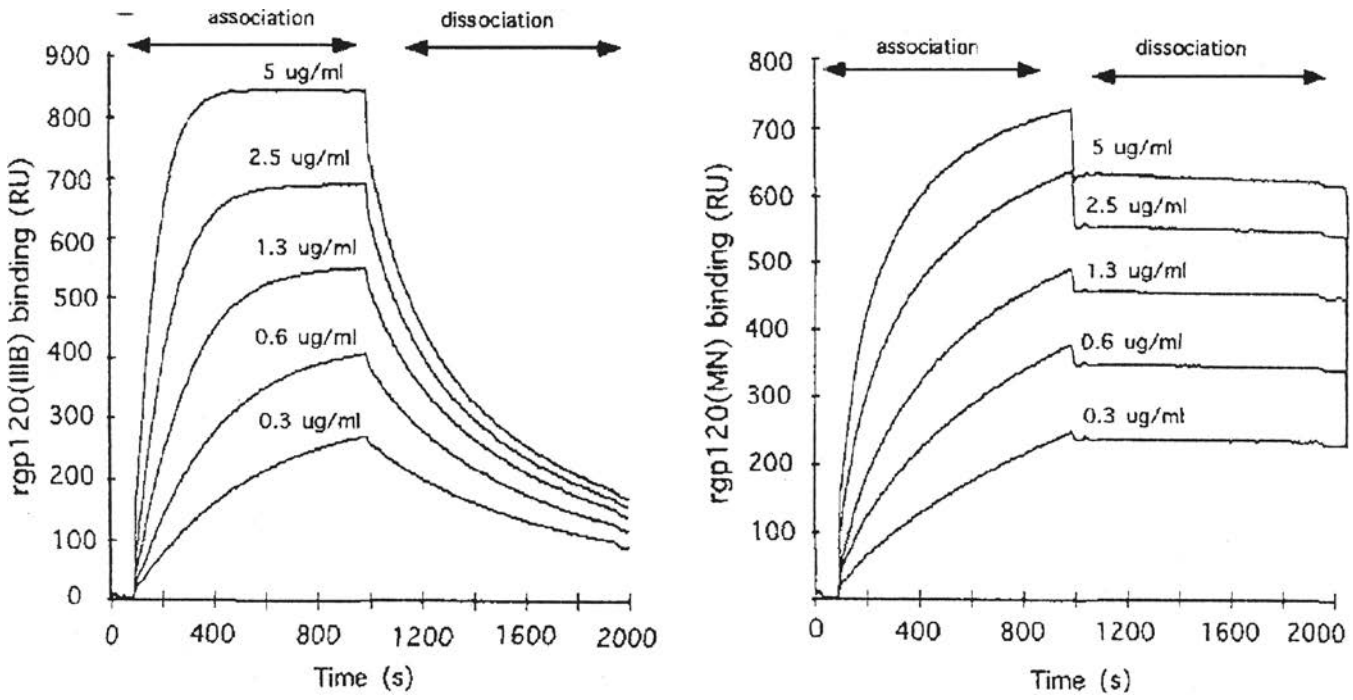

FIG. 7.17. Monoclonal Antibody to gp120 was Introduced into the Flow Cell at Time 0, and Antibody Binding was Followed Over Time as a Change in the Critical Angle, Measured in Response Units. After 1000 seconds, free antibody was washed out, and the release of bound antibody was measured as a decrease in refractive index. Lower affinity binding to gp120 from the IIIB strain *(left)* was shown as a faster "off rate," as compared to the very slow rate of antibody release from the MN strain *(right)*. These results, obtained under nonequilibrium conditions, provide direct measurement of the forward and reverse rate constants for antibody binding, and the ratio of these two gives the affinity constant. Modified from VanCott et al.,[97] with permission.

differences in the refractive index of glass and what is behind it. Above a certain angle, called the critical angle, bending will be so great that total internal reflection will occur. Small changes in refractive index behind the glass can be detected as significant changes in the critical angle, where light reflection occurs, and in the intensity of reflected light at this angle. By reading the reflected light intensity in a diode array detector, the critical angle and intensity can be determined simultaneously. Due to the wave nature of light, the effect of refractive index in the gold film extends about one wavelength beyond the glass or about 300 to 700 nm.[95] Within this layer, if an antigen is covalently attached to the gold, then antibody binding can be detected as a change in refractive index, resulting in a different critical angle and intensity of the reflected light.

SPR systems have three essential features[96]: an optical system that allows determination of the critical angle and light intensity at the same time, a coupling chemistry that links antigen or antibody to the gold surface, and a flow system that rapidly delivers the binding molecule in the mobile phase, so SPR can measure the rate of binding, rather than the rate of diffusion. Because binding causes a physical change in the gold film, there is no need for radioactive labels or enzyme conjugates. Molecular binding interactions can be followed in real time.

A typical SPR experiment is shown in Figure 7.17. HIV gp120 of type IIIB (*left panel*) or MN (*right panel*) were fixed to the gold layer, and various concentrations of monoclonal antibody to gp120 were added to the flow cell.[97] Over the first 1000 seconds, antibody binding was measured as a change in reflected light (in response units), allowing a calculation of the rate constant for the forward reaction of antibody binding. Once the signal reached a plateau, antibody was washed out of the flow cell, and the decrease in SPR signal over time indicated the rate at which antibody came off the antigen. The "on rate" for antibody binding to IIIB gp120 (*left*) was about twice as fast as for MN (*right*) at each antibody concentration. However, the "off rate" was about 50-fold less for MN than for IIIB. Combining these kinetic results indicates much greater binding affinity for gp120 of MN type, which may explain the observation that MN type virus was 10-fold more sensitive to neutralization by this antibody than was the IIIB strain.

MONOCLONAL ANTIBODIES

Homogeneous Igs have long played important roles in immunologic research. Starting in the 1950s, Slater et al.[98] studied sera from human patients with multiple myeloma and recognized the relationship between abnormal myeloma proteins and normal serum globulins. Potter[99] characterized numerous mouse myeloma tumors and identified the antigenic specificities of some of them. Human and mouse myeloma proteins were studied as representative Igs

and recognized for the advantages they had with proteins as diverse as antibodies for studies of Ig structure, function, and genetics. It was not yet possible, however, to induce monoclonal Igs of desired specificity.

This goal was achieved by the introduction of hybridoma technology by Köhler and Milstein[1,100] and by Margulies et al.[101] in the 1970s. Since that time, monoclonal antibodies have come to play an enormous role in biologic research and applications. They offer as advantages the relative ease of the production and purification of large quantities of antibody, the uniformity of antibody batches, and the ready availability of Ig messenger ribonucleic acid and DNA from the hybrid cell lines.

Derivation of Hybridomas

Hybridomas producing monoclonal antibodies are generated by the somatic cell fusion of two cell types: antibody-producing cells from an immunized animal, which by themselves die in tissue culture in a relatively short time, and myeloma cells, which contribute their immortality in tissue culture to the hybrid cell. The myeloma cells are variants carrying drug selection markers so that only those myeloma cells that have fused with spleen cells providing the missing enzyme will survive under selective conditions. Initial work used myeloma cells that secreted their own Ig products, but later such fusion partners were replaced by myeloma variants that fail to express Ig[102,103] so that the fused cell secretes exclusively antibody of the desired specificity. Successful hybridoma production is influenced by the characteristics of the cell populations (immune lymphocytes and myeloma fusion partner), the fusion conditions, and the subsequent selection and screening of the hybrids. A diagrammatic version of the overall process of hybridoma derivation is presented in Figure 7.18.

This section will not attempt to provide a detailed, step-by-step protocol for laboratory use. For that purpose, the reader is referred to monographs and reviews on the subject, including a detailed laboratory protocol with many hints and mention of problems to avoid.[104]

Hybridomas Derived from Species Other than Mice

Laboratory mice are the most common species immunized for hybridoma production, but for a variety of reasons, other animal species often have advantages. If an antigen of interest is nonpolymorphic in the mouse, the mouse component might be immunogenic in other species while mice would be tolerant to it. In the case of hybridomas for clinical use, mouse antibodies have the drawback of inducing antimouse Ig immune responses with possible deleterious effects.

Several approaches have been taken to the derivation of hybridomas in species other than mouse. First, interspecies hybridization can be performed using mouse myeloma fusion partners. The resulting hybrids are often unstable and throw off chromosomes, but clones can sometimes be selected that produce antibody in a stable fashion. Examples of this would be rat–mouse fusion to produce antibody to the mouse Fc receptor[105] and hamster–mouse fusion to produce

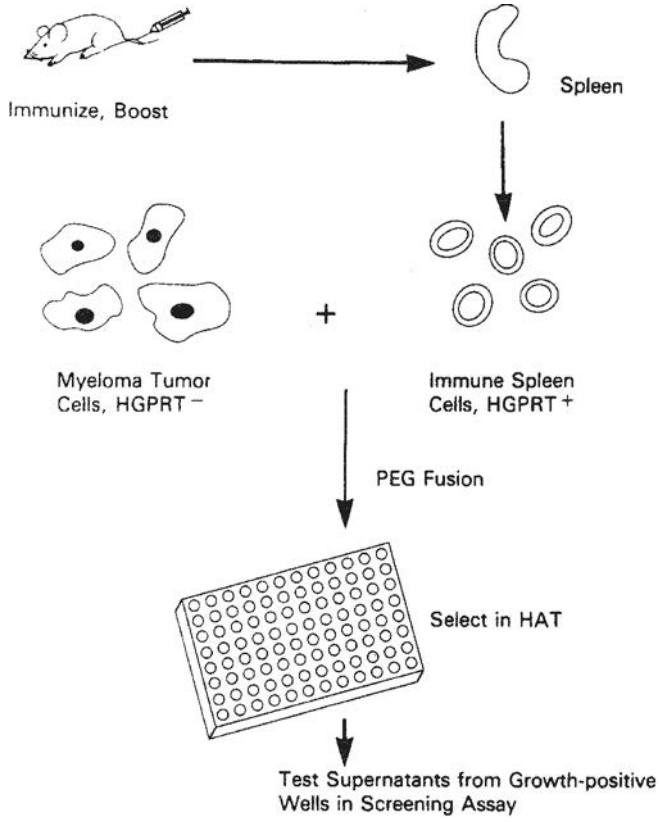

FIG. 7.18. Production of Hybridomas. Steps in the derivation of hybridomas can be outlined as shown. Spleen cells from immunized donors are fused with myeloma cells bearing a selection marker. The fused cells are then cultured in selective medium until visible colonies grow, and their supernatants are then screened for antibody production. HAT, hypoxanthine, aminopterin, thymidine; HGPRT, Hypoxanthine-guanine phosphoribosyltransferase; PEG, polyethylene glycol.

antibody to the mouse CD3 equivalent.[106] Rabbit–mouse hybridomas have also been described.[107]

A second approach is the use of fusion partner cells from the desired species. Myeloma variants carrying drug selection markers are available in a number of species. A rat myeloma line adapted for this purpose, IR983F, was described by Bazin.[108] Production of human hybridomas is of special importance because their use in therapies would avoid the problem of human immune responses to Ig derived from other animal species, as discussed in detail in the later section on applications.

Use of Gene Libraries to Derive Monoclonal Antibodies

Monoclonal antibodies produced by hybridoma technology are derived from B cells of immunized animals. A recent alternative technology uses gene libraries and expression systems instead. This approach has the advantages of avoiding labor-intensive immunizations of animals and the screening of antibody-containing supernatants. Another advantage of the approach is circumventing tolerance. One can derive monoclonal antibodies to antigens expressed in the animal species

that donated the gene library, including highly conserved antigens for which there may be no available responder that does not express the antigen.

The first version of such an approach involved preparation of V_H and Vκ libraries and expression of the libraries in bacteria. Further development of the system led to use of VH and VL libraries (see Chapters 5 & 6) made separately, and then preparation of a combinatorial library by cleaving, mixing, and religating the libraries at a restriction site.[109,110] A linker can be used so that V_H and V_L can both be expressed on one covalent polypeptide; the flexibility of the linker allows association of the V_H and V_L in a normal three-dimensional configuration and thus formation of an antigen-binding site.[110]

Another innovation involves expression of V_H and Vκ genes on the surface of bacteriophage as fusion proteins with a phage protein to permit rapid screening of large numbers of sequences.[110–112] Adsorption of antibody-bearing phage on antigen-coated surfaces allows positive selection of phage containing DNA encoding the desired variable region fragment (Fv) from combinatorial variable region gene libraries.[111,112]

Human antibody gene sequences can be recovered by polymerase chain reaction (PCR) from peripheral blood cells,[113] bone marrow,[114] or human cells reimmunized in severe combine immunodeficiency–hu mice.[115] The phage display technique can then be used to select antigen-binding clones and derive human reagents of desired specificity, such as antibody to hepatitis surface antigen[113] or HIV envelope.[114]

One limitation in the phage library technique initially was low affinity of the monoclonal antibodies derived because they were generated by a random process and not subject to further somatic mutation. Several approaches have now been used to improve affinities. Hypermutation and selection has now been achieved in vitro by a strategy using a bacterial mutator strain.[116] The process involves multiple rounds of mutation followed by growth in nonmutator bacteria and then selection for high-affinity binding led to an overall 100-fold increase in affinity.[116] Improved affinity has also been achieved by use of site-directed mutagenesis to alter residues in hypervariable regions affecting dissociation rates.[117]

Recombinant Monoclonal Antibodies Derived from Single B Cells After Cell Sorting

A new and very productive approach to making monoclonal antibodies is based on amplification of both the V_H and V_L chains of single B cells by reverse transcription–PCR.[118,119] In the first step, B cells are sorted by fluorescence-activated cell sorting, based on antigen specificity or other characteristics, and then distributed in 96 well plates at a dilution of one B cell per well. The cells are lysed, followed by reverse transcription of the messenger ribonucleic acid of a single cell and amplification of the V_H and V_L chains by nested PCR of each chain separately. The two chains are cloned into expression vectors in frame with the appropriate constant regions. Both chains are coexpressed to reconstitute the antibody. The resulting monoclonal antibodies are then analyzed for sequence, antigen specificity, affinity, and biologic activity. The efficiency of antibody production has been as high as 30% to 60% per well in different experiments.

Advantages include the ability to focus on a particular B-cell compartment, such as memory B cells, during the sorting step, the large numbers of monoclonals produced in a single experiment (typically more than 100), and the absence of antigen boosting prior to cloning. Due to the high efficiency of cloning, these monoclonals are thought to represent the expressed B-cell repertoire at a given time, location, and stage of differentiation. For example, human monoclonals had the same ratio of kappa to lambda light chains (60% to 40%) as found in circulating B cells, and they showed allelic exclusion in 95% of wells. Key features of the method include the choice of fluorescent antigen for B-cell sorting, called the "bait," and the choice of primers for nested PCR. The bait should capture as many antigen specific B cells as possible; different bait will capture different monoclonals from similar populations of B cells (compare Pietzsch et al.[120] and Scheid et al.[121]). A complex bait with multiple epitopes, such as HIV gp140, will capture antibodies to different parts of the antigen.[119,122,123] In order to amplify highly variable antibodies, the forward (and reverse) PCR primers should be chosen upstream (or downstream) of the V_H region so they will capture sequence variation near both ends of the coding sequence.

In one study, 1.5×10^5 memory B cells from a chronically HIV-infected subject were sorted based on binding to a stabilized form of gp120.[121] Individual antigen-specific B cells were distributed in microtiter wells, and they produced 576 monoclonal antibodies that bound gp120 by ELISA. Sequencing the V_H and V_L chains showed that each monoclonal was often clonally related to several others, which differed only by somatic mutation. Overall, these monoclonals represented 200 distinct B-cell clones. The monoclonals could be divided into groups, based on binding four distinct epitopes on gp120. Within the same clonally related group, some monoclonals had potent HIV-neutralizing activity while other closely related monoclonals were inactive. The B-cell donor was a long-term nonprogressor. The analysis of a significant sampling of the antibody repertoire may provide insight into how these patients control their circulating virus, and these findings may lead to improved vaccine antigens.

Applications of Monoclonal Antibodies

Because monoclonal antibodies can be made easily and reproducibly in large quantities, they allow many experiments that were not possible or practical previously. Affinity chromatography based on monoclonal antibodies can be used as a step in purification of molecular species that are difficult to purify chemically. Homogeneous antibody can be crystallized and can also be crystallized together with antigen to permit the study of the structure of antibody and of antigen–antibody complexes by x-ray diffraction. Homogeneous antibodies are also valuable in the study of antibody diversity. Such analyses have revealed much about the roles of somatic mutation, changes in affinity, and changes in clonal dominance in antibody responses.

Catalytic Antibodies

One area of recent interest is the use of antibody molecules to catalyze chemical reactions.[124] In this role, antibodies serve as an alternative to enzymes, an alternative that can be customized and manipulated more easily in some cases.

The concept of antibodies as catalysts had been proposed a long time ago by Woolley.[124] Use of homogeneous antibodies permitted identification of some with significant catalytic effects; MOPC167 accelerates the hydrolysis of nitrophenyl phosphorylcholine by 770-fold.[125] Polyclonal antibodies have also been reported to possess detectable enzymatic activity.[126] With the advent of hybridoma technology, purposeful selection of antibodies with potent enzymatic function became possible. Antibodies have been characterized that catalyze numerous chemical reactions, with rates nearing 10^8-fold above the spontaneous rate.[124] One common strategy for elicitation of such antibodies is immunization with transition state analogs,[127] although there are other strategies.[128] Antibodies function as catalysts in a stereospecific manner,[129] a valuable property.

Molecular mechanisms of antibody-mediated catalysis vary, as do enzymatic reactions.[128,130] To accelerate a reaction, an antibody has to lower the activation energy barrier to the reaction, which means lowering the energy of the transition state by stabilizing it. For this reason, an antibody that recognizes the transition state is favorable, and immunizations with analogs of the transition state have advantages.

Antibodies can serve as what has been termed an *entropy trap*[124]; binding to the antibody "freezes out" the rotational and translational degrees of freedom of the substrate and thus makes a chemical reaction far more favorable energetically. Interactions with chemical groups on the antibody can neutralize charges or bury hydrophobic groups, thereby stabilizing a constrained transition state.

Discovery of such catalytic antibodies opens practical opportunities: Antibodies can be customized for an application by appropriate selection and can be produced relatively cheaply and purified easily. Catalytic antibodies can be developed to perform chemical reactions for which no enzyme is available. They can shield intermediates from solvent, for example, allowing reactions that do not occur in aqueous solution.[131] They can form peptide bonds,[132] suggesting a new approach to polypeptide synthesis. Thus, catalytic antibodies will likely have many practical applications.

Bispecific and Bifunctional Antibodies

Antibodies produced naturally by a single B cell have only one binding site specificity, and their effector functions are determined by the structure of the Fc domain. The availability of monoclonal antibodies made possible the generation in quantity of artificial antibodies as cross-linking reagents by linking binding sites of two specificities to form bispecific antibodies. A variety of techniques have been used to prepare such hybrid or bispecific antibodies, and they have been put to a variety of uses. In addition, antibody-binding sites can be linked to other functional domains such as toxins, enzymes, or cytokines to create "bifunctional antibodies."[133]

One of the most powerful uses of hybrid antibodies is in redirecting cytolytic cells to targets of a defined specificity. In one early demonstration of this use,[134] a monoclonal antibody specific for the Fcγ receptor and one specific for the hapten dinitrophenyl were chemically cross-linked. In the presence of this hybrid antibody, FcγR-bearing cells were able to lyse haptenated target cells specifically. The FcγR played a critical role; antibody to MHC class I antigens on the cell could not be substituted. Antibody to the T-cell receptor complex has also been used extensively to redirect T-cell lysis to desired targets. For example, anti-CD3 was cross-linked to antitumor antibodies and mixed with effector cells. These "targeted T cells" were able to inhibit the growth of human tumor cells in vivo in nude mice.[135] Bispecific antibodies have also been used recently to alter the tropism of a viral gene therapy vector to target specific cells.[136]

Cumbersome cross-linking chemistry can now be replaced by genetic engineering for creation of designer antibodies.[133] Bifunctional and bispecific antibodies can be engineered as single chain variable fragment constructs or by specialized strategies using two chains. A wide variety of configurations are possible and can be used to make multivalent reagents as well as reagents with one site of each specificity. Tags can be built in by fusion of additional sequence such as streptavidin, or, as mentioned previously, antibody domains can be combined with other functional domains such as toxins, enzymes, or cytokines.

Clinical Applications

The possible clinical uses of monoclonal antibodies are many. In vitro, they are widely used in RIA and ELISA measurements of substances in biologic fluids, from hormones to toxins. They are also extremely valuable in flow cytometric assays of cell populations using antibodies specific for differentiation antigens expressed on cell surfaces. Monoclonal antibodies plus complement or toxin-conjugated monoclonal antibodies have also been used to remove T cells from bone marrow prior to transplantation.[137]

In vivo, although it took more than two decades for the original promise of hybridoma technology to be translated into widespread clinical applications, a number of monoclonal antibodies are now in use or in trials for a variety of purposes.[138–140] Monoclonal antibody OKT3 directed to a marker on human T lymphocytes is used as a treatment for rejection reactions in patients undergoing kidney transplant.[141] Other monoclonal antibodies, for example, ^{111}In-labeled CYT-103 referred to as Oncoscint (Cytogen Corp, Princeton, NJ),[142] are used as diagnostic tumor imaging reagents. Monoclonal antibodies have now been approved for various therapeutic uses.[140,143] Cancer therapies use either unconjugated monoclonal antibody[138,144–148] or toxin-coupled[149,150] or radiolabeled monoclonal antibody.[144,148,151] Molecules targeted in cancer therapies include CD25 (IL-2 receptor alpha chain) in adult T-cell leukemia,[138,140,148,152–154] CD20 in non-Hodgkin lymphoma using either unlabeled[147,155] or more recently radionuclide-labeled anti-CD20,[156,157] the HER-2/neu oncoprotein in breast and

ovarian cancer,[145,146,158,159] CD22 in hairy cell leukemia,[150] vascular endothelial growth factor to limit angiogenesis in diverse tumors (especially colorectal, renal, and non–small-cell lung cancer),[160–165] antiepidermal growth factor receptor in colorectal carcinoma and others,[166] anti-CD52 in chronic lymphocytic leukemia,[167] and anti-CD33 in acute myelogenous leukemia.[168] Other therapies studied include antilipopolysaccharide for treatment of sepsis, anti–IL-6 receptor for treatment of multiple myeloma and for rheumatoid arthritis, anti-IgE for treatment of allergy,[139] anti–tumor necrosis factor for treatment of arthritis,[169,170] anti–respiratory syncytial virus for prevention of respiratory syncytial virus morbidity and mortality in infants,[171,172] and anti–IL-2 receptor (CD25) for prevention of graft rejection[148,173] as well as for treatment of autoimmune diseases such as uveitis[174] and multiple sclerosis.[175] Other monoclonal antibodies have been developed to modulate immune responses. On the one hand, antibodies to block checkpoint inhibitors in the immune response, especially against cancer, have been developed, such as the first licensed one, ipilimumab, against cytotoxic T-lymphocyte antigen-4 (CD152), an inhibitory receptor expressed on activated T cells,[176–181] and anti-PD-1, another inhibitory receptor on activated T cells that is currently in trials.[182–186] An antibody to transforming growth factor–beta, a soluble inhibitor of immune responses, is also in clinical trials in both cancer and fibrosis.[187–190] On the other hand, monoclonal antibodies are being developed to amplify immune responses and synergize with vaccines, such as agonist anti-CD40 antibodies.[191–193]

In the specialized case of B-cell lymphoma, monoclonal anti-idiotypes against the idiotype expressed by the patient's tumor have been tested as a "magic bullet" therapy.[194] Active immunization of the patient with idiotype[195–199] has the advantage that escape mutants[200] are less likely to emerge because multiple idiotopes are recognized. Another approach under study is immunization using not idiotype as protein but plasmid DNA-encoding patient idiotype.[201] This approach would have additional advantages, such as ease of preparing customized reagents for each patient.

Production of Human or Humanized Monoclonal Antibodies

Many of the side effects of monoclonal antibodies in clinical use are due to the foreign Ig constant regions. Recognition of foreign Ig epitopes can lead to sensitization and so preclude subsequent use in the same individual of different monoclonal antibodies. Thus, monoclonal antibodies with some or all structure derived from human Ig have advantages. Several approaches have been taken employing fusion of human cells with animal myelomas or with human tumor cells of various kinds[202,203] and use of Epstein-Barr virus to immortalize antibody-producing cells.[204] Production of populations of sensitized human cells to be fused presents another special problem, as the donors cannot be immunized at will. In one example, in vitro stimulation of lymphocytes with antigen followed by fusion with mouse myeloma cells has been used to generate a series of antibodies to varicella zoster.[205]

Another approach to production of monoclonal antibodies with human characteristics involves application of genetic engineering. The part of the antibody structure recognized as foreign by humans can be minimized by combining human constant regions with mouse variable regions[206,207] or even just mouse hypervariable segments[208] by molecular genetic techniques. Antigen-binding specificity is retained in some cases, and the "humanized" chimeric molecules have many of the advantages of human hybridomas.

Production of fully human monoclonal antibodies in transgenic mice has now been achieved by multiple laboratories. The strategy has involved insertion into the mouse germ line of constructs containing clusters of human Ig V, D, J, and C genes (see Chapter 6) to generate one transgenic line and targeted disruption of the mouse heavy chain and κ chain loci to generate another transgenic line. From these two lines, mice are then bred that express only human antibodies.

To show feasibility of this approach, cosmids carrying parts of the human heavy chain locus were used to make transgenic mice.[209] The next step was to produce mice carrying human genes for both heavy and light chains to generate a functional human repertoire. Several groups using different technologies constructed heavy chain miniloci containing functional V segments representing several major V region families, D and J segments, constant and switch regions, and enhancers. The κ chain constructs were made that contained multiple functional Vκ segments, the J segments, Cκ, and enhancers.[210,211] Mice were bred that were homozygous both for the transgene loci and for disruption of the mouse heavy chain and κ light chain loci; note that the mouse λ locus was left intact. The human Ig genes could rearrange in the mouse genome, and expression of human Ig resulted. If these mice were immunized with a fragment of tetanus toxin, resulting antibodies included some that were fully human.[211] In one of the studies,[210] serum contained human μ, γ1, and κ as well as mouse λ and γ. Immunization of such mice with various antigens led to class switching, somatic mutation, and production of human antibodies of affinities of almost 10^8.

Ig expression in these mice demonstrates cross-species compatibility of the components involved in antibody gene rearrangement and diversification. The mice also provide a responder able to provide fully human antibodies to clinically important antigens, and they have the advantage that they are not tolerant to human antigens, such as the human IgE and human CD4 used by Lonberg et al.[210]

Nucleotide Aptamers: An Alternative to Monoclonal Antibodies

Antibodies are not the only biologic macromolecules that have evolved to permit an enormous range of specific structures. Oligonucleotides selected for ability to bind a ligand with high affinity and specificity are termed "aptamers" and can be used in many of the ways antibodies have been used. Selection, properties, and uses of aptamers have been reviewed.[212] Aptamers have the advantage that their

production does not require animals or cell culture. These well-defined reagents may be used increasingly in diagnostic testing and are also being tested in clinical trials for use as imaging agents or therapeutics.

Specificity and Cross-Reactivity

Specificity of Monoclonal Antibodies

Because all the molecules in a sample of monoclonal antibody have the same variable region structure, barring variants arising after cloning, they all have the same specificity. This uniformity has the advantage that batches of monoclonal antibody do not vary in specificity as polyclonal sera often do. The most obvious fact about cross-reactions of monoclonal antibodies is that they are characteristic of all molecules and cannot be removed by absorption without removing all activity. An exception would be an apparent cross-reaction due to a subset of denatured antibody molecules, which could be removed on the basis of that binding. The homogeneity of monoclonal antibodies allows refinement of specificity analysis that was not possible with polyclonal sera. A few examples follow.

First, one can use monoclonal antibodies to distinguish closely related ligands in cases where most antibodies in a polyclonal serum would cross-react, and so absorption of a serum would not leave sufficient activity to define additional specificities. This ability is useful in designing clinical assays for related hormones, for example. Such fine discrimination also allows the definition of new specificities on complex antigens. When large numbers of monoclonal antibodies specific for class I and class II MHC antigens were analyzed, some defined specificities that could not be defined with existing polyclonal antisera.[213–215]

On the other hand, monoclonal antibodies are also a powerful tool for demonstrating similarities rather than distinctions between two antigens. In some cases, only a minor portion of an antibody response detects a cross-reaction, and so it is not detected by polyclonal reagents. For example, determinants shared by the I-A and I-E class II MHC antigens in the mouse were demonstrated using monoclonal antibodies[215] while they had been suspected but were difficult to demonstrate using polyclonal sera.

Another type of fine specificity analysis possible only with monoclonal antibodies is the discrimination of spatial sites (epitope clusters) by competitive binding. In some cases, such epitope clusters correspond to specificities that are readily distinguished by other means. However, in other cases, the epitope clusters may not be distinguishable by any serologic or genetic means. An example is the splitting of the classical specificity Ia.7 into three epitope clusters by competitive binding with monoclonal antibodies.[215] The epitopes cannot be distinguished genetically, as all three are expressed on cells of all Ia.7-positive mouse strains. Thus, polyclonal sera cannot be absorbed to reveal the different specificities. Only with the use of monoclonal antibodies were the epitopes resolved from each other.

The importance of this type of analysis is shown by another example, the definition of epitope clusters on CD4, a surface molecule on a subset of human T cells that also functions as the receptor for HIV. Monoclonal antibodies to CD4 can be divided into several groups based on competitive inhibition.[216] The cluster containing the site recognized by OKT4A is closely related to virus infection, as antibodies to this site block syncytium formation. The cluster recognized by OKT4, however, is not related to infection because antibodies to it do not block syncytium formation,[216] and cells expressing variant forms of the CD4 molecule lacking the OKT4 epitope can still be infected by HIV.[217] This information about the sites on the molecule is important in understanding the molecular interactions of virus with its receptor and may be useful in designing vaccine candidates.

While most antibodies are not MHC-restricted in their recognition of antigens, distinguishing them from T-cell receptors, antibodies can be selected that recognize peptide–MHC complexes[218–220] (MHC-restricted antipeptide antibodies or peptide-dependent anti-MHC class I antibodies). Several monoclonal antibodies have been selected that require both a certain MHC class I antigen and a particular peptide for reactivity. Such monoclonal antibodies are useful reagents capable of detecting cells presenting the appropriate peptide-MHC complexes on their surfaces.[218] Such monoclonal antibodies may also be useful in dissection of T-cell responses. In one study, the monoclonal antibodies could inhibit IL-2 secretion by a T-cell hybridoma of corresponding specificity and could also block induction of cytotoxic T lymphocyte recognizing that epitope when given in vivo during priming.[220] Such monoclonal antibodies have been used to address structural questions about antigen recognition by T and B cells.[219] Such antibodies also appeared to skew the repertoire of T cells for this particular HIV peptide MHC complex to specific T-cell receptor Vβ types and T-cell avidities.[221] However, only very rare monoclonal antibodies have this type of specificity and they were purposely selected in the fusions, so they do not provide a general comparison of T-cell receptor and Ab characteristics.

Cross-Reactions of Monoclonal Antibodies

Monoclonal antibodies display many type 1 cross-reactions, emphasizing that antibody cross-reactions represent real similarities among the antigens, not just an effect of heterogeneity of serum antibodies. Even antigens that differ for most of their structure can share one determinant, and a monoclonal antibody recognizing this site would then give a 100% cross-reaction. An example is the reactivity of autoantibodies in lupus with both DNA and cardiolipin.[222]

It should be emphasized that sharing a "determinant" does not mean that the antigens contain identical chemical structures, but rather that they bear a chemical resemblance that may not be well understood, for example, a distribution of surface charges. Antibodies to the whole range of antigens can react with Igs in idiotype anti-idiotype reactions, showing a cross-reactivity of the same antibodies with proteins (the anti-idiotypes) and with the carbohydrates, nucleic acids, lipids, or haptens against which they were raised.

Polyclonal versus Monoclonal Antibodies

When monoclonal antibodies first became available, some people expected that they would be exquisitely specific and would be superior to polyclonal sera for essentially all purposes. Further thought about the issues discussed previously, however, suggests that this is not always the case and depends on the intended use of the antibodies. Not only do monoclonal antibodies cross-react, but when they do, the cross-reaction is not minor and cannot be removed by absorption. A large panel of monoclonal antibodies may be needed before one is identified with the precise range of reactivity desired for a study.

In polyclonal sera, on the other hand, each different antibody has a distinct range of reactivity, and the only common feature would be detectable reactivity with the antigen used for immunization or testing. Thus, the serum as a whole may show only a low-titered cross-reaction with any particular other antigen, and that cross-reaction can be removed by absorption, leaving substantial activity against the immunizing antigen. For the purposes of an experiment, a polyclonal serum may be "more specific" than any one of its clonal parts and may be more useful. This concept is the basis of the theory of "multispecificity" (see previous discussion).

Polyclonal sera also have advantages in certain technical situations such as immunoprecipitation in which multivalency is important. Many antigens are univalent with respect to monoclonal antibody binding but display multiple distinct sites that can be recognized by different components of polyclonal sera. Thus, a greater degree of cross-linking can be achieved.

The ultimate serologic reagent in many cases may well be a mixture of monoclonal antibodies that have been chosen according to their cross-reactions. The mixture would be better defined and more reproducible than a polyclonal antiserum and would have the same advantage of overlapping specificities.

CONCLUSION

In conclusion, antibodies, whether monoclonal or polyclonal, provide a unique type of reagent that can be made with high specificity for almost any desired organic or biochemical structure, often with extremely high affinity. These can be naturally divalent, for example in the case of IgG or multivalent, for example in the case of IgM or can be made as monovalent molecules such as Fab or recombinant Fv fragments. They serve not only as a major arm of host defense playing a major role in the protective efficacy of most existing antiviral and all antibacterial vaccines but also as very versatile tools for research and clinical use. RIAs and ELISAs have revolutionized the detection of minute quantities of biologic molecules, such as hormones and cytokines, and thus have become indispensable for clinical diagnosis and monitoring of patients as well as for basic and applied research. Current solid-phase versions of these take advantage not only of the intrinsic affinity and specificity of the antibodies but also of the implicit multivalency and local high concentration on a solid surface. Cross-reactivity of antibodies often provides the first clue to relationships between molecules that might not otherwise have been compared. Conversely, methods that use antigens to detect the presence of antibodies in serum have become widespread in testing for exposure to a variety of pathogens, such as HIV. Antibodies also provide specific reagents invaluable in the rapid purification of many other molecules by affinity chromatography. They have also become indispensable reagents for other branches of biology, for example, in histocompatibility typing and phenotyping of cells using a myriad of cell–surface markers that were themselves discovered with monoclonal antibodies and for separating these cells by fluorescence-activated cell sorting, panning, or chromatographic techniques. Monoclonal antibodies have also finally emerged as clinically important therapeutics in cancer, arthritis, organ graft rejection, and infectious diseases. Thus, antibodies are among the most versatile and widely used types of reagents today, and their use is constantly growing. Understanding the fundamental concepts in antigen–antibody interactions thus has become essential not only to an understanding of immunology but also to the effective use of these valuable molecules in many other fields.

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CHAPTER 8

B-Lymphocyte Development and Biology

Richard R. Hardy

INTRODUCTION

B lymphocytes constitute one of the major arms of the immune system, being responsible for humoral immunity. B cells in humans and mice are produced throughout life, primarily in the fetal liver before birth and in the bone marrow after birth. Their development from hematopoietic stem cells (HSCs) has been extensively characterized in mice, and the generation of numerous gene-targeted and transgenic lines in many cases has provided crucial information on the role of transcription factors, cellular receptors, and interactions that are critical in their generation. Recently, the role of microribonucleic acids (miRNAs) in regulating hematopoietic development has also emerged. The complexity of this process is now apparent, and B-cell progenitor differentiation into multiple peripheral subsets with distinctive functions is also widely appreciated. This chapter will focus on B-cell development and function in the mouse, touching more briefly on aspects of human B cells that are similar or distinctive, with a focus on immunodeficiency and B-cell neoplasias. It will conclude with a brief description of novel aspects of B-lymphocyte development in other species, highlighting differences from development in mouse and human.

B-CELL DEVELOPMENT IN MICE

In mice, B cells are produced from HSCs through a complex process of differentiation that has been uncovered over the past 30 years or so. One of the goals of classical hematology has been the delineation of differentiation pathways for different lineages of blood cells. There has been considerable progress in utilizing the ordered expression of a diverse set of cell surface and internal proteins, some with known functions, others whose roles are only suspected, to construct a description of the intermediate stages that cells transit as they develop into B lymphocytes. A simplified example of such a description is presented in Figure 8.1. Thus, HSCs with the capacity to generate all the cell types in blood generate progeny with a more restricted capacity, recognizable in this example by expression of the receptor for interleukin (IL)-7. These in turn produce yet more restricted progenitor cells identified by expression of cluster of differentiation (CD)45R/B220 (and, importantly, by absence of CD19).

This kind of pathway can be constructed by isolation and short-term culture of intermediate stages, allowing progression to occur, which helps to define the order. This framework for development serves as a starting point for analysis of the effects of transcription factors, microenvironmental interactions, cytokines, and natural or engineered mutations. It can also be extended by analysis of gene or protein expression at distinct intermediate stages. Critical processes, such as D-J rearrangement and immunoglobulin (Ig) heavy chain expression, can also be mapped onto this framework. Progress in this work facilitates experiments that address additional issues, such as identification of key regulatory interactions, developmental checkpoints, and the mechanism of B-lineage commitment.

The following sections will cover the sites of B-lineage development at different stages of ontogeny, then focus on what is known about their development in the bone marrow of adult mice, highlighting the function of the pre–B-cell receptor and the crucial role of Ig heavy and light chains in guiding development. Later sections will consider their differentiation into various specialized peripheral populations and emphasize insights into B-cell selection gained from various transgenic models of tolerance.

Early Development

Sites of B Lymphopoiesis during Ontogeny

In the mouse, hematopoiesis occurs predominantly in the fetal liver prior to birth, in the spleen just prior to and shortly after birth, and in the bone marrow thereafter. Prior to liver hematopoiesis, the blood islands of the yolk sac (YS) contain the first identifiable hematopoietic cells, nucleated erythrocytes with embryonic forms of hemoglobin.[1] However, these early YS precursors appear incapable of generating other blood cell lineages, and generation of all blood cell types, including lymphocytes,[2,3] initiates at around 9 to 10 dpc in an embryonic region referred to as the splanchnopleura/AGM (or simply Sp/AGM). Cells from this site are capable of long-term repopulation of lethally irradiated adult recipients with all blood lineages.[4,5] These cells colonize the fetal liver at about 11 dpc, initiating hematopoiesis there. Thus, there are two sites of very early hematopoietic precursors, with one in the YS largely limited to erythropoiesis and the other in the Sp/AGM capable of complete (referred to as "definitive") hematopoiesis. However, it may be that precursors in the YS have a broader lineage potential in the fetal microenvironment, as when they are injected directly into the newborn liver.[6]

HSCs capable of developing into all the blood cell types are produced in the Sp/AGM and migrate to the fetal liver at about d10. Thereafter, B-lineage cells develop largely in a wave, with earlier stages present at earlier times and later stage predominating at later times, close to (and shortly after) birth.[7,8] This progression with gestation day is easily visualized by staining with antibodies that delineate B-cell

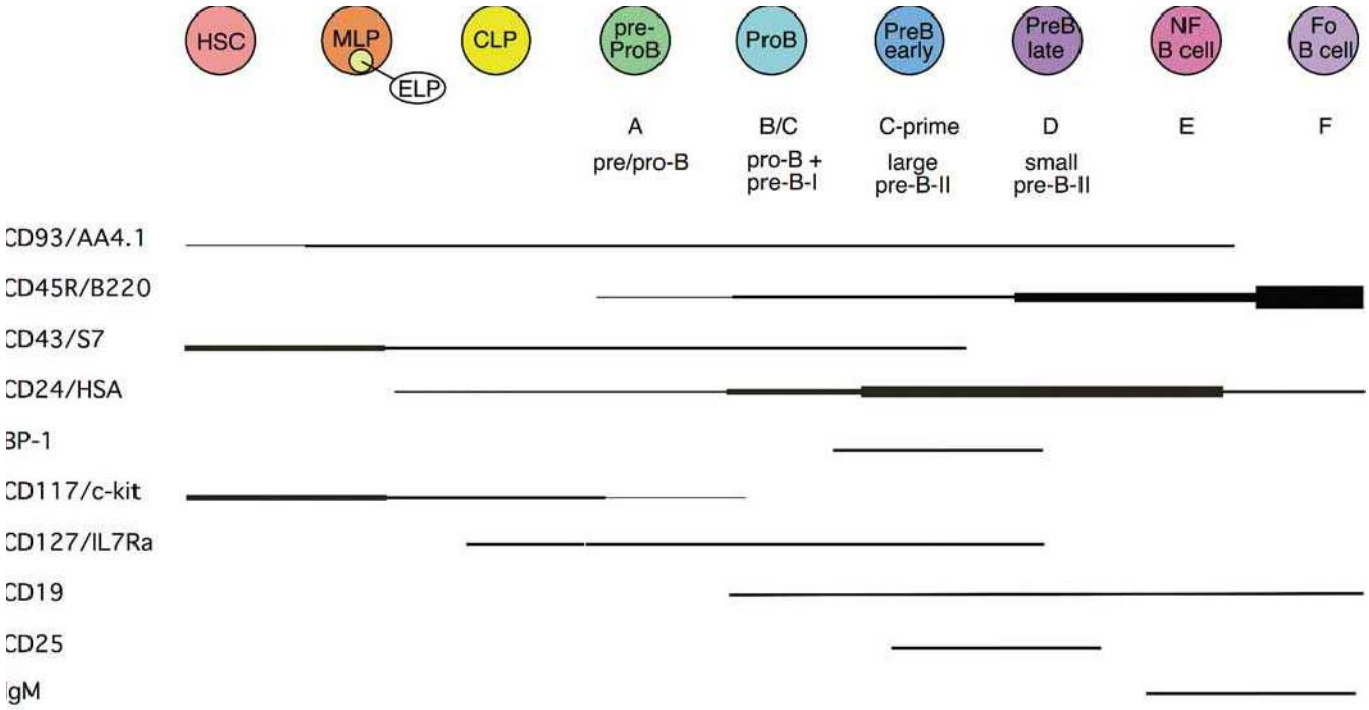

FIG. 8.1. Differentiation Diagram for Development of B Cells from Hematopoietic Stem Cells. Expression of the each surface protein is indicated by a *line*. Changes in level of expression is indicated by line thickness. CLP, common lymphoid progenitor; ELP, early lymphoid progenitor; Fo B, follicular B cell; HSC, hematopoietic stem cell; MLP, multilineage progenitor; NF B, newly formed B cell.

development, as shown in Figure 8.2. Early precursors can also be found in the fetal omentum.[9] In contrast with the bone marrow, cells at most differentiation stages in the fetal liver appear to be rapidly proliferating so that larger and larger numbers of B-lineage cells are detected at progressive days of gestation. Another distinction of fetal liver from bone marrow development in the adult is the absence of terminal deoxynucleotidyl transferase (TdT),[10,11] an enzyme that mediates nontemplated addition of nucleotides at the D-J and V-D junctions of Ig heavy chain.[12–14] Therefore, heavy chains produced during fetal development have little or no N-region addition, and CDR3 diversity is constrained even further by favoring of short stretches of homology at the V-D and D-J junctions.[15,16] Rearrangement of certain V or D elements may also differ between fetal and adult development, as for example, the reported high utilization of the DFL16.1 segment in fetal liver.[17] Differential expression of genes other than TdT also distinguish B-cell development during fetal life from that in the adult including precursor lymphocyte regulated myosin light chain like PLRLC transcripts[11,18] and major histocompatibility complex (MHC) class II.[19,20] Interestingly, although absence of the cytokine IL-7 completely eliminates bone marrow B-lineage development,[21] it nevertheless spares some fetal development,[22] suggesting a difference in growth requirements. The B-cell progeny of this early fetal wave may largely consist of B cells quite distinct from adult-derived cells, populating the B-1 subset.[23]

At birth, B-cell development can also be detected in spleen, but development at this site gradually decreases to very low levels by 2 to 4 weeks of age. Over this same period, B-cell development shifts to the bone marrow and thereafter it continues for the life of the animal. B lymphopoiesis decreases in aged mice. This may be due to diminished responsiveness of precursors to IL-7.[24,25]

Stem Cells, Commitment, and Early B-Cell Progenitors in Bone Marrow

B cells are continually generated from HSCs in the bone marrow of adult mice. Considerable effort has focused on evaluating the functional capacity of fractions of bone marrow cells to repopulate different lineages of blood cells; this work has progressed to the stage of defining a phenotype for such cells, with expression of c-KIT constituting an important marker in the so-called lineage negative subset.[26,27] This is the small fraction of bone marrow cells (<5%) that lacks expression of a panel of "differentiation markers," cell surface molecules that are expressed on later stages of various hematopoietic cell lineages. Careful analysis of this HSC fraction using additional markers has shown that it represent perhaps 1/30,000 of nucleated bone marrow cells with as few as 10 mediating multilineage repopulation in cell transfer assays.[28–30] An important capacity of "true" or "long-term repopulating" HSCs is their ability to give rise to cells in a recipient mouse that can also repopulate all the blood cell lineages upon retransfer into a second host, indicating a capacity for extensive self-renewal without differentiation into more restricted progenitors.

A major focus in research on hematopoiesis has been defining and characterizing lineage-restricted progenitors, such as the common myeloid and common lymphoid

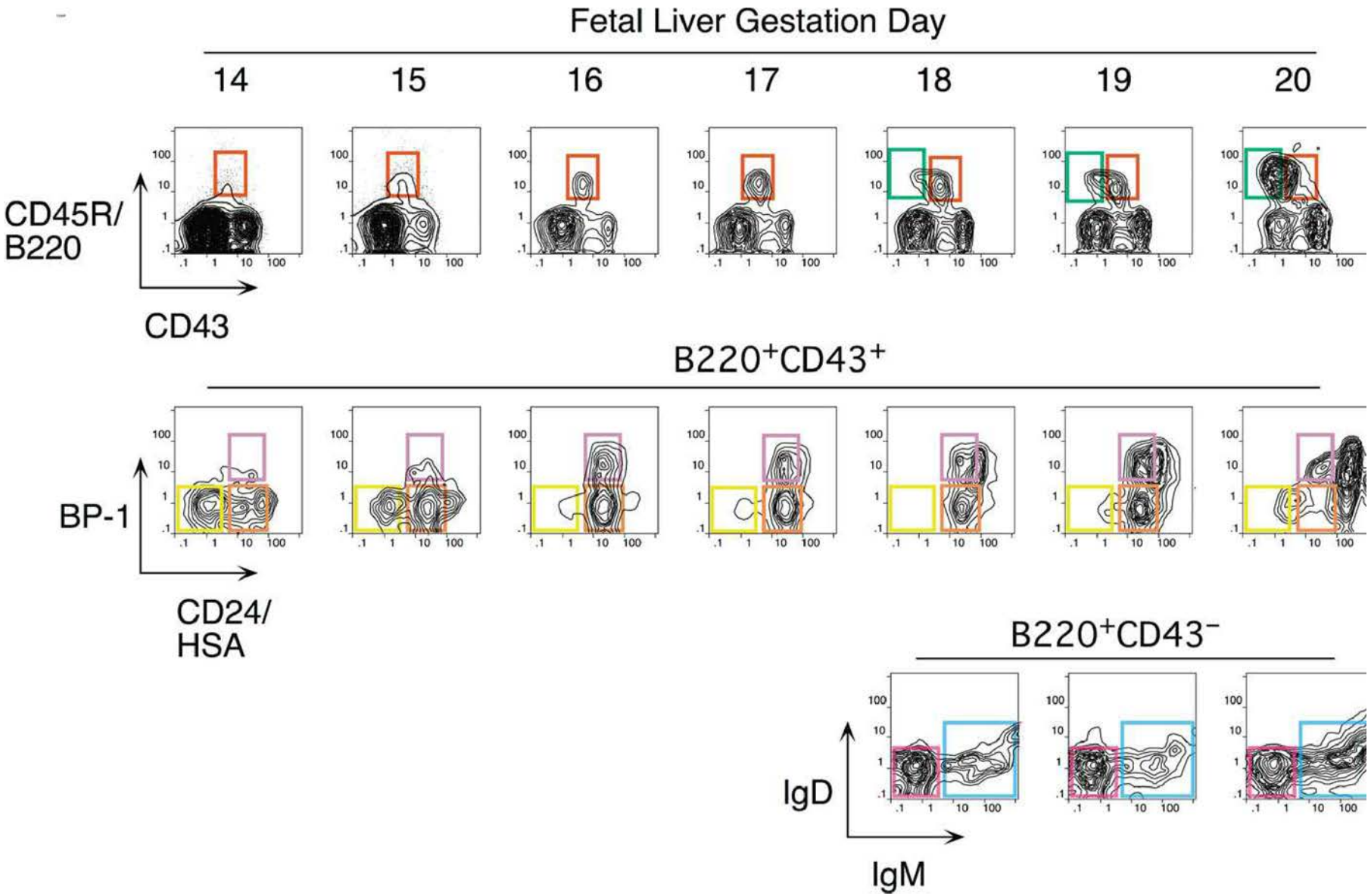

FIG. 8.2. Phenotypic Progression of Developing B-Lineage Cells in Mouse Fetal Liver Analyzed at Different Days of Gestation. Note that B220+cluster of differentiation (CD)43+ cells precede B220+CD43– cells and that within the B220+CD43+ fraction, heat stable antigen (HSA)– cells precede HSA+ cells, and BP-1– cells precede BP-1+ cells. Within the B220+CD43– fraction, the immunoglobulin M+ percentage increases until birth (at about day 20).

progenitors (CLPs).[31–33] The CLP cell fraction was identified by lack of a panel of "lineage markers," expression of the IL-7Rα chain, and distinctive, intermediate levels of c-KIT, compared to higher levels on HSCs. Initial characterization of these cells in various functional assays suggested that these cells could generate B, T, natural killer (NK), and a subset of dendritic cells but no other blood cell lineages. The reason for this restriction has been intensively studied, and downregulation of the receptor for granulocyte–myeloid colony stimulating factor has been suggested to be a key event in this process.[34] Cells with the phenotype of CLP constitute about 1/3000 of bone marrow cells. Prior to the CLP stage, multipotent progenitors exhibit low-level expression of genes characteristic of diverse cell lineages, leading to the idea that such promiscuous expression indicates chromatin accessibility that facilitates flexibility in cell fate decisions.[35]

CLP cells can give rise in short-term cultures to cells of the B lineage, naturally raising the issue of when cells become restricted to the B lineage. Most cells growing in stromal cultures give rise only to B cells upon transfer into mice, and the phenotype of these cells has been well characterized.[36] Most have at least some heavy chain rearrangement and bear the B-lineage marker CD19.[37,38] There is less certainty concerning the cells isolated directly from primary lymphoid tissues, as such cells are quite rare similar to CLPs and HSCs. Most of the CD45R/B220+ cells in bone marrow are also CD19+; such cells are committed to the production of B cells.[39] However, a subset of B220+ cells lacks detectable CD19 expression; cells within this fraction can generate CD19+ cells in short-term stromal culture with IL-7. Such cells are included in the CD43+CD24^{low} fraction (Fr. A, 1% of bone marrow) of B220+ cells in bone marrow, but this fraction also contains other cell types including NK-lineage precursors.[38,40] Thus, it is necessary to exclude cells lacking AA4.1 (about half[38]) and expressing Ly6c.[41] Many of these Ly6c+ cells also express CD4; recent work suggests that these are plasmacytoid dendritic cells.[42,43] A phenotypic approach for enriching and fractionating very early B-lineage subsets is shown in Figure 8.3A.

Careful analysis of the LIN− (including CD19) IL7Rα+cKIT+ CD45/B220− (CLP) and CD45/B220+ (Fr. A), as delineated in Figure 8.3, modified our understanding of the earliest stages of B-cell development in bone marrow.[44] First, while CLP stage cells fail to efficiently generate myeloid cells upon transfer into irradiated hosts, they nevertheless retain significant capacity to produce such cells in short-term cultures, likely due to continued (albeit reduced) expression of receptors for myeloid growth factors. In contrast, this myeloid capacity is greatly reduced as cells begin to express CD45R/B220 (ie, become "Fr. A"), concomitant with reduced expression of receptors for myeloid growth factor receptors. Yet these Fr. A cells, while poorly reconstituting T cells in cell transfer assays, nevertheless retain the capacity to generate T-lineage cells in culture, mediated by engagement of Notch by its ligand DL1.[45] Thus, the potential for alternate hematopoietic lineages appears to be lost somewhat later in progression down the B-lineage pathway in mouse bone marrow than previously thought. On the other hand, it appears that initiation of Ig rearrangement is initiated earlier than some studies have indicated. Determination of the extent of germline deoxyribonucleic acid (DNA) segments lost upon D-J rearrangement, and the formation of such D-J segments in individual cells isolated by electronic cell sorting showed that 30% to 50% of cells in CLPs and more than 80% of cells in Fr. A contained a D-J rearrangement on at least one chromosome.[44] This is consistent with high-level expression of genes important in Ig rearrangement, including TdT, Rag-1, and Rag-2, in CLP[46] and Fr. A stage cells.

An emerging view of CLPs (and possibly even the earlier multilineage progenitor [MLP]) stage cells considers them to be early B-lineage precursors rather than branch points in the production of other hematopoietic cell lineages. Thus, analysis of CD4/CD8/CD3 "triple-negative" cells in thymus failed to identify cells with a surface phenotype comparable to CLPs, and mutant mice lacking CLPs in bone marrow nevertheless have relatively intact thymic development, leading these authors to suggest a distinct "early T progenitor" different from CLPs.[47] Furthermore, cells with T/myeloid potential, but lacking B-lineage capacity, have been described.[48] It seems reasonable to hypothesize that MLP, CLP, and Fr. A stage cells occupy a distinctive microenvironmental niche in bone marrow where they receive signals that guide them along the early stages of B-lineage development culminating in CD19+ pro-B cells that are irreversibly committed to becoming B cells due to expression of PAX5[39] (see following section). Interestingly, cells considered to be progressing down a lymphoid/B-lineage path can be redirected to become dendritic cells by signals through toll-like receptors (TLRs), suggesting that infection can profoundly alter early stages in hematopoietic development.[49]

Additional issues remain regarding the lineage restriction of cells at these early stages in B-cell development. For example, there is evidence that cells restricted to generating B and myeloid/macrophage (but not T) lineage may exist in the fetal liver[50] and even in bone marrow.[51] There is also apparently a different dependence of fetal liver B lymphopoiesis on expression of the transcription factor BSAP compared to bone marrow, as determined by analysis of PAX5 null mice.[52] Further comparison of B-cell development in fetal liver with that in bone marrow is needed to clarify this point. Finally, the precise delineation and characterization of B-cell precursors earlier than CLPs, prior to IL-7 expression, remains imprecise. It seems likely that at least some of the MLP stage cells mentioned previously are initiating a B-lineage program based on their expression of E2A, Rag-1, Rag-2, and TdT.[44] However, potential heterogeneity in this fraction needs to be assessed. Determination of Rag-1 transcriptional activity at the single cell level by a green fluorescent protein reporter, used for identification of the early lymphoid progenitor fraction,[53] may provide a key approach for such studies.

Transcription Factors Regulating B-Lineage Development

The GATA-2 and Runx1/AML1 transcription factors are required for the development of HSCs that are the precursors of all the blood cell lineages, including B cells[54–58] (Fig. 8.4). Experiments with the core-binding factor–associated leukemia fusion protein CBFbeta-SMMHC, whose expression

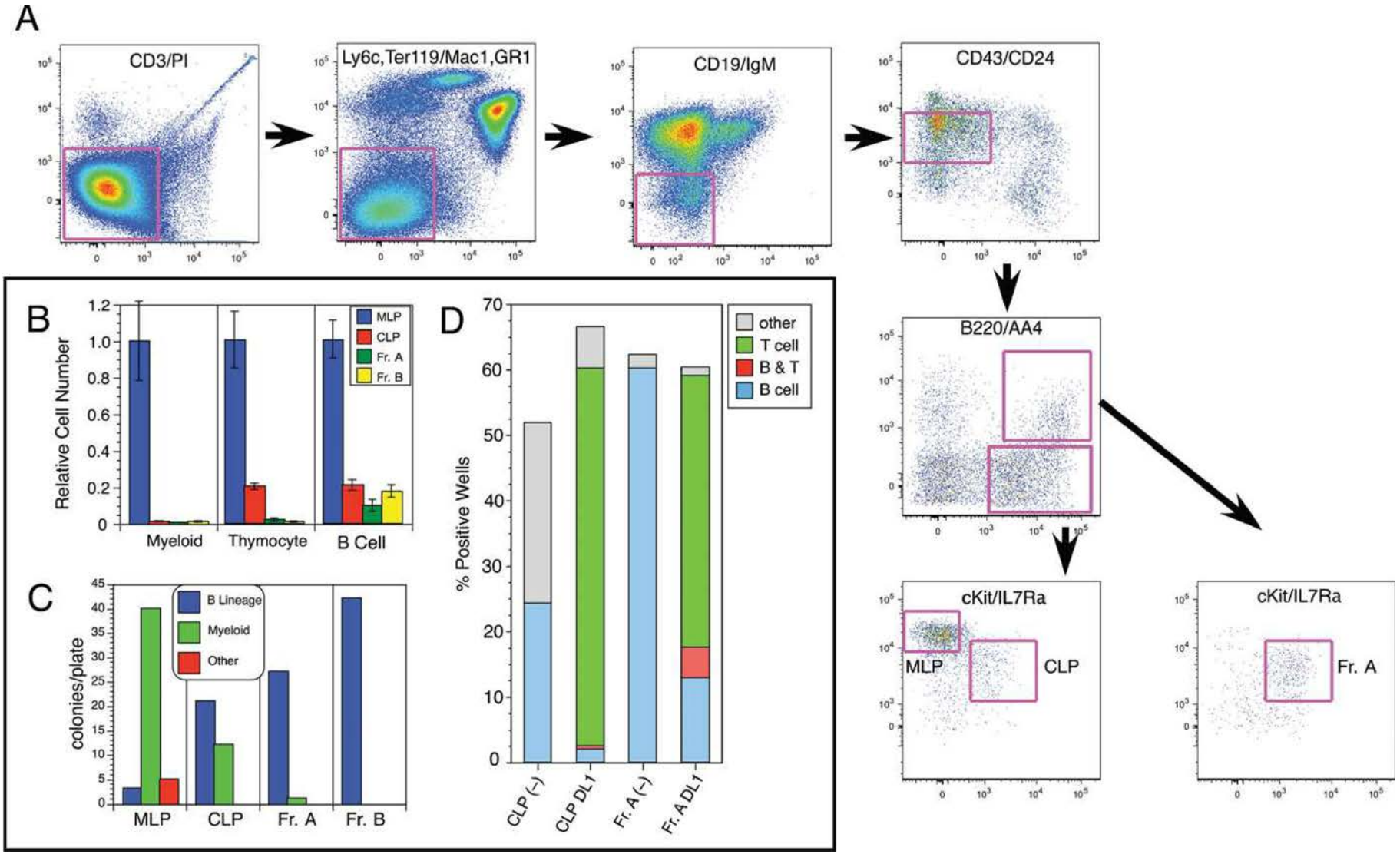

FIG. 8.3. A: An approach for purifying the earliest stage of B-lineage cells in mouse bone marrow. Bone marrow cells expressing cell surface proteins characteristic of differentiated stages of T, myeloid, erythroid, and B lineages are depleted sequentially by electronic gating in the first three panels. Cells with low-level expression of cluster of differentiation (CD)24/heat stable antigen (HSA) and intermediate levels of CD43(S7) are selected in the fourth and the distribution of CD45R/B220 versus CD93/AA4 is shown in the fifth. AA4+B220– cells contain multilineage progenitors (MLPs) and common lymphoid progenitors (CLPs), resolved by analysis for c-Kit versus interleukin (IL)-7Rα in panel six. AA4+B220+ cells, shown in the final panel, are enriched for c-Kit+IL-7Rα+ cells, termed Fr. A. CLP stage cells resemble Fr. A, but lack detectable expression of CD45R/B220. In contrast with CLP and Fr. A, MLP stage cells have higher levels of c-Kit and lack IL-7Rα expression. **B:** Functional analysis of early B-lineage cells by in vivo competition assay, showing absence of myeloid or T-lineage generation, but production of B-lineage cells from Fr. A. In contrast, CLP stage cells generate B and T cells, whereas MLPs repopulate B, T, and myeloid lineage cells. MLP, CLP, and Fr. A as identified in **A**. Fr. B stage cells are DJ/DJ rearranged pro-B cells, identified as CD19+CD43+CD24(HSA)+. **C:** Functional analysis of early B-lineage cells by in vitro S17 stromal cell assay, showing predominant B-lineage colony formation from Fr. A, but some myeloid generation from CLP stage cells. **D:** Functional analysis of early B-lineage cells by in vitro DL1-OP9 stromal cell culture, revealing significant T-lineage potential in Fr. A stage cells.

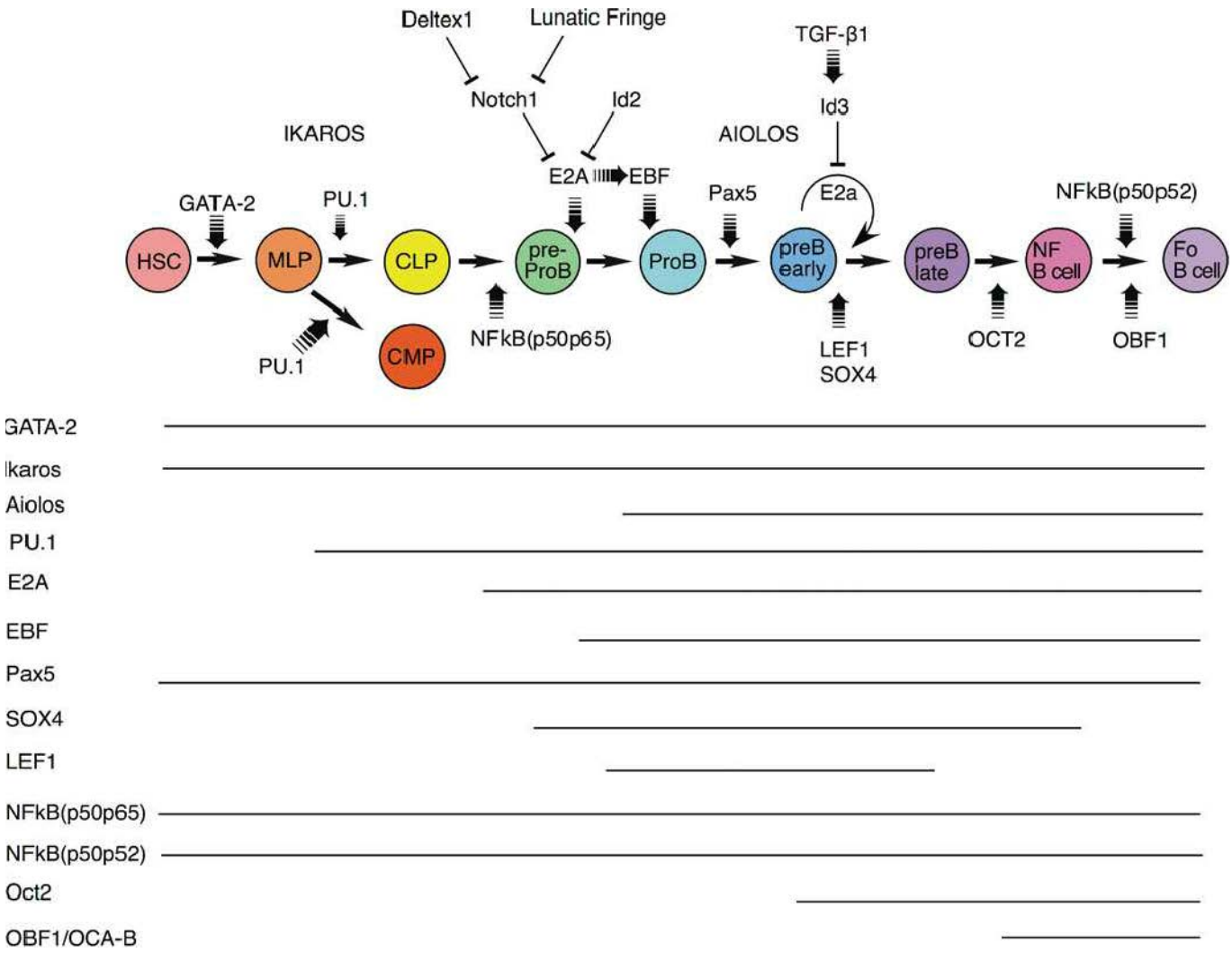

FIG. 8.4. Transcription Factors Important at Different Stages in B-Cell Development in Mouse Bone Marrow. Some regulatory networks are also shown. Positive/activating activity is indicated by *arrows*, whereas negative or blocking activity is indicated by *bars*. The rapidly cycling stage, early pre-B cell, is also indicated. Predominant stages of expression are indicated below the diagram.

inhibits RUNX function, have revealed that its expression negatively impacts pre–pro–B-cell through pre–B-cell populations in bone marrow.[59] Although the frequency of CLP stage cells was unaffected, the expression of B-lineage associated genes (such as CD79a and λ5) was decreased, demonstrating the key importance of early RUNX activity.

Somewhat later acting, but still very early in development, is the Ikaros transcription factor.[60–62] Ikaros and the related transcription factor Aiolos[63] play important roles in lymphocyte development. Ikaros is expressed early in hematopoietic precursors. Ikaros null mice lack B-lineage cells[62]; a different Ikaros mutant that acts as a dominant negative completely blocks lymphoid development.[60] Ikaros activates numerous early B-lineage genes, including TdT, Rag-1, λ5, and VpreB. Expression of EBF1 in Ikaros−/− hematopoietic progenitor cells restored generation of CD19+ cells, but these cells were not committed to the B-cell fate and failed to rearrange IgH genes.[64] Thus, Ikaros acts in a transcription factor pathway, inducing EBF1 expression, and acting in concert with PAX5 to maintain B-lineage commitment, but also altering chromatin compaction around the IgH locus, which together with Rag expression results in heavy chain rearrangement. Aiolos is detected somewhat later in development at about the stage of B-lineage commitment, and its expression increases further at later stages. It is induced by pre–B-cell receptor (BCR) signaling (see section on the role of Ig heavy chain and the pre-BCR), and it acts to downregulate transcription of λ5 (initially induced by action of Ikaros), a part of the pre-BCR complex. In this way, Ikaros family members play key roles both initiating and terminating pre-BCR signaling, a critical checkpoint in B-cell development.

The PU.1, an Ets family transcription factor, is critical for progression to the earliest stage of lymphoid development, as demonstrated by the inability of PU.1 null precursors to generate lymphocytes.[65,66] An important target of PU.1 for B-lineage development is the gene for Igβ, known as MB-1. The level of PU.1 appears to be critical for development

along the B lineage, as, while low-level expression induced in PU.1 null mice allowed B-lineage development, high-level expression blocked this and fostered myeloid lineage development,[67] likely due to differential induction of the IL-7Rα and macrophage colony-stimulating factor receptor chains.[68] In fact, retroviral mediated expression of the IL-7Rα chain complements defective B lymphopoiesis in PU.1 null bone marrow HPCs.[69] Surprisingly, recent work from several groups indicates that some B-cell development can occur in the absence of PU.1 expression.[70] Furthermore, analysis of conditional PU.1 knockout mice showed that expression of this transcription factor was not required after the pre–B-cell stage.[71]

E2A codes for two proteins, E12 and E47, members of the basic helix-loop-helix family of transcription factors; its induction is crucial from the earliest stages of B-lineage development, as all stages after CD19 expression are absent from E2A null mice.[72,73] These mice lack detectable D-J rearrangements, and, interestingly, such rearrangements can be induced in nonlymphoid cells by introduction of the Rag genes and ectopic expression of E2A,[74] implicating this transcription factor in the process of chromatin remodeling of the Ig heavy chain locus that permits accessibility by the recombinase machinery.[75] The regulation of E2A is crucial for B-lineage development, as negative regulators such as Notch1 and ID2 have been show to block this lineage and induce alternate cell fates, the T and NK lineages.[76–79] Consistent with this picture, ectopic expression of genes that negatively regulate Notch1, lunatic fringe, and Deltex-1 induce the B-cell fate.[80–82]

Expression of the early B-cell factor, EBF1, a member of the O/E protein transcription factor family, is requisite for progression of early B-lineage progenitors to the D-J rearranged pro-B stage (Fr. B), as shown in EBF1 null mice.[83] The expression of EBF1 is induced by action of the epigenetic histone H2A deubiquitinse MYSM1, as revealed by targeting this gene.[84] EBF1 and E2A act at a similar stage in early in B-lineage development; these two transcription factors can act together to upregulate a family of early B-lineage–specific genes, including Ig-α/β, VpreB/λ5, and Rag-1/2.[85,86] There is evidence that E2A upregulates expression of EBF1, found by transfection of E2A in a macrophage cell line,[87] suggesting an ordering of these two in development. Furthermore, recent studies showed that there are two distinct promoters for EBF1 that are regulated differently.[88] A distal promoter is activated by IL-7 signaling, E2A and EBF1, whereas a proximal promoter is regulated by PAX5, Ets1, and PU.1. Such complex regulation indicates that B-cell development occurs through the action of several feedback loops in a regulatory network that is becoming understood.[89,90]

BSAP, the product of the PAX5 gene, is expressed throughout B-cell development until the plasma cell stage.[91] PAX5/BSAP transcriptional targets include CD19 and BLNK; expression of this transcription factor acts to upregulate V to D-J heavy chain rearrangement.[52] Analysis of chromatin structure around the Ig heavy chain locus revealed that PAX5 induces V to D-J locus contraction, thereby promoting rearrangement.[92] PAX5 null mutant mice show an arrest in bone marrow development at the pro-B stage, likely due to the lack of complete heavy chain rearrangements and also due to the absence of the critical B-cell adaptor protein BLNK that serves to link the pre-BCR to the intracellular signaling pathway via the tyrosine kinase Syk.[93] BSAP/PAX5 also acts to repress alternate cell fates, as pro-B phenotype cells isolated from PAX5 null bone marrow can generate diverse hematopoietic cell lineages, in contrast with such cells from wild-type mice that are B-lineage restricted.[39,94] This occurs by repression of the myeloid growth factor receptor gene c-fms[95] and by repression of the Notch1 signaling pathway[96] critical for T-cell fate specification.[45,97] Conditional targeting of PAX5 in more mature B-cell stages shows that its continued expression is necessary for maintenance and function of mature B cells.[98] Finally, as mentioned previously, in contrast with bone marrow, the absence of BSAP/PAX5 arrests B-cell development prior to the B220+ stage in fetal liver, suggesting a crucial difference in the early dependence on this transcription factor.[52]

The Forkhead family transcription factor FoxO1 plays important roles at several stages of B-cell development.[99] Early in development, it acts to induce expression of the receptor for IL-7 at the CLP stage. It also functions to regulate Rag-1 and Rag-2 expression during heavy and light chain rearranging stages of development.[100] Finally, in mature B cells, it is needed for normal expression of L-selectin, a homing receptor important for normal recirculation of peripheral B cells through the lymphatics. GA binding protein, a ubiquitously expressed Ets family transcription factor, is another player regulating expression of the IL-7R.[101] Importantly, through interaction with PAX5, it acts in concert to induce expression of critical PAX5 target genes such as CD79a. There is recent evidence that FoxO1 regulates Ikaros activity by altering splicing of its messenger ribonucleic acid (mRNA), rather than altering Ikaros transcription.[102] FoxO1 activation of Ikaros was sufficient for induction of rearrangement of proximal VH genes, but expression of PAX5 was required for rearrangement of distal VH genes. Thus, FoxO1, Ikaros, and PAX5 appear to function in a network to coordinate the ordered rearrangement of Ig genes during B-cell development.

Lymphoid enhancer binding factor (LEF-1) shows a pattern of expression restricted to the pro-B and pre-B stages of B-cell development.[103] Targeted inactivation of the LEF-1 gene allows B-cell development but with reduced numbers.[104] This is because LEF-1 regulates transcription of the Wnt/β-catenin signaling pathway whose activation increases proliferation and decreases apoptosis of early B-lineage cells. In fact, exposure of normal pro-B cells to Wnt protein induces their proliferation.[104] Interestingly, there is a counterproliferative signal that can act at the pre-B proliferative stage, mediated by transforming growth factor-β1.[105] It appears that this occurs due to induction of the ID3 inhibitor that negatively regulates the activity of E2A.[106] Another transcription factor whose expression is similar to LEF-1 is SOX-4; its inactivation also results in the inability of normal early B-lineage cell expansion and a block at the pro-B stage.[107]

Several forms of NF-κb subunits are expressed throughout B-cell development; this transcription factor can regulate kappa light chain expression and also growth factor signaling.[108] Mice lacking the p65 subunit die before birth, so development must be analyzed by transfer of fetal liver precursors into wild-type recipients. Such experiments showed diminished B-lineage cell numbers, but the major defect was in mature B-cell mitogenic responses.[109] Mice lacking the p50 subunit showed relatively normal B-cell development but again poor response to mitogen by mature B cells.[109] However, mice lacking both the p50 and p65 subunits failed to generate any B220+ B-lineage cells. Curiously, when mixed with wild-type fetal liver cells, normal numbers of mature B cells could be generated from the double-defective precursors, suggesting that the defect could be overcome by secreted or membrane-bound signals provided by the wild-type precursors. Another double mutant, p50p52, showed a late stage defect in B-cell development, with a failure to generate mature B cells in spleen.[110]

Inactivation of the Oct-2 transcription factor results in neonatal lethality, but transfers of fetal precursors can reconstitute lymphoid cells in wild-type recipients, allowing assessment of effects on the B lineage. Such studies have shown that fewer mature follicular B cells are generated in these mice and B-1 (CD5+) B cells are completely eliminated.[111–113] Similarly, the Oct binding factor, OBF-1, also known as OCA-B and BOB-1, appears to function in the maturation of newly formed B cells in the bone marrow to become follicular B cells in the periphery, as inactivation of this gene resulted in a significant deficit in mature B cells.[114–116] Both of these transcription factors have been shown to regulate the follicular B-cell chemokine receptor CXCR5, which may explain at least part of the defect.[117] Curiously, unlike Oct-2 null mice, there was reportedly no deficit in B-1 B cells in OBF-1 null mice. Interestingly, when the OBF-1 mutant mouse is crossed with btk-deficient mice, there is a complete lack of peripheral B-cell generation,[118] suggesting that this transcription factor may function in the BCR-mediated selection of mature B cells.

Impact of Microribonucleic Acids on B-Cell Development

The miRNAs are small noncoding ribonucleic acids that facilitate the degradation of mRNAs and thereby act at a posttranscriptional level to regulate gene expression. The generation of mature functional miRNAs requires action of Dicer, a protein that cleaves pre-miRNAs, so the targeting of Dicer allows assessment of the global effect of miRNA on B-cell development. Ablation of Dicer in early B-lineage progenitors results in a block at the pro- to pre-B cell stage, likely due to upregulation of the proapoptotic molecule Bim.[119] Counteraction of Bim function by a Bcl-2 transgene reveal further dysregulation of normal development, including nontemplated nucleotide addition (N-regions) at the Ig light chain V-J junction, due to aberrant expression of the terminal deoxynucleotidyl transferase gene that is normally extinguished at the pre–B-cell stage.

Another approach for assessing the importance of specific miRNAs is direct overexpression or knockdown of expression; such a study with miR-150 reveal its role in regulating c-Myb, a transcription factor that regulates pro-B to pre-B progression and also the survival of mature B cells.[120] Transgenic overexpression of miR-17-92, a miRNA often found to be amplified in lymphoma, resulted in a lymphoproliferative syndrome and autoimmune disease, resulting in premature death.[121] One target of miR-17-92 is Bim; its decrease in the transgenic animals may have resulted in excessive cell survival and a loss of normal tolerance to self-antigens. The potential relevance to normal growth regulation is quite interesting, considering the amplification of this miRNA in some lymphomas. Another miRNA with a cancer association, miR-21, has been studied as a transgene in mice, where it results in tumors with a pre-B malignant lymphoid-like phenotype.[122] The miRNAs that are amplified in cancers and likely contribute to the neoplastic process are now termed "oncomirs."

miRNAs can also influence lineage choice or act at key checkpoints during hematopoietic development. Retroviral provision of miR-34a in bone marrow hematopoietic progenitors blocked B-cell development at the pro-B to pre-B checkpoint, resulting in reduced numbers of mature B cells in mice repopulated with such precursors. A possible explanation for the developmental block is action of miR-34a on Foxp1, a transcription factor that appears to be important at this stage, as cotransfection of FoxO1 lacking its normal 3′ UTR target of miR-34a restored B-cell development. A novel regulator of lineage choice appears to be Let-7, a family of miRNAs regulated by the highly conserved ribonucleic acid–binding protein Lin28.[123] While Lin28 has been studied for its role in pluripotency, developmental timing, and oncogenesis, a recent study indicates that it may regulate a developmental switch in both B and T cells, such that its expression in adult hematopoietic progenitors results in reprograming development toward a more "innate-like" pattern, normally only seen during fetal/neonatal timing.[124]

Bone Marrow Developmental Stages

Functional Definition

Distinct stages of developing B-lineage cells can be delineated based on their capacity for growth under different culture conditions. That is, the earliest precursors require cell contact with the stromal microenvironment in addition to specific cytokines, notably IL-7.[105,125] This stromal cell/precursor adhesive interaction is mediated, at least in part, by binding of VLA-4 to intercellular adhesion molecule-1.[126,127] Later stage cells do not require cell contact but maintain a need for cytokines.[128,129] Both cell types can undergo considerable cell proliferation in culture. Interestingly, the difference between cell contact requirement and independence is linked to the expression of heavy chain protein.[128,130] A population of cytoplasmic heavy chain–expressing B-lineage cells later than either of these, the so-called late or small pre-B cells, does not proliferate in culture. These cells likely require different culture conditions for survival, as they usually do not persist for extended periods, but rather die with a half-life of less than 24 hours unless protected from apoptosis by a Bcl-2 transgene.[131]

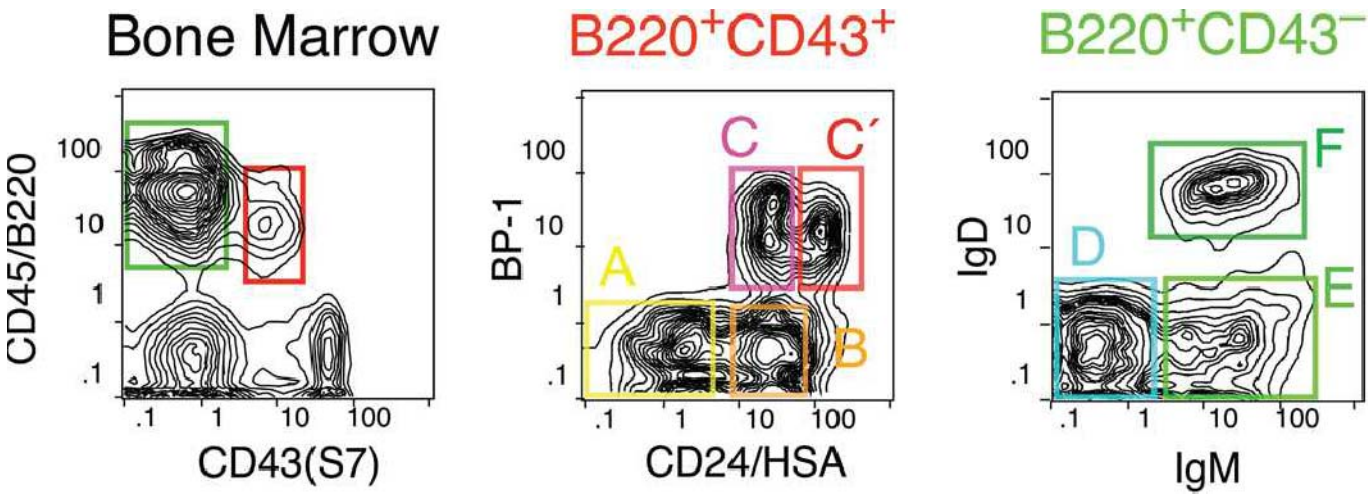

FIG. 8.5. Flow Cytometry Approach for Analyzing Different Stages of Cluster of Differentiation (CD)19+ B-Cell Development in Mouse Bone Marrow. Note that the antibody used for CD24/heat stable antigen (HSA) staining, 30F1, is important, as other monoclonal antibodies that recognize HSA do not resolve high from low-level expression as well. The cells expressing low levels of CD24/HSA, labeled "A", are CD19– and is enriched for very early B-lineage precursors but also is contaminated by other cell types that can be detected by staining for AA4, NK1.1, DX5, and Ly6c; early B-lineage precursors are AA4+NK1.1–DX5–Ly6c–.

Phenotypic Definition

Further clarification of the heterogeneity in bone marrow can be achieved by analysis using fluorescent staining reagents and either microscopic or flow cytometric analysis. For example, the earliest determination that there were both heavy chain surface-positive B cell and cytoplasmic-positive pre-B cells was through microscopic examination using anti-Ig staining.[132,133] Later studies in mouse showed that there were specific surface proteins or "markers" that could be useful in identifying these populations, notably a restricted isoform (CD45Ra) of the common leukocyte antigen, CD45.[134] This largely B-lineage–restricted 200 kDa molecular mass isoform is often referred to as "B220." Some highly B-lineage–restricted monoclonal antibodies, such as RA3-6B2, recognize a specific glycosylation of the CD45Ra isoform.[135] However, as described previously, even highly specific antibodies such as 6B2 may also recognize other cell types, such as particular differentiation stages or subsets of NK or dendritic cells.

The application of multiparameter/multicolor flow cytometry and additional monoclonal antibodies specific for other cell surface proteins differentially expressed during B-lineage development has facilitated delineation of multiple additional intermediate stages in this pathway.[129] For example, the B220+ population in bone marrow can be further fractionated into an earlier subset expressing CD43 (about 3% to 5% of marrow cells) and a later fraction with much lower CD43 expression (20% to 30% of marrow). The precursor/progeny relationship of cells in these two fractions can be readily demonstrated by short-term culture, with CD43+ cells giving rise to CD43– cells. These two populations can be further subfractionated based on additional developmentally regulated surface proteins, such as CD24/heat stable antigen (HSA), BP-1 (a zinc-dependent cell surface metallopeptidase also known as aminopeptidase A[136]), and the surface Ig molecules IgM and IgD.[129] This is shown in Figure 8.5. Again, these cell populations can be isolated and short-term culture used to determine their order in the pathway. Alternative approaches based on other developmental markers can be correlated with this framework of cell stages, notably the system developed by Melchers' group[137] using expression of CD45R/B220, CD19, c-KIT, and the IL-2Rα chain. A diagram summarizing this type of phenotypic subdivision and relating different nomenclatures is shown in Figure 8.6.

Culture Systems and Critical Microenvironmental Interactions

The combination of phenotypic characterization coupled with analysis of growth and differentiation in culture has provided a powerful approach for the further understanding of B-cell development, as employed by many different investigators. Bone marrow cultures developed by Whitlock and Witte,[138–140] and fetal liver cultures developed by Melchers' group[141,142] have allowed determination of the critical cytokines and some of the cell adhesion molecules important in the in vivo development of these cells. Many of these are summarized in Table 8.1. A typical B-lineage colony proliferating on S17 stromal cells in the presence of IL-7 is shown in Figure 8.7.

Survival and growth of the earliest stages of developing B-lineage cells require cell contact with nonlymphoid adherent cells that can be isolated from bone marrow, cells referred to generically as "stromal cells." A number of lines have been derived from primary cultures of bone marrow adherent cells and characterized in terms of their capacity to support B lymphopoiesis in vitro.[143] This work has led to the discovery of adhesion molecules that play important roles in mediating the organization of clusters of developing B-lineage cells on stromal layers, including CD44 interacting with hyaluronate and VLA-4 interacting with vascular cell adhesion molecule 1.[126,127,144,145] Both of these interactions could be disrupted by addition of blocking antibodies

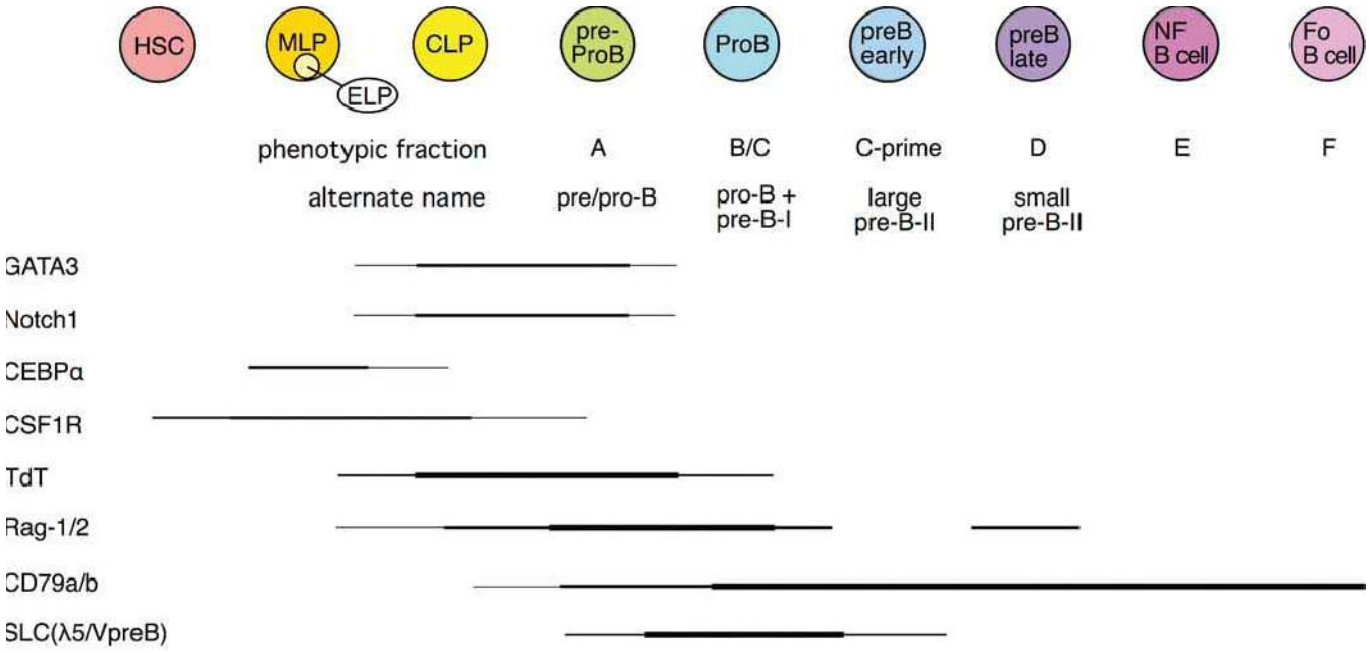

FIG. 8.6. Diagram of Distinct Phenotypic Stages and Characterization of Terminal Deoxynucleotidyl Transferase, Biphasic Rag Expression, Immunoglobulin-α/β, and Surrogate Light Chain Expression. Genes characteristic of myeloid and T-cell lineage are also shown. The cell type descriptions are cross-referenced to the alphabetic phenotypic fraction nomenclature and also to the Basel nomenclature. The early lymphoid progenitor population is identified by activation of the Rag-1 locus in a green fluorescent protein reporter mouse.

to CD44 and VLA-4 on B-cell precursors, resulting in a disruption of normal pre-B proliferation in vitro.[146] Such adhesion interactions may serve to transmit signals directly to the stromal cells or B precursors, or both. There is some evidence that stromal cells are induced to elaborate specific growth mediators after interaction with B-cell precursors or soluble regulators.[147]

Another function of the stromal cells is to produce growth factors critical to B-lineage survival, proliferation, and differentiation; the most important of these for mouse B-cell development is IL-7.[105,125,148,149] IL-7 promotes the survival and proliferation of pro-B and pre-B stage cells, both in vivo and in vitro.[150,151] Neutralizing antibody to IL-7 can block B-cell development in vitro,[129] and IL-7 expressed as a transgene can deregulate normal B-cell development, leading to B-cell lymphadenopathy.[152] The IL-7 receptor consists of a unique IL-7Rα chain[153] paired with the common gamma chain (γc) that is also found in the receptors for

TABLE 8.1 Regulators of Growth of Early B-Lineage Cells

Mediator	Effect	Reference
L-7	Stimulates CLP and B-precursor proliferation	105,148,149,590
TSLP	Alternate IL-7–like cytokine	156,157
IGF-1	Stimulates accumulation of Cμ+ cells in culture	185
FLT-3/FLK2-L	Critical for earliest stages of B-lineage development	168,172,591,592
c-KIT-L	Synergizes in IL-7–induced proliferation	26,166
IL-3	Substitute for IL-7 in proliferation of pre-B clones	176
CXCL12/CXCR4	Crucial chemokine interaction for early B-lineage precursors	194,196,198,199
Hemokinin	Novel regulator of B lymphopoiesis	187
VLA-4/VCAM-1	Adhesive interaction; antibodies to either block B lymphopoiesis	126,127,593
CD44/hyaluronate	Adhesive interaction; mediates association of B-lineage/stromal cells	144,146
TGF-β	Inhibits proliferation stimulated by IL-7	105
Sex steroids	Decrease B-lineage precursors in bone marrow	594,595
Growth hormones	Required for normal B lymphopoiesis	188,189,596
TLRs	Innate immune system regulation	190

CD, cluster of differentiation; CLP, common lymphoid progenitor; IL, interleukin; TGF, transforming growth factor; TLR, toll-like receptor; TSLP, thymic stromal lymphopoietin; VCAM, vascular cell adhesion molecule.

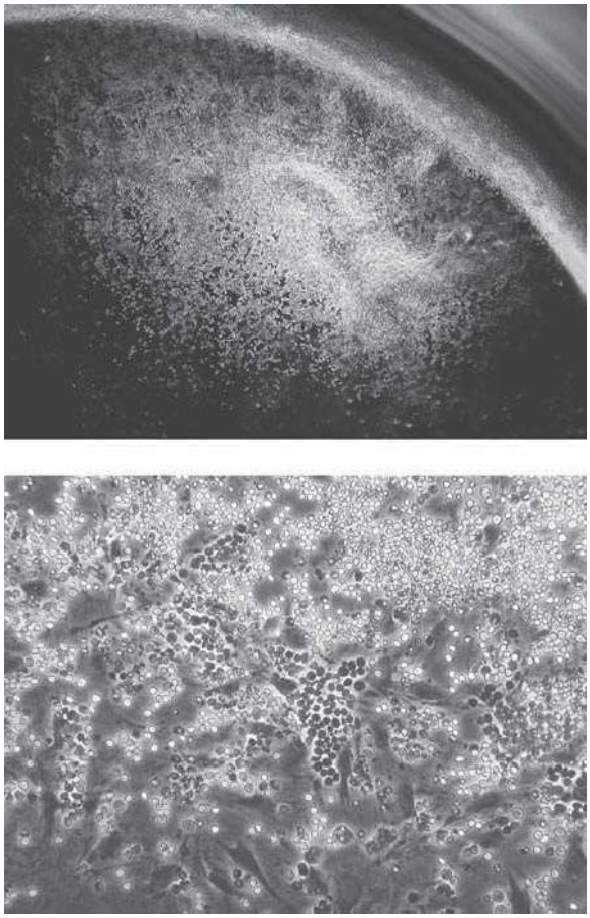

FIG. 8.7. Photomicrographs of B-Lineage Colony Proliferating on S17 Stromal Layer (in the Well of a 96-Well Microplate) in the Presence of Interleukin-7. Day 10 colony derived from a single Fr. A phenotype (see Fig. 8.5) cell. Low power and high power views. All of these cells now express CD19 and many have progressed to BP-1+.

IL-2, IL-4, IL-9, IL-15, and IL-21.[154] IL-7Rα null mice have a severe deficit of both B and T cells in the periphery and lack most B-lineage cells in bone marrow.[155] Mice with targeted inactivation of the γc or IL-7 do have some B-lineage development, suggesting an alternate cytokine; this appears to be thymic stromal lymphopoietin (TSLP). This protein was first identified as a pre–B-cell growth factor produced by a thymic stromal line[156] and shows some of the same effects in culture as IL-7, although possibly inducing less proliferation and more differentiation.[157] Its receptor has been cloned; it consists of two chains, the TSLP receptor and IL-7Rα.[158] The TSLP receptor shares both sequence homology and genomic exon organization with the common gamma chain.[159] Signaling through the IL-7 receptor requires JAK3 and activates the transcription factor STAT5, whereas signaling through TSLP is JAK3 independent but also activates STAT5.[157,160] Unexpectedly, the growth response to TSLP requires synergy with the pre-BCR in bone marrow but not in fetal liver,[161] leading some to propose that this might be a marker for distinctive B1 B-cell development in bone marrow.[162] When TSLP is overexpressed from an inducible transgene, B1 B cells expand, apparently at the expense of marginal zone (MZ) B cells.[163]

The earliest precursors in the B-lineage pathway, probably including cells that are not B-lineage committed but that can efficiently give rise to B cells in a short time in vitro, have receptors for SCF/c-KIT-ligand[26,27,164–167] and FLK2/FLT3-ligand.[168–172] Thus, the most permissive cultures for expanding precursors of B-lineage cells will include these cytokines, in addition to IL-7 and a stromal adherent cell layer, such as S17.[173–175] IL-3 has also occasionally been suggested as playing a support role for pre-B cells in vitro,[176] although its role in vivo may be at a much earlier stage. While culture conditions have been reported that can support B-lineage development in the absence of stromal cells,[177,178] the clear-cut alteration of contact-dependence prior and post heavy chain expression[128–130,179,180] argues that the most physiologic model for early B-lineage growth will include stromal cells. Besides providing important cell–cell contacts that may signal survival, proliferation, and differentiation, it is also likely that stromal cells bind at least some cytokines to their surface, providing higher local concentrations to the clusters of B-lineage precursors that adhere.[181,182]

B-lineage development may be modified by exposure to hormones; considerable interest has focused on sex steroids released during pregnancy that serve to depress B lymphopoiesis, particularly the pre–B-cell pool.[183] This may be important to avoid autoimmune responses by the mother but could have negative consequences due to possible transient immunodeficiency. Interestingly, fetal B lymphopoiesis is not similarly depressed due to the absence of hormone receptors on fetal B-lineage cells.[184] Insulin-like growth factor has been reported to potentiate progression in vitro to the Cμ+ stage,[185,186] and, more recently, there is a report of a bioactive peptide, a type of tachykinin, that synergizes with IL-7 to enhance the growth of IL-7–dependent cultures.[187] Besides insulin-like growth factor, other pituitary hormones, thyroxine, and growth hormone have effects on B lymphopoiesis.[188] For example, thyroxine treatment can restore normal B-cell development in dwarf Pit-1 mutant mice with deficient pituitary function.[189] Recent work has highlighted the effect that activation of TLRs during infection may have on altering development.[190] Thus, it is likely that more detail remains to be filled in to complete our picture of the growth requirements and modulating influences of B-lineage cells in mouse bone marrow.

Another function of cell–cell interaction is cell fate determination during the lineage commitment stage, very early in development of B-lineage cells. The Notch signaling pathway is implicated in cell fate determination in invertebrates and more recently has been shown to function in lymphoid lineage specification.[45,76,191] Notch family transmembrane receptors regulate transcription by being cleaved upon ligand binding to release an intracellular cytoplasmic domain that translocates to the nucleus where it interacts with the transcriptional repressor CSL.[192] Recent studies have shown that Notch1 can play a pivotal role in commitment of common lymphoid progenitors to the T-cell

lineage.[76] That is, expression of Notch1 by retroviral transduction has been shown to redirect B-lineage differentiation in bone marrow along the T lineage. Furthermore, a reciprocal result was found in conditional Notch1 null mice, blocking T-cell development in the thymus to be replaced by B-cell development.[97] Finally, altering the Notch1 modifier lunatic fringe by overexpressing this molecule under regulation of an lck promoter resulted in B-cell development in the thymus.[80] Differentiation of lymphoid precursors to NK or dendritic cell lineages was unaffected in Notch1 null CLP cells, so Notch apparently affects only the B/T-lineage decision.

Role of Chemokines in Migration of B-Cell Precursors

One of the most distinctive features of B-cell development in bone marrow is the migration of developing precursors from early stages nearest the bone endosteum layer to latter stages progressively closer to the central arteriole, where they will eventually exit.[193] This migration is likely due to differential expression of specific adhesion molecules and also to expression of chemokine receptors. Analysis of B-cell migration has identified a critical chemokine that is important in this process, SDF-1, now known as CXCL12,[194,195] and its receptor CXCR4.[196] CXCL12 is expressed by fetal liver and bone marrow stromal cells, whereas CXCR4 is found on hematopoietic precursors and B-cell progenitors.[197] Deletion of either the receptor or ligand results in severely impaired B lymphopoiesis.[198–200] Interestingly, the critical defect appears to be failure to retain precursors in the primary lymphoid organ, as progenitors and precursors can be found in the blood of mutant mice.[201]

Gene Expression and Immunoglobulin Rearrangement

In addition to delineation of developmental stages based on changes in protein surface expression, B-lineage cells can also be characterized for expression of internal proteins related to critical processes in their progression along this pathway, specifically those related to rearrangement and expression of the B-cell antigen receptor (Fig. 8.8). Thus, expression of μ heavy chain constant region, prior to Ig rearrangement, from a cryptic promoter generates a "sterile transcript" that reflects an open chromatin structure important for the onset of rearrangement,[202–204] and so analysis of sterile μ expression can be used to investigate very early stages of B-cell development. Classical northern analysis can be done with transformed lines, but much work analyzing RNA levels in B-lineage cell fractions, whether directly isolated or cultured, has depended on polymerase chain reaction amplification of complementary DNA.[38] For example, using this approach, sterile μ can be detected in a very early fraction of B220+CD43+CD19− (Fr. A) cells. Expression of the recombinase activating genes Rag-1 and Rag-2, which together make the double-strand breaks in DNA required for Ig rearrangement,[205–207] also occurs in Fr. A stage cells, which also have high levels of TdT, the enzyme responsible for adding nontemplated nucleotides at the D-J and V-D junctions of the heavy chain.[12,13] Rag-1 binds in a highly specific fashion to discrete sites within the IgH locus, recombination signal sequences, defining "recombination centers."[208]

The extent of heavy chain or light chain rearrangement can be quantitated either in bulk isolated populations[129] or in individual cells.[137,209,210] At the heavy chain locus, D-J rearrangement occurs prior to V-DJ rearrangement, and cells with extensive D-J but little V-DJ rearrangement can be detected at the B220+CD43+CD19− (Fr. A) stage, where Rag-1/2 and TdT are strongly expressed.[44] V-DJ rearrangements are readily detected in the abundant B220+CD43− (Fr. D) stage small pre-B cells, although productive rearrangement has already completed by the large pre-B (Fr. C-prime) stage (see following discussion). Single-cell sequence analysis of rearrangements in Fr. C stage cells shows a large proportion with nonproductive rearrangements on both chromosomes, suggesting that this may represent a dead-end fraction.[209] Some light chain rearrangement is detectable in early stage B220+CD43+ (Fr. B) cells, and this is consistent with the observation that low-level kappa light chain rearrangement is detectable in bone marrow of mice where μ heavy chain has been crippled by deletion of the membrane exon.[211] However, much higher levels of kappa rearrangement can be detected in B220+CD43− (Fr. D) stage cells consistent with the finding of sterile kappa mRNA increase just prior to this stage likely induced by pre-BCR signaling (see following discussion).

Role of Immunoglobulin Heavy Chain and the Pre–B-Cell Receptor

Many years ago, the analysis of severe combined immunodeficiency (SCID) mouse[212] bone marrow revealed the presence of a population of B220+ cells, all with a very early CD43+ phenotype, suggesting a block in B-cell development at this stage.[213] SCID mice have a defect in the catalytic subunit of the DNA-dependent protein kinase DNA-PKcs,[214,215] and, as a result, B-lineage cells in these mice are very ineffective at completing productive Ig heavy chain rearrangements. This block could be overcome by introduction of an Ig heavy chain transgene, indicating a critical role for μ protein in progressing past an early developmental checkpoint.[216] Furthermore, a gene targeting experiment that eliminated the membrane exon of μ heavy chain (μ-mt) also generated a block at this stage.[217,218]

The μ heavy chain is associated with a set of B-cell–specific peptides at the early pre–B-cell stage,[219] and this complex is referred to as the pre-BCR. It seems clear that pre-BCR mediates a type of signaling function analogous to the BCR in mature B cells. Prior to light chain expression, two peptides known as λ5 and VpreB, originally isolated as B-lineage–specific complementary DNAs,[220,221] associate with heavy chain. The λ5 shows homology to a lambda constant region and VpreB is so-called because it has homology to a variable region domain. Together, these peptides constitute a pseudo- or surrogate light chain (SLC). The critical role of λ5 was demonstrated unambiguously in gene-targeted mice, where B-cell development was blocked at the B220+CD43+ stage.[222] The production of some mature cells that accumulate in this mutant is likely due to early kappa rearrangement, with light chain substituting for SLC, as demonstrated in light chain transgenic experiments.[223]

The μ heavy chain has a very short cytoplasmic region consisting of only three amino acids; signal transduction

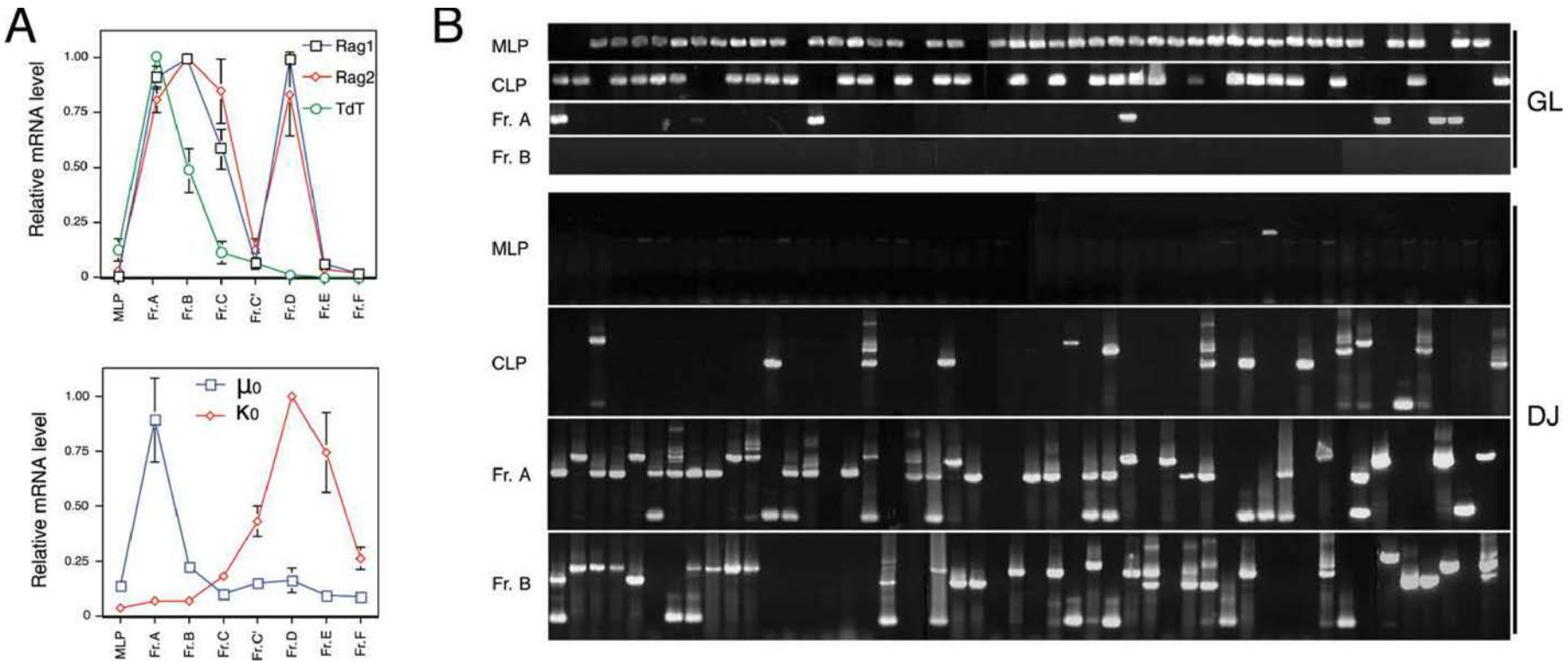

FIG. 8.8. A: Profiles of immunoglobulin (Ig) rearrangement–related gene expression. Cells isolated using fractionation scheme in Figure 8.3 for multilineage progenitors and Fr. A; in Figure 8.5 for Fr. B through Fr. F. Relative messenger ribonucleic acid levels assessed by performing semiquantitative reverse transcription-polymerase chain reaction using limited numbers of cycles, blotting, then probing, and quantitating the probe signal. Note the biphasic expression of Rag genes and the early expression of sterile μ (labeled μ_0) during the first wave, where heavy chain rearranges, and the upregulation of sterile kappa transcripts (κ_0) during the second Rag wave, when most light chain rearrangement takes place. **B:** Single-cell polymerase chain reaction analysis of Ig heavy chain rearrangement in four early stages of developing B-lineage cells. Deoxyribonucleic acid prepared from individual cells isolated following the scheme shown in Figure 8.3 was divided into two aliquots and analyzed for retention of a deoxyribonucleic acid segment lost upon any D-J rearrangement (labeled *GL*, germline) and also for D-J rearrangement. Note that D-J rearrangement initiates at the common lymphoid progenitor stage, where 30% to 50% of cells show a rearrangement.

through the BCR is mediated by accessory peptides, similar to the CD3 components of the T-cell receptor, known as Ig-α and Ig-β.[224–227] Inactivation of Ig-β[228] results in a block at the B220+CD43+ stage in mouse bone marrow, similar to that seen in μ-mt and λ5 null mice. Finally, the Syc tyrosine kinase plays a critical role in transducing BCR cross-linking signals in mature B cells and inactivation of this gene results in a "leaky" block at this same stage.[229,230] Thus, any mutation that affects this pre-BCR complex (see following section; see Fig. 8.10A) precludes efficient progression past the earliest stages of B-cell development.

Careful examination of B-cell development in normal mice shows that heavy chain is first expressed at a late fraction of the B220+CD43+ stage, termed Fr. C-prime (Fig. 8.9A). This fraction is also interesting because it shows a much higher proportion of cells in cycle (revealed by a high frequency of cells with greater than 2N DNA content; Fig. 8.9B), compared with any other B220+ stage in bone marrow.[129] Mice unable to assemble a pre-BCR, due to inability to rearrange heavy chain (Rag-1 null), show a block in development at the CD43+ stage that can be complemented by introduction of a functionally rearranged μ heavy chain as a transgene[180] (Fig. 8.9C). Analysis of several types of pre-BCR defective mutant mice shows a complete absence of Fr. C-prime stage cells, suggesting that pre-BCR signaling results in the upregulation of CD24/HSA and also entry into rapid cell proliferation. Thus, a model of pre-BCR function is that it signals the clonal expansion phase of pre–B-cell development, amplifying cells with in-frame VDJ rearrangements capable of making heavy chain protein.

The precise nature of pre-BCR signaling remains to be completely understood. An early model suggested that cross-linking of heavy chain was mediated through interaction of SLC with a bone marrow expressed ligand. However, subsequent experiments showed that normal light chain could substitute for SLC, and that even a V_H truncated μ heavy chain could mediate progression past this stage. Furthermore, intensive searches for the putative ligand over a 10-year period have been fruitless, leading to the model

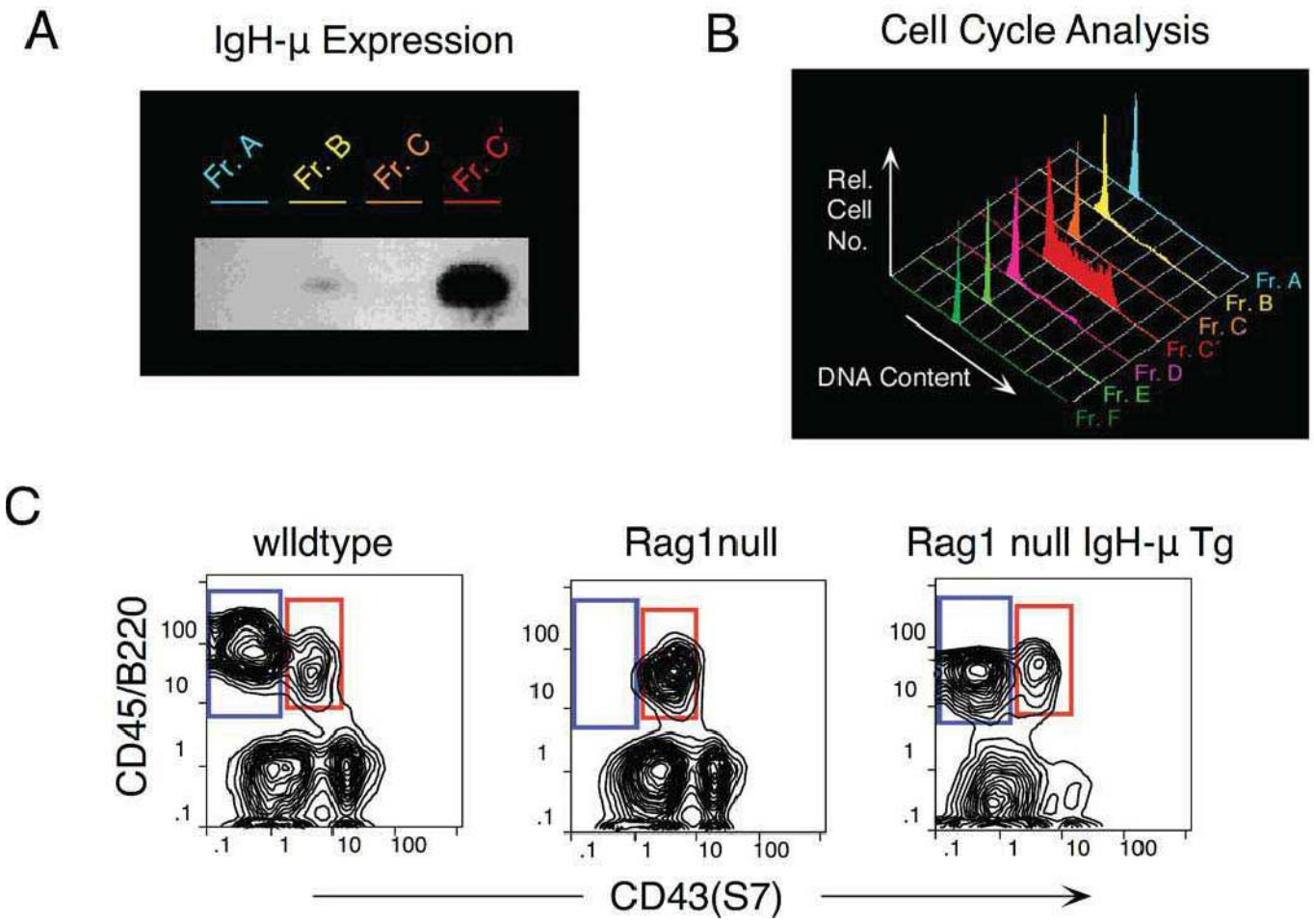

FIG 8.9. A: Western blot of immunoglobulin μ heavy chain expression showing high-level expression in Fr. C-prime. **B:** Cell cycle analysis of individual fractions shows most cells in Fr. C-prime are cycling. Propidium iodide staining of permeabilized sorted cells allows determination of deoxyribonucleic acid content per cell using flow cytometry. **C:** Block in B-cell development in Rag-1–deficient mice can be overcome by introduction of an immunoglobulin μ heavy chain transgene.

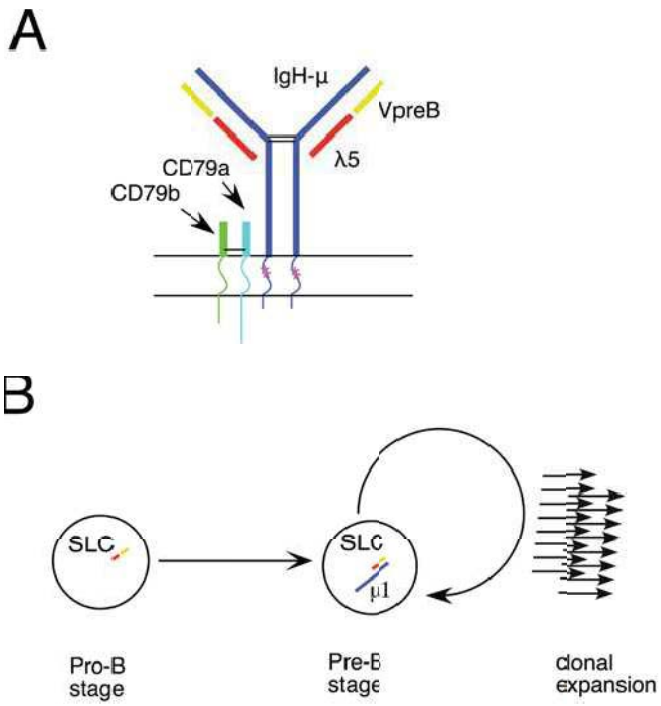

FIG 8.10. A: Diagram of the pre–B-cell receptor (BCR) μ heavy chain with surrogate light chain (λ5 and VpreB) in place of conventional light chain. As in the BCR, immunoglobulin (Ig)-α and Ig-β serve to couple signals between the receptor and cytoplasmic components, such as BLNK and Syk. Starred m transmembrane residues are important in mediating interaction with Ig-α/Ig-β, as mutation of these diminishes BCR function. **B:** Clonal expansion mediated by pre-BCR assembly. Association of newly generated μ heavy chain with preexisting surrogate light chain leads to a burst of proliferation at the pre-B stage.

that pre-BCR signaling is more akin to "tonic" signaling in mature B cells.[231–233] That is, simple assembly of the complex (or possibly some degree of multimerization fostered by the self-aggregating nature of SLC[234]) probably is sufficient for the cell to pass this developmental checkpoint (Fig. 8.10B). One clear-cut finding is that pre-BCR signaling in a transformed pro–B-cell model system can occur in the absence of any additional cell type, suggesting that if a ligand exists, it must be expressed on B-lineage cells rather than stromal cells.[235] Possibly, pre-BCR homodimerization is mediated through glycosylation at a conserved asparagine residue in the first μ constant region domain.[236] Studies of the T-cell analog of the pre-BCR, pre-Tα, provide strong evidence that it signals through spontaneous dimerization, without requirement for an external ligand.[237–239]

Mutations in other molecules in the pre-BCR signaling pathway have been shown to affect B-cell development and pre–B-cell clonal expansion. Although the Btk mutation is less severe in mouse than in human, there nevertheless is an alteration in pre–B-cell expansion in Btk-deficient mice.[240] Also, X-linked immunodeficiency (xid) B cells (deficient in Btk) have been reported to proliferate more in stromal cell cultures, possibly due to decreased differentiation to later nonproliferative stages.[241,242] The role of Btk is thought to modulate BCR signaling strength,[243] and this is probably also the case for pre-BCR signaling, allowing only strongly signaling pre-BCRs to progress in the mutant mice. BLNK/SLP65 serves to link the pre-BCR to the Syk kinase critical in BCR signaling.[244,245] Mutant mice lacking BLNK show a partial block in B-cell development at the pro-B to pre-B transition.[246] Curiously, whereas pre-BCR signaling is thought to mediate allelic exclusion (expression of a single heavy chain allele), this remains intact in BLNK-deficient mice.[247] Animals deficient in both BLNK and Btk develop an extensive pre-B expansion that progresses to lymphoma, leading to study of such mice as a model for human pre-B acute lymphoblastic leukemia (ALL).[248,249]

Syk-deficient mice show a more severe block at the pro-B to pre-B transition and a lack of allelic exclusion.[229,230,247] There is also evidence that pre-BCR signals through Erk to induce proliferation.[250]

Outcomes of pre-BCR signaling, in addition to pre-B proliferation, are downregulation of the Rag genes,[251] downregulation of TdT,[235] and transcriptional activation of the kappa locus, detected as upregulation of sterile kappa transcripts.[252] A control element for regulating Rag expression has been identified.[253,254] Extinction of recombinase activity is probably important for chromosomal stability during the clonal burst period of B-cell development[255–257] and is also at least a part of the mechanism that assures allelic exclusion, the expression of a single heavy chain by any given B cell.[258] There is evidence that pre-BCR selection requires low levels of IL-7[259] and probably occurs naturally as the developing precursors migrate through different stromal cell microenvironments in bone marrow.

The function of the pre-BCR may be more complex than simply to sense whether an in-frame VDJ rearrangement has occurred. This possibility is suggested by the observation that heavy chains with different VDJ segments vary in their capacity to assembly with SLC components.[260–263] V regions are classified into families based on sequence homology, and many members of two of these families, the 7183 and Q52, appear to frequently generate heavy chains that assemble poorly with SLC.[262] A consequence of this will likely be poor pre-BCR signaling and little clonal expansion; such cells will become underrepresented at later stages of B-cell development relative to cells containing heavy chains that signal effectively. One explanation for this SLC assembly-mediated clonal expansion is that it serves a quality control function to test heavy chain V regions for their potential to fold with real Ig light chain, a critical requirement if the cell is to express a complete BCR. An alternative (not necessarily mutually exclusive) explanation is that making pre–B-cell proliferation dependent on pre-BCR expression provides a simple mechanism for regulating the extent of clonal expansion, as an immediate consequence of pre-BCR signaling is to terminate SLC expression. Thereafter, SLC protein levels decay and are diluted by cell division so that after several rounds of proliferation, pre-BCR levels will decrease to below the threshold required to provide the signal to maintain the cell in cycle. Figure 8.11 illustrates a model for bone marrow B-cell development, showing the pre-BCR checkpoint.

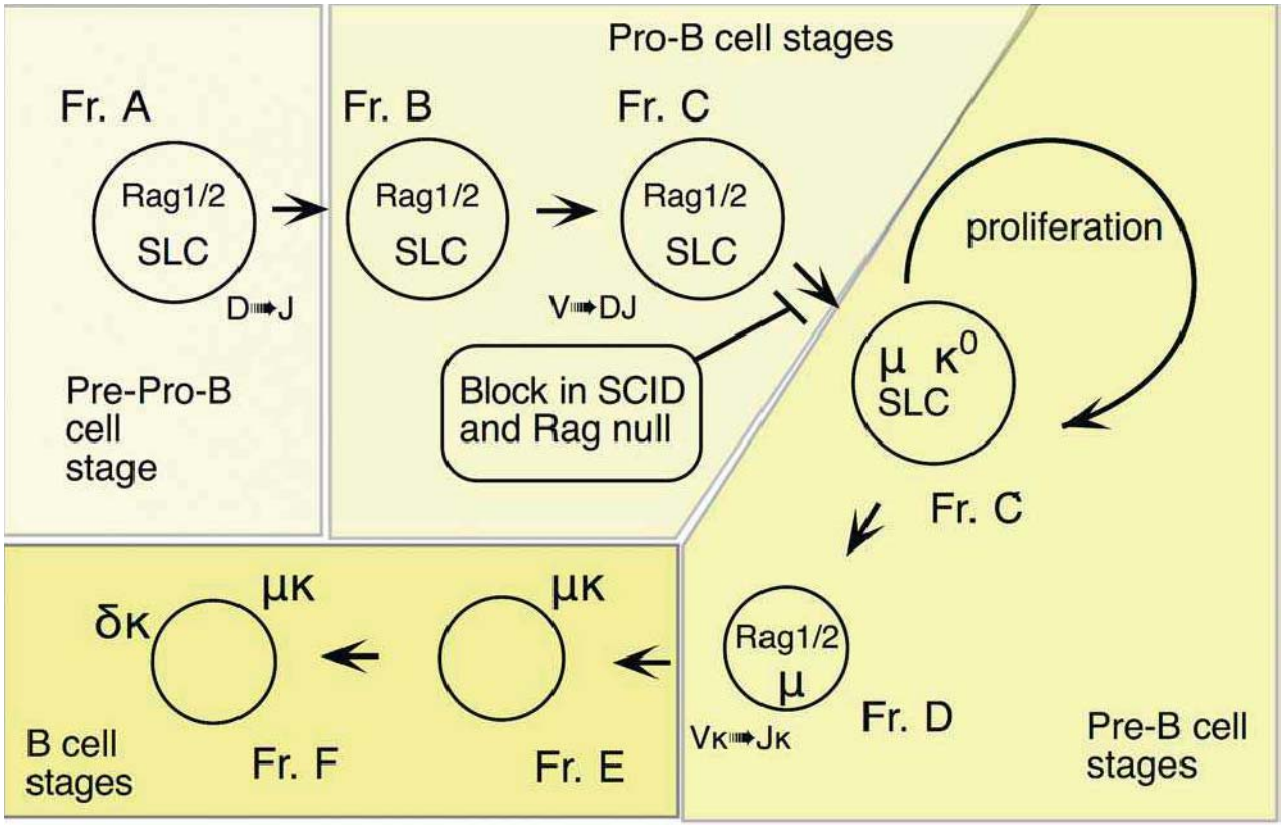

FIG. 8.11. Model of Mouse Bone Marrow B-Cell Development Showing Relationship of Immunoglobulin Rearrangement with Progression and Proliferation.

One of the most striking examples of pre-BCR selection is seen with the D-proximal V_H gene, V_H81X, where early precursors show biased overutilization due to preferential rearrangement of this V_H gene.[11,264,265] In fact, regulatory sequences interspersed throughout the distal VH region, termed PAX5-activated intergenic repeat elements, appear to enhance utilization of distal J558 family VH genes.[266] Furthermore, a recombination regulatory region, the intergenic control region 1, which lies between the VH and D clusters, has been shown to inhibit proximal and promote distal VH gene utilization.[267]

Curiously, although it is abundant in early B-cell precursors, V_H81X is rarely seen in the mature B-cell compartment. The demonstration of the decrease in representation of cells with V_H81X rearrangements at the pre-B clonal expansion phase in bone marrow,[268,269] together with the finding that heavy chains utilizing VH81X frequently fail to assemble functional pre-BCRs,[260,261,270] explained this paradox. Models of pre-BCR three-dimensional structures based on x-ray crystallography may help to explain variation in the ability of different heavy chains to assemble with SLC.[271]

However, it is still puzzling that the most frequently rearranged V_H gene is so strongly selected *against* at the clonal expansion stage. A possible explanation may lie in comparisons of V_H utilization during fetal development. That is, in contrast with bone marrow precursor cultures, the ratio of productive/nonproductive V_H81X does not decrease during cultures of fetal precursors.[272,273] Furthermore, the proliferative burst that pre-BCR assembly provides to bone marrow pre-B cells may instead result in exit from cell cycle in fetal precursors,[263] leading to selection of very different BCR repertoires during fetal and adult B lymphopoiesis. The possible significance of this is discussed subsequently in the section on B1 B cells. Leaving aside such developmental difference, the role of the pre-BCR in B-cell selection remains subject to debate, with evidence suggesting it functions to eliminate[274] or enrich[275] self-reactivity.

Light Chain Rearrangement and Generation of Immature B Cells

Besides termination of TdT and SLC gene expression, pre-BCR signaling also results in the downregulation of Rag-1 and Rag-2 expression, mediated by activity of Gfi1b on Erag, the B cell-specific Rag regulatory element,[254] and the repression of FoxO1.[276] It appears that induction of Bcl-6 by pre-BCR signaling functions in this progression by repressing Myc and Ccnd2, thereby promoting exit from cell cycle.[277,278] As mentioned previously, there is evidence that the pre-BCR signals through ERK and this Ras–MEK–ERK signaling acts to silence transcription of Ccd3, encoding the cell cycle protein cyclin D3, thus promoting cell quiescence.[279] Opposing cell cycle exit, IL-7R signaling activates STAT5, maintaining Ccd3 expression. Eventually, the pre-B cell escapes from

IL-7 signaling, exits the cell cycle, and the Rag genes are reexpressed at high levels. Sterile kappa transcripts become detectable during the cycling stage, likely reflecting chromatin remodeling to make the kappa light chain locus accessible,[252] so induction of the recombinase machinery can initiate kappa light chain V to J rearrangement.

An interesting feature of the Vκ locus is that the approximately 100 genes are in both transcriptional orientations, and so these genes can rearrange either by deletion (generating an extrachromosomal excision circle) or by inversion.[280] The absence of intervening D segments also means that it is possible for upstream V kappa genes to rearrange to downstream J kappa segments, "leapfrogging" the initial rearrangement, assuming it was to any Jκ other than Jκ5. The successive association of different kappa chains with the same heavy chain in a B cell is referred to as BCR "editing" and was originally observed in the context of autoreactivity, which maintains Rag expression even at the B-cell stage[281,282] (see following section on B-cell tolerance). Because assembly and expression of a complete BCR (that is not self-reactive) terminates Rag expression, an additional reason for light chain editing in the bone marrow may be to replace an initial light chain that fails to assemble effectively with the particular heavy chain present in that pre-B cell. This is probably the explanation for multiple light chain rearrangements detected in single early B-lineage cells.[283] Complete failure of kappa rearrangement, possibly after receptor editing to avoid self-reactivity, leads the pre-B cell to a second phase of light chain rearrangement, dependent on IKK-mediated NF-κB signaling, where the λ light chain locus rearranges.[284]

Newly formed B cells can be distinguished from mature B cells on the basis of their inability to proliferate in response to BCR cross-linking (ie, they are functionally immature). This is also the stage where negative selection is reported in transgenic models of autoreactivity.[285,286] Cells at this stage have a short half-life of only a few days, compared to mature follicular B cells with a half-life measured in months. They can be distinguished by surface phenotype from other B cells based on expression of certain combinations of markers, such as IgM+IgD−, absence of CD23, and high-level expression of CD24/HSA.[287] Recently, there have been reports of single markers that are useful in distinguishing newly formed cells from any mature subset, such as the molecules recognized by monoclonal antibodies 493[288] and AA4.1.[289] The AA4.1 target molecule has been cloned and identified as the mouse ortholog of a component of the human C1q receptor.[290]

Cells similar to newly formed B cells can be generated to varying extent during stromal cell culture of B-cell precursors, although the more primitive cycling pre-B or pro-B cells are usually more abundant and tend to increase in frequency with prolonged culture. It is possible to induce differentiation of B cells in these cultures by withdrawing IL-7, which induces a wave of small pre-B and then newly formed B-cell generation. Such cells do not persist for more than a day, unless the cultures are established from Bcl-2 transgenic mice,[131] suggesting that the short half-life of newly formed cells in these cultures, and possibly also in vivo, is due to their low level of antiapoptotic mediators. Both Bcl-2 and Bcl-X_L mRNA are present at only very low levels in these cells, in contrast with other B-lineage stages where either one or the other predominates. Overexpression of Bcl-X_L from a transgene results in accumulation of a population of pro-B phenotype cells with nonfunctional rearrangements,[291] implicating this protein in the process of pre-BCR selection of cells with functional rearrangements.

Peripheral Maturation Stages and Functional Subsets

Transitional B Cells

Newly formed immature B cells migrate to the spleen where they either die or undergo further maturation to a mature B cell. These maturing B cells can be subdivided based on differential expression of several surface proteins, including CD21, CD23, CD24/HSA, and AA4.1. These subdivisions have been referred to as "transitional B cells."[292] One recent subdivision based on CD21, CD23, AA4.1, and IgM levels has shown progression from an AA4.1+CD21−CD23− T1 stage to an AA4.1+CD21−CD23+ T2 stage, followed by downregulation of IgM as a T3 stage, and finally loss of AA4.1 with upregulation of CD21 to yield the mature follicular phenotype.[289] As shown in Figure 8.12, this approach also resolves two AA4.1− subsets that lack CD23, the B-1 subset with low CD21 and the MZ subset with very high CD21 (see following). The transitional stage cells are all short-lived as shown by bromodeoxyuridine incorporation.[289,293] They are also not functionally competent, as shown by inability to proliferate after BCR cross-linking.[287,294] Another well-characterized functional distinction is that B-cell tolerance, rather than an immune response, is induced by BCR cross-linking of immature B cells.[295–297] More recent studies with transgenic models of self-reactivity have shown that these B cells can be deleted, undergo receptor editing, or rendered functionally unresponsive (anergic) by BCR signaling at the immature stage.[281,285,298–302] One group has suggested that the "T3" stage is not an intermediate in production of follicular B cells, but rather a population of autoreactive anergic cells,[303] so additional work needs to be done to establish the identities of all the B-cell subpopulations present in spleen. For example, careful comparison of T1 and T2 B-cell populations shows that a fraction of T2 cells do respond to certain microenvironmental stimuli and include a portion of cycling cells that appear to constitute a branchpoint in generation of MZ B cells.[304]

It is not simply the inability to receive T-cell help due to differences in microenvironment or receptor expression that makes immature B cells incapable of responding as mature B cells. Different from the result with mature B cells, cross-linking the BCR on immature B cells has been shown to induce apoptosis, suggesting distinctions in the signaling pathways between these two stages.[305,306] Studies with transgenic mice suggest that this apoptosis is not mediated through the Fas/Fas-ligand pathway, as central deletion is intact in Fas-mutant mice.[307,308] Prior to induction of apoptosis, immature B cells have been shown to complete

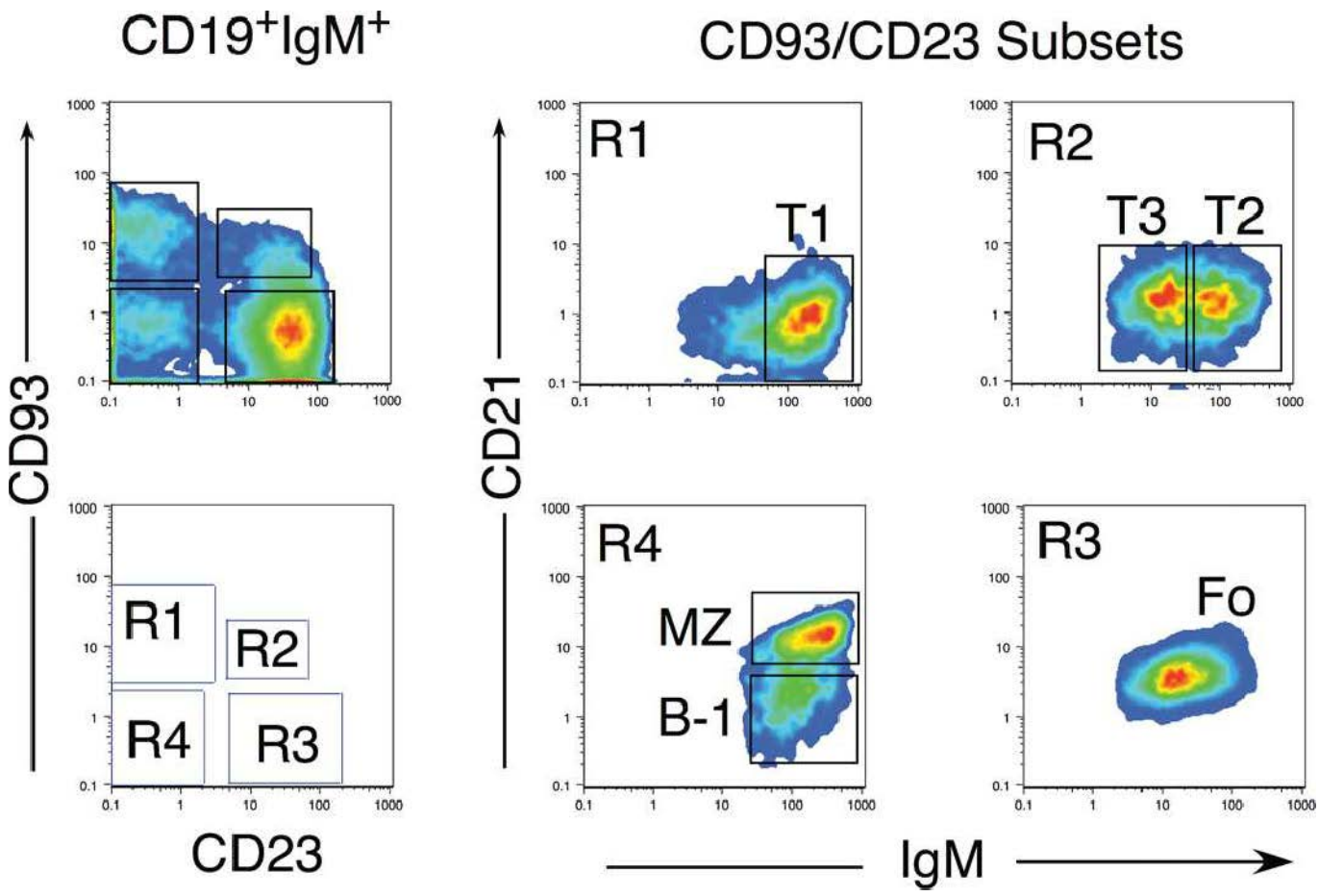

FIG 8.12. Flow Cytometry Approach for Resolution of Transitional (T1–T3) and Mature (Follicular, B1, Marginal Zone) Populations of B Cells in Mouse Spleen. Left panels show the distribution of AA4.1 and cluster of differentiation (CD)23 on B cells (defined as CD19+immunoglobulin [Ig] M+). Cells in the boxed regions are then analyzed for correlated expression of CD21 and IgM, facilitating resolution of three AA4.1+ fractions (T1–T3), the follicular subset (AA4.1–CD23+CD21+), the marginal zone subset (AA4.1–CD23–CD21++), and the B-1 subset (AA4.1–CD23–$CD21^{low}$).

some of the early events associated with entry into cell cycle while failing to complete this program.[309] Distinct stages in maturation appear more or less capable of responding to BCR cross-linking by reinduction (or maintenance) of Rags to facilitate receptor editing.[286] Furthermore, it appears that immature B cells are more sensitive to smaller changes in intracellular free calcium, compared to mature B cells.[310] It is possible that the capacity to upregulate antiapoptotic molecules, such as A1, may play a critical role in the inability of immature B cells to survive and complete a normal response.[311,312] The characterization of signaling pathways in different immature stages of developing B cells is ongoing and should eventually provide insights into the detailed mechanism for immature B-cell tolerance.

Analyses of various normally occurring or engineered mutant mice have provided approaches for investigation of the process of progressing from a newly formed B cell to a mature follicular B cell. B-cell populations and B-cell function has been studied for many years in CBA/N mice bearing the xid mutation. This mouse has a mutation in the Btk gene that produces a milder phenotype than the complete absence of peripheral B cells seen in humans. The Btk gene likely plays a role at several stages of B-cell development and activation, which complicates the analysis, but it appears clear that one major consequence is altered BCR signaling that has a profound effect on progression through the various transitional stages in spleen. A likely consequence of diminished strength of BCR signaling is a compensatory requirement for higher surface BCR expression that eventually produces at decreased frequency a type of "mature" B cell that is still functionally handicapped.[313,314] Several groups have produced xid mice on a nu/nu T-cell less background that results in a more profound absence of mature B cells, suggesting a requirement for T cells or T cell–produced factors in the maturation of xid B cells.[315,316] A more recent variation of this type of investigation is the production of xid/CD40-deficient mice that show a similar deficit in mature B cells, suggesting a role for the CD40/CD40L interaction in the generation of mature B cells from transitional B cells, particularly when the BCR is handicapped by defective Btk.[317]

Lyn is a Src family protein kinase that is associated with the BCR and functions in signaling in mature B cells.[318] Lyn-deficient mice exhibit defects in maturation of immature cells,

suggesting a positive role for BCR/Lyn signaling at this stage, but these mice also develop a severe autoimmune condition, suggesting an additional negative regulatory role for Lyn in maintaining tolerance in mature B cells.[319,320] Cross-linking of the BCR on T2 and mature B cells results in c-Rel activation that upregulates antiapoptotic genes and the prosurvival BAFF receptor.[321] Furthermore, migration of early bone marrow emigrants to areas where T1 and T2 cell mature has been shown to depend on chemokine signaling that is disrupted in Rac1/Rac2 double-deficient mice.[322] Finally, progression of transitional cells to the mature B-cell stage is dependent on "tonic" BCR signaling, as mice engineered to express low-surface BCR levels, and, thereby lower tonic BCR signaling showed decreased generation of mature B cells.[323] Analysis of these mice suggested that maturation was dependent on activation of ERK via a signaling pathway requiring Ras.

CD72 is a predominantly B-lineage restricted C-type lectin, and ligating this molecule was recognized for many years (when it was known as Lyb2) as having functional consequences.[324] Recent analyses of a CD72 null mouse has clarified its function in B-cell development and activation.[325] CD72 has been shown to recruit SHP-1 to the BCR, supporting a negative regulatory role in BCR activation.[326] Consistent with this model for CD72 function, null gene-targeted mice have been shown to produce B cells that are hyperresponsive.[325] Interestingly, late stages of B-cell development are affected, with fewer mature B cells and relatively normal numbers of immature B cells in spleen.[325] Thus, too intense signaling may also delay maturation of immature B cells.

A number of studies have demonstrated the important role that the phosphoinositide 3-kinase (PI3K) signaling pathway plays in the maturation of transitional cells and generation of mature B cells. Under conditions of normal tonic signaling, PI3K signaling inhibitors block the normal downregulation of Rags in immature B cells and also decrease progression past this stage.[327] This BCR signal is dependent on CD19 and BCAP, a BCR complex adapter molecule,[328] and acts through AKT to activate PI3K. The survival of B cells at the mature stage has been shown to depend on an intact BCR. More recent work revealed that the survival of BCR-deficient B cells can be rescued by activation of the PI3K signaling pathway alone.[329]

Follicular B Cells

The major population of mature recirculating B cells in the spleen is located in the B-cell follicle region, hence the term follicular B cells. Entry into this anatomic site appears to constitute a final stage in maturation for developing bone marrow B cells, as competition for this site is compromised in several transgenic models of B-cell tolerance.[330–333] Cells in this compartment do not proliferate but persist in the resting state for several months. A conditional knockout study, eliminating expression of the BCR (by deleting the V region), revealed that expression of the BCR is required for cell survival.[232,233] It is not yet established whether this is due to "tonic signaling" (simple assembly of the BCR signaling complex) or instead reflects signaling by low-affinity binding to cross-reactive self-determinants, a kind of "positive selection." It is interesting to note that ablation of heavy chain from immature B cell, thereby eliminating any possibility of pre-BCR or BCR tonic signaling, results in a "reversion" of cell phenotype to an earlier developmental stage.[334] Finally, the maintenance of follicular B cells has been found to depend on the function of the c-Myb transcription factor due to its role in BAFF signaling[335] (see following section).

The repertoire of the follicular B-cell pool appears to differ from the earlier immature splenic B-cell population, as assessed by sequence analysis of the light chain repertoire in heavy chain transgenic mice.[336] The approach of fixing the heavy chain and then examining the light chain repertoire simplifies the analysis, and the results of this study were interpreted to indicate that BCR-mediated antigen selection is indeed operating. However, the resolution of the analysis probably could not have rigorously excluded populations known to show V gene biases, such as B-1 or MZ B cells (see following sections), so further work will be required to provide convincing evidence of antigenic selection in the follicular B-cell pool.

B-Cell Migration and Maintenance

Newly formed B cells migrate from the bone marrow to the spleen, undergo further maturation in the red pulp, and eventually enter the follicle where they constitute the mature B-cell pool that recirculates. Their migration is dependent on chemokines/receptor interactions, notably the SLC(CCL21)/CCR7 interaction, as demonstrated by the inability of mature B cells to be retained normally in spleens of CCR7 null mice.[337] The role of the CXCR5 receptor on B cells in homing to the lymphoid follicle due a gradient of the B-lymphocyte chemoattractant CXCL13 is also well known, and CXCL13 can directly induce Ltα1β2 on the recruited cells.[338] Finally, it is also possible that the SDF1 (CXCL12)/CXCR4 interaction, critical for normal B lymphopoiesis, may also be important at this later stage, although investigation of this issue is complicated by the early defect. This is an ongoing area of investigation and may eventually be clarified by developmentally regulated gene targeting studies.

Recently, considerable interest has focused on the role of a tumor necrosis factor family member cytokine known variously as BAFF, BLyS, TALL-1, zTNF4, or THANK in the process of peripheral B-cell maturation.[339] BAFF is a tumor necrosis factor family member found to enhance survival of B cells or even produce autoimmunity in transgenic mice constitutively expressing it.[340,341] Initially, two receptors defined for BAFF, BCMA and TACI, provided a complex picture, as targeted inactivation of BCMA yielded no B-cell defect and deletion of TACI had increased B cell numbers (suggesting that TACI might be a negative regulator). This puzzle was resolved by identification of a third receptor, BAFF-R/BR3,[342,343] which was mutated in a strain of mice known to lack most mature B cells, A/WySnJ.[344] A second ligand, APRIL, can bind to BCMA and TACI, but not to BAFF/BR3, and this binding is proliferative rather than survival promoting.[345] Thus, the critical interaction for maintenance of follicular B cells is BAFF/BLyS with its receptor. B-1 cells are not deficient in A/WySnJ,[344] suggesting that their maintenance does not depend on this pathway

but instead is more BCR dependent. Interestingly, excessive levels of BAFF promote survival of highly autoreactive BCR transgenic mouse B cells, consistent with the finding of elevated levels in patients with autoimmune diseases such as systemic lupus erythematosus and Sjögren syndrome.[346]

Mechanism of B-Cell Receptor Signaling

Considering that the structure of the Ig molecule was elucidated more than 40 years ago, it is surprising that the details of initiation of B-cell activation following antigen binding are still not firmly established. Recent work using single-molecule imaging demonstrated that antigen binding altered the mobility of BCRs within microclusters on the B cell, resulting in rigid oligomeric structures that generated a signal.[347] Interestingly, this oligomerization required the membrane proximal domain of IgH-μ (Cμ), and such domains in isolation spontaneously formed aggregates in the membrane, leading to the hypothesis that antigen ligation induces a conformational change in Cμ4 that then promotes assembly of signaling clusters. Other work visualizing simple Ig molecules has demonstrated the importance of the membrane cytoskeleton in limiting diffusion of the BCR and revealed the importance of Igb/CD79b in mediating such restriction, showing that alterations in BCR distribution rapidly result in strong intracellular signaling.[348] Finally, an alternative model has been proposed, based on analysis using quantitative bifluorescence complementation, suggesting that preexisting BCR aggregates are less active in signaling, and that the disruption of such complexes upon antigen binding is responsible for generating a signal.[349] Additional work using such advanced imaging techniques will undoubtedly resolve this apparent contradiction and lead to a more complete understanding of BCR signaling.

B-Cell Turnover

It is estimated that 10 to 20 million B cells are produced in bone marrow of the mouse each day,[350] yet it appears that only about 10% of this number reach the periphery.[293] Thus, there is considerable loss at this bone marrow emigration stage/spleen entry stage, possibly due to elimination of autoreactive cells (B-cell tolerance) or to homeostatic regulation. The latter possibility is supported by the observation that depletion of the mature B-cell population results in a relatively rapid recovery of this pool, suggesting that most of the immature B-cell population can enter the mature follicular subset in this situation.[351]

Once functionally mature B cells are generated, it has been difficult to unambiguously determine their half-life, although accumulating data from several laboratories using bromodeoxyuridine labeling has led to the idea that follicular B cells have a relatively long half-life on the order of months.[293,352] A recent elegant study provided definitive confirmation of this by conditional elimination of Rag-2 expression, allowing termination of B-cell development in adult mice.[353] This study showed that follicular B cells have a half-life of about 4.5 months. This same analysis showed that two other subsets of B cells, B-1 and MZ B cells, did not diminish over time, consistent with their well-known capacity for self-renewal and lifelong persistence.

Germinal Center B Cells

T cell–dependent immune responses usually give rise to anatomically distinctive structures in spleen and lymph nodes that are referred to as germinal centers (GCs) and contain large numbers of rapidly cycling B cells.[354,355] These cells can be recognized in stained sections of spleen by binding of high levels of peanut agglutinin and by the absence of IgD.[356,357] Many of the B cells with this phenotype have downregulated-Bcl-2/upregulated-Fas expression and, in the absence of strong BCR signaling, will likely die by apoptosis.[358–361] The termination of IgD expression means that surface BCR expression decreases at least 10-fold, and so limiting amounts of antigen will favor the cells with increased affinity for antigen, generated by a process termed *somatic hypermutation*. Recently, a miRNA, miR-155, has been found to be critical for the generation of GCs by regulating cytokine production.[362]

A major advance in understating the GC response has been the discovery that activation-induced cytidine deaminase (AID), an RNA-editing enzyme that can induce class switch recombination in fibroblasts,[363–365] is also a key player in the process of hypermutation.[366,367] In fact, a third means for Ig gene diversification used in nonmammalian species, V gene conversion, is also dependent on AID.[368,369] AID is now thought to be responsible for genetic mutations outside the Ig loci in lymphomas that arise from GC B cells.[370]

The precise mechanism of selection for higher affinity B cells generated by hypermutation of the BCR V regions remains to be fully understood, but the regulation of proapoptotic and antiapoptotic genes likely plays a major role. B cells able to bind antigen with high affinity can present antigen to specialized CD4+ T cells (follicular helper T cells) that then signal the B cell through a CD40/CD40L interaction, resulting in the upregulation of Bcl-X_L.[358–360] Most GC B cells have sharply downregulated levels of Bcl-2 and upregulated levels of Fas,[360,361] and so in the absence of rescue by expression of the alternative antiapoptotic mediator Bcl-X_L, cell death by apoptosis will be the fate of most B cells in the GC. Careful regulation of selection is critical to affinity maturation, and elimination of self-reactive cells that potentially could be generated during this process must also occur efficiently to avoid the potential of autoimmune disease.

Recently, the role of the immunoinhibitory receptor PD-1, present at high levels on follicular helper T cells, in GC B-cell selection has been described.[371] Furthermore, high-resolution imaging of fluorescent reporters is revealing details of cell dynamics within the GC, indicating that competition for T-cell help is a key limiting factor in the selection of higher affinity B cells.[372]

Memory B Cells

Memory B cells were initially defined functionally as cells that could respond rapidly by production of high-affinity antibody when challenged in a host reconstituted with B cells and T cells from a primed animal.[373–378] Subsequently, such cells have been purified based on their antigen-binding properties[379] and shown to consist primarily of isotype-switched (IgG+) B cells that continue to express CD45R/B220 and have distinctively

lower levels of cell surface BCR.[380] They arise during the T cell–dependent immune response, probably only from follicular B cells, in the GC. They are very long-lived or self-regenerating, as cell transfer assays have shown that memory responses can be detected for long periods after the primary immunization.[380,381] More recent work has focused on examining the heterogeneity of secondary B-cell subsets, examining cell surface proteins that may aid in distinguishing them.[382] In terms of genes that distinguish memory B cells, it appears that phospholipase C gamma 2 is important both for the generation and maintenance of memory B cells.[383]

It is not clear whether all B cells are capable of giving rise to memory B cells. Memory or "secondary" B cells were originally described as expressing distinctively low levels of CD24/HSA, recognized by the monoclonal antibody J11d.[384] Some years later, fractionation of naïve spleen B-cell precursors into J11d^{low} and J11d^{high} subsets in a spleen focus assay system showed that while rapid antibody secretion derived from J11d^{high} cells, memory came largely from the J11d^{low} subset.[385] Subsequent experiments demonstrated that GCs (the site where most memory B cells are generated) were only produced in cell transfers of J11d^{low} B cells and not with J11d^{high} or CD5+ B cells.[386] Considering the rapid Ig secretory response of B1 B cells and MZ B cells, both contained in the CD24/HSA^{high} fraction, and the fact that most other CD24/HSA^{high} cells are immature (transitional) B cells, likely to be highly susceptible to apoptosis, it seems quite reasonable that memory B cells would not be a major product of this fraction. Rather, the most likely candidate for the memory B-cell precursor is the follicular B-cell subset (Fo), which has variable but lower expression of CD24/HSA. Whether there is heterogeneity for GC formation or memory B-cell generation within the Fo population remains to be determined.

Whether the maintenance of memory requires periodic restimulation by antigen has been a longstanding controversial issue. On the one hand, transfer of B cells and T cells into irradiated recipients usually required simultaneous challenge with antigen in order to elicit the full response and maintain B-cell memory in recipients.[387] Antigenic fragments can persist for very extended periods on follicular dendritic cells, which are very potent antigen-presenting cells, and, so in this model, the memory B cells are periodically triggered to self-renew by interaction with antigen on follicular dendritic cells. However, on the other hand, memory B cells can be maintained in the apparent absence of T cells or follicular dendritic cells.[388,389] Furthermore, analysis by bromodeoxyuridine labeling of memory B-cell populations showed that they were nondividing.[381] This issue has been addressed in elegant experiments using inducible Cre recombinase to switch the BCR on memory cells away from the immunizing antigen.[390] Such antigen-negative memory cells still persisted for extended periods, clearly demonstrating that this was a physiologic property of the cell type, independent of the presence of antigen.

B1 B Cells

B1 B cells, initially described as Ly-1/CD5+ B cells, are distinguished from follicular B cells by phenotype, anatomic distribution, and function.[23,391] The B1 B-cell phenotype encompasses both CD5+ and CD5– B cells that are IgM^{high}, IgD$^{low/-}$, CD23–, and CD43+. They constitute a large proportion of the B cells found in the peritoneal and pleural cavities (30% to 50% of B cells, around 10^6 cells), but are also found in spleen where they are present at numerically similar levels, but constitute a much lower proportion of the total B-cell pool (2% of B cells, around 10^6 cells). The B1 B cells in the peritoneal cavity are also CD11b/Mac-1+ unlike those in spleen. They appear early in ontogeny, representing 30% or more of the B cells in spleen of 1-week-old animals. They also have a distinctively higher frequency of λ light chain usage compared to follicular B cells (20% vs 5%). Also unlike follicular B cells, they maintain their population in adult animals largely by self-renewal (possibly dependent on periodic stimulation by self-antigen; see following discussion), rather than by input from precursor cells, as shown in cell transfer studies.[392] There is also a recently described population of IgM-secreting cells in bone marrow with a B1 cell surface phenotype.[393]

Perhaps the most distinctive feature of CD5+/B1 B cells is their enrichment of certain self-reactive specificities, notably for branched carbohydrates, glycolipids, and glycoproteins, including phosphorylcholine, phosphatidylcholine (PtC), the Thy-1 glycoprotein, and bacterial cell wall constituents.[394–397] These antibodies, although autoantibodies, are not pathogenic, but rather referred to as "natural autoantibodies" whose existence has been recognized in serum for several decades.[398–400] Their function is still under active investigation, but at least some natural autoantibodies are thought to function in clearance of senescent cells or proteins and to provide an initial immunity to common bacterial or viral pathogens, serving as a kind of "hardwired" memory B-cell population.[401–404]

Under physiologic conditions in normal mice, most of the CD5+ B cells in the B1 population (so called "B1a B cells") arise from precursors in fetal liver, as cell transfer studies showed many years ago that B cells with this phenotype were inefficiently generated from bone marrow precursors in adult mice, compared to fetal or neonatal precursors.[405] This is shown by repopulation of SCID mice by pro-B stage cells isolated from fetal liver and adult bone marrow (Fig. 8.13). Part of the reason for this difference may be that novel BCRs are enriched by distinctive mechanisms of fetal B lymphopoiesis, including recombination in the absence of TdT (thereby favoring rearrangement of certain D-J and V-D junctions possessing short regions of homology[16]) and distinctive pre-BCR selection.[263,273,406] Forced expression of transgenic BCRs cloned from CD5+ B cells can give rise to CD5+ B cells from bone marrow of adult animals, but the physiologic relevance of this remains to be carefully assessed. B1 B cells that lack CD5, termed B1b B cells, can be generated from bone marrow and a distinctive phenotype for a precursor capable of producing such cells has been described,[162] although the developmental relationships between conventional ("B2") B-cell precursors and these cells are complex.[407] Embryonic day 9 YS hematopoietic progenitors have been sown to give rise to B1 and MZ B cells but not conventional follicular B cells.

The development of B1/CD5+ B cells is thought to be more dependent on antigenic selection compared to follicular B cells. This idea was first suggested by the finding of particular specificities enriched in this population and

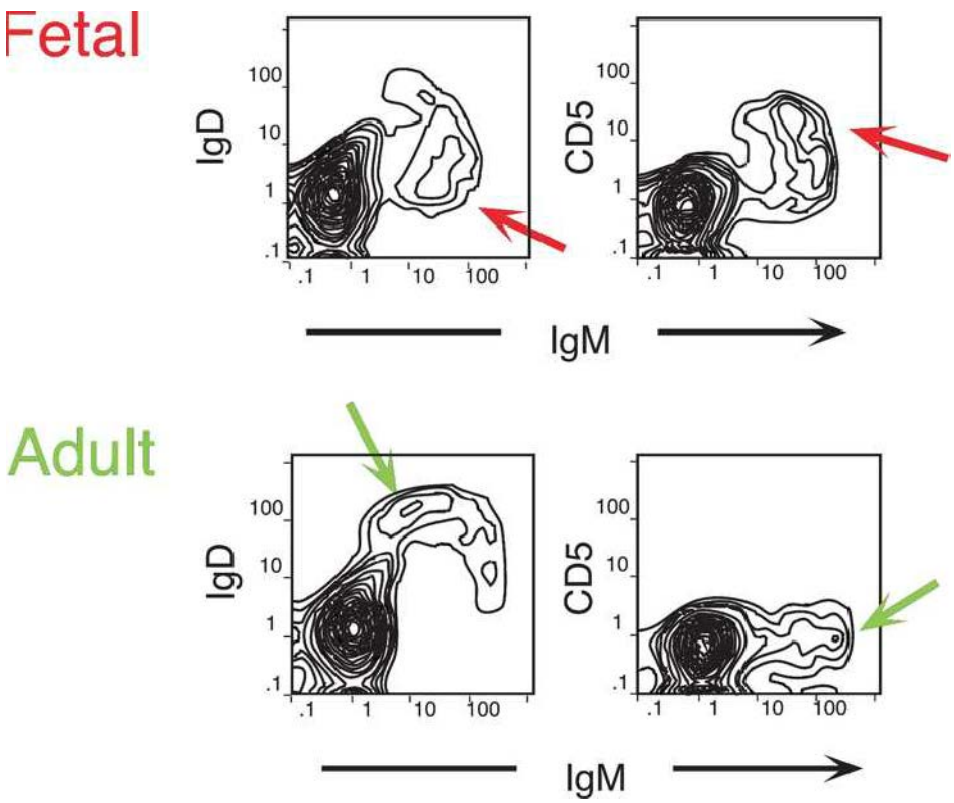

FIG 8.13. Generation of B Cells in Severe Combined Immunodeficiency (SCID) Mice Shows the Distinctive Phenotypes Produced from Fetal and Adult Pro-B Cells. Similar numbers of pro-B cells, isolated as in Figure 8.5, Fr. B/C, were injected intravenously into sublethally irradiated adult SCID mice. Recipients were analyzed 3 weeks later for spleen cell lymphocytes by staining as shown in the figure.

strengthened by the observation of repeated occurrences of particular V_H/V_L pairs.[408] Thus, for example, the anti-PtC specificity is predominantly encoded by $V_H11V_\kappa9$ and $V_H12V_\kappa4$ utilizing two V_H genes rarely found in conventional T-dependent immune responses.[395,409] These cells appear to participate in T-independent responses, but in normal physiology may in fact provide an initial low-affinity "first wave" response to many pathogens that eventually will also elicit a T-dependent response.[404,410]

The observation of their self-reactive bias, their capacity for self-renewal, and their restricted repertoire of distinctive BCRs all are consistent with an important role for BCR–antigen interaction in generation and maintenance of this population. This has been formally confirmed recently by studying mice bearing a BCR transgene specific for a glycosylation present only on the Thy-1 membrane protein.[411] In these mice, transgene-encoded serum anti–Thy-1 autoantibody[396] was readily detected, and there was a corresponding accumulation of a population of CD5+ transgene BCR+ B cells in the peritoneal cavity. Importantly, in Thy-1 null mice generated by gene targeting, neither serum autoantibody nor the B-cell population was found, demonstrating the critical role for antigen in selection of this B-cell population.[412]

Consistent with the importance of antigen selection in the generation and/or maintenance of these cells, this population is often severely affected in mice bearing mutations that alter BCR signaling intensity. The loss of negative mediators such as PTP1C/SHP-1 in "moth-eaten" mice,[413] of CD72 that recruits this phosphatase,[413] and of the CD22 coreceptor[414,415] all result in an increased frequency of B1/CD5+ B cells relative to follicular B cells. On the other hand, the loss of critical BCR signaling components or positive mediators in this pathway such as an Ig-α tail mutant,[416] Btk deficiency (in xid or Btk null mice),[417,418] CD19 null mice,[419,420] CD21 null mice,[421] CD45 null mice,[410] and vav null mice[422] all negatively impact this B-cell population. A further indication of a signal-dependent selection model is the accumulation of B1-type B cells with high-level expression of a BCR surrogate, LMP2A, but only B2 B cells and MZ B cells with low-level expression.[423]

The functional role of B1 B cells is still incompletely understood. Whereas it is clear that they produce natural autoantibody, there also is evidence for their participation in immune responses. B1 B cells have also been shown to accumulate in draining lymph nodes of the respiratory tract during influenza infection of mice.[393] Treatment of mice with *Francisella tularensis*–derived lipopolysaccharide protects them from subsequent exposure to the live bacteria (the etiologic agent of tularemia) by activating a small fractions of B1 B cells with this antigen specificity.[424]

IL-10 is a cytokine with anti-inflammatory properties that also enhances B-cell survival and antibody production. Thus, it can function to damping down cellular immune responses and promote humoral immunity. Many years ago, B1 B cells were identified as the B-cell source of this cytokine that is also secreted by monocytes, mast cells, and regulatory T cells.[425] Recently, based on observations of alterations in the level of inflammation in mice lacking or overexpressing CD19, a CD1d(high) subset of B-1 cells expressing IL-10 has been identified.[426] Considering the anti-inflammatory regulatory effects of IL-10, this subset has been referred to as "B Regs" or "B10" B cells. Investigation of this subset in the experimental autoimmune encephalomyelitis mouse

model of multiple sclerosis showed that initiation and progression of disease was influenced reciprocally by depletion of B cells. Because experimental autoimmune encephalomyelitis is considered a T cell–mediated disease, this provides strong evidence for the capacity of B1 cells in modulating T cells during the initiation of disease. Curiously, depleting B cells after experimental autoimmune encephalomyelitis was already established actually diminished symptoms, indicting that B cells were required for persistence of autoreactive T cells. This suggests that simple depletion of all B cells in certain autoreactive pathologies may have different consequences, depending on the progression of the disease. Finally, examination of B10 function in the autoimmune lupus-prone NZB/W mouse F1 indicated that these cells were responsible for inducing regulatory T cells that could ameliorate the course of disease in these animals.[427]

Marginal Zone B Cells

Another B-cell subset distinct from follicular B cells is the MZ B-cell population. MZ B cells are localized in a distinct anatomic region of the spleen that represents the major antigen filtering and scavenging area (by specialized macrophages resident there). It appears that they are preselected to express a BCR repertoire similar to B1 B cells biased toward bacterial cell wall constituents[428] and senescent self-components (such as oxidized low-density lipoprotein [LDL]).[429,430] Similar to B1 B cells, they respond very rapidly to antigenic challenge, likely independently of T cells, but participating in the early phase of T-dependent responses.[431–433] Uniquely, among all populations of B cells, MZ B cells are dependent on Notch2 signaling for their development.[434,435]

There are similarities and differences in the cell surface phenotype of MZ and B-1 B cells. Thus, they both are IgM+++IgD−/+, CD23−, and CD9+.[436] However while B1/CD5+ B cells express CD5 and CD43, MZ B cells do not, and MZ B cells express distinctively high levels of CD21, while B1 cells have distinctively low levels (see Fig. 8.12). Also, MZ B cells have high levels of CD1d, while most B1 B cells do not,[437] the exception being the "B10" subset mentioned previously. Certain mutant mice show similar effects on MZ and B1 B cells, distinct from follicular B cells. For example, most of the mutations described for B1 B cells that alter BCR signaling have similar consequences for MZ B cells,[438] although cells that resemble MZ B cells are present in xid/Btk-deficient mice, leading to some controversy in their origins.[439] Both are decreased by a mutation in the Ig-α tail that weakens overall BCR signaling.[440] They are also both decreased in the Aiolos transcription factor null mouse.[63] Interestingly, deletion of the Pyk-2 tyrosine kinase results in elimination of MZ B cells while B1 cells are still found.[441]

MZ B-cell development can be studied in a heavy chain transgenic mouse model system where large numbers of such cells are produced.[428,442] In this V_H81X heavy chain mouse, B cells with a specific light chain accumulate with an MZ phenotype. This MZ population is eliminated by deletion of CD19, Btk, or CD45, all genetic changes that weaken BCR signaling.[438] MZ B cells are generated in mice even when B-cell development is blocked shortly after birth by conditional deletion of Rag-2,[353] showing that like B1 B cells, a significant portion of MZ B cells are produced during fetal development. Consistent with this fetal origin, many MZ B cells have Ig heavy chains with little or no TdT-mediated N-regions at their V-D and D-J junctions.[443] Interestingly, irradiated mice repopulated with bone marrow precursors still accumulate significant numbers of MZ B cells with low levels on N-addition, indicating that the strong BCR-mediated selection guiding entry into this subset can select such cells from adult bone marrow–generated B cells.

In recent years, there has been considerable progress in understating some of the non–BCR-related pathways that regulate the generation of MZ B cells. For example, the cannabinoid receptor 2 appears important in localization of MZ B-cell precursors to the anatomic region where Notch signals are available.[444] Notch ligand expression for generation of MZ B cells is regulated by the E3 ubiquitin ligase Mind bomb 1, which also is critical for T-cell development.[445] Furthermore, Notch2 activation in MZ B cells is potentiated by expression of the fringe glycosyltransferases lunatic fringe and manic fringe.[446] Notch activation requires its proteolysis, and this is now known to occur through activity of a disintegrin and metalloproteinase 10 (ADAM10).[447]

B-Cell Tolerance and Receptor Editing

A number of transgenic mouse models have been developed for the study of B-cell tolerance (Table 8.2). In two of these systems, high-affinity BCRs are expressed as IgM–IgD transgenes, one specific for the antigen hen egg white lysozyme (HEL), the other for a specific polymorphic determinant on MHC class I.[281,298–300,448–450] The advantage of these systems is that they utilize antigens that can be regulated: Transgenic B cells can develop in either the presence or absence of antigen, and cell transfer experiments can be employed to alter the B cell's antigenic milieu. These have been used initially to confirm ideas on B-cell tolerance that originated in work with nontransgenic B cells, namely that exposure to antigen is generally deleterious to developing B cells, resulting in their elimination or failure to mature (instead entering an "anergic" state). However, the resolution of these systems, coupled with advances in gene targeting and other molecular technologies, have uncovered important new details regarding the way that self-reactive B cells develop (or fail to develop). For example, studies in the anti–H-2 model uncovered an alternative to deletion in response to immature B-cell encounter with antigen, BCR editing to escape autoreactivity.[281]

In the HEL system, differences have been discovered in immature B-cell responses to soluble versus membrane-bound antigen, suggesting that the extent of BCR cross-linking can influence cell fate.[299] Furthermore, studies with this system on different mutant backgrounds that shift BCR signaling thresholds up or down has shown that such alterations can result in striking alterations in selection outcomes.[451,452] Work in this system has shown that one consequence of B-cell tolerance may be arrest of B-cell migration so that follicular entry is inefficient.[330,331] Presumably, failure to reach such follicular niches contributes to handicapping the autoreactive B cells, resulting in their relatively speedy elimination.

TABLE 8.2 Transgenic Models of B-Cell Tolerance

Ig Transgene	Antigen	Background	Effect	Reference
3-83 μk	MHC class I H-2Kk,b	H-2Kk	Deletion, receptor editing	281,300,450
		H2-Kd	Normal development	
3-83 μk	MHC class I H-2Kk,b	H-2Kk lpr autoimmune-prone	Deletion unaffected	308
anti-HEL μδ	HEL	sHEL-Tg	Anergy	298,448,449
		Wild-type	Normal development	
anti-HEL μδ	HEL	mHEL-TG	Deletion	299
anti-HEL μδ	HEL	HEL-Tg lpr autoimmune prone	Deletion unaffected	307
anti-HEL μδ	HEL	sHEL-Tg CD45 null	Self-antigen promoted development	452
anti-HEL μδ	HEL	sHEL-Tg motheaten	Deletion by lower valency autoantigen	451
3H9 μ-only	ssDNA with many light chains	BALB/c	No anti-DNA autoantibodies	301
3H9 μ-only	ssDNA with many light chains	lpr Autoimmune-prone	Anti-DNA autoantibodies	597
3H9 μκ	dsDNA	BALB/c	Deletion, editing	302
3H9 μκ	dsDNA	J_H^-/J_κ^-	Deletion	285
3H9 μλ	dsDNA	Wild-type and lpr Autoimmune prone	Anergy	332,333
3H9-R/Vκ4-R 3H9-R/Vκ8-R	dsDNA ssDNA	BALB/c	Deletion, editing anergy	598
3H9-R/Vκ4-R 3H9-R/Vκ8-R	dsDNA, ssDNA	Rag-2$^-$	Deletion, activation	599
3H9/56R 3H9/56R76R	dsDNA, ssDNA	BALB/c	Deletion, editing	600
Antierythrocyte	Red blood cell	Wild-type	Tg^+ cells only in peritoneal cavity	457–459
$V_H11\mu$, $V_H12\mu$	PtC, BrMRBC	Wild-type	Increased number of B1 B cells	601,602
6C10μ	ATA determinant (glycosylation of Thy-1)	Wild-type	ATA B cells ATA serum	412
		Thy-1 null	Normal development; no ATA	
ATA μκ (6C10μVκ21c)	ATA determinant	Wild-type	Receptor editing or development block in bone marrow; ATA in serum	464,465
		Thy-1 null	Normal development	

ATA, antithymocyte autoantibody; DNA, deoxyribonucleic acid; HEL, hen egg white lysozyme; Ig, immunoglobulin; MHC, major histocompatibility complex.

Another major line of investigation has focused on a more "physiologic" example of pathogenic autoreactivity, the anti-DNA antibodies produced in lpr (Fas-deficient) mice that are generally considered to model the human disease of systemic lupus erythematosus. Analysis of transgenic mice bearing a heavy chain transgene known to be capable of generating anti-dsDNA reactivity with numerous light chains showed that only light chains with ssDNA activity were tolerated in the periphery, and even these did not contribute to the serum antibody pool.[301] Follow-up work uncovered receptor editing in this model[302,453–455] and also showed that when such editing was blocked, the B cells were eliminated in the bone marrow at an immature stage.[285]

Receptor editing, due to failure of Rag downregulation, in replacement of the original light chain by a different one, resulted in decrease or elimination of autoreactivity. Although discovered in several BCR transgenic models, it also occurs at some level in the newly formed B-cell stage in bone marrow, as random pairing of heavy and light chains will result in autoreactivity by some of these cells. Studies with a NF-κB–dependent IκB alpha gene reporter mouse revealed that autoreactive BCRs resulted in activation of the reporter and that reporter+ cells have elevated levels of IRF4, indicating that BCR cross-linking by self-antigen signals through a NF-κB pathway and acts through IRF4 to regulate receptor editing.[456]

Later work analyzing transgenic B cells expressing λ light chain (or in kappa null mice) where B cells have dsDNA binding has shown the failure of follicular entry previously described in the HEL system.[332,333] Interestingly, similar analyses on an "autoreactive" (Fas-deficient) background have shown that this follicular exclusion is lost and production of pathogenic autoantibodies ensues, providing a powerful model for the further characterization of the development of autoimmunity due to breakdown of B-cell tolerance.

A different model of a pathogenic antierythrocyte autoantibody has shown another possible mechanism whereby

self-reactive B cells may avoid deletion or receptor editing by sequestration from self-antigen.[457,458] In this system, the transgenic B cells are largely absent from spleen but instead survive in the peritoneal cavity where exposure to the distinctive microenvironment may also contribute to the persistence of these cells. Eventual activation of the B cells by mitogen or antigen can lead to an autoimmune condition in these mice.[459]

B cells may express BCR coreceptors or modulators of signaling pathways that change the response to self-antigen cross-linking. For example, it appears that enzymatic acetylation and deacetylation of a cell surface carbohydrate by sialate: O-acetylesterase alters B-cell responsiveness, by regulating the function of CD22, a sialic acid binding Ig-like lectin (siglec) that inhibits BCR signaling.[460] As mentioned previously, the level of CD19 also modulates BCR signaling and selection, such that altering CD19 expression can alter the frequency of B1 B cells. Recent work has revealed that mutation of the Wiskott–Aldrich syndrome protein selectively in B cells results in severe autoimmunity due to BCR and TLR hyperresponsiveness.[461]

A common thread in all of the studies described here is the negative impact that the B cell experiences upon interaction with self-antigen, an expected result for systems that model the regulation of pathogenic autoantibodies. However, a class of autoantibodies is produced in healthy individuals, and these "natural autoantibodies" may play a role in early responses to certain classes of pathogens.[401,402,404,462] Such a natural autoantibody has been used to construct a transgenic model system where the self-antigen can be regulated. Most natural self-antigens are common glycosylations or cell constituents such as PtC that cannot be eliminated, but a class of natural autoantibodies binds to thymocytes (antithymocyte autoantibody [ATA]) and many of these recognize a glycosylation that is only present on the abundant thymocyte cell surface glycoprotein CD90/Thy-1.[412] Thy-1–null mice have already been generated,[463] so production of ATA–BCR μ-only transgenic mice enabled the study of the role of antigen in the generation of this natural autoantibody. Interestingly, both production of serum ATA and accumulation of B cells with the appropriate light chain (by rearrangement of endogenous Ig light chain locus) for the ATA–BCR required the presence of Thy-1 self-antigen.[412] Thus, at least some B cells are selected for binding to self-antigen, although these may belong exclusively to specialized B-cell compartments, such as the B1 B-cell subset.

This ATA–BCR/Thy-1 tolerance model has been extended by the production of ATA μκ transgenic mice, where most developing B cells express the natural autoreactive specificity.[464] In such mice, most of B cells developing from bone marrow become arrested in spleen and either cease maturation or else "edit" their BCR by replacing the transgene-encoded light chain by a different light chain as a consequence of rearranging the endogenous light chain locus. This resembles typical "negative selection," as seen with other transgenic models.[281,298–300,448,450] Nevertheless, some B1 B cells are detected, and high levels of serum autoantibody are produced, suggesting that some B cells with this BCR are capable of maturing, perhaps in specialized microenvironments or at distinctive (fetal/neonatal) times.

The role of BCR signaling intensity in directing B cells to various specialized subpopulations has been studied using the ATA μκ BCR transgenic model, by altering the levels of Thy-1 self-antigen and then examining B-cell development.[465] This analysis observed follicular B-cell development in the absence of antigen, but MZ B-cell production in the presence of very low levels of autoantigen. As shown previously, normal physiologic levels of the Thy-1 autoantigen results in a block in bone marrow development, but allowed B1 production, with concomitant generation of ATA detected in serum. This work led the authors to propose a model of B-cell development where strong BCR signaling produces B1 B cells (particularly during fetal development), intermediate BCR signaling yields MZ B cells, and weak (or negligible) BCR signaling results in follicular B-cell generation.

Role of Complement, Serum Antibody, and Cluster of Differentiation 5 in B-Cell Tolerance and Response

The importance of complement in the immune system has long been recognized based on classic experiments showing that cobra toxin decreased responses.[466–468] Over the past 10 years, the importance of complement in immune responses, explaining the function of adjuvants, has become clearer.[469] In the context of B-cell development, components of the complement system also play a role in modulating BCR responses and negative selection of B cells. At least in the HEL–Ig/sHEL system, altering the strength of the BCR signal, by CD45R or PTP1C/SHP1 inactivation, could either reduce or enhance B-cell deletion.[452] Similar results could be obtained by altering the BCR-associated chain CD19.[470] Considering the role of complement as a coreceptor for modulating BCR signaling thresholds, the finding that HEL double transgenic mice that also are Cr2 (CD21/CD35) null develop peripheral B cells that are apparently not fully anergized, as they could still respond to antigen challenge.[471] This could be due to an inefficient retention of antigen (sHEL) on stromal/dendritic cells and, therefore, weaker signaling by a monomeric soluble antigen to developing/transitional B cells in the bone marrow or spleen. Potentially, a major role for complement would be to localize self-antigens on bone marrow stromal and dendritic cells facilitating tolerance at the newly formed B-cell stage. An implication of these findings is that complement deficiency could result in accumulation of functional autoreactive cells, possibly leading to autoimmunity.

Natural autoantibody, a major component of the serum, may have a role (together with complement) in maintaining tolerance to highly conserved self-antigens.[469] Some such IgM antibodies, highly conserved phylogenetically, have been shown to recognize conserved carbohydrates beta-glucan and chitin structures present on fungi and so serve to enhance host defense.[472] It also appears that it plays a role in amplifying immune responses, as suggested by studies with a mutant mouse lacking the μ heavy chain secretory exon.[473] The deficit due to a lack of serum IgM was particularly severe in an acute peritonitis model, where some restoration of responsiveness could be obtained by injection of a monoclonal IgM antibody derived from CD5+ B cells.[473] Finally, in infection with influenza virus, it appears that early presence

of natural autoantibody was equally as important as antibody induced during the course of infection in mediating viral clearance and survival.[404] This work has led to the proposal of a two-phase description of immune responses, with an early T cell–independent phase dominated by natural autoantibody, likely critically dependent on innate immune recognition and activation of complement, leading to a later T cell–dependent phase culminating in the GC reaction, producing high-affinity antibody and memory B cells.[474]

The presence of CD5 on many of the B1 B cells has led to the question of whether it plays a direct role in maintaining such self-reactive B cells, particularly in light of its role in altering T-cell selection thresholds as demonstrated in the CD5-null mouse.[475] Analysis of B-cell responses to BCR cross-linking in CD5-null mice suggested that the presence of CD5 makes the normal population less likely to respond by secreting IgM,[476] effectively "raising the threshold" for their response, possibly by promoting interaction of the BCR with SHP-1.[477] Analysis of the effect of CD5 expression on tolerance in the HEL-Ig/sHEL system also suggested a modulating role, with absence of CD5 leading to a loss of B-cell tolerance and production of serum anti-HEL autoantibody.[478]

B-CELL DEVELOPMENT IN HUMANS: SIMILARITIES AND DIFFERENCES FROM MOUSE

Fetal Development

As described for mouse, human B-cell development can be broadly divided into that taking place prior to birth and that operating after birth and throughout life. The liver and spleen are major sits of fetal B lymphopoiesis in humans and, as in mouse, the bone marrow is the predominant site in adults, with production continuing over most of an individual's lifespan, with some decrease in the aged. The fetal omentum has also been described as a site for B-cell development similar to mouse.[9,479] There is some indication that VH gene usage is more restrictive in fetal development.[480] TdT, absent from mouse fetal development, is similarly missing very early in human fetal development but is expressed by the eighth or ninth week of gestation based on detection of N-region addition.[480] In general, there is more N-addition (and consequently longer CDR3 regions) in human antibodies compared to mouse at all stages of development, including the adult, although the significance of this is not known. Analysis of human T-cell development indicates that fetal and adult T cells arise from distinct developmental pathways, with fetal cells allowing more self-reactivity to persist[481] similar to mouse.[482] Finally, as recently described in mouse, B cells with the capacity to rapidly secrete IL-10 have also been identified in humans, and such cells appear to be increased in patients with autoimmune disease, such as rheumatoid arthritis.[483]

Bone Marrow Development

B-cell development can be detected in human bone marrow from 20 weeks of gestation and continues throughout life.[484,485] Phenotypic subdivisions similar to those in the mouse have been described for developing bone marrow cells[486] (Fig. 8.14). Thus, CD19 identifies B-lineage cells at all stages, with the earliest stages also expressing CD34, a molecule found on MLPs.[487] These CD19+CD34+ cells have been shown to express TdT, Rag-1 and Rag-2, the SLC orthologs,

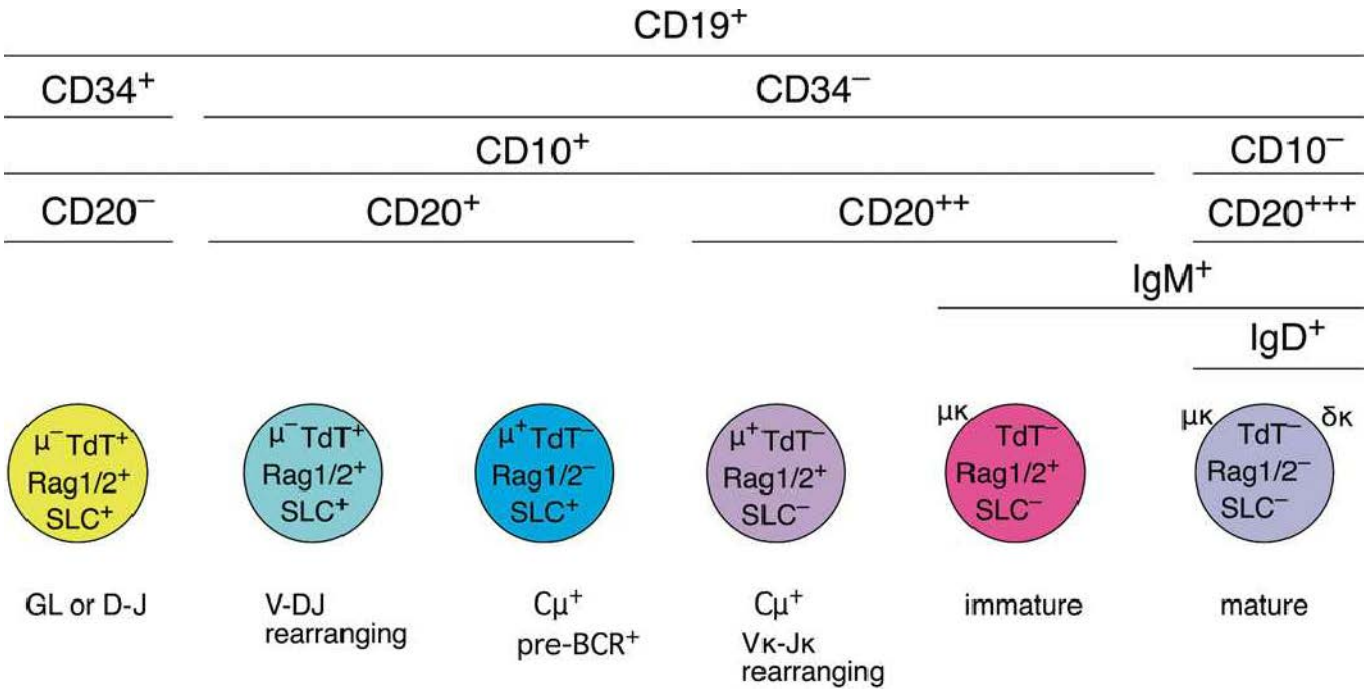

FIG. 8.14. Stages of Developing B-Lineage Cells in Human Bone Marrow can be Delineated, Based on Expression of Combinations of Cell Surface Proteins, Similar to Mouse.

and Ig-α/Ig-β.[486] They contain D-J, but not productive VDJ rearrangements. The next stage can be defined by loss of CD34, with a fraction that is still IgM– (pre-B cells) and another subset that is IgM+. The pre-B cells express heavy chain in their cytoplasm and have downregulated expression of TdT.[486] All of the IgM+ cells express a marker lost upon final maturation, CD10, so they are immature B cells. A recent study examined the B-lineage potential of cells expressing different levels of CD10 and found that increasing levels of this molecule were found on more differentiated B-lineage cells, suggesting its potential for identifying distinctive stages in B-cell development in humans.[488]

Human early B-lineage cells have proven much more difficult to grow in culture than mouse cells, although conditions have now been defined that allow their expansion in vitro. One of the most surprising differences between mouse and human B precursor growth has been much less dependence on IL-7, suggesting that a different cytokine may substitute in humans.[489–491] The finding that lack of the cytokine common gamma chain (γc) in mouse blocks B-cell development but spares T-cell development, whereas the reverse is the case in humans,[154,492] demonstrates clear differences in cytokine dependence between mouse and human lymphopoiesis.

Human B precursors also express orthologs of λ5 and VpreB, although their organization and number differ from the mouse.[493–495] Their expression occurs at a corresponding stage and analysis of the human EBF gene has shown that its targets are similar to those of mouse EBF, including Ig-α, Ig-β, and 14.1 (the human ortholog of λ5).[496] Importantly, mutations in components of the pre-BCR lead to immunodeficiency diseases in humans, pointing out the similar and crucial role that pre-BCR signaling plays in human B-cell development.[497–499]

Germinal Center Differentiation

As in mouse, GCs are anatomic structures in secondary lymphoid organs where T-dependent immune responses occur, selecting high-affinity clones during the process of somatic hypermutation and eventually generating memory B cells. The GC consists of B cells at a variety of differentiation states, from early activated B cells through the plasmablast stage. Therefore, study of this process has benefited significantly from resolution of intermediate stages using multiparameter flow cytometry and carefully chosen cell surface markers. In one study, this allowed definition of two IgD+ stages, two GC stages, and a memory stage in tonsillar B cells.[500] This work focused on the levels of somatic mutations accumulating as cells passed through this pathway, finding that it was first detected in the initial GC stage (centroblast). Subsequent work has shown that IgD+ somatically mutated cells can be detected in peripheral blood, based on expression of CD27, suggesting that they are IgM+IgD+ memory B cells.[500]

Recent work has found an interesting link between somatic hypermutation and certain Bcls. Bcl-6 is a transcriptional repressor that is linked to both GC B cells and to B lymphomas that likely derive from GC B cells.[501,502] Bcl-6 expression is high in GC B cells and is required for formation of the GC.[503] Strikingly, its 5′ regulatory region is mutated as a consequence of hypermutation in GC B cells, the first example of hypermutation targeted outside the Ig regions,[501,502,504] and links this process to deregulated cell growth and lymphomagenesis. These findings have focused interest on Bcl-6, and recent work has shown that it binds to the promoter of the antiapoptotic proto-oncogene Bcl-2 by interacting with the transcriptional activator Miz1, also suppressing Miz1 activation of Bcl-2.[505] In lymphomas, Bcl-6–mediated suppression of Bcl-2 is lost, either by chromosomal translocations to Bcl-2, mutation of Bcl-2, or Miz1 deregulation.

Abnormalities of Development

A key discovery in the past decade has been the finding that a well-known immunodeficiency, xid, characterized by inability to respond to bacterial infections and a severe deficit in peripheral B cells,[506] is due to mutations in Btk.[507] Shortly after this finding, the mouse ortholog of Btk was shown to be the cause of murine xid, an extensively studied mutation originally identified in CBA/N mice.[508] Btk deficiency in humans is more severe than xid, with little B-cell development past the early B-cell stage, in contrast with an absence of normal peripheral B-cell development in mouse and inability to respond to certain types of T-independent antigens.[509] This difference is not simply due to specific difference in the mutations, as a complete null mutation in mouse is indistinguishable from xid.[418] Thus, human B-cell development, likely at the pre-BCR signaling stage, is much more dependent on Btk.

While xid is by far the most common B-cell deficiency, amounting to more than 80% of those identified, non–X-linked mutations have also been observed. These correspond to mutations in the pre-BCR signaling complex, and, in most cases, similar effects had been observed in the mouse. For example, deletions or mutations in the μ constant regions accounted for another 5%.[499] Examples have been found of mutations in the 14.1 gene, the human ortholog of the mouse surrogate light chain λ5 protein,[497] and also in Ig-α.[498] In both of these cases, early B-lineage cells, identified as CD19+CD34+ were present in normal numbers in bone marrow, but CD19+CD34– Cμ+ cells (pre-B cells) and all later stages were absent. Finally, a patient with a mutation in BLNK, an adaptor protein that links pre-BCR signaling from syk to the rest of the signaling cascade, showed a similar phenotype.[510]

Very few peripheral B cells are detected in any of these disorders, which suggests that pre-BCR signaling is more critical in human than in the mouse, where mutations in λ5 and BLNK allow the generation of variable numbers of peripheral B cells. The reason for this difference is not yet understood. Interestingly, common variable immunodeficiency has been shown to result from mutations in the common gamma chain[154] or in the JAK3 gene[511] consistent with IL-7 playing a much less critical role in human pre–B-cell growth than in mouse, as had already been determined from culture studies.[489,490] B-cell development is relatively

intact, whereas T cells are ablated. The reverse is true for mouse.[512,513]

Analysis of B cells with mutations in genes required for normal TLR signaling has revealed the importance of such signaling for maintenance of B-cell tolerance.[514] This study examined the binding specificity of BCRs isolated from B cells of patients with defects in IL-1 receptor-associated kinase 4, myeloid differentiation factor 88, and UNC-93B. As all TLRs but TLR3 depend on IL-1 receptor-associated kinase 4 and because TLR3 depends on UNC-93B, this work assessed the contribution of all TLR signaling. Autoreactive BCRs were found at high frequency in the newly formed and mature B-cell pools in all patients, establishing a critical role for TLR signaling in establishing and maintaining B-cell tolerance. Interestingly, none of these patients had serum autoantibody or autoimmune disease, suggesting that some further signal, possibly TLR mediated, is needed for disease progression.

Insights into B-Cell Malignancies

One of the principle reasons for studying the regulation of B-cell development is that defects in this process may result in lymphoma. Human B-lineage neoplasias can be viewed as transformed counterparts of normal B-cell developmental stages, such as pro-B, pre-B, immature B, mature B, or plasma cell, based on rearrangement status and surface phenotype.[493] In some cases, transformed cells may even retain growth characteristics of the normal counterpart. This type of classification scheme, correlating features of lymphomas and leukemias with their normal counterparts, has been useful in diagnosis and prognosis of B-lineage neoplasias. For example, B-precursor ALL, the most common type of ALL in children (ALL accounts for 25% of childhood cancer), is a clonal expansion of a cell defined by surface phenotype and Ig rearrangement status as representing the pro-B stage.[515–517] Recent analyses of B-precursor ALL suggests that they can be subdivided into a pro-B type, predominant in pediatric patients, and a pre-B type, more frequent in adults.[518] A subtype of adult pre-B ALL is Philadelphia chromosome-positive, being defined by the oncogenic BCR–ABL1 kinase and showing deletion of the Ikaros gene in most cases. Pre-BCR signaling mediated cell cycle arrest has been shown to depend on normal Ikaros function,[519] providing an explanation for the high incidence of Ikaros deletion in this disease.

Whereas traditional chemotherapeutic agents typically target proliferating cells without specificity for malignant cells, the identification of molecular abnormalities that result in the abnormal survival and proliferation of leukemic cells may lead to the design of novel therapies that specifically target these cells. For example, the successful treatment of mice carrying human B-precursor leukemias with antisense strategies and tyrosine kinase inhibitors holds promise for efficacy in humans.[520,521] The identification of novel translocations in B-precursor ALL also may identify mechanism(s) responsible for this disease at the molecular level.[522–525]

Another example is diffuse large B-cell lymphoma (DLBCL) that appears to arise from GC B cells[526] that are undergoing somatic hypermutation of their IgV genes mediated through activity of AID.[366,367] As mentioned previously, aberrant somatic hypermutation in DLBCL has been shown to induce mutations in the Bcl-6 gene.[501,502,504] As mentioned previously, Bcl-6 is often found to be overexpressed in DLBCL by either hypermutation of its promoter or chromosomal translocation, but investigation of DLBCLs lacking these genetic alterations has revealed that Bcl-6 is targeted for ubiquitylation and proteasomal degradation by a SKP1-CUL1-F-box protein ubiquitin ligase complex that contains the orphan F-box protein FBXO11.[527] FBXO11 was found to be mutated or deleted in DLBCL cell lines and also in primary leukemias, thus revealing another mechanism for Bcl-6 dysregulation, leading to lymphoma.

AID in DLBCL likely is responsible for widespread genome instability,[528] and using a mouse model system, AID was clearly identified as responsible for c-myc to Ig V-J(H) translocations.[529] Examination of known non-Ig targets of AID also identified a high rate of mutation of PAX5 in both classic and nodular lymphocyte-predominant Hodgkin lymphoma.[530] Microarray gene profiling showed that the NF-κB signaling pathway is constitutively activated in DLBCL and as normal NF-κB activation by BCR cross-linking requires CARD11, a cytoplasmic scaffolding protein, this gene was examined for mutations. Ten percent of DLBCL tested had missense mutations, providing a rationale for development of new therapeutic agents targeting this pathway.[531] Other work has identified a negative regulator of NF-κB signaling, A20, as a frequent target in B-cell lymphomas, by using genome-wide analysis of genetic lesions.[532] Thus, early "whole genome" analyses of leukemias and lymphomas by microarray gene profiling has been followed up by high-resolution determination of gene copy and targeted sequence analysis. Now, the availability of whole exome or even whole genome high-throughput sequence determination will undoubtedly identify as yet unsuspected key driver mutations in these diseases, potentially leading to new and more personalized therapies.

Another therapeutic approach makes use of knowledge of the surface phenotype of the transformed cell, as in the recent development of anti-CD20 therapy for several types of B lymphomas.[533] CD20 is a 33 kDa phosphoprotein expressed highly on the surface of mature B cells.[534–536] Antibodies to CD20, originally called B1, were initially characterized by their stimulatory and inhibitory effects on human B cells, indicating the importance of CD20 in regulating B-cell proliferation and differentiation.[537–539] Therapeutic anti-CD20, called rituximab, is a chimeric antibody derived by fusing the V regions of a mouse antibody to the human IgG1 constant segment.[540] The precise mechanism of depletion is not yet fully understood but likely include contributions from antibody-dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity, and direct antibody binding effects, including sensitization to apoptosis.[533]

As most of the cells in many indolent B-cell neoplasms, such as non-Hodgkin lymphoma or chronic lymphocytic leukemia (CLL), are not predominantly in cycle, the problem may be more a failure to die appropriately, rather than a failure to regulate proliferation. A greater understanding of the

growth and sensitivity to apoptosis of different types of lymphomas and leukemias may allow more specific targeting using this approach.[541] For example, while CLL cells express relatively low levels of CD20, likely requiring higher doses of anti-CD20 for a response, a combination with another antibody recognizing a molecule that is highly expressed on CLL, CD52 has shown promise.[542] Alternatively, treatment with anti-CD20 may render the cells generally more sensitive to apoptosis so that use in combination with more conventional chemotherapeutic agents will be efficacious. These combination therapies are already in clinical trials. Finally, new insights into distinctive growth properties of transformed B cells, such as the role of Wnt signaling in CLL,[543] may suggest new approaches to treat such disorders.

ALTERNATIVE STRATEGIES FOR B-CELL DEVELOPMENT

The broad outlines and even many of the details of B-cell development are quite similar in mouse and man, but there are striking differences in other species. There is a notable common alternative approach that involves generation of Ig+ cells during fetal/neonatal development with a relatively restricted repertoire that is then diversified by novel approaches (gene conversion or somatic hypermutation) in specialized lymphoid organs that are associated with the gut. In these species, most development from Ig− precursors appears to cease by birth, and the B-cell population is maintained by self-renewal of mature B cells. Here, we consider the development and diversification of B cells in chicken and rabbit.

Chicken

B-cell development in the avian occurs in the bursa of Fabricius.[544,545] In fact, the term "B" cell refers to "bursa derived," reflecting the historic origins of research in lymphocyte development. That is, removal of the bursa just after hatching eliminated the ability to mount an antibody response, demonstrating the importance of this organ in generating cells capable of antibody formation.[544] In contrast with the bone marrow, the bursa, being associated with the gut,[546] facilitates exposure of developing cells to external antigens and bacterial flora. B-cell development in chicken is usually divided into three stages: prebursal, bursal, and postbursal.[547]

During prebursal development, at day E5, early precursors can be identified in the para-aortic foci,[548] likely corresponding with similar precursor stages localized in this anatomic site in mammals.[2] B-lineage commitment, as indicated by D-J rearrangements, is detected in the YS at day E5/6, and V gene rearrangement is found 3 days later.[549] Unlike mammalian ordered development, light chain rearrangement is detected at about the same time as heavy chain, and light chain can precede heavy chain.[550] This means that there is no pre-B stage, per se, and also probably no requirement for SLC. Rearranging B-lineage precursors migrate into the bursal mesenchyme at about day E12, and, thereafter, these cells begin to proliferate in bursal epithelial buds. This proliferation selectively expands cells that have BCRs. These receptors have limited diversity, as the heavy chain is formed by rearrangement of a single VH, several Ds, and a single JH pairing with a light chain generated by rearrangement of a single VL with a single JL.[551,552] As in mouse fetal development, there is no TdT-mediated N-region addition at the junctions.[552]

This "prebursal" receptor is diversified by gene conversion by a set of V pseudogenes during this proliferative phase. At about hatching, these cells become exposed to the contents of the bursal lumen that is connected to the gut lumen via the bursal duct similar to the appendix. Thus, these proliferating B cells are exposed to the contents of the digestive tract, and there is also reverse peristalsis at the end of the gut that transports external antigens into the bursal duct.[546] At about this time, the level of apoptosis increases dramatically, and it is possible that only 5% of the cells generated in the bursa eventually emerge.[553] This death may be due to generation of nonfunctional receptors during the course of gene conversion or it may reflect antigenic selection.

At hatching, emigration of B cells from the bursa increases, but most of these cells constitute a population with a relatively short half-life measured in days.[554] The long-lived pool colonizes the peripheral lymphoid organs over several weeks as the bursa atrophies. By 3 weeks after hatching, bursectomy no longer results in agammaglobulinemia, indicating that the postbursal phase has become established.

Rabbit

B-cell development in rabbit is similar to chicken, in that B cells initially are produced with a limited BCR diversity[555–557] during fetal life, with little new production after birth.[558,559] This repertoire is then expanded through gene conversion[555,560] and somatic hypermutation[560,561] in a specialized gut-associated organ, the appendix.[562] However, unlike chicken, this diversification process is dependent on antigen availability.[563–565]

Pre-B cells can be found in rabbit before birth in the liver, bone marrow, and omentum,[558,566–568] but B lymphopoiesis decreases at birth and is negligible in adult animals.[559] Ig rearrangement during fetal and neonatal times is dominated by usage of the most D-proximal V_H gene paired with multiple V_L genes in the light chain.[556,557,569–572] In contrast with chicken and mouse, there is significant N-addition.[557,571] From 4 to 8 weeks after birth, there is a striking increase in the diversity of this primary repertoire that occurs during proliferation of B cells in the gut-associated lymphoid tissues (GALTs).[559,560] V genes are diversified by both gene conversion[555] and also by somatic hypermutation.[560,561] Importantly, surgical removal of GALT organs, appendix, sacculus rotundus, and the Peyer patch, from neonatal rabbits led to unresponsiveness to many antigens, suggesting that this diversification was crucial for normal immune function.[573] This finding has been confirmed by sequence analysis, demonstrating that removal of the GALT blocks diversification.[562]

Considering the relatively late diversification in rabbit compared to the chicken, these gut areas will provide a milieu

of microbial antigens, and this appears to be a critical aspect of the diversification process.[574] For example, surgery to prevent access of intestinal flora to the appendix blocked diversification in this organ[575,576] that could be restored by reversing the ligation.[575] Furthermore, analysis of rabbits reared in germfree conditions revealed abnormal cellular development in the GALT[564] and a lack of responsiveness to certain antigens.[563] The dependence of V gene diversification on antigen has been directly demonstrated in animals where the sacculus rotundus and Peyer patches were removed at birth and the appendix ligated. Testing the peripheral blood B cell V gene repertoire showed an absence of diversification in contrast to controls.[565] Although the mechanism for this stimulation remains to be established, possibilities include B-cell activation by a BCR superantigen[577–579] or through a B-cell TLR.[580]

Two B-Cell Developmental Pathways

The similarities of chicken and rabbit B-cell development with that in other species such as sheep, swine, and cow suggests that the initial production of a limited BCR repertoire during fetal/neonatal life, diversification at a later time, and maintenance of the B-cell pool in adult life by self-renewal (rather than de novo generation from unrearranged precursors) is a major pattern in the design of the immune system (Fig. 8.15). This pattern contrasts with that described for mouse and man, where ordered heavy and light chain rearrangement, pre-BCR selection, and replacement of senescent B cells by newly generated B cells are major aspects of development. This raises the interesting question of whether there is an analogous B-cell development pathway in humans or mouse, perhaps as a vestige.

The idea of two pathways in bone marrow development was suggested previously based on the observation that heavy and light chain ordering is not absolute: Light chain rearrangements are detected in mice incapable of making heavy chain rearrangements and can be detected in cells early in B-cell development.[581] In one pathway, heavy chains rearrange first and the pre-BCR is assembled and signals downregulation of Rag-1/2 expression (ending heavy chain rearrangement), clonal expansion, and accessibility of the kappa locus. Cessation of proliferation is coincident

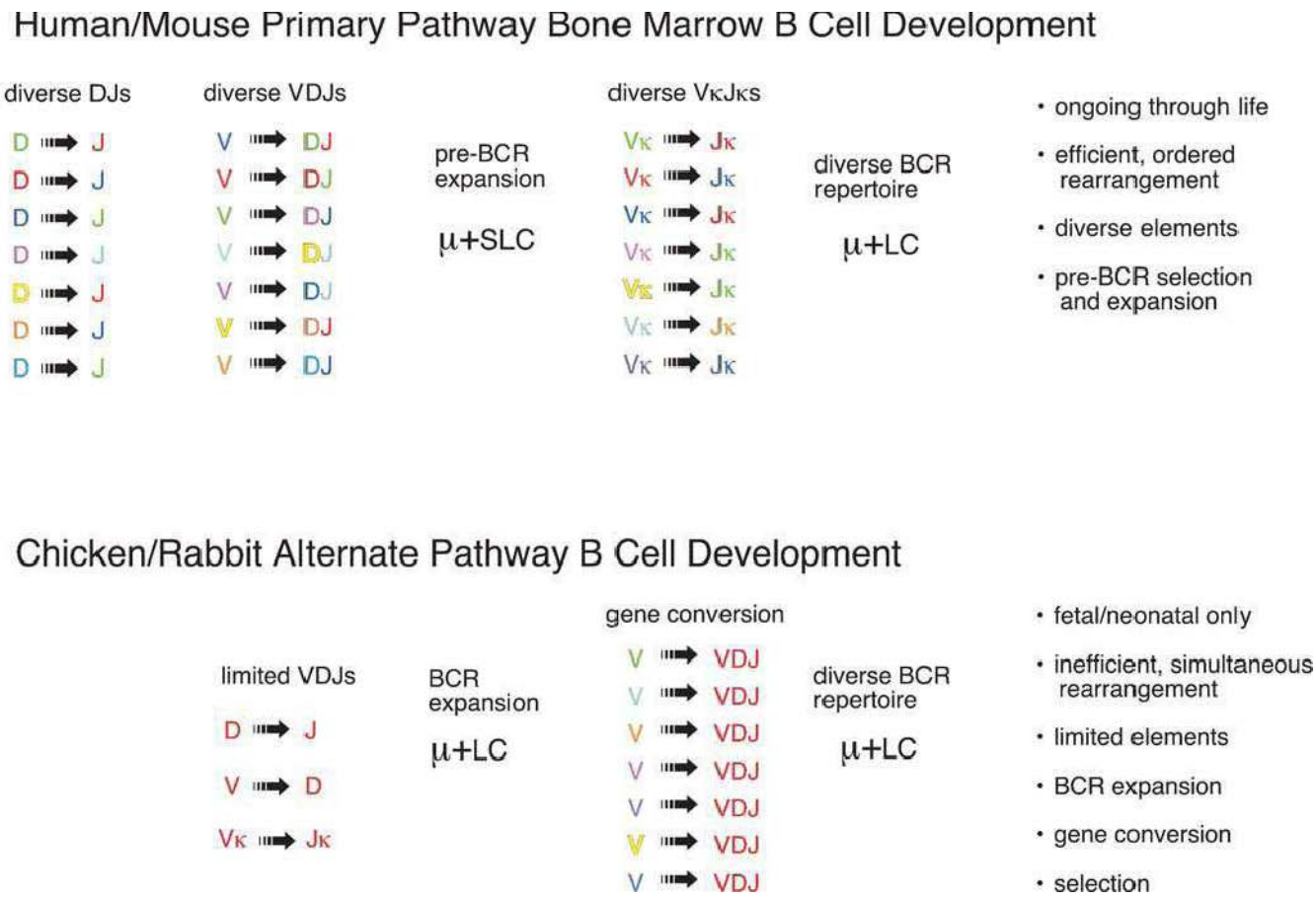

FIG. 8.15. Alternative Strategies of Repertoire Diversification and B-Cell Development. In both mouse and human bone marrow B-cell development, ordered rearrangement predominates, with a pre–B-cell receptor (BCR_ selection phase dividing heavy and light chain rearrangement. The eventual outcome is a population of newly formed B cells with a diverse set of BCRs produced throughout life. In chicken and rabbit, immunoglobulin rearrangement appears less efficient (usually the other allele is germline) and much less diverse, with a single BCR for chicken. However, these cells proliferate and undergo gene conversion during fetal/neonatal life, followed by selection, eventually generating a set of B cells with a more diverse repertoire that persist for the life of the animal.

with reinduction of Rag-1/2 and light chain rearrangement. Expression of a complete BCR signals a final termination of Rag expression. In this model, pre-BCR and BCR mediates allelic exclusion by a feedback mechanism regulating Rag expression. Thus, most B cells have D-J or even VDJ rearrangements on both alleles. In the alternative pathway, heavy and light chains rearrange stochastically, and allelic exclusion is mediated by relative inaccessibility of the loci (and thereby low frequency of rearrangement). If this is the major pathway in chicken and rabbit, it is consistent with the observation that most chicken B cells have only one allele rearranged.[552,582,583] Furthermore, the careful ordering of TdT expression, being downregulated at the light chain rearranging stage, is apparently not the case for rabbit, where a large percentage of light chains have N-regions.[572]

Most rabbit B cells express CD5.[584] In mouse, much of the CD5+ B-cell population is generated during fetal/neonatal development and persists in the adult through self-renewal.[392] Furthermore, these cells express a relatively restricted BCR repertoire that is dependent on antigen selection.[23] Finally, the pre-BCR selection phase of several heavy chains abundant in CD5 B cells appears to follow different rules than classical pre–BCR-mediated expansion.[263] In fact, the process of development in mouse fetal liver may predominantly follow this alternative pathway.[406] Thus, in mouse, fetal development, culminating in production of B1 B cells, may represent a type of alternative or "primitive" B-cell development, as has been proposed previously.[585] It is less clear whether a similar distinction exists in human B-cell development, as data are lacking. Nevertheless, one can envision a primordial pathway, randomly combining heavy and light chains and simply selecting cells that express BCRs with weak reactivity to self or environmental antigens as an alternative to ordered rearrangement, pre-BCR selection, and BCR selection—an elaborate process fine tuned to generate more variation.

CONCLUSION

There has been considerable progress over the past decade in understanding the mechanisms that regulate B-cell development, with important insights coming from application of the novel genetic approaches of transgenesis and gene targeting. In a sense, this has allowed progression from research with simple model systems using cell lines to analysis of "normal B cells" in whole animals. The use of such mutant mouse "reagents," together with much higher resolution of normal development made possible by multiparameter flow cytometry, has proven a powerful combination for unraveling much of the complexity of this process. It is daunting to attempt to predict where new advances in the field will come, but undoubtedly, the completion of the genome sequence for both mouse and human will provide impetus to large-scale gene profiles of B-cell development, as have already begun.[586,587] A complementary approach to gene targeting based on such profiling will involve characterizing new mutant mice generated by chemical mutagenesis.[588,589] Considering the recent unanticipated discoveries of AID in isotype switching and BAFF in peripheral B-cell development, it seems likely that the coming decade will provide many surprises. A goal will be to eventually understand how the interplay of the innate and adaptive immune systems generates protective responses while avoiding autoimmune pathologies at the organism level.

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CHAPTER 9

B-Lymphocyte Receptors, Signaling Mechanisms, and Activation

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INTRODUCTION

The primary function of mature B lymphocytes in response to foreign antigens is to proliferate and differentiate into antibody-producing plasma cells that secrete large quantities of antibodies. Effective antibody responses are highly specific and of high affinity for the inducing antigen and of the appropriate isotype to allow the antibodies to carry out the effector functions, as described in Chapter 5, that best serve to eliminate an antigen-containing pathogen. For B cells to produce the most effective antibodies, they must have mechanisms that allow them to discern their specificity and affinity for antigens and to produce plasma cells that secrete antibodies of the appropriate isotype. In this chapter, we explore what we know and are learning about these mechanisms. Understanding how B cells recognize and respond to antigens will aid in our efforts to develop effective vaccines and to target therapies to block hyper-B-cell responses as in systemic autoimmune diseases and in some B-cell tumors.

B cells are activated by antigen by a process of clonal selection, a fundamental feature of the adaptive immune response. Each B cell expresses a membrane form of a single heavy (H) chain (V_HC_H) and a single light (L) chain (V_LC_L) that are assembled together with a noncovalently bound immunoglobulin (Ig)α–Igβ heterodimer into an antigen receptor, the B-cell receptor (BCR), which is expressed on the B-cell surface (Fig. 9.1). It is conservatively estimated that during a lifetime, an adult human expresses more than 10^{13} unique clonally distributed BCRs. This estimate is based, in part, on the diversity of H and L chain variable (V) regions that can be generated given the germ line encoded V gene segments and the molecular combinational and mutational mechanisms that create the V genes described in Chapter 6. When an antigen enters the immune system, it selects from among this extraordinarily large array those B cells whose receptors fit it best and signal these to proliferate and differentiate into antibody-secreting plasma cells. Even very simple antigens are predicted to find hundreds of B cells that express BCRs with sufficient specificity and affinity to be activated. This process of antigen selecting best fits from among the enormous array of preexisting B cells to proliferate and differentiate into antibody-secreting cells is a complicated process that takes place in highly specialized microenvironments in the secondary lymphoid organs and involves the functions of both T cells and innate immune system cells, as will be described in Chapter 10. Here, we focus on the events that follow antigen binding to the BCR that initiate signaling, an essential, critical first step in B-cell activation.

Antigen binding to the BCR triggers several signaling cascades that lead to the transcriptional activation of a variety of genes associated with B-cell activation (see Fig. 9.1). The BCR is also an internalizing receptor; under the influence of the signaling cascades, the BCR and bound antigen are actively endocytosed into the cell and trafficked to specialized intracellular compartments in which the antigen is processed and presented on major histocompatibility complex (MHC) class II molecules, as described in Chapter 22. The MHC-peptide complexes then move to the B-cell surface where they activate antigen-specific helper T cells to provide T-cell help, a complex process involving both direct interactions between B cells and T cells as well as the release of soluble cytokines by the T cells.

A considerable amount of what we know about the biochemical nature of the signaling pathways triggered by antigen binding to the BCR comes from studies in which multivalent antigens are provided to the B cell in solution (see Fig. 9.1). Although B cells can respond to antigens in solution, a variety of recent studies suggest that antigens are presented to B cells on the surface of antigen-presenting cells (APCs) including macrophages and dendritic cells present in the local microenvironments of the lymph nodes and spleen in which B cells are activated in vivo (see Fig. 9.1). Antigens may be presented on macrophages and dendritic cells as part of complement-fixed complexes bound to the complement receptors or immune complexes bound to FcRs expressed by these cells. We do not yet know if the molecular mechanisms by which antigens in solution and antigens on cell surfaces initiate signaling are identical, even though it appears in both cases that the engagement of antigen by the BCR results in the triggering of similar signaling cascades.

In this chapter, we describe what we are learning about the events that occur following the binding of the BCR to antigen that trigger signaling cascades that lead to B-cell activation. The question that will be addressed is how is the BCR's specificity and affinity for an antigen read by the BCR and transduced across the B cell's membrane to trigger intracellular signaling cascades that induce the B cell's response. The signaling cascades that are set off upon antigen binding have been described in considerable biochemical detail and will be reviewed in this chapter. However, biochemical analyses do not capture the spatial and temporal dynamics of the events that are triggered by antigen binding to the BCR. The recent applications of live cell imaging technologies are providing the first views of B cells as they encounter antigen with the resolution to follow individual BCRs. These images are showing us

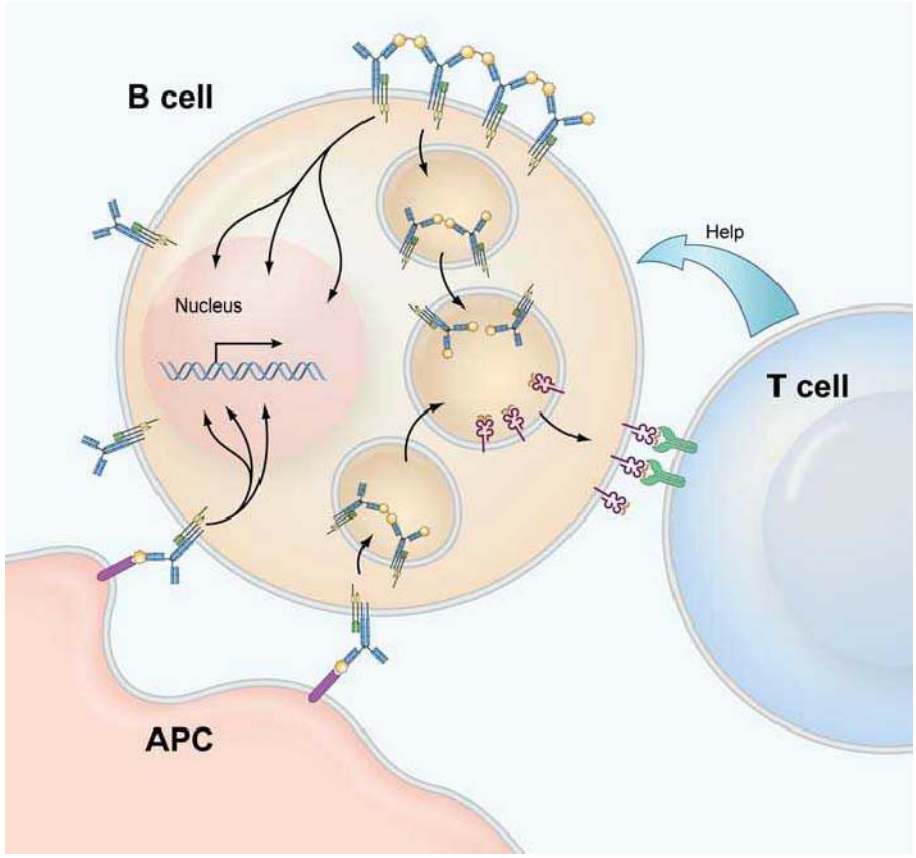

FIG. 9.1. Overview of the Activation of B Cells by Antigen. B-cell activation is initiated by the binding of antigen to the B-cell receptor (BCR). B cells encounter antigen either in solution or on the surface of an antigen-presenting cell (APC), likely as complement coupled antigens or as immune complexes bound to APC complement receptors or FcRs. Antigen binding triggers signaling cascades that lead to the activation of a variety of genes associated with B-cell activation. The BCR is also a trafficking receptor; under the influence of BCR signals, it transports antigens into intracellular compartments where the antigens are processed and presented on major histocompatibility complex class II molecules for recognition by helper T cells.

that the activation of B cells by antigen is far more dynamic than originally thought. It has been possible to infer from the behavior of the B cells and BCRs in these images the mechanisms underlying antigen-driven events that lead to B-cell activation. Such images are changing our view of BCR-mediated B-cell activation and are providing the tools to gain an understanding of the mechanisms underlying diseases that result from inappropriate B-cell activation including systemic autoimmune diseases and certain B-cell tumors.

As we consider the mechanisms by which antigens trigger B cells through BCRs, we need to be aware that B cells encounter antigen in both developmental and environmental contexts, and that these contexts greatly influence the outcome of antigen engagement. Consider, for example, that B cells express BCRs throughout their development from immature B cells to memory B cells. However, engagement of the BCR by antigen at different developmental stages has different outcomes. The binding of self-antigens to the BCRs expressed on immature B cells signals these cells, but the signals do not result in activation. This means that the developmental state of the B cell dictates the outcome of the BCR's engagement of antigen, either driving proliferation and differentiation in mature B cells or triggering mechanisms that lead to elimination or silencing of self-reactive immature B cells, as described in Chapter 8. The basic structure of the BCR does not change from the immature to the mature B-cell stage, but the BCR signals differently in different developmental cellular contexts. The outcome of BCR signaling is also influenced by the environmental context in which the antigen is encountered. Antigens enter the immune system in various contexts as relatively simple vaccines to complex microorganisms including viruses, bacteria, and parasites. Both vaccines and pathogens bring with them materials that can activate B cells through coreceptors other than the BCR as well as activate T cells and cells of the innate immune system to secrete products that bind to B cell coreceptors that influence how the BCR signals in response to the antigen. The molecular form of the antigen itself can influence the outcome of BCR antigen binding, as described in Chapters 10 and 23. Bacteria and some viruses display rigid arrays of antigens on their surface that induce antibody responses in the absence of helper T cells (coined T-independent antigens), as do polysaccharides on bacteria in which the carbohydrate moieties are arrayed as multimers. Thus, in predicting the outcome of BCR-antigen binding, we need to consider not only the antigen's interaction with the BCR but also the developmental state of the B cells, the environment in which the B cell is activated, and the nature of the antigen itself. In other words, BCR signaling always occurs in a context; to predict the outcome of antigen binding to the BCR, we need to be aware of that context.

The BCR also signals during development to provide survival signals to the B cell, often referred to as tonic signaling, and to keep B cells in nonresponsive or tolerant states, termed *anergic signaling*; however, we do not yet know how these signals relate to those that activate mature B cells.

Concerning the impact of the environmental context in which B cells are activated, this chapter will focus on the effect of B-cell coreceptors that directly affect BCR signaling. We will provide a comprehensive list of the coreceptors and what they respond to and describe how two of the best studied coreceptors, the enhancing cluster of differentiation (CD)19/CD21 complex and the inhibitory FcγRIIB receptor, function to influence B-cell responses. We will also comment on the interactions between the innate immune system's toll-like receptors (TLRs) and the BCR that appear to serve to regulate each other's signaling.

We hope that this chapter leaves the reader with a clear view of the early events that follow the engagement of antigen by the BCR and the signaling cascades that are triggered that ultimately activate B cells to proliferate and differentiate into antibody-secreting cells. We also hope that the reader gains an appreciation that B-cell activation occurs in both a developmental and environmental context that dictates the outcome of antigen binding to the BCR. Lastly, we consider the repercussions of uncontrolled BCR signaling that may result in B-cell tumors and in systemic autoimmune diseases.

THE STRUCTURE OF THE B-CELL RECEPTOR

The BCR belongs to the multichain immune recognition receptor (MIRR) family that includes the T-cell receptor for antigen and the high-affinity receptor for IgE. MIRR family members contain ligand-binding chains, which for the BCR is a membrane form of Ig (mIg) (Fig. 9.2). B cells express BCRs composed of Igs of all isotypes that are expressed in a developmentally controlled fashion beginning with IgM-BCRs in immature B cells, IgM- and IgD-BCRs in mature B cells, and then isotype switched BCRs, containing IgGs, IgAs, and IgEs in memory B cells. The mIgs have short cytoplasmic tails of 3 to 28 amino acids that, with the exception of IgG- and IgE-BCRs, do not connect directly with the cell's signaling apparatus.[1–3] Rather, the MIRRs ligand binding chains noncovalently associate with membrane proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. For the BCR, the mIg associates with a disulfide linked heterodimer, Igα and Igβ, each chain of which has a single ITAM in its cytoplasmic tail (see Fig. 9.2).[4] The stoichiometry of the BCR complex is 1mIg:1Igα-Igβ determined by biochemical analysis as well as by live cell imaging (see Fig. 9.2).[5,6]

In considering how the BCR's engagement of antigen triggers signaling, it is helpful to consider what we know about the structure of the BCR. From x-ray crystallographic studies, we know that the antibody Fab does not undergo large conformational changes between the free and antigen-bound states that could transduce the information that the BCR has bound antigen to the BCR's cytoplasmic domains to initiate signaling.[7] However, the BCR is a complicated multichain complex and to date, there is no structure of a complete BCR containing mIg, Igα, and Igβ. Thus, a full understanding of the molecular basis of the antigen-induced initiation of BCR signaling will await the determination of the structure of the BCR. Recently, as an effort toward this goal, the structure of the disulphide-linked homodimer of Igβ was solved, which allowed the modeling of an Igα–Igβ heterodimer with the existing structure of the Cα4 domain.[8] These results predicted an unexpectedly extensive contact surface between the ectodomains of mIg and both Igα and Igβ through multiple charged residues that could potentially be sensitive to antigen-induced changes in the BCR's mIg.

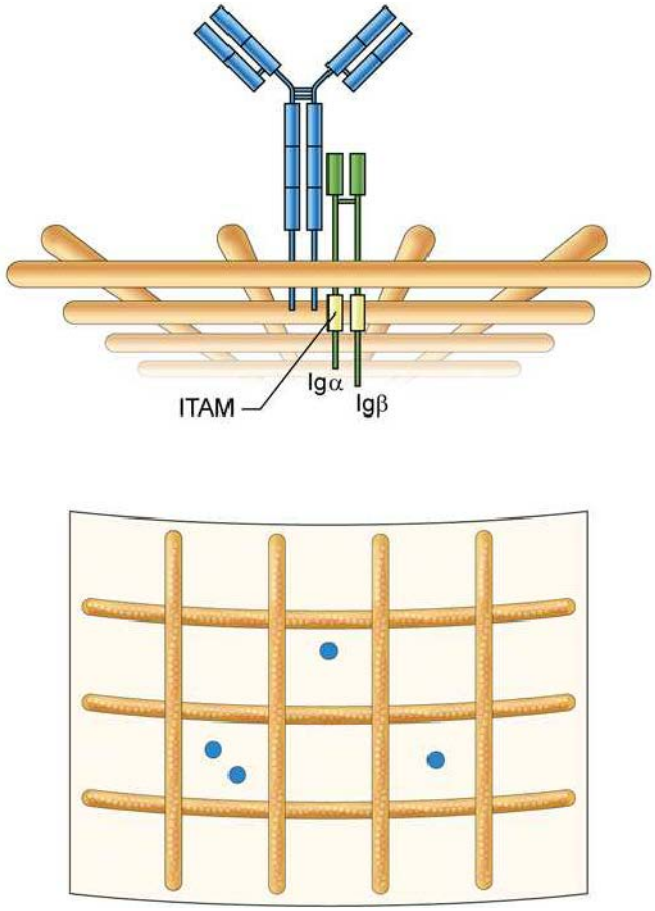

FIG. 9.2. The Structure and Organization of the B-Cell Receptor (BCR). The immunoglobulin (Ig)M BCR is composed of a membrane form of IgM associated with a covalently linked heterodimer of Igα and Igβ that contain in their cytoplasmic domains immunoreceptor tyrosine-based activation motifs. The BCR is depicted compartmentalized on the plasma membrane by actin cytoskeleton "fence"[9] as proposed by Batista et al.[21] Two different views are provided: a side view showing the BCR short cytoplasmic tail and the Igα-Igβ tails in the cytosol and a view from above showing the BCRs compartmentalized by actin "fences."

In considering how the BCR's engagement of antigen triggers signaling, it is also useful to consider the organization of the plasma membrane in which the BCR resides (see Fig. 9.2). Our current understanding of the plasma membrane is that it is not simply a fluid mosaic structure of freely diffusing proteins in a phospholipid bilayer, but rather, the plasma membrane appears to be partitioned into highly dynamic compartments formed by actin cytoskeleton "fences" and actin-anchored protein "pickets."[9,10] These compartments serve to organize the plasma membrane to control the diffusion of membrane proteins and concentrate proteins in one compartment or conversely segregate proteins into separate compartments. The fences and pickets are also

dynamic structures that can disassemble and reassemble during B-cell activation.

THE ANTIGEN-INDUCED CLUSTERING OF THE B-CELL RECEPTOR

Like other MIRRs, the BCR has no intrinsic receptor kinase activity, but upon antigen binding, the ITAM tyrosines in the Igα and Igβ chains are phosphorylated by the membrane-tethered kinase Lyn.[11,12] The phosphorylation of the BCR ITAMs leads to the recruitment of the Src homology 2 (SH2) domain-containing kinase Syk and the initiation of a variety of downstream signaling pathways described later in this chapter. The focus of this section is on the early events following antigen binding that lead to the phosphorylation of the BCR.

Until recently, most of what we learned about the responses of B cells to antigens came from biochemical studies of B cells responding to antigens in solution. Such studies showed that BCR signaling can only be initiated by the binding of multivalent antigens. As an example, only the bivalent $F(ab')_2$ fragments, but not the monovalent Fab fragments, of anti-IgM antibodies trigger BCR signaling.[12] By immunofluorescence imaging, following multivalent antigen binding, BCRs were observed to form microscopic clusters or patches on the cell surface that then move to one pole of the B cell to form a cap. Based on these biochemical studies, a widely accepted concept emerged that the physical aggregation of the BCR by multivalent antigens promoted the patching and capping of BCR that initiated signaling.

However, there is growing evidence both from studies in vitro as well as from intravital imaging in live animals that B cells are activated by membrane-bound antigens and not by antigens in solution. B cells were shown to be efficiently activated by antigens expressed on the surfaces of APCs in vitro resulting in the formation of a polarized bull's eye–like structure in which the BCRs are concentrated in the center, surrounded by the adhesion molecule lymphocyte function–associated antigen, which engages intercellular adhesion molecule on the APC surface.[13] Recent intravital imaging studies showed that B cells interacted with antigens on the surfaces of APCs in lymph nodes in vivo.[14–18] Antigens in lymphatic fluid enter lymph nodes through efferent vessels and gain access to B cells through various mechanisms.[19] Small soluble antigens move through follicular conduit networks and are presented to B cells within the follicles. Particle-like antigens, including viruses and immune complexes, are captured by macrophages lining the subcapsular sinuses and are then transported into the cortex of the lymph node where they are presented to B cells. In addition, B cells are also able to engage antigens on dendritic cell surfaces in the lymph nodes.[20] These findings provided a new view of the initiation of antigen-driven BCR signaling in which BCR signaling is initiated at the contact interface between B cells and APCs. Live cell imaging technologies are providing the tools to observe B cells as they first engage antigen on membrane surfaces. To facilitate these studies and gain high-resolution images, B cells are often activated by antigens incorporated into fluid planar lipid bilayers as surrogate APCs. These studies are revealing the B cells' engagement of membrane-bound antigens to be a remarkably dynamic event[21,22] that contrasts with the view of the patching and capping of BCRs that resulted from the simple physical cross-linking of BCRs by multivalent antigens in solution.

B cells first touch the antigen-containing bilayer through finger-like protrusions of their plasma membrane from which the BCRs engage antigen and form microclusters (Fig. 9.3).

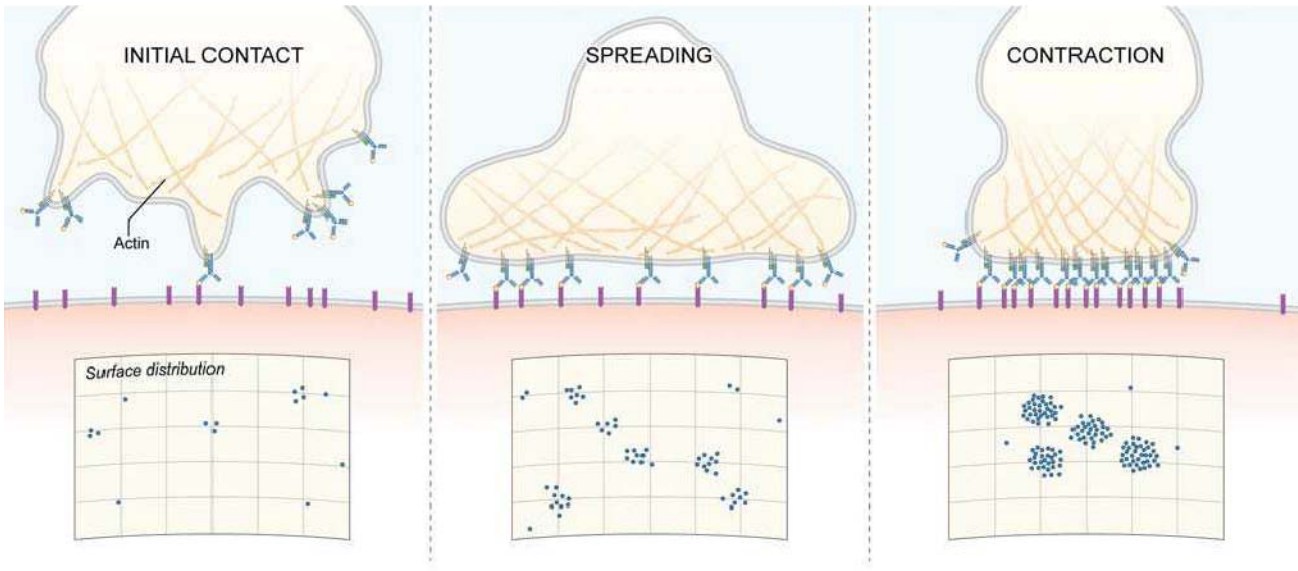

FIG. 9.3. The Activation of B Cells on Antigen-Presenting Cell (APC) Surfaces. The initial contact of the B cell with an antigen-containing APC surface is through finger-like protrusions of the membrane. Antigen binding induces B-cell receptor (BCR) clustering and signaling that triggers the B cell to spread over the APC surface, forming additional BCR clusters as antigens are encountered. After maximal spreading, the B cell contracts, actively moving BCR clusters to the center of the contact area ultimately forming an immune synapse.

These BCR microclusters are enriched in tyrosine phosphorylated proteins and are the site of Lyn and Syk recruitment and thus appear to be the elemental signaling units. B cells subsequently spread over the bilayer allowing the engagement of additional antigens (see Fig. 9.3). Following maximal spreading, the BCR-antigen microclusters are actively moved toward the center of the contact area, and the B cell contracts to form an immunologic synapse. This dynamic process of touching, spreading, and contraction occurs within minutes of the BCR's first contact with the bilayer.[23] These observations bring us back to the fundamental questions: How are BCR microclusters formed, and how do BCR microclusters trigger BCR signaling? Our understanding of these processes is still incomplete. However, based on current data, several models have been proposed that address these questions.

One model, the conformation-induced oligomerization model, is based on evidence from live cell imaging at the level of individual BCRs. Single BCR tracking provided evidence that BCRs are dispersed and freely diffusing in the plasma membrane of resting B cells. Upon binding antigens presented on lipid bilayers, BCRs formed immobile oligomers that grew into microclusters by trapping additional antigen-bound BCRs.[24] Remarkably, contrary to soluble antigens, monovalent antigens presented on fluid lipid bilayers, which could not physically cross-link BCRs, activated B cells equivalently to multivalent antigens. This observation led to the conclusion that the physical cross-linking of BCRs by multivalent antigens was not a requirement for BCR oligomerization or clustering. Early oligomerization and microcluster formation that occurred within 60 seconds of antigen binding were shown to be BCR-intrinsic events that did not require the signaling apparatus of the BCR.[25] Mutagenesis studies showed that the membrane-proximal Cμ4 portion of the ectodomain of mIgM (or the Cγ3 membrane-proximal domain of mIgG) was both necessary and sufficient for BCR oligomerization and signaling.[25] Thus, the membrane proximal domain of the mIg appeared to contain an oligomerization domain that was not exposed in the absence of antigen binding. It was hypothesized that the B cell's binding antigen presented on an APC surface exerted a force on the BCR that revealed the oligomerization domain, allowing oligomerization of antigen-bound BCRs as they randomly bumped on the membrane. This model provides a mechanism by which the universe of foreign, structurally distinct antigens can bring BCRs into a precise signaling-active oligomer by the monovalent binding of BCRs to epitopes on APC-presented antigens. This model also suggests how BCRs are able to so exquisitely discriminate their affinity for antigen by monovalent binding, avoiding the affinity obscuring effects of the avidity contributed by multivalent antigen binding to bivalent BCRs.

Another model for the initiation of BCR signaling based on live cell single molecule imaging focuses on the role of the actin cytoskeleton in signaling.[26–29] In this model, the membrane cytoskeleton fences and pickets restrict BCR mobility and interactions with signaling molecules and coreceptors. BCR signaling results in the disruption of the cytoskeleton barriers and increases the likelihood that the antigen-engaged BCR will encounter activated kinases and coreceptors.[29] The actin cytoskeleton may also segregate BCRs from kinases and phosphatases at steady state. Current evidence suggests that the cytoskeleton fences and pickets confine the BCR and inhibitory phosphatases to the same areas, and that BCR signaling serves to disrupt the cytoskeleton and allow the BCRs and phosphatases to diffuse away, promoting B-cell activation.

A third model, the dissociation activation model, is based on biochemical studies.[6,30] In this model, in resting B cells in the absence of antigen most BCRs exist on the B-cell surface as signaling-inactive, autoinhibited oligomers in equilibrium with a small number of signaling-active BCR monomers.[30] The binding of multivalent antigen disrupts the oligomers shifting the equilibrium toward BCR active monomers, which are then clustered by the multivalent antigen in a manner that prevents the formation of inactive oligomers within the cluster. This model also accounts for the ability of structurally diverse antigens to activate B cells by proposing that antigens keep BCRs apart rather than bringing them together into well-ordered oligomers.

THE ROLE OF B-CELL RECEPTOR AFFINITY AND ISOTYPE IN SIGNALING

Affinity maturation and class switching are two hallmark features of humoral immune responses.[31] In a typical T-cell–dependent antibody response, antigen-specific antibodies become increasingly higher in affinity and predominantly of the IgG isotype through the linked molecular processes of somatic hypermutation and class switching. Because the B-cell immune response functions through clonal selection, it is presumed that affinity maturation and class switching reflect an advantage of B cells expressing high-affinity, class-switched BCRs in the selection process. Indeed, adoptive transfer studies provided clear evidence that high-affinity B-cell outcompete low-affinity B-cell clones for survival in vivo.[32–38] Similarly, when comparing IgM and class-switched BCRs, several seminal studies showed that class-switched B cells outcompete IgM B cells for survival in vivo, and that this survival advantage can be attributed to the 28 amino acid cytoplasmic tail of mIgG,[1,2] missing in mIgM and mIgD. The question is at what point in the antigen-selection process does a high-affinity, class-switched BCR gain an advantage?

Biochemical experiments suggested that the signals that are triggered through high-affinity BCRs are qualitatively different from signals through low-affinity BCRs.[39] At an earlier point in B-cell activation, the affinity of the BCR for antigen was shown to determine the degree to which B cells spread over antigen-containing fluid lipid bilayers, allowing increased accumulation of antigen into the immune synapse and subsequent enhanced responses.[23] The IgG-BCR cytoplasmic tail is responsible for the enhanced B-cell proliferative responses to antigen in vitro and signaling cascades that have been shown to be qualitatively different from those triggered by IgM-BCRs.[3,40–42] Enhanced signaling through IgG-BCRs is dependent on the phosphorylation of a tyrosine in the IgG tail that serves to recruit growth factor-receptor-bound protein 2 (Grb2), resulting in sustained kinase activation and enhanced B-cell proliferation.[3]

Recent studies using high-resolution imaging to follow single BCRs showed that the earliest events that occur following antigen binding to the BCR are also highly sensitive to both the affinity of the BCR for antigen and the isotype of the BCR. Comparing BCRs that differed 50-fold in their affinity for antigen, it was observed that high-affinity BCRs more readily formed BCR microclusters that grow more rapidly, thereby resulting in larger microclusters that recruit more Syk and signal for more robust calcium responses.[24] These imaging experiments also demonstrate that IgG-BCRs are dramatically enhanced in their ability to oligomerize and to grow microclusters, ultimately leading to increased recruitment of Syk and more robust calcium responses as compared to IgM-BCRs of the same affinity.[43] The enhanced function of IgG-BCRs was mapped to a novel membrane proximal 15 amino acid region of the cytoplasmic tail. These studies place the effects of both affinity maturation and class switching at the earliest steps in the initiation of BCR signaling.

HOW B-CELL RECEPTOR CLUSTERING TRIGGERS SIGNALING

Another fundamental yet still open question related to the initiation of BCR signaling is how BCR clustering in response to antigen recruits Lyn to phosphorylate the antigen-bound BCRs and triggers signaling. One simple explanation is that Lyn is constitutively associated with the cytoplasmic domains of some monomeric BCRs, but only phosphorylates BCRs in trans when the BCRs are clustered. However, recent studies suggest that the mechanism for recruitment of Lyn may be more complicated. Lyn is lipidated and tethered to the inner leaflet of the plasma membrane. BCR clustering has been shown to perturb the local lipid environment leading to the transient coalescence of lipid rafts around the BCR oligomers followed by a more stable association of the BCR microcluster with lipid tethered Lyn.[44] Thus, the BCR's perturbation of the membrane may serve to recruit Lyn to the BCR cluster. BCR clustering has also been shown to alter the way the Igα and Igβ chains' cytoplasmic domains associate. In the absence of antigen, the domains are in close proximity in a "closed" conformation and upon antigen binding, the domains "open"; the opening is simultaneous with Lyn's phosphorylation of the ITAMs.[5] Thus, BCR clustering may serve to reveal the ITAMs for phosphorylation by Lyn.

B-CELL RECEPTOR–TRIGGERED SIGNALING CASCADES

BCR signaling is a multistep process that involves the initiation of signaling by the activation of protein tyrosine kinases (PTKs), serine-threonine kinases, and lipid kinases; amplification of signaling by recruiting adaptors; generation of second messengers; and finally activation of the transcription of genes involved in B-cell responses.

Initiation of B-Cell Receptor Signaling—Protein Tyrosine Kinase Activation

Following BCR clustering, three different families of PTKs, Src, Syk, and Tec, are activated sequentially. This sequential activation of the members of the three different PTK families is essential to trigger and regulate downstream signaling. The importance of these PTKs in the B-cell signaling is underscored by the fact that deficiencies in any one of the three families result in aberrant B cell development and function (Table 9.1). The first kinases that are activated following BCR cross-linking are the Src family PTKs, primarily Lyn, but also Blk and Fyn followed by the activation of Syk and the Tec family kinase, Btk[45] (Fig. 9.4). ITAM phosphorylation of Igα-Igβ by Lyn generates phosphotyrosine motifs that allow the binding of the SH2 domains (Box 9.1) of the second kinase, Syk, resulting in rapid Syk activation. Upon binding to phosphorylated ITAMs, Syk undergoes autophosphorylation at multiple tyrosines within its linker regions that not only prolongs Syk's activation but also creates SH2 binding sites on Syk for the recruitment of downstream signaling molecules, including PLC-γ2,[46] leading to a positive feedback of BCR signaling and the concomitant influx of calcium.[12] Btk is the third PTK that is activated upon BCR cross-linking.[47] The importance of Btk in BCR signaling for the development, activation, and differentiation of B cells is underscored by the fact that the loss-of-function mutations of the gene encoding Btk lack circulating B lymphocytes, are unable to generate Igs, and cannot mount humoral immune responses.[48] This primary immunodeficiency is named X-linked agammaglobulinemia.[49,50] Similarly, a spontaneous mutation in the mouse Btk gene leads to X-linked immunodeficiency.[51] Btk consists of multiple protein domains including PH, SH2, SH3, and kinase domains (see Box 9.1), which define its subcellular location and regulate its activity. For Btk activation, plasma membrane localization is important, which is governed by the interactions between the PH domain of Btk with phosphatidylinositol (PI)(3,4,5)P_3, the product of PI3K activity and between the SH2 domain of Btk with phosphorylated BLNK, an adaptor protein. Mutation in the PH domain of Btk (R28C) leads to classical X-linked agammaglobulinemia,[52] substantiating the importance of plasma membrane localization for Btk activation. Following BCR cross-linking, Btk translocates from the cytosol to the plasma membrane where it is activated by phosphorylation at Y551 in its catalytic domain by Lyn[53–55] followed by an autophosphorylation of its SH3 domain.[54,56] Once activated, Btk triggers a cascade of signaling events that culminate in calcium mobilization through phosphorylation of PLC-γ2, cytoskeletal rearrangements, Vav activation, and transcriptional activation involving NF-κB.

Amplification of B-Cell Receptor Signaling—Recruitment of Adaptors

BCR-mediated signaling is a complex process in which each phosphorylation event is linked with another in a regulated manner, thereby generating a large number of protein–protein interaction networks ultimately resulting in the formation of a large, well-ordered structure often referred to as a signalosome. Interactions between signaling networks are regulated by a number of scaffolding or adaptor proteins, which regulate BCR-mediated signaling cascades not only by recruiting multiple signaling intermediates to the proper location but also by controlling interactions between signaling components. Recent studies have identified the roles of

TABLE 9.1 The Phenotype of Mice Deficient in Key B-Cell Signaling Components

Target Protein	Major Phenotype	Reference
Igα cytoplasmic domain	Normal pre-B-cell development; completely impaired mature B-cell development	184
Igβ	Complete block at the pro-B-cell stage	185
Igμ	Deletion of mature B cells; increased Fas expression	186
Lyn	Normal B-cell development in the bone marrow; reduced number of peripheral B cells; increased proportion of immature B cells; enhanced BCR induced ERK activation and hyperproliferative responses	187
Syk	Block in transition of the pro-B- to the pre-B-cell stage; intact Igα-Igβ ITAM phosphorylation in remaining B cells; abolished BCR-induced calcium influx; failure to transmit downstream signals	188,189
Btk	Reduced number of peripheral B cells; increased immature B cells; complete loss of B1 B cells; fail to respond to TI-II antigens	190–193
BLNK	Block in transition of the pro-B- to the pre-B-cell stage; incomplete block in B-cell development; fail to respond to both TD and TI antigens	194–196
PLC-γ2	Decreased mature B cells; block in pro-B-cell differentiation; B1 B–cell deficiency; block in BCR-induced calcium influx and proliferation	197,198
BCAP	Reduced number of B cells; B1 B–cell deficiency; reduced serum IgM and IgG3 levels; abolished TI antibody response; reduced BCR induced calcium influx and proliferation	82
PI3K, p110 subunit	Reduced numbers of B1 and marginal zone B cells; reduced serum Ig levels; defective primary and secondary response to TD antigens; diminished response to TI-II antigens; reduced BCR, CD40, and LPS induced proliferation	199,200
PI3K p85 subunit	Reduced number of peripheral B cells and B1 cell; reduced serum Ig levels; reduced BCR- and CD40-induced proliferative response; abolished TI antibody response	201,202
PKC-β	Failure to activate IKK and degrade IκB; failure to upregulate NF-κB–dependent survival signals; impaired humoral immune responses and reduced cellular responses	203,204
Bam32	Normal B-cell development; impaired TI-II antibody responses; reduced responses to BCR cross-linking	89
Vav1/2	Reduced number of B cells; defects in formation of germinal centers and class switching; defective immune responses against TD and TI antigens; impaired BCR-induced calcium influx and proliferation	205,206
Rac	Reduced number of mature and marginal zone B cells and B1-a cells and IgM secreting plasma cells; increased number of peripheral B cells in blood; reduced serum IgM and IgA concentration	207
CD19	Defective response to TD antigens; failure to form germinal centers and undergo affinity maturation; lack of B-1, marginal zone B cells; reduced BCR- and CD40-induced proliferative responses	191,208,209
CD22	Decreased surface IgM levels; augmented BCR-induced calcium; compromised marginal zone B-cell compartment	210,211
PIRB	Increased number of peritoneal B1 cells; constitutive activation of follicular B cells; increased BCR-induced proliferation	212
Calcineurin	Reduced number of B1 cells; reduced plasma cell differentiation; decreased TD antibody response; BCR-induced proliferation defects	102
IKKα	Reduced mature B-cell population; impaired basal and Ag-specific Ig production; disrupted splenic architecture including germinal center formation; decreased expression of NF-κB target genes	213,214
IKKβ	Disappearance of mature B cells	215
NEMO	Disappearance of mature B cells	215

BCR, B-cell receptor; CD, cluster of differentiation; Ig, immunoglobulin; ITAM, immunoreceptor tyrosine-based activation motif; LPS, lipopolysaccharide; TD, T-dependent; TI, T-independent.

adaptors in B-cell activation, leading to important insights into how they integrate BCR signaling. Although adaptors generally lack any enzymatic activity, they consist of multiple protein–protein or protein–lipid interaction domains, including SH2, SH3, PH and PX homology domains (see Box 9.1). Each of these domains has the potential to interact with a number of proteins that are critical to amplify BCR signaling by facilitating the coupling of multiple downstream signaling pathways. Essentially, the adaptors define where and when macromolecular complexes are assembled, allowing both spatial and temporal regulation of signaling cascades. To illustrate the importance of adaptors, we describe the functions of three: B-cell linker protein (BLNK); B-cell adaptor for PI3K (BCAP), and B-cell adaptor molecule of 32 kDa (Bam32).

BLNK—Integrating Protein Tyrosine Kinases and PLC-γ2

BCR clustering activates Lyn and Syk that regulate a variety of effectors including PLC-γ2, an essential phospholipase for

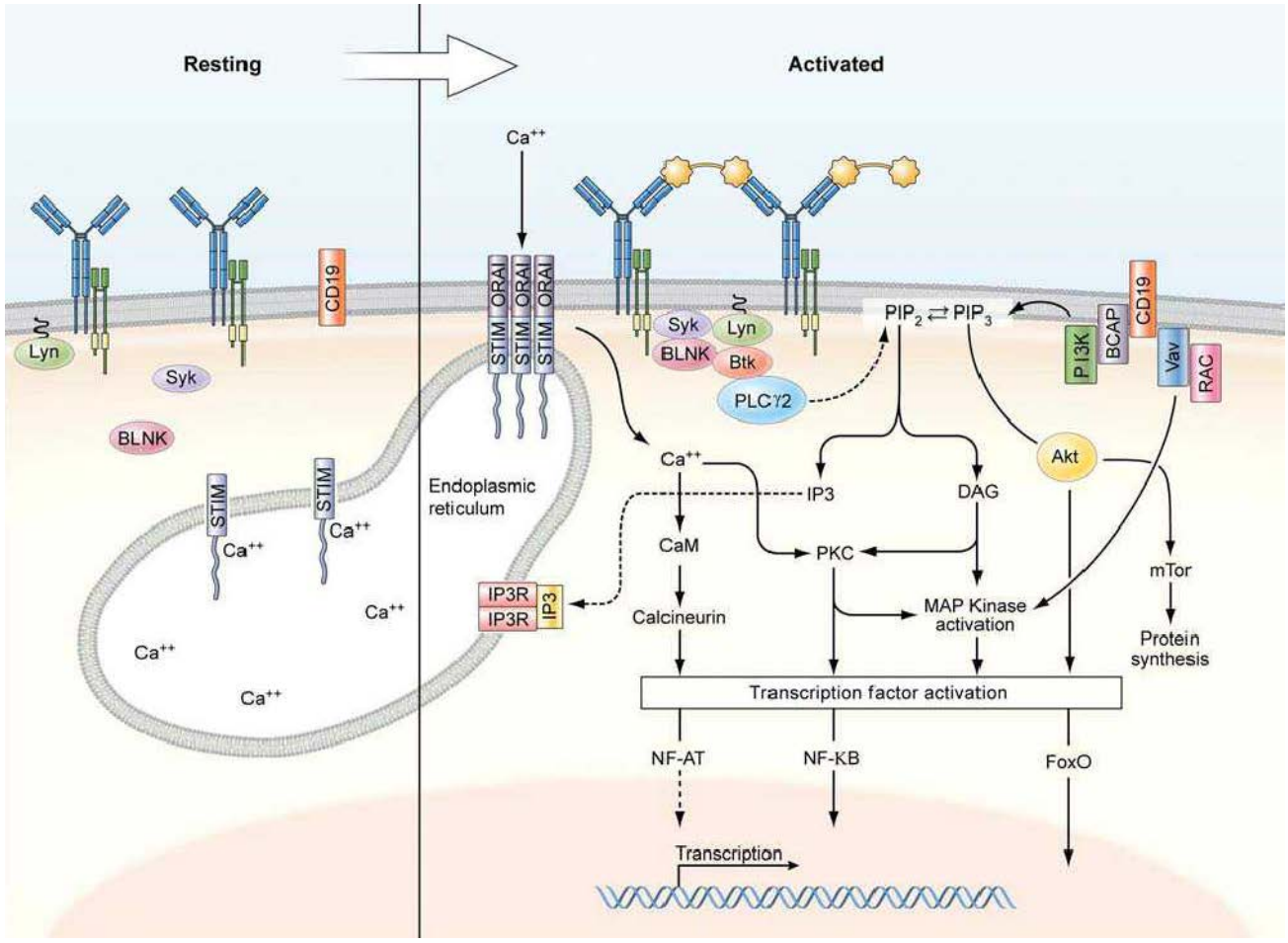

FIG. 9.4. Schematic Depiction of B-Cell Receptor (BCR) Signaling. Presented is a highly simplified version of the signaling pathways triggered by antigen-induced BCR clustering. Antigen binding to the BCR leads to the formation of signalosome consisting of BCR, protein tyrosine kinases, adaptors, and effector molecules. BCR clustering results in PI3K activation, leading to the production of PIP3 from PIP2 and PLC-γ2 activation resulting in the production of IP3 and diacyl glycerol (DAG) from PIP2. Binding of IP3 to the IP3 receptor on the endoplasmic reticulum (ER) opens channels in the ER membrane leading to calcium release and depletion of ER calcium. Calcium depletion is sensed by STIM, a calcium-binding protein, which results in its oligomerization and movement to regions of the ER membrane in close proximity to the plasma membrane where it interacts with Orai opening the calcium release–activated channel, resulting in calcium influx. Calcium, DAG, and PIP3 trigger signaling cascades leading to mitogen-activated protein kinase and transcription factor activation and activation of the Akt pathways promoting cell survival and growth.

calcium responses. Although Syk can directly phosphorylate PLC-γ2 in vitro,[57] expression of a functional BCR, Lyn, and Syk in nonlymphoid cells did not induce PLC-γ2 phosphorylation or calcium mobilization,[58] leading to the discovery of the B-cell–specific adaptor protein, BLNK. BLNK (also known as SLP-65 or BASH) is a cytoplasmic protein consisting of an N-terminal region containing a short leucine zipper motif, a proline-rich region within the middle third of the molecule, and a C-terminal SH2 domain. BLNK contains 13 tyrosine residues, of which 6 are in putative SH2 binding motifs and are phosphorylated upon BCR clustering. BLNK is recruited to the clustered BCR by binding through its SH2 domain to the phosphorylated non-ITAM tyrosine 204 in Igα. Mice with a Igα Y204 mutation exhibit reduced BLNK phosphorylation and calcium fluxes.[59] Once translocated to the plasma membrane, BLNK is phosphorylated by Syk, which creates docking sites for SH2 domains of multiple effector molecules facilitating interactions among them and allowing them to phosphorylate and activate their respective signaling pathways. One of the best studied examples of how BLNK bridges multiple signaling components is provided by the activation of PLC-γ2. PLC-γ2 activation is completely abolished in Syk-deficient B cells.[60] In addition, BCR-induced PLC-γ2 activation is diminished in Btk-deficient B cells, suggesting that both Syk and Btk are required for PLC-γ2 activation.[61] However, the mechanisms underlying how these two families of PTKs regulate PLC-γ2 activation became clear only after the discovery of BLNK. After phosphorylation by Syk, BLNK creates binding sites for the SH2 domains of both Btk and PLC-γ2, bringing them into close proximity with each other, and hence facilitating PLC-γ2 phosphorylation at Tyr753 and Tyr759 by Btk, which is required for PLC-γ2 activation.[62–68] Cells expressing a mutant BLNK lacking either Btk or PLC-γ2 binding sites

BOX 9.1. PROTEIN MODULES INVOLVED IN SIGNAL TRANSDUCTION

Src homology 2 (SH2) domain. The SH2 domain is a structurally conserved protein domain present in the Src oncoprotein from which its name is derived and in many signaling molecules including those involved in B-cell signaling. SH2 domains bind with high affinity to peptide sequences within target proteins that contain phosphorylated tyrosine residues but have no affinity for the unphosphorylated sequence. This allows tyrosine phosphorylation to act as a molecular switch, recruiting SH2 domain–containing proteins to activated receptors and initiating signaling cascades. Important SH2 domain–containing proteins involved in B-cell signaling include Lyn, Syk, Btk, PLC-γ2, BLNK, and Grb2.

Src homology 3 (SH3) domain. SH3 domains are protein modules that recognize proline-rich sequences, in particular those containing a PxxP motif, which do not require phosphorylation. However, recent studies have suggested that the SH3 domains also bind to nonproline-rich sequences accounting for diverse functions they mediate. Some examples in B cells are Lyn, Btk, and Grb2.

Pleckstrin homology (PH) domain. The PH domain is a lipid binding module that recognizes phosphoinositides phosphorylated at the 3 position of the inositol ring, the most important of which is $PI(3,4,5)P_3$, the product of PI3K's phosphorylation of $PI(4,5)P_2$. This property allows PH domain–containing proteins to bind to the inner leaflet of the plasma membrane following PI3K activation where they function. PH domain–containing proteins in B cells include Btk, Vav, Akt, Gab, and Bam32.

Phox homology (PX) domain. The PX domain is another phosphoinositide-binding domain that was originally identified in two cytosolic components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, p40phox and p47phox. In a majority of cases, PX domains contain a proline-rich motif, PxxP, in the middle that serves as SH3 binding motif, suggesting that PX domains might directly interact with SH3 domains as well. PX domains have been shown to play diverse cellular functions including vesicle docking and fusion, cell signaling, and protein sorting. An example in B cells is phospholipase D.

show significant reduction in calcium activation, further suggesting that BLNK acts as a scaffold for bridging Btk to PLC-γ2.[69] A single BLNK molecule can bind three PLC-γ2 molecules, thus BLNK also functions as an amplifier of BCR signaling.[62] Following BCR clustering, both BLNK and Btk are also translocated to the plasma membrane bringing along PLC-γ2 and allowing it to gain access to its substrate $PI(4,5)P_2$ in the inner leaflet of the plasma membrane.[66–68] In addition to phosphorylating PLC-γ2 and recruiting it to the plasma membrane, Btk also recruits PIP5 kinase to the plasma membrane, which catalyzes $PI(4,5)P_2$ synthesis from PIP(4)P to ensure that activated PLC-γ2 does not run out of its substrate.[70] Once activated, PLC-γ2 hydrolyzes $PI(4,5)P_2$ to generate two important second messengers: inositol trisphosphate (IP_3) and diacyl glycerol (DAG). BLNK not only couples Btk and PLC-γ2, but it also binds to the SH2 domains of Vav and Nck, resulting in activating the mitogen-activated protein (MAP) kinase pathway, cytoskeletal rearrangements, and BCR internalization.[71]

BCAP—Integrating Protein Tyrosine Kinases and PI3K

PI3K is a heterodimeric enzyme consisting of a catalytic subunit (p110) and a regulatory subunit (p85) that phosphorylates phosphatidylinositol lipids generating lipid derivatives at the inner leaflet of the plasma membrane that serve as second messengers for downstream signaling.[72,73] Of particular interest is the generation of $PI(3,4,5)P_3$ from $PI(4,5)P_2$, which serves as a ligand for the proteins containing PH domains and tethers these to the membrane. Although multiple isoforms of PI3K are known, p110δ and p85α are the predominant isoforms in B cells. Gene targeting experiments have established the role of these subunits in the development and activation of B cells (Table 9.2). Deletion or inactivation of either of the two subunits of PI3K demolish BCR-induced phosphorylation of Akt, FoxO, and protein kinase D and results in reduced calcium flux, impaired cell cycle progression, and reduced glucose metabolism.[74–80] Despite the critical role of PI3K in BCR signaling, the mechanisms by which the BCR-associated PTKs regulate the PI3K pathway is unclear. It has been proposed that tyrosines in the cytoplasmic tail of CD19, which are phosphorylated upon BCR clustering, provide binding sites for the SH2 domains of the p85 subunit of PI3K, recruiting it to the plasma membrane and increasing the specific activity of the enzyme. However, the observation that defects in p85α KO mice are more severe than those in CD19 KO mice suggested that additional adaptor molecules may participate in bridging BCR-associated PTKs and PI3K, leading to the discovery of another B-cell–specific adaptor molecule, BCAP.[81] BCAP contains multiple tyrosines, which are phosphorylated after BCR stimulation through the concerted actions of Syk and Btk, thereby creating binding sites for SH2 domains of the p85 subunit of PI3K. BCAP was shown to regulate PI3K signaling in CD19-deficient B cells, but in BCAP KO mice, PI3K signaling was largely unaffected in B cells,[81,82] suggesting that in the absence of BCAP, CD19 functions as a major adaptor. Indeed, CD19/BCAP double knockout mice show an almost complete block of BCR-mediated Akt activation and severe defects in the generation of immature and mature B cells.[83] Recently a GTPase, TC21, was also identified as an adaptor for PI3K activation by binding directly to Igα-Igβ.[84]

Bam32—Linking B-Cell Receptors to Mitogen-Activated Protein Kinase Activation and Cytoskeletal Rearrangements

Bam32 bridges clustered BCRs to two important pathways, namely, Rac and cdc42, which trigger cytoskeletal rearrangement leading to cell adhesion, polarization, and motility and to activation of the MAP kinase c-Jun N-terminal kinase (Jnk) and extracellular signal-regulated kinases (ERK)

TABLE 9.2 B-Cell Coreceptors that Influence B-Cell Receptor Signaling

Coreceptor	Activating (+) or Inhibitory (–)	Phosphorylated Motif(s)	Ligand(s)	Signaling Molecules Recruited upon Motif Phosphorylation	Expressed in Humans (H) or Mice (M)	References
CD19	+	Tyr residues	C3-coated antigen	PI3K + Vav + Lyn + PLC-γ2	H + M	126,127,216
FcμR (FAIM3/TOSO)	+	Ser + Tyr residues	IgM	PI3K + PLCγ-1	H + M	131,217
FcRL1 (FcRH1)	+	ITAM	Unknown	Unknown Tyr kinase?	H + M	132,218,219
PIRA	+	ITAM on associated FcRγ	MHC-1	Unknown	M	133,134,220,221
CD45R (B220)	+	PTP domain	CD22?	C-terminal Tyr of SFKs	H + M	135,136,222,223
CD148 (HPTPh)	+	PTP domain	Unknown	C-terminal Tyr of SFKs	H + M	137,138
FcγRIIB (CD32B)	–	ITIM	IgG-IC	SHIP	H + M	139–141,224
CD22 (BL-CAM)	–	ITIM	Sialic acid	SHP-1	H + M	223–227
PIRB	–	ITIM	MHC-1	SHP-1 (+ SHP-2?)	M	133,220,221,224
ILT-2	–	ITIM	HLA-A, -B + -G (MHC-1)	SHP-1	H	224
CD72 (Lyb-2)	–	ITIM	CD100, CD5	SHP-1	H + M	224,227,228
CD5 (Ly1)	–	ITIM	CD72 (+ others?)	SHP-1	H + M	224,229
PD-1	–	ITIM	IgSF protein	SHP-2	H + M	224
CD66a (BGP-1)	–	ITIM	CD66a	SHP-1 + SHP-2	H + M	224
FcRL4	–	ITIM	Unknown	SHP-1 + SHP-2	H	132,219
FcRL2 (FcRH2)	–	ITAM/ITIM	Unknown	SHP-1	H	132,153
FcRL3 (FcRH3)	–	ITAM/ITIM	Unknown	SHIP + SHP-1 + SHP-2	H	132,154
FcRL5 (FcRH5)	–	ITAM/ITIM	Unknown	SHP-1	H + M	132,155
FcRL6 (FcRH6)[a]	–	ITIM	HLA-DR (MHC-2)	SHP-2	M	132,230,231

CD, cluster of differentiation; HLA, human leukocyte antigen; Ig, immunoglobulin; IC, immune-complex; IgSF, immunoglobulin superfamily; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; MHC, major histocompatibility complex; PTP, protein tyrosine phosphatase; SFK, Src-family kinase; SHIP, SH2-containing inositol 5′-phosphatase; SHP, SH2-domain-containing protein tyrosine phosphatase.
[a] FcRL6 motif, ligand, and signaling molecule information was obtained from studies of human T and natural killer cells.

that play central roles in the activation of transcription factors that regulate the expression of genes involved in B-cell proliferation, differentiation, and survival.[85–88] Following BCR clustering, Bam32 translocates to the plasma membrane in a PI3K-dependent mechanism through its PH domain[88] where it binds to Rac and cdc42 leading to their activation, triggering downstream signaling to Jnk and ERK. Mice deficient in Bam32 show diminished Jnk and ERK phosphorylation.[89] Rac activation is diminished in Bam32-deficient B cells, whereas overexpression of Bam32 results in enhanced Rac activation.[90]

Generation of Second Messengers

As described previously, binding of the PTKs and various signaling intermediates such as PLC-γ2 and PI3K on various adaptors results in the activation of these enzymes resulting in the generation of second messengers including calcium, DAG, and phosphoinositides. These second messengers play a crucial role in regulating BCR signaling by virtue of their ability to affect downstream signaling initiated by the BCR.

Calcium as a Second Messenger

Calcium mobilization is a multistep process that is initiated with the activation of PLC-γ2 that hydrolyzes $PI(4,5)P_2$ present in the inner leaflet of the plasma membrane to generate IP3 and DAG. A receptor for IP3 (IP3R) is present as a tetramer on the ER membrane that forms a calcium channel.[91] The binding of IP3 to the IP3R activates the receptor and opens the channel resulting in the release of calcium from the ER into the cytosol. Depletion of intracellular ER calcium stores results in the oligomerization of the ER calcium sensor, STIM1, an ER membrane protein that contains in its cytoplasmic domain an EF-hand calcium-binding domain.[92,93] STIM1 oligomers rapidly redistribute into clusters and move to junctions of the ER and plasma membrane where STIM1 interacts with Orai, a calcium release–activated channel, and activates Orai to open, triggering the entry of extracellular calcium into the cytosol.[94–96] A single missense mutation (R91W) in human Orai or a truncation mutation in STIM1 causes severe combined immune deficiency supporting the functional roles of STIM1 and Orai in calcium signaling in the immune system.[97,98] The increase in cytosolic calcium is sensed by various calcium-binding proteins, ultimately leading to activation of several downstream pathways including activation of protein kinase C (PKC) and transcription factors nuclear factor of activated T cells (NFAT) and NF-κB. One of the major cytosolic calcium-binding proteins, calmodulin, functions as a calcium sensor by binding to calcium with its two EF hands. Calcium-bound calmodulin activates several molecules including calcineurin, which is a phosphatase that dephosphorylates NFAT resulting in NFAT's translocation into the nucleus where it activates the transcription of target genes. One of the targets of NFAT in B cells is interferon

regulatory factor 4, a critical transcription factor for the differentiation of plasma cells.[99–101] Indeed, calcineurin-deficient B cells show reduced plasma cell differentiation and decreased antigen-specific antibody responses to antigens.[102]

Diacylglycerol as Second Messenger

The second product of $PI(4,5)P_2$ hydrolysis by PLC-γ2 is DAG that together with calcium activates PKC-β that regulates the NF-κB pathway. NF-κB in resting B cells is retained in the cytoplasm by binding to its inhibitor, IκB. BCR clustering induces IκB phosphorylation, through a PKC-β–initiated pathway, ubiquitination, and subsequent degradation, allowing translocation of NF-κB into the nucleus.[103–105] Aberrant activation of NF-κB has been linked to defective B-cell activation leading to multiple immune disorders. Ablation of PKC-β, which leads to the defective activation of the NF-κB pathway, results in defective B-cell activation and maturation.[106] In humans, mutations in components of the NF-κB pathway are associated with the formation of lymphomas.[107–113] In addition to activating PKC-β, DAG also recruits the DAG binding Ras guanine nucleotide releasing protein 3 (RasGRP3) to the plasma membrane, thus initiating the Ras to Raf to MEK to ERK pathway.[114] Deletion of the DAG-binding domain from RasGRP blocks the movement of RasGRP3 to the plasma membrane and the activation of Ras. The Ras–Raf–MEK–ERK signaling pathway plays a critical role in antigen-induced proliferation of mature B cells.[115] Ras is also involved in developing B cells, as transgenic expression of dominant negative forms of Ras blocks developmental progression to the pre–B cell and immature B-cell stages.[116,117] Major targets of ERK in B cells are Ets-family transcription factors, resulting in the expression of early response genes including Egr1 that has been shown to promote expression of adhesion molecules.[118]

Phosphoinositides as Signaling Mediators—A Diverse Group of Molecules Regulating Diverse Pathways

PI is unique among membrane lipids as it can undergo reversible phosphorylation at multiple sites on its inositol head group by lipid kinases and phosphatases to generate a variety of phosphorylated PI lipids called phosphoinositides. Phosphoinositides play crucial roles in cell signaling and membrane trafficking. As described previously, BCR clustering leads to the accumulation of the phosphoinositide, $PI(4,5)P_2$, the substrate for PLC-γ2, which generates two crucial second messengers, IP3 and DAG. Another important phosphoinositide is the PI3K-generated $PI(3,4,5)P_3$ that provides a ligand for recruiting important PH domain–containing proteins to the plasma membrane, including Btk, PLC-γ2, and Akt. Akt is activated at the membrane by phosphorylation and in turn phosphorylates several proteins and transcription factors that regulate protein synthesis, cell survival, and proliferation. Some of the targets of Akt in B cells are mammalian target of rapamycin, glycogen synthase kinase 3, forkhead family transcription factors, Caspase-9, and proapoptotic protein, BAD.[119–121] Given the crucial role of $PI(3,4,5)P_3$ in B-cell activation, its levels are tightly regulated by two different lipid phosphatases that oppose the activity of PI3K, phosphatase and tensin homolog, which removes a phosphate from the D3 position generating $PI(4,5)P_2$, and SH2-domain-containing inositol 5′ phosphatase (SHIP), which generates $PI(3,4)P_2$ by removing a phosphate from the five position and essentially shuts down signaling. One of the pathways that activates SHIP in B cells is the ligation of the inhibitory FcR FcγRIIB,[122] as described in the next section of this chapter.

The Spatial and Temporal Dynamics of B-Cell Receptor Signaling Cascades

Advanced fluorescence-based imaging techniques are just now providing the first view of the spatial and temporal dynamics of the recruitment of individual signaling molecules to BCR microclusters following BCR oligomerization in response to membrane-bound antigens. Each newly formed BCR cluster recruits the earliest PTKs, Lyn and Syk[24,123,124]; as the BCR moves to form an immune synapse, Syk is lost and PI3K is recruited. It has also recently been possible to use imaging to follow signaling as the BCR is internalized into the B cell. Such studies showed that BCR signaling is initiated at the plasma membrane with the recruitment and phosphorylation of Lyn and Syk and continues after the BCR is endocytosed and trafficked through early endosomes then to late endosomes and multivesicular antigen processing compartments with the recruitment and phosphorylation of downstream kinases, initially cRaf and subsequently the MAP kinases, ERK, p38, and Jnk.[125] Internalization of the BCR is necessary for proper signaling as when internalization is blocked, phosphorylation of kinases become dysregulated as does gene transcription. The continued application of imaging technologies to describe B-cell activation should lead to an increasingly detailed spatial and temporal view of BCR signaling.

CORECEPTOR REGULATION OF B-CELL RECEPTOR SIGNALING

The outcome of signaling through the BCR is carefully regulated not only by the developmental stage of the B cells but also by a variety of B-cell coreceptors that both promote and attenuate BCR signaling in response to antigen. Ideally, coreceptors ensure that sufficient amounts of antigen-specific antibodies are produced to control an infection, and that once controlled, antibody production is turned off. We have provided a list of B-cell coreceptors and have indicated whether they function to enhance or inhibit BCR signaling and, when it is known, what they recognize and the molecular basis of their effect on BCR signaling (see Table 9.2). Presumably, all B-cell coreceptors respond to clues from the environment as to the course of the immune response and relate this information to the B cells by enhancing or inhibiting BCR signaling. The best understood examples of the role of coreceptors in B-cell responses are the activating CD19/CD21 complex and the inhibitory FcγRIIB. Both coreceptors become physically cross-linked to the BCR through the recognition of antigen-containing complexes. The CD19/CD21 complex binds complement fixed antigens (C3d-modified antigen, as described in Chapter 36) through CD21, a complement receptor that binds to C3d. Complement modification of antigens can be viewed as a sign that the antigen is dangerous, having the ability to

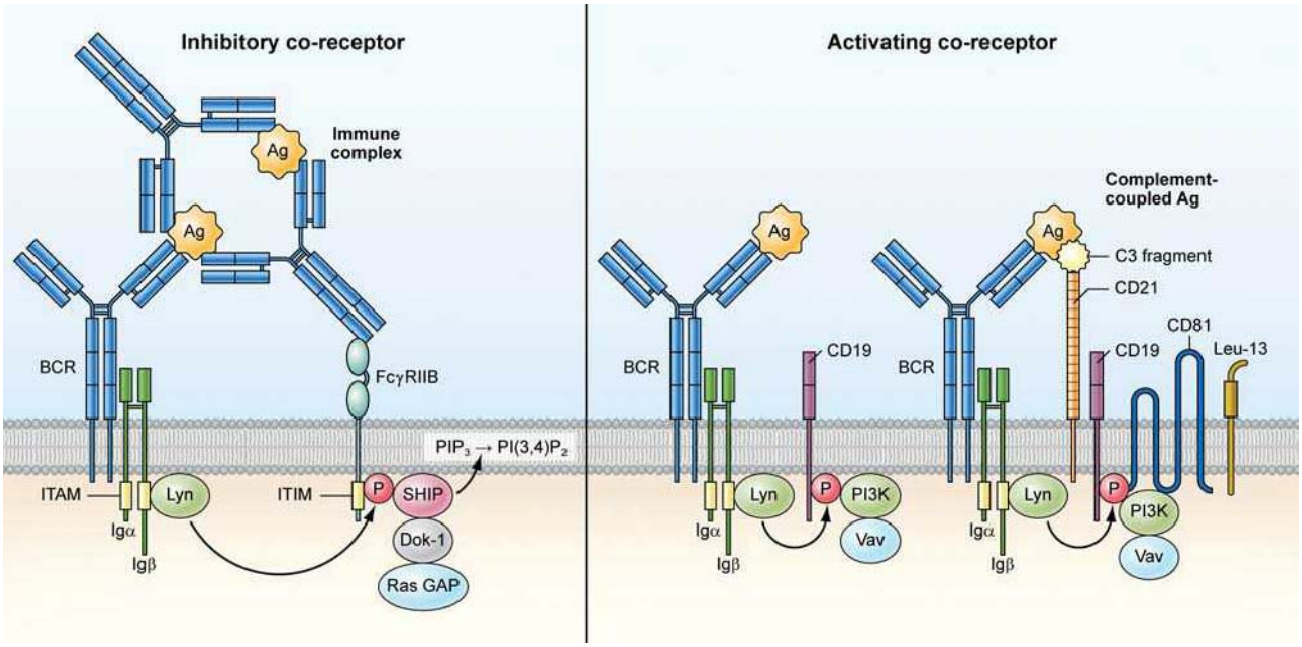

FIG. 9.5. Inhibitory and Activating B-Cell Receptor (BCR) Coreceptors. Antigen-containing immune complexes engage both the BCR and the inhibitory coreceptor FcγRIIB **(Left)**. Lyn associated with clustered BCRs phosphorylates the immunoreceptor tyrosine-based inhibitory motif of FcγRIIB, recruiting SH2-domain-containing inositol 5′ phosphatase that dephosphorylates $PI(3,4,5)P_3$, the product of PI3K activation, to $PI(3,4)P_2$, blocking downstream signaling. Antigen binding to BCRs induces their clustering and signaling that results in the phosphorylation of CD19 by BCR-associated Lyn, recruiting PI3K and Vav **(Right)** and enhancing BCR signaling. Enhancement of BCR signaling is stronger when complement conjugated antigens engage both the BCR and the complement receptor, CD21, recruiting CD19 to the signaling complex where it is phosphorylated by Lyn recruiting PI3K and Vav **(Right)**.

activate the complement pathway, and thus, the CD19/CD21 complex serves to enhance BCR signaling. The FcγRIIB binds to antigen–antibody immune complexes (described in Chapter 24) through the Fc portion of the IgG antibody complexed with the antigen. Immune complexes form when antigen-specific antibodies reach sufficient levels relative to the antigen, an indication that further B-cell activation should be curbed, and thus, the FcγRIIB inhibits BCR signaling.

CD19/CD21, a transmembrane glycoprotein complex expressed on the surface of all B cells, is the best understood activating coreceptor for BCR signaling. On mature B cells, the CD19 component can exist alone or as part of a tetrameric complex with CD21 (complement receptor type 2), the tetraspanin family protein, CD81, and the small interferon-induced transmembrane protein 1, Leu-13[126] (Fig. 9.5). CD21 links this complex to the BCR in response to complement-coupled antigens and boosts BCR signaling and ultimately antibody production. Upon binding of complement-coupled antigen, the BCR and CD19/CD21 complex are coligated, and several tyrosine residues in the cytoplasmic tail of CD19 are phosphorylated by Lyn, providing binding sites for the SH2 domains of the p85 subunit of PI3K, Vav and Lyn, thus enhancing the signaling triggered by BCR antigen binding.[127] It is hypothesized that the association of Lyn with CD19 may counteract the autoinhibition of Lyn, which is maintained by interaction between its SH2 domain and its carboxy-terminal phosphotyrosine, thus stimulating the enzymatic activity and phosphorylation of Lyn during BCR signaling.[128] CD81 may aid in the stable association of the BCR with saturated lipids that coalesce around the antigen-bound BCRs to sustain antigen-induced signaling when the CD19-CD21 complex is coengaged with the BCR.[129] CD81 and Leu-13 may also be involved in mediating cell–cell interactions during the B-cell response to antigen.[130]

In addition to the CD19-CD21 complex, B cells express other activating coreceptors whose ligands and mechanisms by which they affect BCR signaling are less well characterized (see Table 9.2). These activating coreceptors include the recently identified Fc receptor for IgM (FcμR), which contains several conserved tyrosine and serine residues in its cytoplasmic tail that are targets for phosphorylation after receptor ligation with IgM-containing immune complexes.[131] Although the physiologic role of FcμR in B-cell responses is not known, it has been hypothesized that FcμR may become coligated to the BCR and CD19/CD21 complex through the recognition of complement-coupled IgM-antigen immune complexes and drive the B cell response toward isotype-switched antibodies. Other activating coreceptors are Fc receptor-like 1 (FcRL1)[132] and paired Ig-like receptor A (PIRA)[133,134] that respectively contain or associate with cytoplasmic ITAMs. The tyrosine residues within these ITAMs are phosphorylated by one of the Src-family tyrosine kinases of B cells (Lyn, Fyn, or Blk) upon receptor engagement by their ligands, mediating costimulatory signals by creating docking sites

for other proteins involved in the B-cell signaling pathway. Instead of ITAMs, the cytoplasmic domains of the activating coreceptors CD45R and CD148 contain an active protein tyrosine phosphatase domain that dephosphorylates a key regulatory tyrosine at the C-terminal end of Src kinases during BCR signaling.[135–138]

While various coreceptors promote BCR signaling, there are also numerous coreceptors that are poised to counter B-cell activation. FcγRIIB, the best characterized inhibitory coreceptor for BCR signaling, is expressed on the surface of all B cells and is composed of two extracellular Ig-like domains: a transmembrane domain and a cytoplasmic domain that contains a single immunoreceptor tyrosine-based inhibitory motif (ITIM)[139] (see Fig. 9.5). When a B cell binds IgG-antigen immune complexes, FcγRIIB and the BCR are coengaged. Serum contains a relatively high concentration of soluble IgG, but the affinity of the FcγRIIB for soluble IgG is low and such that it is only able to stably engage multimeric IgG-antigen immune complexes. The presence of IgG immune complexes indicates that sufficient antigen-specific antibody has been made to counter any foreign threat, and that antibody production can be attenuated or stopped, depending on the amount of IgG-immune complex present. Coengagement of FcγRIIB and the BCR promotes phosphorylation of the ITIM of FcγRIIB by BCR-associated Lyn, recruiting the lipid phosphatase SH2-containing inositol 5′-phosphatase (SHIP).[140] SHIP dephosphorylates PI(3,4,5)P_3 to PI(3,4)P_2, preventing the recruitment of PH domain–containing kinases (eg, BTK and PLC-γ2) to the cell membrane, thereby downregulating downstream signaling and proliferation.[141] SHIP also recruits RasGAP via the adaptor Dok-1, which inactivates Ras and thus further downregulates proliferation.[142,143]

Besides affecting downstream BCR signaling, it has also been shown that coengagement of FcγRIIB with the BCR greatly destabilizes newly formed BCR oligomers that are essential for the initiation of signaling.[144] This early point of inhibition is not influenced by interactions between the cytoplasmic tails of the two receptors but instead by the perturbation of the local lipid environment by FcγRIIB, which destabilizes BCR association with saturated lipids and Lyn.[144,145] It is of interest that a loss-of-function mutation in the transmembrane domain of FcγRIIB that is associated with the autoimmune disease systemic lupus erythematosus (SLE) prevents the association of ligated FcγRIIB with saturated lipids[146,147] and thus inhibits the ability of FcγRIIB to block BCR oligomerization and signaling. FcγRIIB may also function when engaged by immune complexes independently of the BCR. Cross-linking FcγRIIB by immune complexes that do not contain the antigen for which the BCR is specific leads to apoptosis through a c-Abl–dependent pathway.[148,149] Recent evidence indicates that this pathway may be important in eliminating preexisting FcγRIIB-expressing plasma cells in the bone marrow after infection or immunization, creating space in the limited niches in the bone marrow for newly formed plasma cells.[150]

In addition to FcγRIIB, B cells express a variety of other coreceptors that inhibit BCR signaling via a similar ITIM-mediated mechanism, such as those shown in Table 9.2. In general, the Lyn-phosphorylated ITIMs recruit lipid phosphatases (eg, SHIP) or protein phosphatases (eg, SH2-domain-containing protein tyrosine phosphatase-1 [SHP-1]) that effectively block BCR signaling.[151] SHP-1 likely downmodulates BCR signaling by dephosphorylating early signaling components that include BLNK.[152] Coreceptors such as FcRL2, 3, and 5 have both ITAM-like and ITIM sequences in their cytoplasmic tails, but the inhibitory effects of their ITIMs appear to dominate their regulatory function.[153–155] Negative modulators of BCR signaling other than FcγRIIB do not require coligation to the BCR by immune complexes to attenuate signaling but are instead regulated functionally by binding various ligands on the B-cell surface.

A recurrent theme throughout the discussion of the mechanism by which the CD19/CD21 complex and FcγRIIB function is the requirement for coligation to the BCR by complement-coupled antigens or immune complexes. The requirement for joint binding of the BCR and coreceptors to antigen-containing complexes ensures that only the antigen-specific B-cell response is regulated. However, some coreceptors that function to regulate BCR signaling do not appear to require physical cross-linking with the BCR to affect BCR signaling. In fact, for several such coreceptors their ligands, if they exist, are not known. These coreceptors raise a number of yet unanswered questions concerning how they sense that a BCR is activated and the mechanism by which they regulate BCR signaling. For example, CD19 regulates BCR signaling independently of CD21. In response to antigen presented on fluid lipid bilayers as surrogate APCs, CD19 was shown by live cell imaging to transiently associate with signaling-active BCR microclusters, mediating the recruitment and activation of associated signaling molecules and, therefore, amplifying signaling within the individual microclusters.[28,124] CD19-deficient B cells are unable to form BCR microclusters and undergo a spreading response or flux calcium in response to membrane-bound antigens, suggesting that the BCR-CD19 complex may be a basic signaling unit, essential for BCR activation. Notably, B-cell spreading is not abrogated in the absence of CD21, suggesting that CD19 can function independently of the CD19/CD21 complex. As evidence toward an independently functioning CD19, mice lacking CD19 exhibited a more severe phenotype than those lacking CD21.[156] It is interesting that a CD19 deficiency in mouse B cells does not impair the BCR response to soluble antigens,[127] suggesting that the process by which BCRs cluster in response to membrane-associated antigens provides a mechanism for recruiting CD19 to the BCR, resulting in enhanced signaling. In addition to affecting early BCR signaling, a CD19 deficiency is also implicated in impaired pre–BCR-dependent development and responses to antigen in vivo.[157,158] Another potential example of ligand-independent coreceptor regulation of BCR signaling is FcRL4, a member of the FcR-like family of proteins, which has no known ligand.[132] FcRL4 is expressed on a subpopulation of atypical memory B cells that are expanded in individuals with chronic infections, including

acquired immunodeficiency syndrome and malaria, and are hyporesponsive to BCR triggering. Antigen binding leads to association of FcRL4 with the BCR and a block in BCR signaling at the point of Syk phosphorylation[159] and eliminating FcRL4 expression in atypical memory B cells from individuals infected with human immunodeficiency virus restores BCR signaling.[160] An important unanswered question is in the absence of coligation, by what mechanism do these coreceptors associate with the BCRs? Possible suggested mechanisms are through intracellular adaptor molecules or through membrane perturbations that facilitate BCR-coreceptor interactions. In the absence of a mechanism that triggers the association of these coreceptors with the BCR, it is also not easy to guess what environmental clues these receptors are responding to.

Another family of innate immune system receptors that receive information from the B cell's environment and can affect signaling through the BCR is the TLR family. TLRs are distinctive in that they recognize highly conserved motifs present in microorganisms, including bacteria, viruses, fungi, and protozoans, referred to as pathogen-associated molecular patterns, as described in Chapter 15.[161] As for the BCR, TLR-initiated signaling results in the activation of MAP kinases and NF-κB[162]; thus, TLR and BCR signaling have the potential to synergize. Of particular interest to BCR signaling are TLR7 and TLR9, which reside in intracellular compartments of B cells and respectively recognize pathogen-associated molecular patterns in single-stranded ribonucleic acid (ssRNA) derived from RNA viruses and unmethylated CpG-containing deoxyribonucleic acid (DNA) of bacterial and viral origin. The ssRNA-induced TLR7 signaling and CpG-containing DNA–induced TLR9 signaling have been shown to synergize with antigen-induced BCR signaling in NF-κB activation.[162] It was recently shown that synergistic signaling in response to CpG-containing DNA–containing antigens is mediated by a novel mechanism in which, following antigen binding, the BCR signals from the plasma membrane to recruit TLR9 from small endocytic intracellular vesicles to autophagosomal compartments into which the BCR traffics the CpG-containing antigen and from which synergistic signaling occurs.[163] By this mechanism, TLR9 can alert the BCR to the presence of pathogenic DNA in the environment and heighten BCR signaling. Similar mechanisms may be at play for BCR and TLR7 signaling in response to ssRNA-containing antigens.

ABNORMAL B-CELL RECEPTOR SIGNALING IN HUMAN DISEASE

In this chapter, we have provided an overview of our current understanding of the molecular mechanisms underlying the early BCR-intrinsic events that lead to normal BCR oligomerization and subsequent signaling and have emphasized that early events in the initiation of BCR signaling are tightly regulated. It might be predicted that alterations in any step of the initiation of signaling could drive the BCR to hyperactivation. For example, the outcome of antigen engagement could be affected by mutations in the BCR itself, changes in the composition of the membrane lipids that stabilize BCR oligomers, or mutations affecting the activity of coreceptors or the kinases in the BCR signaling pathway. Hyperactivation of the BCR is associated with both B-cell tumors and autoimmune disease. In the future, it may be possible to develop new therapeutic strategies that target spontaneous BCR oligomerization clustering and chronic active signaling to treat B-cell tumors and systemic autoimmune disease.

The BCR is required for the survival of the activated B cell-like (ABC) subtype of diffuse large B-cell lymphomas (DLBCLs).[164] Using live cell imaging, the BCRs on these activated DLBCLs were found to form immobile oligomers within microclusters similar to those observed on normal antigen-stimulated B cells. In contrast, the BCRs on Burkitt's lymphoma, mantle cell lymphoma, and germinal center B cell-like DLBCL tumors, which are not dependent on the BCR for survival, show no clustering. Somatic mutations affecting the cytoplasmic domains of Igα and Igβ are detected frequently in ABC DLBCL biopsy samples but rarely for other DLBCLs and never for Burkitt lymphoma or mucosa-associated lymphoid tissue (MALT) lymphoma, although the contributions of these mutations to chronic BCR clustering is not known.

Interestingly, the spontaneous BCR clustering on ABC DLBCLs is similar to that observed for human H chain disease in which ligand-independent BCR self-aggregation and constitutive activation are a consequence of BCR H chain gene mutations that cause misfolding and disrupt antigen binding.[165] Impaired glycosylation and folding of the μ-H chain are implicated in chronic lymphocytic leukemia, which has symptoms similar to those of μ-H chain disease; however, it has not yet been determined whether this misfolding leads to enhanced BCR ligand-independent, or chronic active, signaling.

In addition to the BCR itself, other BCR signaling molecules that are key regulators of the NF-κB pathway are also known targets of mutations found in B-cell lymphomas. Specifically, MALT1 and Bcl10 are independently associated with chromosomal translocations in MALT lymphoma, whereas CARD11 has activating point mutations in a percentage of ABC DLBCLs that result in constitutive NF-κB activation.[113,165–167] The ABC DLBCLs are also dependent for their survival on MyD88, the adaptor that mediates TLR signaling.[168] Remarkably, nearly 30% of ABC DLBCLs have a single gain-of-function mutation in MyD88 that promotes cell survival by activating the NF-κB pathway. The dual dependence on both the BCR and TLR pathways suggest that these work in concert to drive B-cell tumorigenesis.

B cells from patients with systemic autoimmune disease exhibit B-cell hyperactivation that may be due to alterations in the BCR signaling pathway or alterations in coreceptor function.[169] In SLE, stimulating peripheral blood B cells with BCR ligand leads to increased intracellular calcium flux and increased phosphorylation of various cytosolic proteins as compared to healthy individuals and patients with other rheumatic diseases.[170] In most of patients with SLE analyzed, expression of Lyn is also significantly decreased in resting, and BCR stimulated peripheral blood B cells[171]; the ability of Lyn to associate with saturated membrane lipids is reduced in SLE B cells as compared with healthy donors.[172]

Decreased Lyn expression negatively impacts its ability to inhibit BCR signaling via phosphorylation of FcγRIIB and other inhibitory coreceptors that contain ITIMs. In addition, a loss-of-function mutation in the transmembrane domain of FcγRIIB that is associated with SLE susceptibility prevents BCR-ligated FcγRIIB from associating with saturated membrane lipids[146] and consequently inhibits the ability of FcγRIIB to block BCR oligomerization, clustering, and subsequent signaling. The synergistic engagement of the BCR and TLR7 and TLR9 in response to antigens containing ssRNA or CpG-containing DNA has also been implicated in the activation of autoimmune B cells. Many of the antigens targeted in SLE contain DNA and RNA that are thought to be released from apoptotic cells. Indeed, genetic variations in TLR9 are linked to SLE susceptibility[173]; in a mouse model of SLE, multiple copies of a normal TLR7 gene are sufficient to drive SLE.[174] Overall, it seems that enhanced B-cell signaling as a result of a multiple genetic abnormalities is the defining pathogenic event of SLE and provides a therapeutic target for treating autoimmune disease.

BCR signaling can also be altered in infectious diseases by viruses that commandeer B cells for their own replication. Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is implicated in Burkitt's lymphoma, Hodgkin disease, nasopharyngeal carcinoma, and various lymphoproliferative disorders that arise in immunocompromised individuals.[175] EBV infects B cells of the oral epithelium and establishes a lifelong latent infection in a subset of those cells.[176] Latent membrane protein 2A (LMP2A) messenger RNA is readily detected in the peripheral blood B cells of healthy individuals, and LMP2A is often found in tumors from EBV-associated malignancies.[177] To subvert immune responses and consequently maintain viral latency in B cells, EBV blocks signaling and antigen-trafficking functions of the BCR through the activity of the cytoplasmic tail of LMP2A[178–180] that, when phosphorylated, binds to Lyn (Y112) and Syk (Y74, Y85),[181,182] thus blocking BCR signaling. LMP2A also activates the PI3K/Akt pathway, which normally provides a survival signal in response to BCR signaling.[183] Overall, LMP2A maintains viral latency by preventing normal BCR activation, which initiates viral replication, while sustaining survival pathways in latently infected B cells.

CONCLUSION

B-cell activation is initiated by the binding of antigen to the BCR resulting in the triggering of a number of signaling pathways that ultimately drive B-cell proliferation and differentiation to antibody-secreting cells. At present, we understand the biochemical nature of the signaling pathway in some detail. What remains less well understood is the nature of the events following antigen binding to the BCR that trigger these signaling cascades. New tools of live cell imaging both in vitro and in vivo are anticipated to provide an increasingly detailed spatial and temporal picture of events that initiate signaling in both time and space. Our increased understanding of B-cell activation is likely to lead to new approaches for developing therapies for diseases caused by hyper–B-cell activation.

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CHAPTER 10

B-Lymphocyte Responses

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INTRODUCTION

B-lymphocyte responses provide the effective and long-lasting immune protection induced by most vaccines in use today. We measure antibody molecules as the circulating agent of immune protection but now understand much more about the underlying B-lymphocyte response that progressively matures in response to foreign antigen exposure. This chapter will focus on the highly-regulated cellular and molecular development of antigen-specific B-lymphocyte responses.

The three cardinal features of effective B-cell immunity are antigen specificity, antibody class, and antigen binding affinity. To be effective, antibodies must bind vulnerable antigens on the targeted pathogen. During infection, this is a struggle between the evasion mechanisms of the pathogen and preexisting diversity within the adaptive immune system. Different classes of antibodies engage distinct mechanisms of antigen clearance. These various classes of antibody provide either more or less protection depending on the pathogen's portal of entry at infection. Finally, the right antigen-specific antibody still requires induction of sufficiently high binding affinity to provide adequate sensitivity for long-term immune protection. Many promising antigens fail to achieve adequate immunogenicity in contemporary vaccine strategies due to poor affinity maturation. Here, we focus on the sequential mechanisms that program these central attributes of effective antigen-specific B-cell memory.

In the past few years, experimental access to immune response biology has dramatically shifted with the advent of multiphoton laser-based intravital imaging techniques. These studies provide direct access to the mechanics and cell dynamics of antigen-specific cognate regulation in vivo. This information serves to integrate existing knowledge in the field using a real-time scaffold for developmental progression in vivo. Importantly, follicular helper T (T_{FH}) cells have recently emerged as a new class of antigen-specific immune regulator that controls multiple stages of high-affinity B-cell immunity. Understanding antigen-specific T_{FH}-cell development and function remains an active research challenge with great potential for the rational design of future vaccines. Current information regarding the role of antigen-specific T_{FH} cells will be integrated into this chapter to provide a regulatory dimension to B-lymphocyte responses.

Following initial exposure to antigen, T_{FH}-cell–regulated B-cell immunity progresses in three separable stages of antigen-specific development. Each stage is characterized by a B-cell antigen-recognition event followed by contact with cognate T_{FH} cells that determines subsequent B-cell fate. Naïve B cells recognize foreign antigen and then present antigenic peptides to specific T_{FH} cells to progress in development across two major pathways (pre–germinal center [GC] development). Extrafollicular development permits antibody class switch and plasma cell differentiation while entry into the GC reaction is the major pathway to high-affinity B-cell memory (GC cycle). GC B cells in this pathway can switch antibody class and affinity mature their expressed B-cell receptor (BCR) following access to antigen and presentation to GC T_{FH} cells. Upon antigen reexposure, memory B cells recognize, uptake, and then present antigen to memory T_{FH} cells to promote rapid memory B-cell responses and boost circulating high-affinity antibody (memory B-cell response). Antigen recall is the least studied facet of B-lymphocyte responses but provides an important developmental juncture for vaccine-based prophylactic or therapeutic intervention.

This chapter mainly focuses on what is known of antigen-specific B-lymphocyte responses in mouse models with reference to work conducted in humans. Further, there is an emphasis on the response to model antigens that provides a greater understanding of how to manipulate adaptive immunity for preventative vaccination rather than a focus on the immune response to infection.

PRE–GERMINAL CENTER DEVELOPMENT

The initial antibody response to many infectious agents is largely based on the rapid T-cell independent (TI) expansion of B cells and their subsequent differentiation into plasma cells. B1 and marginal zone B cells are largely responsible for these rapid TI humoral responses suggesting that some level of "natural memory" function resides in these B-cell subsets and may be predetermined in an evolutionarily conserved manner. TI antigens can be separated into two broad categories based on their ability to polyclonally activate B cells (TI-1) or require BCR recognition of multivalent epitopes to induce B-cell differentiation in the absence of T-cell help (TI-2). TI-2 antigens can activate BCR signaling but require accessory signals to promote the development of antigen-specific plasma cells. In contrast, monovalent protein antigens require antigen-specific helper T-cell regulation to promote high titer antibody responses and the development of B-cell memory. These T_{FH}-cell–dependent antibody responses take longer to emerge and display a spectrum of BCR affinities and antibody isotypes as multiple strategies for antigen-specific clearance in vivo. Immune responses to

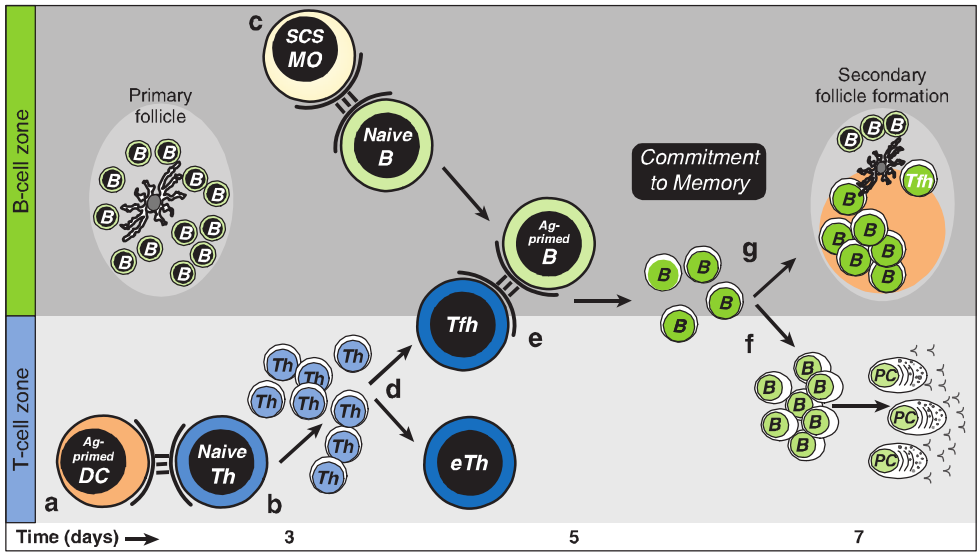

FIG. 10.1. Pre–Germinal Center (GC) Development. **A:** Local protein vaccination induces dendritic cells (DC) maturation and migration to the T-cell zones of draining lymph nodes (LNs). **B:** Peptide major histocompatibility class II expressing DCs will engage naive antigen-specific helper T (T_H) cells to induce proliferation and effector T_H-cell differentiation. **C:** Whole antigen will be trapped by subcapsular sinus macrophage and presented to naive follicular B cells. Antigen-specific B cells will become activated and uptake, process, and present antigenic peptides and migrate toward the T-B borders of the draining LN. **D:** Effector T_H cells emerge in multiple forms with emigrant T_H cells exiting the LN to function at distal tissue sites and T_{FH} cells relocating to T-B borders and interfollicular regions. **E:** Cognate contact between pre-GC T_{FH} cells and antigen-primed B cells is required for multiple programming events in the pathway to B-cell immunity. **F:** Clonal expansion, antibody class switch, and non-GC plasma cell development proceeds within extrafollicular regions of LNs. **G:** Secondary follicle formation and antibody class switch precede formation of the GC reaction as the dominant pathway to memory B-cell formation.

model protein antigens provide experimental access to this complex cascade of cellular and molecular events that underpin long-term protective immunity.

B-cell immunity is initiated on two fronts. Initially, naïve B cells are activated through cell-associated antigen to uptake antigen, process and present peptide major histocompatibility complex [MHC] class II (pMHCII) complexes to enable cognate contact with pMHCII-specific T_{FH} cells. These antigen-primed B cells relocate to T-B–cell borders in lymphoid tissues to increase the likelihood of contact with antigen-primed T_{FH} cells. On the second front, populations of dendritic cells (DCs) also take up antigen, process and present pMHCII, and migrate to draining lymphoid tissues to initiate T_{FH}-cell responses. One outcome of contact with pMHCII-expressing DCs is differentiation of the T_{FH}-cell lineage and the productive contact between pMHCII-specific T_{FH} cells and antigen-primed B cells. This pre-GC cell contact and bidirectional exchange of molecular programming is central to the development of effective B-cell immunity. Antibody class switch, extrafollicular plasma cell differentiation, and initiation of the GC reaction are major B-cell fates associated with this initial phase in B-cell immunity (Fig. 10.1).

Antigen Presentation to B Cells

B cells can acquire soluble antigen by free diffusion into lymphoid follicles[1] or through the lymphoid system of conduits.[2] However, populations of lymph node (LN) subcapsular sinus (SCS) macrophages appear most effective at presenting cell–associated antigen to follicular B cells.[3] B cells take up noncognate antigen presented by SCS macrophages through complement receptors and transfer it into follicular regions and onto follicular DCs (FDCs), which can serve as a source of antigen to prime naïve B cells.[4] In contrast, priming with the cognate antigen at first contact with SCS macrophages results in movement of antigen-specific B cells to the T-B–cell borders and antigen-specific B-cell responses to captured antigens.[4–6] Hence, the SCS macrophages filtering the lymphatic fluid not only protect from systemic infection,[7] but also effectively initiate T helper cell–regulated antigen-specific B-cell immunity.

DCs can also effectively prime B-cell immunity. Injection of DCs pulsed with protein antigen can induce isotype switch and promote efficient B-cell responses. B cells can form synapse-like interactions with antigen-pulsed DCs.[8,9] More recently, two-photon imaging revealed that naïve B cells entering local LNs surveyed protein antigen-pulsed DCs before entering the follicular areas.[10] In this model, engagement of BCR led to calcium flux, migration arrest, and the local accumulation of the antigen-specific B cells. Furthermore, there is a reticular network of collagen fibers that physically connects the subcapsular and paracortical sinuses of LNs to blood vessels and separates these regions from T- and B-cell areas.[11] This organization facilitates the efficient delivery of

soluble antigen toward the lumen of high endothelial venules without entering the LN parenchyma. While resident DCs can access this conduit transport system, follicular B cells may have difficulty accessing soluble antigen.

Nevertheless, in the context of protein antigens, B cells must recognize their cognate antigen and internalize, process, and present peptides from this antigen in the context of MHCII in order to receive pMHCII-specific T-cell help. If the antigen is cell associated, in the presence of adequate costimulation, some aspects of the B-cell response can proceed in a TI manner. B-cell proliferation and plasma cell development can occur in the absence of cluster of differentiation (CD)40-CD40L interactions with some residual isotype switch induced by the action of TACI and BAFF-R.[12] Thus, B cells can respond in a TI manner to protein antigens but do not express the characteristic range of outcomes and the full extent of protective immunity that is found with cognate regulation and T-cell help.

Antigen-Specific B-Cell Activation

Initial activation of naïve B cells through the BCR triggers multiple gene expression programs that enable effective contact with cognate T helper cells. Dynamic contacts with membrane-associated antigens determines the amount of antigen naïve B cells accumulate following antigen exposure.[13] Effective cell contacts require expression of the signaling adaptor dedicator of cytokinesis 8 (DOCK8).[14] Mutations in DOCK8 disrupted integrin ligand accumulation in the immune synapse without altering BCR signaling events. B-cell–specific conditional ablation of the calcineurin regulatory subunit 1 (CNB1),[15] myocyte enhancer factor 2c,[16,17] and stromal interaction molecule 1 and stromal interaction molecule 2[18] have shown that calcium responsiveness is necessary for cell cycle progression in these early pre-GC stages of B-cell responses. Hydrogen voltage-gated proton channels 1, which are internalized with the BCR, have been recently implicated in early B-cell programming events.[19] Single-pulsed BCR signaling[20] that only partially activated NF-κB increased CC chemokine receptor 7 and MHC class II expression, and responsiveness to CD40, indicating some of the early facets of B-cell activation. Severe defects in early B-cell proliferation have also implicated integrin-binding CD98hc[21] and extracellular signal-regulated kinase activation[22] in preparing the antigen-primed B cells to receive cognate T-cell help in vivo. Hence, initial antigen recognition, uptake, processing, and presentation critically impact the early B-cell developmental fate.

High-resolution dynamic imaging has provided substantial insight into the earliest events associated with initial BCR engagement on naïve B cells. The membrane cytoskeleton controls BCR diffusion; disruption of this organization initiates signaling.[23,24] Discrete microclusters form upon antigen binding[25] and recruit multiple components of the intracellular BCR signaling network to initiate signal transduction.[26] One rapid response involves B-cell spreading to increase surface contact with antigen on the presenting cells.[13] Central clustered antigen at the cellular interface is then internalized into antigen-processing lysosomes for presentation with MHCII[8] as the cognate point of T_{FH} contact. Curiously, antigen presentation appears asymmetrically segregated with one daughter retaining larger antigen stores thereby more able to contact pMHCII-specific T_{FH} cells.[27] Whether these developmental outcomes are directed by initial context of antigen presentation or stochastically assorted[28] remains an interesting fundamental issue with an early impact on antigen-specific B-cell development.

Dendritic Cell Maturation

DCs are essential antigen-presenting cells (APCs) for initiating adaptive immunity. Multiple DC subsets exist prior to antigen challenge. Different phenotypic schemes can be used to characterize DC subsets with the origins and developmental relatedness of different DC subsets still subject to debate.[29,30] In the murine system, three main bone marrow–derived DC subsets enter all secondary lymphoid organs via the blood and reside at different levels. These blood-derived DCs all express CD11c and are distinguishable as $CD11b^{hi}CD8a^{neg}$ DCs, $CD8a^{hi}$ DCs, and $6B2^{hi}$ plasmacytoid DCs. In LNs draining the skin, there are at least two further CD11c+ DC subsets, Langerhans cells (LCs) and dermal DCs, that emigrate from the skin, even at homeostasis.[31] Thus, before antigen challenge, multiple subsets of DCs are available to differentially process and present antigen to the adaptive immune compartment.

Protein antigen administration in the absence of inflammation induces immune tolerance. In contrast, coadministration of an immune adjuvant activates facets of innate immunity, induces inflammation, and primes antigen-specific adaptive immunity. Sensing pathogens involves pattern recognition receptors such as the evolutionarily conserved toll-like receptors[32,33] and the more recently described nucleotide oligomerization domain–like receptors.[34] Most forms of antigen and innate stimulators require DC priming at some level for B-cell immunity, whereas particulate antigen in virus-like particles are more reliant on B-cell innate receptor activation.[35] Nevertheless, both families of innate receptors recognize different types of microbial components initiating programs of DC maturation that promote immediate local inflammation and innate effector clearance mechanisms.

Temporal and spatial constraints on DC maturation provides another layer of regulation for the innate system that can impact adaptive immunity. In the steady state, DCs form dense networks at the T-B borders of LNs. Interestingly, motile lipopolysaccharide-activated DC immigrants will rapidly coalesce with this preexisting network in vivo.[36] In separate studies using genetically tagged LCs, the emigrants of LCs and dermal DCs were shown to emerge separately in time and colonize separate regions of the T-B border.[37] A similar temporal regulation was seen using antibodies to specific pMHCII complexes with resident DCs presenting an early wave of pMHCII and dermal DCs emerging later in a second wave.[38] In each of these studies, the immune stimulus was varied and the coordinated response of the innate system also qualitatively and quantitatively different.

Clonal Selection in Helper T Cells

The initial outcome for pMHCII+ DC interactions with naive T_H cells is T-cell receptor (TCR)-driven clonal selection. T_H-cell responses that focus the specific T_H-cell response to a set of dominant TCRs provide access to the mechanisms that underpin T_H clonal selection.[39–41] Earlier studies indicate the importance of TCR-pMHCII affinity in determining T_H cell fate.[39,42–44] In this model, selective expansion of clonotypes expressing higher-affinity TCRs would rely on competition for pMHCII complexes on APCs. Alternatively, in vitro studies using altered peptide ligands suggested that the duration of TCR-pMHCII contact was critical to cell fate and therefore defined "best fit" in vivo.[45–48] In support of this model, T_H cells expressing TCR with fast off rates were lost over time in vivo.[49] Further, there is evidence for different peptides stabilizing the pMHCII to create a hierarchy of dominant peptides.[50] Each and all of these variables would impact what is considered "best fit" and influence the outcome of clonal selection. More recently, we provide evidence for a model of T_H clonal selection that is based on a TCR affinity threshold.[51] Multiple affinity-based thresholds underpin cell fate and T_H clonal expansion[52] with evidence that the highest affinity TCR assort into the T_{FH} compartment.[53] Hence, selection thresholds generate antigen-specific clonal diversity in ways that impact the development of effector T_H cell function in vivo.

Follicular Helper T Cells

There were early reports of effector T_H cells specialized to regulate B-cell responsiveness. CXCR5 expression was first reported on CD4+CD45RO+ cells in the peripheral blood and secondary lymphoid tissue in humans.[54] Gene ablation studies emphasized the role of CXCR5 and CC chemokine receptor 7 in the correct positioning of T and B cells in secondary lymphoid tissue that was also needed to support effective T_H cell–dependent B-cell immunity.[55,56] Blocking CD28 and OX40 interactions in vivo blocked the development of CXCR5+ T_H cells and the GC reaction.[57] Further, CXCR5 expression was induced in an antigen-specific manner on T_H cells in vivo, and these cells relocated to follicular areas and the GC of responding lymphoid tissue.[58] CXCR5+ T_H cells were sorted from human tonsil and shown to support antibody production in vitro.[59,60] The tonsillar CXCR5+ T_H cells expressed high levels of CD40L and ICOS and were found in both the follicular mantle and GC. Adoptive transfers distinguished CXCR5+ B cell helper activity (T_{FH}) from P-selectin ligand^{hi} tissue homing inflammatory mediation (DTH-promoting T_H) that emerged together from the same set of precursors in vivo.[61] The term T_{FH} cells was coined to categorize this functionally and phenotypically distinct effector T_H cell compartment.[59,60]

As the name implies, the cardinal characteristic of all T_{FH} cells is their repositioning into the follicular regions of secondary lymphoid tissues. Early assessments of cytokine production by in vitro restimulated CXCR5+ T_{FH} cells indicated interleukin (IL)-2, interferon (IFN)-γ, and IL-10 from human peripheral blood[60] with evidence for IL-4 and IFN-γ from TCR transgenic mouse T_{FH} cells.[61] Early microarray analyses suggested separable gene expression programs for T_{FH} cells and other known T_H-cell subsets. CXCL13 was highlighted early[62] with evidence for ICOS, IL-21,[63,64] IL-21R,[65] and the differential expression of Bcl-6[63] being used as the most reliable attributes of T_{FH} function in vivo. Thus, acquisition of special pre-GC T_{FH}-cell functions was associated with the programming of a separate T_H-cell lineage.

Molecules important in the development of normal B-cell immunity were implicated in early studies of T_{FH} development. CD28 deficiency or treatment with blocking CD28 antibodies led to profound defects in B-cell immunity.[66] CD28 was required early to initiate naive T_H-cell responsiveness to pMHCII+ CD80- and CD86-expressing DCs. In contrast, CD40-CD40L interactions were central to the delivery of T-cell help to B cells.[67–69] The CD28 family member ICOS[70] was implicated in T_{FH}-cell function in pre-GC interactions with pMHCII-expressing B cells.[71–73] ICOS-deficient humans and mice[74–76] and ICOS-L–deficient mice had marked deficits in all aspects of B-cell immunity. ICOS deficiency was associated with decreased T_{FH}-cell development and considered an important molecule in the delivery of effector T_{FH} function.[77,78] Conversely, overexpression of ICOS in mice with a regulatory defect in ICOS expression[79] resulted in an overproduction of CXCR5+ T_{FH} cells and breakthrough autoimmune disease.[64] Recent studies indicated that ICOS can substitute for CD28 and rescue the T_{FH} defects and B-cell defects in CD28-deficient mice.[80] Furthermore, the abundant CXCR5+ T_{FH} cells in this model act in a T-cell autonomous manner to promote autoantibody production.[81] PD-1, another CD28 family member that has been implicated in the negative regulation of chronically activated T cells, was also found on GC T_{FH} cells in human tonsil and mouse.[82] Positive and negative influences of antigen-specific T_{FH}-cell costimulation are balanced in ways that remain poorly understood in vivo and are an active avenue of current research in this field.

BCL6 is required for development of the T_{FH} program[83–85] and is reinforced within T_{FH} cells upon pre-GC B-cell contact.[86,87] IL-21 also plays a major role in T_{FH} function with substantial loss of B-cell immunity in its absence.[88,89] Recent studies have identified BCL6 in antigen-specific T_{FH} cells and BLIMP1, which has an opposing function, in non-T_{FH} cells.[53] BCL6 and BLIMP1 expression was mutually exclusive across these two T_H-cell subtypes, already evident by the second cell division in vivo.[29] More recently, transcription factors c-Maf and BATF were shown to act with BCL6 to program T_{FH} development.[90,91] Hence, distinct transcriptional programming of unique cellular functions directs the early T_{FH}-cell development that is central to subsequent B-cell immunity (Fig. 10.2).

Pre-Germinal Center Follicular Helper T-B–Cell Contact

First contact between antigen-specific T_{FH} and antigen-primed B cells has also been captured through dynamic imaging.[92] Stable "monogamous" interactions between one antigen-specific T_{FH} cell and one B cell can last for a duration of 10 to 60 minutes in the follicular regions of the LNs. These interactions were accompanied by highly dynamic movements, with the B cells migrating extensively and leading

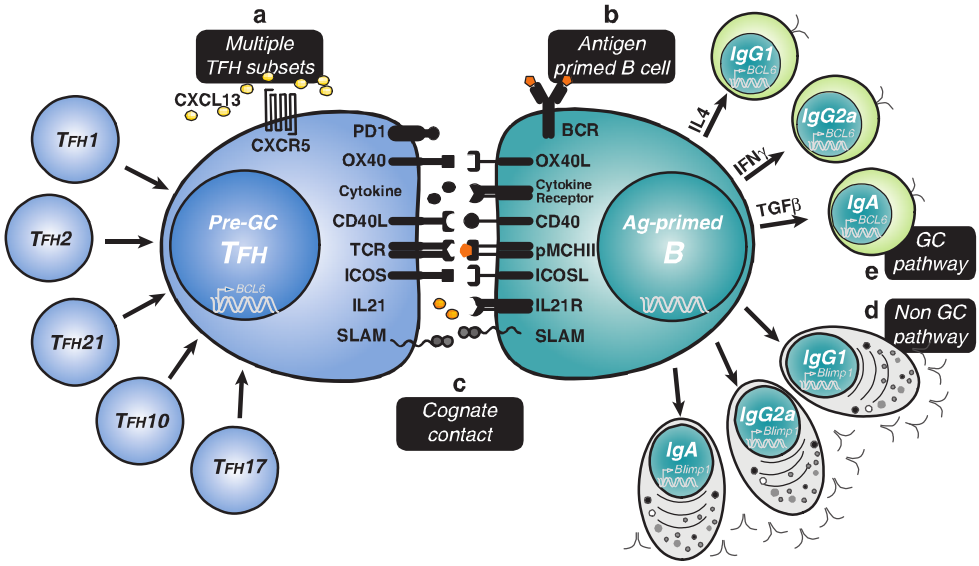

FIG. 10.2. Pre–Germinal Center (GC) Follicular Helper T (TFH)-B-Cell Contact. **A:** Multiple subsets of antigen-specific pre-GC T_{FH} are produced to regulate B-cell immunity. The organization of function within T_{FH} subsets remains speculative. There is evidence for different cytokine-secreting T_{FH} cells that regulate commitment to separate antibody class as well as different types of T_{FH} cells that regulate non-GC plasma cell differentiation. Bcl-6 and CXCR5 expression is thought to be a common feature of all T_{FH} cell subsets. **B:** Antigen-primed B cells must process and present peptide major histocompatibility class II to receive cognate help from pre-GC T_{FH} cells. Upregulation of molecules involved in helper T cell contact is a poorly resolved component of early antigen-driven B-cell maturation. **C:** Cognate contact between antigen-specific T-cell receptor and peptide major histocompatibility class II complexes focus pre-GC T_{FH}-B cell intercellular exchange of molecular information. Modifying interactions at first contact are known to involve costimulatory molecules (eg, cluster of differentiation [CD]40L-CD40, ICOS-ICOSL), accessory interactions (eg, signaling lymphocytic activation molecule family interactions, OX40-OX40L), cytokine and cytokine receptors (eg, IL4-IL4R, interferon [IFN]γ-IFNγ receptor, IL21-IL21R). Distribution of these functional attributes within pre-GC T_{FH} compartments is not yet well resolved in vivo. **D:** The non-GC pathway to plasma cell development permits antibody class switch recombination (CSR) without somatic hypermutation (SHM) depending largely on the cytokine stimulus provide by pre-GC T_{FH} cells. Blimp-1 expression is required for plasma cell commitment across all antibody classes. **E:** The GC pathway to memory B-cell development begins with extensive B-cell proliferation within secondary follicles that will polarize into dark and light zone to initiate the GC reaction. The GC pathway is associated with Bcl-6 upregulation and activation-induced cytidine deaminase expression to supports both antibody CSR and SHM. These GC features emerge across all antibody classes and require productive and long duration pre-GC T_{FH} contact.

the T_{FH} cells.[92] A recent study demonstrated that expression of the adaptor molecule signaling lymphocytic activation molecule–associated protein (SAP) was needed to form long duration contacts with antigen-primed B cells.[93] In these studies, there was no role for SAP in early T_{FH}-DC contacts, and the SAP-deficient T_{FH} cells reached the follicular regions and expressed all the hallmarks of pre-GC T_{FH} cells ($CXCR5^{hi}$, CD40L+, $ICOS^{hi}$, and $OX40^{hi}$).[93] Furthermore, in the absence of SAP, T_{FH} cells were still capable of cytokine production[94] but unable to promote GC formation.[95,96] More recently, dynamic imaging places ongoing critical pre-GC contacts in the interfollicular zones of LNs[97] with a requirement for persistent BCL6 expression in B cells to maintain effective cognate contact.[98] Therefore, early T_{FH}-cell developmental programs establish the capacity for cognate contact needed to promote ongoing antigen-specific B-cell immunity.

It has been unclear how differential BCR affinity can impact the early fate of antigen-primed B cells. Very-low-affinity B cells are capable of forming GCs[99] but fail to do so in the presence of high-affinity competition.[100] In contrast, there is evidence for the highest-affinity B cells preferentially entering the non-GC plasma cell pathway, leaving lower-affinity B cells to mature within the GC cycle.[101] This issue has been addressed more recently using intravital imaging to examine the early pre-GC selection events.[102] In this model, access to antigen was not impacted by BCR affinity, but the differential capacity to present antigen to pre-GC T_{FH} cells assorted with BCR affinity. Increased T-cell help promoted greater access to both the plasma cell pathway and the GC reaction. Thus, BCR affinity thresholds regulate B-cell fate at the earliest pre-GC junctures of antigen-specific T_{FH}-B interactions.

Overall, the precise function of different costimulatory molecules and their combinatorial impact on antigen-specific B cell fate remains an exciting area of current interest. Quantitative differences in cell surface molecules in combination with mixtures of cytokines will likely synergize in

predetermined ways to skew pMHCII-expressing B cells into separate pathways of B-cell immunity. Unraveling these molecular combinations will help to define the rules of molecular control for antigen-specific B-cell immunity.

Controlling Antibody Class

Pre-GC cognate T_{FH}-cell contact controls multiple antigen-specific B-cell differentiation options. Beyond this early pre-GC developmental juncture, antibody class switch appears in both in the extrafollicular pathway to plasma cell differentiation and the GC pathway to memory development.[103] Class switch also requires multiple rounds of cell division in the target cell before expression of non-immunoglobulin (Ig)M antibody.[104] Hence, it is likely that initial commitment to antibody class occurs at the first pre-GC point of cognate contact and is propagated in a lineal manner across each developmental option (see Fig. 10.2).

Class switch recombination (CSR) is an intrachromosonal deletional process between the switch (S) regions that reside 5′ of each constant region gene in B cells (except Cδ).[105] Signaling through CD40 and cytokine receptors induces germline transcription through the targeted S regions providing activation-induced cytidine deaminase (AID; also known as Aicda) access to deaminate cytosines in the single stranded template. AID is required and sufficient for the initiation of the CSR reaction in the activated locus.[106] AID-deficient animals[107] and humans[108] display no CSR or somatic hypermutation (SHM) of the Ig genes. Recent evidence indicates that following antigen stimulation, AID expression is regulated in B cells by paired box gene/protein 5, E-box proteins,[109] homeobox C4,[110] and forkhead box O1.[111] Removal of the resulting uracils and deoxyribonucleic acid (DNA) cleavage generates double strand breaks that trigger the recruitment of DNA damage machinery, mismatch repair, and nonhomologous end joining to complete the CSR event.[112] The adapter protein 14.3.3 is recruited with AID to switch regions[113] and polymerase ζ has been implicated in the repair process associated with CSR.[114] Peripheral B cells undergoing CSR in the absence of the x-ray–repair cross-complementing protein 4 component of the double strand break repair machinery are also highly susceptible to translocation events and oncogenic transformation.[115] Antibody class switch is a destabilizing and potentially dangerous cellular event that can proceed without SHM in the extrafollicular pathway.

Induced CD40L on pre-GC T_{FH} cells and the receipt of this signal through CD40 on B cells is required for antibody class switch.[67,116] Animals and humans lacking CD40 display a hyper-IgM syndrome with profound defects in class switch, GC formation, and the development of affinity matured B-cell memory.[116] ICOS expression on activated T_{FH} cells is thought to act upstream of CD40L in this temporally orchestrated set of events.[117] ICOS-deficient animals also have clear defects in antibody class switch, GC formation, and the development of B-cell memory.[74–76] Some residual class switch in the absence of CD40/CD40L interactions may be explained by the action of TACI and BAFF-R.[12] OX40/OX40L interactions also quantitatively impact class switch while CD27-CD70 interactions promote plasma cell (PC) production.[118] Thus, the range of molecules expressed at the pre-GC T_{FH} cell surface influences the developmental impact of TCR-pMHCII contact on antigen-primed B cells.

Considering the range of antibodies that can be produced, there must be multiple subtypes of pre-GC T_{FH} cells that control antibody class. Different classes of pre-GC T_{FH} would vary in production of T_{FH} cell–derived cytokines to control antibody isotype. IL-4 and IFN-γ are reciprocal regulators of IgG1 and IgG2a production.[119] Animals lacking IL-4 or Stat 6 have decreased IgG1 levels and no IgE.[120,121] IL-4 also acts together with IL-21 to control IgG subtypes and IgE levels.[88] In contrast, transforming growth factor (TGF)β is implicated in the induction IgA, while IL-2 and IL-5 augment IgA production.[122] Similarly, IL-6 may selectively support IgG2a and IgG2b expressing B cells in vivo.[123] Each of these factors can exert their effects in vitro or in a bystander manner in vivo. However, it is thought that the directed delivery of these soluble molecules toward points of TCR-pMHCII contact allows soluble signals to focus locally in an antigen-specific cognate manner.

Within antigen-responsive B cells, the molecular machinery that regulates CSR is deployed in an antibody class–specific manner. The global CSR machinery is targeted by transcription factors downstream of the cytokine receptors that control specific antibody classes. For example, IFNγ activates signal transducer and activator of transcription downstream of the IFNγ receptor to induce T-bet and promote IgG2a class switching.[124,125] Similarly, TGFβ signals through TGFβ-receptor to activate SMAD and RUNX transcription factors to promote IgA class switch.[126] Furthermore, the transcriptional regulator BATF required for T_{FH} development is also required in B cells to generate germline switch transcripts and promote AID expression.[91] Finally, Ikaros regulates antibody class decisions by differentially controlling transcriptional accessibility of constant region genes.[127] How the initial commitment to antibody class is maintained and propagated during clonal expansion, BCR diversification, and affinity-based selection within the GC reaction remains an important but unresolved issue. Therefore, it remains plausible that functional reprogramming accompanies CSR creating separable lineages of class-specific memory B cells in vivo.

Extrafollicular Plasma Cell Development

Under the cognate regulation of pre-GC T_{FH} cells, a cohort of antigen-primed B cells clonally expand within the T-cell zones of secondary lymphoid organs and rapidly give rise to PCs. Within the first few days after antigen exposure, small foci of B-cell blasts can be seen within the T-cell zones.[103] This plasmablast stage appears transitional and defines pre-PCs that may secrete antibody but also retain the capacity to proliferate. In contrast, PCs are typically considered terminally differentiated and in a postmitotic state.[128,129] PCs display a marked increase in IgH and IgL messenger ribonucleic acid and prominent amounts of rough endoplasmic reticulum to accommodate translation and secretion of abundant Ig. They have reduced or lost numerous cell surface molecules

including B220, CD19, CD21, and CD22 with an increase in the proteoglycan syndecan-1 (CD138),[130,131] often used as a distinguishing marker for PCs. We recently demonstrated that class-switched plasma cells retained robust antigen-presentation capacity as a negative feedback loop for regulation of T_{FH} cells.[132] These studies further highlight the bidirectional programming between antigen-primed B cells and pMHCII-specific T_{FH} cells during developing immune responses.

In mice deficient for SAP, the extrafollicular pathway to PC development, remains largely intact but the GC pathway is blocked.[95] Recent studies indicate that SAP-deficient T_{FH} cells exhibit heightened CD40L expression but decreased ICOS induction that alone can account for this defect.[94] More recent studies indicate that SAP-deficient T_{FH} cells can only form short duration cognate contact with antigen-primed B cells, which are insufficient for GC entry but adequate for PC formation. It will be important to dissect the separable signals and cellular subsets of pre-GC T_{FH} cells that regulate the rapid but short-lived effector B-cell response in vivo.

Extrafollicular PCs have half-lives of 3 to 5 days[133] and express germline-encoded antigen-specific antibodies.[131,134] Newly formed PCs migrate via the marginal zone bridging channels into the red pulp of the spleen and into the medullary cords of LNs. Migration patterns in vivo appear controlled by increased responsiveness to the CXCR4 ligand CXCL12 and decreased expression of CXCR5 and CC chemokine receptor 7.[135] There is evidence for BCR affinity-based selection even at this early stage in the response.[131] In some studies, B cells expressing low affinity BCR remain non-GC and high-affinity B cells preferentially enter the GC pathway.[100] The converse can also be seen with high-affinity B cells preferentially entering the non-GC short-lived PC pathway.[101] While seemingly contradictory, these different outcomes in vivo may reflect the plasticity of intercellular control at the T_{FH}-B point of contact.

The transcriptional control of PC development also exists in multiple layers controlling a cascade of developmental change. The transcriptional repressor prdm-1 (encoding Blimp-1) plays a central role in the regulation of PC development.[136] B cells lacking Blimp-1 do not differentiate into extrafollicular PC or post-GC PC, with TI and TD antibody responses profoundly diminished in vivo.[137] These Blimp-1–deficient animals also display defective levels of serum antibody, suggesting that spontaneous production of antibody by B-1-B cells also requires Blimp-1 expression. Structure-function analysis indicates the modular action of Blimp-1 integrating a variety of environmental cues that lead to PC development.[138,139] Blimp-1 represses proliferation through c-myc as one direct target among many others involved in cell cycle control. Blimp-1 also induces antibody secretion by repressing the transcription factor Pax-5, thereby derepressing Xbp-1. The transcription factor Xbp-1 controls the unfolded protein response, and many facets of the cellular secretory mechanism that are critical to PC function and survival.[140–142]

The transcriptional coactivator OBF-1 also appears important for B cells to complete the PC program.[143] In the absence of OBF-1, Bcl-6, Pax-5, and AID are not repressed, blocking the induction of Blimp-1 and PC development. In contrast, ablation of the transcription factor MitF leads to the spontaneous development of PC that appears independent of antigen stimuli.[144] Interestingly, reexpression of bcl-6 and its cofactor MTA3 re-activates the B-cell program and increased CD19 and MHC II, and decreased CD138 in plasma cell lines.[145] This remarkable study suggests that PC fate is not as terminal and passive as it has been thought to be, and that the PC fate remains subject to dynamic gene expression programs. Regulation of the unfolded-protein response by X-box-binding protein 1 is not needed for plasma cell development but is necessary for antibody secretion.[146,147] Epstein-Barr virus-induced molecule 2 also appears essential for B-cell movement to extrafollicular sites and the non-GC plasma cell response.[148,149] In addition, Epstein-Barr virus-induced molecule 2 guides recently activated B cells to interfollicular LN regions and then to outer follicular areas as a prelude to GC formation. Furthermore, there appears to be an early pre-GC proliferative phase at the perimeter of follicles that also precedes GC formation and BCR diversification.[150] Interestingly, recent dynamic imaging studies indicate migration of T_{FH} cells to the follicle interior, even before accumulation of GC B cells.[97]

THE GERMINAL CENTER CYCLE

Movement of antigen-primed B cells into the follicular regions of lymphoid organs after effector T_{FH}-cell contact initiates the GC pathway to memory B-cell development. These pre-GC B cells expand rapidly within the follicular region to form areas of B220+IgD^{low} antigen-specific B cells referred to as secondary follicles. Secondary follicles polarize into T-cell proximal dark zones of cycling centroblasts and opposing light zones of largely noncycling centrocytes among the dense processes of FDC and sparse presence of GC T_{FH} cells.[151,152] This broad anatomical distribution of the GC reaction orients the activities of clonal expansion, BCR diversification, and clonal variant selection that underpin the evolution of high-affinity memory B cells and post-GC plasma cells in this pathway (Fig. 10.3).

Under normal physiologic conditions, the GC reaction emerges as the most efficient means to control affinity maturation and memory B-cell development. However, in the disorganized or absent preimmune lymphoid subcompartments of mice lacking LTa, LTb, tumor necrosis factor receptor I, and LTbR, the activities of the GC reaction remain disorganized but largely intact.[153–156] Vestiges of the GC or small cell aggregates manage the expansion, diversification, and selection steps required for affinity maturation and memory B-cell development.

Germinal Center Cellular Dynamics

Dynamic imaging of the GC reaction in situ has provided outstanding clarity to the kinetics of GC B cell[157,158] and GC T_{FH}-cell movements and interactions.[159] There was evidence for interzonal movement of GC B cells indicating bidirectional migration between light and dark zones,[157–160] with one study emphasizing the majority of movement to be

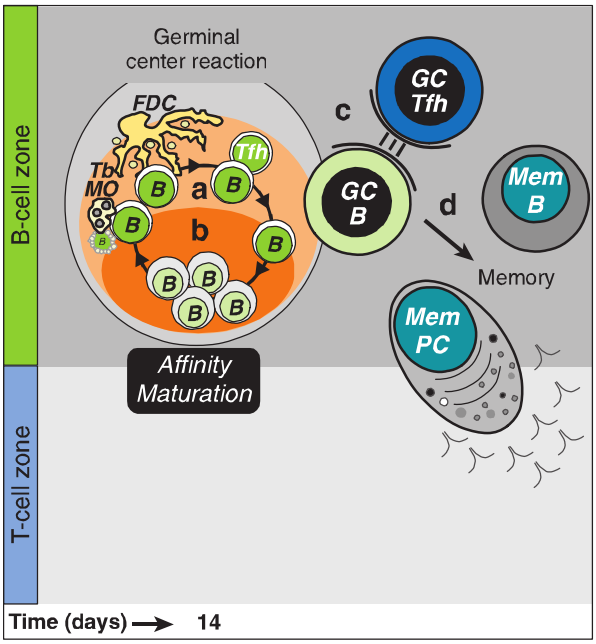

FIG. 10.3. The Germinal Center (GC) Reaction. **A:** Polarization of the secondary follicle anatomically signifies initiation of the GC cycle with the dark zone supporting GC centroblast expansion, class switch recombination and B-cell receptor (BCR) diversification through somatic hypermutation. **B:** Noncycling GC centrocytes move to the light zone and continually scan follicular dendritic cell networks. Centrocytes that lose antigen binding will undergo apoptosis while those that express variant BCR with higher affinity can compete for binding to antigen-specific GC T_{FH} cells. **C:** Cognate contact with GC T_{FH} cells requires peptide major histocompatibility class II expression by GC centrocytes that can promote reentry into the dark zones and the GC cycle or exit from the GC. **D:** Entry into the affinity-matured memory B cell compartments of nonsecreting memory B cells and post-GC plasma cells.

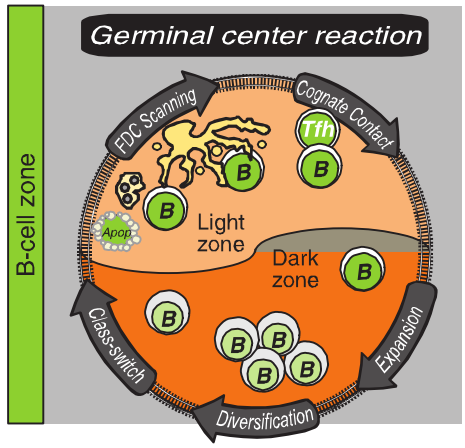

FIG. 10.4. The Germinal Center (GC) Cycle. The GC cycle is initiated through pre-GC contact with cognate follicular helper T (T_{FH}) cells that promotes extensive proliferation of antigen-primed B cells. The GC cycle is thought to begin when immunoglobulin D– secondary follicles polarize into micro-anatomically distinguishable dark zones (DZ) (containing centroblasts) and light zones (LZ) (containing centrocytes, antigen-laden follicular dendritic cell [FDC] networks, and antigen-specific GC T_{FH} cells). Antigen-specific GC B cells' clonal expansion in the DZ is accompanied by B-cell receptor (BCR) diversification through SHM and antibody class switch. Both SHM and CSR are associated with transcriptionally active gene loci, are associated with deoxyribonucleic acid replicative and repair machinery, and occur during cell cycle. Hence, we associated these activities with the DZ phase of the GC cycle. Exit from cell cycle coincides with relocation of noncycling GC B cells to the LZ. Continual scanning of immune complex–coated FDC is observed in the LZ and has been associated with the potential for GC B cells to test variant BCR for antigen binding. Loss of antigen binding can lead to death by apoptosis and clearance of dead cells by tingible body macrophages in the LZ. Positive signals through the BCR during FDC scanning program GC B cells to compete for contact with cognate GC T_{FH} cells. Productive contact with GC T_{FH} cells can induce reentry into the GC cycle through movement back into the DZ and induction of cell cycle and BCR rediversification. Alternatively, affinity-matured GC B cells can exit the GC as either a nonsecreting memory B cell precursors for the memory response, or secreting long-lived "memory" plasma cells that contribute to serologic memory.

intrazonal.[157,160] Surprisingly, cell division appeared in both zones of the GC in contrast to classical models.[157–159] There was also evidence for the ability of naive B cells to traverse the GC environment with evidence that high-affinity B cells could also enter and dominate existing GCs.[158] All studies identified highly motile GC B-cell movements with evidence for continuous uninterrupted movement over FDC processes. This movement contrasted with the capacity of GC B cells to form frequent short-term contacts but infrequent stable contacts with GC T_{FH} cells.[159] The latter group suggested that these infrequent cognate contacts play a dominant role in the selection of high-affinity BCR expressing GC B cells.[161] Hence, a unique model for affinity-based selection emerges with uninterrupted access of GC B cells to antigen-coated FDCs followed by the ability of "reprimed" B cells to express pMHCII complexes to amounts that gain competitive access to pMHCII-specific GC T_{FH} cells.

More recently, labeling of B cells based on GC zonal location with a photo-activatable green fluorescence protein tag provided more conclusive evidence for these activities in vivo.[162] These elegant studies indicated that proliferation was largely restricted to the dark zone followed by a net movement to the light zone. Importantly, movement back into the dark zone and reinitiation of proliferation was controlled by antigen presentation to GC T_{FH} cells.[162] These studies provide experimental evidence for the reiterative cycles of BCR diversification and positive selection as central events during affinity maturation driving clonal evolution in the antigen-specific memory B cell compartment (Fig. 10.4).

Memory B-Cell Evolution

The GC reaction requires T-cell help as it is absent in athymic nude mice, CD40- and CD40L-deficient mice, and is diminished using reagents that block or deplete T_H-cell function such as anti-CD4, anti-CD40, and anti-CD28.[163–165] Mice deficient in ICOS also display profound defects in all aspects of T-cell–dependent B-cell responses.[74–76] Conversely, mice deficient in Roquin, an inhibitor of ICOS expression, produce excessive numbers of T_{FH} cells and GCs and are prone to autoimmunity.[64] However, TI antigens can also promote GC

reactions, but they collapse within the first week after priming with no evidence of BCR diversification.[166] In contrast, marginal zone B cells can be recruited into T-cell–dependent responses and form GCs that diversify and affinity mature.[167] Interestingly, in these studies, the marginal zone B cells responding to antigen challenge expressed a preimmune BCR repertoire distinct from that of the follicular B cells responding in the same animals. Hence, there is some redundancy to the control of GC formation and flexibility to the origins of the B cells recruited into the GC reaction.

The transcriptional repressor Bcl6 is highly expressed in the GC and is necessary for GC formation.[168] However, IgM and IgG1 antigen-specific memory B cells can develop in the absence of Bcl6.[169] These studies were based on direct labeling with antigen and their capacity to respond to soluble low-dose antigen recall. However, there were no GC formed, and the memory B cells expressed no SHM or evidence for affinity maturation. Bcl6 has been shown to repress p53, the tumor suppressor gene that controls DNA-damage–induced apoptosis.[170] Regulation of p53 in this manner may protect GC B cells to allow for the DNA breaks that are necessary intermediates in SHM and CSR. Bcl6 also directly represses Blimp-1[171] and hence must be lost at some point during the GC reaction to allow subsequent development of memory B cells and post-GC PCs in vivo.[137]

The GC cycle of activity regulates clonal evolution within antigen-primed B-cell responders. All pre-GC B cells express some measure of antigen specificity and appear preselected into the memory B-cell pathway based on germline BCR expression. Massive and rapid clonal expansion with doubling times of 6 to 8 hours underpins secondary follicle formation and drives clonal expansion in the GC reaction. It is now clear that GC B cells in the light zone also have the capacity to proliferate.[172] Intravital studies emphasize the more open nature of the GC itself demonstrating the capacity of naive B cells to traverse the follicular region occupied by the antigen-responsive GC.[173] Importantly, antigen-specific B cells could also be recruited into ongoing GCs if they expressed sufficiently high-affinity BCR. While these studies use high frequencies of BCR transgenic B cells pulsed into the ongoing response, it is intriguing to consider the ramifications of such an open network of affinity-based selection and its impact on the composition of the memory B-cell compartment.

Somatic Hypermutation

BCR diversification is dependent on DNA replication and largely restricted to GC B cells in the pathway to memory. Upon expansion in the GC reaction, antigen-specific B cells downregulate their germline BCR and diversify their variable region genes through SHM. Single base substitutions, rare insertions, and deletions are introduced into a region spanning 1.5 to 2.0 kb downstream of the transcription initiation site; however, activity peaks within the V(D)J region and decreases within the J-C intronic region of IgH and IgL variable genes.[174] The mutation rate approaches 10−3 per base pair per generation, which is approximately six orders of magnitude higher than spontaneous mutation frequencies. Thus, approximately one mutation is introduced with each cell division. Analysis of mutation in "passenger" Ig transgenes that are not under selection pressure indicate intrinsic sequence hot spots for the mutator mechanism.[175] Hence, BCR diversification accompanies extensive clonal expansion within the GC reaction generating progeny that express variant antigen-binding BCR (Fig. 10.5).

AID is the central component of the SHM mechanism. Originally discovered through complementary DNA subtraction focused on novel genes in GC B cells,[176] it was then found to be defective in an autosomal-recessive form of hyper-IgM syndrome.[108] Mice deficient in AID were able to form the GC reaction but were unable to undergo CSR or SHM.[107] AID deaminates cytosine to uracil in single-stranded DNA that can be processed by a mutagenic repair pathway.[105,177] The initial changes target sequence-dependent hotspots within rearranged variable region genes of antibodies. Uracil excision by uracil DNA glycosylase is then processed by error-prone DNA replication to introduce point mutations within the actively transcribed Ig locus. Error-prone processing using mismatch repair and base excision repair factors is selectively offset with high-fidelity processing to protect genome stability.[178] The targeting spectrum of AID associated with its active site can be altered to modify variable region gene SHM[179] and rate of antibody diversification.[180] AID stability within the cytoplasm of Ramos B-cell lines can be regulated by heat shock protein 90 with specific inhibition leading to destabilized AID[181] providing a means to modify the rate of antibody diversification. The details of this mutating complex, as well as its action and regulation within the GC reaction, are active areas of research that have been reviewed in detail elsewhere.[182]

Cell Cycle Arrest and Apoptosis

The control of cell cycle in the GC environment is of fundamental importance to the evolution of high-affinity B-cell memory. Variant GC B cells exit the cell cycle and move toward the light zone to undergo selection. Dysregulated cell cycle arrest promotes enlarged GCs in situ. This phenotype is found in multiple genetic knockout models that also influence the composition of the memory B-cell compartment. Large GCs form in the absence of AID and are thought to indicate a negative feedback mechanism that follows SHM and/or CSR.[107] The absence of the CDK inhibitor $p18^{INK4c}$ more directly impacts GC size through blocking cell cycle arrest and also decreases the formation of long-lived PCs.[183] The large GCs in the absence of Blimp-1[137] may also be related to the lack of cell cycle control that may contribute to the post-GC PC defect in these mice. The absence of the main regulatory subunit of calcineurin, CnB1, results in the large GC phenotype, the loss of late stage antibody production, and the diminution of the memory B-cell response to antigen recall.[184] It will be important to assess whether these putative cell cycle defects directly impact high-affinity memory B-cell evolution or indirectly modify GC B-cell fate and the onset of apoptosis.

It is generally believed that the majority of GC B cells expressing variant BCR will die in situ. Extensive local apop-

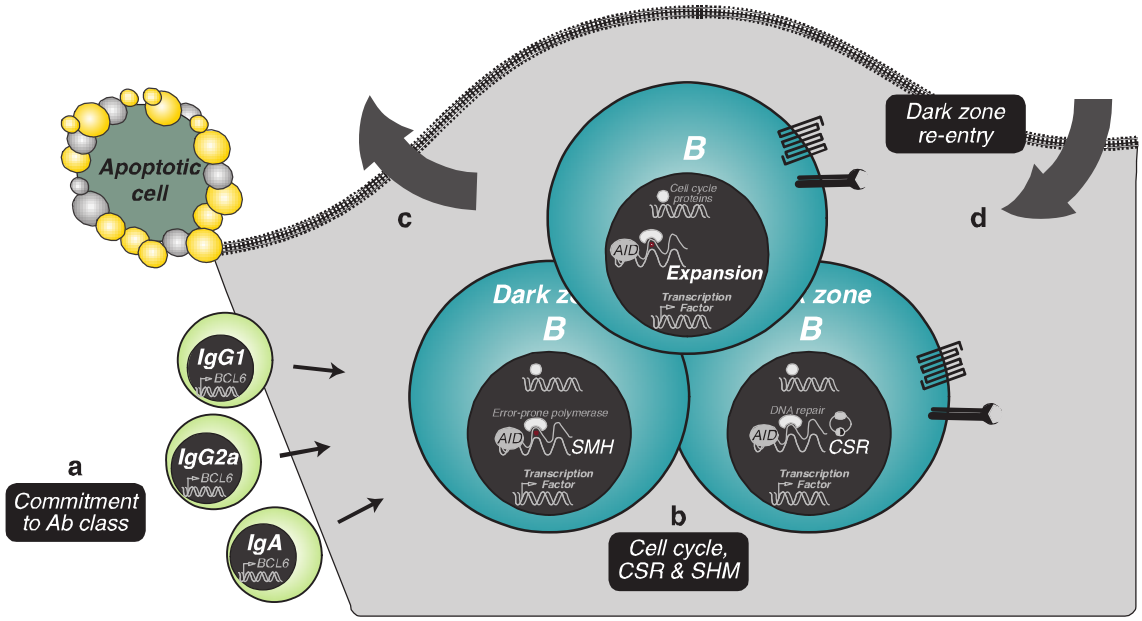

FIG. 10.5. The Germinal Center (GC) Dark Zone (DZ). **A:** Pre-GC cognate follicular helper T cues instruct antigen-primed B cells to initiate the GC reaction. It is likely that commitment to antibody class is preprogrammed at this initial juncture and all classes of B cells can seed the primary GC response. **B:** Molecular control of the cell cycle is an integral component of DZ B-cell dynamics and Bcl6 expression involved in ways that remain poorly resolved. Activation-induced cytidine deaminase (AID) expression, cytosine deaminase activity, and uracil deoxyribonucleic acid (DNA) glycosylase initiate the somatic hypermutation (SHM) machinery targeted to single-stranded DNA. Uracil excision is then processed by error-prone DNA replication to introduce point mutations into the rearranged antibody variable genes. Class switch recombination (CSR) can also occur during this DZ phase using AID to target DNA cleavage to antibody switch regions, generate double strand DNA breaks that trigger the DNA damage machinery to complete the CSR event. The interrelatedness of cell cycle control, SHM, and CSR is not clearly resolved in vivo. **C:** DZ GC B cells will reexpress diversified B-cell receptors and enter the light zone (LZ) of the GC reaction to undergo antigen-based selection. **D:** Following antigen-based selection and cognate contact with GC follicular helper T cells, LZ GC B cells can reenter the DZ regions and rediversify the BCR to initiate secondary rounds of affinity maturation.

tosis is characteristic of the GC reaction. Furthermore, overexpression of antiapoptotic molecules such as Bcl-2 or Bcl-xL prolongs survival of GC B cells without improving selection efficiency.[185] In the absence of CD95 (Fas), there is also a dilution of high-affinity clonotypes in the memory B-cell compartment.[186] Cell death is a prevalent outcome of the GC cycle.[187] Fas receptor expression on B cells regulates negative selection in the GC.[188] Moreover, myeloid cell leukemia sequence 1 has emerged as a major antiapoptotic factor controlling GC B-cell formation and survival.[189] Positive selection of variant GC B cells must be a major driving force within the GC and is based on the increased capacity of the mutated BCR to bind antigen. Mutations in the comodifiers of BCR signal also impact GC B-cell dynamics. Unlike the CnB1 mutation, defects in CD45 and CD19 reduce proliferation and survival of GC B cells arguing for a more global impact of these molecules on BCR signal integration.[172,190,191] Initiation of the GC development program may also be altered to differing degrees in these animal models in ways that are difficult to dissect. Driving the expression of Cre recombinase at a stage in development when germline Cγ1 has been transcribed has helped to overcome these problems.[192] This model allows the conditional deletion of alleles at a late stage in antigen-driven B-cell development that occurs after the first T_H-B–cell checkpoint in development.

Affinity Maturation

Affinity maturation requires the positive selection of GC B cells expressing high-affinity variant BCRs. The details of this process are still poorly understood. Receipt and integration of signals through the BCR are clearly involved in positive selection and must be based on the affinity for antigen. Ablation of BCR signals such as calcineurin-dependent signals, mutations in the CD19 signaling pathway, and loss of CD45 interferes with positive selection and memory B-cell development.[172,184,190,191] However, as discussed previously, it is difficult to dissect the GC BCR interactions with antigen from the initial BCR triggers that recruited naive B cells into the primary response. Most models suggest immune complex (IC) trapping on FDCs as the most likely means for variant BCRs to receive a rescuing signal from native antigen. Antigen appears rapidly in this location and can persist for extended periods of time focused to FDC in lymphoid tissue draining the site of antigen administration.[193] In support of this notion, complement receptors CD21/CD35 on FDCs are needed to generate long-term serum antibody responses.[194] However, animals that

do not secrete antibody and therefore cannot form ICs can still support affinity maturation.[195] In the absence of inhibitor of NF-κB kinase 2–dependent activation of the NF-κB pathway, FDCs can still capture ICs but are not stimulated to upregulate vascular cellular adhesion molecule-1 and intercellular adhesion molecule-1.[196] In the absence of these adhesion molecules, GC B cells appear more susceptible to apoptosis with evidence for altered gene expression and decreased affinity maturation.

Evidence connecting BCR signal strength within the GC B-cell compartment and affinity maturation has been lacking. BCR signaling and antigen presentation are required to initiate the GC reaction and hence are difficult to manipulate specifically within the GC. In the absence of DOCK8, which causes compromised early immune synapse formation, early GCs still develop.[14] However, without DOCK8, these GCs do not persist and GC B cells do not undergo affinity maturation. Calcium influx as a consequence of BCR signaling also appears dispensable for affinity maturation under multiple T cell–dependent priming conditions in vivo. Although B cells deficient for stromal interaction molecule 1 and stromal interaction molecule 2 or for CNB1 exhibit profound defects in proliferation in vitro,[15,18] these signaling molecules are dispensable for the maturation of antibody responses in vivo. Downstream of BCR signals, the transcription factor myocyte enhancer factor 2c is necessary for early B-cell proliferation and GC formation,[16,17] but dissecting the pre-GC versus GC function remains unresolved. B cell–specific deletion of nuclear factor of activated T cells, cytoplasmic 1 also compromises B-cell responses in vivo[197] but the level of the defect remains unclear. Nevertheless, as BCR signal strength must drive affinity maturation at some level, it remains important to resolve the B-cell intrinsic mechanisms that also help to shape the affinity of the memory B-cell compartment.

Antigen Scanning on Follicular Dendritic Cell Networks

Polarity in the GC microenvironment is partly controlled by the differential expression of chemokines and their receptors.[198] Higher expression of CXCL12 in the dark zone assorts CXCR4-expressing centroblasts, while higher CXCL13 in the light zone attracts CXCR5-expressing centrocytes. This study used flow cytometry and cell cycle status to identify GC B-cell subsets and demonstrate the aberrant behavior of GC B cells from various genetically modified host animals. Recent two-photon analysis revealed the movement of GC B cells along FDC processes within the GC.[159] This study emphasized the lack of dwell time for GC B cells on FDC processes with little change in GC B-cell velocity upon FDC contact. Therefore, if antigen binding is associated with GC B cell–FDC contact, there appears to be no evidence for interclonal competition between different GC B cells. These studies describe when variant GC B cells are most likely to contact antigen with the opportunity to test the binding properties of their expressed mutated BCR (Fig. 10.6).

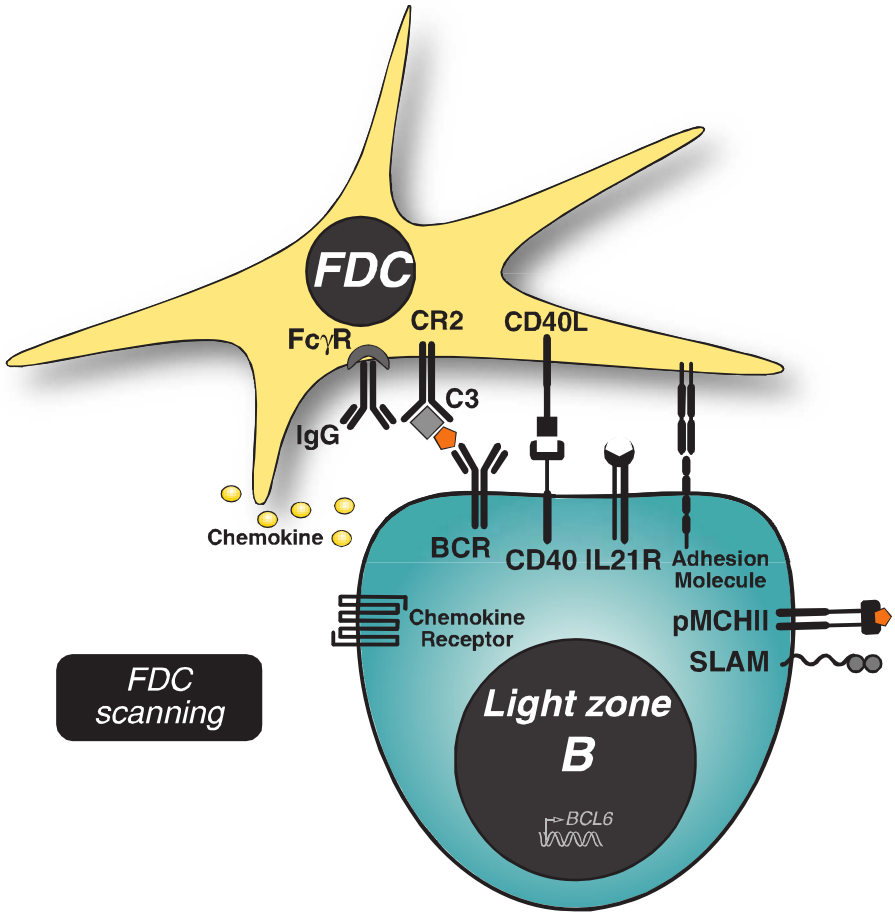

FIG. 10.6. Antigen Scanning on Follicular Dendritic Cell (FDC) Networks. FDC scanning appears as continuous movement of germinal center B cells along mature immune complex-laden FDC processes. These contacts are more similar to stromal cell-associated trafficking behavior than stable immune synapse-like interactions. Differential B-cell receptor (BCR) affinity for antigen may influence antigen-uptake and peptide major histocompatibility class II presentation at this juncture of development. Programs of gene expression for molecules able to modify cognate contact may also be differentially induced due to BCR signal strength through FDC scanning.

As with all in vivo studies, there are substantial influences associated with the choice of models, affinities of BCR, type of antigen, and method of immunization that will impact the observations presented in the studies. The issues of antigen receptor monoclonality are self-evident in BCR transgenic models in the context of repertoire studies, but are less clearly understood in association with the expression of diverse effector functions in vivo. More importantly, in the context of T-cell responses, it is now apparent that elevated precursor frequency deviates the dynamics of clonal expansion[199,200] and can alter the development of T-cell memory in vivo.[201] Changing the balance or affinity of regulator populations will impact cell fate of the target populations in ways that remain difficult to assess. Nevertheless, new insights offered by dynamic imaging continue to buttress our appreciation for the workings of the immune system and serve to challenge existing dogma in powerful and productive ways.

Germinal Center Follicular Helper T Cells

In contrast to pre-GC effector T_{FH} cells discussed previously, CXCR5+ T_H in GC will be referred to as GC T_{FH} cells. Many CXCR5+ T_{FH} cells were found very early in the immune response (day 3), at the T-B borders and within follicular regions of LNs.[58] These early studies also provided evidence for a circulating compartment of CXCR5+ T_H cells. Hence, it is clear that CXCR5 expression is not a reliable marker of GC T_{FH} cells only. Further, the expected function of GC T_{FH} cells would be broadly different from those described for pre-GC effector T_{FH} cells. The presence of GC T_{FH} cells among the dense FDC networks of the GC light zone predict a role in the propagation of high-affinity variant GC B cells into the long-lived memory B-cell compartment[198] (Fig. 10.7).

Subsequent to FDC scanning, some GC B cells were shown to make more stable, immune synapse-like contacts with GC T_{FH} cells, as determined by two-photon imaging.[159] These early images gave rise to the notion that competition for GC T_{FH} cells may be the limiting factor in GC B cell selection of variant high-affinity BCRs.[161] More recently, antigen presentation by GC B cells without engaging the BCR was shown to dominate the selection apparatus within GCs.[162] GC B cells capable of presenting more antigen rapidly exited the GC reaction and produced more post-GC plasma cells. These studies indicated similar mechanisms to the pre-GC selection event[102] and argued strongly that antigen presentation to GC T_{FH} cells was the rate-limiting event during affinity maturation in the GC cycle.

It remains technically difficult to manipulate cellular and molecular activities within the GC cycle without interfering with the developmental programs that initiate the GC reaction in the first place. Many of the molecules associated with pre-GC T_{FH} function may also function within the GC. BCL6 expression itself is reinforced within T_{FH} cells upon contact with pre-GC B cells.[98] ICOS–ICOSL interactions are important throughout this pathway at early DC contact,[202] pre-GC contact,[78] and likely during the GC reaction itself. IL-21 and its receptor appear of continued importance at the pre-GC stage and during the GC reaction.[86,87] Sphingosine-1 phosphate receptor 2 has an important role in confining GC B cells to the GC niche in vivo.[203] Elevated programmed cell death 1 expression also correlates with GC localization of the T_{FH} compartment[82] and the absence of programmed cell death ligand 2 on B cells impacts PC production and affinity maturation.[204] Most interestingly, cytokine production and class-specific GC B cells appear to be associated in the GC well beyond the original CSR event.[205] This surprising functional pairing between GC T_{FH} cells and switched GC B cells (eg, IL-4+ T_{FH} cells with IgG1+ GC B cells; IFNγ T_{FH} cells with IgG2a+ GC B cells) hints at the extended level of heterogeneity that exits within the GC cycle of memory B-cell development. Hence, it is likely that each separable class-specific GC B-cell compartment requires cognate contact with separate class-specific GC T_{FH} cells.

During antiprotein immune responses, the T_{FH} cells within the light zone of the GC express pMHCII-specific TCRs. There appears to be sequential movement of antigen-specific T_{FH} cells from the T-cell zones into the GC microenvironment[40,206] with evidence that suggests GC T_{FH} cells can also move between different GCs.[207] Interestingly, all antigen-specific T_{FH} cells responding to a protein antigen are not represented within the GC reaction, indicating pre-GC functional differentiation for responders T_{FH} cells.[208] Furthermore, non-GC T_H cells can reemerge in a memory response, indicating that the GC is not required for memory T_H-cell development.[40] Downregulation of CD90 (Thy-1) has been used as a marker of GC T_{FH} cells in the mouse, although when this occurs and how well it discriminates only GC T_{FH} cells is not clear.[207] Interfering with CD40L-CD40 interactions disrupts the GC reaction, while blocking B7-2 interaction impairs memory B-cell development.[209] Hence, the cognate control of GC B-cell fate appears to be modified by the cellular and molecular context of pMHCII presentation.

The work on human T_{FH} cells use expression of CD57, as well as CXCR5, to define T_{FH} cells that are contained within the GC of tonsils.[63,210] Microarray analysis highlights the distantly related functional programs of CD57+ GC T_{FH} cells as compared to naive T_H cells, central memory T_H cells, and effector memory T_H cells from the peripheral blood.[210] Differences in adhesion molecules, chemokine receptors, cytokines, and transcription factors appear as distant as different lineages. Interestingly, the CXCR5 ligand, CXCL13, is highly expressed in GC T_{FH} cells. In vitro–derived T_H1 and T_H2 effector T_H cells also appear distant in gene expression program to GC T_{FH} cells.[63] This analysis associates CD84, CD200, IL-21, and BCL6 with CD57+ GC T_{FH} cells. A separate analysis of T_{FH} cells by function and gene expression demonstrates that T_{FH} activity in human tonsil is independent of CD57 expression.[65] These studies highlight $ICOS^{hi}CXCR5^{hi}$ T_{FH} as CXCL13 secretors and the most potent inducers of antibody production in vitro. Therefore, it is clear in situ that all GC T_{FH} cells express CXCR5 and many express CD57; however, these molecules are not exclusively expressed on GC T_{FH} cells nor expressed on all T_{FH} cells in the tonsil. Nevertheless, it remains important to clarify GC T_{FH} phenotype so that the details of their function can be pursued in vivo.

MEMORY B-CELL RESPONSE

It is now clear that centrocytes can undergo proliferation in the light zone,[191] and there is direct evidence for movement

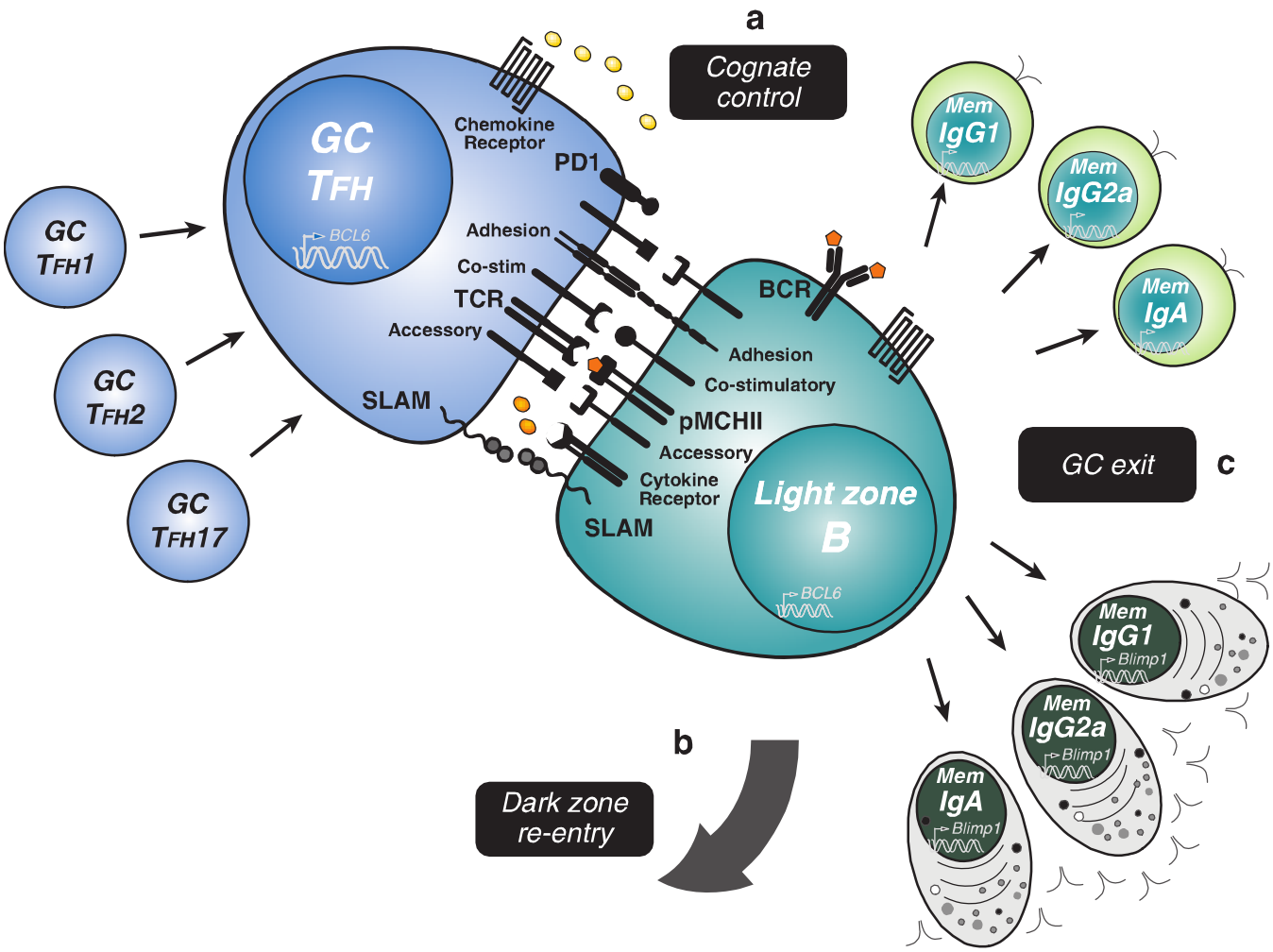

FIG. 10.7. Germinal Center (GC) Follicular Helper T (TFH)-B-Cell Contact. **A:** Longer duration cognate contact with GC T_{FH} cells in the light zone can be visualized directly in vivo. Similar to earlier pre-GC events these contacts must focus around T-cell receptor–peptide major histocompatibility class II interactions and can be modified by a multitude of intercellular exchanges of molecular information. There is still little detailed analysis of these interactions in vivo. We depict the class of molecules that can be associated with this critical programming event but do not appreciate the organization of these interactions or their precise developmental imprint as yet. **B:** Antigen presentation by B cells can influence dark zone reentry and reinitiation of B-cell receptor diversification with cell proliferation, somatic hypermutation, and class switch recombination. **C:** GC cognate contact can also initiate GC exit into separable nonsecreting memory B cell and post-GC long-lived memory plasma cell compartments.

of centrocytes back into the dark zone of GCs.[159,173] Thus, it appears that one outcome of GC B cell-GC T_{FH} interactions is reentry into the GC cycle permitting reiterative rounds of expansion, diversification, and selection. In this model, each subsequent round of clonal expansion and BCR diversification is applied to GC B-cell variants that have been positively selected based on increased BCR affinity. Hence, the introduction of a few mutations in each clonal progeny of selected variants is less likely to destroy BCR specificity and leaves room for further increases in an already high-affinity variant BCR.

The second major developmental outcome of cognate contact in the GC is exit from the GC cycle and entry into the memory B-cell compartment. The GC reaction produces at least two broad categories of affinity-matured memory B cells.[187] The most typical memory B cells are the precursors for a memory response to antigen recall. These memory B cells are easily distinguished functionally from the second cellular compartment of memory, the post-GC PCs.[211] The post-GC PCs are terminally differentiated cells that continually produce high-affinity antibody and will not be drawn into a secondary response. In both categories of memory B cells, the expression of antibody isotype distinguishes separable memory B-cell compartments. By definition, these subsets differ in the antibody they can produce and hence their developmental program and cellular function is distinct. However, these "class-specific" memory B-cell subsets may also differ in migration patterns, survival needs, and the requirements for reactivation at the time of antigen recall. These issues define a level of heterogeneity that has re-

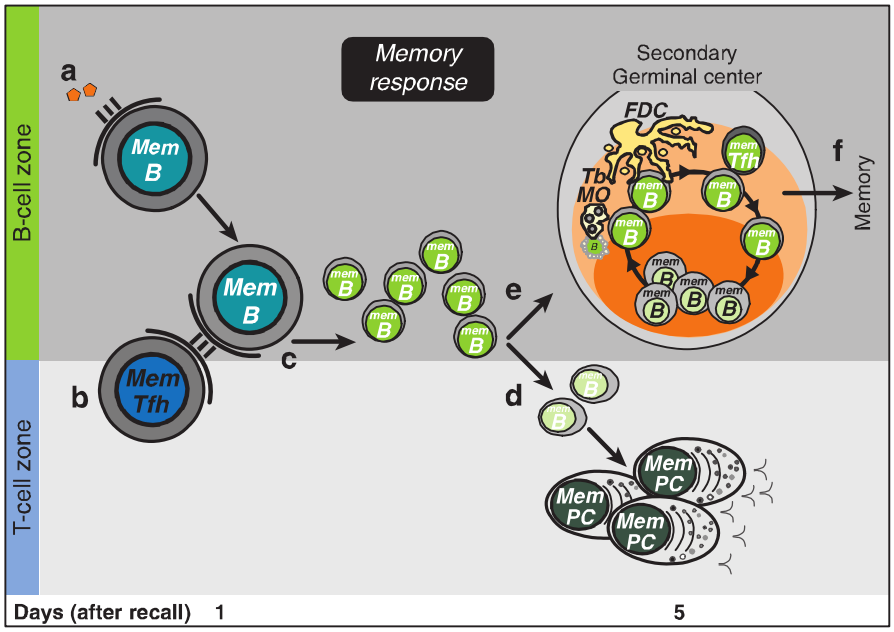

FIG. 10.8. Memory Response to Antigen Recall. A: Memory B-cell responses can emerge in the absence of innate inflammatory stimuli. In this case, the central antigen presenting cells are the affinity-matured memory B cells themselves. B: Memory B-cell response to T-cell–dependent antigens still require helper T (T_H)-cell regulation upon antigen recall. When priming and recall antigens are identical, memory T_H cells are the rapid responders and are thought to emerge preferentially over their low-frequency naïve counterparts. Regarding the regulation of memory B-cell responses, antigen-specific memory T_{FH} cells are the most likely candidate for rapid cognate regulation. C: Cognate contact at this developmental juncture occurs across sets of memory B cells and memory T_{FH} cells with an organization and kinetics that remain poorly resolved in vivo. There is rapid and vigorous local clonal expansion within the first 2 to 3 days in both memory B and memory T_{FH} compartments. D: Expansion of affinity-matured memory B response plasma cells occurs very quickly with evidence that most memory-response plasma cells already appear affinity-matured. E: There is evidence for memory B-cell subsets that express a germinal center (GC) phenotype and create GC-like structures upon antigen recall. Whether these structures are residual from the primary response GC or reemerge with GC activities upon recall has not been resolved. F: Increased numbers of memory B cells and memory-response plasma cells persist after antigen recall. It remains unclear whether these cells are the product of memory GC reactions or extrafollicular non-GC memory response outcomes.

ceived very little attention at the current time and remain important features in the organization of B-cell memory in vivo (Fig. 10.8).

Memory Response Precursors

There are reports of non-GC early memory B-cell development,[86,169] although how well these germline BCR-expressing memory B cells compete with post-GC memory B cells in the response to antigen recall remains to be evaluated. Affinity-matured IgM+ memory B cells can emerge from the GC reaction and persist for long periods in vivo.[212] These nonswitched memory cells appear more active in secondary responses in the absence of circulating antibodies. Genetic labeling of AID-expressing cells with yellow fluorescent protein allowed memory B cells to be monitored over long periods.[213] Surprisingly, primary response GC reactions were persistent for extended periods of time (over 8 months after priming) depending on the form of antigen used to immunize. In these studies, class-switched memory B cells rapidly promoted plasma cell differentiation, whereas their IgM+ counterparts promoted secondary GC reactions. Depending on the form of antigen delivery and the combination of innate stimuli, B-cell responses can be skewed toward memory formation with extended GC reactions, which can last over 1.5 years.[214] Hence, it is possible that persistent GCs can continuously produce nonsecreting memory B cells well after the initial priming event.

Memory response precursors are more typically affinity-matured post-GC B cells that have switched to non-IgM antibody isotypes and can respond, in a T_H-cell–dependent manner, to low-dose soluble antigen recall. The long-term persistence in vivo of antigen-binding B cells, after the decline of the primary response, provides the single best indicator for all memory response precursors. Beyond this particular attribute, antibody isotype and

stage of development within the memory B-cell compartment introduces a level of cellular heterogeneity that is only recently being appreciated.

As soluble antigen is the ligand for the BCR, most experimental approaches use variations of labeled antigen to identify antigen-specific B cells. While panning techniques used gel-associated antigen to detect antigen-specific B cells,[215,216] flow cytometry provided the most reliable access to antigen-specific B cells.[217,218] These earliest studies[217,218] coupled cell sorting technology and direct labeling to enrich antigen-specific B cells for adoptive transfer. These early studies helped to demonstrate that B cells with receptors for antigen were the precursors for antibody-forming cells. This approach has been adopted by many groups,[219–222] with the subsequent evaluation of specificity and purity demonstrated by the frequency for antibody forming cells or the enrichment for production of antigen-specific antibodies in vitro.

Early studies indicated antigen-binding B-cell populations of long-lived cells with slow or no turnover in vivo.[223] These memory B cells can survive independent of their expressed BCR specificity and hence do not require persistent antigen depots in vivo.[224] Loss of surface IgM/IgD and expression of downstream isotype are also indicative of antigen experience but are not required for memory-cell development.[218,220,225] Further, the expression of mutated BCRs with evidence for affinity increasing changes is the most useful molecular marker for the memory B-cell compartment.[131,226–228] However, there are also abundant examples of germline-encoded BCRs expressed by memory response precursors. Location has also been a reliable means for isolating memory B cells from the blood very soon after intentional priming.[226] The combination of location, phenotype, genotype, and time after intentional priming has many elements of a comprehensive definition for memory B cells.

Post–Germinal Center Plasma Cells

High-affinity antibody-producing PCs that emerge from the GC reaction can also be considered an integral part of antigen-specific B-cell memory. High-affinity GC B cells preferentially assort into the PC compartment giving rise to high-affinity circulating antibodies.[229] In LNs, affinity-matured PCs dwell in paracortical areas to mature[230] then migrate toward the medullary regions prior to export.[231] CD93 is expressed at this early stage and is required for PC survival in the bone marrow.[232] Clearly, the circulating antibody that is produced by post-GC PCs contributes to ongoing serologic immune protection.[233]

Post-GC PCs do not self-replenish through turnover, secrete isotype-switched antibody, and display evidence of SHM with affinity increasing mutation patterns.[211,234,235] This post-GC antigen-specific B-cell compartment appears during the second week after initial antigen exposure[131] and preferentially homes to the bone marrow for growth factor support of stromal cells.[236] Based on gene ablation studies, these cells use a variety of redistribution mechanisms such as upregulation of CXCR4, α4β1 integrin binding to its ligand vascular cellular adhesion molecule-1[135] to get to the bone marrow, where they can persist for the life of the animal.[133,237–241] In the bone marrow, long-lived PC need signals through the tumor necrosis factor receptor family member, BCMA, for survival.[242] It has also been proposed that a pre-PC precursor[243] or memory cells themselves[233] produce PCs in a non–antigen-dependent manner as a means of maintaining serum antibody levels for extended periods.

The extended longevity of the post-GC PC can be demonstrated using BrdU incorporation and adoptive transfer.[238–241] The extinguished gene programs associated with PC development[142,244,245] support a terminally differentiated end-stage cell that needs to arrest cell cycle progression[183] and will not be reactivated on antigen recall. Nevertheless, based on the evidence of a GC phase in development, the extended longevity of these PCs and the continued production of high-affinity antibody, it is reasonable to consider that these end-stage B cells belong to the memory B-cell compartment. We have recently demonstrated that post-GC antibody-secreting B cells not only express BCR, but also present antigen and can modulate cognate T_{FH}-cell responses.[132] These surprising studies further demonstrate that PCs negatively regulate BCL6 and IL-21 expression in antigen-specific T_{FH} cells.[132] Thus, PCs are not only the producers of antibody, but can also engage in antigen-specific immune regulation. Signals through the BCR or MHC class II on post-GC PCs may serve to regulate the ongoing production of high-affinity antibodies in the serum. The long-term antigen presenting or regulatory function of post-GC PCs has not yet been elucidated.

Based on variability in memory B-cell formation across different antigen models, it appears likely that different sets of initiating stimuli modify the balance of cells within the different memory B-cell compartments. The memory cell balance may be a quantitative "interpretation" of the quality of the initial immunizing signals. Ultimately, memory B-cell fate is likely to be controlled by interactions in the GC itself. The strength of BCR signal and costimulatory context serve to select variant BCR and initiate changes in memory B-cell development. Cognate interactions with GC T_{FH} cells may consolidate these functional outcomes. Understanding the role of the different memory B-cell subsets in long-term protection and the rules that govern their development in vivo remain important unresolved areas in this field.

Memory Response to Antigen Recall

Persistent high-affinity serum antibody provides the first layer of protection against antigen recall. While binding to antigen is a clearance mechanism, it also serves to increase the efficiency of antigen presentation to memory B cells through rapid IC formation and binding to FcR or complement receptors on cells of the innate system. In this manner, antibody may amplify the sensitivity of the memory B-cell response to antigen recall. However, the boost with antigen rechallenge also seems necessary to establish adequate long-term protection following protein vaccination. The cognate cellular dynamics and molecular regulation of the memory response boost are important factors in the consolidation of B-cell memory that remain poorly understood and inadequately optimized in most vaccine regimes.

Primary response GCs persist for about 3 weeks after initial priming, but this timing may vary substantially depending on the immune stimuli.[151,152] There is evidence for continued selection in the memory B-cell compartment even after the demise of the GC reaction.[235] This selection process represents more typical interclonal competition without further BCR diversification.[246] Where this secondary selection occurs and how it relates to the selection mechanisms in the GC remains to be determined. Nevertheless, secondary selection events appear capable of reshaping the memory compartment toward higher-affinity clonotypes that can substantially influence the quality of the secondary response and consolidation of the memory compartment.[233] Recent evidence indicates that memory B cells can reinstitute a GC reaction upon antigen recall. The form of antigen appears to have a role in primary response GC persistence with particulate antigen more likely to promote GC longevity.[213] Moreover, innate system stimuli differentially impact persistent GC structures with combinations of toll-like receptor-4 and toll-like receptor-7 more effective than single stimuli.[214] Whether the secondary GC is a continuation and reexpansion of a primary GC activity remains unclear. More importantly, whether these secondary or persistent GC-like structures support the rediversification of affinity-matured BCR and the selection of even higher-affinity clonotypes remains to be determined. These issues are central to the future management of prime-boost vaccination protocols with substantial practical impact in this field.

Using priming doses of antigen and adjuvant, antigen-specific memory T_H-cell responses[40,41,247] and memory B-cell response[248] emerge more rapidly than their naive response counterparts. Memory T_H-cell responses reach peak levels more rapidly but to similar levels as the primary response. In contrast, memory B cells display accelerated kinetics and reach substantially higher maximal levels compared to the primary response. Even in the presence of priming doses of antigen and adjuvant, the memory responders dominate the recall response,[40,130] outcompeting naive lymphocytes that may express specific Ag-R.

Antigen recall promotes accelerated clonal expansion and rapid differentiation to high-affinity PCs. Initiation and microclustering of IgG1 BCRs is enhanced at the single cell level due to membrane proximal regions in the cytoplasmic tail of IgG1.[249] The cytoplasmic tails of class-switched memory B cells can contribute substantially to the expanded clonal burst associated with retriggering by antigen.[250] There is evidence for distinct changes in BCR signaling pathways.[251–254] Increased affinity of the BCR on memory cells must also contribute to memory B-cell sensitivity to low-dose soluble antigen that does not induce a primary immune response. In addition to these intrinsic attributes, circulating high-affinity antibody contributes to differential management of antigen in vivo. Rapid presentation of immune complexes to the memory B cells maybe enhanced. Furthermore, memory B cells require antigen-specific T_H-cell regulation to initiate secondary immune responses.[255] These issues have not been well studied but remain central to the capacity of memory B cells to expand, self-replenish, and boost high-affinity PCs and circulating antibody levels that provide long-term immune protection.

Antigen-Specific Memory Follicular Helper T Cells

Memory B-cell response to protein antigens require antigen-specific T-cell help.[187] Under normal physiologic circumstances, this secondary T-cell help must be antigen-specific and is most likely delivered by memory T_H cells that are specific to the immunizing antigen. Under typical low-dose antigen rechallenge regime in the absence of immune adjuvant, antigen-specific B cells are likely to be the predominant APC for the memory response. Hence, the cognate interactions of pMHCII+ memory B cells and antigen-specific memory T_H cells define a major developmental checkpoint in the evolution of long-term immune protection. Alternately, circulating antibody and innate immune activation induce an accelerated memory response due to increased frequencies and heightened responsiveness of memory response precursors. In this scenario, the memory T_H cell–memory B-cell checkpoint may be preceded by an independent series of antigen presentation events prior to cognate memory T_{FH}-memory B-cell contact.

The major cellular outcome of the secondary boost is rapid and exaggerated memory B-cell expansion and the production of large numbers of high-affinity PCs. While high-affinity B cells can be drawn into GC reactions,[158] it is not clear that the GC pathway is operative at the time of the secondary boost. Regardless, there is clear evidence for secondary selection events upon antigen rechallenge that are more likely to be driven by cellular selection without somatic BCR diversification.[187] The nature of the selection mechanism for B cells at this stage of the response is also poorly understood. In contrast, the accelerated local reexpansion of antigen-specific memory T_H cells occurs with little change in the expressed TCR repertoire[256] or cytokine-secreting potential.[247] Hence, the antigen-specific memory T_H-cell compartment appears to conserve and reexpress many of the functions associated with the initial primary immune response.

There is evidence for a division of labor in the memory T-cell compartment that involves the migratory capacity of memory T_H-cell subsets.[257] Recirculating central memory T cells and tissue-homing effector memory T cells further divide on their readiness to reexpress effector function upon reexposure to antigen. It is reasonable to propose that each antigen-specific effector T_H-cell subset will produce a memory T_H-cell counterpart.[247] However, the range of T_H memory effector attributes required to control antigen-specific memory B-cell responses is not well understood. Compartments of tissue-localized memory T_H cells provide rapid "reactive" immune protection that is antigen-specific.[257] For example, the focal placement of effector memory T-cell subsets at sites of original antigen entry preempts the behavior of the pathogen. In this manner, location provides an opportunity to accelerate the recall response enhancing the capacity of the immune system to block overt infection. As the precursors of the memory response to antigen recall,

high-affinity memory B cells belong to a similar "reactive" set of memory cells. However, in contrast to local hypersensitivity responses by T_H cells, antigen reexposure promotes rapid and exaggerated memory B-cell responses in the lymphoid tissue that drains the site of rechallenge. In this context, we can refer to the memory T_H-cell compartment that controls memory B cell responses as memory T_{FH} cells.

We have provided evidence for the local persistence of an antigen-specific memory T_{FH} cell compartment. CXCR5+ T_{FH} cells bind pMHCII tetramers, express lower levels of ICOS, and have lost the capacity to express messenger ribonucleic acid for a series of cytokines.[53] These putative memory T_{FH} cells rapidly upregulated cytokine signals following specific peptide restimulation in vivo. We propose that these locally confined memory T_{FH} cells are the cognate regulators of the memory B-cell response. Many of the antigen-specific memory T_{FH} cells retain expression of CD69, which suggests recent contact with pMHCII complexes and provides a plausible mechanism for local retention. It is likely that memory B cells with high-affinity BCR will rapidly capture minute levels of secondary antigen and present this antigen to the cognate memory T_{FH} cells. Hence, memory B cells may be the primary APC in the memory response, further accelerating the memory B-cell response to recall.

It is not clear whether memory T_{FH} cells would necessarily express CXCR5+ like their primary response T_{FH} compartment. Furthermore, it is not clear whether both effector T_{FH} and GC T_{FH} produce memory T_{FH} counterparts. Nevertheless, it will be important to unravel the programs of cognate control used by antigen-specific memory T_{FH} to regulate the memory B-cell response to antigen rechallenge. It is plausible that manipulating cellular and molecular interactions at this developmental juncture can alter the shape of antigen-specific B-cell memory.

Antigen Persistence In Vivo

Tonic signaling through the BCR and the downstream activation of phosphoinositide 3-kinase, together with B cell-activating factor signaling through the B cell-activating factor receptor are required for survival of naïve B cells in the periphery.[224,258] Similarly, inducible deletion of phospholipase Cγ2 after the formation of antigen-specific B-cell memory substantially depleted the memory B-cell compartment and suggested a BCR signaling requirement in memory.[259] Nevertheless, earlier genetic studies indicated that cognate BCR specificity was not required after formation of B-cell memory to provide the tonic survival signal.[224] Thus, persistent antigen appears not to be required for the survival of antigen-specific memory B cells, although memory B-cell function has not been addressed in this model.

More recently, there has been evidence of persistent pMHCII complexes in the context of antiviral responses in vivo,[260] leading to local activation of naïve T_H cells even after clearance of virus. We recently demonstrated a similar persistence of pMHCII complexes for longer than 100 days following protein antigen vaccination in a non-depot adjuvant.[261] The depots of pMHCII complexes were restricted to the LNs draining initial vaccination site and persistent antigen presentation induced naïve T_H-cell proliferation.[261] We proposed that pMHCII complexes on immunocompetent APCs had a role in confining the antigen-specific memory T_{FH}-cell compartment to LNs draining the site of initial priming.[262] Although it has been known for some time that FDC networks are capable of trapping whole antigen as immune complexes for extended periods of time,[161] the nature of the long-lived local APC remains unresolved.

Unlike CD8 T cells, CD4 T_H cells appear to require continued presence of antigen to reach maximal clonal expansion in vivo. The conditional induction and abrogation of pMHCII molecules demonstrate that as soon as a lower threshold level of antigen is breached, T_H cells cease to divide.[263] Surprisingly, this model induces no form of inflammation and would otherwise be considered a model of tolerance induction. Recent intravital imaging studies also indicate that antigen-responsive T_H cells engage multiple DCs at successive early stages after priming.[264] These multiple DC–T_H-cell contacts also impact the development of effector T_H-cell function in vivo. Most surprisingly, there is evidence for persistent depots of pMHCII complexes up to 3 weeks after viral infection.[265] These pMHCII depots are present at times after viral clearance in vivo. The same persistent depots can be demonstrated for pMHCI with the capacity for local activation of viral-specific naive CD8 cells.[266] While persistent antigen is not required for the maintenance of antigen-specific T-cell memory,[267,268] there may be a role for persistent depots of pMHC complexes as local guidance cues for antigen-specific memory T cells.

CONCLUSION

Understanding the cellular and molecular control of antigen-specific memory B-cell development remains a high priority for basic research with important application to future vaccine design. The largest growing class of pharmaceutical agents today is the antigen-targeting antibodies with an endless spectrum of biologic impact. Producing potent antibodies with high affinity is an important facet of this growing new industry.

There has been substantial progress in our understanding of this complex developmental cascade in vivo with many of the cellular processes being more carefully defined in their most relevant in vivo context. The sophisticated manipulation of mouse genetics provides a powerful set of tools that begin to unravel the regulatory mechanisms operative across each developmental checkpoint controlling cell fate in vivo. Real-time intravital imaging provides a major advance to our appreciation of cell dynamics and intercellular communication as it proceeds in the crowded confines of secondary lymphoid tissue. Most importantly, a surge in research activity has revealed many of the mechanisms controlling T_{FH}-cell development and function in ways that have changed the conceptual landscape surrounding our appreciation of cognate regulation in B-cell memory.

Beyond antigen recognition, antibody class determines immune function and binding affinity controls sensitivity within memory B cells. Antigen contact initiates presentation by B cells and a program of events, which is consolidated following B-cell contact with antigen-specific T_{FH} cells. We have outlined these events as a progressive developmental program across three related but distinct phases of antigen encounter. We remain cautious of manipulating critical adaptive immune functions but quietly optimistic. The molecular regulation of antigen-specific cellular events initiates a complex but finite set of regulatory programs that can be modified both indirectly, following vaccination with innate stimuli, and perhaps directly, during the acquisition of high-affinity B-cell memory. The vaccine boost is the most readily accessible phase of this strategy that can directly target antigen-specific adaptive responses. Unraveling the molecules and programs that control each phase of memory B-cell development provides a plethora of new targets for vaccine-based modification in vivo.

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SECTION IV

T-Lymphocytes

CHAPTER 11

T-Cell Antigen Receptors

Mark M. Davis • Yueh-Hsiu Chien

T-lymphocytes expressing αβ or γδ T-cell antigen receptors (TCRs) are found together with B-lymphocytes in all but the most primitive vertebrate animals. These three cell types are the only ones that use random variable (V), diversity (D) in the case of TCR-β and -δ, joining (J), gene rearrangement to generate diverse antigen receptors. During the last two decades, there has been a great deal of progress in identifying the molecules and genes of TCRs, and there is considerable information on their biochemistry and structure. While TCRs share structural and genetic similarities with B-cell antigen receptors (immunoglobulins [Igs]), they also possess a number of unique features pertinent to their specific functions. The first major difference was suggested by the experiments of Zinkernagel and Doherty, who found that cytotoxic cells specific for a viral antigen could only lyse infected cells that expressed a particular major histocompatibility complex (MHC) molecule.[1,2] This phenomenon of "MHC-restricted recognition" is in marked contrast to the recognition of intact antigens by Igs.[3,4] Later work demonstrated that what was being recognized by these T cells, which were of the αβ TCR type, were fragments of antigens or peptides bound to a characteristic groove in MHC molecules.[5] These αβ TCRs are expressed on classical helper and cytotoxic T cells, which predominate in most lymphoid compartments (90% to 95%) of humans and mice.[6] They are also expressed on natural killer (NK)T cells,[7] regulatory T cells,[8] and T cells in the mucosal sites such as the intestinal epithelial compartment (IEL).[9] In most cases, the αβ TCR ligand is a peptide antigen bound to a class I or class II MHC molecule; but in the case of NKT cells, the antigen is a glycolipid bound to a nonclassical class I MHC molecule, cluster of differentiation (CD)1d.[7]

T cells bearing γδ TCRs are less numerous than the αβ type in most cellular compartments of humans and mice (<5%). However, they make up a substantial fraction of T-lymphocytes in cows, sheep, and chickens.[10] γδ T cells coexist with αβ T cells but seem to be better represented in the mucosal compartments.[10,11] Although αβ T cells perform most of the functions classically attributed to T cells, mice lacking γδ T cells are clearly have a compromised immune defense indicating that γδ and αβ T cells contribute to host immune defense differently.[11,12] γδ TCRs also recognize antigens directly, like antibodies, with no apparent need for antigen processing,[13] at least in the most thoroughly studies cases. During the past few years, there have been considerable advances in our understanding of antigen recognition by γδ T cells. This should lead to a better understanding of how γδ T cells contribute to immune competence.

T-CELL ANTIGEN RECEPTOR POLYPEPTIDES

The search for the molecules responsible for T-cell recognition first focused on deriving antisera or monoclonal antibodies specific for molecules on T-cell surfaces. Ultimately, a number of groups identified "clonotypic" sera[14] or monoclonal antibodies.[15–19] Several of these antibodies were able to block antigen-specific responses by the T cells they were raised against or, when coated on a surface, could activate the T cells they are specific for. They were also able to immunoprecipitate 85,000 to 90,000 molecular weight (MW) disulfide-bonded heterodimers from different T-cell clones or hybridomas consisting of two 40,000 to 50,000 MW glycosylated subunits referred to as α and β. Peptide mapping studies showed that there was a striking degree of polymorphism between heterodimers isolated from T cells of differing specificity, thus suggesting that these antigen recognition molecules might be akin to Igs.[20,21]

Work in parallel to these serologic studies exploited the small differences (approximately 2%) observed between B- and T-cell gene expression,[22] and isolated both a mouse[23,24] and a human[25] T cell–specific gene that had antibody-like V, J, and C region sequences and could rearrange in T-lymphocytes.[24] This molecule was identified as TCR-β by partial sequence analysis of immunoprecipitated materials.[26]

Subsequent subtractive cloning work rapidly identified two other candidate TCR complementary deoxyribonucleic acids (DNAs) identified as TCR-α[27,28] and TCR-γ.[29] It was quickly established that all antigen-specific helper or cytotoxic T cells expressed TCR-αβ heterodimers. Where TCR-γ fit in remained a puzzle until work by Brenner et al.[30] showed that it was expressed on a small (5% to 10%) subset of peripheral T cells together with another polypeptide, TCR-δ. The nature of TCR-δ remained unknown until it was discovered within the TCR-α locus, between the V_α and J_α regions.[31] Formal proof that the TCR-α and -β subunits were sufficient to transfer antigen/MHC recognition from one T cell to another came from gene transfection experiments,[32,33] and equivalent experiments have also been done with γδ TCRs.[34]

As shown in Figure 11.1, all TCR polypeptides have a similar primary structure, with distinct V, D in the case of TCR-β and -δ, J, and constant (C) regions exactly analogous to their Ig counterparts. They also share many of the amino acid residues thought to be important for the characteristic variable and constant domains of Igs.[35] The C_β region is particularly homologous, sharing 40% of its amino acid sequences with C_K and C_λ. The TCR polypeptides all contain a single C region domain (versus up to four for Igs) followed by a connecting peptide. These usually contain the cysteine for the disulfide linkage that joins the two chains of the heterodimer (some human TCR-γδ isoforms lack this cysteine and consequently are not disulfide-linked[36]). *N*-linked glycosylation sites vary from two to four for each polypeptide with no indications of O-linked sugar addition. C-terminal to the connecting peptide sequences are the hydrophobic transmembrane regions. These have no similarity to those of the *IgH* genes but instead have one (TCR-β and -γ) or two (TCR-α and -δ) positively charged residues. As discussed later, these charged residues are critical for the association of the ligand-binding TCR polypeptides with the CD3 signaling polypeptides. This is important because the TCR polypeptides have very short cytoplasmic regions with no known role in signaling.

A more recent member of the TCR polypeptide family is the pre-Tα chain, which serves as a chaperone for TCR-β in early thymocytes, similar to the role of λ5 in pre-B cells.[37] It was first identified and cloned by von Boehmer and colleagues.[38] It has an interesting structure that consists of a single Ig constant region-like domain followed by a cysteine-containing connecting peptide, a transmembrane region

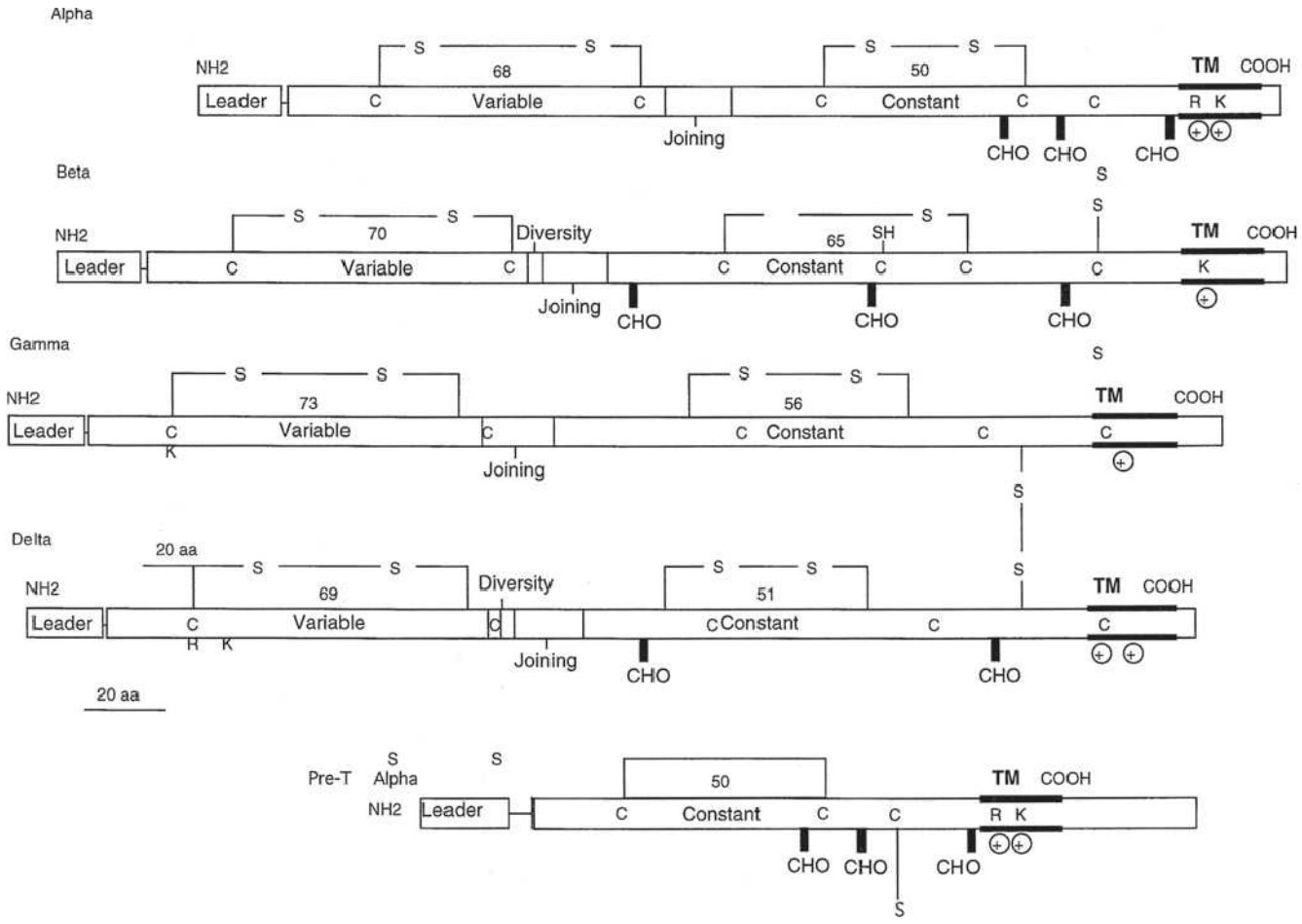

FIG. 11.1. Structural Features of T-Cell Receptors and Pre-T α Polypeptides. Leader (L), variable (V), diversity (D), joining (J), and constant region (C) gene segments are indicated. *Transmembrane* and *bold horizontal lines* delineate the putative transmembrane regions; *CHO* indicates potential carbohydrate addition sites; *C* and *S* refer to cysteine residues that form interchain and intrachain disulfide bonds; *R* and *K* indicate the positively charged amino acids (arginine and lysine, respectively) that are found in the transmembrane regions.

containing two charged residues: an arginine and a lysine spaced identically to the TCR-α transmembrane region. The cysteine in the connecting peptide is presumably what allows heterodimer formation with TCR-β and the similarity to TCR-α in the transmembrane region is most likely to accommodate the CD3 polypeptides. Recently, Rossjohn and colleagues have presented a structure of the pre-Tα chain that is comprised of a single Ig-like domain that is distinct from the C domain of the TCR-α chain; nevertheless, the mode of association between pre-Tα and TCR-β mirrored that mediated by the Cα-Cβ domains of the αβTCR.[39] The pre-TCR has a propensity to dimerize in solution, and the molecular envelope of the pre-TCR dimer correlated well with the observed head-to-tail pre-TCR dimer. This mode of pre-TCR dimerization enables the pre-Tα domain to interact with the Vβ domain through residues that are highly conserved across the Vβ and Jβ gene families, thus mimicking the interactions at the core of the αβ TCR's Vα-Vβ interface. Disruption of this pre-Tα-Vβ dimer interface abrogates pre-TCR dimerization in solution and impaired pre-TCR expression on the cell surface. Their work suggests a mechanism of pre-TCR self-association that allows the pre-Tα chain to simultaneously "sample" the correct folding of both the V and C domains of any TCR β-chain, regardless of its ultimate specificity,[39] which likely represents a critical checkpoint in T-cell development.

In both the mouse and humans, the cytoplasmic tail of pre-Tα is much longer than any of the TCR chains (37 and 120 amino acids, respectively), and the murine sequence contains two likely phosphorylation sites and sequences homologous to an SH3 domain binding region. These are not present in the human sequence, however, and so their functional significance is unclear.[38] Thus, at least in principal, the murine pre-Tα molecule could function as signaling intermediate independent of the CD3 polypeptides, and it has recently been shown that at least one CD3 component (δε, see the following) is not required for it to function normally in early thymocyte differentiation.[40]

CLUSTER OF DIFFERENTIATION 3 POLYPEPTIDES

Immunoprecipitation of the human TCR with anti-idiotypic antibodies after solubilization with the nonionic detergent, noniodet P-40 (NP-40), initially revealed only the α- and β-chain heterodimer. However, the use of other detergents, such as digitonin or Triton-X100, revealed four other proteins.[41–44] These are known as the CD3γ, δ, ε, and ζ. γ and δ form distinct heterodimers with ε within the TCR/CD3 complex (γε and δε), and ζ usually occurs as a disulfide-linked homodimer. In mouse T cells, NP-40 does not dissociate TCR heterodimers from CD3 molecules.[44,45] In some cases, the ζ-chain can be part of a heterodimer in at least two forms. In mouse T cells, the ζ-chain can disulfide bond with a minor variant called the η (eta) chain.[46,47] This latter chain is an alternate splicing variant of the ζ-chain gene.[48] This alternatively spliced species of the ζ-chain is not found in significant quantities in human T cells.[48] The second type of ζ-chain containing heterodimer contains the γ-chain associated with the $F_cεRI$ ($F_cεRI$ γ) and $F_cγRIII$ (CD16) receptors.[49,50] These CD3 subunits, in their various forms, are an integral part of TCR-mediated T-cell recognition because only they possess the immunoreceptor tyrosine-based activation motifs (ITAMs) that are necessary for cellular activation when the TCR engages ligand.

Characterization and Structural Features of the Cluster of Differentiation 3 Polypeptides

Figure 11.2 illustrates the principal structural features of the CD3 γ, δ, ε, and ζ polypeptides as derived from gene cloning and sequencing,[42,51] and more recently by protein crystal structures of the extracellular domains of γ, δ, and ε.[52–55] The extracellular domains of the γ, δ, and ε chains show a significant degree of similarity to one another. These domains retain the cysteines that have been shown to form intrachain disulfide bonds and each consists of a single Ig superfamily domain. The spacing of the cysteines in these domains produces a compact Ig-fold, similar to a constant region domain. The γ and δ subunits form distinct heterodimers with ε via highly conserved residues at the dimerizing interface.[52–55] The connecting peptides of the CD3 γ, δ, and ε chains all contain highly conserved, closely spaced cysteines just before the membrane-spanning regions. These residues are likely candidates for the formation of interchain disulfide bonds and appear to play a role in the assembly of the CD3 and TCR polypeptides.[52,56] The extracellular domain of the ζ chain consists of only nine amino acids and contains the only cysteine, which is responsible for the disulfide linkage of the ζζ homodimer or the ζ $F_cεRIγ$ heterodimer. Each of the γ, δ, ε, and ζ polypeptides contain a conserved, negatively charged amino acids in their transmembrane region complementary to the positive charges seen in the TCR transmembrane regions.[57–60]

The cytoplasmic regions of the γ, δ, ε, and ζ chains are the intracellular signaling "domains" of the TCR heterodimer. Each of these molecules contains one or more amino acid sequence motifs that can mediate cellular activation.[61] The intracellular sequences responsible for this activation are contained within an 18 amino acid conserved ITAM[62] with the sequence $X_2YX_2L/IX_7YX_2L/I$. Both of the tyrosines in this motif are absolutely required to mediate signal transduction as mutation of either completely prevents the mobilization of free calcium or cytolytic activity.[63] This sequence occurs three times in the ζ chain and once in each of the CD3 γ, δ, ε, and $F_cεRI$ γ chains. There are also pairs of tyrosines present in the cytoplasmic domains of the γ, δ, ε, and ζ chains. This sequence motif is also present in the mβ-1 and B29 chains associated with the Ig β-cell receptor and in the $F_cεRI$ β-chain but there are many more[10] in TCR/CD3 than in any other receptors which use ITAMs. The tyrosines in these cytoplasmic sequences are substrates for the tyrosine phosphorylation that is one of earliest steps in T-cell signaling[61] and is thought to occur aberrantly in nonproductive T-cell responses (eg, antagonism; see following). Serine phosphorylation of the CD3γ also occurs upon

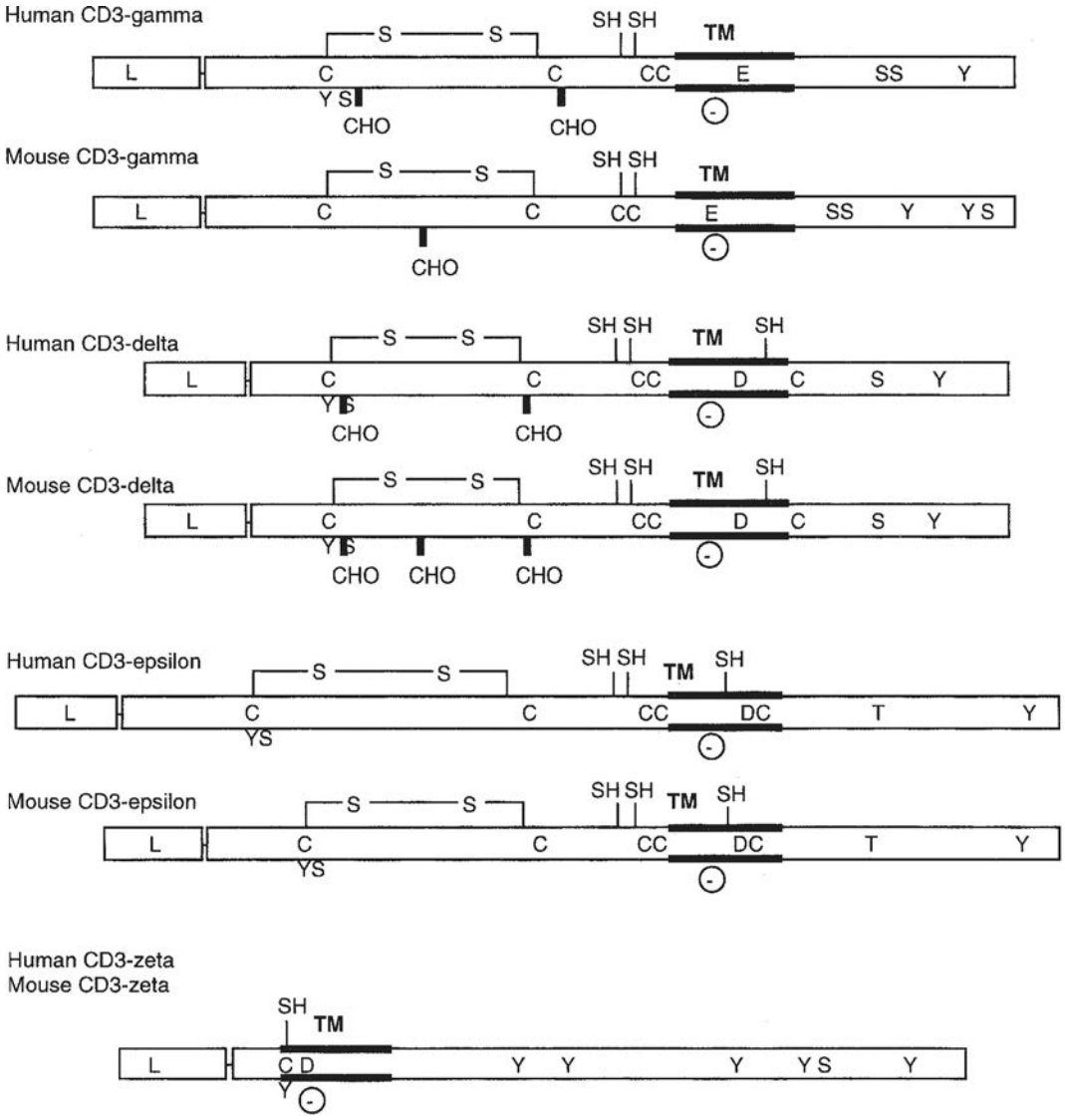

FIG. 11.2. Structural Features of the CD3 Molecules. As in Figure 11.1, transmembrane regions, carbohydrate addition sites, and cysteine residues are indicated. In addition, negatively charged transmembrane residues (*D* for aspartic acid and *E* for glutamic acid) and putative phosphorylation sites are shown.

antigen or mitogenic stimulation of T cells[64] and may play a role in T-cell activation as well.

Recently, live-cell imaging studies have shown a close interaction of the CD3epsilon cytoplasmic domain of the TCR with the plasma membrane, using fluorescence resonance energy transfer (FRET) between a C-terminal fluorescent protein and a membrane fluorophore.[65] Electrostatic interactions between basic CD3epsilon residues and acidic phospholipids enriched in the inner leaflet of the plasma membrane were required for binding. Nuclear magnetic resonance studies of the lipid-bound state of this cytoplasmic domain revealed a deep insertion of the two key tyrosines of the ITAM into the hydrophobic core of the lipid bilayer, likely preventing their phosphorylation.[65] Similar studies of CD3 zeta have confirmed that this is also the case for that component of the TCR/CD3 complex as well.[66] This then likely defines the "off" state of the TCR/CD3 complex and is a novel explanation for how it can prevent tyrosine phosphorylation by lck. What is still mysterious is how ligand binding by the TCR disengages these signaling modules from the plasma membrane and triggers the kinase cascade needed to activate T cells.

Assembly and Organization of the T-Cell Receptor/Cluster of Differentiation 3 Complex

The assembly of newly formed TCR-α and -β chains with the CD3 γ, δ, ε, and ζ chains and their intracellular fate have been studied in detail.[39–41,61,63] Early studies have focused on mutant hybridoma lines, which fail to express TCR on their cell surface, and on transfection studies using complementary DNA for the different chains in the receptor; but recently, Wucherpfennig and colleagues have developed an elegant in vitro translation and assembly system that has clarified a number of important issues.[54,55]

Experiments in a nonlymphoid cell system[67] have shown that TCR-α can assemble with CD3 δ and ε but not CD3 γ

and ζ. In contrast, the TCR-β chain can assemble with any of the CD3 chains except the ζ chain. When the ζ chain was transfected with either α or β chain genes, or any of the three CD3 chains, no pairwise interaction occurred. Only when all six complementary DNAs were cotransfected was it shown that the ζ chain could be coprecipitated with the other chains.[67] Based on these data, a model has been proposed that suggests that TCR-α pairs with CD3 δ and ε chains and that TCR-β pairs with the CD3 γ and ε chains in the completed molecule. The ζ chain is thought to join the TCR and other CD3 polypeptides in that last stage of assembly.

Pulse-chase experiments have shown that all six chains are assembled in the endoplasmic reticulum (ER), transported to the Golgi apparatus, and then transferred to the plasma membrane. It also appears that the amount of ζ chain is rate limiting, as it is synthesized at only 10% the level of the other chains. This results in the vast majority of newly synthesized α, β, or CD3 components being degraded within 4 hours of their synthesis. The remaining nondegraded chains are long lived due to the formation of complete TCR/CD3 complexes with the limiting ζ chain.[68] TCR/CD3 lacking CD3 ζ chains migrate through the ER and Golgi intact but then are transported to and degraded in the lysosomes. The immunologic significance of this pre-Golgi degradation pathway is most evident in CD4+CD8+ thymocytes where, despite high levels of synthesis of both messenger ribonucleic acid and protein for all the TCR, CD3, and ζ chains, surface expression is relatively low. The TCR chains in immature thymocytes seem to be selectively degraded.[68] Thus posttranslation regulation appears to be an important means of controlling the cell surface expression of TCR heterodimers.

The TCR and CD3 γ, δ, and ε chains contain ER retention signals.[68,69] If the γ and δ signals are removed, then the chains are transported through the Golgi and rapidly degraded in the lysosomes. In contrast, removal of the CD3 ε ER retention signal allows this chain, and any associated chains, to be transported to the cell surface. Thus, association of the TCR and other CD3 chains with ε renders their ER retention signals inoperative. However, the ε ER retention signal remains functional. This prevents the surface expression of partial complex intermediaries until CD3 ζ is incorporated into the complex, which then masks the ε ER retention signal and allows the transport of mature complexes to the cell surface.

The overall stoichiometry of the αβ TCR/CD3 complex is controversial. The work of Call and colleagues[57] has shown the relationships between different CD3 dimers (γε, δε, and ζ ζ) and a single TCR αβ heterodimer (as shown in Fig. 11.3). Using mutagenesis, they found very specific interactions based on the positive charges in each TCR transmembrane domain with complementary negative charges in the transmembrane domains of the different CD3 components. The two positively charged residues of TCR-α mediate interactions with the negatively charged residues of the of CD3δε and CD3ζζ dimers, while the single positively charged residue of TCR-β mediates interactions with the negatively charged residues of CD3γε. In addition, their data suggest a highly ordered assembly process as they found that the TCR/CD3δε association facilitates the assembly of CD3γε into the complex and that the association of the TCR and CD3 heterodimers was a prerequisite for incorporation of CD3ζζ into the complex. Importantly, the data show that there is only one TCR heterodimer per nascent TCR/CD3 complex in their in vitro expression system.

In contrast, a number of groups have found evidence that there can be two TCR heterodimers in a given TCR/CD3 cluster on T-cell surfaces. In particular, Terhorst and colleagues[42] showed that in a T-T hybridoma, a monoclonal antibody against one TCR-αβ pair could comodulate a second αβ heterodimer. In addition, sucrose gradient centrifugation of TCR/CD3 showed a predicted molecular weight of 300 kDa, more than 100 kDa larger than expected from a minimal δ subunit complex (α, β, γ, δ, ε_2, ζ_2).[70] Another study suggesting that there are least two TCRs in a given CD3 complex is the Scatchard analysis indicating that the number of CD3ε molecules on a T-cell surface equals the number of αβ TCRs.[71–73] Finally, there is the work of Fernandez-Miguel et al.,[74] who showed that in T cells which have two transgenic TCR-β chains, antibodies to one Vβ can immunoprecipitate the other. It was also found that they are often close enough to allow fluorescence energy transfer, meaning that the two TCR-βs in a cluster are within 50 Angstroms of each other.[74] Interestingly, it appears that the TCR complexes with CD3 either have CD3 γ or CD3 δ, but not both, and these two

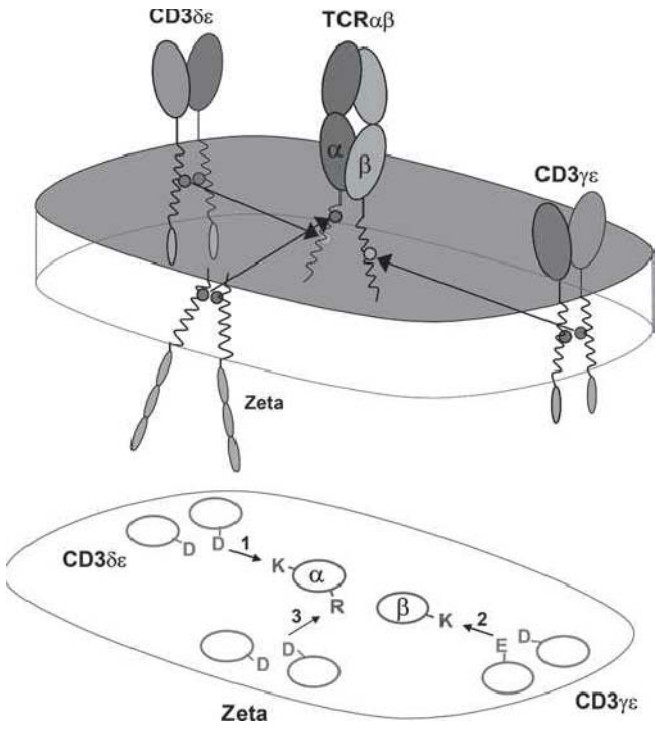

FIG. 11.3. A Model of the *αβ* T-Cell Receptor (TCR)-Cluster of Differentiation (CD)3 Complex. This shows the approximate positions of the *αβ* TCR chains and the CD3*α*, *δ*, *ε*, and *ζ* chains based on the membrane reconstitution and mutagenesis experiments of Call and Wucherpfennig.[55] More recent experiments suggest that the CD3 polypeptides may be somewhat more clustered on the side of the TCR heterodimer.[299]

receptor types are expressed in different ratios in different cells. These data may not be irreconcilable, because while the initial TCR/CD3 assembly may involve only one TCR, these may dimerize or multimerize later on the cell surface.[50,75]

The composition of TCR/CD3 complexes on γδ T cells is distinct from that of αβ T cells and changes with the activation state of the cell. Biochemical analysis showed that most murine γδ TCRs contain only CD3 γε dimers. Interestingly, a differentially glycosylated form of CD3 γ was found to associated with γδ TCRs dependent on the activation state of the cells.[76] In addition, while C3ζζ is incorporated into the complexes of naïve cells, activation results in the expression and incorporation of F_cεRI γ into the γδTCR complex.[76] Using quantitative immunofluorescence, Hayes and Love have derived data and proposed a model of murine γδTCR stoichiometry in which there are two CD3γε dimers, as well as one CD3ζ dimer in each TCR complex.[77] Taken together, these findings strongly suggest that signal transduction through the TCR will occur differently in γδ versus αβ T cells.

T-CELL RECEPTOR GENES

As shown in Figure 11.4, TCR gene segments are organized similarly to those of Igs and the same recombination machinery is responsible for joining separate V and D segments to a particular J and C. This was initially indicated by the fact that the characteristic seven and nine nucleotide conserved sequences adjacent to the V, D, and J regions with the 12 or 23 nucleotide spacing between them, first described for Ig genes, are also present in TCRs.[78] The most conclusive evidence of this common rearrangement mechanism is that both a naturally occurring recombination-deficient mouse strain (severe combined immune deficiency[79]) and mice engineered to lack recombinase activating genes 1[80] or 2[81] are unable to rearrange either *TCR* or Ig gene segments properly. Many of the other molecules involved in Ig gene rearrangement serve the same function in TCRs as well.[82] As with Igs, if the V region and J region gene segments are in the same transcriptional orientation, the intervening DNA is deleted during recombination. DNA circles of such material can be observed in the thymus,[83,84] the principal site of TCR recombination (see the following). In the case of *TCR*-β and *TCR*-δ, there is a single V region 3′ to the C in the opposite transcriptional orientation to J and C. Thus, rearrangement to these gene segments occurs via an inversion. Variable points of joining are seen along the *V*, *D*, and *J* gene segments as well as random nucleotide addition (N regions) in postnatal TCRs. The addition of several nucleotides in an inverted repeat pattern, referred to a P element insertion, at the V-J junction of the TCR-γ chains has also been observed.[85]

Organization of the T-Cell Receptor α/δ Locus

In humans and in mice, there is a single α-chain C-region gene that is composed of four exons encoding: 1) the constant region domain, 2) 16 amino acids including the cysteine that forms the interchain disulfide bond, 3) the transmembrane and intracytoplasmic domains, and 4) the 3′ untranslated region (see Fig. 11.4). The entire α/δ locus spans about 1.1 MB in both mice and humans. There are 50 different J-region gene segments upstream of the C-region in the murine locus. At least eight of these J-regions are nonfunctional because of in-frame stop codons or rearrangement and splicing signals that are likely to be defective. A similar number of α-chain J-regions are present in the human locus. This very large number of J-regions compared to the Ig loci may indicate that

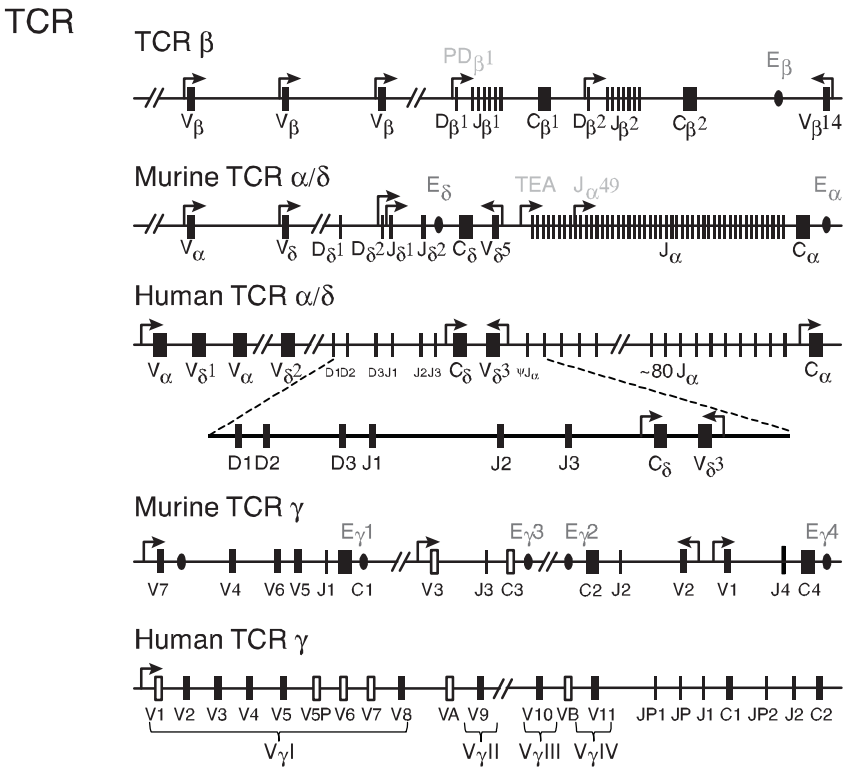

FIG. 11.4. T-Cell Receptor Gene Organization in Mice and Humans. Schematic of V, D, J, and C elements of the T-cell receptor genes. Transcriptional orientation is from *left to right*, except where noted. *E* designates enhancer elements, and *S* are silencer elements.

the functional diversity contributed by the J segment of the TCR (which constitutes a major portion of the complementarity-determining region [CDR]3 loop) makes an important contribution to antigen recognition (see the following).

In both the murine and human loci, the Cδ, Jδ, and two Dδ gene segments are located between the Vα and Jα gene segments. In the murine system, there are two Jδ and two Dδ gene segments on the 5′ side of Cδ and the Cδ exons are approximately 75 kb upstream of the Cα gene and approximately 8 kb upstream of the most 5′ known Jα gene segments. The human organization is similar, with three Dα gene segments and three Jδs. Surprisingly, in both species all of the D elements can be used in one rearranged gene rather than alternating as is the case with TCR-β or IgH. That is, in mice one frequently finds Vδ D_1, D_2, and Jδ rearrangements,[86] and in humans Vδ, D_1, D_2, D_3, and Jδ.[87] This greatly increases the junctional or CDR3 diversity that is available, especially because of the potential for N-region addition in between each gene segment. This property makes TCR-δ the most diverse of any of the antigens receptors known, with approximately 10^{12} to 10^{13} different amino acid sequences in a relatively small (10 to 15 amino acid) region.[86] The implications for this and comparisons with other antigen receptor genes are discussed subsequently.

The location of Dδ, Jδ, and Cδ genes between Vα and Jα gene segments suggests that TCR-δ and -α could share the same pool of V gene segments. While there is some overlap in V gene usage, in the murine system, four of the commonly used Vδ genes (Vδ1, Vδ2, Vδ4, Vδ5) are very different than known Vα sequences and they have not been found to associate with Cα.[88] The other four Vδ gene families overlap with or are identical to Vα subfamilies (Vδ3, Vδ6, Vδ7, and Vδ8, with Vα6, Vα7, Vα4, and Vα11, respectively).

The mechanisms that account for the preferential usage of certain gene segments to produce δ versus α chain are not known. While some Vδ genes are located closer to the Dδ and Jδ fragments than Vα genes (such as Vδ1), other Vδs (such as Vδ6) are rarely deleted by Vα Jα rearrangements and thus seem likely to be located 5′ of many Vα gene segments.

One of the Vδ gene segments, Vδ5, is located approximately 2.5 kb to the 3′ of Cδ in the opposite transcriptional orientation and rearranges by inversion. Despite its close proximity to Dδ Jδ gene segments, Vδ5 is not often found in fetal γδ T cells. Instead, the Vδ5 → Dδ rearrangement predominates in adult γδ T cells.

An implicit characteristic of the α/δ gene locus is that a rearrangement of Vα to Jα deletes the entire D-J-C core of the δ-chain locus. In many αβ T cells, the α-chain locus is rearranged on both chromosomes and thus no TCR δ could be made. In most cases, this is due to Vα → Jα rearrangement, but evidence suggesting an intermediate step in the deletion of TCR-δ has been reported.[89] This involves rearrangements of an element termed T early alpha (TEA) to a pseudo-Jα 3′ of Cδ. The rearrangement of TEA to this psuedo-Jα would eliminate the δ-chain locus in αβ T cells. Gene targeting of the TEA element resulted in normal levels of αβ and γδ T cells, but usage of the most Jαs was severely restricted,[90] suggesting that its function is to govern the accessibility of the most proximal 5′ Jαs for recombination.

Organization of the T-Cell Receptor β Locus

The entire human 685 kb β chain gene locus was originally sequenced by Hood and coworkers[91] (Fig. 11.5). One interesting feature is the tandem nature of J_β-C_β in the *TCR*-β locus. This arrangement is preserved in all higher vertebrate species that have been characterized thus far (mouse, human, chicken, frog). The two C_β coding sequences are identical in the mouse and nearly so in humans and other species. Thus it is unlikely that they represent two functionally distinct forms of C_β. However, the J_β clusters have relatively unique sequences, and thus this may be a mechanism for increasing the number of J_β gene segments. Together with the large number of Jα gene segments, there is far more combinatorial diversity (Jα × Jβ = 50 × 12 = 600) provided by J regions in αβ TCRs than in Igs.

Most of the V-regions are located upstream of the joining and constant regions and in the same transcriptional orientation as the D and J gene element, and rearrange to DβJβ gene via deletion. Similar to the case of Vδ5, a single Vβ gene, Vβ14 is located 3′ to C-regions and in the opposite transcriptional orientation, thus rearrangements involving Vβ14 occur via inversion.

In the NZW strain of mouse, there is a deletion in the β chain locus that spans from Cβ1, up to and including the Jβ 2 cluster.[92] In SJL, C57BR and C57L mice, there is a large deletion[93] in the V-region locus from Vδ5-Vβ9. These mice

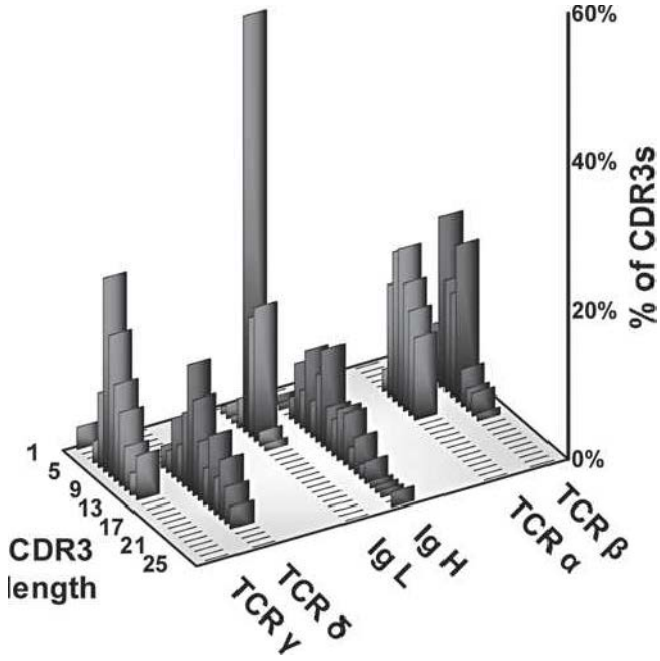

FIG. 11.5. Complementarity-Determining Region (CDR)3 Length Analysis of T-Cell Receptor (TCR) Polypeptides versus Immunoglobulin (Ig) Heavy and Light Chains. These data, modified from Rock et al.,[129] show that whereas TCR-α and -β CDR3 regions are relatively uniform with respect to each other, the other antigen receptor pairs show a marked asymmetry. Specifically, both Ig light chains (κ and λ) show very short CDR3s, as do TCR-γ chains. In contrast, both IgH and TCR S TCRs are quite heterogeneous and tend to be longer. These data suggests that $\gamma\delta$ TCRs have a more antibody-like structure and binding properties. This has been borne out by subsequent analysis (see text).

also express a V gene, Vβ17, that is not expressed in other strains of mice. Deletion of about half of the V genes (in SJL, C57BR and C57L mice) does not seem to have any particular effect on the ability of these mice to mount immune responses whereas mice that have deleted the $J_\beta 2$ cluster show impaired responses.[94]

Organization of the T-Cell Receptor γ Locus

The organization of the mouse and human γ-chain loci are shown in Figure 11.4. The human γ genes span about 150 kb[85] and are organized in a fashion similar to that of the β chain locus with two JγCγ regions. There is more than one nomenclature commonly used to describe the γ chain genes.[85,96–98] Here, we use that of Lefranc and Rabbitts[99] and Tonegawa and colleagues[85] for the human and mouse γ chains, respectively. The organization of the human γ chain genes consists an array of Vγs in which at least six of the V-regions are pseudogenes is located 5′ to these JγCγ clusters, and each of the V genes are potentially capable of rearranging to any of the five J-regions. The sequences of the two human Cγ regions are very similar overall and only differ significantly in the second exon. In Cγ2, this exon is duplicated two or three times and the cysteine that forms in the interchain disulfide bond is absent. Thus, Cγ2-bearing human T cells have an extra large γ-chain (55,000 MW) that is not disulfide bonded to its δ-chain partner.

The organization of the murine γ chain genes is very different than that of the human genes in that there are three separate rearranging loci that span about 205 kb.[100,101] Of four murine Cγ genes, Cγ3 is apparently a pseudogene in BALB/c mice, and the Jγ3 Cγ3 region is deleted in several mouse strains including C57 Bl/10. Cγ1 and Cγ2 are very similar in coding sequences. The major differences between these two genes is in the five amino acid deletion in the Cγ2 gene that is located in the C II exon at the amino acid terminal of the cysteine residue used for the disulfide formation with the δ chain. The Cγ4 gene differs significantly in sequences from the other Cγ genes (in 66% overall amino acid identity). In addition, the Cγ4 sequences contains a 17 amino acid insertion (compared to Cγ1) in the C II exon located at similar position to the five amino acid deletions in the Cγ2 gene.[101a] Each Cγ gene is associated with a single Jγ gene segment. The sequences of Jγ1 and Jγ2 are identical at the amino acid level, whereas Jγ4 differs from Jγ1 and Jγ2 at 9 out of 19 amino acid residues.

The murine Vγ genes usually rearrange to the Jγ Cγ gene that is most proximal and in the same transcriptional orientation. Thus Vγ1 rearranges to Jγ4; Vγ2 to Jγ2; and Vγ4, Vγ5, Vγ6, and Vγ7 to Jγ1. Interestingly, some Vγ genes are rearranged and expressed preferentially during γδ T-cell ontogeny and in different adult tissues as well.[101] In particular, Vγ5+ and Vγ6+ T cells are generated in the fetal thymus with very limited/no junctional diversity. Instead, the adult thymus produces γδ T cells expressing Vγ1, Vγ2, Vγ4, and Vγ7 gene segments with highly diverse junctional sequences. Moreover, γδ T cells that localize to the secondary lymphoid organs tend to express Vγ4, Vγ1, and Vγ2, whereas those that localize to the intestinal epithelium express Vγ7.[88,102–106] There are also reports suggesting that some intestinal epithelial γδ T cells develop extrathymically.[107] Regardless, it has been suggested that the Vγ gene rearrangement is a programmed process.[108,109]

Control of Transcription and Rearrangement

It has become increasingly apparent that transcriptional accessibility and rearrangement of TCR and Ig loci are closely linked, following the early work of Alt and Yancopoulous.[110] Factors governing accessibility and rearrangement include histone methylation,[111–114] DNA methlyation, and the presence of enhancer and specific promoter elements.[115] Even specific variations in the recombination signal sequences have been shown to elicit specific biases in V(D)J joining.[86] With respect to enhancer elements in the TCR loci, these were first identified in the TCR-β locus, 3′ of Cβ2,[116,117] and subsequently for the other TCR loci as well,[115] as indicated in Figure 11.4. These TCR enhancers all share sequence similarities with each other. Some of the transcriptional factors that bind to the TCR genes are also found to regulate Ig gene expressions. It has been shown that TCR-α enhancer (Eα) is not only important for normal rearrangement and expression for the α chain locus but also is required for the normal expression level of mature TCR-δ transcripts.[118] Also interesting is the work of Lauzurica and Krangel,[119,120] who have shown that a human TCR-δ enhancer-containing minilocus in transgenic mice is able to rearrange equally well in αβ T cells as in αδ T cells but that an Eα-containing construct was only active in αβ lineage T cells. Similar to Ig genes, promoter sequences are located 5′ to the V gene segments. Although D → J_β rearrangement and transcription occur fairly often in B cells and in B-cell tumors,[121] V_β rearrangement and/or transcription appears highly specific to T cells. In addition to enhancers, there also appear to be "silencer" sequences 3′ of Cα[122,123] and in the Cγ1 locus.[124] It has been suggested that these "repressor sites" could turn off the expression of either of these genes, influencing T-cell differentiation toward either the αβ or the γδ T-cell lineage.

The murine TCR Cγ1 gene cluster comprises four closely linked Vγ gene segments, in the order Vγ7, 4, 6, and 5, which rearrange to a single common downstream J gene segment, Jγ1 (see Fig. 11.4). In early fetal thymocytes, rearrangements of Vγ5 and Vγ6 genes predominate, and the resulting Vγ3+ and Vg4+ cells migrate to the skin or reproductive tissue, respectively. Later in ontogeny, Vγ4 and Vγ7 rearrangements predominate, and cells expressing these V regions migrate from the adult thymus to the secondary lymphoid organs and the intestinal epithelium.[100,101] At least two *cis*-acting, enhancer/locus control region (LCR) elements are present in the Cγ1 cluster. One is a T cell–specific transcriptional enhancer, 3γEγ, located 3 kb downstream of the Cγ1 gene segment.[125] A second element, "has," was found between the Vγ7 and Vγ4 genes, based on DNAse I hypersensitivity.[126] Similar enhancers have also been found to be associated with the Cγ2 and Cγ3 genes.[95] Experiments suggest that simultaneous deletion of both enhancer elements in Cγ1 cluster severely diminishes TCR-γ transcription, but only modestly reduces TCR-γ gene rearrangement, while deletion of each element

separately has little effect.[127] In contrast to these results in thymocytes, deletion of "has" alone reduces transcription of one Vγ gene specifically in peripheral γδ T cells. Thus, the two elements not only exhibit functional redundancy in thymocytes but also have unique functions in other settings.

Allelic Exclusion

In Igs, normally only one allele of the heavy chain locus and one of the light chain alleles is productively rearranged and expressed, a phenomenon termed "allelic exclusion." With respect to αβ TCR expression, while TCR-β exhibits allelic exclusion,[128] TCR-α seems much less constrained,[129,130] and many mature T cells express two functional TCR-α chains. As the chances of forming an in-frame joint with any antigen receptor is only one in three, the probability that a T cell would have two productively rearranged TCRαs is only ⅓ × ⅓ = ⅑, or 11%. However, even when this happens, the two TCR-α chains may not form heterodimers equally well with the single TCR-β that is expressed; thus, only one heterodimer may be expressed. But this simple calculation is complicated by the likelihood that only thymocytes that make at least one productive TCR rearrangement of each type will have a chance at maturation, which would eliminate almost half of the T cells (four-ninths, which is the product of a two-thirds chance of failure on one chromosome, followed by the same failure rate on the second). Secondly, it has been found that there is a mechanism called receptor editing, which means that a given rearrangement that is not productive, either because of an out-of-frame joint or for reasons of self reactivity, can induce a V region further 5′ or "upstream" of the initial VJ joint to rearrange to one of the remaining Jas.[131] In any event, it has been reported that one-third of human T cells express two TCRs,[132] which is slightly higher than what is expected from the probabilities discussed previously (approximately 20%), perhaps reflecting the effect of receptor editing.

There also appears to be an important role for the pre-TCR heterodimer (eg, pre-Tα:TCR-β) in blocking further TCR-β rearrangement and thus ensuring allelic exclusion at that locus.[133,134] In particular, pre-Tα–deficient mice had a significant increase in the number of cells with two productive TCR=β rearrangements, compared with wild-type mice.[133]

T-Cell Receptor Diversity

Although the basic organization and V(D)J recombination machinery are shared between TCR loci and Igs, there are a number of striking differences. One of these is somatic hypermutation. In antibodies, this form of mutation typically raises the affinities of antigen specific Igs several order of magnitude, typically from the micromolar (10^{-6} M) to the nanomolar (10^{-9} M) range.[135,136] We now know that most cell-surface receptors that bind ligands on other cell surfaces, including TCRs, typically have affinities in the micromolar range (see later section) but that they compensate for this relatively low affinity by engaging multiple receptors simultaneously (eg, increasing the valency) and by functioning in a confined, largely two-dimensional volume (eg, between two cells). Cells employing such receptors require weak, but highly specific, interactions so that they can disengage quickly.[137,138] The rapid off-rates seen with TCRs (see later section) may even amplify the effects of small numbers of ligands.[139,140]

There has also been no enduring evidence for a naturally secreted form of either an αβ or γδ TCR. Here again, it can be argued that such a molecule would have no obvious use as the affinities are too low to be very useful in solution. Thus, for most TCRs, the concentration of protein would have to be extremely high in order to achieve an effect similar to soluble antibodies (in the milligram/milliliter range).

A third mechanism seen in antibodies but not TCRs is C_H switching, which allows different Ig isotypes to maintain a given V region specificity and associate it with different constant regions that have different properties in solution (such as complement fixation, basophil binding, etc.). As there is no secreted form of the TCR, this feature would also lack any obvious utility.

Where TCRs are equal—and in fact generally superior—to Igs is in the sheer number of possible receptors that can be generated through recombination alone. Table 11.1 summarizes the potential V region diversity that TCRs are capable of when the number of V region gene segments is multiplied by D, J, and N region diversity. It can be seen from this table that while the V region number is generally lower in murine TCRs, particularly TCR-δ and TCR-γ, this is more than compensated for by the degree of junctional diversity (where V and J or V, D, and J come together) and chain combinations, such that overall TCRs have orders of magnitude greater potential diversity than Igs. This junctional region corresponds to CDR3 as originally defined by Kabat and Wu for Igs.[141] With respect to αβ TCRs, the concentration of diversity in this region (in both chains) can be explained by the key role that these sequences play in recognizing diverse peptides in MHC molecules (see later section), as supported by mutagenesis and structural studies. For γδ TCRs and Igs, however, the diversity is almost all in just one chain (TCRδ and IgH, respectively), and the implications of this are discussed subsequently. Recent work using high throughput sequencing techniques are in remarkable agreement with these crude early estimates of TCR-β diversity.[142]

The Complementarity-Determining Region 3 Length Distributions of γδ T-Cell Receptors are More Similar to Those of Immunoglobulin than to Those of αβ T-Cell Receptors

Because CDR regions are loops between different β strands of an Ig or TCR V region (see later section), the configurations they adopt are generally very sensitive to their length, such that a difference of even one amino acid may produce a significant change in the overall structure.[3,143] A comparison of CDR3 length distributions between the αβ TCRs, γδ TCRs, and Igs (see Fig. 11.5)[144,145] showed that those of TCR-α and -β have a very constrained distribution of lengths and that these are nearly identical in size. These length constraints may reflect a requirement for both the α

TABLE 11.1 Sequence Diversity in T-Cell Receptor and Immunoglobin Genes

	Immunoglobulin		TCR-α/β		TCR-γ/δ	
	H	κ	α	β	γ	δ
Variable segments	250–1,000	250	100	25	7	10
Diversity segments	10	0	0	2	0	2
Ds read in all frames	Rarely	—	—	Often	—	Often
N-region addition	V-D, V-J	none	V-J	V-D, V-J	V-J	V-D1, D1-D2, D1-J
Joining segments	4	4	50	12	2	2
Variable region combinations	62,500–250,000		2,500		70	
Junctional combinations	$\sim 10^{11}$		$\sim 10^{15}$		$\sim 10^{18}$	

Calculated potential amino acid sequence diversity in TCR and immunoglobulin genes without allowance for somatic mutation. The approximate number of V gene segments are listed for the four TCR polypeptides and contrasted with immunoglobulin heavy and light chains. CDR1 and CDR2 are encoded within the V gene segments. The pairing of random V regions generates the combinatorial diversity listed as "variable region combinations." Because there are fewer TCR V gene segments than immunoglobulin V gene segments, the combinatorial diversity is lower in TCRs than in immunoglobulins. Estimates for the number of unique sequences possible within the junctional region are contrasted for TCRs and immunoglobulins. Amino acids within CDR3 are encoded almost entirely within the D and/or J region gene segments. (The last few amino acids encoded by a TCR V gene segment can contribute to diversity within the TCR CDR3-equivalent region, but the effects of these residues on junctional diversity are not included in these calculations.) The mechanisms for generation of diversity within the junctional region that are used for this calculation include usage of different D and J gene segments. N region addition up to six nucleotides at each junction, variability in the 3′ joining position in V and J gene segments, and translation of D region in different reading frames. Numbers are corrected for out-of-frame joining codon redundancy and N-region mimicry of germ-line sequences. Modified from Elliott et al.[88]

CDR, complementarity-determining region; TCR, T-cell receptor.

and β chains of TCRs to contact both the MHC molecules and bound peptides on the same plane, as borne out by structural studies (see later section). In contrast, the CDR3s of Ig heavy chains are long and variable, whereas those of Ig light chains are short and constrained. This may reflect the fact that Igs recognize both very small molecules (eg, haptens) as well as very large ones (eg, proteins). Surprisingly, γδ TCR CDR3 length distributions are similar to those of Igs in that the CDR3 lengths of TCR-δ chains are long and variable, whereas those of the TCR γ chains are short and constrained. Thus, on the basis of this measure of ligand recognition, one might expect γδ TCRs to be more similar to Igs than to αβ TCRs. This has been validated in subsequent biochemical and structural studies (see later sections).

Chromosomal Translocations and Disease

The chromosomal locations of the different TCR loci have been delineated in both mouse and humans, and the results are summarized in Table 11.2. One significant factor in cancers of hematopoietic cells are chromosomal translocations that result in the activation of genes normally turned off or the inactivation of genes that are normally turned on. Thus, B- or T-lymphocyte neoplasia is frequently associated with inter- or intrachromosomal rearrangements of Ig or TCR loci or in some cases both.[146,147]

These translocations seem to mediated by the V(D)J recombinase machinery, indicating the inherent danger and need for tight regulation of this pathway. Such rearrangements are particularly common in the α/δ locus, perhaps because this locus spans the longest developmental window in terms of gene expression, with *TCR*-δ being the first and *TCR*-α the last gene to rearrange during T-cell ontogeny (as discussed in more detail in the following). In addition, the α/δ locus is in excess of 1 mb in size, and this provides a larger target for rearrangement than either TCR-β or TCR-γ. Interestingly, in humans, *TCR*-α/δ is on the same chromosome as the *IgH* locus and $V_H \rightarrow J_\alpha$ rearrangements (by inversion) have been observed in some human tumor material.[148,149] The functional significance of this is not known.

Particularly frequent is the chromosome 8–14 translocation [t(8;14) (q24;q11)] that joins the α/δ locus to the c-myc gene, analogous to the c-myc → IgH translocation in many mouse myeloma tumors and in Burkitt lymphomas in humans. In one cell line, a rearrangement occurred between the Jα-region coding sequences, and a region 3′ of c-myc.[150] In both B- and T-cell malignancies, the translocation of c-myc into IgH or TCR-α/β appears to increase the expression of c-myc and may be a major factor in the unregulated cell growth that characterizes cancerous cells. Other

TABLE 11.2 Chromosomal Locations of T-Cell Receptor, Immunoglobulin, and Related Loci in Mouse and Human

	Mouse Chromosome	Human Chromosome
TCR-α	14	14(q11–q12)
TCR-δ	14	14(q11–q12)
IgH	12	14(qter)
TCR-β	6	7(q35)
CD4	6	12
CD8	6	2(p11)
Ig*k*	6	62(p12)
TCR-γ	13	7(p14)
CD3-γ	9	11(q23)
CD3-δ	9	11(q23)
CD3-ε	9	11(q23)
CD3-ζ	1	1
Thy-1	9	11(q23)
Ig-λ	16	22(q11.2)
MHC	17	6(p21)
Pre-Tα	17	6

CDR, complementarity-determining region; Ig, immunoglobulin; MHC, major histocompatibility complex; TCR, T-cell receptor.

putative proto-oncogenes that have been found translocated into the TCR-α/β locus are the LIM domain–containing transcription factors Ttg-1[151] and Ttg-2,[152,153] which are involved in neural development; the helix-loop-helix proteins Lyl-1[154] and Scl,[155] which are involved in early hematopoietic development; and the homeobox gene Hox 11,[156] which is normally active in the liver. How these particular translocations contribute to malignancy is unknown, but they presumably causes aberrations in gene expression that contribute to cell growth or escape from normal regulation. In patients with T-cell leukemia infected with the human T-cell lymphotrophic-I virus, there are large numbers of similar translocations; it is thought that human T-cell lymphotrophic-I itself is not directly leukemogenic but acts by causing aberrant rearrangements in the T cell that it infects, some of which become malignant.

Another disorder, which frequently associates with TCR and Ig locus translocation, is ataxia telangiectasia, an autosomal recessive disorder characterized by ataxia, vascular telangiectasis, immunodeficiency, increased incidence of neoplasia, and an increased sensitivity to ionizing radiation. Peripheral blood lymphocytes from patients with ataxia telangiectasia have an especially high frequency of translocations involving chromosomes 7 and 14.[157] These sites correspond to the TCR-γ, -β, and -α loci, and the Ig heavy chain locus. Thus, it appears as though one of the characteristics of patients with ataxia telangiectasia is a relatively error-prone rearrangement process that indiscriminately recombines genes that have the TCR and Ig rearrangement signals.[158]

The Structure of αβ and γδ T-Cell Receptors

As discussed previously, the sequences of TCR polypeptides show many similarities to Igs, and thus it has long been suggested that both αβ and γδ heterodimers would be antibody-like in structure.[24,25,159] The similarities between TCRs and Igs include the number and spacing of specific cysteine residues within domains, which in antibodies form intrachain disulfide bonds. Also conserved are many of the inter- and intradomain contact residues and, in addition, secondary structure predictions are largely consistent with an Ig-like "β barrel" structure. This consists of three to four antiparallel β strands on one side of the "barrel" facing a similar number on the other side, with a disulfide bridge (usually) connecting the two β "sheets" (sets of β strands in the same plane). All Ig variable and constant region domains have this structure, with slight variations in the number of β strands in variable region domains (by convention including V, D, and J sequences) compared with constant domains.

αβ T-Cell Receptor Structure

Efforts to derive x-ray crystal structures of TCR heterodimers and fragments of heterodimers presented many technical hurdles.[160] One difficulty is that structure determination required engineering the molecules into a soluble form. A second problem is that many of the TCRs are heavily glycosylated, and it was necessary to eliminate most or all of the carbohydrates on each chain to achieve high-quality crystals. An alternative is to express soluble TCRs in insect cells, where they have compact N-linked sugars, or in *Escherichia coli*, where they are unglycosylated. The first successes in TCR crystallization come from the laboratory of Mariuzza and collaborators who solved the structure of first a Vβ Cβ polypeptide[161] and then a Vα fragment.[162] In the following year, the first complete αβ TCR structures were solved.[163,164] The structure of the 2C TCR, by Garcia and colleagues, is shown in Figure 11.6.[163] In general, as predicted from sequence homologies, these domains are all Ig-like, with the classical β-barrel structure in evidence in all three domains. At each end of the barrel in each V-region domain there are four loops between the β sheets, three of which form the CDRs of Igs, which are numbered in Figure 11.6. The fourth loop, between the D and E strands, has been implicated in superantigen binding. The six CDR loops from the two variable domains form the antigen-binding surface in both Igs and TCRs. The major anomaly in terms of similarity of TCRs to Igs is the structure of the Cα.[165] Cα consists of one-half of the classical β-barrel, that is, one set (or "sheet") of β strands while the rest of the partially truncated domain exhibits random coils. This type of structure is unprecedented in the Ig gene family. The functional significance of such a variant structure in unknown, but it has been suggested that this incompletely formed Ig-like domain may be responsible for the observed lability of TCR-α, and this may allow greater flexibility in the regulation of its expression. Another possible explanation is that this configuration is designed to accommodate one or more of the CD3 molecules.

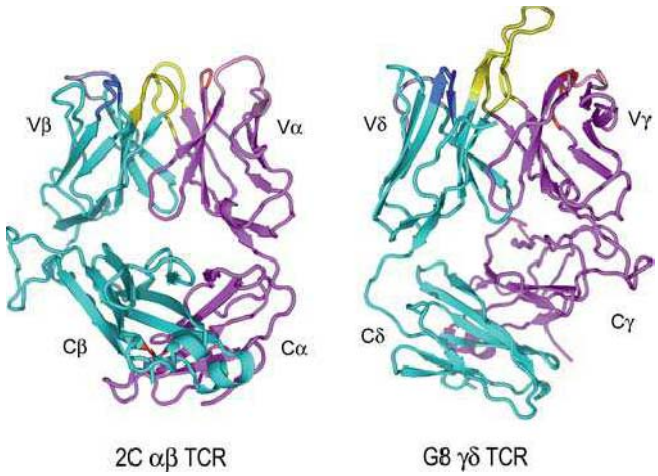

FIG. 11.6. Ribbon Diagrams of the T-Cell Receptor (TCR) Structures. This shows the structures of the 2C*αβ* TCR[150] versus the G8*γδ* TCR.[153] The TCR-*β* and the -*γ* chains are in *cyan*, and the TCR-*α* and -*γ* chains are in *vermillion*. The complementarity-determining regions (CDRs) of both are in *yellow*. The very long TCR-*δ* CDR3 in G8, which binds the T10/T22 ligands, is very apparent here but is shorter in most other *γδ* TCRs. Note the different C-region interactions with these TCRs and the deviations from the classic "*β* barrel" structure in both C*α* and C*δ*. The prominent C*β* loop to the left is also unusual and may mediate interactions with CD3 or other molecules on the T cell surface. (Figure courtesy of Dr. K.C. Garcia)

The now large number of solved αβ TCR structures can be compared to the three γδ heterodimers (discussed in more detail in the following), and while these also resemble the Fab fragment of an antibody, there are several features that are unique to the αβ molecules, which may be significant. These include the following:

1) In one structure,[165] four out of seven N-linked sugars diffracted to high resolution, indicating that they are not free to move very much and thus are likely to play a structural role, particularly in Cα:Cβ interactions. This correlates with mutagenesis data indicating that certain Cα sugars cannot be eliminated without abolishing protein expression[166] and the disordered state of a Cα domain in the structure of a TCR lacking glycosylation.[164]
2) There is significantly more contact between Vβ and Cβ and between Vα and Cα than in the equivalent regions of antibodies.
3) The geometry of the interaction of Vα and Vβ more closely resembles that of the C_H3 domains of antibodies than V_HV_L.
4) Between the CDR3 loops of Vα and Vβ, there is a pocket that can (and does in at least one case[165]) accommodate a large side chain from the peptide bound to an MHC.

γδ T-Cell Receptor Structure

There are now three γδ heterodimer structures: a γδ TCR from a human T-cell clone G115,[167] which can be activated by natural or synthetic pyrophosphomonoesters, a γδ TCR from the murine T cell clone G8 together with its ligand, the nonclassical MHC class I molecule T22,[168] and a human MHC class I chain–related–reactive γδ TCR (δ1A/B-3),[169a] which was determined as a single-chain Fv construct. The G8 structure is shown in Figure 11.6, alongside the 2C αβ TCR. The structure of a single human Vδ domain also has been determined.[169] The Vδ domain of the G115 structure is similar to the isolated Vδ domain and the quaternary structure of G8 is similar to that of G115.[168]

The most distinctive feature of both the G115 and the G8 TCR, when compared with αβ TCRs and Igs, is that the C domains "swing out" from under the V domains. This unusual shape is highlighted by both a small elbow angle of 110 degrees, defined as the angle between the pseudo twofold symmetry axes that relate V to V and C to C, and a small V-C interdomain angle. This contrasts with an average of 149 degrees for αβ TCR structures. The small angle between the Vγ and Cγ domains shifts both Cδ and Cγ to one side. Moreover, the molecular surfaces of the constant domains are different than those of αβ TCRs with no clear similarities either in the shape or the nature of the CαCβ and CγCδ surfaces; there are only a few solvent-exposed residues that are conserved in both Cβ and Cγ domains as well. Thus, it is unclear where or how the extracellular domains of the CD3 subunits interact with the extracellular portions of γδ TCRs compared with αβ TCRs. This may explain why the CD3 components of αβ TCRs are so different from those of γδ TCRs.

In terms of ligand binding surfaces, we note that the Vδ CDR3 of G8 protrudes significantly away from the other CDRs, as shown in Figure 11.6. This has significance in that this is the major region of contact with the T22 ligand (see later section). In the case of G115, both Vδ and Vγ CDR3 loops protrude from the rest of the putative binding surface and create a cleft between them. Portions of the CDR1γ and δ and CDR2γ combine with the clefts between the CDR3 loops to form a pocket, which is surrounded by positively charged amino acid residues contributed by CDR2γ and δ, and CDR3γ. The jagged surface of this TCR resembles the surface of an antibody that binds a small-molecule antigen. Although this would be consistent with the supposition that this TCR binds the negatively charged phosphate compounds,[170] direct binding between the TCR and phosphoantigen including crystal-soaking and cocrystallization experiments have not been successful. Instead, a soluble G115 was found to bind a soluble form of adenotriphosphate (ATP) synthase F1 and apolipoprotein A-1.[171]

While the δ1A/B-3 TCR maintains an overall fold similar to the other γδ TCR structures, it was noted that unlike the G115 and G8 CDR3 regions, which are protruding out, the δ1A/B-3 CDR loops together generate a nearly flat surface on the combining site. This difference is anticipated, as the CDR3 length distribution of the TCRδ chains is quite variable as discussed previously, and like antibodies should have a broad range of binding site shapes.

αβ T-CELL RECEPTOR–LIGAND RECOGNITION

Binding Characteristics

Although it has long been established that this type of T cell generally recognizes a peptide bound to an MHC molecule, a formal biochemical demonstration that this was due to TCR binding to a peptide/MHC complex took many years to establish. Part of the difficulty in obtaining measurements of this type has been the intrinsically membrane-bound nature of MHC and TCR molecules. Another major problem is that the affinities are relatively low, in the micromolar range, which is too unstable to measure by conventional means.

To some extent, the problem of measuring the interactions of membrane-bound molecules can be circumvented by expressing soluble forms of TCR and MHC, which is also essential for structural studies (see previous discussion). For TCRs, many successful strategies have been described, including replacing the transmembrane regions with signal sequences for glycolipid linkage,[172] expressing chains without transmembrane regions in either insect or mammalian cells,[173] or a combination of cysteine mutagenesis and *E. coli* expression.[164] Unfortunately, no one method seems to work for all TCR heterodimers, although the combination of insect cell expression and leucine zippers at the c-terminus to stabilize heterodimer expression has been successful in many cases.[174] The production of soluble forms of MHC molecule has a much longer history, starting with the enzymatic cleavage of detergent solubilized native molecules[175] as well as some of the same methods employed for TCR such as glycophosphatidylinositol (GPI) linkage,[176] *E. coli* expression and refolding,[177,178] and insect cell expression of truncated

(or leucine zippered) molecules.[179] One interesting variant that seems necessary for the stable expression of some class II MHC molecules in insect cells has been the addition of a covalent peptide to the N-terminus of the β chain.[180]

The first measurements of TCR affinities binding to peptide/MHC complexes were performed by Matsui et al.[181] and Weber et al.[182] Matsui and colleagues used a high concentration of soluble peptide/MHC complexes to block the binding of a labeled anti-TCR Fab fragment to T cells specific for those complexes, obtaining an equilibrium binding affinity (K_d) value of approximately 50 μM for several different T cells and two different cytochrome peptide/I-E^k complexes (as shown in Table 11.3). Weber and colleagues used a soluble TCR to inhibit the recognition of a flu peptide/I-E^d complex by a T cell and obtained a K_D value of approximately 10 μM. While these measurements were an important start in TCR biochemistry, they gave no direct information about the kinetics of TCR-ligand interactions. Fortunately, the development of surface plasmon resonance instruments, particularly the BIAcore™ (Pharmacia Biosensor, Uppsala, Sweden) with its remarkable sensitivity to weak macromolecular interactions,[183] has allowed rapid progress in this area. In this technique, one component is covalently cross-linked to a surface and then buffer containing the ligand is passed in solution over it. The binding of even approximately 5% of the surface-bound material is sufficient to cause a detectable change in the resonance state of gold electrons on the surface. This method allows the direct measurement of association and dissociation rates, that is, kinetic parameters, and also has the advantage of requiring neither cells nor radioactive labels. Recently, microcalorimetry has also been used to measure some TCR ligand affinities; and these analyses have confirmed the surface plasmon resonance (SPR) values,[184] but do not allow kinetic measurements. These and other data[138,160] showed definitively that TCR and peptide-loaded MHC molecules alone are able to interact and also that expression in a soluble form has not altered their ability to bind to each other. As shown in Table 11.3, SPR measurements show that while the on-rates of TCRs binding to peptide/MHC molecules vary from very slow (1,000 M sec) to moderately fast (200,000 M sec), their off-rates fall in a relatively narrow range (0.5 to 0.01 sec^{-1}) or a $t_{½}$ of 12 to 30 seconds at 25°C. This is in the general range of other membrane bound receptors that recognize membrane molecules on other cells,[137] but it has been noted that most TCRs have very slow on-rates,[138] which reflects a flexibility in the binding site that might help to foster cross-reactivity (see the following). In the case of a class I MHC–restricted TCR, 2C, this relatively fast off-rate may be stabilized (10-fold) if soluble CD8 is introduced,[165] but this result is controversial.[185] CD8 stabilization of TCR binding has been seen by Luescher et al. in cell-based TCR labeling assay[186]; however, no enhancement of TCR binding has been seen using soluble CD4[187] (see the following for more discussion of CD4 and CD8).

More recently, new methodologies have been utilized to measure TCR binding in its native state, that is, on a T-cell surface and interacting with peptide-MHC ligands on either an artificial bilayer[188] or on the surface of a red blood cell[189] "reporter." These types of measurements are important because studies of TCRs and peptide/MHCs binding in

TABLE 11.3 T-Cell Receptor-Ligand Binding

T Cell	Ligand	K_D (mM)	k_{on} ($M^{-1}s^{-1}$)	K_{off} (s^{-1})	Method	Reference
T_H Cells						
5C.C7	MCC/E^k	50	—	—	Anti-TCR comp.	166
2B4	MCC/E^k	50	—	—	Anti-TCR comp.	166
2B4	MCC/E^k	30	—	—	Anti-P/MHC comp.	166
2B4	MCC/E^k	90	600	0.057	BIA1	166
228.5	MCC 99E/E^k	50	—	—	Anti-TCR comp.	166
14.3d	Flu H1N1/E^d	~10	—	—	Sol. TCR	167
14.3d	SEC 1,2,3	5.4–18.2	>100,000	>0.1	BIA1	251
HA1.7	HA/DR1	>25	—	—	BIA1	300
HA1.7	SEB	0.82	13,000	0.001	BIA1	300
Tc cells						
2C	p2Ca/L^d	0.5	11,000	0.0055	Anti-TCR comp.	176
2C	p2/Ca/L^d	0.1	21,000	0.026	BIA1	301
2C	OL9/L^d	0.065	53,000	0.003	Labeled MHC	176
4G3	pOV/L^d	0.65	22,000	0.02	Labeled MHC	176
42.12	OVA/K^b	6.5	3,135	0.02	BIA4	179
2C	p2Ca/L^d	3.3	8,300	0.027	BIA1	208
HY	M80/D^b	23.4	6,200	0.145	BIA1	208
HY	CD8 α/β + M8/D^b	2.0	5,100	0.01	BIA1	208
2/C	CD8 α/β + p2Ca/L^d	0.32	1,200	0.0038	BIA1	208
T$\gamma\delta$ cells						
G8	T10/T22	0.13	65,000	0.0081	BIA1	274

BIA1, TCR amine coupled; BIA2, TCR cysteine coupled; BIA3, MHC-peptide amine coupled in competition experiment; BIA4, TCR coupled by using H57 antibody and MHC coupled via amine chemistry; MHC, major histocompatibility complex; P, peptide; sol., soluble; TCR, T-cell receptor.

solution only partially reflect what is happening between two membranes. This is because of the very severe constraints on binding that are present in this specialized environment. For one, it is likely that TCRs on T cells and peptide/MHCs on antigen-presenting cells are fixed into a fairly rigid alignment that maximizes their ability to bind to each other, as opposed to the same molecules in solution that are diffusing in all directions and for which only a fraction of collisions will result in a productive binding event. Secondly, binding is happening in a very small space/volume of liquid, making the effective concentration very high. While this environment is not strictly two-dimensional, Dustin and others have used this as a simplifying assumption with relative success. But a direct measurement of TCR affinity and kinetics has been made by Huppa and colleagues,[188] who used a fluorescent probe on an anti-TCR antibody to create a system in which this fluorophore could form a FRET pair with a complementary label on the c-terminus of the peptide that could emit photons of the appropriate wavelength only when the TCR bound its peptide-MHC ligand. This enabled precise measurements of the dissociation rate (koff) and affinity (KD) within an immunologic synapse, and these measurements could also be used to estimate an average association rate (kon). Another advantage of these measurements is that they can be done at 37°C, whereas most of the SPR measurements cited previously were performed at 25°C due to instrument limitations. Dissociation rates at higher temperatures are significantly faster. As expected from the previous discussion, the association rate was greatly accelerated approximately 100× over the solution rate, presumably because of the ideal orientation of the TCR and peptide/MHC with respect to each other. Not expected was an accelerated dissociation rate of 4 to 10 times faster. This turns out to be a function of actin polymerization and depolymerization activity within the T cell, as shown dramatically in the actin dynamics studies of Vale and colleagues.[190] Once this was inhibited, the dissociation rates within the synapse were quite close to the solution values.[191] Still, it is worth noting that TCR binding to peptide/MHC is even more unstable within an active synapse than the solution measurements have suggested, with half lives of less than 1 second in many cases.

A second experimental system that has been applied to the question of TCR binding in the context of cell-cell contact has been that of Chung and colleagues,[189] in which red blood cells coated with peptide-MHC complexes are brought into repeated contact with a T cell of the appropriate specificity. By visualizing the response of the peptide/MHC–coated red blood cell to different frequencies and durations of T-cell contact, one can infer parameters of TCR affinity, at least in the two-dimensional aspect.[192] While this system has worked well in describing integrin binding, it is indirect and it some ways contradicts results obtained with the FRET system of Huppa et al.[188] and even some of the basic properties determined in solution measurements. Nonetheless, it may be telling us important properties of cell-cell contact that are more difficult to discern with more directly molecular methods.

To what extent are we now able to predict a T-cell response based on the binding characteristic of its TCR to a ligand? One of the most intriguing discoveries concerning T-cell reactivity has been the phenomenon of altered peptide ligands. These are single amino acid variants of antigenic peptides that either change the nature or degree of the T-cell response (partial agonists) or prevent a response to a normally stimulating ligand (antagonists).[193,194] Discussions concerning the mechanism of these "altered peptide" responses have centered on whether they are due to some conformational phenomenon involving TCRs and/or CD3 molecules or to affinity or kinetic characteristics. With the data now available, we can now say that most, but not all, T-cell responses correlate well with the binding characteristics of their TCRs. In particular, Sykulev et al.[195] first noted that higher-affinity peptide variants elicited more robust T-cell responses. Subsequently, Matsui et al.[196] found that in a series of three agonist peptides increasing dissociation rates correlated with decreasing agonist activity. Lyons et al.[197] found that this correlation extended to antagonist peptides in the same antigen system (moth cytochrome c/I-E^k). They also showed that while an antagonist peptide might differ only slightly in affinity compared with the weakest agonist, its dissociation rate differed by 10-fold or more. This data in a class II MHC–restricted system is largely supported by the studies of Alam et al. in a class I MHC system,[198] who also saw a drop-off in affinities and an increase in off-rates (with one exception as noted in Table 11.2) with antagonist versus agonist ligands. In the cell-based TCR labeling system of Luescher and colleagues, a survey of related peptide ligands of varying potency also found a general, but not absolute, correlation between receptor occupancy and stimulatory ability.[199] Thus, while there is a general trend toward weaker T-cell responses and faster off-rates and lower affinities, this does not seem to be an absolute rule, and thus other factors may be important in some cases. Alternatively, Holler[200] has suggested that some or all of the discrepancies may derive from differences in peptide stability (in the MHC) between the relatively short (minutes) time scale of BIAcore analysis at 25°C compared with the much longer (days) cellular assays at 37°C. But this explanation probably only applies to a fraction of the anomalous cases in which TCR ligands fail to adhere to the "$t_{½}$ rule." In particular, Krogsgaard et al.[201] performed a very comprehensive survey of both known and newly derived cytochromic peptide antigens and found that almost half of the peptide-MHC ligands analyzed failed to exhibit a linear relationship between $t_{½}$ and T-cell stimulatory ability (Fig. 11.7). Extensive thermodynamic analyses of these ligands showed that one particular parameter, the change in heat capacity ($-\Delta Cp$), which reflects changes in conformation or flexibility upon binding, seems to be synergistic with $t_{½}$ in enhancing a ligand's stimulatory capacity. In fact, as shown in Figure 11.7, when ΔCp values are combined with $t_{½}$, the x axis values for the range of ligands correlate much better with T-cell stimulation. This suggests that ΔCp may be the "missing" variable in correlating ligand binding to stimulation.

How could a negative change in heat capacity synergize with the stability of binding? One possibility is that large conformational changes at the binding surface of a TCR that can occur when it engages peptide/MHC ligands (as

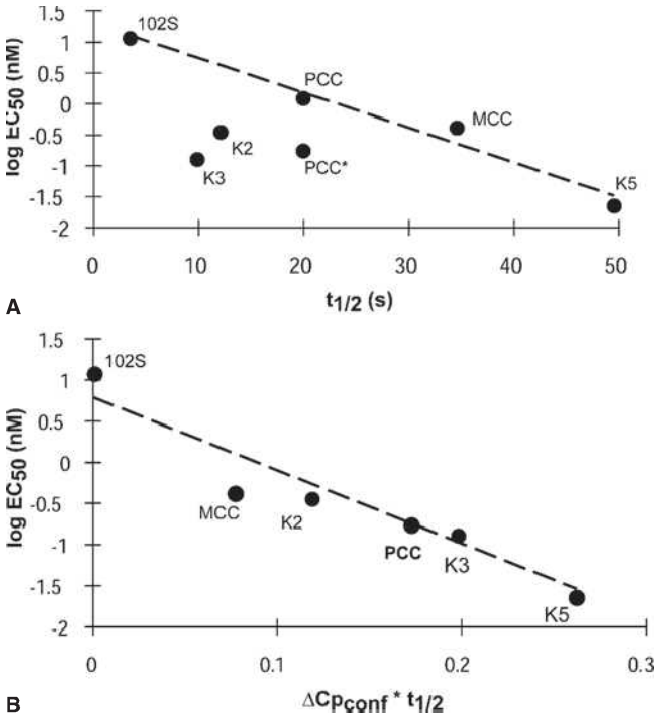

FIG. 11.7. Dissociation Rate and Heat Capacity with Peptide–Major Histocompatibility Complex (MHC) Ligands Both Influence T-Cell Activation. Data from Krogsgaard et al. show that where deviations from the general dependence of T-cell activation on dissociation rate occur, they may be compensated for by another factor, namely the heat capacity (ΔC_P) of the T-cell receptor (TCR)-peptide/MHC interaction. This is a measure of changes in mobility or conformation during the binding interaction and suggests that other binding parameters can influence TCR-mediated activation besides half-life.[184] **A:** The relationship between the half-maximal peptide concentration needed for T-cell activation (EC50) and the half-life ($t_{½}$) of the TCR binding to that particular ligand. In this series, three of the seven peptides tested (K2, K3, and PPC) do not seem as dependent on $t_{½}$ as the others (PCC* represents a correction for a lack of stability when bound to the MHC at 37°C). **B:** When ΔC_P is factored in, all of the peptides can be plotted on a line.

shown by structural studies; see the following) can translocate the TCR deeper into the membrane—as suggested by the "piston"[52,202] and "twist cap"[184] models—and trigger conformational changes in the CD3 signaling domains. Chakraborty and colleagues recently suggested another possibility for how conformational changes at the binding surface might exert their effect.[203] They found that in the context of membrane-membrane interactions, conformational changes in the surfaces of relatively rigid proteins (such as all but the CDR3 regions of TCRs appear to be) would act to increase the effective half-life of TCR-peptide/MHC interactions. Thus $t_{½}$ and ΔCp may be equivalent in the unique environment between two cell surfaces.

How might the relatively small differences in the binding characteristics of the ligands cause such different T-cell signaling outcomes as agonism or antagonism? As McKeithan[204] and Rabinowitz[205] have noted, a multistep system such as T-cell recognition has an inherent ability to amplify small differences in signals that are received on the cell surface to much larger differences at the end of the pathway, in this case gene transcription in the nucleus. Thus antagonism may occur at one threshold and an agonist response at another. Alternatively, an antagonist ligand may traverse the activation pathway just far enough to use up some critical substrate, as proposed by Lyons et al.[197] Yet another possibility that has also been suggested is that some antagonists may act even earlier by blocking TCR clustering at the cell surface.[206] Lastly, Germain and colleagues have found evidence that a feedback loop involving the phosphatase SHP-1 may act as an alternative pathway to inactivate TCR signaling of insufficient strength.[207,208]

Another controversy that relates to TCR binding characteristics is the "serial engagement" model of Valittutti and Lanzavecchia and colleagues,[139,209] which proposed that one way in which a small number of peptide/MHC complexes can initiate T-cell activation is by transiently binding many TCRs in a sequential fashion. While the dissociation rates reviewed here show that TCR binding is likely to be very transient, they do not in fact support the statement that more interactions are better. This is because, in most cases, improvements in TCR-peptide/MHC stability within any one system result in a more robust T-cell response. This has been shown by the work of Kranz and colleagues,[210] who selected a nanomolar affinity TCR from a mutagenized library expressed in yeast. With an approximately 100-fold slower off-rate than the original, this TCR should have been only poorly stimulatory based on the serial engagement model. Instead, T cells bearing it are considerably more sensitive to antigen, which casts considerable doubt on this aspect of the model. Similar work of Allen and colleagues on another TCR-ligand interaction produced similar results.[211] Furthermore, recently Xie et al. cross-linked peptide-MHCs to a cognate TCR and found that when aggregated they can still signal efficiently.[212] But the concept that the rapid dissociation rates of TCRs for peptide-MHC ligands could serve to amplify signaling under limiting conditions is still very likely and has been cited in the context of the "pseudodimer" model,[140,213] as discussed in the following.

Topology and Cross-Reactivity

As discussed previously, TCR sequence diversity resides largely in the region between the V and J region gene segments, which corresponds to the CDR3 regions of antibodies.[214] This has led to models in which the CDR3 loops of Vα and Vβ make the principal contacts with the antigenic peptide bound to the MHC.[214] Support for this model has come from many studies in which it has been shown that the CDR3 sequences of TCRs are important predictors of specificity,[214] as well as elegant mutagenesis studies which showed that a single CDR3 point mutation could alter the specificity of a TCR[215] and also a CDR3 "transplant" could confer the specificity of the donor TCR onto the recipient.[216] In addition, a novel approach to TCR-ligand interactions was developed by Jorgensen et al.,[217] who made single amino acid changes in an antigenic peptide at positions that affect T-cell recognition but not MHC binding. These variant peptides are then used to immunize mice that express either α or β chain of a TCR that recognizes the original

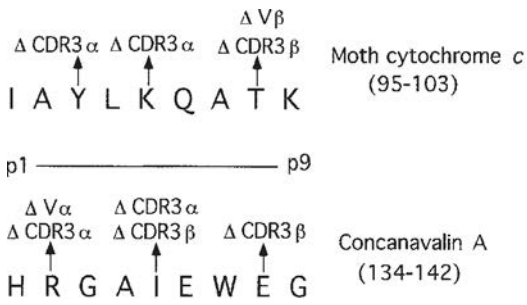

FIG. 11.8. Sensitivity of T-Cell Receptor (TCR) Complementarity-Determining Region 3 (CDR3) Sequences and Vα/Vβ Usage to Changes in the Antigen Peptide. This figure summarizes the data of Jorgensen et al.[196,198] and Sant'Angelo et al.,[197] who immunized single-chain transgenic mice (TCR-α or TCR-β) with antigenic peptides (MCC or CVA) altered at residues that influence T-cell recognition but not major histocompatibility complex binding. These data show that such changes invariably affect the CDR3 sequences of Vα or Vβ or both, and that there appears to be a definite topology in which Vα governs the N-terminal region and Vβ seems more responsible for the C-terminal portion of the peptide.

peptide and the responding T cells are analyzed. Using these "hemitransgenic" mice allows the resulting T cells to keep one-half of the receptor constant, while allowing considerable variation in the chain that pairs with it. The results from this study and work in another system by Sant'Angelo et al.[218] are very similar in that every mutation at a TCR-sensitive residue triggered a change in the CDR3 sequence of Vα, Vβ, or both, and in some cases, changed the Vα or Vβ gene segment as well (as summarized in Fig. 11.8). One of the more striking examples of a CDR3-peptide interaction occurred in the cytochrome c system where a Lys → Glu change in the central TCR determinant on the peptide triggered a Glu → Lys charge reversal in the Vα CDR3 loop, suggesting a direct Lys → Glu contact between the two molecules.[217] This prediction has recently been confirmed in the structural work of Newell et al.[219] and extended to a closely related TCR, 2B4, which contacts the central lysine on the peptide with an oppositely charged residue in CDR3β.

Another interesting finding was the order of Vα → Vβ preference going from the N-terminal to the C-terminal residues of the peptides. This led Jorgensen to a proposed "linear" topology of TCR-peptide/MHC interaction in which the CDR3 loops of Vα and Vβ line up directly over the peptide.[217,220] Sant'Angelo et al.[218] proposed an orientation of the TCR in which the CDR3 loops are perpendicular to the peptide. This was partially based on intriguing data they found suggesting an interaction between the CDR1 of Vα and an N-terminal residue of the peptide.

This controversy regarding orientation has been largely resolved by the numerous crystal structures of TCR-peptide/MHC complexes.[160,221] These studies show that TCRs bind in roughly diagonal to 90-degree configurations, ranging over 30 degrees. In these structures, one of which is shown in Figure 11.9,[165] the CDR3 loops are centrally located over the peptide, but the Vα CDR1 and the Vβ CDR1 are also in a position to contact the N-terminal and C-terminal peptide residues, respectively. The confined nature of TCR recognition constitutes a major departure from antibody-antigen interactions and may reflect a need to accommodate other molecules into a particular configuration that is optimal for signaling, such as CD4, CD8, and/or CD3 components. Particularly relevant to this possibility are the recent observations by Garcia and colleagues[222] suggesting that TCR signaling can be limited by the docking geometry of the TCR to the peptide/MHC complex. They used a yeast library in which millions of different peptides bound to a class I MHC can be interrogated by a single TCR. Structural analysis showed that three of the peptides isolated bound to the TCR with the typical orientation and induced TCR signaling. But a fourth peptide bound the TCR not in the typical diagonal mode but in a parallel fashion, and failed to induce signaling.[222] This suggests that the typical TCR-MHC docking mode is selected for in the thymus and may be critical for signaling.

As αβ TCR heterodimers are first selected in the thymus for reactivity to self-peptides bound to MHC molecules, all foreign-peptide reactive TCRs could be considered to be inherently cross-reactive. Indeed, a number of T cells have reactivity to very different peptide sequences, as shown by Nanda and Sercarz.[223] It has also been argued by Mason[224] that the universe of peptides is so large that each T cell must on average be cross-reactive to approximately 10^6 different peptides (although many of the differences in peptide sequence in this calculation would not be accessible to the TCR, being buried in the MHC binding groove). Several large-scale screens of a random 9-mer peptide library with different T cells did turn up a great many stimulatory

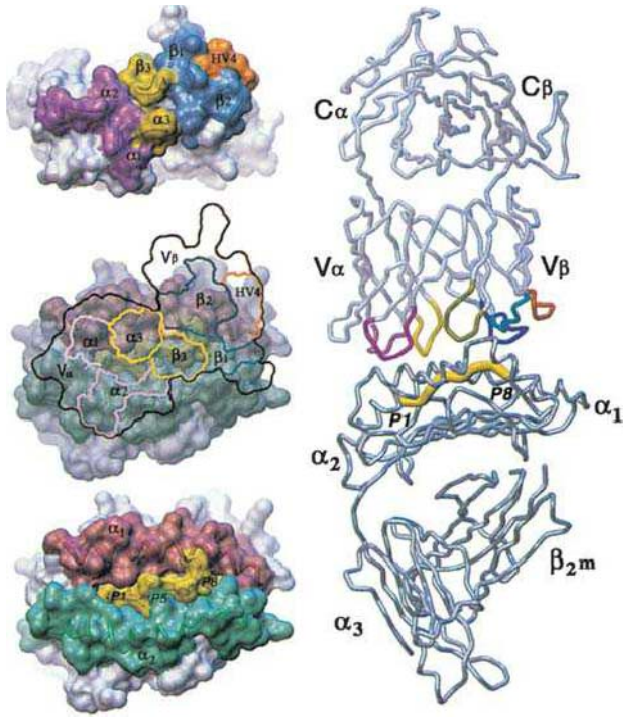

FIG. 11.9. T-Cell Receptor–Peptide/Major Histocompatibility Complex Crystal Structure of a T-Cell Receptor–Peptide/Major Histocompatibility Complex Complex. Peptide and complementary-determining regions are portrayed in different colors.[150]

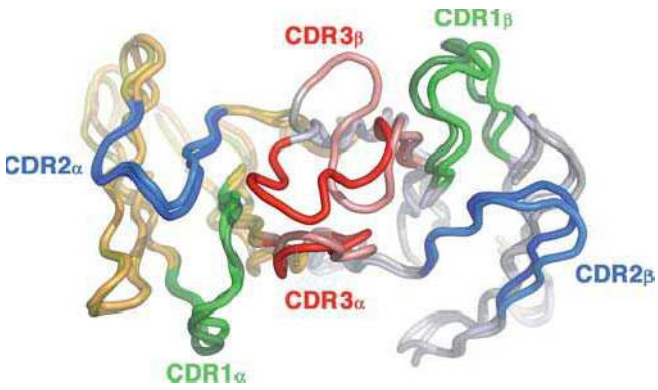

FIG. 11.10. Complementarity-Determining Region (CDR)3 Movement in $\alpha\beta$ T-Cell Receptor (TCR) Binding. In most cases where there is a structure for an $\alpha\beta$ TCR as well as for one (or more) for that TCR in complex with a peptide–major histocompatibility complex, there is a marked movement of one or the other of the Vα or Vβ CDR3s. This example from Mallissen et al.[209] shows a particularly large (14 A) movement of CDR3β with binding. This meshes well with thermodynamic data showing an "induced fit" binding mechanism for most TCRs.[173,211]

peptides, but very few have changes in the two to four key TCR-sensitive residues.[225] This led Garcia and colleagues to conclude that cross-reactivity to different agonist peptides may not be a general feature of T-cell specificity.[226] Instead, the major requirement for cross-reactivity may be in thymic selection[227] and/or in the use of endogenous peptide-MHCs to augment T-cell sensitivity to agonist ligands.[207,213,228]

Direct evidence of TCR plasticity was first obtained by Garcia et al.,[229] who in comparing the x-ray crystal structures of the same TCR bound to two different peptide-MHC ligands found a large conformational change in the CDR3 loop and a smaller one in the CDR1α loop. An even larger conformational change (15 Å) has been found in the CDR3β residue of another TCR as it binds to a peptide/MHC complex,[230] as shown in Figure 11.10. That each TCR may have many different conformations of its CDR3 loops is suggested by the two-dimensional nuclear magnetic resonance studies of Reinherz and colleagues, who found that the CDR3 regions of a TCR in solution were significantly more mobile than the rest of the structure.[231] That this may be a general feature of most TCRs is supported by thermodynamic analyses of various TCRs binding to their peptide/MHC ligands, both class I and class II. Here, the binding is often accompanied by a substantial loss of entropy (Table 11.4) and, in most cases, an "induced fit" mechanism.[184,192,232] This seems to be a situation where an inherently flexible binding site achieves greater order upon binding. This is a mechanism which is also employed by DNA recognition proteins, and Boniface et al.[192] have suggested that it might represent a common mechanism of "scanning" an array of very similar molecular structures (MHCs or DNA) rapidly for those few that "fit" properly. We have seen previously that the association rates are often remarkably slow, with K_as ranging from 1,000 to 10,000 $M^{-1}s^{-1}$ (see Table 11.3). This indicates that either a multistep process is occurring before stable binding can be achieved or that only a fraction of the TCRs in

TABLE 11.4 Thermodynamic and Structural Parameters for T-Cell Receptor Peptide–Major Histocompatibility Complex Interactions

TCR	pMHC	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔCp (kcal/mol)	TCR Conformational Change	pMHC Conformational Change	Reference
2C	dEV8-H2-K^b	−6.3	−22.7	−1.1	CDR3α(6 Å)	None[a]	169,208
2C	p2Ca-H2-K^b	−6.1	−29	−1.5	ND	ND	169
2C	SIYR-H2-K^b	−7.2	−8.4	−1.1	CDR3α(3.9 Å)	None	169,208
2C	dEV8-H2-K^{bm3}	−5.8	ND	ND	CDR3α(6 Å)	None	305,308
JM22z	MP(58–66)-HLA-A2	−7.1	−23	ND	—	Q155-A2(2.4Å)	211,307
KB5-C20	PKB1-H2-K^b	ND	ND	ND	CDR3β(15 Å)	None	209
LC13	EBNA3A-HLA-B8	−6.8	ND	ND	CDR3α(2.5 Å); CDR1α(1.9 Å):Cα	(Q155)-HLA-B8	304
A6	Tax-HLA-A2	−8.2	ND	ND	CDR3β(4.4 Å)	A2-α2(1.4Å)	149,209
BM3.3	VSV8-H2-K^b	−5.4	ND	ND	CDR3α(5.3 Å)	None	303
D10	CA-I-A^k	−7.0	ND	ND	CDR3β	None	172,210
F5z	AM9-H2-K^b	−6.7	−19	ND	ND	ND	211
2B4	MCC-I-E^k	−6.9	−13	−0.6	ND	ND	169,173
2B4	K2-I-E^k	−6.9	−9.4	−2.1	ND	ND	169
2B4	K3-I-E^k	−6.1	−30.5	−4.0	ND	ND	169
2B4	K5-I-E^k	−7.5	−8.0	−1.2	ND	ND	169
2B4	102S-I-E^k	−5.6	−13.2	−0.3	ND	ND	169
2B4	PCC-I-E^k	−6.1	13.3	−1.8	ND	ND	169
2B4	PCC-103K-I-E^k	−7.0	−8.4	−1.0	ND	ND	169
172.10	MBP(1–11)-I-A^u	−6.9	−21.2	−0.16	ND	None	305
1934.4	MBP(1–11)-I-A^u	−6.0	−15.7	−1.2	ND	ND	305
D3	SL9-HLA-A2	−7.5	−10.4	−0.4	ND	ND	309

The ΔG values were derived from the equation $\Delta G = -RT \ln(K_A)$ where R = 0.001987 kcal/mol/K. CDR, complementarity-determining region; ND, not determined; p/MHC, peptide/major histocompatibility complex; TCR, T-cell receptor.

[a] No major conformational change observed after ligand recognition.

solution have the correct conformation. Just how such a scanning mechanism might work for TCRs is seen in the analysis of Wu et al.,[233] who found that a cytochrome c/MHC II–specific TCR derived most of its stability of binding from antigenic peptide residues, but very little of its initial activation energy from these residues. In contrast, MHC residues contributed by far the most of the initial binding, but had relatively modest effects on stability. This indicates that "scanning" may be a process (as shown in Figure 11.11) that first involves contact with (and orientation by) the α-helices of the MHC and then a "fitting" process with and stabilization by peptide residues that involves a substantial loss of entropy. This model of TCR binding might help to explain the striking efficiency and sensitivity of T-cell recognition with the MHC helices guiding the TCR into the correct orientation. It might also be the structural basis for cross-reactivity in which structurally very different peptides bind to the same TCR, as the CDR3 regions of TCR could "fold" into the peptide in many possible configurations. While attractive, there remain caveats about this "two-step binding" model; one is the existence of some important human class I MHC antigens that "bulge" out of the binding groove, creating a barrier to "scanning" as a mechanism of binding to those antigens.[234] A second issue is that one would expect a reproducible "footprint" for MHC binding of particular TCR V regions, which until recently has been elusive. But Garcia and colleagues,[235] as well as Kappler and colleagues,[236] have documented such specific footprints that correlate to particular Vβ usage, although it does seem that there are multiple ways way in which a particular V region can contact a particular MHC.

The rules for the conserved reaction of αβ TCRs with MHC proteins plus peptides are addressed by evaluating the contact residues between TCR and MHC in cocrystal structures of TCR/peptide/MHC complexes. Thus it has been suggested that each TCR variable-region gene product engages each type of MHC through a "menu" of structurally coded recognition motifs that have arisen through coevolution.[235,236]

Role of Cluster of Differentiation 4 and Cluster of Differentiation 8

What is the role of CD4 and CD8 with respect to the T-cell response to agonist and antagonist peptides? Clearly, their expression greatly augments activation, and in some cases, determines whether there is a response at all.[237] In addition, the results of Irvine et al.[238] and Purbhoo et al.,[239] using a single-peptide labeling technique, found an appreciable T-cell response to even one agonist peptide in all four T cells analyzed, resulting in a "stop" signal for the T cell and a small, but detectable rise in intracellular calcium. In CD4+ T cells, these effects are attenuated by antibody blockade of CD4, such that many more (25 to 30) peptides

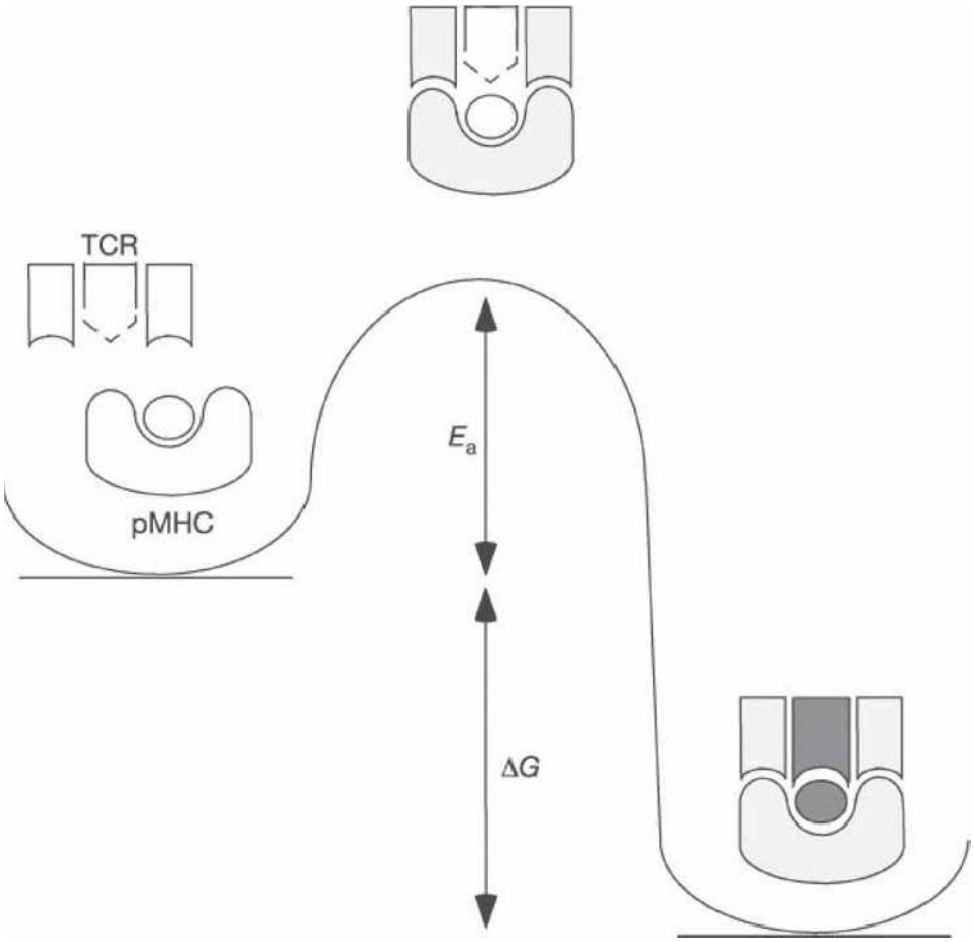

FIG. 11.11. As Shown by Wu etal.,[212] Mutational Analysis of T-Cell Receptor (TCR)-Peptide/Major Histocompatibility Complex (MHC) Binding Indicates that the TCR First Contacts MHC Residues (in the Transition State), and the Peptide has Very Little Influence. Subsequently, however, the peptide residues contribute greatly to the stability of the complex. Thus, we have proposed that the transition state largely involves TCR-MHC contact followed by stabilization of mobile complementarity-determining region 3 residues into a stable state, usually involving significant conformational change and loss of entropy.

are required in order to elicit a stop signal and a calcium flux.[238] Much of this effect likely comes from the recruitment of lck to the TCR/CD3 complexes via the cytoplasmic tails of either molecule. But in addition, there is also a significant positive effect even with CD4 molecules that are unable to bind lck and thus there appears to be an effect on TCR-ligand interaction as well. Nonetheless, while a weak binding of CD4 to class II MHC has been observed,[187] most recently in a complete crystal structure of CD4-TCR-peptide/MHC,[240] there was no apparent stabilizing effect on TCR binding to peptide-MHC in measurements of binding within a synapse by Huppa et al.[188] This is in contrast to the earlier data of Luescher and colleagues,[241] who found that CD8 had a measureable stabilizing effect on TCR binding to ligand on T cells expressing that molecule. This later result is consistent with measurements of CD8 affinity for class I MHCs that are very similar, or even superior, to that of many TCRs for peptide/MHC.[242] So, are CD4 and CD8 playing very different roles with respect to TCR binding to peptide/MHC? Chakraborty and colleagues suggest not in a model they have developed that indicates that the differences in CD4 and CD8 affinity for MHCs contribute at best marginally to activation and that delivering lck to productive TCR-peptide/MHC complexes is their most important property.[243] In this view, the specificity of these molecules for their respective MHC molecules is more of a targeting mechanism than the stabilization function implicit in the ubiquitous illustrations originally proposed by Janeway[116] and featured in many subsequent models.

How could CD4 be facilitating the recognition of small numbers of peptides? Irvine et al.[238] proposed a "psuedodimer" model, which suggests that a CD4 molecule associated with a TCR binding to an agonist peptide-MHC could bind laterally to an endogenous peptide-MHC complex that is also being bound by an adjacent TCR. This takes advantage of the apparent abundance of endogenous peptide/MHCs that can be bound by a given TCR[244] and uses two weak interactions (CD4 → MHC II and TCR → endogenous peptide-MHC) to help create a dimeric "trigger" for activation. This is supported by the work of Krogsgaard et al.,[213] who also showed that disabling CD4 binding to the endogenous peptide/MHC in a heterodimer had a much a greater effect on activation than the same mutation on the agonist peptide/MHC.

What about the timing of CD4 or CD8 binding with respect to TCR-peptide/MHC? The first hints of this came from the work of Hampl et al.,[245] who found that antagonist peptide/MHCs showed no apparent influence of CD4 versus agonists, and because these interactions are much briefer, suggested that CD4 binding was subsequent to TCR engagement with a ligand. That this is likely to be a general effect was shown recently by Jiang et al.,[246] who found that whereas very brief T-cell contacts with peptide/MHC loaded red blood cells involved TCR but not CD8, longer contact periods involved both molecules. Thus it seems that the TCR must first bind to a high quality (eg, agonist) ligand and then CD4 or CD8 comes in to deliver lck and dissociates (based on the lack of stabilization, at least in the case of CD4). But more direct measurements are needed, as well as further definition of the role of endogenous ligands.

Superantigens

One of the most interesting and unexpected areas to emerge from the study of αβ T-cell reactivities is the discovery of "superantigens." Whereas a particular antigenic peptide might only be recognized by 1 in 100,000 or fewer T cells in a naive organism, a given superantigen might stimulate 1% to 20% of the T cells.[247,248] As will be discussed in more detailed in the following, the physical basis for this is that the superantigen binds to a Vβ domain of the TCR on T cells while simultaneously binding to an MHC class II molecule on an antigen-presenting cell (although not in the peptide-binding groove). This allows a single superantigen, such as staphylococcal enterotoxin A (SEA) in Table 11.5, to stimulate virtually every murine T cell bearing Vβ 1, 3, 10, 11, 12, or 17 (about 15% of all αβ T cells), in most cases regardless of what Vα it is paired with or what CDR3 sequence is expressed. Clearly, this is a unique class of T-cell stimulatory molecules.

The first indication of a superantigen effect was the discovery of minor lymphocyte stimulating determinants by Festenstein in the early 1970s.[249] Many years later, Kappler and colleagues characterized a mouse strain–specific deletion of T cells expressing a specific TCR Vβs that were attributable to these loci.[250] It emerged that these effects were due to endogenous retroviruses of the mouse mammary tumor virus family.[251–255] Different family members bind different TCR Vβ domains (as shown in Table 11.5) and stimulate T cells expressing them. Meanwhile, Janeway and colleagues[256] had shown earlier that staphylococcus enterotoxins could polyclonally active naive T cells in a Vβ-specific manner without a requirement for antigen processing. Many of these enterotoxins have been characterized extensively.[247,248,257,258] Unlike the MMTV proteins, which are type II membrane proteins, the enterotoxins are secreted. Subsequently, proteins having similar properties have been isolated from other bacteria (*Yersinia pseudotuberculosis*,[259,260] streptococcus,[261] and from mycoplasma[262,263]). There is also evidence of superantigen-like activities in other mammalian viruses such as rabies,[264] cytomegalovirus,[265] herpes virus,[266] Epstein-Barr virus,[267] and also in *Toxoplasma gondii*.[268] As so many pathogenic or parasitic organisms possess these molecules, apparently by convergent evolution, there must be some selective advantage, but in most cases there is no conclusive evidence as to what this might be. The one exception is the case of the MMTV superantigens, where it has been shown that polyclonal T-cell stimulation allows the virus to more efficiently infect the B-lymphocytes that are activated by the T cells.[269,270] This may be a special case, however, and most authors have suggested that superantigens primarily serve to confuse and occupy the immune system while the pathogen escapes specific targeting and elimination. Large doses of superantigens have also been implicated in various "shock" syndromes, such as food poisoning or "toxic shock,"[247] but this is probably not their everyday purpose, as it would violate the general rule that the host and parasite should coexist.

It has also been suggested by Stauffer et al.[271] that superantigens might be involved in triggering autoimmune diseases. Here the hypothesis is that a large number of some Vβ-bearing T cells are activated by a pathogenic superanti-

TABLE 11.5 Vβ Specificity of Exogenous and Endogenous Superantigens

Bacterial Superantigen	Human Vβ Specificity	Reference	Murine Vβ Specificity	Reference
				(as referenced in 235,314)
SEA	ND		1,3,10,11,12,17	310,311
SEB	3,12,14,15,17,20	315,316	(3), 7,8.1,8.3, (11), (17)	310,311,312
SEC_1	12	316	7,8.2,8.3,11	310
SEC_2	12,13,14,15,17,20	315,316	8.2,10	310
SEC_3	5,12	316	(3), 7,8.2	310
SED	5,12	316	3,7,(8.2),8.3,11,17	310
SEE	5.1,6.1–6.3,8,18	315,316	11,15,17	310
TSST-1	2	316	15,16	310
ExFT	2	315	10,11,15	310
Strep M	2,4,8	239	ND	

Endogenous Proviruses	Vβ Specificity	Mls Type[a]	Chromosome	Reference
				(as referenced in 235,236)
Mtv-1	3	c, 4a	7	
Mtv-2	14	NA	18	
Mtv-3	3,17	c	11	
Mtv-6	3,17	c, 3a	16	
Mtv-7	6,7,8.1,9	a, 1a	1	
Mtv-8	11,12	f, Dvbll.1	6	
Mtv-9	5,11,12	f, Etc-1	12	
Mtv-11	11,12	f, Dvbll.3	14	
Mtv-13	3	c, 2a	4	
Mtv-43	6,7,8.9,9	Mls-like	ND	

Exogenous Viruses	Vβ Specificity	Mls Type	Chromosome	Reference
MMTV-C3H	14,15	NA		231
MMTV-SW	6,7,8.1,9	Mls-like		247
Rabies	ND			242
EBV	ND	HERV-K18		245
CMV	ND			243
Herpesvirus				244

Other Pathogens	Vβ Specificity	Name	Chromosome	Reference
Mycoplasma arthritidis	h17,6,8.1,8.3	MAM		240,241
Toxoplasma gondii	5			246
Yersinia enterocolitica	ND			237
Yersinia pseudotuberculosis	ND			238

Vβ in parentheses are reactive with commercial but not recombinant enterotoxins.
CMV, cytomegalovirus; EBV, Epstein-Barr virus; MMTV, mouse mammary tumor virus; NA, not applicable; ND, not determined.
[a]The nomenclature in use before the discovery that the phenotype resulted from endogenous retroviruses.

gen and that subsequently self-reactive T cells within those activated cells are more easily stimulated by a particular tissue antigen. That this may occur in some cases is supported by their work on a human endogenous retrovirus which specifically stimulates Vβ7 T cells and is implicated in the initiation of type I diabetes.[271] Another report implicates a superantigen in Crohn disease, another autoimmune disorder.[272]

While the biochemistry of superantigen binding to TCR and MHC is similar to that of TCR peptide/MHC interactions,[273] mutagenesis, and particularly x-ray structural data, has shown that the topology is both quite different and variable.[248] In particular, it has been found that Mls-la presentation to T cells is most affected by mutations on the "outside" surface of the Vβ domain, which do not affect peptide/MHC recognition.[162] In contrast, CDR1 and CDR2 of regions of Vβs are involved in bacterial superantigen reactivity.[274]

An example of the structural data is shown in Figure 11.12, which shows how a model TCR-SAg-MHC complex (derived from separate structures) would displace the TCR somewhat (but not entirely) away from the MHC binding groove,[275] thus making the interaction largely insensitive to the TCR/peptide specificity. Other TCR-SAg-MHC complexes have very different geometries.[276,277]

Why do all the many independently derived superantigens interact only with the TCR β-chain? One possibility is that the β-chain offers the only accessible "face" of the TCR, perhaps because the CD4 molecules hinders access to the Vα side, as suggested by the antibody blocking studies of Janeway and colleagues.[278,279]

ANTIGEN RECOGNITION BY γδ T CELLS

γδ T cells together with αβ T cells are present together in all but the most primitive vertebrates. This finding argues that γδ T cells functions are different from those of αβ T cells. Yet, most γδ T cells produce cytokines that are similar to

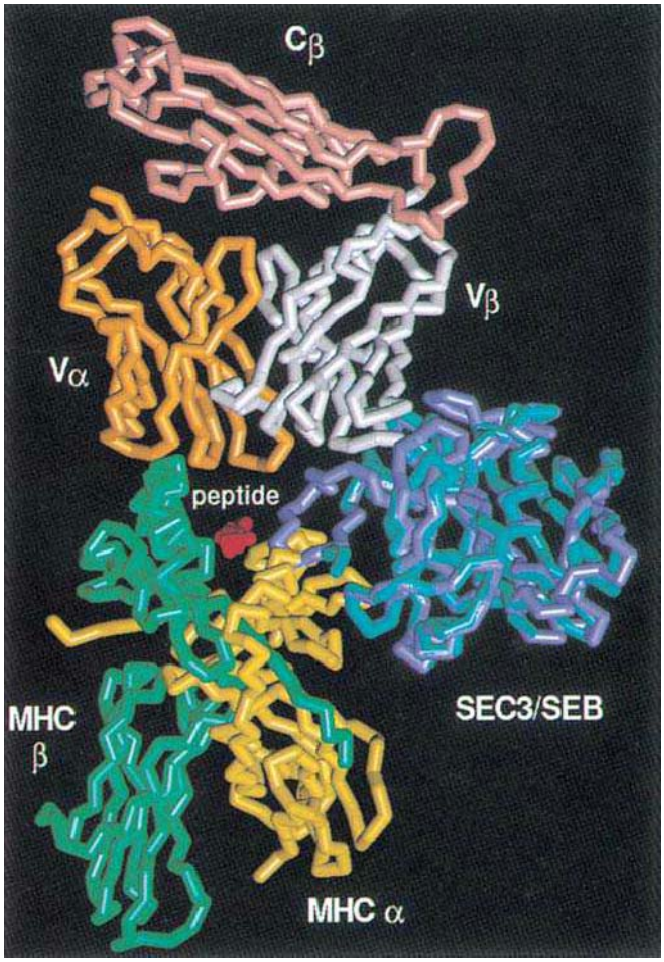

FIG. 11.12. Crystal Structure of a T-Cell Receptor (TCR) *β*/Superantigen (SAg) Complex. Fields et al.[253] crystallized TCR-SAg complexes and from the structure of the same superantigens with a class II major histocompatibility complex (MHC) molecule and were able to deduce the relative spatial arrangement of the three molecules. This model suggests that TCR does not contact the MHC very strongly, which is consistent with the relative peptide insensitivity of SAg activation.

those produced by αβ T cells. γδ T cells also can mount cytotoxic responses upon activation like CD8+ αβ T cells. These results, together with the fact that αβ T cells are almost always found alongside and usually in excess of γδ T cells, suggest that the difference in how γδ T cells and αβ T cells contribute to host immune competence is less likely because of differences in effector functions or in tissue distribution but rather because of differences in how these two types of cells are triggered. Thus, to understand how γδ T cells function, it is essential to know the antigens and the target cells recognized by γδ T cells.

Antigen Recognition Requirements and γδ T-Cell Ligands

Because the study of γδ T cells is relatively recent and does not stem from any knowledge of their biologic function, experiments designed to characterize their specificity and function had drawn heavily on our knowledge of αβ T cells. Because αβ T-cell recognition requires antigen presentation by MHC and related molecules, it was assumed that γδ T-cell recognition would also follow the same rules. Even in cases where classical MHCs are clearly not involved, it has been suggested that nonclassical MHC molecules or some as yet to be identified surface molecules might play a similar role. These possibilities are difficult to test experimentally. Thus, analysis of γδ T cells specific for MHC molecules were carried out to determine the antigen recognition requirement of γδ TCRs. This approach asks the following questions: When γδ T cells recognize MHC molecules, what kind of antigen processing is required, is any of the specificity conferred by bound peptide, and which part of the MHC molecule is recognized? This approach took advantage of the detailed knowledge of the molecular structure of MHC class I[280,281] and MHC class II molecules,[282] thus making T-cell epitope mapping feasible and interpretable. In addition, and more importantly, the biosynthetic pathways and antigen-processing requirements for both MHC class I and MHC class II molecules had been extensively studied. Mutant cell lines defective in either pathway were readily available and could be transfected with various MHC class I and class II genes. Therefore, potential antigen processing and presentation requirements for alloreactive γδ T cells could be studied with precision and compared with those of αβ T cells.

The γδ T cell LBK5[283,284] recognizes I-E^{b,k,s} but not I-E^{d}. An analysis of the fine specificities of LBK5 showed that the peptide bound to the I-E molecules does not confer specificity and that no known antigen-processing pathways were required in the recognition of I-E by LBK5. All variations in the ability of different stimulator cells to activate LBK5 can be attributed solely to their level of surface I-E expression and are independent of their species origin (mouse, hamster, human) and cell type (B cells, T cells, fibroblasts). Modifications of the repertoire of peptides loaded onto MHC molecules also showed no effect because native I-E^{k}, GPI-linked I-E^{k}, I-E^{k} expressed with or without invariant chains, and the presence or absence of functional MHC class I or class II antigen-processing pathways all stimulated LBK5 similarly.[285] Thus, LBK5 recognizes native I-E^{k} molecules with a variety of different bound peptides or in the case of GPI-linked I-E^{k}, which most likely does not complex with a peptide. In addition, LBK5 recognizes *E. coli*–produced I-E^{k} *α* and *β* chains folded with a single peptide.[285]

The functional epitope on I-E for LBK5 recognition maps to the β67 and β70 residues.[285] This explains why LBK5 recognizes I-E^{b,k,s} but not I-E^{d} and why peptide bound to I-E^{k} does not confer the specificity. Surprisingly, I-E^{k} mutants with an altered carbohydrate structure on the α84 position are not recognized by LBK5.[286] Although the binding interface of the LBK5 γδ TCR and I-E has yet to be determined, it is reasonable to assume that it will not deviate much from those reported for antibodies, αβ TCRs and the G8 γδ TCR. From the coordinates of a published crystal structure of I-E^{k},[287] the distance between β67–70 (the LBK5 epitope) and the carbohydrate attachment site at position α82 was estimated to be around 28–33Å. Hence, it is likely that the carbohydrate structure is peripheral to the core of the LBK5/I-E interaction. This type of interaction may be similar to that described for human growth hormone and the extracellular

domain of its receptor[288] where a central hydrophobic region at the contact site, which is dominated by two tryptophan residues, accounts for more than three-quarters of the binding free energy and where peripheral electrostatic contacts contribute substantially to the specificity of binding but not to the net binding energy. This type of protein-protein interaction has been postulated to ensure the specificity of an interaction without requiring a high affinity.[289]

Two independently derived γδ T-cell clones, KN6[290] and G8,[291] are found to recognize T10 and T22, two closely related, nonclassical MHC class I molecules that have 94% amino acid identity. T22 appears to be expressed constitutively on a variety different cell types, whereas the expression of T10 is inducible on cells of the immune system. Among strains of mice tested so far, all express T22. However, mice of the H-2^d or H-2^k MHC haplotypes (eg, BALB/c and C3H, respectively) lack functional T10 molecules.[291,291a] T10 and T22 have also been identified as natural ligands for murine γδ T cells. Approximately 0.2% to 1% of the γδT cells in normal, unimmunized mice are T10/T22-specific (Fig. 11.13).[292]

The primary sequences of T10 and T22 suggest that the necessary structural features that enable classical MHC class I molecules to bind peptides are absent. Indeed, x-ray crystallography has shown that T10 and T22 adopt a severely modified MHC-like fold that lacks a classical peptide-binding groove and exposes part of the β sheet "floor" of the α1/α2 platform.[293,294] Consistent with the structural data, no endogenous peptides can be eluted from chimeric T10/L^d molecules that are expressed by transfected cells.[295,295a] This indicates that these molecules can reach the cell surface devoid of peptide. Importantly, the *E. coli*–produced, in vitro–folded

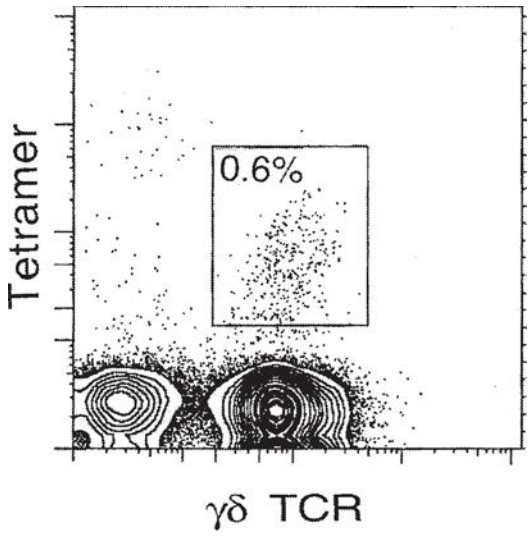

FIG. 11.13. T10/T22-Specific γδ T Cells can be Detected in Normal Mice Through Use of a Tetrameric T22 Staining Reagent. As shown by Crowley et al.,[274] a T22 reagent that was generated by similar methods as tetrameric peptide/major histocompatibility complex reagents stained approximately 0.6% of splenic γδ T cells in normal animals. More than 90% of these cells are cluster of differentiation (CD)4–CD8–; the rest are either CD4 or CD8 single positive (about 3% to 4% each). A similar frequency of tetramer-positive γδ T cells was also found in the intestinal intraepithelial lymphocyte population (data not shown).

T10/β_2m and T22/β_2m molecules can stimulate the G8 γδ T cell,[295,296] which provides unequivocal evidence that these peptide-free molecules retain their immunologic function.

Direct binding between soluble G8 γδ TCRs and T10/β_2m and T22/β_2m complexes has been measured.[297] Surface plasmon resonance showed that the dissociation rates for the interaction between G8 and $T10^b$ and $T22^b$ were similar ($k_d = 8.1 \pm 2.3 \times 10^3 s^{-1}$) and slower than those that had been observed for most interactions between αβ TCRs and peptide/MHC complexes. The association rates ($k_a = 6.53 \pm 1.73 \times 10^4\ M^{-1}s^{-1}$) are among the fastest that had been reported for αβ TCRs and their ligands. Therefore, compared to αβ TCRs, the affinity between G8 γδ TCRs and their ligand is rather high ($K_D = 0.13 \pm 0.05\ \mu M$). A Scatchard analysis of equilibrium binding generated a similar affinity of 0.11 ± 0.07 μM for this interaction.[297]

There are only two other pairs of ligand-TCR interaction have been reported. The binding between the soluble form of G115 and its ligand F1-ATPase and the apolipoprotein A-I complex showed a K_D of 1.5 μM and 0.8 μM, respectively.[171] These affinities are within the range of soluble αβ TCRs and their peptide/MHC ligands. Surprisingly, a K_D of 110 to 900 μM was reported for δ1A/B-3:MICA interactions.[299] This affinity is one to two orders of magnitude weaker than the NKG2D:MICA interaction, more on a par with MHC class I:CD8 interactions (from 11 to ≥ 1,000 μM).

It should be noted that the same human γδ T-cell clones that recognize AS/ApoA-I complexes are activated by a set of nonpeptidic pyrophosphomonoesters that are collectively referred to as phosphoantigens (phosphoAgs).[170] It has been observed for well over a decade that some human peripheral blood Vγ9Vδ2 cells can be stimulated by phosphoAgs such as isopentenyl pyrophosphate and dimethylallyl pyrophosphate. These cells show in vitro responses to tumors, phosphoAgs produced by eukaryotes and prokaryotes, natural and synthetic alkylamines, and aminobisphosphonates. However, repeated attempts to show interactions between phosphoAgs and Vγ9Vδ2 TCRs have failed. The identification of AS/ApoA-I complexes as antigens of some of these T cells further challenges this notion.

As indicated in Table 11.6,[145] other than toward phosphoAgs, the reactivities of Vγ9Vδ2-expressing T cells to other challenges such as tumors and AS/ApoA-I are far from homogeneous. Further, these cells mount a much more robust response to phosphoAgs than to CD3 cross-linking. Thus, it is possible that isopentenyl pyrophosphate and other isoprenoid intermediates enhance antigen-specific responses of Vγ9Vδ2 cells without being TCR antigens themselves. Isopentenyl pyrophosphate and dimethylallyl pyrophosphate are metabolites of the mevalonate pathway that regulates the biosynthesis of cholesterol as well as of isoprenoids that mediate the membrane association of certain GTPases. The addition of isoprenoid intermediates has been shown to augment antigen-specific αβ T-cell responses and to alter their cytokine profiles.[300]

These experimental observations together with the CDR3 length distribution analysis described previously make it clear that the molecular nature of γδ T-cell antigen recognition is fundamentally different than that of αβ T cells. While MHC and MHC-related molecules are recognized by

TABLE 11.6 Partial List of γδ T-Cell Reactivities[a]

Name (referred to as)	Source	Reported Reactivities	Comments	Reference
Murine				
DGT3	Lymph node cells from DBA2 mouse primed with poly ($Glu^{50}Tyr^{50}$)	Qa-1/($Glu^{50}Tyr^{50}$)		317
KN6	Double-negative thymocytes from C57Bl/6	$T10/T22^{b,k\ not\ d}$		318
Tgl4.4	Lymph node of HSV-infected C3H mouse and restimulated with L cells transfected with HSV-gl	HSV-gl	Can be stimulated by gl protein alone	319,320
G8	BALB/c nu/nu immunized with B10.BR APCs	$T10/T22^{b,k\ not\ d}$	Direct binding and cocrystal structure have been shown	153,274,323
LBK5	C57Bl/10 nu/nu immunized with B10.BR splenocytes	$I\text{-}E^{b,k,s\ not\ d}$	Can be stimulated by I-E proteins alone; reactivity is not peptide-specific	322,323
LKD1	B10.BR immunized with B10.D2 splenocytes	$I\text{-}A^{d}$		322
69BAS-122	C57Bl/10, adult splenocyte	HSP-60 peptide, cardiolipin, β2-glycoprotein 1	Transferring TCR transfers reactivity	324,325
BNT-19.8.12	C57Bl/10, newborn thymocyte	Mycobacterium PPD, cardiolipin, β2-glycoprotein 1	Transferring TCR transfers reactivity	325,326
7–17 and other dendritic epidermal T cells	γδT cells from murine epidermis	Keratinocytes	Transferring TCR transfers reactivity	33
Human				
Panels of T cell clones expressing Vγ 9Vδ2	PBMC stimulated with irradiated PBMCs and PHA	Tumor cells (eg, Molt-4), MT, metabolites in the mevalonate pathway	20% of Vγ9Vδ2 clones do not react to MT; only slightly more than 50% of MT- or Molt-4–specific clones recognize the other specificities	327,328
Panels of T-cell clones expressing Vγ9Vδ2 including G42 and G115	γδT cells from PBM cultured with irradiated PBLs and lymphoblastoid cells	phosphoAg, tumor cells (eg, Daudi), AS/ApoA-I(G115TCR)	Close correlation between Daudi and mycobacterial reactivity; direct binding between AS/ApoA-I; heterogenous reactivity to ApoA-I	156,329,330
DG.SF13 (Vγ9Vδ2)	γδT cells isolated from RA synovial fluid stimulated with sonicate of *Mycobacterium tuberculosis*	Daudi cells, *Mycobacterium tuberculosis*, MEP	Transferring TCR transfers reactivity	331
Panels of T-cell clones expressing Vγ 9Vδ 2 including CP1.15 and DG.SF68	PBMC γδT cells stimulated with *Mycobacterium tuberculosis* extract	phosphoAg, alkylamines, aminobisphosphonates	Reactivities require cell-cell contact	332,333
Clones 1,2,3,4, 5 (Vδ1)	Lymphocytes extracted from human intestinal epithelial tumors cultured with irradiated CIR-MICA and CIR-MICB cells	MICA, MICB	Transferring TCR transfers reactivity; Vδ1-Jδ1 with diverse CDR3 are used; not all Vδ1-Jδ1 cells are MIC-specific	334,335
JR.2 and XV.1 (Vδ1)	PBL stimulated with autologous $CD1^{+}$ DC and M. tuberculosis extract	CD1c expressing cells	Transferring TCR transfers reactivity; many Vδ1 positive γδ T cells are not CD1c-reactive	335
Vγ1.3Vδ2 expressing BW5147	γδ TCR chains from muscle-infiltrating T cells of a polymyositis patient and transfected into TCR-deficient BW5147	Muscle cell extract and *Escherichia coli* extract		336,337
4–29 and 5–3 (Vδ2 negative)	PBMC from CMV-infected transplant recipients stimulated with irradiated PBMC and PHA	CMV-infected fibroblasts, Hela, HT-29, Caco-2		338

[a]The names of the T cells, the way they are generated, and their reported reactivities.

CMV, cytomegalovirus; HSA, herpes simplex virus; MEP, monoethylphosphate; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; PPD, microbacterium purified protein derivative; RA, rheumatoid arthritis.

γδ T cells, γδ T-cell antigens need not be MHC or MHC-related molecules. Moreover, in all cases, the antigens are recognized directly. This suggests that pathogens and damaged tissues can be recognized directly and cellular immune responses can be initiated by γδ T cells without a requirement for antigen degradation and specialized antigen-presenting cells, such as B cells, macrophages, and dendritic cells. This would allow for greater flexibility than is present in classical αβ T-cell responses. T10/T22, I-E, as well as MICA and MICB expression can be induced under certain physiologic/pathologic conditions.[301] ATP synthase F1 is normally localized in the membranes of mitochondria, but it has also been found on the surfaces of tumors, hepatocytes, and endothelial cells.[302–303] Thus, γδ T-cell activation may be regulated through the level of protein expression. In addition, the recognition of I-E by LBK5 is acutely sensitive to changes in the glycosylation of the I-E molecule. This suggests a novel way by which antigen recognition by γδ T cells can be regulated. Changes in the posttranslational modifications of surface glycoproteins often indicate that tissues have become infected, have undergone neoplastic transformations, or have experienced other types of cellular stress. For example, it has been shown that the infection of mice with *Listeria monocytogenes* impairs the addition of sialic acid to host cell glycoproteins that include MHC molecules.[304] Additionally, whereas the surface glycoprotein mucin is heavily glycosylated in normal cells, it is underglycosylated in breast, ovarian, and pancreatic carcinomas such that the peptide backbone is unmasked.[305] Thus, both the quantity and the quality of the ligand could contribute significantly to the specificity of γδ TCR recognition.

Thymic Ligand Recognition and γδ T-Cell Repertoire and Function Development

γδ T cells, like αβ T cells, develop in the thymus before entering the periphery. In the case of αβ T cells, thymic development entails endogenous ligand–driven positive and negative selection, which determine what αβ T cells can recognize and whether these T cells will develop into CD4+ helper or CD8+ cytolytic T cells. However, the role of ligand-mediated selection in γδ T-cell development and function has been less clear. Recently, this issue has been reexamined and the emerging picture is fundamentally different from what we know about αβ T-cell development and differentiation. These work suggest that thymic development does little to constrain γδ T cell antigen specificities, but determines γδ T cell effector fate.[306] In particular, γδ thymocyte development and exit into the periphery is not contingent on encountering cognate antigen in the thymus and TCR dimerization may be sufficient to induce signaling for γδ T cells to develop in the thymus.[306a] Thus, once the antigen specificity repertoire is generated by V(D)J rearrangement, it is only marginally modified by thymic selection. In fact, a large fraction of peripheral γδ T cells have not encountered ligand either in the thymus or in the periphery, and this antigen-naïve population is actively maintained by rapidly turning over, and cells that have encountered self-ligands do not accumulate.[306a]

While ligand expression does little to constrain antigen specificities of the γδ T cell repertoire, it does play a role in endowing γδ T cells with different functional programs, in that TCR signaling strength experienced by γδ thymocytes seem to determines the functional specification of γδ T cells: γδ thymocytes that have not encountered cognate ligands make IL-17, γδ thymocytes that have encountered thymic ligands make IFN-γ,[306a] and those that are strongly self-reactive make IL-4. Importantly, regardless of ligand experience, some γδ T cells in normal mice are able to make cytokines immediately upon TCR engagement. IL-17 is a cytokine, which regulates the expansion and recruitment of neutrophils and monocytes to initiate the inflammatory response.[306b] In acute inflammation, a swift IL-17 response must be elicited without prior antigen exposure. Therefore, γδ T cells may be uniquely suited to produce IL-17 at the onset of the inflammatory response to initiate an acute inflammatory response to pathogens and to host antigens revealed by injury. In addition, by acting early in the inflammatory response, γδ T cells may modulate and shape the subsequent αβ T cell and B cell responses that develop during the inflammatory process and thus may play a much larger role in the adaptive immune response than previously recognized.

Antigen Recognition Determinants of γδ T-Cell Receptors: V Genes Versus Complementarity-Determining Region 3 Regions in γδ T-Cell Receptor Ligand Recognition

In part because γδ T cells from different anatomic sites show preferential V gene expression, γδ T cells are commonly been divided into subsets based on V gene usage.[309,310] Numerous studies have also reported that γδ T-cell functions segregate with Vγ or VγVδ usage.[311–314] These observations have led to suggestions that the bias in V gene usage enables γδ T cells to respond to antigens that are specific to their resident tissues.[11,312,315] If this were the case, then γδ TCRs would function like innate immune receptors even though VDJ recombination of the TCR δ chain leads to orders of magnitude higher potential junctional (CDR3 region) diversity than is found in Ig and αβ TCRs (as discussed previously).

To resolve this issue, it is essential to understand the basis of γδ TCR antigen recognition. Based on the analysis of T22-specific γδ T cells isolated from normal mice, the majority of T22-specific γδ TCRs use Vγ1 and Vγ4 in the spleen and a sizeable number use Vγ7 in the IEL compartment.[316] Thus, at least for T22 specificity, Vγ usage reflects tissue origin and not antigen specificity. Consistent with this, it has been demonstrated that the preferential usage of Vγ7 by γδ T cells that can migrate into the IEL compartment primarily results from interleukin-15–driven control of Vγ7 accessibility during thymic VJ rearrangement.[317]

While different Vγs and Vδs were associated with T22-specific TCR sequences, there is one defining feature that is common among them.[316] This is a prominent CDR3δ motif shown in Figure 11.14 that consists of a Vδ or Dδ1-encoded Trp (W); a Dδ2-encoded sequence of Ser, Glu, Gly, Tyr, and Glu (SEGYE); and a P-nucleotide-encoded Leu (L). Gene transfer experiments established that TCRs with the W-(S)

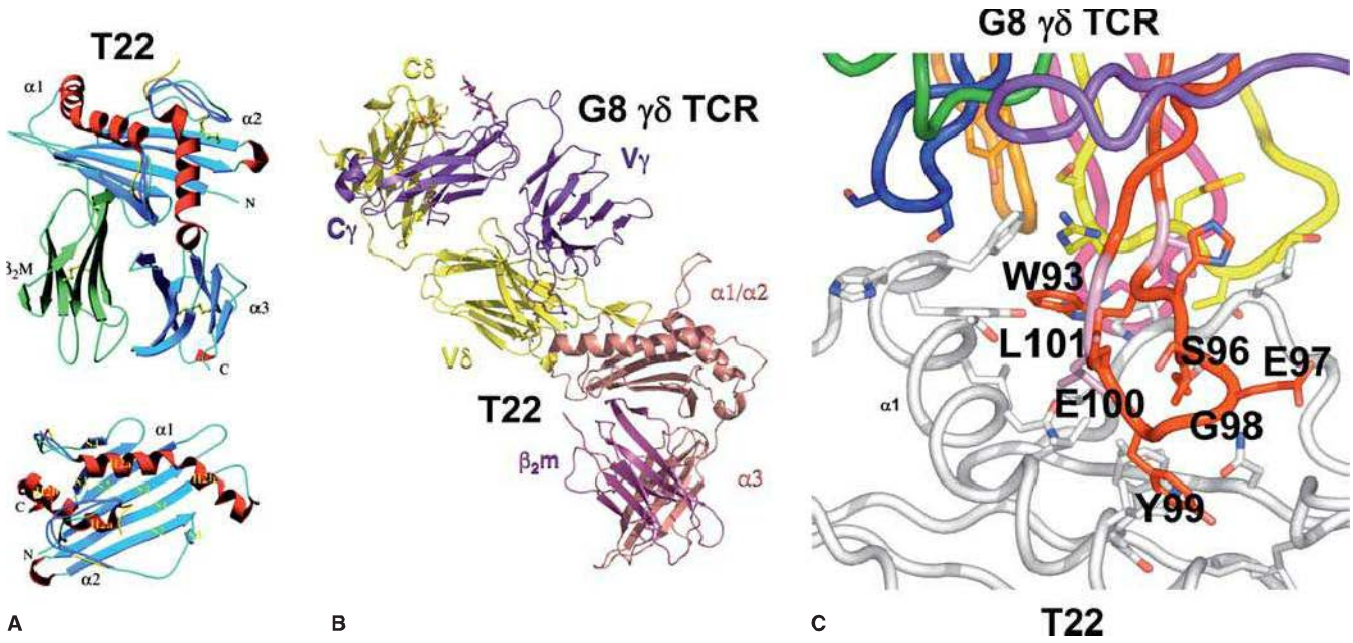

FIG. 11.14. The T22 Structure and the Interaction with the G8 γδ T-Cell Receptor (TCR). A: The structure of a T22 molecule, a nonclassical class I MHC that is missing half of an alpha helix and does not bind peptides.[270] B,C: The intersection with the G8 TCR, where the long TCR δ CDR3 accounts for almost all of the contact residues.[153]

EGYEL motif bound T22 while those lacking the motif did not[316] (see Table 11.6).

Proof that the W- (S)EGYEL motif is the antigen contact site came from the crystal structure of the G8 γδ TCR bound to T22.[168] As discussed in the previous section, T22 has an MHC class I–like fold, but one side of what would normally be a peptide-binding groove is severely truncated and exposes the β-sheet "floor."[296] G8 binds T22 at a tilted angle that contrasts with the essentially parallel alignments of the long axes of the αβ TCR and the peptide/MHC when in complex (Fig. 11.15). The majority of the contact residues are contributed by the β-sheet floor and the α1 helix of T22, and the fully extended CDR3d loop with Trp anchoring at the N-terminal end and residues Gly, Tyr, Glu, and Leu together with a Thr residue encoded in the J region anchoring the loop at its C-terminal end. This is similar to Ig, where antigen specificity in non-somatically mutated antibodies resides predominantly in the CDR3 of the heavy chain.[318] This mode of antigen recognition

	Vδ	P/N	Dδ1 (GTGGCATATCA)	P/N	Dδ2 (ATCGGAGGGATACGAG)	P/N	Jδ1 (CTACCGACAAACTC)
G8	TGT GCT GCT GA C A A D	C AC T	G TGG CAT AT W H I		A TCG GAG GGA TAC GAG S E G Y E	CTC GG L G	T ACC GAC AAA CTC T D K L
KN6	TGT GCC TCG GGG C A S G		TAT Y	TG W	G GAG GGA TAC GAG E G Y E	CTG L	ACC GAC AAA CTC T D K L
Vδ6	TGT GCT CTC TGG GAG CTG C A L W E L	GAG E			TCG GAG GGA TAC GAG S E G Y E	CTC G L A	CC GAC AAA CTC D K L
	TGT GCT CTC TGG GAG CTG C A L W E L	GTT A V M	TG GC A		A TCG GAG GGA TAC GAG S E G Y E	CTA L	ACC GAC AAA CTC T D K L
	TGT GCT CTC TGG GAG CTG C A L W E L		AT I		A TCG GAG GGA TAC GAG S E G Y E	CTA L	ACC GAC AAA CTC T D K L
Vδ4	TGT GCT CTC ATG GAG C A L M E	GGT ATA G I	TGG CAT AT W H I		A TCG GAG GGA TAC GAG S E G Y E	CTT G L A	CT ACC GAC AAA CTC T D K L
	TGT GCT CTC ATG GAG CG C A L M E R	A	AT I		A TCG GAG GGA TAC GAG S E G Y E	CTG GA L D	C GAC AAA CTC D K L
Vδ5	TGT GCC TCG GGG T C A S G S	CC CCC P	CAT AT H I	A TGG CTC GG W L G	A TCG GAG GGA TAC GAG S E G Y E	CTC CTC G L L A	CT ACC GAC AAA CTC T D K L
	TGT GCC TCG GGG TAT C A S G Y	AT M	G TGG W	AGC AT S I	A TCG GAG GGA TAC GAG S E G Y E	CTT G L A	CT ACC GAC AAA CTC T D K L

A

Vγ1	Vδ6	$t_{1/2}$ (min)	K_D (nM)
CAVWI LS GTSWVKIF	CALWEL E SEGYEL A DKL	64 ± 7	12.0 ± 0.5
CAVWI P GTSWVKIF	CALWEL VMA SEGYEL T DKL	86 ± 22	17.2 ± 2.8
CAVWI T GTSWVKIF	CALWEL I SEGYEL T DKL	109 ± 19	13.8 ± 2.8

B

FIG. 11.15. Conserved T-Cell Receptor (TCR) δ Complementarity-Determining Region (CDR)3 Sequences Correlate with T10/T22 Specificity in γδ T Cells. Sequence data from Shin et al.[290] showing that both established cell lines (G8 and KNG) and γδ T cells from clonal cultures that are specific for the T10/T22 share highly conserved TCRδ CDR3 sequences. These are largely derived from the Dδ2 gene segment and from the structural data.[Adams et al., color plate 4, 153] These conserved sequences constitute the main interaction between these γδ TCRs and their ligands.

also fits well with analyses of CDR3 length distributions of all immune receptor chains that first suggested that γδ TCRs bind antigens more similarly to Igs than to αβ TCRs.[144]

There are two G8/T22 complexes in the asymmetric unit.[168] While the contact residues are similar at the interface of the CDR3/T22 β sheet, the two complexes differ by a relative rotation between the G8 TCRs. This shift alters the contacts formed between the CDR1, CDR2, HV4, and CDR3 loops and T22 in each TCR, suggesting that the CDR3 loop acts as a pivot point for G8 binding, with some flexibility in the interaction between the other CDR loops and T22. The hinge-like flexibility around this pivot point stands in stark contrast to interactions seen in antibody/antigen and αβ TCR-peptide/MHC complexes. In those cases, the relatively straight-on docking mode results in multipoint (ie, multi-CDR) attachment of the receptor to the ligand, essentially rigidifying the intermolecular orientations between the two binding partners. In most cases, the αβ TCR CDR1 and CDR2 loops provide a perimeter of contacts with the MHC helices surrounding the CDR3 loops, and so far, no variation has been seen in the docking angle of TCR to MHC in cases where multiple complexes exist in the asymmetric unit. Thus, the CDR3 motif of G8 and other γδ TCRs may be thought of as a somewhat autonomous binding entity that is presented by a variety of germline-encoded variable domain scaffolds without strong preference for particular CDR1 and 2 sequences. It would be interesting to see other examples of γδ TCR/ligand binding. CDR3 regions were found to be important for γδ T-cell recognition of MICA/MICB. In this case, the reactivity correlates only with a junction between Vδ1 and Jδ1. Aside from the W-(S)EGYEL motif, the T22-specific CDR3γδ sequences were diverse and were encoded by various Vδs, N- and P-nucleotides, and Dδ1s of different lengths and reading frames. Importantly, it was shown that sequence variations in the CDR3 regions around this motif modulated the affinity and the kinetics of T22 binding.[313] In fact, the T22-specific repertoire in normal mice covers a range of affinities, as is evident by the large range of T22 tetramer staining intensities.[300,316] This allows for the selection of T cells with the "most optimal" antigen-binding capabilities during an immune response, which is a hallmark of the adaptive immune response.

Nonetheless, such a repertoire that is created mainly by V, D, and J region–derived germline-encoded nucleotides, despite requiring VDJ recombination, would be "innate" in character, because the antigen specificities would be predetermined and the repertoire would be much less variable among individuals of the same species.

Analysis of the formation of T10/T22-reactive repertoire indicates that biases linked to the recombination machinery influence the generation of a γδT-cell repertoire toward certain specificities. A repertoire that is generated by recombination but conferred by a limited set of germline or germline-like residues at the CDR3 region will be created at a much higher frequency than one whose specificity is conferred primarily by N-nucleotide additions, as is the case with that of αβ TCRs. Indeed, the 1 in 100 frequency of T22-specific γδ T cells in normal mice is much higher than the estimated 1 in 10^5 to 10^6 frequency of naïve peptide/MHC-specific αβ T cells.[315–317] This could provide a solution to the apparent paucity of γδ T cells and could allow for a significant response without an initial need for clonal expansion as is required for most αβ T-cell responses. It was shown that rearrangements at the TCR δ locus are biased towards full-length Dδ2 sequences rather than extensive D region nucleotide deletion, as is the case for the TCR-β locus.[313] Thus, different reading frames of Dδ2 may contribute to the recognition of other ligands by γδ TCRs in a manner similar to that of T22-specific γδ TCRs and would lead to a repertoire that is biased toward a relatively small number of ligands, more on the order of hundreds to thousands versus millions as estimated for αβ T cells, but with highly variable antigen-binding affinities. A repertoire of this type would allow more flexible and efficient responses to changes in ligand expression. These are testable hypotheses, especially once more γδ TCR ligands have been identified.

GENERAL FEATURES OF T-CELL RECEPTOR AND IMMUNOGLOBULIN DIVERSITY

A Dominant Role for Diverse Complementarity-Determining Region 3 Regions in Antigen Specificity

One interesting observation that emerges from a detailed analysis of the gene rearrangements that create both TCR and Igs is how the diversity of the CDR3 loop region in one or both of the chains in a given TCR is so much greater than that available to the other CDRs. A schematic of this skewing of diversity is shown in Figure 11.16 for human Igs and for αβ and γδ TCR heterodimers. In the case of αβ TCRs, this concentration of diversity occurs in both Vα and Vβ CDR3 loops, and numerous TCR-peptide/MHC structures[160] have confirmed that these loops sit largely over the center of the antigenic peptide (see previous section). While this concentration of diversity in αβ TCRs in the regions of principal contact with the many possible antigenic peptides seems reasonable, it is much harder to explain for Ig or γδ TCRs. Clearly, there must be some chemical or structural "logic" behind this phenomenon. A clue as to what this might be comes from the elegant work of Shin et al.[313] in the demonstration that for at least one γδ T-cell specificity, the antigen recognition determinants are encoded by germline V or D region residues, with remaining sequence diversity modulating the affinity. While this may be a feature of some or many γδ TCRs, it could not explain the much broader repertoire of Igs. Instead, one possible explanation comes from the studies of Wells and colleagues,[288] who systematically mutated all of the amino acids (to alanine) at the interface of human growth hormone and its receptor as determined by x-ray crystallography. Interestingly, only a quarter of the 30 or so mutations on either side had any effect on the binding affinity, even in cases where the x-ray structural analysis showed that the amino acid side chains of most of the residues were "buried" in the other. This study illustrates an important caveat to the interpretation of protein crystal structures, which is that while they are invaluable for identifying which amino acids could be important in a given interaction, they do not indicate which ones are the most important. This is presumably because the "fit" at that many positions is not "exact" enough to add significant binding energy to the interaction.

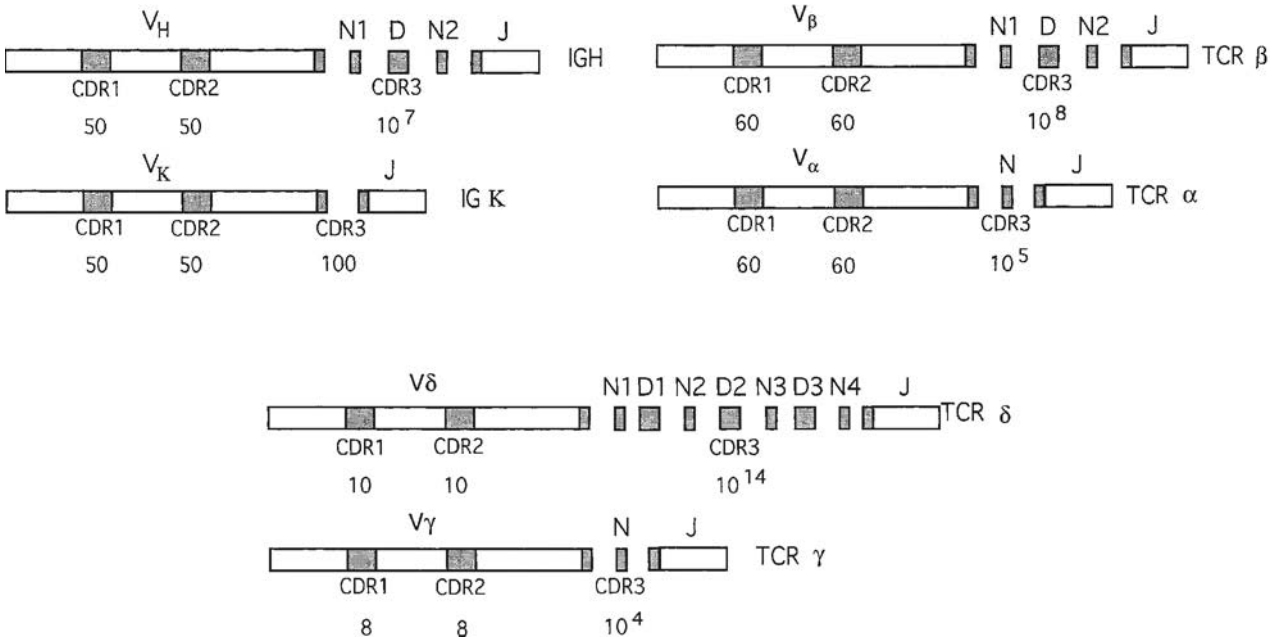

FIG. 11.16. Diversity "Map" of Immunoglobulins and T-Cell Receptors, Calculated Potential for Sequence Diversity in Human Antigen Receptor Molecules.[Adams et al., 153] The N region addition is assumed to contribute zero to six nucleotides to the junction of each gene segment, except for immunoglobulin K chains, in which this form of diversity is seldom used.

In this context, we have proposed a new model[319,320] in which the principle antigen specificity of an Ig or TCR is derived from its most diverse CDR3 loops. In the case of antibodies, we imagine that most of the specific contacts (and hence the free energy) with antigen are made by the V_H CDR3 and that the other CDRs provide "opportunistic" contacts that make generally only minor contributions to the energy of binding and specificity. Once antigen has been encountered and clonal selection activates a particular cell, somatic mutation would then "improve" the binding of the CDR1s and 2s to convert the typically low-affinity antibodies to the higher-affinity models as observed by Berek and Milstein,[135] and also by Patten et al.[136] As a test of this model, Xu et al.[315] analyzed mice that have a severely limited Ig V region repertoire, consisting of one V_H and effectively two V_Ls ($V\lambda_1$ and $V\lambda_2$). These mice are able to respond to a wide variety of protein and haptenic antigens, even with this very limited complement of V regions. In several cases, hybridomas specific for very different antigens (ovalbumin versus 2,4-dinitrophenol [DNP], for example) differ only in the V_H CDR3. A limited V region repertoire also seemed no barrier to deriving high-affinity antibodies with somatic mutation, as repeated immunizations produced IgG monoclonals with very high affinities (10^9 to 10^{-10} M). The major immune deficit in these mice was in their inability to produce antibodies to carbohydrates, which may require a special type of binding site or specific V region. Thus, while these experiments only involved one V_H, the results are highly suggestive about the inherent malleability of V_HV_L in general, at least with respect to protein and haptenic epitopes. With respect to αβ TCRs, we expect that most of the energy of the interaction with a typical ligand will reside in the CDR3-peptide contacts and here again the CDR1 and 2 regions will make less energetically important contacts. For γδ TCRs, it is not yet clear whether the T10/T22 specificity[313] is an isolated case or the general rule. From the hypothesis discussed here, if there are γδ TCRs that use the very large inherent diversity in the Vδ CDR3 directly for antigen recognition, it may be that the lack of somatic mutation forces it to provide more diversity in the initial repertoire (versus Igs).

CONCLUSION

Because TCR genes were first identified in the early 1980s, information about their genetics, biochemistry, structure, and function has accumulated to become almost a field unto itself. Despite this very real progress, many issues still remain unsolved such as: What do γδ T cells normally "see," and what function do they serve? What do superantigens actually do during the course of a normal response and how is this of benefit to the pathogen/parasite? What is the structural/chemical basis of TCR specificity? What sort of rearrangements or conformational charges occur in the TCR/CD3 molecular ensemble upon ligand engagement? These and other questions will require many more years of effort.

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CHAPTER 12

Mechanisms of T-Lymphocyte Signaling and Activation

Takashi Saito

INTRODUCTION

The immune system protects us against attack by pathogens through two separate but interacting systems: innate immunity and acquired (or adaptive) immunity. Whereas innate immunity exhibits rapid and transient responses by recognition of conserved molecular patterns of pathogens, adaptive immunity distinguishes any small difference/heterogeneity of a huge number of pathogens and antigens and mediates a rather slow but long-lasting response. For this purpose, as is discussed elsewhere (see Chapter 6) in this volume, our body prepares a vast variety of receptors on T cells and B cells (immunoglobulins [Igs]) to be able to recognize virtually all potential antigens. Because each lymphocyte possesses single antigen specificity, antigen-specific responses begin with the recognition by and activation of a single cell and consequently the cells proliferate, a process called clonal expansion and selection many years ago by Macfarlane Burnet.[1] To understand the mechanism of antigen-specific activation of lymphocytes, it is necessary to study how a single cell of relevant specificity recognizes the antigen and induces its activation to mediate various functions and protection against pathogens. Recognition of antigen only on the cell surface by the T-cell receptor (TCR) of a T cell is unique and totally different from that of Ig on a B cell. Such differences in T-cell antigen recognition force the establishment of extensive cell-cell interactions/communications in the body to achieve protective immunity and to maintain immune homeostasis. In this chapter, we describe the unique feature of antigen recognition of T cells through their unique antigen receptor complex and the mechanisms that trigger T-cell activation.

T-CELL RECEPTOR COMPLEX

Antigen specific recognition is mediated by the TCR, which is composed of a heterodimer of either αβ or γδ polypeptides. This chapter will focus on signaling mediated through the αβ TCR. As described in Chapter 11, both TCRα and β chains contain variable regions, which together are responsible for binding to the complex of antigen peptide-major histocompatibility complex (MHC). After a decades-long search for the biochemical nature of TCR, the TCR genes were finally cloned and found to be quite similar to those encoding Ig genes.[2–6] TCRα and β chains both possess very short cytoplasmic domains without any particular protein-binding motif, and thus cannot transduce antigen-recognition signals into the cell. It is necessary, therefore, for the TCR to assemble with signal-transducing components, the cluster of differentiation (CD)3 complex, to transduce antigen-recognition signals. The initial finding that CD3 chains are responsible for transducing activation signals into T cells came from the observation that treatment of T cells with a crosslinking anti-CD3ε antibody (OKT3) induces strong T-cell polyclonal activation[7] similar to that induced upon stimulation with mitogens such as concanavalin A. It was also shown that there is a physical but noncovalent association between TCR dimers and the CD3 complex. It is now known that the association of the TCR dimer and the CD3 complex is required first for cell surface expression and then to transduce antigen-recognition signals into T cells.[8,9] The CD3 complex is composed of four different chains. Three of them, γ, δ, and e, are closely related in their protein structure, gene structure, biosynthesis, and chromosome localization. While the γ, δ, and ε are genes are located within a neighboring region of the chromosome (chromosome 11 in human and 9 in mouse), ζ is located on chromosome 1 in both species (Fig. 12.1). The CD3 complex is composed of three distinct dimers, γε, δε, and ζζ, which associate with the TCRαβ dimer. Whereas γε and δε are noncovalently assembled, ζ–ζ is a covalently linked dimmer with a disulfide bond. The ζ chain has an isoform termed η, which is derived from alternative splicing of the ζ transcript and is found only in rodents but not in humans. The cell surface expression of the TCRαβ dimer requires its assembly with the CD3 complex due to a stringent quality control checkpoints in the endoplasmic reticulum (ER) that prevent the release of incompletely assembled TCR-CD3 complexes. Studies of the biosynthesis of these components of the this complex showed that CD3γ, δ, and ε as well as TCRα and β chains are synthesized in great excess and readily degraded in the ER in the absence of assembly with the other chains. The assembly with the partner chain protects the complex from ER degradation. CD3ζ is synthesized at limiting levels, and even the TCRαβ-CD3γδε complex cannot be transferred to the cell surface without assembly with CD3ζζ and is degraded in lysosome.[10] Therefore, the assembly with ζ–ζ defines the fully functional TCR-CD3 complex, and the entire TCRαβ-CD3γδεζζ complex is now allowed to transport to the plasma membrane where it is expressed as the complete and functional TCR-CD3 complex.

THE IMMUNORECEPTOR TYROSINE-BASED ACTIVATION MOTIF AND INITIAL SIGNALING

The cytoplasmic tails of all CD3 chains contain a common signaling motif, immunoreceptor tyrosine-based activation motif (ITAM), which has the consensus sequence YxxL/I x6-8 YxxL; composed of two repeats of YxxL/I motif (Y; tyrosine, L/I; leucine/isoleucine) with a spacer of six to eight

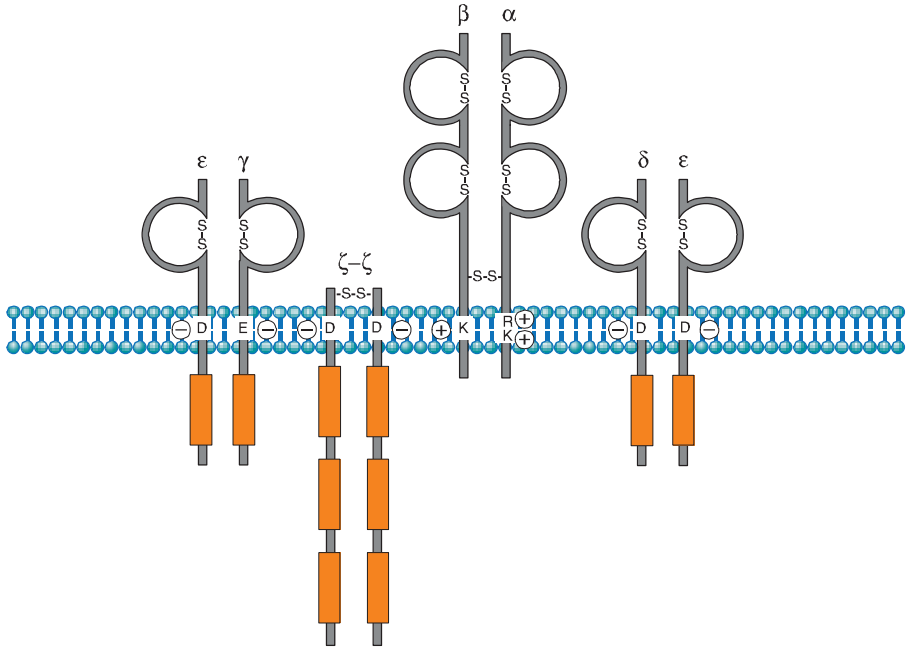


FIG. 12.1. Structure of the T-Cell Receptor (TCR)-Cluster of Differentiation (CD)3 Complex. The TCRαβ dimer is associated with three dimers of CD3 chains: CD3εγ, CD3εδ, and CD3ζζ. Whereas TCRα and β possess positively charged amino acids within the transmembrane region, all CD3 chains contain negatively charged amino acids, which are necessary to be assembled as the complex. CD3 chains possess immunoreceptor tyrosine-based activation motif (ITAM) within their cytoplasmic tails as shown in *red boxes* (γ, δ, and ε have one; ζ has three).

amino acids. CD3δ, γ, and ε have a single and CD3ζ has three tandem ITAMs within the intracellular region. The ITAM was first identified as a signature sequence present in several cell surface receptors on immune cells.[11] ITAM-containing molecules have since been found to be widely distributed among various important immune receptors, not only TCR (CD3s) but also in B-cell antigen receptors (Igα and Igβ), various types of Fc receptors (FcRγ), adaptor proteins associated with MHC-recognizing receptors, or paired receptors on natural killer (NK) cells (DAP12 and DAP10) (Fig. 12.2) and further in various pattern-recognizing receptors of innate cells such as macrophages and dendritic cells (DCs).[12] The ITAM turns out to be specific motif capable of transducing receptor–mediated recognition signals into cellular activation.[13] The function of the ITAM in transducing T-cell activation signals has been shown first by analyzing the capacity of a chimeric protein composed of the extracellular domain of CD8 and the intracellular domain of CD3ζ to

		Amino acid sequence
TCR	CD3γ	EQLYQPLKD–REYDQYSHL
	CD3δ	EQLYQPLRD–REDTQYSRL
	CD3ε	NPDYEPIRK–GQRDLYSGL
	CD3ζa	NQLYNELNL–GRREEYDVL
	CD3ζb	EGVYNALQKDKMAEAYSEI
	CD3ζc	DGLYQGLST–ATKDTYDAL
BCR	Igα	ENLYEGLNL–DDCSMYEDI
	Igβ	DHTYEGLNI–DQTATYEDI
FcR	FcRγ	DAVYTGLNT–RSQETYETL
	FcεRIβ	DRLYEELNH–VYSPIYSEL
NKR	DAP12	ESPYQELQG–QRPEVYSDL
Virus	BLV gp30	DSDYQALLP–SAPEIYSHL
	EBV LMP2A	HSDYQPLGT–QDQSLYLGL
	consensus	Dx x Yx x Lx x –x x x x x Yx x L E I I

FIG. 12.2. Structure and Signaling Function of Immunoreceptor Tyrosine-based Activation Motif (ITAM). Cluster of differentiation 3s (T cells), Ig-α/β (B cells), FcR (myeloid cells; macrophages, dendritic cells, neutrophils, etc.), NK cells, as well as some viruses possess ITAM within the cytoplasmic domain. There are increasing numbers of ITAM-bearing or ITAM$^+$ adaptor–associated receptors in innate systems. The consensus sequence of the ITAM is composed of YxxL/I- (seven or eight spacers)- YxxL/I where x is any amino acid. The tyrosine residues within ITAM are phosphorylated by src family kinase Lck upon TCR engagement, followed by the binding of the tandem two SH2 domains of ZAP-70 to both phosphorylated tyrosines in T cells.

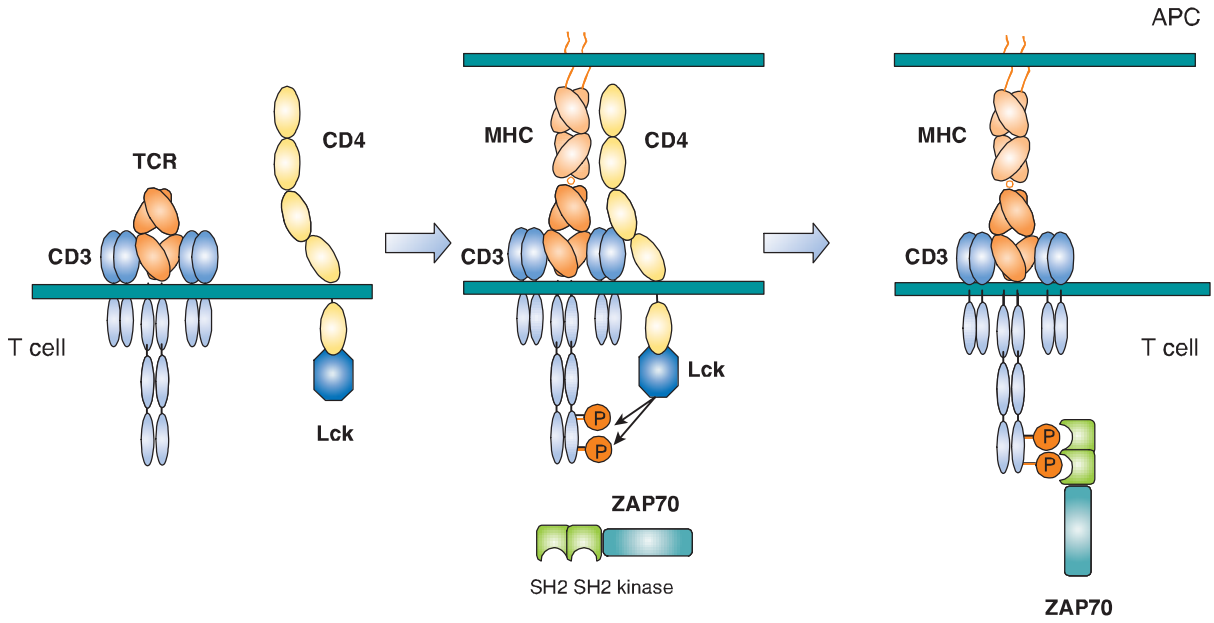


FIG. 12.3. Initial Activation of T Cells by Sequential Two Tyrosine Kinases. Left: The T-cell receptor (TCR)-cluster of differentiation (CD)3 complex and the complex of CD4 and the Src kinase Lck are located separately in resting T cells. **Middle:** Upon antigen peptide-major histocompatibility complex (MHC) recognition by TCR, the TCR complex and the CD4-Lck complex get near through the CD4-MHC binding, which induces Lck close to the CD3 chains. The immunoreceptor tyrosine-based activation motif (ITAMs) of CD3 chains are tyrosine-phosphorylated by Lck. **Right:** The phosphorylated ITAM of the CD3 chains became the binding sites for the tandem two Src-homology 2 domains of the second tyrosine kinase ZAP-70. ZAP-70 is phosphorylated by Lck and by itself and activated, and then transduces activation signals by phosphorylating downstream signaling molecules.

induce T-cell activation.[14] Antibody crosslinking of the CD8 domain showed that the cytoplasmic tail of CD3ζ can induce almost all the events observed during normal T-cell activation, including tyrosine phosphorylation of various proteins, induction of intracellular calcium mobilization and inositol phosphate metabolism, induction of activation markers, cytokine production, and cell proliferation. Mutational analyses of the ITAM sequences within the CD3ζ cytoplasmic region revealed that both tyrosine and leucine (isoleucine) residues are critical for transducing a T-cell activation signal.[15–16]

Biochemical analyses to define the downstream signaling molecules recruited upon TCR stimulation resulted in the discovery of several important proteins that are involved in tyrosine phosphorylation and activation of several adaptor molecules including a Src family tyrosine kinase, Lck, and a Syk family kinase, Zeta-associated protein-70 (ZAP-70).[17] The ITAM is phosphorylated by Src kinase Lck upon TCR engagement by antigen. Once tyrosines are phosphorylated, they become susceptible to binding in general by proteins possessing Src-homology 2 (SH2) domains. The phosphorylated ITAMs recruit the second tyrosine kinase ZAP-70, one of Syk family kinases. ZAP-70 is contains two tandem SH2 domains in its N terminus and a tyrosine kinase region in the C-terminus.[18,19] The tandem SH2 domains with exactly the right spacing can bind the two phosphorylated tyrosines within an ITAM[20] (Fig. 12.3). Because TCR and CD3 chains do not have any intrinsic effector function, unlike tyrosine kinase-containing receptors such as the epidermal growth factor receptor or the insulin receptor, the binding of ZAP-70 to the phosphorylated CD3ζ ITAM changes the TCR-CD3 complex, making it competent for signal transduction. ZAP-70 assembled with the CD3 phospho-ITAMs must then be enzymatically activated by phosphorylation of otherwise inhibitory tyrosines in its juxtamembrane region by Lck as well as by itself. The binding and activation of ZAP-70 may be associated with the structural changes of CD3 chains induced by phosphorylation, a topic that will be described under the section Triggering Mechanism. Activated ZAP-70 induces phosphorylation of tyrosine residues of various downstream target molecules.[21] These include adaptor molecules LAT and SLP-76. These phosphorylation events lead to further activation of several adaptor and effector molecules and subcellular assembly as multimolecular signaling complex.

REGULATION OF PROXIMAL SIGNALING

Regulation of Tyrosine Kinases

Src family tyrosine kinases (Lck, Fyn in T cells) are composed of an N-terminus unique region, SH2, SH3, and a kinase domain at the C-terminus.[22] The N-terminus unique region contains palmitoylation sites by which Lck is localized in lipid rafts on the inner leaflet of the plasma membrane. Because Lck specifically associates with the cytoplasmic regions of CD4 and CD8 coreceptors, Lck accumulates at the site of TCR engagement because CD4 or CD8 are also present within the engaged site through binding to MHC.[23,24] As a result, Lck is recruited to the vicinity of the CD3 chains where it is able to phosphorylate them.

The activity of Lck is regulated allosterically by phosphorylation of a tyrosine in the carboxyl terminus by the

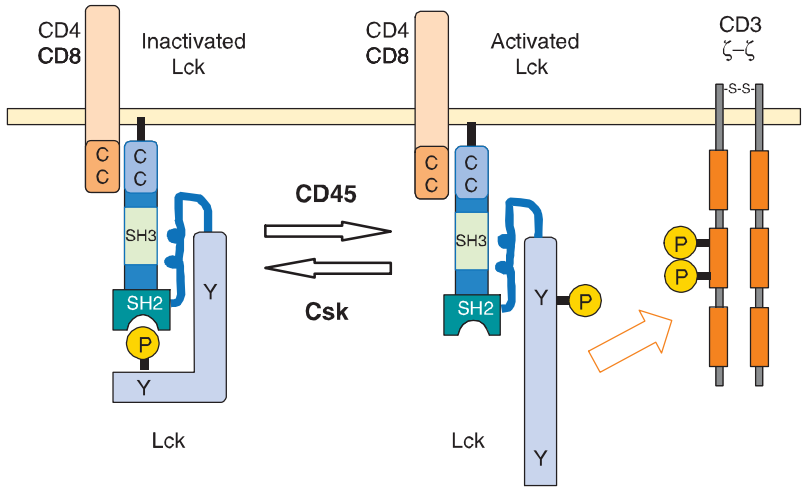


FIG. 12.4. Regulation of the Src Kinase Lck Activation by the Tyrosine Kinase Csk and the Phosphatase Cluster of Differentiation (CD)45. In resting T cells, Lck has a closed configuration through the intra-association between the phosphorylated tyrosine at the C terminus and the Src-homology 2 domain. C-terminus src kinase (Csk) phosphorylates this regulatory tyrosine residue. The association between CD4 and Lck is mediated by the interaction of the cysteine-containing regions of both molecules. CD45 dephosphorylates the C-terminus tyrosine, which alters the configuration from the closed structure as the "inactivated Lck" to an open shape as the "activated Lck" with active kinase function.

C-terminal kinase (Csk).[25] This phosphotyrosine interacts with the SH2 domain of Lck, and this induces a conformational change to maintain Lck in a catalytically inactive status. Csk normally and continuously acts to reduce Lck activity and thereby attenuate TCR signaling. Indeed, in the absence of Csk, peripheral T cells become autonomously activated. Dephosphorylation of the C-terminus tyrosine releases Lck from its inactive status. In addition, in order to fully activate Lck, the tyrosine phosphorylation in the catalytic domain is also required. Because the tyrosine phosphatase CD45 induces the dephosphorylation of both of these regulatory tyrosines, CD45 functions as both a positive and negative regulator of T-cell activation[26] through the C-terminal and kinase-domain tyrosines, respectively. The balance between Csk and CD45 regulates the status of T-cell activation[27] (Fig. 12.4). A considerable fraction of Lck in naïve T cells is catalytically active because of this dynamic balance. Such active Lck may be responsible for constitutive activation signals including phosphorylation of CD3ζ and help in initiating T-cell activation.[28] A related src-family kinase Fyn is also expressed in T cells and is weakly associated with the TCR complex. Fyn appears to have some signaling role because T cells deficient in both Lck and Fyn have a more complete block in T-cell development than Lck-deficient mice.

Adaptor-Mediated Signaling

Among the targets of ZAP-70–mediated phosphorylation, a transmembrane adaptor protein, linker for the activation of T cells (LAT),[29] and an intracellular adaptor protein, SH2 domain–containing leukocyte phophoprotein of 76 kDa (SLP-76),[30] are the most important substrates (Fig. 12.5). The discovery of these two adaptor proteins revealed the connection between the ZAP-70 tyrosine kinase and PLCγ activation. These adaptor proteins create an important signal assembly to induce downstream activation signals. The critical roles of these two adaptors are evidenced by the observation that T cells deficient in either LAT or SLP-76 exhibit complete defects in TCR activation signaling.

LAT is a transmembrane protein with a short extracellular region and resides in lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids, via two palmitoylation sites within the juxtamembrane region. Mutation of these palmitoylation sites results in the failure of LAT to be expressed on the plasma membrane or to transduce T-cell activation, indicating that the association within lipid rafts regulates trafficking to the membrane. Upon TCR stimulation, LAT becomes tyrosine-phosphorylated on multiple tyrosine residues (five tyrosines among nine conserved residues), and these serve as the docking sites for several SH2-containing molecules. Important effector molecules containing SH2 domains that bind to phosphorylated LAT include PLCγ, growth factor receptor binding protein 2 family members, Grb2 and Gads, and a Tec family kinase, inducible T-cell kinase Itk.[31] Because Grb2 constitutively binds to son of sevenless (Sos), which is a guanine exchange factor (GEF) that mediates guanine triphosphate (GTP) binding of Ras, this LAT-Grb2-Sos complex induces Ras activation; however, RasGRP plays a more critical role for Ras activation than the Grb2-Sos pathway in T cells. Gads binds to phosphor-LAT upon T-cell activation, and Gads associates with SLP-76. Although all tyrosine mutations of LAT lost the function for T-cell activation, the LAT136 mutant mouse exhibits a lymphoproliferative disorder and induction of strong Th2-type cytokine production, suggesting the possibility of a LAT-independent pathway to activate T cells.[32,33]

SLP-76 is a cytosolic adaptor with three domains: an amino terminal region containing three major tyrosines that are phosphorylated upon TCR ligation and become binding sites for various SH2-containing proteins, a central proline-rich domain that binds to SH3-containing proteins, and a carboxy-terminal SH2 domain by which SLP-76 can bind to phosphorylated tyrosines of other proteins.[34] SLP-76 constitutively associates with Gads through its SH3 region, and the SLP-76/Gads complex is recruited to the phosphorylated LAT upon TCR engagement (Fig. 12.6). Both LAT and SLP-76/Gads bind to PLCγ1 independently, and formation of this tetramolecular assembly stabilizes the complex. Because LAT also binds to the Itk Tec family kinase,

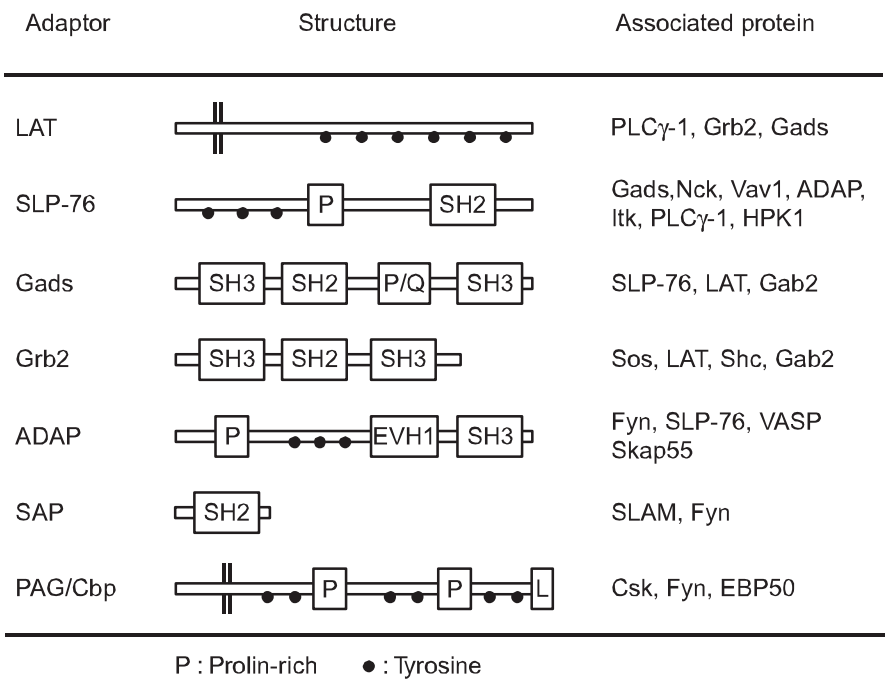


FIG. 12.5. Adapter Molecules Functioning Downstream of T-Cell Receptor Signals. The structure of several adapters described in the text are depicted. These adapter molecules are composed of several modules critical for signal transduction such as Src-homology 2 (SH2) and Src-homology 3 (SH3). Whereas tyrosine residues (*black dot*) functions as the docking sites for SH2 upon phosphorylation, the prolin-rich regions (*P*) interact with SH3. Some of these adaptors with the transmembrane regions are localized on the plasma membrane as "membrane adaptors" such as LAT and PAG/Cbp, whereas others within the cytosol as "intracellular adaptors." ADAP, adhesion- and degranulation-promoting adapter protein; Gads, Grb2-related adapter downstream of Shc; LAT, linker for activated T cells; PAG/Cbp, phosphoprotein associated with glycosphingolipid-enriched domains/csk-binding protein; SAP, signal lymphocyte activation molecule–associated protein; SLP-76, SH2 domain–containing leukocyte-specific phosphoprotein of 76 kDa.

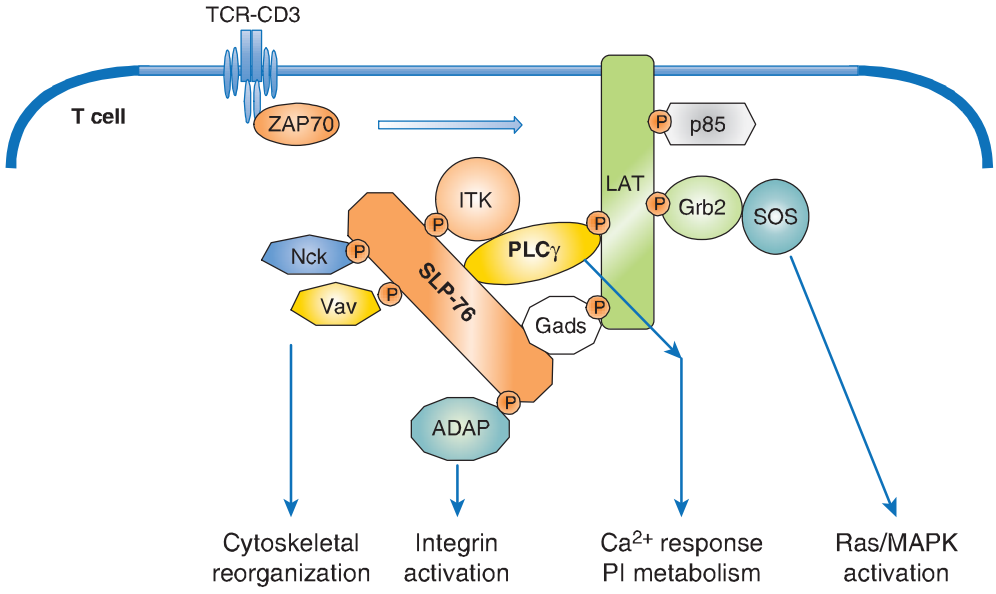


FIG. 12.6. Formation of Multimolecular Signaling Complex Critical for Initial T-Cell Receptor (TCR) Activation Signaling Pathways. Sequential activation of two tyrosine kinases Lck and ZAP-70 upon antigen recognition by TCR induces phosphorylation of two critical adapter proteins LAT and SLP76. Gads constitutively associates with SLP76 and is phosphorylated by TCR activation and recruited to LAT as the Gads-SLP76 complex. Thereafter, several adapter proteins form a physical complex centralized by LAT and SLP-76. Further recruitment of various adapters and effector molecules to the complex leads to the activation of differential signaling pathways. Phospholipase γ1 (PLC-γ1) and Gads link between LAT and SLP76 and a Tec-kinase family ITK, which binds to SLP76, is responsible to phosphorylate/activate PLCγ1. Association of these molecules generates a stable complex of LAT-Gads-SLP76-PLCγ1-ITK critical for further calcium and phosphatidylinositol (PI) responses (as in Fig. 12.7). SLP76 also binds Nck, Vav1, and ADAP, which links to cytoskeletal reorganization (as in Fig. 12.11). LAT binds Grb2/Sos, which induces Ras/MAPK activation (as in Fig. 12.8).

which is responsible for phosphorylating PLCγ1, the stable complex of Itk-LAT-SLP-76-PLCγ1 induces PLCγ1 activation. Besides PLCγ1 activation, SLP-76 induces other functions through its association with several other important molecules. At its N-terminus, SLP-76 binds to Vav, a GEF, and Nck, an adaptor protein, both of which are critical for TCR-mediated cytoskeletal changes, and at its C-terminus it binds adhesion- and degranulation-promoting adapter protein (ADAP),[35,36] an adapter protein coordinating TCR signals with integrin activation (see Fig. 12.12).

DOWNSTREAM SIGNALING PATHWAYS

The next step of the T-cell activation pathway after ZAP-70–induced phosphorylation of the adaptor LAT and SLP-76 upon TCR ligation is the activation of the key signaling enzyme PLCγ1. PLCγ1 is recruited to the plasma membrane through the binding of its PH domain to PIP3, which is generated within the membrane by the phosphorylation of PIP2 by PI3-kinase. PLCγ1 is activated upon phosphorylation by Itk,[37,38] which is composed of PH, SH2, SH3, and kinase domains, and is also recruited to the plasma membrane through its PH domain by interacting with PIP3. Phosphorylated and activated PLCγ1 then cleaves the membrane bound PIP2 to generate two critical products: the membrane-bound lipid diacylglycerol (DAG) and the diffusible inositol 1,4,5-triphosphate (IP3), both of which function as second messengers for further inducing downstream signaling.

Calcium–Nuclear Factor of Activated T Cells

Upon TCR stimulation, the levels of intracellular free calcium are regulated in two phases: there is an initial and transient induction of calcium release from storage in the ER, and this is followed by the induction of a strong influx of high levels of calcium from outside of the cells by opening a calcium channel called the calcium release–activated calcium channel (CRAC) in the plasma membrane. IP3 generated by PLCγ1 catalysis diffuses into the cytoplasm and binds to IP3 receptors on the ER membrane. IP3 receptors are calcium channels and, after IP3 binding, they open and allow the release of the calcium stored within ER into the cytosol. The released low level of calcium induces cluster formation by the calcium-binding transmembrane protein, stromal interaction molecule-1 (STIM1), within the ER.[39] STIM1 is an ER-residential protein that contains an N-terminal sterile motif and paired EF hands that function as a calcium-binding motif and a coiled-coil motif at the C terminus. A critical CRAC called Orai1 has been identified from the analysis of a patient with severe combined immunodeficiency who had a major defect in lymphocyte activation.[40] Clustered STIM1 on the ER membrane is colocalized and assembled with Orai1 on the nearby plasma membrane to open the channel and introduce extracellular calcium into the cytosol (Fig. 12.7). Recent analysis has shown that oligomerization of STIM1 is sufficient to induce CRAC activation independent of ER calcium store depletion, but the mechanism by which STIM1 induces Orai1 oligomerization remains unknown.

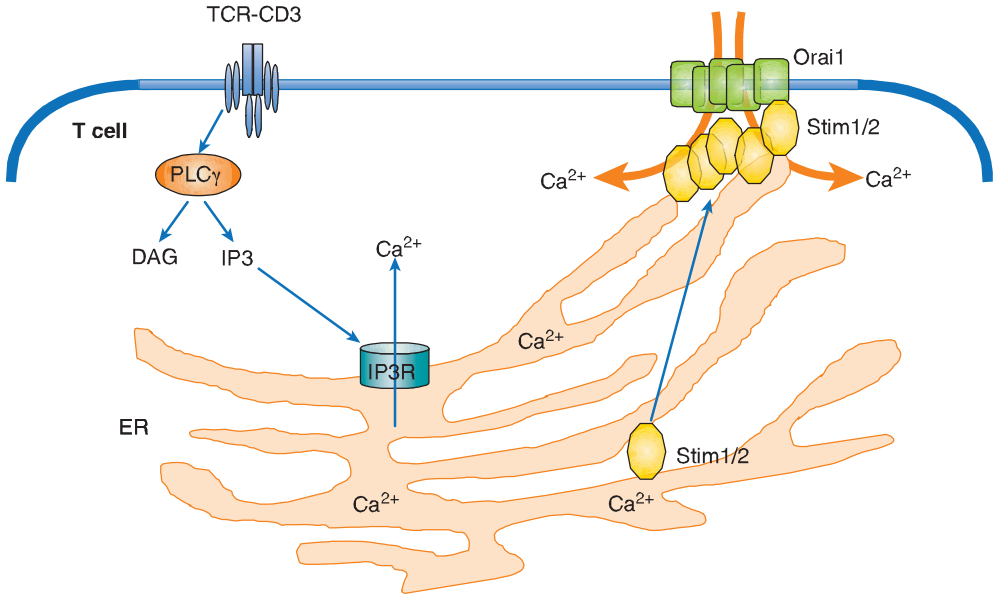


FIG. 12.7. The Mechanism of Calcium Response in T-Cell Activation. T-cell receptor engagement induces activation of PLCγ1 and generates inositol trisphosphate (IP_3), which binds to the IP_3 receptors (IP3R) present on the endoplasmic reticulum (ER) and stimulates the calcium release from intracellular calcium pool within the ER. Increase of intracellular calcium level activates a calcium-binding paired EF hands-bearing protein Stim1/2, which then translocates to the vicinity of the plasma membrane. Stim1 localizes with and induces dimerization of ORAI calcium release–activated calcium modulator 1 (Orai1), resulting in opening functional calcium release–activated calcium channel. The assembly between Orai1 and Stim1/2 induces the activation pathway dependent on intracellular calcium concentration.

Surprisingly, STIM1, STIM2 (another family member), and Orai1 are dispensable for T-cell development as deficiency of these molecules does not affect this process. Because calcium flux is also induced and is important in thymocytes, it is assumed that there must be other functionally redundant and critical calcium channels in thymocytes.[41]

Upon T-cell engagement, intracellular calcium binds to a protein, calmodulin, and induces a conformational change that allows the protein to bind to various target proteins. An increase of cytosolic calcium induces activation of signaling molecules including calcium calmodulin-dependent kinase and a phosphatase calcineurin. Activated calcineurin acts on a critical transcription factor, nuclear factor of activated T cells (NFAT), to dephosphorylate the protein, which induces its translocation into the nucleus.[42] There are five NFAT family members (NFATc1, c2, c3, c4, and NFAT5), and they are expressed in many different tissues.[43] NFAT is present in the cytoplasm in the resting state in T cells. Cytosolic serine/threonine kinases such as glycogen synthase kinase 3 and casein kinase 2 phosphorylate the nuclear localization signal on NFAT to prevent its translocation into nucleus. Upon antigen stimulation, activated calcineurin dephosphorylates this critical phosphorylation site, which allows NFAT to enter into the nucleus, where it triggers various gene expression programs including various cytokine genes by cooperating with other transcription factors. The most well-characterized gene among NFAT targets is the interleukin (IL)-2 gene. NFAT binds to the IL-2 promoter by forming a cooperative complex with activator protein (AP)-1, which is a heterodimer composed of members of the Jun and Fos family, to induce IL-2 transcription. NFAT and AP-1 represent two signaling pathways of calcium and Ras/MAPK, respectively. It has been shown that T-cell activation in the absence of the induction of AP-1 results in anergic or unresponsive T cells.[44]

Ras–Mitogen-Activated Protein Kinase

In parallel with IP3, DAG is generated by PLCγ and remains localized within the plasma membrane where it recruits various signaling molecules that contain a specific DAG-binding motif (called the C1 domain), including members of the protein kinase C (PKC) family[45] and RasGRP,[46] a guanine-nucleotide exchange factor for Ras. DAG binding activates PKC and promotes RasGRP to activate Ras by exchange from the guanosine diphosphate (GDP) to GTP-binding form.[47] Ras is active in the GTP-bound form, and this activation step is mediated by GEFs and suppressed by GTPase-activating proteins (GAPs). RasGRP and Sos are two major GEFs in the TCR activation pathway (Fig. 12.8). In T cells, RasGRP functions dominantly for early activation of Ras. RasGRP is inducibly associated with the plasma membrane through binding of its C1 domain to DAG and is phosphorylated by PKCθ. On the other hand, Sos constitutively associates with an adaptor protein Grb2 and the Grb2 SH2 domain binds to phosphorylated LAT upon T-cell activation; Sos-Grb2 binds to LAT-SLP76, which results in its recruitment to

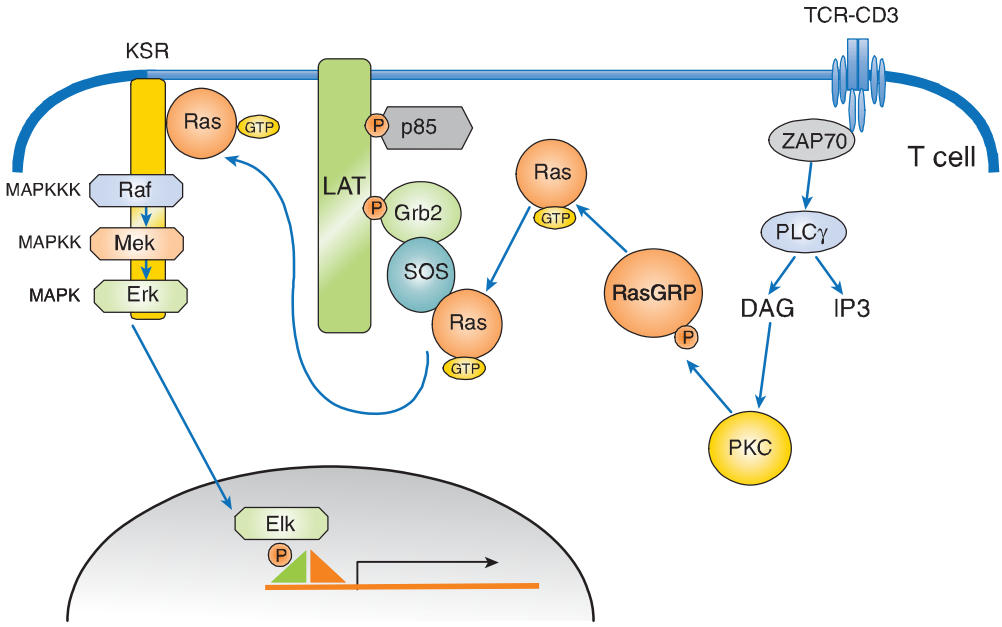


FIG. 12.8. Ras Activation Pathways by T-Cell Receptor (TCR) Stimulation. Activation of Ras in T cells following antigen engagement involves activation of two different guanine triphosphate (GTP) exchanging factors: one is Ras-GRP, and the other is son of sevenless (Sos). TCR activation induces diacylglycerol generation by PLCγ1, which recruits Ras-GRP to the membrane where Ras-GRP is activated by PKCθ. Activated Ras-GRP induces Ras activation by exchanging guanosine diphosphate (GDP) to GTP, which then binds to Sos, because Sos constitutively assembles with Grb2 and the Grb2-SOS complex is recruited to LAT upon TCR activation. Activated Ras then induces the activation cascade of mitogen-activated protein kinase which sequentially contains MAPK kinase kinase (Raf), MAPK kinase (Mek), and MAPK (Erk).

the vicinity of the TCR. The relationship between RasGRP and Sos appears to be cooperative rather than competitive as RasGRP-mediated Ras activation enhances Sos activity as a positive feedback to induce strong Ras activation.[48]

Ras activation triggers a cascade of kinase activation, which finishes by activating a serine/threonine kinase known as a mitogen-activated protein kinase (MAPK). The MAPK cascade is composed of three kinases: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and the MAPK itself. In the case of the TCR signaling pathway, the first MAP3K is Raf, which is a serine/threonine kinase that phosphorylates the next MAP2K, which in T cells is MEK1. MEK1 is called a dual-specificity kinase because it can phosphorylate both a tyrosine and a threonine of the last member of the cascade, the MAPK extracellular signal-regulated kinase 1 (Erk1) and Erk2 in T cells and B cells, respectively. Erk1/2 induces the activation of Elk1, which in turn activates the AP-1 transcription factor complex composed of Fos and Jun.

PKC-CARMA1/Bcl-10/Malt1-Nuclear Factor of KappaB

Historically, it has long been known that phorbol ester (phorbol 12-myristate 13-acetate) plus a calcium ionophore, a molecule that allows calcium ions to cross cell membranes, induce strong activation signals similar to TCR-induced signals.[49] Phorbol ester is known to activate PKC; thus, PKC is a critical component in T-cell activation. T cells predominantly express the PKCθ isoform of protein kinase C. PKCθ has a DAG-binding domain, is recruited to the plasma membrane through binding to DAG, and is activated upon generation of DAG by PLCγ1. One of the main pathways activated by PKCθ is NF-κB. PKCθ plays a critical role in initiating several cascades of the classical pathway of NF-κB activation downstream of the TCR. NF-κB is a family of transcription factors composed of homo- and heterodimers of five members of the Rel family including NF-κB1(p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (Rel).[50] In resting T cells, NF-κB is found in the cytosol associated with one of a family of inhibitors of NF-κB (IκB) that prevents NF-κB from translocating into the nucleus. Upon T-cell activation, IκB is phosphorylated by the IκB kinase (IKK) complex, ubiquitinylated and degraded, which allows NF-κB to translocate into nucleus, where it activates various genes involved in survival, homeostasis, and activation of T cells and inflammatory responses.

Whereas this signaling pathway to activate NF-κB is common among many cell types and has been known for quite some time, the specific pathway by which PKCθ induces NF-κB activation has only been elucidated in the past decade. The critical molecule responsible for mediating the activation signal that connects PKCθ and NF-κB is now known as the CBM complex (Fig. 12.9). It consists of three proteins; a scaffold protein, CARMA1 (caspase recruitment domain [CARD] and membrane-associated guanylate kinase–containing scaffold protein), a CARD-containing

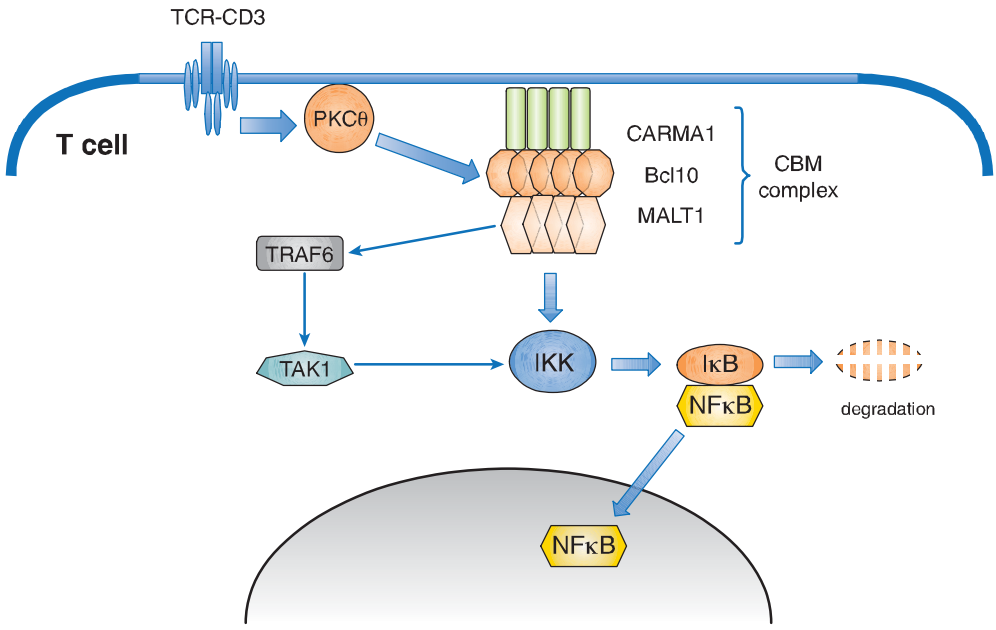


FIG. 12.9. Signaling Pathway for T-Cell Receptor (TCR)-Mediated NF-κB Activation. Upon TCR stimulation, PKCθ is activated and induces phosphorylation and aggregation of caspase-recruitment domain-containing membrane-associated guanylate kinase protein-1 (CARMA1). Aggregated CARMA1 forms a complex (as the CBM [CARMA1-Bcl-10-Malt1] complex) with two other proteins: B cell lymphoma 10 (Bcl-10) and mucosal-associated lymphoid-tissue lymphoma-translocation gene 1 (Malt1). Malt1 induces the degradation of the regulatory component of IκB kinase (IKK) complex (IKKγ) by ubiquitination via activation of tumor necrosis factor receptor–associated factor-6. IKKγ degradation induces phosphorylation and subsequently degradation of the inhibitor of NF-κB (IκB), which results in activation and nuclear translocation of NF-κB.

adaptor protein, B-cell lymphoma 10 (Bcl10), and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (Malt1).[51–53] Upon TCR stimulation, this trimolecular complex is induced by PKCθ-mediated phosphorylation of CARMA1, which is required for CARMA1 oligomerization and association with Bcl10. Malt1 binds to Bcl10 and mediates the degradation of the IKKγ subunit (also called NEMO) by inducing polyubiquitination through activation of an E3 ubiquitin-ligase, tumor necrosis factor receptor–associated factor 6 (TRAF6).[55,56] TAK1 recruited by TRAF6 phosphorylates IKK, which in turn induces IκB phosphorylation. This event, together with degradation of NEMO, induces consequently IκB degradation and release and translocation of NF-κB to the nucleus, and thereby induces gene activation.

Overexpression of the CBM complex induces spontaneous NF-κB activation without T-cell (or B-cell) activation. Recently, it has been shown that a high proportion of diffuse large B-cell lymphomas are induced by specific oncogenic mutations of CARMA1, which induce its spontaneous oligomerization of CARMA1, formation of the CBM complex, and subsequent continuous chronic activation of NF-κB, ultimately resulting in tumor induction.[57] This illustrates the importance of maintaining appropriate control of the CBM complex–mediated NF-κB activation for appropriate immune responses without inducing excess inflammation and diseases.

THE IMMUNOLOGIC SYNAPSE

T-cell activation is induced upon recognition of antigen peptide/MHC on antigen-presenting cells (APCs) such as DCs or B cells by the TCR on T cells upon the antigen-specific interaction between these two cell types. The interaction induces a specific structure with molecular segregation at the interface between the two cells, which is called the immunologic synapse (IS) or supramolecular activation cluster (SMAC).[58–59] In the IS, several surface receptors and intracellular signaling molecules are accumulated and segregated to form the concentric "bulls-eye"–like structure composed of three discrete regions (Fig. 12.10); there is enriched accumulation of molecules such as TCR, CD28, CD2, and PKCθ in the central region (cSMAC), adhesion molecules such as integrin LFA1 in the peripheral region (pSMAC), and large molecules such as CD43 and CD44 in the distal region (d-SMAC). The IS, particularly the cSMAC, was initially thought to be the structure responsible for transducing signals that lead to

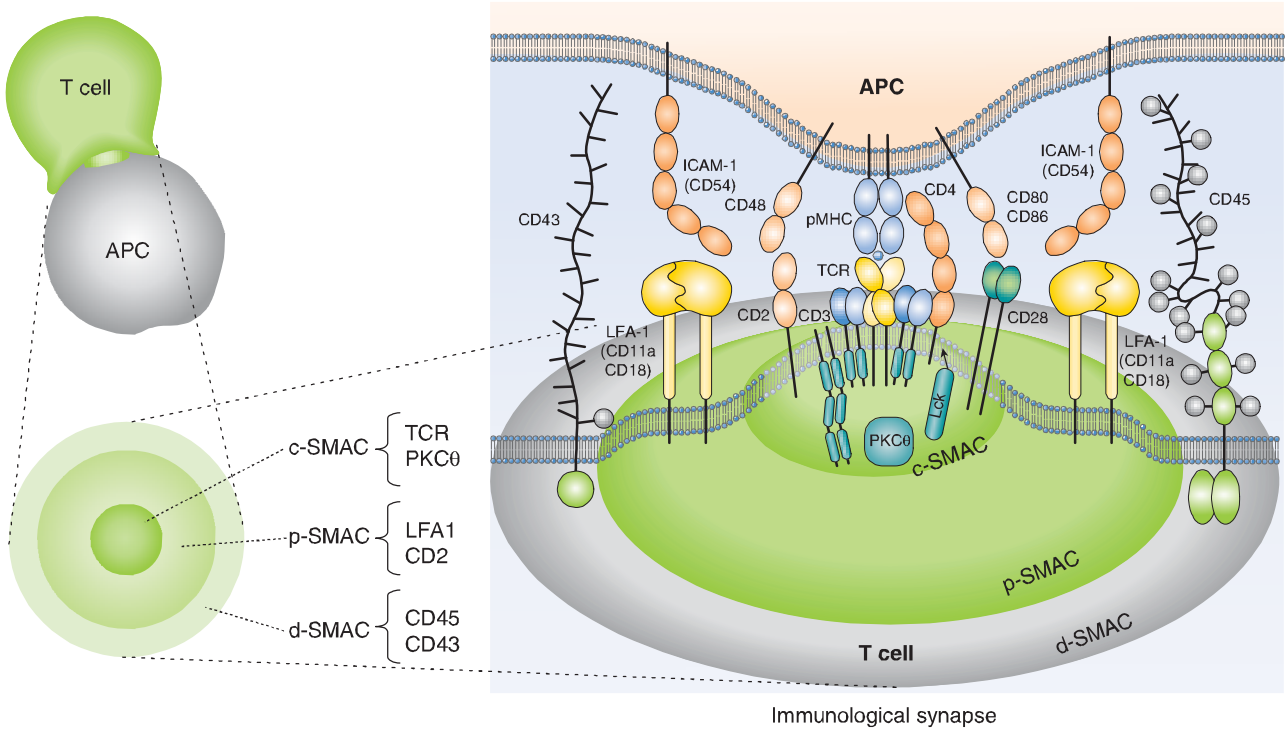


FIG. 12.10. Schematic Structure of the Immunologic Synapse. Antigen recognition and activation of T cells starts by the specific interaction between T cells and antigen-presenting cells (APCs) (**left**). In the interface of two cells, special structure with segregated arrangement of receptors and signaling molecules is induced where T-cell receptros (TCRs) and major histocompatibility complexes (MHCs) are accumulated in the central region termed as the central supramolecular activation cluster (SMAC), the adhesion molecule leukocyte function-associated antigen (LFA)-1–intercellular adhesion molecule 1 is localized at the surrounding area around the TCR-MHC (as the peripheral SMAC), and further large molecules such as cluster of differentiation (CD)45 are located outside peripheral region SMAC as distal SMAC (**right**). This "bulls eye–like" structure is dynamically induced after T cell-APC contact; LFA-1 moves outside and the TCR-CD3 moves into the center within 10 minutes. Activation signals are induced not at the synapse but at TCR microclusters, which are generated at the interface between T cell and APCs prior to central SMAC formation, which is composed of TCR, kinases, and adaptors.

T-cell activation. However, the formation of IS takes as long as 10 minutes after cellular conjugate formation, whereas the initial activation events such as intracellular calcium mobilization and protein phosphorylation occur within 1 minute, indicating that the activation signal is initiated much earlier than the IS is formed.[60] Recent studies revealed that the real signal transducing structure, called the TCR microcluster, is generated immediately after the T cell–APC contact and prior to IS formation.[61–63] TCR microclusters contain the TCR, kinases such as Lck and ZAP70, adaptor proteins such as LAT and SLP76, and several effector molecules such as PLCγ and PI3K. TCR microclusters function as the signalsome to induce T-cell activation because all TCR microclusters induce tyrosine phosphorylation of various proteins including CD3ζ, ZAP-70, and PLCγ, and their generation occurs in parallel to the initiation of the intracellular calcium flux. Therefore, the TCR microcluster is thought to be the minimal functional unit for TCR signaling. TCR microclusters are generated initially in the periphery of the interface between T cells and APCs, and are then translocated into the central region of the interface to make the cSMAC.[64]

Contrary to the initial idea that the cSMAC is the site for signal transduction, it is now thought that the cSMAC is the place where the accumulated TCR complex is internalized and degraded. In contrast to the negative function of the cSMAC for TCR degradation, costimulation receptors such as CD28 and cytotoxic T-lymphocyte antigen (CTLA)-4 accumulate in the cSMAC, suggesting that the cSMAC also serves a signaling function for costimulation.[65] In addition, T-cell activation results in the polarization of the T cells and movement of the microtubule organizing center toward the TCR-MHC/peptide contact sites. The microtubule organizing center movement appears to be driven by localized accumulation of DAG[66] and also involves ADAP, both of which recruit the microtubule motor protein dynein. cSMAC formation is closely regulated by cytoskeletal arrangement and leads to the polarization of T cells for polarized secretion of secretory vesicles including cytokines, chemokines, and lytic factors such as granzyme B. Various cytokine-containing vesicles are accumulated at the TCR engaged site and are released directionally and locally. During the T cell–APC interaction, whereas some of cytokines such as interferon γ, IL-2, and IL-4 are secreted directionally toward the interface, some as tumor necrosis factor (TNF)α are randomly secreted.[67]

TRIGGERING MECHANISM

Signaling mechanisms for T-cell activation have been extensively analyzed and are now well understood, as described previously. It has been known for a long time that the antigen recognition event by the TCRαβ dimer is transmitted to the ITAM phosphorylation of the CD3 chains, which induce signal transduction for T-cell activation. However, the actual mechanism by which antigen recognition by the TCR initiates the initial activation event of the signal transduction cascade is still widely debated and controversial. There are several different models to explain the first events of T-cell triggering, and the proposed models can be divided into two main conceptual categories. One is through the receptor clustering by ligand binding, and the other is through the induction of conformational changes within the TCR-CD3 complex. None of these models or their variations (see the following discussion) can satisfactorily account for the diverse experimental observations regarding TCR triggering.

1. Kinetic proofreading model. This is an early quantitative model that attempted to explain a T-cell response by the half-life of the TCR-MHC/peptide interaction.[68] The length of time between the initial ligand binding and receptor signaling was proposed to induce qualitatively differential signaling. Longer and shorter half-life would induce a stronger and weaker response by either strong or weak agonist, respectively.[69]
2. Serial triggering model. T-cell activation requires a sustained signal that lasts for several hours. However, TCR affinity for MHC/peptide is very low, and activation of TCR induces only a brief spike of intracellular signals. This model attempts to resolve this paradoxical requirement for T-cell activation. The model states that sustained signaling is accomplished by the concerted action of multiple TCRs that are sequentially engaged with and triggered by the MHC/peptide complex.[70] A single complex could serially engage and trigger up to approximately 200 TCRs.
3. Kinetic segregation model. Signal initiation takes place by excluding inhibitory molecules such as the phosphatase CD45 from the tight contact area (cSMAC of the IS) between T cell and the APC, thereby shifting the enzymatic steady state toward an activating status. Such segregation is induced by sizes of the ectodomains of the excluded proteins.[71] This model is supported by the structure and kinetics of IS, as shown by the segregated regions.
4. Permissive geometry model. Binding between TCR dimers and MHC/peptide dimers induces rotational scissor-like conformational changes in the CD3 chains that reveal previously hidden intracellular activation motifs. This model integrates receptor clustering and conformational change models, together with the existence of preformed oligomeric receptors, providing a mechanism to explain TCR signal.[72]
5. Multimerization model. This model proposes that the minimum unit of T-cell activation is a dimer and is based on antibody-induced dimerization of B-cell receptors and MHC structure studies. A single MHC/peptide could induce initial and transient calcium signaling, but monomeric MHC/peptide in solution did not induce stimulation of T cells. Obviously, more multioligomeric ligands induce more intense T-cell activation.[73]
6. Conformational change model. Extensive analyses of the crystal structures of TCR-MHC/peptide have not revealed any significant structural changes upon MHC/peptide binding by the ectodomains of TCRαβ dimers. However, biochemical analyses demonstrated structural changes of TCR complex, particularly in CD3ε. A

proline-rich region of CD3ε is a hidden determinant in the resting state but is exposed and recruits Nck upon stimulation to induce downstream activation signals.[74]

7. Safety model. This model is based on recent findings that the cytoplasmic domains of CD3ε and CD3ζ have affinity for the acidic lipids present on the plasma membrane, which may result in prevention of ITAM phosphorylation. Conversely, these chains in free aqueous solution may be readily phosphorylated by Lck. These results have led to the following model: whereas CD3εζ tails are tightly associated with the lipid-rich inner membrane of plasma membrane in resting T cells, they are released from this membrane-bound configuration and can be phosphorylated upon TCR ligation.[75]
8. Pseudodimer model. Only a few MHCs carry foreign cognate peptides for the specific TCRs, whereas a great majority of MHCs carry endogenous self-peptides. This model proposes that endogenous MHC/peptide amplify signals produced by agonist MHC/peptide by promoting TCR aggregation. Agonist MHC/peptide-TCR recruits a second TCR in a CD4-dependent manner, which binds endogenous MHC/peptide, stably forming a pseudodimer that triggers T-cell activation.[76]

The clustering models have been claimed to be incompatible with the presence of preformed oligomeric receptors on the surface of resting cells. However, the recent finding that TCR microclusters are the functional unit to transduce activation signals shows that aggregation of preformed TCR "nanoclusters" may form microclusters to induce signals.[77–79] On the other hand, models based on CD3 conformational changes that induced as a direct effect of ligand binding are not consistent with the requirement for multivalent ligand to initiate TCR signaling. Structural analyses of the complete molecular complex, including transmembrane regions and cytoplasmic tails, will provide a definite answer to this still confusing issue.

CYTOSKELETAL REGULATION

T-cell stimulation also induces a program for alteration of the cytoskeleton: actin polymerization, a microtubule rearrangement, which induces polarization and activation of T cells, and consequently changes the shape and regulates adhesion and movement of T cells. T cell–APC interaction results in morphologic changes: the cell becomes round and accumulates actin filaments at the stimulatory interface. These changes reflect the increase of fluidity of plasma membrane and a decrease of cellular motility. Accumulation of filamentous actin at the T cell–APC interface is the results of TCR-induced localized activation of multiple actin regulatory and polymerizing pathways. T-cell activation induces SLP76 phosphorylation, which recruits actin cytoskeleton regulatory proteins, Itk, Vav-1, Nck, and Cdc42 as the regulatory proteins. Localized activation of Cdc42 stimulates the actin-related protein 2/3 (Arp2/3)-regulating protein WASp, Wiskott-Aldrich syndrome protein, which interacts with Arp2/3 and activates actin polymerization.[80] WASp is recruited to the TCR activation complex by association with Nck and is activated by assembly with Vav-1, a GEF for the Rho family of GTP binding proteins and Vav-1, a central regulator of cytoskeleton, migration, and adhesion (Fig. 12.11). WASp functions as a regulator of the Arp2/3 complex that is critical for actin polymerization. Itk recruits Vav-1 to the immune synapse. Cdc42 regulated by the Itk-Vav-1 complex directly stimulates WASp actively, and the Nck-WASP complex regulates actin dynamics. Vav-1-mediated activation of a second Rho family, GTPase Rac1, results in the activation of WAVE2.

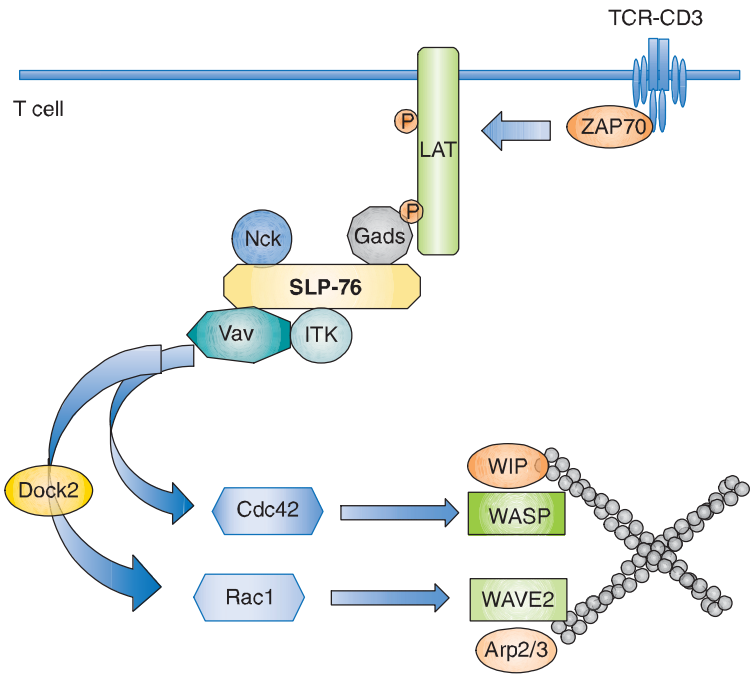


FIG. 12.11. Cytoskeletal Regulation by T-Cell Receptor (TCR) Signaling. TCR stimulation induces actin reorganization, which is required for the alteration of cell shapes, cell movement, and receptor clustering. Actin-related proteins such as Arp2/3, WASp, and WAVE2 regulate accumulation of F-actin. The LAT-SLP-76 signaling cluster induced upon TCR engagement recruits Nck, Vav1, and Itk. Whereas WASp binds to the adapter protein Nck and is activated by the Rho-family GTPase Cdc42, WAVE2 binds to Vav1 and is activated by another Rho-family GTPase Rac1 through Dock2.

CELL ADHESION REGULATION—INSIDE-OUT SIGNALING

Antigen stimulation of T cells induces their strong adhesion to APCs. This adhesion is mediated mainly by activation of integrin on T cells such as leukocyte function-associated antigen (LFA)-1, increasing its affinity and avidity for the ligand on APCs. Integrin activation is induced by a signaling cascade initiated by the TCR engagement and is a process termed inside-out signaling (Fig. 12.12). The inside-out signaling by TCR engagement results in integrin clustering and an increase in affinity and avidity for the ligands, which then induces high-affinity ligand binding.[81] It has been shown that the integrin LFA-1 goes through three conformational changes to achieve high affinity ligand: resting state, transitional state, and active conformation.[82] The integrins predominantly expressed on T cells are LFA-1 and very late antigen-4, which bind to their ligands, intercellular adhesion molecule (ICAM)-1 and 2, and vascular cell adhesion molecule and fibronectin, respectively. The process of inside-out signaling from TCR to integrin activation has been shown to include downstream signaling and actin-cytoskeletal rearrangement. Several critical adaptor molecules, particularly Rap1, ADAP, SKAP55, and RIAM, are necessary to translate TCR engagement to integrin activation.

Ras proximity 1 (Rap1), which belongs to the Ras superfamily of GTPases, plays a critical role in the inside-out signaling for integrin activation. Active Rap1 induces increased cell adhesion through integrin activation without changing their expression levels on T cells.[83] TCR stimulation and chemokines induce Rap1 activation, which then induces clustering of high-affinity LFA-1 on the leading edge of the membrane.

Following TCR ligation, SLP-76 inducibly interacts with ADAP, which then forms a complex with SKAP55. The SLP-76-ADAP-SKAP55 pathway leads to regulation of T-cell adhesion.[84] Both ADAP- and SKAP55-deficient T cells showed defective LFA-1 clustering and adhesion. ADAP-induced T-cell adhesion involves Rap1-GTP-interacting adaptor molecule (RIAM), which is required for Rap1 localization at the membrane.[85] Because RIAM associates with integrin, the complex with ADAP-SKAP55-RIAM makes a complex with LFA-1, resulting in adhesion to ICAM-1 upon TCR ligation. RIAM also binds to a cytoskeletal binding protein, Talin, and the Talin-RIAM-Rap1 complex may induce Talin to bind LFA-1 with high affinity. RIAM is also the ligand of Ena/VASP and profiling, which bind to actin cytoskeleton. In addition, similar to Talin, other cytoskeletal proteins such as Vinculin, WAVE2, and the Arp2/3 complex are also involved in the TCR-induced integrin activation.

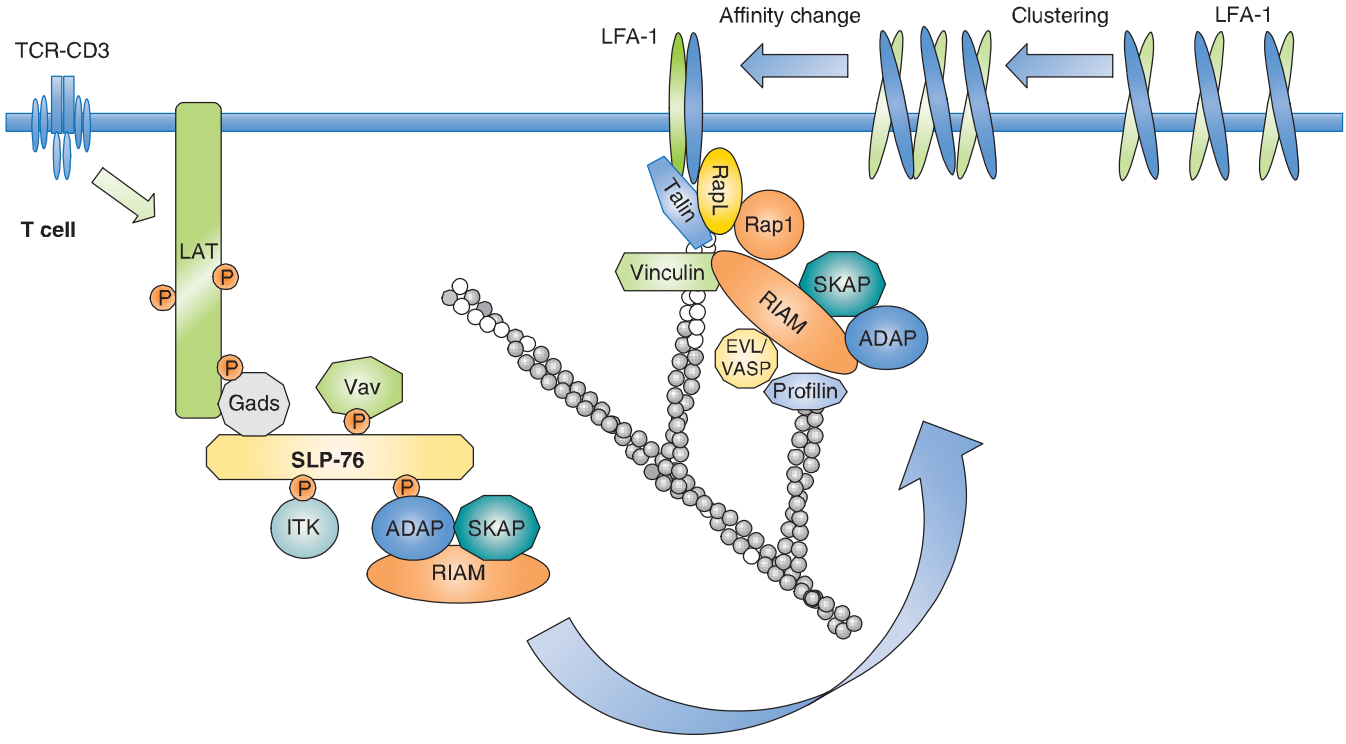


FIG. 12.12. Inside-out Signaling for T-Cell Receptor (TCR)-Mediated Integrin Activation as the Mechanism of TCR-Induced Cell Adhesion. TCR engagement induces signaling complex of linker for the activation of T cells and SLP-76. SLP-76 recruits Vav1 and Itk important for actin reorganization, as in Figure 12.11, and also adhesion- and degranulation-promoting adapter protein (ADAP), which constitutively associates with Src kinase–associated phosphoprotein of 55 kDa (SKAP55) and Rap1-GTP–interacting adapter molecule (RIAM). The complex of ADAP-SKAP55-RIAM associates with and activates Rap1 upon TCR activation and then translocates Rap1 to the membrane. The Rap1-RIAM/SKAP/ADAP complex induces the association of talin with the tail of integrin β. The local accumulation of talin is required for high-affinity binding by integrin. Integrin changes the status by clustering and then affinity maturation through structural changes. Integrin clustering involves Rap1 and Rap1-binding molecule RAPL that also associates with RIAM. RIAM associates with F-actin through the association with EVL/VASP proteins and the actin-binding protein profilin. PKD binds to the β1 integrin tails and recruits and activates Rap1 by Rap1-GEF C3G.

As to the effector mechanism of Rap1 signaling, Rap-1 binds to RAPL, the effector regulator of cell adhesion and polarization enriched in lymphoid tissues.[86] The interaction induces the membrane localization of RAPL and the binding to the αL subunit of LFA-1, which is critical for LFA-1 clustering and increasing its affinity. RAPL associates with a kinase Mst-1 as an effector molecule. Inside-out signaling induced by TCR engagement results in integrin clustering and increased affinity and avidity for the ligand, which then induces high-affinity binding to the ligand. Further analysis of the precise signaling cascade for inside-out signaling downstream of TCR is required.

COSTIMULATION

Cluster of Differentiation 28–Mediated Costimulation

T-cell activation is influenced by signals through several surface receptors including costimulation receptors (see following discussion), cytokine receptors, and adhesion molecules. T cells cannot be fully activated to proliferate and mediate cytokine secretion in the absence of costimulation signals through these costimulation receptors. TCR engagement induces various early activation signals such as an increase in the levels of intracellular calcium, tyrosine phosphorylation, and inositol metabolism, but these are not sufficient to induce full activation including cytokine production, cell proliferation, and effector functions. Instead, in the absence of costimulation, T cells fall into a state of unresponsiveness or anergy where they do not respond to antigen stimulation. Therefore, two signals are required for efficient T-cell activation: "signal 1" is defined as the antigen-specific TCR signal by the binding of peptide/MHC complex, and "signal 2" refers to the additional costimulation signal through a costimulation receptor. This "two signal model" for T-cell activation, which was proposed in the early study of T cells,[87,88] has been proved by various observations, particularly of the peripheral tolerance induction in the absence of "signal 2."[89,90] Although there are a number of receptors that can induce a costimulatory signal, the most physiologic, critical and well characterized is CD28[91] (Fig. 12.13A).

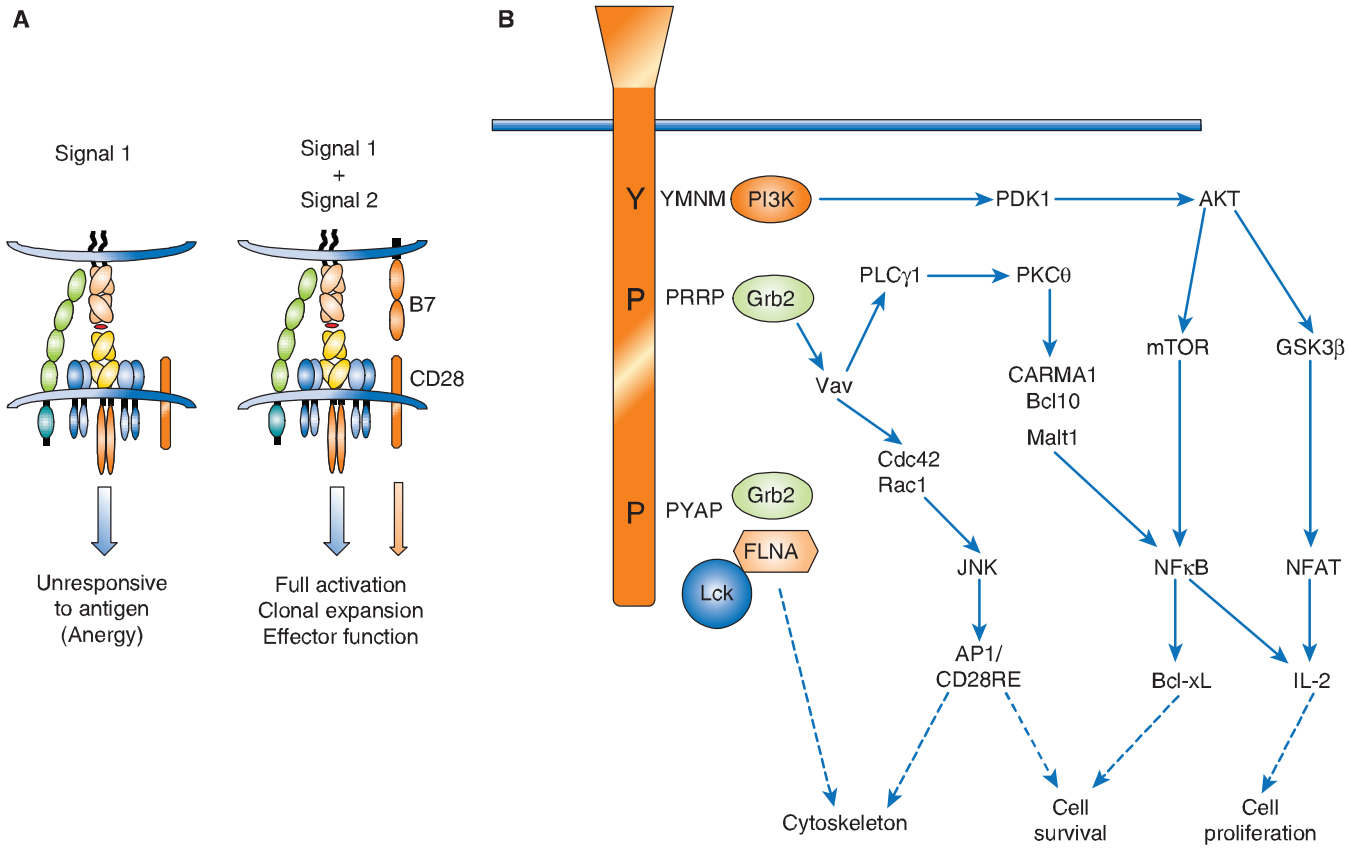


FIG. 12.13. Costimulation and Cluster of Differentiation (CD)28-Mediated Signals in T Cells. **A:** Full T-cell activation such as cell proliferation and cytokine secretion requires two signals: one (signal 1) through the T-cell receptor–CD3 complex as antigen-recognition signal, and the other (signal 2) through costimulation receptors such as CD28 as costimulatory signals. In the absence of costimulation signal, T cells become unresponsive to antigen stimulation (called anergy). **B:** Detailed signal pathway of CD28-mediated costimulation. The cytoplasmic tail of CD28 has major three motifs: N-terminus tyrosine motif YMNM associates with PI3K leading to the activation of Akt, which induces cell survival through mammalian target of rapamycin–mediated NFκB activation and cell proliferation, the upper Prolin-rich motif binds to Grb2 leading to Vav1 activation, which mediates cytoskeletal regulation and NFκB activation, and the last proline region associates with Lck and Filamin and Lck, which leads to actin-cytoskeletal organization.

CD28 is constitutively expressed on the surface of all T cells as a homodimer. The ligands for CD28 are the B7 family molecules, CD80 (or B7-1) and CD86 (B7-2), which are expressed on APCs. While CD80 is constitutively expressed, CD86 is expressed at low level on APCs in the resting state but is highly induced upon activation by innate signals, such as those derived from pathogens, in parallel with the induction of high level expression of MHC class II.[92] The induction of MHC class II and B7 molecules on activated APCs such as mature DCs are critical for T-cell stimulation because they can deliver a strong signal 1 and signal 2, respectively.

CD28 induces a profound increase in IL-2 production by both transcriptional and posttranscriptional regulatory mechanisms, as well as increased proliferation and cell survival, which is partly due to the induction of the antiapoptotic gene Bcl-xL (Fig. 12.13B). Whether CD28 induces costimulation totally independently of TCR engagement has been a controversial question for a long time. CD28-specific signals responsible for the costimulation signal have been extensively analyzed. CD28 has a short cytoplasmic tail (41 amino acids in human, 38 amino acids in mouse) and no intrinsic enzymatic function. It contains several tyrosines including an YxxM motif and two PxxP motifs. The YxxM motif, which is conserved in CD28, CTLA-4, and inducible costimulator (ICOS), is a consensus motif for the binding of the p85 subunit of the lipid kinase phosphatidyl-inositol 3-kinase (PI3K). Functional relevance of the YxxM motif is suggested by the fact that PI3K activation is enhanced by CD28 signaling.[93,94] PI3K activation generates PIP2, which recruits various molecules containing pleckstrin homology domains such as Tec family kinases, a serine/threonine kinase Akt, phospoinositide-dependent kinase 1, Vav, and WASP to the plasma membrane. Akt can be phosphorylated by phospoinositide-dependent kinase 1 and plays an important role in cell survival, which is one of the benefits of CD28-mediated costimulation.[95] The PxxP motif, which ICOS and CTLA-4 do not have, also has binding specificity for Grb2/Gads. Grb2 recruits Sos and Vav, which in turn phosphorylates and activates Rac1 and Cdc42, which then activates the MEKK1 cascade to ultimately activate the Jun kinase JNK.[96] Whereas the TCR signal predominantly activates ERK as an MAPK family member, CD28 engagement induces activation of the other member of the MAPK family, JNK, and the balance between the activity of ERK and JNK as a consequence of CD28-mediated costimulation may regulate cell survival.

Early studies showed that the distal PxxP (PYAP) motif in CD28 may recruit Lck, and it was recently shown that filamin A also binds to this motif to connect to the cytoskeleton. Although in vitro analysis and an overexpression system indicated a critical function for these motifs in costimulation, in vivo analysis with specific knock-in mutations revealed that mice with mutated YMNM motifs that failed to bind PI3K and activate Akt had no overt phenotype. By contrast, mice with the mutant distal motif had evidence of impaired CD28-mediated costimulation, such as reduced proliferation and IL-2 production.[97,98]

Enhancement of IL-2 production by CD28 engagement is regulated at both transcriptional and posttranscriptional levels.[99] A specific region responsible for CD28-mediated transcriptional activation (termed a CD28-response element, CD28RE) was found in the IL-2 promoter.[100] The binding of transcription factors to CD28RE is dependent on CD28 signal.

In addition to enhancement of the transcription of the IL-2 gene, CD28-mediated costimulation augments IL-2 production by increasing the stability of IL-2 mRNA.[101] Many cytokine transcripts including IL-2 contain adenylate-uridylate (AU)-rich elements within the 3′ untranslated region that stabilizes the message. Upon TCR stimulation, an AU-binding protein, TTP, binds to the AU-rich elements within the 3′ untranslated region and induces degradation of the mRNA. By contrast, CD28 costimulation induces other proteins, such as NF90, a transcription factor associated with NFAT, which may compete with TTP for binding at the AU-rich elements and enhance IL-2 mRNA stability.

CD28 signaling activates I-κB and consequently NF-κB. CD28 regulates the IKK activation step, and IKK activation results in I-κB activation/degradation and induces NF-κB activation. Extensive analysis to define the connection between CD28 signaling and IKK activation resulted in the discovery of CARMA1 as caspase recruitment domain–containing membrane-associated guanylate kinase protein-1, followed by identification of its associated proteins, Bcl-10 and Malt-1, which together form the CBM complex.[102,103] CARMA1 is phosphorylated by PKCθ and binds to Bcl10 through a CARD-CARD domain interaction.[104] The CBM complex is responsible for activation of NF-κB, thus T cells defective in any of the CBM components show impaired NF-κB activation and impaired T-cell proliferation. CD28-mediated activation of the CBM complex and NF-κB dramatically enhances IL-2 production.

Given its powerful activity as a second signal for T-cell activation, CD28 agonists and antagonists have been seriously considered for clinical application in humans. CD28 superagonist monoclonal antibodies were shown to polyclonally activate T cells in vivo, but such treatment ultimately led to the expansion of regulatory T cells, and several studies showed therapeutic benefit in autoimmune and inflammatory disease models in mice and rats. After preclinical safety testing in nonhuman primates, six healthy human volunteers were injected with the TGN1412 CD28 superagonist monoclonal antibodies, and the results were disastrous.[105] All six individuals suffered an immediate and life-threatening release of systemic proinflammatory cytokines, a "cytokine storm" that is now termed cytokine release syndrome. Recent studies suggest that the source of these cytokines was the effector memory population of T cells and have clarified why no such adverse effects were seen in rodents and nonhuman primates. Rodents do not accumulate a large population of effector memory population of T cells because the generation of these cells requires repeated exposure to infections; these experimental animals are kept in extremely clean conditions. The CD4 T

cells in macaques, the nonhuman primate used in the preclinical studies, unlike human T cells, lose CD28 expression when they differentiate into effector memory population of T cells.

COSTIMULATION THROUGH RECEPTORS OTHER THAN CLUSTER OF DIFFERENTIATION 28

CD28 is most prominent costimulation receptor and thus CD28-deficient mice have a general impairment in immune responses. However, not all immune response are dampened by CD28 deficiency, which indicates that other costimulation molecules can compensate for some of the remaining functions. These costimulation receptors include CD2, CD5, CD30, the Ig gene superfamily member ICOS, the TNF receptor family members CD137 (4-1BB) and CD134 (OX40), and LFA-1. These receptors are expressed on the T-cell surface to induce costimulation signals upon crosslinking with their ligands (Fig. 12.14).

In contrast to CD28, which is constitutively expressed on T cells, ICOS is expressed at a very low level on resting T cells and is inducibly expressed on activated T cells upon TCR stimulation, which is the major difference in its function from CD28 on resting T cells.[106] As ICOS-deficient mice have impaired immune responses similar to that seen with CD28 deficiency, ICOS must play an important role in immune regulation.[107] ICOS binds to a novel B7 family member, ICOS ligand, which is expressed rather broadly as compared to CD80/86 including nonhematopoietic tissues upon stimulation with inflammatory cytokines. ICOS is expressed on the cell surface as a dimer similar to CD28 and shares some structural features with CD28, including the YMXM motif in the cytoplasmic tail that binds p85 of PI3K. ICOS-mediated PI3K activation is stronger than that mediated by CD28, whereas other signals through CD28 that are not triggered through ICOS include Grb2 binding.[108] Such differences in signaling between ICOS and CD28 lead to differential function. Most notably, unlike CD28, ICOS does not induce IL-2 gene transcription. Instead, ICOS-mediated costimulation is critical for B-cell help for Ig production and germinal center formation. ICOS deficiency causes one of the multiple forms of common variable immunodeficiency, and these patients suffered from impaired B-cell function and germinal center formation and from hypogammaglobulinemia including IgA deficiency.[109]

Among TNF receptor family molecules, the molecules with costimulatory function represent CD27, OX40 (CD134) and 4-1BB (CD137), CD30, herpes virus entry mediator A (HVEM), and glucocorticoid-induced TNF-receptor (GITR). Among them, OX40 and 4-1BB mediate the most prominent functions. These molecules augment T-cell activation upon engagement of their ligands, OX40L and 4-1BBL, respectively. Engagement of OX40 and 4-1BB with their ligand induces activation of signaling pathways similar to CD28: PI3K and Akt, and NF-κB and MAPKs (JNK and p38), but this is through activation of the TNF receptor–associated factor (TRAF) family of adaptor molecules, which is different from CD28.[110]

One of the most important functional differences between CD28 and others such as ICOS, OX40, or 4-1BB is that CD28 is constitutively expressed on naïve T cells and plays a critical role to stimulate naïve T cells for proliferation, effector function, and functional differentiation, whereas all the others are inducibly expressed upon T-cell activation and therefore play roles to induce costimulation on activated

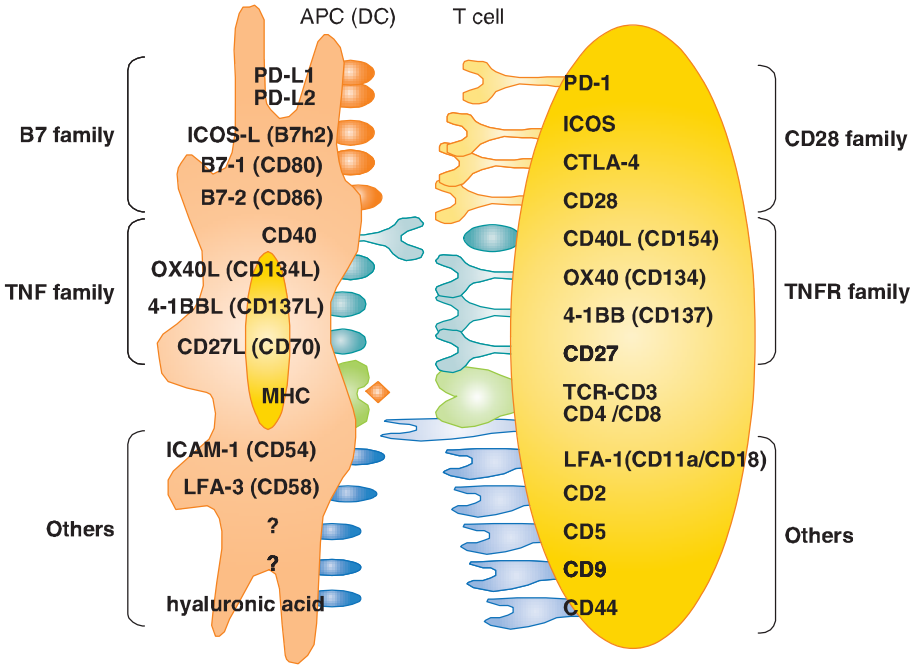
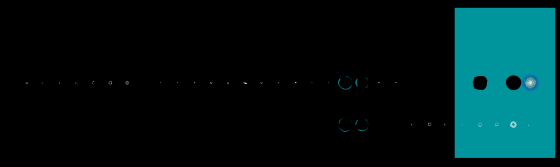
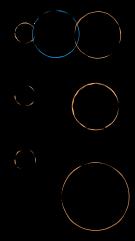


FIG. 12.14. Various Costimulation Receptors and Their Ligands. Various adhesion molecules between T cells and antigen-presenting cells (APCs) function to mediate costimulation signals for T-cell activation. These molecules are divided into three groups: cluster of differentiation (CD)28 family, tumor necrosis factor receptor family, and others including integrin leukocyte function-associated antigen-1. CD28 family molecules consist of two positive (CD28, inducible costimulator) and two negative (cytotoxic T-lymphocyte antigen [CTLA]-4, programmed death-1) receptors and the individually corresponding ligands. Whereas some receptors are constitutively expressed such as CD28, others are expressed upon T-cell stimulation such as CTLA-4 and OX-40. Whereas some of the ligands are expressed exclusively on APCs, others are widely expressed on variety cell types.





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4) Between the CDR3 loops of Vα and Vβ, there is a pocket that can (and does in at least one case[165]) accommodate a large side chain from the peptide bound to an MHC.

γδ T-Cell Receptor Structure

There are now three γδ heterodimer structures: a γδ TCR from a human T-cell clone G115,[167] which can be activated by natural or synthetic pyrophosphomonoesters, a γδ TCR from the murine T cell clone G8 together with its ligand, the nonclassical MHC class I molecule T22,[168] and a human MHC class I chain–related–reactive γδ TCR (δ1A/B-3),[169a] which was determined as a single-chain Fv construct. The G8 structure is shown in Figure 11.6, alongside the 2C αβ TCR. The structure of a single human Vδ domain also has been determined.[169] The Vδ domain of the G115 structure is similar to the isolated Vδ domain and the quaternary structure of G8 is similar to that of G115.[168]

The most distinctive feature of both the G115 and the G8 TCR, when compared with αβ TCRs and Igs, is that the C domains "swing out" from under the V domains. This unusual shape is highlighted by both a small elbow angle of 110 degrees, defined as the angle between the pseudo twofold symmetry axes that relate V to V and C to C, and a small V-C interdomain angle. This contrasts with an average of 149 degrees for αβ TCR structures. The small angle between the Vγ and Cγ domains shifts both Cδ and Cγ to one side. Moreover, the molecular surfaces of the constant domains are different than those of αβ TCRs with no clear similarities either in the shape or the nature of the CαCβ and CγCδ surfaces; there are only a few solvent-exposed residues that are conserved in both Cβ and Cγ domains as well. Thus, it is unclear where or how the extracellular domains of the CD3 subunits interact with the extracellular portions of γδ TCRs compared with αβ TCRs. This may explain why the CD3 components of αβ TCRs are so different from those of γδ TCRs.

In terms of ligand binding surfaces, we note that the Vδ CDR3 of G8 protrudes significantly away from the other CDRs, as shown in Figure 11.6. This has significance in that this is the major region of contact with the T22 ligand (see later section). In the case of G115, both Vδ and Vγ CDR3 loops protrude from the rest of the putative binding surface and create a cleft between them. Portions of the CDR1γ and δ and CDR2γ combine with the clefts between the CDR3 loops to form a pocket, which is surrounded by positively charged amino acid residues contributed by CDR2γ and δ, and CDR3γ. The jagged surface of this TCR resembles the surface of an antibody that binds a small-molecule antigen. Although this would be consistent with the supposition that this TCR binds the negatively charged phosphate compounds,[170] direct binding between the TCR and phosphoantigen including crystal-soaking and cocrystallization experiments have not been successful. Instead, a soluble G115 was found to bind a soluble form of adenotriphosphate (ATP) synthase F1 and apolipoprotein A-1.[171]

While the δ1A/B-3 TCR maintains an overall fold similar to the other γδ TCR structures, it was noted that unlike the G115 and G8 CDR3 regions, which are protruding out, the δ1A/B-3 CDR loops together generate a nearly flat surface on the combining site. This difference is anticipated, as the CDR3 length distribution of the TCRδ chains is quite variable as discussed previously, and like antibodies should have a broad range of binding site shapes.

αβ T-CELL RECEPTOR–LIGAND RECOGNITION

Binding Characteristics

Although it has long been established that this type of T cell generally recognizes a peptide bound to an MHC molecule, a formal biochemical demonstration that this was due to TCR binding to a peptide/MHC complex took many years to establish. Part of the difficulty in obtaining measurements of this type has been the intrinsically membrane-bound nature of MHC and TCR molecules. Another major problem is that the affinities are relatively low, in the micromolar range, which is too unstable to measure by conventional means.

To some extent, the problem of measuring the interactions of membrane-bound molecules can be circumvented by expressing soluble forms of TCR and MHC, which is also essential for structural studies (see previous discussion). For TCRs, many successful strategies have been described, including replacing the transmembrane regions with signal sequences for glycolipid linkage,[172] expressing chains without transmembrane regions in either insect or mammalian cells,[173] or a combination of cysteine mutagenesis and *E. coli* expression.[164] Unfortunately, no one method seems to work for all TCR heterodimers, although the combination of insect cell expression and leucine zippers at the c-terminus to stabilize heterodimer expression has been successful in many cases.[174] The production of soluble forms of MHC molecule has a much longer history, starting with the enzymatic cleavage of detergent solubilized native molecules[175] as well as some of the same methods employed for TCR such as glycophosphatidylinositol (GPI) linkage,[176] *E. coli* expression and refolding,[177,178] and insect cell expression of truncated

RORγt to promote Th17 function, Bcl6 for follicular helper T-cell function, and Foxp3 to promote T_{reg} function. Of particular interest is the fact that most of these factors also play pivotal earlier roles in the generation of T cells in general.

The differentiation of particular effector subsets is usually considered to start with antigen-inexperienced (naïve) mature T cells, after they have finished with their intrathymic development. The specialization itself is part of the mature T cell's portfolio of possible responses to antigen, especially among CD4 T cells. Conventionally, one may consider these antigen-driven events to be a completely different type of developmental process from the intrathymic events that mold hematopoietic precursors into T cells. However, we will see in the following that effector programming can also originate in the thymus for subsets like NKT cells, certain classes of TCRγδ cells, and some T_{reg}s: the distinction is not absolute.

Narrative Summary: Major Stages of Intrathymic T-Cell Development

The major known site for T-cell development in all jawed vertebrates is the thymus.[1] The thymus does not harbor its own long-term source of precursors but is continuously populated throughout life by new precursors that are replaced as their descendants complete maturation and selection. These precursors migrate to the thymus from bone marrow or fetal liver when they are still multipotent. The thymus not only enforces their commitment to a T-cell fate and T-lineage differentiation but also provides a proliferation-inducing environment. It is estimated that the numbers entering the thymus per day are quite modest, on the order of < 100 per day for a typical young adult mouse.[2,3] The descendants of that small cohort of input cells expand over a 2-week period to a cohort of cells that have undergone T-lineage commitment, TCR gene rearrangement, and TCR expression, eventually yielding ~50×10^6 new thymocytes a day before their proliferation stops.

Major Subdivisions Based on CD4, CD8, and T-Cell Receptor-αβ Status

The steps of T-cell differentiation in the mouse thymus are summarized in Figure 13.1.[3–6] Important landmarks for the process are provided by the expression patterns of the TCR coreceptors, CD4 and CD8, and the timing of rearrangement of the TCR-coding genes themselves.

Cells in the early stages of development in the thymus are CD4− CD8− ("double negative" [DN]) and cannot yet express TCR. While the DN stages can be subdivided (described subsequently), the cells in all the DN stages are mostly determining their commitment to a T-cell fate, proliferating, and preparing for their first expression of TCR complexes. With successful expression of a TCRβ chain, they turn on CD4, CD8α, and CD8β genes together. The resulting CD4+ CD8αβ+ "double positive" (DP) cells are highly distinctive to the thymus, not found among mature T cells in peripheral lymphoid organs, and are diagnostic of a pivotal stage in T-cell development and survival. The DP cells in steady state constitute by far the major fraction of thymocytes (~80%), and many of them come to express complete TCRαβ receptors as well. However, the overwhelming majority of these cells are fated never to go further in their differentiation. Only a small fraction are allowed to progress further through positive selection, to become either CD4+ CD8− TCRαβ+ cells ("CD4 cells") or CD4− CD8αβ+ TCRαβ+ cells ("CD8 cells"). It is estimated that the cells have only about 3 days within the DP stage in which to achieve this success before their window of opportunity closes. Left in the cortex, the other DP cells of their cohort die "of neglect," to be replaced by progeny of the next cohort of precursors, at turnover rates up to 50×10^6 per day.

T-Cell Receptor-αβ Rearrangement and Selection Checkpoints

The regulated process of TCR rearrangement and selection helps to explain why so many DP cells must be produced. TCR coding genes are assembled through a highly regulated but highly imprecise recombination process mediated by the recombinase complex RAG1/RAG2, the same recombinase that rearranges immunoglobulin VDJ gene segments in B cells. The imprecision is useful to add to the diversity of the eventual T-cell recognition pool, but it also results in many gene rearrangements that are out of reading frame or otherwise defective. To winnow out the useful cells, TCR gene rearrangement occurs in two sequential bursts, each followed by a quality control checkpoint at which all the cells with defective receptors can be eliminated.

The timing of different phases of recombination competence is determined by the specific activation and deactivation of the RAG1 and RAG2 gene products; the genetic loci on which their effects can be focused at any given time are determined by developmentally regulated unmasking via localized chromatin opening. For TCRαβ cells, the most numerous T cells in mice and humans, the TCRβ gene must rearrange first, while the cells are still in the DN stage. The cells carrying out this rearrangement need to pause proliferation in order to allow the RAG complex to work, and most often they are never allowed to divide again unless they succeed in generating a good TCRβ coding sequence through rearrangement at one allele. This strict condition is the "β-selection checkpoint." Only cells passing this checkpoint are allowed to become DP cells, receiving a bonus of multiple rounds of cell division to expand the winners and dilute the stalled, dying losers. Through the process of β-selection, then, the cells also shift the gene loci that are accessible for rearrangement, losing the ability to rearrange their TCRβ genes (and TCRγ and TCRδ genes) any further and acquiring the ability to rearrange their TCRα genes.

Once they become DP cells, TCRα rearrangement gets underway. As a successful in-frame TCRα chain rearrangement occurs, the heterodimer TCR complexes of the new TCRα chains with the previously generated TCRβ chains become the next subject of testing at a second checkpoint. This second checkpoint is more draconian than the first, as the criterion for success here is not simply successful expression of a TCRαβ heterodimeric protein, but also the quantitative details of the recognition specificity of the new

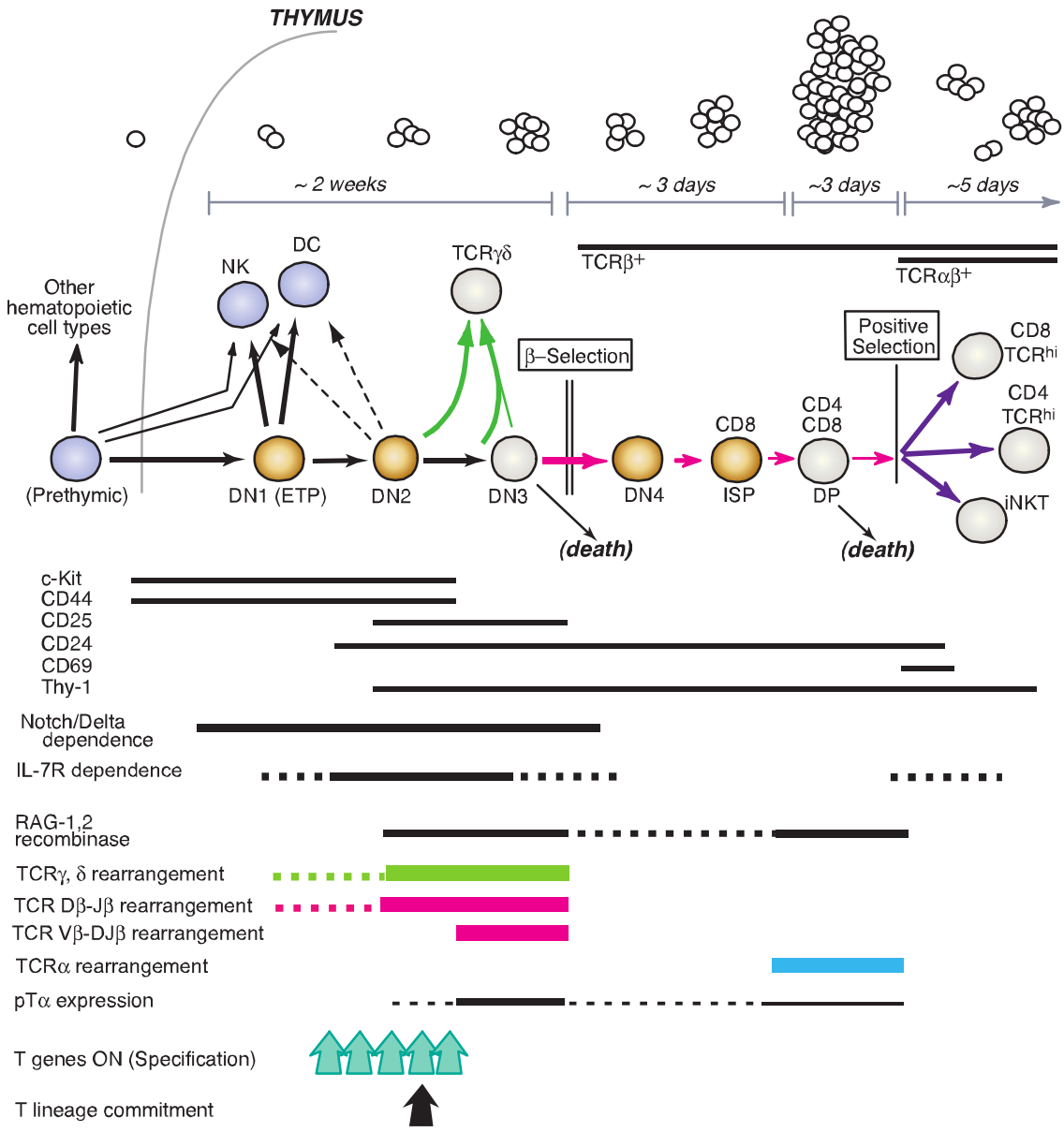

FIG. 13.1. Schematic Summary of T-Cell Developmental Stages. *Top:* Cartoons depict the approximate transit times and population expansion through the different stages. *Middle:* Recognized major stages of mouse T-cell development are shown, indicating CD4, CD8, and T-cell receptor (TCR) phenotype, and phases that are TCRβ-dependent or TCRαβ-dependent. Development through positive selection of CD4, CD8, and natural killer T cells is shown; not shown are pathways to regulatory T cells, CD8αα innate-type cells, alternative γδ pathways, or late-stage negative selection. *White circles:* nondividing or slowly dividing stages. *Yellow circles:* stages of extensive proliferation. *Blue circles:* prethymic cells and non-T developmental alternatives. *Green arrows:* enabled by TCRγδ expression. *Magenta arrows:* enabled by TCRβ expression. *Purple arrows:* enabled by TCRαβ expression and selection. *Lower:* timing of expression of additional markers, phases of TCR gene rearrangement, and specification and commitment events (see text for details).

receptor, as measured by its interaction with ligands in the thymic environment. The newly expressed TCR could fail to recognize anything, making it useless, or it could confer on the cell a dangerous autoreactivity that could lead to autoimmune disease. All of these failures must be eliminated before they appear in the mature T-cell population. The only successful cells are the ones that happen to have heterodimeric receptors with the right combination of functionality and low affinity for self-ligands. These are the ones that are allowed to become CD4 or CD8 cells through a process termed "positive selection," with the CD4 cells generally becoming cytokine-producing "helpers" and the CD8 cells generally becoming "killers." The positively selected cells permanently silence their RAG gene expression and undergo further maturation steps but little if any additional cell division before they are sent out to the periphery.

The effects of the ordered TCR gene rearrangements and quality control checkpoints are seen clearly in the effects of different mutations on the progression of T-cell development. Figure 13.2A,B depicts the CD4/CD8 profiles and DN subsets in a normal young mouse thymus of about 2 to 3 × 10^8 cells. Figure 13.2C,D shows the effects on thymus populations in mutant mice in which TCR gene rearrangement is impossible (eg, via mutation of *Rag1* or *Rag2* or *Prkdc*, the gene that encodes a deoxyribonucleic acid [DNA]-dependent protein kinase needed for rejoining of the gene segments during rearrangement [a very instructive mutant, *Prkdc*^{scid}, causes severe combined immune deficiency]). Because TCRαβ-lineage cells are so predominant in the mouse thymus, the effect of mutations of *Tcrb* alone appears very similar (Fig. 13.2E). In contrast, mutations of the *Tcra* locus alone allow production of a full complement of DP cells, although CD4 and CD8 SP cells are completely missing (Fig. 13.2F).

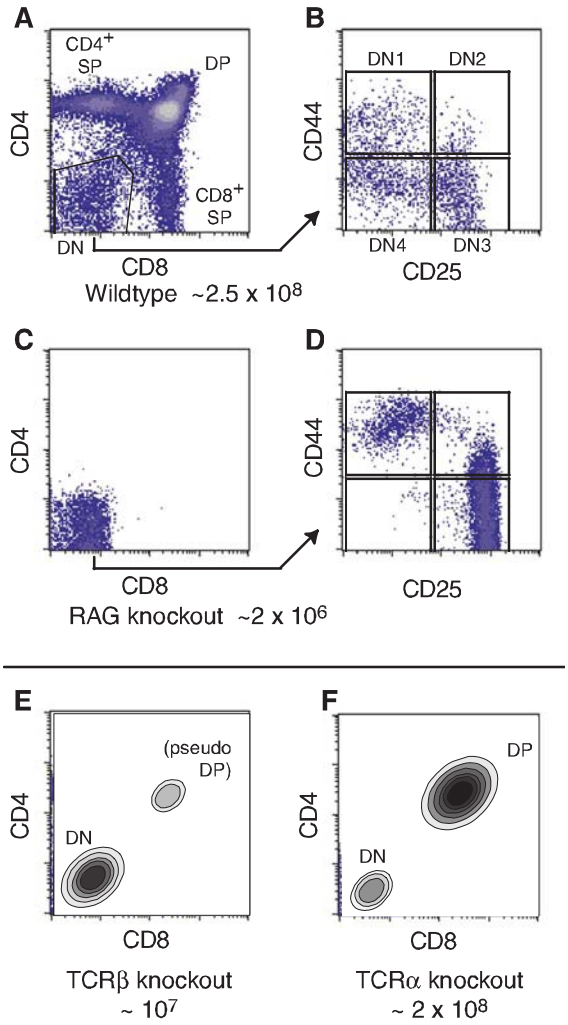

FIG. 13.2. T-Cell Developmental Progression and Thymus Cellularity Controlled by T-Cell Receptor (TCR) Assembly. A,B: Normal wild-type mouse thymocytes: cluster of differentiation (CD)4/CD8 phenotype of all thymocytes **(A)**, CD44/CD25 phenotype of double negative (DN) thymocytes only **(B)**. **C,D:** Recombination-deficient mouse thymocytes: CD4/CD8 phenotype of all thymocytes **(C)**, CD44/CD25 phenotype of the cells, which are all DN **(D)**. Data for *Rag2* knockout cells are shown, but *Rag1* knockout phenotype is the same and similar phenotypes are seen for *Prkdc*^{scid} or *Tcrb*−/− *Tcrd*−/− thymocytes. **E:** Cartoon depicting CD4/CD8 phenotype of TCRβ knockout (γδ still available). A few pseudo–double positive cells are generated through transient use of a γδ TCR, but the great majority of TCR+ cells in these populations are TCRγδ$^+$ DN. **F:** Cartoon depicting CD4/CD8 phenotype of TCRα knockout: development through β-selection is unimpaired, but no single positive populations are formed. Note the effects of each genotype on the cell numbers per thymus.

T-Cell Receptor–based Lineage Diversification for T-Cell Receptor-αβ Cells

The first functional distinctions between TCRαβ cell subsets apparently arise only during emergence from the DP stage. Positive selection itself probably triggers these divergences through distinctive modes of positive selection signaling, as we discuss in detail in a later section. The three main outputs of TCRαβ cell positive selection are CD4 cells, CD8 cells, and NKT cells, so called because they share some functional qualities and surface receptors with natural killer (NK) cells. DP cells first experiencing positive selection signals immediately turn on the activation marker CD69, start increasing the surface density of their newly validated TCRαβ complexes, upregulate CD5, and then begin the process that results in shutting off either CD8αβ or CD4 to generate TCRαβ-high "CD4 single positive" and "CD8 single positive" cells, respectively. As the cells arrive at one of these "single positive" (SP) phenotypes, they still retain expression of another marker of immaturity, the CD24 "heat-stable antigen" that is expressed from the early DN stages onward. However, they turn off both CD69 and CD24 as they reach completion of their functional maturity, in a process that takes 3 to 7 more days.[7] NKT cells undergo a distinctive maturation sequence that is discussed separately in the following, but they too lose CD69 and CD24 as they mature.

The selection of these different subsets is based on TCR and coreceptor interactions with different ligands in the thymic environment. CD4 cells are positively selected by TCR and CD4 joint interaction with MHC class II molecules. CD8 cells are positively selected by TCR and CD8αβ joint interaction with MHC class I molecules. NKT cells are positively selected by nonclassical class I MHC molecules, using non-CD4, non-CD8 accessory molecules (signaling lymphocytic activation molecule [SLAM] family molecules) to assist in signal triggering. Although the primary trigger in each case is TCRαβ-ligand recognition, the effector programs of these different major lineages diverge significantly, through the consequences of using these different types of coreceptors to assist in "interpreting" the TCR signals. How this signal-dependent feature of T-cell maturation works is a major focus of later sections of this chapter.

Negative Selection of Autoreactive T-Cell Receptor-αβ Cells

Many cells with TCR that might be dangerously autoreactive can be eliminated at the DP stage, but another crucial period for this aspect of quality control is after positive

selection. As newly generated single-positive cells display increased surface density of their TCRαβ, raising their avidity for MHC-bound ligands, those that have strongly autoreactive receptors are generally killed off by interaction with specific subsets of thymic stromal cells in a process called "negative selection." This continual purging of the newly made T-cell recognition repertoire is crucial to avoid autoimmune disease, as important mouse mutations and human disease models reveal. An alternative pathway to defeat autoreactivity, at least among CD4+ cells, is to alter the functional subtype of the autoreactive cells, to program them for function as tolerogenic regulatory T cells before they even have a chance to leave the thymus. Generation of such "natural T_{regs}" (nT_{regs}) is but one case where TCR interaction properties direct the cells to a particular T-cell functional class.

Generation of T-Cell Precursors in the Double Negative Compartment

For years, the least well understood set of thymocytes was the DN cells. The DN-stage cells comprise only a small fraction of thymocytes in steady state, generally less than 5%, but in fact represent the generative compartment of the thymus in which the largest number of total cell cycles takes place. Profound developmental transformations also occur during transit through the DN stages.

Understanding the basis of T-cell specification became possible only through the discovery of markers that could subdivide the DN compartment into successive stages (reviewed in refs. 2,3). The earliest stages of precursor differentiation within the thymus are represented by DN (CD4− CD8− TCR− or sometimes $CD4^{low}$) cells that express high levels of the growth factor receptor Kit and the activation/adhesion molecule CD44 but low levels of CD25, termed Kit-high DN1 cells or early T-cell precursors (ETPs). Then, these cells turn on expression of CD25, signaling their definitive entry into the T-cell pathway, and the resulting Kit+ CD44+ CD25+ cells are called "DN2" cells. Both the DN2 and ETP stage cells proliferate, and IL7R is increasingly expressed in the DN2 stage. The DN2 cells then progress into the "DN3" stage ($CD25^{high}$ but now $CD44^{low}$ and Kit^{low}), and here proliferation slows or stalls, RAG protein levels rise, and efficient TCRβ rearrangement can proceed. Development terminates at the DN3 stage unless the cells can pass β-selection or γδ-selection.

Successful TCRβ expression and passage of β-selection enable the cells to restart proliferation, shut off expression of CD25, and pass through a transitional "DN4" or "preDP" stage ($CD25^{low}$, $CD44^{low}$, Kit^{low}). They then proceed to acquire CD8 and CD4 on the cell surface, sometimes CD8 slightly before CD4 ("immature SP"), and finally culminate their differentiation into DP cells by finishing their proliferation (large DP to small DP transition). This extended β-selection-dependent transition from DN3 to small DP cells creates a unique regulatory state in the resulting DP cells that prepares them for selection. Notably, although some of these cells may be positively selected as described previously, only a minority may ever divide again until after they have left the thymus.

Distinctive Paths for T-Cell Receptor-αβ Cells

T cells that will use TCRγδ instead of TCRαβ actually follow a special program or set of program options from the DN2 stage on. Whereas TCRβ rearranges strictly before TCRα and mostly in DN3 stage, the TCRγ and TCRδ genes can rearrange in parallel within the DN2 or DN3 stage. Thus, a RAG1/RAG2+ cell can in some cases acquire successful in-frame rearrangements of both TCRγ and TCRδ genes before it is successful with TCRβ alone. As a result, newly TCRγδ+ cells arising within the DN2 or DN3 population leave the TCRαβ lineage mainstream, undergoing a γδ-selection process that is different from β-selection. The γδ-selected cells generally keep CD4 and CD8 silent, shut off RAG1/RAG2 permanently without promoting TCRα rearrangement, and limit their proliferation to a much smaller number of cell cycles than β-selected cells.

It is not clear yet whether all TCRγδ cells must go through two sequential checkpoints analogous to β-selection and positive selection. Like TCRαβ cells, TCRγδ cells are now understood to emerge in multiple functional subtypes, and we will discuss in a separate section possible ways that these subtypes become differentially programmed.

CONTEXT OF T-CELL DEVELOPMENT

Anatomical Organization of T-Cell Development

Major Thymic Domains

The structure of the thymus plays a substantial role in the ordering and tempo of the steps in T-cell development.[3] The epithelial framework of the thymus is a derivative of the fetal pharyngeal endoderm, emerging from the third branchial pouch in midgestation and only later descending from the neck region to its later position lying just over the heart. At its peak of function, the thymic epithelium looks very different from most endodermal epithelia, as it has become a highly porous, "lacy" three-dimensional lattice with few tight junctions, no obvious apical or basal polarity, and most of the spaces between epithelial cells crammed with developing lymphocytes. The structure is not uniform, as each lobe of the thymus is organized into cortical (outer) and medullary (inner) regions. The epithelial lattice appears quite open in the cortex but more compact in the medulla, where the lymphocyte:epithelial cell ratio is much lower. These different regions contain distinct epithelial cell types and different nonlymphoid hematopoietic cell types in addition to the developing T cells (Fig. 13.3).

Ordered and Checkpoint-Controlled Migration Pathways for Developing T Cells

Multipotent precursors follow a distinctive traffic pattern through the thymus[8] (see Fig. 13.3). They enter through postcapillary venules at the cortical/medullary border, and they spend the bulk of the ETP stage proliferating quite close to this boundary. Then, as they enter the DN2 stage, they begin to migrate centrifugally, toward the outer cortex. They are thought to reach DN3 stage in the outer sector of the cortex and undergo the burst of proliferation triggered by β-selection at the extreme periphery of the cortex, just under the capsule and far away from the initial site of

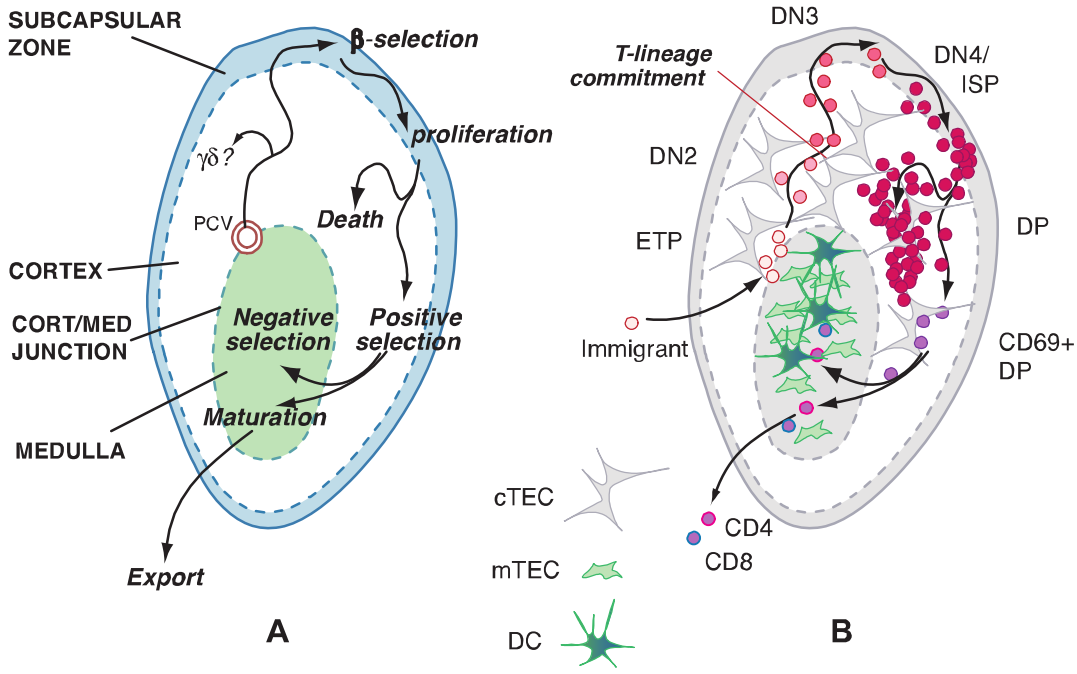

FIG. 13.3. Anatomical Compartments of the Thymus and Migration Pathways of Developing T-Cell Precursors. **A:** Major compartments and migration pathways are depicted with key checkpoints in T-cell development indicated. *Cort/med junction*, cortical-medullary junction; PCV: postcapillary venule (immigration portal). **B:** Phenotypes of T-cell precursors (*pink, red, violet*) and stromal cell types in different compartments. cTEC, cortical thymic epithelial cells; DC, dendritic; mTEC, medullary thymic epithelial cells. For clarity, the "outbound" path from ETP to DN3 is separated from the "inbound" path from DN4 to CD4 or CD8 single positive maturation, but in vivo the "outbound" transit is surrounded with the "inbound" cells.

precursor entry. Then, the DP cells generated during this proliferation start to fall back down through the cortex, covering the same ground as the DN cells that were their precursors, but now traveling in the opposite direction. The sheer number of these DP cells dominates the population in the cortex: in a healthy young adult mouse thymus, for the ~2×10^4 DN2 cells migrating toward the outer cortex, there are over 1.5×10^8 DP cells falling back toward the medulla. Thus, the DN cells must work their way up through the vast excess of DP cells as they acquire T-cell character, and every other cell type in the cortex is embedded in a slow-moving sea of DP cells. As discussed in the next major section, this countercurrent migration may play an important role in helping the earliest precursors to receive inductive signals efficiently from the epithelium without competition.

Despite their huge numbers, the DP cells are normally denied entry into the medulla. Positive selection signals are needed to induce new chemokine receptor expression (CCR7) on the fortunate minority of DP cells, and these are the only ones that can plunge into the medulla to continue their differentiation.[2,9] After further maturation, once all signs of TCR signaling subside, the cells are finally allowed to upregulate emigration receptors that will allow them to leave the thymus entirely. It is also in the medulla, however, that even more stringent testing for TCR autoreactivity and purging of autoreactive cells takes place. Ultimately, less than 5% of the cells from each cohort of DP cells emerge from the thymus alive.

Thymic Anatomy Orders the Presentation of Inductive and Selective Signals

The pathway of T-cell precursors through the thymus helps to order their exposure to key differentiation-inducing stimuli.[2,3,9] The cortical epithelium expresses high levels of integral membrane cell surface ligands for the Notch signaling receptor, mostly Delta-like 4 (DLL4, or DL4). This is important because Notch-Delta interactions play a central role in specification of T-cell progenitors from uncommitted precursor cells, as discussed in detail in the following section. In addition, the epithelium closest to the cortical-medullary junction makes the highest concentrations of the cytokine IL-7, which supports both early and late T-cell development. Epithelial cells expressing Kit ligand appear to be present in multiple domains of the thymus. As the precursors enter the thymus, therefore, they can rapidly engage in Notch-DLL4 signaling interactions in the presence of maximal concentrations of IL-7 and Kit ligand. These are ideal conditions for proliferation in the ETP and early DN2 stages. As the cells move outward, they encounter decreasing IL-7, which enables them to slow proliferation and begin to carry out efficient TCR gene rearrangement.

Thymic epithelium is one of the rare epithelia in the body that can express MHC class II molecules, which are normally limited to hematopoietic APCs, as well as the more broadly expressed MHC class I cell-surface molecules. Because TCR interactions with class II MHC are crucial for positive selection to CD4 fates and TCR interactions with class I MHC

usually direct selection to CD8 fates, this expression pattern makes it possible for the cortical epithelium to support positive selection to both CD4 and CD8 fates. At the cortical/medullary junction, there is a high concentration of thymic macrophages, which can rapidly dispose of the dead cells that are generated in large numbers by TCR-dependent selection every day.

Medullary thymic epithelium is distinctive in an additional way.[9a] Not only does it express class I and class II MHC, but also it is specialized for the detection and elimination of highly autoreactive cells. Medullary epithelial cells mature to an end stage in which they lift the repression of multiple silent loci in their genomes, genes that have no function in the thymus per se and would normally be expressed only in other organs in the body. These diverse tissue-specific self-antigens, presented on class I or class II MHC, are offered as "bait" to any newly positively selected cells whose receptors might recognize them. The presentation of these molecules is further enhanced by special populations of dendritic cells (DCs) in the thymic medulla, and these help to stimulate any autoreactive T cells to commit suicide. As we will see in the following sections, these medullary cell types can also suppress autoreactive T-cell precursors through an alternative mechanism, by directing their differentiation into nT_{regs}.

Variations of T-Cell Development: Ontogeny and Species Differences

T-cell development in the thymus is common to all jawed vertebrates.[10] A thymus, originating embryologically in the neck region and presenting Notch ligands to RAG1/RAG2+ lymphocyte precursors that turn on TCR gene expression, is found from cartilaginous fish to bony fish and amphibia through birds and all mammalian species. There is even an apparent equivalent structure newly discovered in the lamprey, a jawless vertebrate that probably shared its last common ancestor with mammals shortly after the Cambrian explosion (~500 million years ago).[11] Despite important commonalities, there are significant differences between T-cell development in mice and in other vertebrates, and even between mice and other mammals. In addition, even within the mouse, T-cell development undergoes significant changes between the first wave of production that occurs in the fetus and later waves of T-cell development in fetal and postnatal life, described subsequently. Thus aspects of T-cell development can be flexible even though they produce similar T-cell populations as outputs.

Ontogeny: Distinctive Features of the Mouse Embryonic Thymus

When the thymus rudiment is first formed in midgestation in the developing neck region of the embryo, it is not vascularized. Thus, the first wave of hematopoietic precursors that enter the thymus do so by migrating across the mesenchyme and penetrating the thymic rudiment from the outside.

There are two distinctive things about this first wave of thymic lymphopoiesis. First, the precursors of the first wave are intrinsically somewhat different from the cells that will populate the thymus in later waves. Descendants of early intraembryonic hematopoietic precursors, they have a unique capability to give rise to certain specialized types of TCRγδ cells as well as more conventional kinds of TCRγδ cells and TCRαβ cells.[12,13] Second, the thymus epithelium itself is naïve in the sense that it has not yet been affected by signals from lymphocyte gene products, nor dilated into an open mesh by lymphoid proliferation. The medulla is not evident yet as a distinctive domain, and the spatial distribution of cortical epithelial products like IL-7 and Notch ligands is not necessarily ordered as it will later become.[14,15]

One result is that differentiation occurs much faster in the fetal mouse thymus than in the adult. Passage from DN1 to DN3 stage occurs within ≤ 2 days from embryonic gestation days E12.5 to E14.5 instead of 7 to 10 days in adults.[16,17] Passage to DP occurs just 2 days later, requiring about 4 days instead of ~2 weeks overall.[3] The fetal-specific types of TCRγδ cells are in fact produced as the first wave of mature T cells, by E15.5, even before DP cells emerge. Finally, the gene expression requirements for T-cell development in the fetal thymus are subtly different than in the adult (eg, a more stringent requirement for transcription factors Ikaros and E2A, and a reduced requirement for transcription factor TCF-1 and the IL-7R).

It is interesting that there is generally reduced TCR sequence diversity in the fetal T cells as compared to those made after birth. Normally, TCR sequences have two sources of variation: the combinatorial diversity based on the particular V, D, and J segments that are rearranged, and the sloppiness of cleavage and addition of untemplated nucleotides at the recombination breakpoints (ie, junctional diversity). One enzyme, terminal deoxynucleotidyl transferase (encoded by *Dntt*), is responsible for much of the junctional diversity. It is normally turned on in the earliest stages of prethymic precursor development and expressed throughout T-cell development until positive selection. However, the waves of lymphoid precursors developing in the fetus do not express it, and this situation only changes around the time of birth. As a result, some TCR types expressed in fetal thymocytes including the "first wave" TCRγδ receptors are essentially invariant, with highly predictable reading frames, even though the genes that code for them are assembled by RAG-mediated recombination. It seems likely that these TCR specificities have some evolutionary selective advantage for the young mouse.

The production of TCRγδ cells as the initial wave in the fetal thymus is remarkably shared between different classes of vertebrates: one of the initial reports of first-wave TCRγδ cells (then called "TCR1 cells") was in chickens.[18] In the mouse, there is considerable evidence that the first-wave cells are distinctive in more ways than their use of a particular TCRγδ receptor complex, as discussed in the section on γδ cells.

Evolutionary Conservation of Diverse T-Cell Lineages but Flexibility of Roles

Focus on the mouse system may in fact handicap our ability to perceive the roles of TCRγδ cells, because mice and humans are the two known mammalian species with the

most limited systemic use of TCRγδ cells. In fact, an αβ/γδ distinction between T-cell subclasses is an ancient feature of vertebrate lymphopoiesis. Even cartilaginous fish like skates and sharks have well-defined γδ and αβ TCR complexes that are used by T cells in different anatomical domains.[19] This suggests that two TCR-defined branches of T-cell development have been coselected for persistence over > 400 million years of vertebrate evolution. Major divisions within the TCRαβ lineages, between CD4+ helpers and CD8+ killers, are similarly conserved among all bony vertebrates. Thus although the genes for immunological receptors themselves undergo rapid evolutionary change and reorganization, the underlying programs for lymphocyte development appear to be ancient and conserved.

Evolutionary Flexibility at Close Range: Mouse versus Human

Most comparative information is only available for mouse and human T-cell development. The outlines of T-cell development in the thymus are strikingly similar in these closely related mammalian species.[20–22] However, many details of T-cell development have proven to be different between man and mouse. The cell surface markers that define stages of human T-cell development are different from their mouse counterparts, and even some of the same molecules are expressed in different developmental patterns, as shown in Table 13.1. One surprising difference concerns the quantitative influences of different doses of Notch signaling on TCRαβ as compared to TCRγδ cell production.[23] While most of the major features of development are similar, this kind of variation is a caveat against overgeneralizing data from the mouse.

TABLE 13.1 Comparison of Markers in Human and Murine T-Cell Development

Stage	Mouse Phenotype	Human Phenotype
ETP-like: Intrathymic T/DC/NK precursors	CD44+ c-Kit+ CD25–	CD34+ CD38^{lo} CD7– CD1a–
Specification stage (DN2-like)	CD44+ c-Kit+ CD25+	CD34+ CD38+ CD7+ CD1a–
Committed T precursors	CD44$^{+/low}$ c-Kit^{int} CD25+	CD34+ CD38+ CD7+ CD1a+
TCRδ, γ, β rearrangement: αβ/γδ precursors	CD44+/– c-Kit$^{+/-}$ CD25+ (DN2, DN3)	CD34+ CD38+ CD1a+ CD4–/+
β-selection	CD44– c-Kit– CD25+ CD4-CD8– (DN3) to CD25– CD4– CD8–	CD34+ CD38+ CD1a+ CD4+to CD4+ CD8α+ (CD4 ISP, early DP)
TCRα rearrangement	CD25– CD4+ CD8αβ+ (DP)	CD4+ CD8α+ CD8β+ (early DP to DP)
Mature cells	TCR/CD3^{hi} and CD4+ or CD8αβ+	CD1a– TCR/CD3^{hi} and CD4+ or CD8αβ+

CD, cluster of differentiation; DC, dendritic cell; DN, double negative; DP, double positive; ETP, early T-cell precursor; NK, natural killer cell; T, T cell.
Data from reviews by Blom & Spits[283] and Taghon & Rothenberg.[22]

Creating an In Vitro Context for T-Cell Development

The three-dimensional structure of the thymus was for many years a challenge to the full definition of T-cell developmental mechanisms. It was clear after many attempts that dissociated thymic epithelial cells could not reconstitute thymic function if plated in monolayer culture, and thus it seemed that the migration of precursors through organized three-dimensional domains would be a crucial aspect of T lymphopoiesis. Redox conditions also seemed crucial for thymic function. There were two early breakthroughs in experimental dissection of T-cell development. First was the generation of fetal thymic organ cultures, which were found to require a high oxygen concentration to work, either by plating a thymic lobe at an air-medium interface, or by pumping up to 70% oxygen into the incubator in which a submerged fetal thymic lobe was cultured.[24,25] Second was the discovery that in these excellent culture conditions, even dissociated, purified thymic stromal elements could regenerate a competent thymic microenvironment if mixed together in a reaggregate fetal thymic organ culture. The development of reaggregate fetal thymic organ culture has allowed the separate roles of distinct epithelial and hematopoietically derived elements of the cortical and medullary environments in positive and negative selection to be rigorously demonstrated, as discussed in later sections.[26] Both fetal thymic organ culture and reaggregate fetal thymic organ culture systems promote T-cell development from prethymic precursor stages all the way through positive and negative selection to generate functional mature T cells.

The fetal and reaggregate thymic organ cultures both make use of natural thymic stromal elements, which makes them close to physiological conditions, but also a logistical challenge. Furthermore, the organ cultures still re-form into closed structures, making the thymocytes developing within them hard to count, track phenotypically, or experimentally manipulate. Finally, these organ cultures remain very small, hampering use for molecular biology.

Against this background, the development of stromal monolayer coculture systems for T-cell differentiation has revolutionized the field. Two lines of research converged to make this possible. First, Nakano, Kodama, and Honjo developed OP9 bone marrow stromal lines from myeloid colony stimulating factor-deficient mice (*Op*, osteopetrotic mice),[27] which proved to be ideal for supporting lymphoid development. However, only B and NK lymphocytes, not T cells, develop on these original OP9 stromal cell lines. Second, crucial experiments by the groups of Freddy Radtke and Warren Pear showed that environmental activation of Notch signaling is crucial to induce hematopoietic precursors to become T cells.[28,29] Engineering OP9 cells to express a potent Notch ligand, Schmitt and Zuniga-Pflucker developed the OP9-DL1 stromal line to support T-cell development.[30]

This created robust, highly efficient, high-yield conditions for driving and supporting T-lineage development in vitro from a wide range of uncommitted hematopoietic progenitor types, while keeping the cells accessible, easily monitored, and easily transferable throughout.

The OP9-DL1 system and variations of it have made possible enormous advances in understanding of the early, Notch-dependent stages of T-cell development. This system exposes to experimental dissection all the stages from initiation of the T-cell developmental program through commitment and up to the DP stage (ie, all the stages that DN precursors would normally undergo during their "outward bound" migration through the thymic cortex). It has also opened up the ability to track and experimentally perturb early stages of human T-cell development, starting from cord blood precursors, in a direct parallel to early mouse T-cell development.[23] However, the "inward bound" phases of T-cell development after β-selection or γδ-selection appear to depend on special properties of thymic epithelial cells that remain to be duplicated by OP9-DL1 or any stromal coculture.

EARLY LINEAGE CHOICES AND COMMITMENT

Notch and the Specification of T-Cell Identity

Notch signaling is crucial for induction of the T-cell program, for progression from stage to stage of the specification process, and for maintenance of viability of the developing T cells throughout the DN stages, all the way to β-selection. DN thymocytes gain privileged access to Notch ligands in the environment despite the large excess of DP cells. This is because the DP cells "deny themselves" the chance to bind efficiently with Delta-class Notch ligands in the environment by downregulating key Notch modifier genes, *Lfng* and *Mfng*, during β-selection,[31] which are needed to compete for stromal DLL4 binding. Thus, in a healthy normal adult thymus, the masses of DP cells actually insulate individual DN cells from competition with each other, allowing Notch triggering to be renewed repeatedly from ETP to DN3 stage.

Notch-DLL4 signaling activates the regulatory cascade that turns on T-lineage genes from ETP stage to DN2 stage to DN3 stage. T-lineage–specific signaling mediators and invariant TCR components are upregulated starting by the DN2 stage or in the DN2-DN3 transition. The IL-7R (*Il7r* gene) is fully turned on by DN2 stage to support proliferation of the precursors as they are acquiring their T-cell identity. Between DN2 and DN3 stage, RAG1 and RAG2 recombinases and multiple TCR signaling complex components and mediators of TCR signal transduction, including the kinases Zap70 and Itk, are all induced or upregulated. The specialized surrogate T-cell receptor α-like chain, pre-TCRα (pTα, encoded by the *Ptcra* gene), is also turned on to provide a partner for complex formation with any successfully-encoded TCRβ chain. Thus, the cells that reach the DN3 stage are already fully armed with substantial levels of the T-cell specific components that will be needed to transduce signals through the TCR, as soon as they have made a productive TCR gene rearrangement.

Notch signaling accomplishes this large-scale transcriptional mobilization by first activating the expression of several T-lineage transcription factors that can help the cells to open up previously silent genetic loci.[32–34] The most important T-cell–specific members of this group are GATA-3 (encoded by the *Gata3* gene) and TCF-1 (encoded by the *Tcf7* gene). These T-cell factors collaborate with the Notch-activated transcription factor RBPJκ (also called CBF1 – suppressor of hairless – Lag-1 = CSL), and together they interact with a set of transcription factors that is already present and active in multilineage hematopoietic progenitors. These preexisting factors include "E protein" basic helix-loop-helix factors E2A (encoded by *Tcf3* = *Tcfe2a*) and HEB (*Tcf12*), Ikaros family members like Ikaros (*Ikzf1*) and Helios (*Ikzf2*), Runx family factors like Runx1 and Runx3, Ets family factors including PU.1 (*SPI1* in human, *Sfpi1* in mouse) and Ets1, and other key hematopoietic factors like Myb and Gfi1.[35] The knockout of any one of these factors or factor families is sufficient to abort early T-cell development.

This complexity is important to acknowledge because despite the essential role of Notch, no one transcription factor alone turns on the whole T-cell program in a single stroke. In fact, different factors collaborate with each other in a balanced way throughout the DN stages, to coordinate differentiation with the right degree of proliferation before the cells reach the DN3 stage. Furthermore, the E proteins E2A and HEB not only regulate T-cell identity genes, but also serve throughout T-cell development to enforce TCR signaling checkpoints, undergoing cycles of inhibition and reactivation in response to TCR stimulation. We will see others of these factors that also have different, dose-dependent roles later on, working with or against each other to cause one T-cell lineage to diverge from another.

Options, Precursors, and Stages

The cells that populate the thymus are not yet committed to a T-cell fate when they arrive. Elegant single-cell culture experiments using either adapted organ culture systems or stromal coculture systems show that the same individual cells that can give rise to T cells would still be able to generate NK cells, myeloid cells, and DCs under other conditions, when they arrive in the thymus.[36–39] Several lines of research also imply that many of them are initially able to give rise to B cells.[40,41] The commitment process is the process through which the developing T cells decide permanently against these other options.[42]

Given how similarly T and B cells rearrange their immunoreceptor genes and observe developmental checkpoints, it is not surprising that B-cell potential should be present in T-cell precursors. It is notable how early B-cell potential is lost—soon after the thymus-settling precursors enter the thymus; many experiments even imply that access to the B-cell fate can be lost even before thymic entry.[43–45] However, after losing the B-cell option, DN thymocytes maintain a clearly demonstrable ability to shift to a myeloid fate, either macrophage or granulocyte, and this persists into the DN2 stage.[37–39] As long as the cells remain in an intact thymus,

these possibilities remain latent and development progresses overwhelmingly toward a T-cell fate, but all that is needed to reveal these options is to remove the cells from the thymus and offer them cytokines that support myeloid cell survival. Even into the DN2 stage, some T-cell precursors also appear to be able to develop into mast cells, yet another nonlymphoid cell type.[46] The cells that start the T-cell program thus begin with many alternative possibilities.

A great deal of work has been carried out to link the developmental repertoire of newly arrived thymic immigrants with uncommitted cells in the bone marrow that are likely to be their precursors. This lively field of research has shown that thymic immigrants can be derived not only from cells that were mostly committed to some kind of lymphoid fate prethymically (ie, common lymphoid precursors), but also from cells that were broader-spectrum lymphoid-myeloid multilineage precursors (lymphoid-primed multipotent precursors).[43,47–50] Furthermore, even cells that have already begun to be restricted to a nonlymphoid, myeloid pathway (granulocyte-macrophage precursors) can also develop into T cells, if transferred from the bone marrow into a thymic environment or OP9-DL1 coculture.[51] Many T-cell precursors in the thymus in fact show evidence of having earlier activated a myeloid lineage differentiation gene, lysozyme M.[52] Thus, the dominant developmental program imposed by the thymic environment is a kind of boot camp that can force cells of different developmental origins to converge on a common T-cell fate, erasing signs of their previous distinct identities.

Mechanisms of Commitment

The thymus uses different mechanisms to eliminate various fate alternatives for the cells. One of these is the environmental signal through Notch itself. As long as thymic immigrants and even ETP and DN2 cells are being exposed to Notch ligands in the thymic microenvironment, they cannot exhibit any alternative potentials efficiently. As differentiation progresses, however, the cells also give up the ability to access these alternatives under any conditions (Fig. 13.4).

Early B-cell development in particular is intensely inhibited by Notch signaling. Not only are precursors prevented from initiating B-cell development in the presence of Notch-Delta signals, but also exposure to Notch signals rapidly strips them of the capability to enter the B-cell pathway even if Notch-Delta signaling is removed.[40] Perhaps because early B and T cells share many other early developmental requirements—the cytokine IL-7, the role of stroma, prominent use of E proteins among their crucial lymphoid transcription factors—the Notch signal is uniquely important to make these two developmental programs mutually exclusive. The thymus is too good an environment for B-cell development in every other way; in fact it fills up with developing B cells if Notch-DLL4 signaling is defective.[41,53,54]

The choice against other alternatives takes longer and involves more conditionality. The myeloid and DC-enabling transcription factor PU.1 is normally expressed into the DN2 stage and probably explains the access to myeloid potential up to this point. Under normal conditions, however, the thymus is not a good source of myeloid-cell growth factors or differentiation factors, and so myeloid development is a low-efficiency path in the thymus regardless of the programming of the cells. In addition, active Notch signaling appears to add a further conditional obstacle to myeloid development through interference with the action of myeloid transcription factors.[52,55] This mechanism still allows cells to keep myeloid potential as a latent option through several cell divisions, accessible if they are removed from the thymus. But eventually the T-cell developmental program reaches a stage when the myeloid-enabling transcription factors themselves are repressed. This occurs in the later DN2 stage, and it marks the point when the myeloid paths too are permanently cut off.

The most persistent fate alternative is the NK fate, which falls into a somewhat different category. The choice against becoming an NK cell appears to be made about the same time as the choice to forgo the DC fate option,[36,56,57] but in fact one could argue that many CD8+ T cells never fully give up the NK possibility. There are many differences between NK cells and T cells: most obviously, the NK cells do not rearrange their TCR genes and utilize a substantially different antigen recognition system than T cells. Thus, any TCR+ cell would be classified as T rather than NK no matter how NK-like it was in other respects. In an apparent dichotomy, too, developing T cells require a class of transcription factors, the E proteins (E2A, HEB) that NK cells must inhibit: the E protein antagonist Id2 is an NK developmental requirement. However, NK cells mobilize effector programs that are almost completely congruent with those of effector CD8+ killer T cells. On persistent antigen stimulation, CD8 killer T cells turn on expression of NK-cell activation receptors as well as TCR and behave even more like NK cells in terms of promiscuity of killing. A recent gene knockout model suggests that only one transcription factor, Bcl11b, may stand between mature CD8 cells and NK-like behavior normally throughout their lifetimes as mature T cells.[58] The loss, or reduction, of access to the NK program occurs at the specific stage of T-cell development, also in the DN2 stage, when Bcl11b is turned on for the first time.[58–60]

Bcl11b is turned on in a process that requires Notch signaling and TCF-1,[61,62] possibly restrained until DN2 stage by other timing factors. DN2 cells that cannot turn on Bcl11b do not reach a normal DN2b or DN3 stage: instead, they keep prolonged access to myeloid fates and ongoing self-renewal programs as well as the NK program as long as cytokine levels permit.[59,60] They are profoundly defective in generating TCRαβ-lineage thymocytes, DP, and later stages, as well.[63,64] Thus, in addition to its special role in suppressing the NK-cell program, Bcl11b also appears to work in a gatekeeper capacity at the crux of the commitment process.

Commitment, therefore, involves at least three distinct mechanisms: the suppression of B-cell potential, the silencing of myeloid transcription factors, and the induction of Bcl11b. The last two of these happen roughly at the same point, in a transition that is now recognized to split the DN2 stage between uncommitted DN2a cells and committed

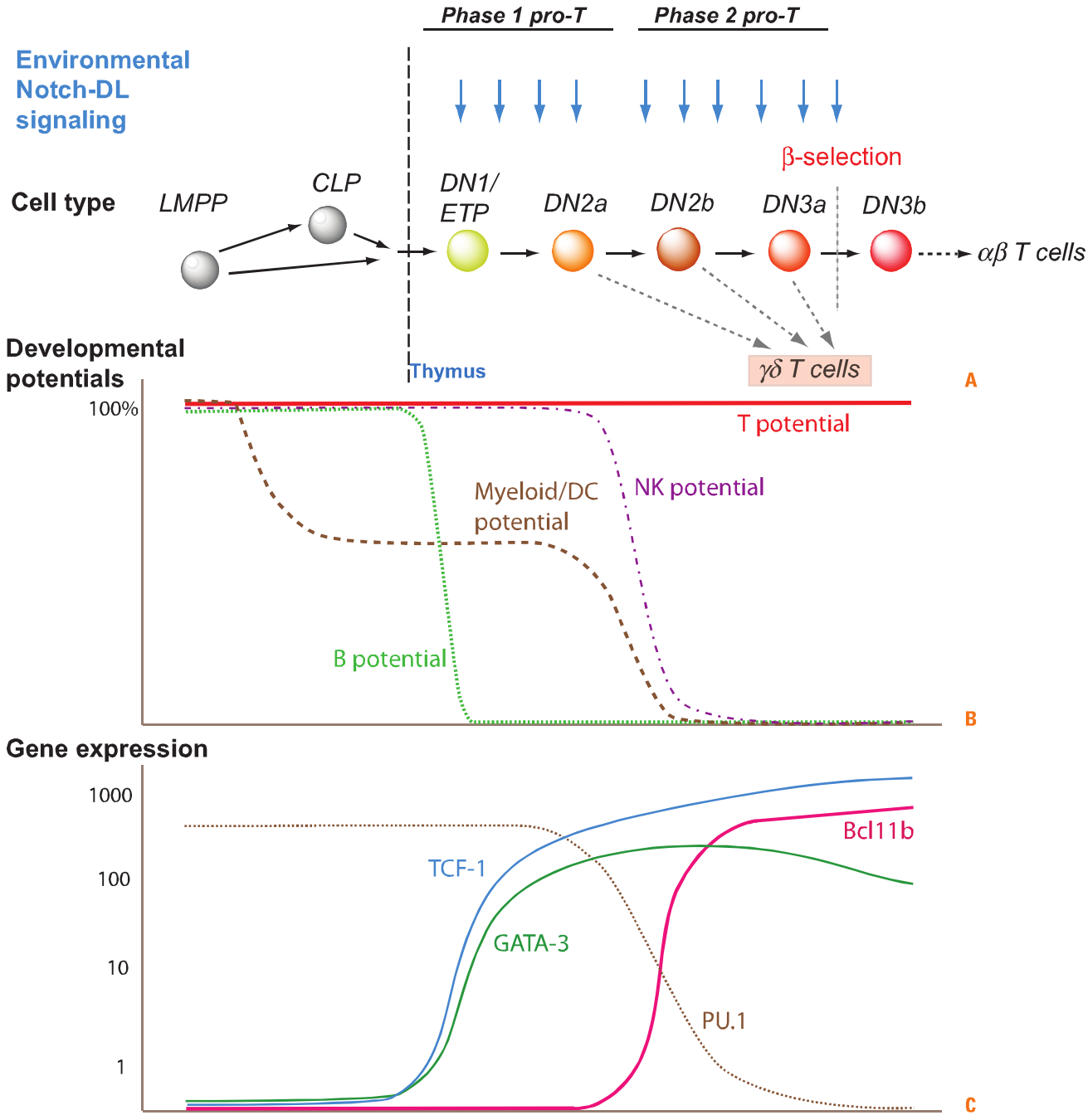

FIG. 13.4. T-Lineage Commitment and Expression Patterns of Contributing Factors. **A:** Prethymic and intrathymic stages involved in T-lineage commitment. The thymus can be seeded by cells that retain myeloid potential (lymphoid-primed multipotent precursor) as well as by cells that have already reduced their access to this option (common lymphoid precursor). As shown in the figure, Notch-Delta signals are delivered to the cells repeatedly from their time of entry into the thymus until β-selection. **B:** Approximate times at which distinct cell-lineage options for T-cell precursors are excluded. Y axis represents remaining ability of T-cell precursor cells to enter alternative pathway at the indicated stages (relative units). **C:** Expression patterns of key regulatory genes noted in the text. Factors used in the T-cell program are depicted with *solid lines*, while PU.1, a factor used in multipotent progenitor, myeloid, and B-cell programs, is shown with a *dotted line*. Adapted from figures in Rothenberg[42] and Rothenberg et al.[279] Evidence for gene expression patterns reviewed in Rothenberg et al.[32]

DN2b cells[56,65] (see Fig. 13.4). DN2a cells have access to other fates and can survive when removed from Notch ligand-rich environments; DN2b cells are committed and become acutely Notch signaling–dependent for viability. From commitment on, DN cells cannot survive deprivation of Notch ligands until they have successfully undergone β-selection or γδ-selection. Transition to the DN2b stage also appears to prepare the way for shutting down precursor expansion and establishing the molecular conditions for enforcing the β-selection checkpoint.

PROTOTYPE OF A T-CELL RECEPTOR–DEPENDENT CHECKPOINT: β-SELECTION

β-Selection: Proliferation, Allelic Exclusion, and Creation of the Double Positive State

All TCRαβ cells, regardless of functional subset, are survivors and products of β-selection. The β-selection checkpoint is the first TCR-dependent control checkpoint that these cells encounter, and the β-selection process is the first TCR-dependent signaling response that they undergo. The outcome shapes the whole population upon which later selection and differentiation mechanisms must operate.

TCRβ rearrangement and TCR-complex signaling are crucial for both the proliferation and differentiation events of β-selection. Thus recombinase-deficient (*Rag1* or *Rag2* knockout), DNA repair-deficient (eg, *Prkdc*^{scid}, "SCID"), TCRβ constant region knockout, or CD3γ or ε knockout mice all leave T-cell development blocked at DN3 stage (see Fig. 13.2). In normal development, successful TCR Vβ joining to Dβ-Jβ joints in a productive reading frame is the limiting event, as CD3 chains begin to be synthesized and expressed on the surface at a low level irrespective of rearrangement. It is the ability to deliver a signal through these complexes that really drives β-selection, as both in vivo and in vitro a response that strongly mimics natural β-selection can be triggered even in RAG-deficient thymocytes if monoclonal antibodies are used to crosslink the CD3 chains on their surfaces to deliver an artificial signal.

Fulfilling the Requirements for β-Selection

By the DN3 stage, developing T cells are already under proliferative restraint, in part to make further development conditional and in part to enable the RAG1/RAG2 complex to work efficiently. Although many ETPs already have limited Dβ-Jβ TCR gene rearrangements due to low-level prethymic RAG expression,[66] these partial rearrangements cannot encode functional receptors. By the DN3 stage, however, efficient recombination of Vβ segments to the joined Dβ-Jβ products begins, and some of these rearrangement products now encode functional TCRβ chains.

The success of TCRβ rearrangement is sensed by the cell when the newly made β-chain is assembled into a complex at the cell surface and transduces back a low-intensity signal through essentially the same kinase and adaptor mechanisms used in mature TCR signaling. Isolated TCRβ chains cannot assemble with CD3 and TCRζ chains on their own, and there is no TCRα available yet, but the invariant pre-TCRα chain provides a surrogate. The *Ptcra* gene does not require rearrangement and is turned on strongly as a direct Notch signal–induced gene by the DN3 stage. The pre-TCRα/TCRβ heterodimer thus forms a "pre-TCR" containing a full complement of CD3 and TCRζ, which is readily transported to the cell surface to trigger signaling.

Unlike all later uses of the TCRβ, this signaling does not depend on any component of ligand recognition from its heterodimer partner, but the pre-TCRα chain can mediate spontaneous clustering that may help to trigger signaling.[67–69] The rate-limiting criterion for successful β-selection is thus simply the structural integrity of the TCRβ chain that allows it to participate in a well-formed signaling complex.

The β-Selection Response

Pre-TCR spontaneous signaling triggers a dramatic cascade of events. In hours, the cells not only begin to proliferate again but also set in motion a profound transformation. The cells become large blasts, almost 10 times the volume of most thymocytes. As they turn on CD4 and CD8αβ expression, and express the coreceptor CD28 for the first time, they shed their DN cell markers and everything that had sustained their viability as DN cells, losing CD25, IL-7R, and the last vestiges of Kit expression and also inactivating the Notch pathway as well.

The proliferation unleashed by β-selection is intense. In their 2 to 3 days of proliferation, the selected DN3 cells generate approximately 10^2-fold more DP cells. During this expansion, there are ample opportunities for clearing the cell surface of obsolete membrane proteins (eg, old CD25 molecules), clearing the nucleus of obsolete transcription factors (eg, the Notch-induced transcription factor Hes-1), and clearing the genome of obsolete states of chromatin modification.

During the proliferation triggered by β-selection, TCR gene rearrangement is temporarily suspended, as the cells downregulate *Rag1* and *Rag2* gene expression and inactivate RAG2 protein by cell cycle-dependent phosphorylation.[70] RAG1 and RAG2 expression is restored by the time proliferation arrests in the DP stage, but by that time the cells have transformed the chromatin landscape in which the RAG1/RAG2 recombinase must find its targets. TCRβ is now closed, establishing rigorous allelic exclusion for this TCR chain. TCRα is now open for rearrangement, pioneered by some germline transcription as early as the late DN3 stage. At the same time, the potential for expressing TCRγδ receptors is eliminated. TCRγ genes become inaccessible, so that both germline transcripts from unrearranged loci and transcripts from any previously rearranged TCRγ genes are silenced.[71–73] TCRδ genes, whether previously rearranged or not, are deleted as the TCRα segments that straddle them rearrange. Thus, by the time proliferation stops, the DP cell is irreversibly confirmed in a TCRαβ lineage.

Creation of the Double Positive State: Setting the Stage for Positive and Negative Selection

With the first productive rearrangement of TCRα, the developing DP cell will acquire the capacity to recognize unpredictable antigenic targets, including self-antigens. Until the new receptor specificity has passed quality control, a DP cell is therefore functionally disabled and "contained," both within the thymic cortex and within a short default lifespan. DP cells not only lack emigration receptor expression but also acquire features that would cause them to be killed quickly if they did escape. As they pass through β-selection,

they downregulate their own MHC class I molecules and their surface glycoprotein sialylation[74] pathways: they can even be purified on the basis of their resulting low MHC class I expression and preferential binding by the lectin peanut agglutinin. These changes make them into potential NK cell targets and ensure that they would be scavenged by macrophages via asialoglycoprotein receptors if they escaped. Also as β-selection begins, the antiapoptotic factor Bcl2 is repressed, replaced with the weaker or more conditional survival factors Bcl-xL and Bcl2A1.[75,76] Even while they are still rapidly dividing, the emerging cells acquire an intense vulnerability to death signals, both glucocorticoid signaling and other strong signals.[77]

Cell surface expression of TCRαβ complexes are kept low on DP cells even when TCRα chains are first expressed, partly due to uniquely high levels of Src-like adaptor protein (*Sla* gene product), which targets TCRζ protein for rapid destruction.[78] Nevertheless, DP thymocytes are, if anything, more sensitive to TCR-ligand interactions than mature cells with the same TCRαβ if an appropriate assay is used. Contributing factors may include their relative lack of sialic acid residues, thus reducing electrostatic repulsion, and their low expression of the negative feedback molecule CD5 that could otherwise damp their response to TCRαβ signals.[79–82] Even though it is sensed, TCR signaling cannot turn on functional response genes like *Il2* in DP cells. In part, this is because these cells have downregulated key activation-transducing transcription factors, c-Jun and c-Rel (for more information, see www.immgen.org/).[83] β-selection also induces expression of the transcription factor, RORγ, from a distinctive promoter (RORγt, also called RORC2), which actively blocks conventional effector responses even while maintaining Bcl-xL expression throughout the DP stage.[84,85] RORγt expression sets DP cells apart from all other major classes of thymocytes; however, it provocatively links them with Th17 cells in the periphery, with lymphoid tissue–inducing cells that organize lymph nodes in fetal life, and certain classes of newly recognized innate effector cells.[86–89] The DP cells that emerge from the complex regulatory events of β-selection thus acquire a highly specialized physiology, and they are poised for selection.

The β-selection response is driven by the same major signaling pathways used in the activation of mature T cells. Lck, Ras pathway, PI3-kinase, and protein kinase C activation all play roles in the process, using virtually all the same adaptor molecules [LAT, Slp76 (Lcp2), GADS (Grap2)] that are used to coordinate signaling in positive selection later and in mature T cells.[90–92] As in later stages of T-cell development, TCR signaling temporarily antagonizes effective activity of E proteins (E2A/HEB). A transient wave of immediate-early Egr factor activation results in transient upregulation of the E protein antagonist Id3, a response that will be echoed in positive selection later.[93–96] As the cells reach the resting DP state, high-level E protein activity is restored.[97] These factors cooperate with some of the same regulatory factors that are used in early T-cell development: initially Notch signaling, then the important T-cell transcription factor TCF-1.[98–101] The fact that the cells emerging from this signaling response are idiosyncratic, functionally disarmed, short-lived DP thymocytes rather than robustly activated effector T cells must be explained as the result of the characteristic transcriptional and epigenetic regulatory state of the cells in this stage—the context within which the signaling pathways are sensed—rather than a unique biochemical pathway for the signals themselves.

A Clear and Present Danger: Creation and Enforcement of the β-Selection Checkpoint

The cell biology of the β-selection response is an obvious hazard for the organism. Intense polyclonal proliferation of this magnitude brings the cells close to a malignant state, and the program must include brakes to stop the proliferation as well as constraints to prevent it from being triggered inappropriately. In fact, most T-cell acute leukemias have precisely the phenotype of cells that cannot stop the β-selection response, continuously generating immature DP cells or DN-DP intermediates. Under normal conditions, a limiting factor for the process is the programmed downregulation of Notch responsiveness, as the extent of population expansion during β-selection is strongly influenced by Notch signaling.[102,103] Indeed, by far the most dominant and consistent feature of T-cell acute lymphoblastic leukemias is the presence of gain of function mutations in the *Notch1* locus, especially mutations that make Notch signaling ligand-independent.[104] The result is particularly grave when the cells can join viability signals from pre-TCR complexes and Notch alike to proliferate without restraint.

Another dangerous combination is if the very rapid proliferation program induced by β-selection is superimposed on any continuing expression of hematopoietic progenitor self-renewal genes, inherited from earlier stages of T-cell specification, including *SCL* (*Tal1*), *Lyl1*, *Lmo1*, *Lmo2*, and *Hhex*.[105,106] These genes are normally turned off during the lineage commitment transition, between DN2a and DN2b, but may easily act as T-cell oncogenes if kept on. Thus, β-selection may not be safe until these genes have been successfully repressed. The implication is that progressing into a normal DN2b-DN3 stage sequence may be important to establish the correct control over future growth.

Nonmalignant αβ T-cell development depends on a robust mechanism that can enforce a clear developmental arrest checkpoint, before the cells have a chance to receive the pre-TCR signal that triggers β-selection. The two most important regulators known for this process are transcription factors: E proteins (E2A and/or HEB) and Ikaros. Both types of factors are expressed fairly stably throughout early T-cell development, but they become indispensable for enforcement of the β-selection checkpoint in addition to their other roles. E proteins promote *Notch1* and *Rag* gene expression as well as expression of a number of T-cell genes, but they also tend to slow or stop proliferation.[107] Ikaros is important for the initial production of T-cell precursors but may also serve as a competitor for Notch/RBPJ at certain common DNA target sites.[108] Losses of function of either class of checkpoint enforcer lead to malignancy with very high efficiency.

THE FIRST T-CELL RECEPTOR–DEPENDENT PROGRAM CHOICE: αβ VERSUS γδ FATES

Making T-Cell Receptor–dependent Developmental Decisions: General Questions, Different Answers

The problem of how TCR recognition specificity becomes linked to irreversible activation of different transcriptional programs is the core issue of T-cell development. For most developing T cells themselves, this problem is first encountered during the decision to become a TCRαβ cell or a TCRγδ cell. As they mature, both TCRαβ cells and TCRγδ cells fan out into a variety of distinct effector cell types or committed lineages. But TCR specificity most commonly emerges as the result of stochastic processes that determine not only which V, D, and J segments will be joined but also exactly which nontemplated sequences will happen to be added at the coding joints, as the latter end up determining antigen-binding specificity as well as whether the rearrangement is in-frame. How can the cells coordinate any coherent transcriptional regulatory programs for development together with such a randomly determined outcome?

Most models that have been considered as possible answers include different balances between "instruction" and "selection." Instructive models propose ways that the randomly determined TCR specificity can result in transduction of distinct signals back to the cell that actually direct specific developmental choices. Selective models postulate that developmental choices are made independently, but that the randomly determined TCR specificity may be more "appropriate" for one choice than another. Then, the signals transduced back from the TCR need make only a simple determination: whether or not the cell should be allowed to live or die. As described in the following sections, there is substantial evidence for roles of both kinds of mechanisms in later TCRαβ cell development.

However, with reference to the TCRαβ versus TCRγδ decision, a third kind of model needs to be considered as well. The selection of TCR gene segments to be rearranged in this case is not random; it is itself under significant developmental and environmental control.[109] Specific aspects of T-cell programming not only influence which TCRγ segments are used, but also may influence whether a cell will choose TCRγ and TCRδ gene segments to rearrange at all, before or in preference to TCRβ gene segments. Thus, developmental programming can directly influence TCR specificity in this case. The effect of preprogramming must be considered together with the effect of TCR specificity to explain the ways that β-selection and γδ-selection, in various versions, can diverge from each other.

Cytokine-dependent and Stage-dependent Access to the T-Cell Receptor-γδ Loci

T-Cell Receptor-γ and T-Cell Receptor-δ Gene Loci

The ability to make a successful TCRγδ complex actually depends on the regulated status of rearrangements at as many as five distinct TCR J-C target loci in the mouse, because of the structure of the TCRγ and TCRδ gene complexes (Fig. 13.5A, B). First, much of the combinatorial diversity of the mouse TCRγδ complex comes from rearrangements of TCRVδ, Dδ, and Jδ segments. However, because all the TCRδ elements are sandwiched into the TCRα genomic locus between the Vα segments and the Jα segments, any TCRα rearrangement on the same chromosome would delete the whole TCRδ complex. Thus γδ cells need both to rearrange δ successfully and to suppress any rearrangement of the TCRα locus, immediately and permanently. Second, a special "enhanced allelic exclusion" mechanism is needed to establish expression of a unique TCRγ in a given T cell, because there are actually three different "subloci" that can serve as rearrangement sites to encode TCRγ in the mouse, albeit closely linked on the same chromosome. Each sublocus has its own Jγ and Cγ that can serve as a rearrangement target for its own dedicated Vγ segment or segments. Two of the subloci, those containing Cγ4 and Cγ2, each "own" only a single V segment and a single J segment, so that the only source of diversity in their rearrangements is junctional. The third sublocus, the one containing Cγ1, also has only one Jγ but can rearrange it to any of four Vγ's. In order to establish a unique TCRγδ, the ordering of rearrangements at each of these distinct subloci must be controlled so that only one yields a productive rearrangement. This split locus organization also makes it noteworthy that TCRγ RNA expression from any of these loci is repressed if the cell should adopt a DP fate instead.[71–73]

Special Role for Interleukin-7R Signaling

One major regulatory unifier for the TCRγ complex is the positive input all its subloci appear to require from IL-7R signaling. In mutant mice defective in IL-7 or IL-7R, one of the first abnormalities noted was their profound defect in TCRγδ cell generation. While IL-7R signaling contributes to survival and expansion of αβ-lineage cells generally, it also has an indispensable role in specification of γδ-lineage cells. At least part of the molecular explanation is that IL-7/IL-7R-activated Stat5 transcription factor is needed to bind to regulatory sites in the TCRγ locus, to open the gene segments for rearrangement.[110–113]

γδ Selection

Like β-selected cells, TCRγδ cells are selected by TCR complex triggering within the DN2-DN3 stages. Some emerge from precursors that had also opened their TCR Vβ loci for rearrangement and might thus have been β-selected instead. Under some conditions, as described subsequently, there even seems to be some overlap, with TCRγδ complexes occasionally being able to trigger a low-level β-selection response, and some "TCRγδ lineage" cells revealing a cryptic productive TCRβ rearrangement as well.[114] Both the eligible cells and the signals that trigger the two kinds of selection responses can therefore be extremely similar. However, the fate of cells undergoing TCRγδ-selection is considerably different from that of cells undergoing β-selection, and this remains only partially explained.

The γδ-Selection Program

The consequences of γδ-selection for rearrangement and expression of different TCR loci are reversed from those of β-selection, with TCRα rearrangement continuing to

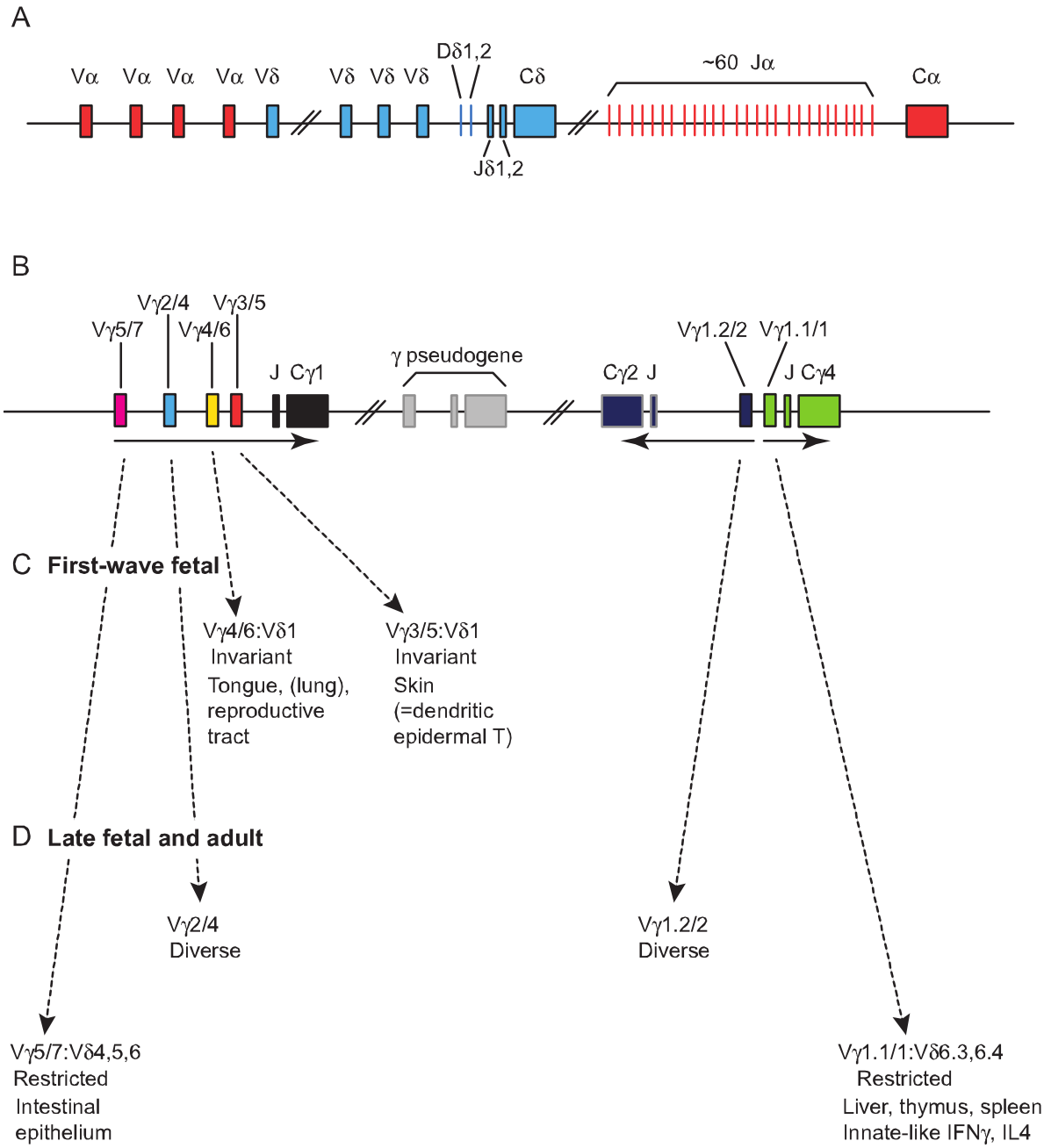

FIG. 13.5. Genetic Loci Involved in T-Cell Receptor (TCR)γδ Cell Generation and Distinct Developmental Assignments. TCRγδ cell generation in the mouse depends on the rearrangement status of the TCRδ/TCRα composite locus and the three functional TCRγ subloci. **A:** TCRδ/TCRα loci in the mouse: only a few of the many Vα, Vδ, and Jα segments are shown. **B:** TCRγ subloci: the V and J segments within the locus are shown in full, though distances are not to scale. Directions of transcripts are shown by *arrows*. Two different nomenclatures are commonly used for the TCRγ gene segments, those of Heilig and Tonegawa[280] and of Garman, Doherty, and Raulet.[281] Here, V segments are identified with both numbers, separated by a slash: (number from Garman et al.[281])/(number from Heilig and Tonegawa[280]). **C:** Fetal-specific timing of rearrangement and specific developmental fates of cells expressing invariant receptors using the indicated Vγ segments from the JCγ1 cluster. These TCRγ chains are paired with invariant TCRδ chains using only the Vδ1 segment for rearrangement. **D:** Specific developmental assignments for cells using other Vγ segments for rearrangement at later fetal and postnatal stages. Although cells using the Vγ5/7 together with Vδ4, 5, or 6 and cells using Vγ1.1/1 together with Vδ6.3 or 6.4 TCRs show some variations in specificity, they are considerably restricted compared to other adult γδ T cells, correlating with their specific functional properties.[109,138]

be blocked and TCRγ transcription being allowed to continue in the γδ-selected cells in contrast to DP cells. Unlike β-selected cells, TCRγδ cells undergo only restricted proliferation, with no induction of RORγt, much rarer induction of CD4 or CD8αβ genes, and maintenance of a much higher ratio of Bcl2 to Bcl-xL. Selection of most γδ cells in mice is also much less dependent on sustained Notch signaling at the DN3 stage than β-selection, and entails much stronger upregulation of immediate-early response transcription factor Egr3 (a zinc finger transcription factor) and the E protein antagonist Id3.[72,102,103,115] The consequence may be greater or more durable viability: in one transgenic model TCRγδ-selection depends on the presence of the presumed target antigen in the environment[116,117]—a situation that would normally induce deletion of nascent β-selected cells.

Strength of Signal

One important model to explain these program divergences focuses on the difference between the TCRγδ complex and the pre-TCR complex in terms of the strength of signals that they transduce when they are assembled in the developing cells.[72,118] In mice, the TCRγδ complex appears to deliver stronger spontaneous signals than the pre-TCR complex, and this seems to have an "instructive" effect on the way the cell responds. There is evidence that experimentally reducing the signaling through the TCRγδ complex now enables the TCRγδ to direct DN3 cells to a β-selection program. Correspondingly, precocious (transgenic) expression of a full TCRαβ heterodimer at the DN3 stage delivers stronger signals than a pre-TCR complex, and this can direct many of the cells into a γδ cell fate, skipping the CD4, CD8, and RORγt expressions that define the DP stage.[119–121] Strength of signal appears to be more important than the identity of the TCR itself. More recent work has elaborated on this to imply that the ratio of TCR signal strength to Notch pathway signal strength may be the true deciding factor.[122]

These observations apply to the divergence between DP cell selection and selection of most γδ cells in the young adult mouse. However, there is more than one way to become a γδ cell, and the γδ fates have great developmental and evolutionary diversity. Furthermore, there is evidence that many of the cells undergoing γδ-selection need not start from the same developmental state as the cells that embark on the β-selection program.

More than T-Cell Receptor Signal Strength: Developmental Factors in T-Cell Receptor-γδ Cell Specification

Stage-dependence of T-Cell Receptor-γδ Specification

If all T cells were undecided until the DN3 stage, when RAG1/RAG2 activity reaches its first peak and most Vβ-DJβ rearrangement occurs, then there should be no difference in the ratio of αβ:γδ progeny from individual T-cell precursors tested from DN2a stage until late DN3 stage. However, Kang, Volkmann, and Raulet pointed out that a subset of DN2-DN3 cells with high IL-7R expression was much more likely than the average to produce TCRγδ progeny as well as TCRαβ progeny; cells with lower IL-7R made TCRαβ progeny only.[123] These highly efficient TCRγδ progenitors are mostly DN2, not DN3. Thus, by the time they reach DN3 stage, even before productive TCRβ rearrangement, many cells have already lost much of their potential efficiency as γδ precursors. In part, this may reflect the declining IL7R signaling intensity that cells appear to experience from DN2 to DN3 stage, as a precondition for TCRαβ lineage development.[100,124,125] Not just signal strength, therefore, but also developmental stage and cytokine signaling context at the time when the first TCR signal is received may distinguish most β-selected cells from most γδ-selected cells. It is interesting that the time at which the "TCRγδ window" closes, together with the relative effects of Notch signaling on the two fates, are evolutionary variables that are different in human and mouse αβ/γδ lineage choice.[21,22,122]

Distinct Genetic Requirements for β-Selection and γδ-Selection

This implies that TCRγδ precursor activity is maximal close to the time of T-lineage commitment itself. Recall that one event that normally divides uncommitted DN2a cells from committed DN2b cells is the induction of Bcl11b. Bcl11b function is indispensable to make cells eligible for β-selection and to sustain their survival into the DP stage.[63,64,126] However, it is not essential for expression of *Rag1* and *Rag2*, nor for the expression of IL-7R. Remarkably, Bcl11b-knockout cells retain the ability to generate a modest number of T-cell progeny, provided that these are TCRγδ cells.[63,127] Thus, there must be some pathway to make at least certain kinds of TCRγδ cells that is open so early that it can bypass the standard commitment machinery, perhaps never using the functions that would be needed to set up a true TCRαβ versus TCRγδ lineage choice.

Indeed, γδ cells can be generated in a number of mutant conditions that are prohibitive for T-cell development.[22] Severe inhibition of E protein activity[128,129] or withdrawal of Notch signaling[102,115,130–133] as already noted can block αβ lineage development much more severely than γδ-cell development in mice. There appear to be broader differences in central features of cell biology and epigenetic regulation between TCRαβ and TCRγδ programs, as well. Deletion of key epigenetic regulators Brg1 and DNA methyltransferase 1 by Lck-Cre (in DN2-DN3 stage) was found to inhibit αβ lineage cells more than γδ lineage cells, though in these cases deletion might have occurred too late to affect all γδ precursors.[134,135] More remarkably, γδ but not αβ lineage cells are generated in mice with germline mutations affecting the Polycomb Group chromatin regulator Bmi1 or the ribosomal protein L22.[136,137] Some of these differences may reflect the special requirements for extensive proliferation at β-selection that do not apply to the milder response of γδ-selection. However, the effect of Bcl11b loss can also hint that the differences could trace back to different times and modes of commitment.

Multiple Lineages of T-Cell Receptor-γδ Cells

TCRγδ fate can be any one of multiple different developmental programs[138] (Fig. 13.5C,D). The first evidence for this related to the "first wave" fetal thymocytes. These are

not a generalized TCRγ population; they comprise two sublineages that share an invariant TCRδ rearrangement and pair it with an invariant TCRγ rearrangement at the Cγ1 sublocus, in one case using Vγ3/5 and in the other case using Vγ4/6 in Figure 13.5 (see figure legend for nomenclature of Vγ segments). These two TCRγδ receptor T-cell classes have distinct assignments in the body, the Vγ3/5 cells emerging from the thymus to patrol the skin epidermis (as "dendritic epidermal T cells") and the Vγ4/6 cells to patrol the tongue and reproductive tract. Both types of TCR rearrangements are limited to fetal life. A third Vγ segment at the Cγ1 sublocus (Vγ5/7) is used in a similar way in cells targeted to the gut epithelium, as a major class of TCRγδ+ intraepithelial lymphocytes. These Vγ5/7 TCRγδ intraepithelial lymphocytes are also distinct in that they can arise not only from intrathymic progenitors but also from progenitors developing in a nonthymic site.[139] Clearly, these early waves of TCRγδ cells have distinct homing specificities as well as notably sublineage-specific, invariant recognition potentials.

The Vγ3/5 cells are preprogrammed to use this Vγ segment for rearrangement because the earliest fetal thymocytes maintain a high level of the E protein antagonist Id2, and this Vγ segment is specifically opened in cells that have low E protein activity.[140] Normally, a high Id2:E protein ratio is a hallmark of NK cells, which these cells are not. Nevertheless, they support their growth with another distinctive NK-like feature, namely the IL-2/IL-15R complex (a dimer of CD122:CD132, also called IL-2/IL-15Rβ:γc).[141] In the Vγ3/5 first-wave cells, signals through this receptor complement and synergize with IL-7R signaling to promote their robust early dominance. As a result, in these cells and in the intestinal-targeted Vγ5/7 cells only, the Stat5 needed for TCRγ rearrangement can be activated by the response to IL-15 via IL-2/IL-15R:γc, in place of IL-7/IL-7R interaction.[142] It is interesting that cells of this type are especially enriched in populations derived from Bcl11b-knockout precursors.[59]

In the past 5 years, distinctive sublineages have also been recognized among adult TCRγδ cells (see Fig. 13.5D). Two other TCR Vγ segments, those used with Cγ4 and Cγ2, can also be associated in a preferential way with particular γδ functional subsets. The Vγ1.1/1-JCγ4 TCRγ chain is associated with an IFNγ-producing, NK1.1+ functional subset, whereas the Vγ1.2/2-JCγ2 TCRγ is often associated with an IL-17–producing, CCR6+ subset.[138] Cells using Vγ1.1/1-JCγ4 chain tend to pair it with a TCRδ chain that uses Vδ6.3 or Vδ6.4 with limited junctional diversity, most likely indicating yet another stereotypical antigen-recognition specificity. Strikingly, the cells that use this receptor have a distinctive set of developmental requirements that split them from all other TCRγδ cells. In contrast to other murine γδ cells that emerge preferentially from conditions of low E protein activity, due to high Id3 (most γδ cells) or Id2 (fetal first-wave γδ cells), these "Vγ1.1/Vδ6.3" cells cannot develop unless there is ample E protein activity. Furthermore, they resemble the NKT cells of the TCRαβ type in other aspects of their developmental requirements. They express both IFNγ and NK1.1 and also have a specific requirement for the transcription factor PLZF (promyelocytic leukemia zinc finger [PLZF], also called Zbtb16).[143–146] As discussed in a later section, this could represent an "innate-like" subset of TCRγδ cells with its own distinctive set of roles.

The demarcation of these different TCRγδ cell subsets is new enough so that how much their different properties are results of maturation from different precursors and how much they are effects of particular TCR-mediated signaling experiences during selection and antigen responses is still unresolved.[138,144,147–149] To the extent that many TCRγδ cells use TCR of very limited diversity, it is likely that they are pretargeted to interact with particular structures of evolutionarily selective importance. However, it is clear that the choice of being a TCRγδ cell rather than a TCRαβ cell opens the door to a rich spectrum of developmental pathways and immunological roles.

POSITIVE VERSUS NEGATIVE SELECTION OF T-CELL RECEPTOR-αβ CELLS: MORE THAN A BINARY SWITCH

The highly diverse TCRαβ repertoires generated in DP cells pose a different challenge to the goal of producing self-tolerant mature T cells that can recognize foreign antigens and produce appropriate effector molecules to orchestrate the immune response. Because TCRαβ specificities are randomly generated, developing T cells must be selected for their ability to respond appropriately to host MHC molecules. This is achieved through positive selection in the cortex, a process in which cortical thymic epithelial cells (cTECs) deliver a survival signal to thymocytes that recognize self-peptide and MHC complexes. Thymocytes that are unable to recognize these complexes fail to receive the survival signal and thus die by a process known as death by neglect. Interestingly, DP cells selected on a particular class of MHC molecules maintain their recognition specificity as they mature and migrate to the periphery.

Positive selection is a test for the basic structural integrity of the TCRαβ complex made as an outcome of productive TCRα rearrangement and for its ability to interact with self-MHC. However, beyond these criteria it is not stringent in its requirement of TCR specificity. A single peptide-MHC complex can select DP cells with several TCR specificities, and several different peptide-MHC complexes can select a single TCR as long as they all have a low-affinity interaction with the selecting complex.[150,151] This is important as all positively selecting cells only see self-antigens in the thymus, and it is possible that they could react strongly and be activated when a foreign peptide is displayed in the same MHC context in the periphery. The MHC exhibits both polymorphism and polygeny, and this is thought to allow the immune system to respond to several different, fast-evolving pathogens. Moreover, the next step of negative selection will weed out any cells that are excessively activated in response to self-antigen presentation in the thymic medulla. In fact, as we will see later, positive selection of some self-reactive cells is imperative for the formation of T_{reg}s. Thus, positive selection ensures that only immunologically useful TCRs (ie, those that recognize the particular combination of MHC molecules expressed in an individual) are selected for further development.

An inevitable outcome of the random generation of TCR diversity and the relatively promiscuous positive selection process is the survival of cells that can be highly reactive to both foreign and self-peptides bound by self-MHC. TCR-positive cells go through an additional checkpoint known as clonal deletion or negative selection in the medulla, which shapes the final TCR repertoire by pruning out self-reactive cells. In the process, they repetitively test their TCRs for self-reactivity through interactions with medullary epithelial cells (mTECs) and DCs expressing a huge range of self-peptide-MHC complexes. Negative selection results in the apoptotic death of cells with high-affinity TCRs for at least one of the self-antigen-MHC complexes. However, not all thymocytes that react with self-peptide-MHC with high affinity die during negative selection. Instead, some self-reactive thymocytes survive and develop into T_{reg}s that are potent dominant suppressors of autoimmune T cells, which also arise in the thymus (see following discussion). Negative selection and T_{reg} development, together known as central tolerance mechanisms, ensure that the final TCR repertoire is self-tolerant.

Timing and Anatomical Sites

DP thymocytes have only a 3-day lifespan on average before they die by neglect, and this creates an effective deadline for positive selection. Throughout this time they actively carry on V-J rearrangement of the TCRα locus. This starts as soon as RAG expression resumes after β-selection and sometimes even before the cells finish proliferating. Expression of an in-frame TCRα chain replaces pre-TCRα in complexes with TCRβ, enabling the cells to be auditioned for positive selection immediately based on the interaction with ligands on the cortical epithelial cells. There is no allelic exclusion of TCRα gene rearrangement; the process is terminated either by positive selection signaling, which finally shuts off RAG expression, or by cell death. Individual cells can rearrange the α-chain genes on both chromosomes, not only once but many times, because the locus offers more than 50 possible Jα segments as well as Vα segments in a permissive topology.[152] Rearrangement tends to begin with Vα and Jα elements centrally located near the TCRδ complex, working outward to rearrange more 5′ Vα and 3′ Jα segments until a product of rearrangement is made that mediates positive or negative selection, or until the cell dies.

Although TCRα gene rearrangement is halted by the first successful TCR engagement, the process of positive selection is more than the initial contact with MHC ligand, and in fact TCR interactions with peptide-MHC must continue for several days in order for the process to be completed. As we will see later, this requirement for ongoing or repeated TCR signaling after the first responses to positive selection signals is a crucial element of the mechanism that determines the CD4/CD8 lineage choice.

The sequence of events triggered by positive selection includes little if any proliferation, in contrast to β-selection. CD69 is upregulated, RAG genes are turned off permanently, CD5 levels increase, and TCR/CD3 complexes are stabilized so that their surface expression increases in parallel. Concomitantly, expression of the chemokine receptor CCR7 is turned on. This enables the cells to migrate from the cortex to the medulla, where they can be screened by negative selection as well as maturing to acquire effector function.[2,9]

While in the medulla, thymocytes undergo several changes that lead to their final maturation. Several peculiar features of DP cells, imposed during β-selection, are reversed. Glycoprotein processing is altered back to a more "normal" pattern, so that new glycoproteins are once again fully sialylated. The cells regain the Bcl-2 and class I MHC expression that they had lost at β selection, and they begin to recover the functional responsiveness that had disappeared at that time. As this proceeds, the cells eventually become resistant to negative selection, and they downregulate CD24 (heat-stable antigen) as TCR/CD3 levels rise even higher and CD69 expression finally subsides. Importantly, activation of PI 3-kinase and Akt diminishes, allowing the quiescence factor FoxO1 to localize to the nucleus, where it can activate an emigration program.[2] FoxO1 target genes include the transcription factor Klf2, which turns on expression of L-selectin and the emigration receptor itself, a G-protein coupled receptor for the glycolipid sphingosine 1-phosphate.[153–156] Thus, the cells are enabled to leave the medulla and migrate out to the periphery. The TCR upregulation, rescue from death by neglect, release to the medulla, and functional maturation events appear to be common to all positively selected thymocytes.

The exact stage at which negative selection is initiated has been a matter of debate. Using TCR transgenic model systems, clonal deletion was originally found to occur at a range of stages from the DN or DP stage of T-cell development in the thymic cortex to the SP stage in the medulla. However, the interpretation of these results for normal thymocytes remained controversial because of the early timing and/or high level of transgenic TCR expression relative to normal T cells.[157,158] Several lines of evidence suggest that under normal conditions, negative selection may be predominantly separated from positive selection both developmentally and spatially. In a transgenic TCR model system where the TCR was expressed with normal timing, deletion was found to predominantly occur in SP cells in the medulla.[159] Moreover, CCR7-directed migration to the medulla was recently shown to be necessary for negative selection against tissue-restricted antigens (TSAs).[160]

During positive and negative selection, cells migrate through the cortex and medulla in distinct patterns that reflect selection and progression to the next stages of development. Thus in the cortex, positively selecting cells survey self-antigen presenting cTECs by a "random walk" at low speeds of 3 to 8 μm/minute. Once a positively selecting signal is received and thymocytes upregulate CCR7, they stop surveying the cortex and, presumably pulled by the chemotactic attraction to CCR7 ligands expressed by mTECs, migrate in a highly directed manner to the medulla. CCR7 is not only important for the directional migration of SP thymocytes, but it is also important for the accumulation of SP cells in the medulla. In the medulla, they resume their random walk but at a much higher speed of 20 to 25 μm/minute. Any particular self-antigen may be expressed only by 1% to

5% of mTECs, and this high-speed scanning is thought to help the thymocytes in seeing all possible self-peptides expressed in the medulla. Upon encountering a negatively selecting antigen, SP cells slow down, give up their random walk again, and restrict their movement to confined areas but continue to survey additional peptide MHC complexes. Thus, developing thymocytes effectively scan both the cortex and the medulla to increase their chance of encountering positively and negatively selecting ligands.[161,162]

Signal Strength and Signal Quality

Both positive and negative selection involve TCR- and Lck-mediated signaling cascades triggered in response to self-peptide-MHC complexes, but these processes result in very different outcomes (Fig. 13.6). While it has been known for a long time that cells that react with self-peptide:MHC with low affinity (and thus receive weaker TCR signal) are positively selected while those that react too strongly are negatively selected, most of our knowledge has come through transgenic TCR expression, and it has been difficult to demonstrate the signal strength perceived by polyclonal thymocytes undergoing selection in vivo. Recently, Moran et al. reported the development of a BAC transgenic Nur77-GFP knock-in reporter mouse that has made it possible for the first time to look at TCR signal strength on a stage-by-stage and cell-by-cell basis.[163] Nur77 is an orphan nuclear receptor that is a product of the *Nr4a1* gene, an immediate early response gene transcribed downstream of TCR engagement.[164,165] Importantly, upregulation of Nur77 is directly proportional to strength of the activating TCR signals. Earlier work has shown that while positive selection causes a 2-fold increase in Nur77 expression, negatively selecting cells expressed Nur77 at 10-fold higher levels.[166] Similarly, Nur77-GFP expression in vivo was found to be much higher in cells undergoing negative selection as compared to positively selecting cells, thus demonstrating directly that negative selection is the result of very strong TCR signaling.

While it is not fully understood how these signals give such different outcomes, several factors contribute. Combined triggering of TCR and costimulatory receptors such as CD28 and CD40L strongly favors a cell death response by purified DP cells and incompletely mature single-positive cells alike.[6] Such costimulation may activate distinct signaling mediators in the cell, or provide an altered balance of signals, not only act to amplify perceived TCR signal intensity. For example, research over the past several

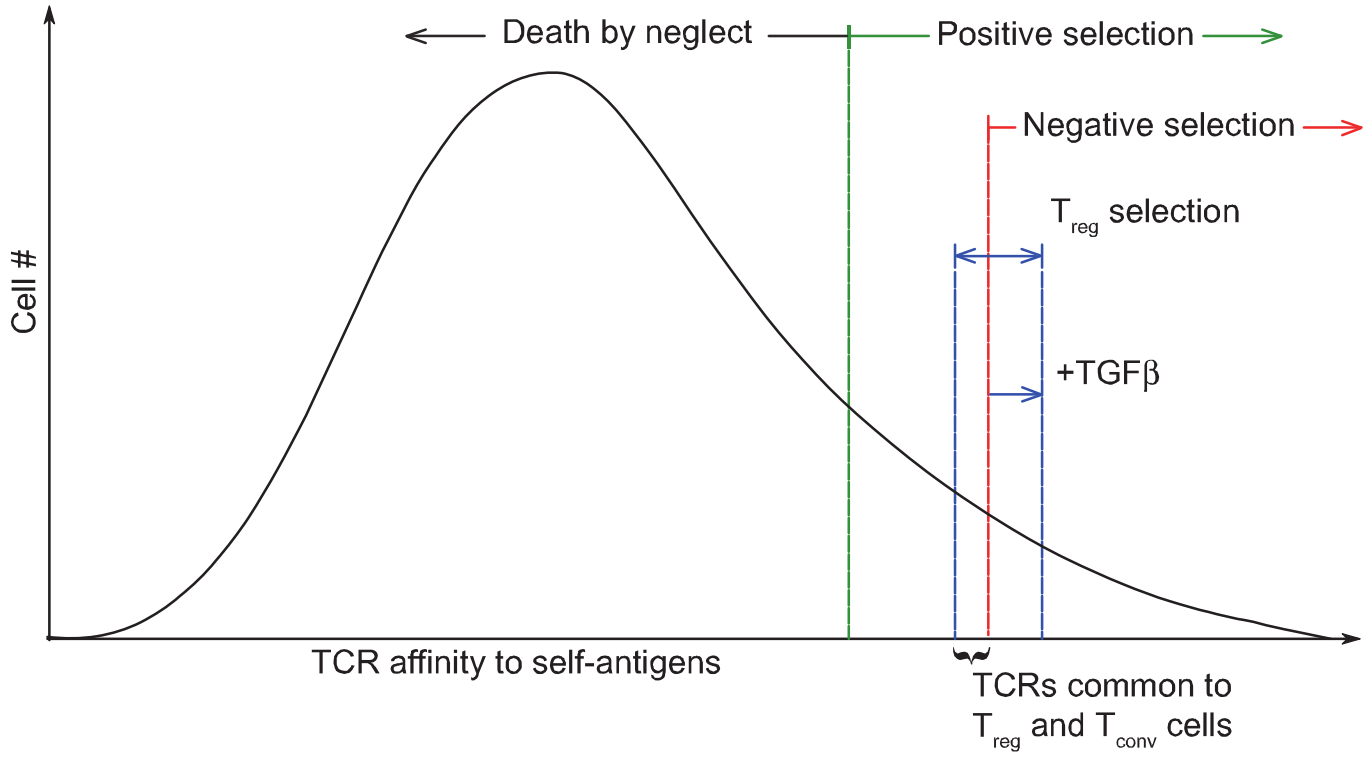

FIG. 13.6. T-Cell Developmental Fate is Determined by the Affinity to Self-Antigens. Majority of thymocytes in normal wild-type mice express T-cell receptors (TCRs) that do not recognize self-antigen:major histocompatibility complex complexes with sufficient affinity and hence die due to neglect while a minority are positively selected (*dashed green line*). However, only those positively selected cells that escape later negative selection can mature into conventional effector T cells (T_{conv}). During negative selection, thymocytes that recognize self-antigens with strong affinity (*dashed red line*) undergo clonal deletion. T_{reg} selection occurs in a narrow window of TCR affinity, (*dashed blue lines*), which shows some overlap with net positive selection giving rise to TCRs represented in both conventional CD4+ T cell (T_{conv}) and regulatory T (T_{reg}) cell populations.[245,246,248] As shown in the figure, TGFβ is important in rescuing T_{reg} cells as well as thymocytes in general from negative selection.[221]

years continues to highlight the essential role of differential activation of the mitogen-activated protein kinase (MAPK) pathways during positive and negative selection. Signals transduced during positive selection appear to be particularly dependent upon calcineurin and ERK, while negative selection depends more upon JNK and p38 activation, although ERK also appears to be activated. However, it seems that weak TCR signals during positive selection result in low, sustained ERK activation, while strong negative selection signals result in a transient burst of ERK activation and JNK and p38 activation. It is also reported that during positive selection, activated Erk remains compartmentalized in the Golgi membrane as opposed to its negative-selection-specific localization at the plasma membrane. Differential ERK activation patterns are functionally important, not just reporters for the difference between positive and negative selection signaling, for double knockout of Erk1 and Erk2 severely reduced positive selection although it did not show any effect on negative selection.[153,167,168,168a] Additionally, while the loss of Grb2, an adaptor protein that mediates Ras-MAPK activation downstream of TCR engagement, results in a severe defect in both positive and negative selection, the absence of Grb2-associating protein, Gasp (also known as Themis: thymocyte-expressed molecule involved in selection) causes a defect only in positive selection.[169–171] Identifying signaling molecules that act exclusively or differentially during positive or negative selection will be important for discovering specific differences in the pathways that lead to such strikingly different fates.

The difference may not simply be based on signal strength. The physiological context changes between positive selection, occurring in the more immature DP cells in the thymic cortex, and negative selection, occurring typically, but not exclusively, in more mature positively selected SP T cells in the thymic medulla. These SP T cells are negatively selected within a network of mTECs and DCs that not only express an expanded array of self-peptides and MHC complexes but also express costimulatory molecules such as CD80, CD86, and CD40 that are rare in the cortex. Furthermore, as a result of positive selection, these SP T cells have undergone major changes in differentiation programming as CD4 or CD8 cells. The higher levels of surface TCR and other changes as a result of positive selection, such as upregulation of CD5 and restored sialylation of surface glycoproteins are likely to change sensitivity of SP cells to high-affinity TCR signals. Thus, the different developmental states (DP versus SP), levels of surface TCR and other signaling components, gene expression programs, and microenvironment of the T cells, as well as the differing arrays of presented antigens in the cortex versus medulla, are all factors that likely contribute to the different outcomes of high- versus low-affinity TCR-peptide-MHC interactions leading to negative versus positive selection.

Anatomical Differences in Antigen Presentation

Cortical epithelial cells provide a rich source of MHC class I and class II surface complexes with a notable lack of costimulatory molecules for T cells. These features of the cTECs are important because at the DP stage as already noted, costimulation leads to clonal deletion instead of activation. Thus, the cortical epithelial microenvironment provides a uniquely forgiving testing ground for newly generated TCR recognition specificities. In addition, however, it may present a qualitatively distinctive set of antigens for positive selection. Recent work has revealed that cTECs uniquely express a novel catalytic subunit "β5t" of the 20S proteosome, a multisubunit protein complex that cuts proteins into peptides, some of which will eventually be displayed on MHC I molecules. While cTECs also express a low level of the immune-cell-specific β5i subunit, the expression of the β5t subunit is essential for the selection of immunocompetent CD8 T-cell repertoire. Peptides that bind MHC I molecules in other cells are generated by the chymotrypsin activity of the canonical β5 subunit, which generates peptides with hydrophobic residues at their C terminus. This is important because they form a hydrophobic anchor and ensure that the peptides fit tightly in the peptide-binding groove of the MHC. The β5t subunit has significantly reduced chymotrypsin activity, and it is not clear whether self-peptides that are processed in cTECs fit snugly in MHC I peptide-binding groove at all.[172–176] Additionally, the crystal structure of TCR-MHC complex shows that TCR V regions that make contacts with MHC are encoded by the germline Vβ and Vα elements themselves, in contrast to the sequences generated during VDJ recombination, which encode the TCR regions that contact the antigenic peptide. These reports have important implications for positive selection, because suboptimal binding of self-peptides to MHC I molecules on cTECs and the possibility that MHC recognition may be encoded in the genomic sequence of the TCRs together imply that the weak signal required for positive selection could be generated by TCR-MHC rather than TCR-peptide-MHC contacts.[177] A cTEC-specific difference is also seen in the peptide processing machinery responsible for loading MHC II molecules. Thus, unlike other bone marrow–derived APCs that express the protease cathepsin S, cTECs express cathepsin L, and the expression of this protease is crucial for the positive selection of CD4 cells.[178,179]

Negative selection depends on the presentation of self-peptides to deliver a strong TCR signal that initiates apoptosis in self-reactive thymocytes, i.e. cells that might react against self peptides if presented in the periphery by professional antigen-presenting cells. Accordingly, it is not surprising that an mTEC-specific difference in the peptide processing and presentation machinery has not yet been described for the medulla. mTECs do have special properties that make the medulla a highly efficient site of negative selection, and these are discussed in a later section.

Thus it is possible that the mechanisms described previously generate positively selecting peptides that could be entirely different from those that trigger negative selection, accounting for the difference in signal strength. A detailed investigation of the nature of MHC class I and II associated peptides displayed by the cTECs should therefore help in a better understanding of the differences between positive and negative selection.

POSITIVE SELECTION: CD4/CD8 LINEAGE CHOICE

Major Models for Functional Lineage Divergence

The mechanism that regulates the choice between CD4 and CD8 lineages has been the focus of intense research as well as debate for the last two decades. The central question has been how DP cells, which express both CD4 and CD8 co-receptors, make the decision to differentiate into CD4 or CD8 SP thymocytes. The CD4/CD8 fate decision is probably made in the cortex, during the iteration of positive selection signals. By the time the cells reach the medulla, their CD4 or CD8 identities appear to be set. Research from several groups has now provided a better understanding of how and when the CD4 versus CD8 lineage choice is made.

It is now clear that MHC class I– and II–restricted thymocytes experience positive selection signals differently. Firstly, it was observed that the affinity of Lck, a downstream kinase crucial for transmitting the TCR signal, for the CD4 and CD8 coreceptors is different. Thus, because CD4 has more Lck associated with it than CD8, DP cells expressing an MHC II-restricted TCR receive a stronger signal. The second difference lies in the duration of Lck signaling over subsequent developmental time. All positively selected cells are preprogrammed to downregulate CD8 irrespective of their MHC restriction and progress to the CD4+ $CD8^{lo}$ stage. These cells are still uncommitted, and their lineage fate is determined based on whether or not they receive further TCR signals. Downregulation of CD8 results in interruption of TCR signaling in MHC I–restricted thymocytes because now there is no CD8 to stabilize the class I MHC and TCR interaction. On the other hand, class II–restricted cells still express CD4, which allows continued TCR signaling upon subsequent contact with MHC II molecules and results in the development of CD4 SP thymocytes. Thus, while CD4 cells develop in a straightforward manner by downregulating CD8, CD8 SP development involves the downregulation of the CD8 receptor itself. In the subsequent stages, in the absence of further TCR signaling and presence of IL-7R-Stat5 signaling, MHC I–restricted cells downregulate CD4, undergo "coreceptor reversal" to upregulate CD8 and mature to the CD8 SP stage. A set of five enhancers, regulated in a stage specific manner, control the expression of CD8α; it is interesting to note that different enhancers are involved in the CD8 downregulation in postselection thymocytes and its re-expression in CD8 SP cells. This allows for coreceptor reversal by the induction of lineage-specific factors downstream of IL-7R signaling that are silent in uncommitted cells and thus adds another layer of transcriptional regulation to the lineage choice decision.[180–182]

The major hypotheses originally proposed to explain the lineage choice decision can be classified into two main categories. 1) The "instructive" models were based on the proposal that MHC class I and II signals are qualitatively different and that TCR and CD8 or CD4 interaction with these molecules "instructs" the cells to differentiate to CD8 and CD4 lineage, respectively. 2) The "selective" or "stochastic" models posited that CD4 and CD8 lineage choices are made even before the cells interact with MHC molecules and that the interaction with MHC merely selects the precommitted cells by delivering a survival signal. In one version, stochastic events were thought to give rise to CD8 and CD4 T cells with mismatched TCR coreceptor pairs, which would eventually die due to neglect. However, the experimental evidence required to support either of these models was lacking. Firstly, in certain TCR transgenic models, MHC I–restricted cells could develop into CD4 cells and conversely CD8 cells could be class II MHC–restricted based on the selecting antigen, thus discounting the exclusivity of the signals these subsets received. Secondly, the efficiency of selection of TCR repertoire can be much higher than that predicted by stochastic matching of TCR specificity and co-receptors.

The experiments that resulted in the abandonment of these hypotheses gave rise to newer, more refined models that focused on the strength and more importantly on the duration of TCR signaling. Thus it was demonstrated using fetal thymic organ cultures in which, depending on whether TCR signaling was either sustained or interrupted, that thymocytes expressing the same transgenic TCR could be differentiated into CD4 and CD8 lineage, respectively. Finally, when the CD4 gene was placed under the control of CD8 regulatory elements so that CD4 instead of CD8 was downregulated in postselection thymocytes, MHC II–restricted thymocytes developed into CD8 SP cells. These experiments thus showed that signal strength was secondary to the more important factor, the duration of TCR signaling, in deciding CD4- versus CD8-lineage fate.[183,184]

Kinetic Signaling

The most recent refinement to the lineage choice hypothesis came in the form of the kinetic signaling model, which provides an explanation of the pathway (outlined previously) through which majority of thymocytes make the CD4/CD8 decision[182,185–188] (Fig. 13.7). Importantly, kinetic signaling agrees with previous models in that the duration of TCR signaling is important in lineage choice. It was formulated principally to incorporate the following experimental observations not explained by previous models: 1) the silencing of CD8 transcription in positively selected DP thymocytes resulting in the development of CD4+ $CD8^{lo}$ thymocytes that exhibit unequal surface expression of co-receptor molecules, and 2) the lineage noncommitted state of postselection CD4+ $CD8^{lo}$ thymocytes. Thus, it was proposed that transcriptional silencing of CD8 expression in thymocytes following a positive selection signal is employed as a mechanism to assess changes in the TCR signaling environment, which would distinguish between TCR/coreceptor complexes with class I MHC and those with class II MHC. If TCR/coreceptor signaling persists, the cells will differentiate into CD4 SP. If TCR/coreceptor signaling begins strongly but is then reduced, then upon receiving IL-7R or other γ_c chain mediated signals, positively selected MHC I–restricted thymocytes differentiate to the CD8 lineage. Recent experiments have now shown that IL-7R-Stat5 mediated signaling is crucial for coreceptor reversal and CD8 development. Elegantly, the emphasis

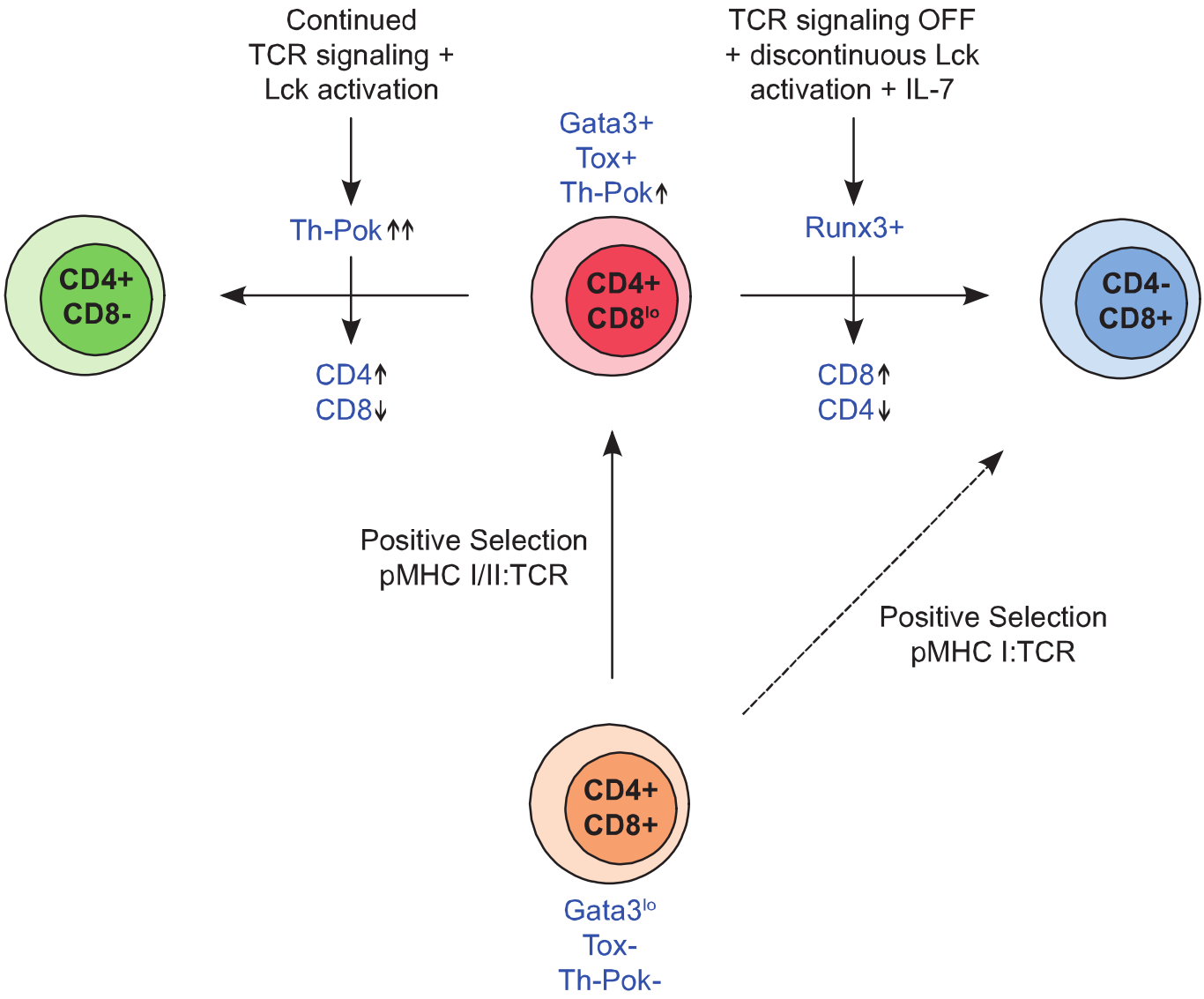

FIG. 13.7. CD4 and CD8 Lineage Differentiation. The majority of the positively selected DP cells (*peach*) differentiate into CD4 (*green*) and CD8 single positive (SP; *blue*) cells through the common CD4+ CD8^{lo} (*red*) uncommitted intermediate.[185] Transcription factors and coreceptor gene expression are shown in *blue*, whereas increases or decreases in their expression are denoted by *upward and downward arrows*, respectively. A small number of major histocompatibility complex I–restricted cells directly mature to the CD8 SP stage (*dashed arrow*) after positive selection. Transcription factor expression and signaling differences that enable these cells to take a more direct route to the CD8 SP stage remain to be identified.

on signal continuity or discontinuity rather than absolute strength enables the CD4/CD8 decision-making mechanism to operate independently of the decision to be positively or negatively selected.

The understanding of the genetic basis for CD4 commitment came about through the discovery of *Th-Pok*, a gene that enforces CD4 commitment in CD4+ CD8^{lo} thymocytes that experience sustained TCR signaling. Study of mice deficient in *Th-Pok* function also showed for the first time that positive selection and lineage commitment are two separate events[189,190] (see Fig. 13.7). The molecular circuitry that executes the CD4/CD8 lineage choice is becoming one of the best understood aspects of T-cell development.

Th-POK and GATA3 as CD4-Specific Drivers

Work from two groups independently led to the discovery of T-helper–inducing POZ/Kruppel-like factor (Th-POK, also known as c-Krox or Zfp67), a BTB-POZ domain containing transcription factor encoded by the *zbtb7b* gene, which is crucial for CD4 lineage commitment and fidelity. Kappes et al. identified a point mutation in the DNA-binding Kruppel-like zinc finger of Th-POK to be responsible for the complete absence of mature CD4+ T cells in the "helper-deficient" mouse model.[189] In these mice, while MHC II–restricted cells were positively selected, they did not mature into CD4 SP cells but were efficiently redirected to the CD8 lineage instead. Similar results were seen in a *Th-Pok* knockout mouse, thus identifying a central role for

Th-POK in CD4-lineage commitment and blocking CD8-lineage development. Bosselut et al. also identified Th-POK as a differentially expressed gene, which is highly expressed in the CD4 lineage as compared with the CD8-lineage cells.[190] Forced expression of Th-POK was able to block CD8 T-cell development completely and redirect MHC I–restricted cells into the CD4 lineage instead. Interestingly, lineage diversion in the absence or forced expression of Th-POK was accompanied by the acquisition of a gene expression pattern specific to the diverted lineage, indicating that Th-POK does more than simply regulate CD4 and CD8 expression.

Th-POK can enforce CD4-lineage commitment in a dominant fashion and is also required to maintain CD4-lineage–specific gene expression even after commitment. Continuous *Th-Pok* expression is required for maintaining CD4 lineage fidelity of developing as well as mature cells, as its absence leads to the upregulation of CD8-specific transcription factors and effector genes, including *Runx3*, *eomesodermin*, *perforin*, and *granzyme B*.[191] Thus the results obtained so far indicate that Th-POK represses CD8-lineage specific gene expression, in addition to providing positive input into several helper-specific genes, to enforce the CD4 fate on developing thymocytes.[192–197]

GATA-3, another transcription factor that is important for T-lineage specification of early DN-stage thymocytes, also plays a major role in CD4 development. GATA-3 is normally downregulated during the DP stage, and its expression can be reactivated by a strong TCR signal typically experienced by DP cells expressing an MHC II–restricted TCR. Several important observations point toward an important role for GATA-3 during CD4 specification and commitment. Firstly, DP-specific knockout of *Gata3* results in the blockade of cells at the CD4+ $CD8^{lo}$ stage, and these cells showed some low-level redirection to the CD8 lineage even in a β2-microglobulin–deficient environment where MHC class I surface expression is profoundly impaired. Furthermore, forced expression of GATA-3 blocks CD8 T-cell development and promotes CD4 development instead. On the other hand, on its own, it cannot redirect developing class I–restricted CD8 cells to the CD4 lineage, and thus is not sufficient to mimic Th-POK in gain of function experiments. A link between GATA-3 and Th-POK was established when positively selected GATA-3 knockout DP cells were found to be deficient in *Th-Pok* expression, and GATA-3 appears to regulate Th-POK directly because a GATA-3 binding site in the second intron of the *Th-Pok* gene was also detected. As expected, strong TCR signals are required in addition to GATA-3 expression for optimal upregulation of Th-Pok. GATA-3 seems to be upstream of Th-Pok because its increased expression in positively selected DP cells does not depend on Th-POK. However, because Th-POK overexpression cannot rescue CD4 development in GATA-3 knockout DP cells, it is clear that GATA-3 is not just required to upregulate *Th-Pok* expression. Instead, like Th-POK, GATA-3 has other important functions of its own in promoting the CD4 fate. Thus, in summary, while GATA-3 acts as a specification factor, Th-POK is the major commitment factor for the CD4 lineage.[192,198–201]

Runx3 as a CD8-Specific Driver

In the CD8 lineage, Runx3, a Runt domain–containing transcription factor expressed only in CD8 SP cells in the thymus, has emerged as the major factor required for lineage commitment. Another family member, Runx1, is expressed in DP thymocytes as well as in CD4 SP cells, but its expression is downregulated in CD8 SP thymocytes. Single knockout of Runx3 shows only a reduction in the number of mature CD8 SP cells in the thymus, but Runx1 was found to be upregulated in these cells, indicating that there may be some functional redundancy among these proteins. Indeed, double knockout of *Runx1* and *Runx3* displayed a severe and complete block in CD8 SP development, highlighting the crucial role of Runx activity in promoting this lineage. In this model, CD8 T cells were redirected to the CD4 lineage instead, and interestingly *Th-Pok* expression was found to be upregulated in DP cells at a stage even before positive selection. Thus part of the mechanism of Runx-mediated CD8 development may be through its role in repressing *Th-Pok* expression to a level that does not allow it to dictate the CD4 fate in these cells (see following discussion). There are some asymmetries between the roles of Runx3 and Th-POK in CD8 SP and CD4 SP development, respectively. Forced expression of Runx3 does not redirect committed CD4 cells to the CD8 lineage. Thus Runx3 seems to promote the CD8 fate by repressing *Th-Pok* expression, but not after high-level *Th-Pok* expression is already established.[202–205] This asymmetry makes sense in light of the different signal duration requirements for CD4 SP and CD8 SP development.

While both mature CD4 and CD8 SP cells express Runx3 messenger RNA, it is translated much more efficiently in the CD8 lineage. The difference seems to be in promoter usage: Runx3 is transcribed from the distal promoter in the CD8 lineage, which encodes an efficient translational start site, while the poorly translated proximal promoter isoform is expressed in the CD4 lineage. The distal isoform of Runx3 is transcribed in CD4+ $CD8^{lo}$ cells only after they receive an IL-7R–Stat5 mediated signal, and this may be important in activating the distal promoter.[182,192,202,206] Runx3 and Th-POK thus drive cells to the CD8 and CD4 lineages, respectively, and the gene networks that steer precursors to adopt these fates will be described in the following section.

Architecture of a Signal-Duration–Dependent Lineage Choice Network

The research that highlighted the role of Th-Pok and Runx3 as the major drivers of the CD4 and CD8 lineage fates, respectively, has shown that these factors antagonize each other's function and expression to establish lineage-specific gene expression profiles. However, these are not the only players, and indeed several other proteins play an important role in translating TCR and cytokine dependent signals to drive lineage choice[4,193,194,196,197,207–210] (Fig. 13.8).

For the CD4 lineage, a strong positively selecting TCR signal leads to the upregulation of *Gata3* in DP cells, which in turn activates the expression of Th-Pok. *Gata3* expression itself is mediated by Ras/MAPK and calcineurin pathways, and depends on the activity of Myb, a transcription factor

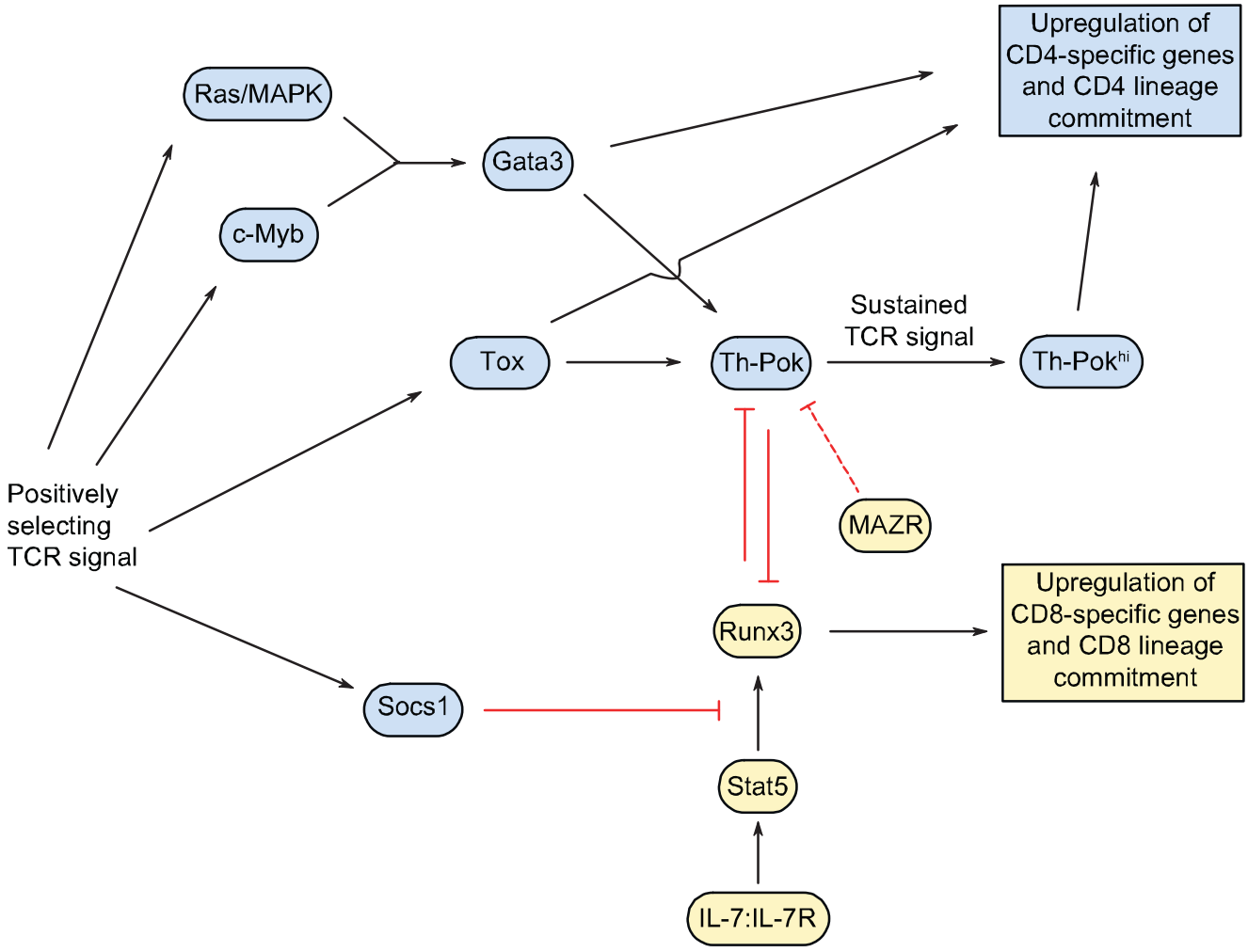

FIG. 13.8. Gene Network Involved in Regulating Commitment to the Cluster of differentiation (CD)4 and CD8 Lineages. Important components of the network that drive CD4 and CD8 lineage commitment following positive selection are shown in *blue and yellow boxes*, respectively. *Red lines* show direct or indirect antagonistic or repressive interactions, whereas *black arrows* denote positive input into specific genes. The mechanism by which MAZR leads to the repression of Th-Pok (*dashed red line*) is not well understood. See text for details.

activated downstream of TCR signaling, as in the absence of *Myb*, *Gata3* is not upregulated in CD69+ postselection thymocytes. Myb was also shown to regulate Gata3 expression directly by binding to the upstream regulatory region of *Gata3*.[211,212] Together with GATA-3, the high mobility group protein Tox is also important for optimal *Th-Pok* upregulation in CD69+ cells. *Tox* deficiency blocks development of all CD4+ lineages selectively while permitting CD8 development. Additionally, positively selected *Tox*-deficient thymocytes upregulate *Gata3* normally but fail to maintain normal Th-POK and surface CD4 expression. Thus it seems that Tox and GATA-3 work in parallel in the presence of strong TCR signaling to upregulate *Th-Pok* expression and advance the cells to the CD4+ $CD8^{lo}$ stage, which as stated previously, is still uncommitted to either lineage.[213]

From here on, presence or absence of continued TCR signaling determines the lineage fate. If these cells receive further TCR signals, it leads to an even greater upregulation of Th-POK whose activity ensures a total shutoff of the CD8 program. Firstly, Th-POK binds to the CD4 silencer element making sure that Runx-mediated repression of *Cd4* cannot take place. *Th-Pok* itself has two upstream Runx binding sites, through which it can be silenced in the CD8 lineage. When expressed at a high level, Th-POK binds to this element, an event that neutralizes Runx mediated silencing of *Th-Pok*. In addition to this, Th-POK weakens Runx3 gene expression by repressing the distal isoform of Runx3 that is crucial for its optimal translation and CD8 lineage promoting function. Lastly, Th-POK also maintains CD4 lineage fidelity by dominantly maintaining repression of important CD8-lineage–specific genes like *CD8α*, *Itgae*, *Nkg7*, *Cd160*, and *Prf1*, giving rise to stable, lineage-committed CD4 SP cells.[192,195]

It is not clear whether Th-POK expression is ever initiated in wild-type positively selected MHC I–restricted thymocytes. However, in TCR transgenic mice, Th-POK is upregulated to a much higher level in class II–restricted thymocytes.[190] Even if some Th-POK was induced in class I–restricted thymocytes in the wild-type situation, its effects could be reversible because class II–restricted CD4+ $CD8^{lo}$

thymocytes that express relatively higher levels of Th-POK still remain uncommitted to the CD4 lineage. Thus, positively selected CD4+ $CD8^{lo}$ thymocytes that do not receive any further TCR signal upregulate Runx3 expression, which drives them to the CD8 lineage. Activation of Runx3 expression in these cells depends on the signaling through the IL-7R, which is not expressed on DP cells but is upregulated in positively selected CD69+ thymocytes. Stat5 (and under some conditions Stat6 also), which are downstream of IL-7R signaling, were shown to be crucial for induction of the CD8-lineage–specific distal isoform of Runx3. This process in turn is negatively regulated by SOCS1, which extinguishes signaling downstream of the cytokine receptor and may play a part in keeping developing CD4 SP thymocytes refractory to IL-7R mediated signaling.[182] Once activated, Runx3 sets off a series of events that in turn block CD4 development. Most importantly, it silences *Th-Pok* and *Cd4* expression by binding to the silencer elements in these genes.[204,214] However, since Runx complexes are bound to the *Th-Pok* silencer even in Th-POK expressing cells, other as yet unidentified factors may be crucial to enforce Runx-mediated *Th-Pok* repression. The transcription factor MAZR or Zfp238, product of the gene *Patz1*, may also cooperate with Runx3 in repressing Th-POK expression, as *Patz1* knockout CD8 SP cells re-express some Th-POK and a small number of them are redirected to the CD4 lineage.[215] Lastly, Runx3 also promotes the expression of CD8-specific genes, thus driving the cells' commitment to that lineage.[192]

Thus, it is the outcome of the antagonistic interplay between Th-POK and Runx3 that determines the lineage choice of uncommitted precursors (see Fig. 13.8). However, the execution of these fates depend on some of their collaborators and target genes, and more factors contributing directly to CD4- and CD8-lineage differentiation probably remain to be identified. This is prompted by the striking observation that deficiency of both Runx activity and Th-POK still allows some CD4 and CD8 development to take place.[192]

NEGATIVE SELECTION

The phenomenon of negative selection of self-reactive T cells in the thymus was first demonstrated in response to endogenous superantigens and self-MHC.[216] Strong TCR signals transduced by self-reactive TCRs trigger apoptosis that appears to be mediated by upregulation and/or phosphorylation of a proapoptotic Bcl-2 family member, Bim, in collaboration with Bax and Bak.[217,218] Upregulation of the orphan steroid receptor, Nur77, also plays a role in this apoptosis.[219] The negative selection program is not only facilitated by TCR+CD28 costimulation in vitro; it has also recently been shown to require CD28 in vivo.[219a] Distinctive signaling pathways from TCR and costimulatory molecules that may activate these mediators are under intense investigation. In knockdown experiments using small interfering (si)-RNA, the serine-threonine kinase MINK has been reported to be involved in negative selection specifically, potentially linking TCR signals with JNK and p38, as well as Bim activation.[220] Recent data has also shown that TGFβ signaling provides a survival signal to thymocytes during the negative signaling window. TGFβRII−/− thymocytes showed an upregulation of the proapoptotic Bim, Bax, and Bak proteins, and this had an adverse effect on conventional T cell as well as T_{reg} maturation.[221]

The Special Role of the Medulla

The medulla is the specific site of two kinds of APCs, which are potent inducers of negative selection: mTECs and DCs. These cells express high levels of MHC and costimulatory molecules, both of which greatly facilitate negative selection, as well as being highly specialized for the presentation of a large array of peripheral self-antigens acquired by several unique mechanisms. The critical importance of the thymic medulla to central tolerance, but not SP cell maturation, was demonstrated in experiments showing that when T cells are prevented from migrating from the cortex to the medulla by the lack of the chemokine receptor CCR7 or its ligand, cells undergo normal maturation and export from the thymus, but the animals develop autoimmunity.[222] The autoimmune phenotype resembles a failure of negative selection, although faulty regulatory T-cell development (see subsequent discussion) has not been ruled out. This showed that central tolerance is, at least in part, dependent upon T-cell passage through the medulla and interactions with medullary cells.

The discovery of the transcription factor gene, *Aire*, which causes a rare autoimmune disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome, when mutated in humans and mice, confirmed the critical role of the medulla in negative selection.[9a,223–226] *Aire* expression is restricted to the $CD80^{hi}$ $MHCII^{hi}$ mature mTECs in the thymus where it regulates the transcription of several tissue restricted antigen genes and may also contribute to the development and turnover of these mTECs.[227,228] The importance of *Aire* function was shown by experiments in which the deletion of a single *Aire*-regulated tissue-restricted gene, coding for Interphotoreceptor Retinoid Binding Protein (*Irbp3*), led to an eye-specific autoimmune response when the deficient thymus was transplanted into an athymic, *Irbp3*-sufficient host.[228] AIRE interacts with several other transcription factors and related proteins, but the exact transcriptional mechanism through which it regulates its target genes is not known. Several lines of evidence indicate that *Aire* may control chromatin accessibility to allow transcription of repressed genes. First, the expression of tissue specific antigens (TSAs) in mTECs does not require transcription factors that are normally essential for their expression, and TSA levels are much lower than in the tissue to which their expression is normally restricted. Also, AIRE has the ability to bind H3K4me0, a histone mark associated with silent chromatin, which may help its recruitment and the subsequent activation of transcription of silent TSA genes.[229–232] Finally, a new population of *Aire*-expressing cells (extrathymic *Aire*-expressing cells) important in maintaining peripheral tolerance in adult mice was recently reported to be present in secondary lymphoid organs. Interestingly, AIRE does not upregulate the exact same set of TSAs in extrathymic *Aire*-expressing cells as it does in

mTECs, indicating that target specificity is determined by the transcriptional state of the cell in which it is expressed.[233] Thus *Aire*-mediated promiscuous expression of TSA genes by mTECs provides essential self-peptides required for the clonal deletion of self-reactive thymocytes. Recently, it was shown that *Aire* function is also required for the cross-presentation of TSAs expressed by mTECs.[234] Thus, while the expression level of an *Aire*-regulated transgenic ovalbumin construct was directly correlated with the negative selection of CD8 SP T cells with cognate TCRs, the deletion of ovalbumin-specific CD4 SP T cells was independent of its activity to regulate transgene expression. Interestingly, *Aire* seemed to regulate transfer of expressed ovalbumin from mTECs to intrathymic DCs that are important for CD4 SP negative selection.

DCs are more abundant in the thymic medulla than in the cortex and are very potent mediators of negative selection, even more so than mTECs. They do not express *Aire* and so do not express the huge range of tissue-specific genes that mTECs do. However, DCs have the ability to cross-present antigens obtained from various other cells. Medullary DCs can cross-present antigens expressed by mTECS; as stated previously, *Aire* expressed in mTECs plays an important role in the antigen transfer. DCs are also capable of acquiring antigens from the periphery, migrating to the thymic medulla for antigen presentation to developing T cells and inducing apoptosis in antigen-specific T cells. Thus, DCs in the medulla have access to antigens obtained in the periphery and indirectly from promiscuous gene expression by mTECs, as well as those they express themselves, for presentation to T cells.

In addition to their extraordinary capacity for acquiring and presenting self-antigens, mTECs and DCs also express costimulatory molecules such as B7 and CD40. CD28/B7 or possibly CD40/40L interactions appear to play an important role in negative selection, although neither is absolutely required for deletion. It is possible that in the absence of these molecules, very strong TCR signals and/or other costimulatory molecules may be able to provide the additional signal.[6,235,236]

T_{reg} Selection as an Alternative

T_{reg} cells are a naturally occurring population of rare Foxp3+ CD25+ thymocytes that are activated by self-antigens in the periphery and function to suppress autoimmune responses in a dominant fashion. Foxp3 is the lineage-determining factor for these cells, and its expression in conventional Foxp3- peripheral CD4+ T cells lead to their differentiation into functional T_{reg} cells. *Foxp3*, a forkhead winged-helix transcription family member, was initially identified as the mutated gene causing a severe autoimmune disease in humans, immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome, and a similar disease in scurfy mice. These mutations, as well as knockouts of the *Foxp3* gene, result in complete loss of T_{reg} cells.[237–241] Although the cortex can fully support the development of T_{reg} cells, it is thought that T_{reg}-cell differentiation correlates temporally and spatially with negative selection in the medulla.[225]

The linkage between T_{reg} development and negative selection is based on the common requirement of both processes for high-affinity TCR interaction with its cognate ligand (see Fig. 13.6). T_{reg} cells do not develop in mice that express a transgenic TCR specific for an exogenous protein unless the cognate ligand is also simultaneously expressed, and T_{reg} development in these mice is also accompanied by massive negative selection of self-reactive thymocytes.[242–244] Also, as compared to conventional T-cells, T_{reg} cells also have higher levels of the surface markers CD5, CTLA4, and CD25, whose expression level is directly proportional to the TCR signaling intensity. In the Nur77-GFP mouse model, T_{reg} cells show two-fold higher GFP levels as compared to conventional T cells, further supporting the inference that strong TCR signals are needed for T_{reg} development.[163] Thus, it is currently thought that TCR affinities for self-antigen that are required for T_{reg} differentiation are higher than those required for positive selection but lower than the affinity threshold that triggers negative selection (see Fig. 13.6). However, this presents a dilemma: if negative selection leads to the deletion of self-reactive thymocytes, how does this allow for the development of T_{reg} cells? While the mechanism is not fully understood, recent experiments have revealed many details of this process.

Sequencing of TCRs from transgenic mice that can generate limited TCR diversity has shown that the regulatory and conventional cells have equally diverse but largely distinct repertoires.[245–247] These experiments seem to suggest an instructive role for TCR signals in the development of T_{reg} cells.[248,249] However, competition between developing T cells with different TCRs and a limiting supply of selecting ligands may also play a role. Transgenic mice that only express a single TCR that was isolated from a mature naturally occurring T_{reg} develop only very low numbers of T_{reg} cells. However, when these TCR-transgenic bone marrow cells were injected into Rag knockout mice together with wild-type precursor cells, T_{reg} frequency was inversely proportional to the fraction of injected cells that were transgenic. Thus, intraclonal competition within transgenic thymocytes for rare selecting ligands could limit normal T_{reg} differentiation.[250] Furthermore, Nur-77 GFP induction in individual cells was also shown to be inversely proportional to the fraction of transgenic cells in a similar competitive assay as previously discussed.[163] This indicates that low number of thymocytes expressing a particular self-reactive TCR normally facilitates their interaction with rare T_{reg}-selecting ligands, and that availability of selecting ligands seems to be another important factor in T_{reg} development. Finally TCR avidity (TCR affinity × number of interactions) may also play a role in T_{reg} differentiation, as reducing the self-antigen–presenting capacity of mTECs by knocking down the expression of MHC II transcriptional activator CIITA resulted in decreased efficiency of negative selection but an increase in the number of regulatory T cells.[251]

Putting several lines of evidence together, a two-step model was proposed for intrathymic T_{reg} development[252,253] (Fig. 13.9). The simultaneous delivery of TCR and CD28 signals to CD4+ Foxp3− T_{reg} precursors advances their development to the CD4+ CD25+ Foxp3− stage. This stage is the

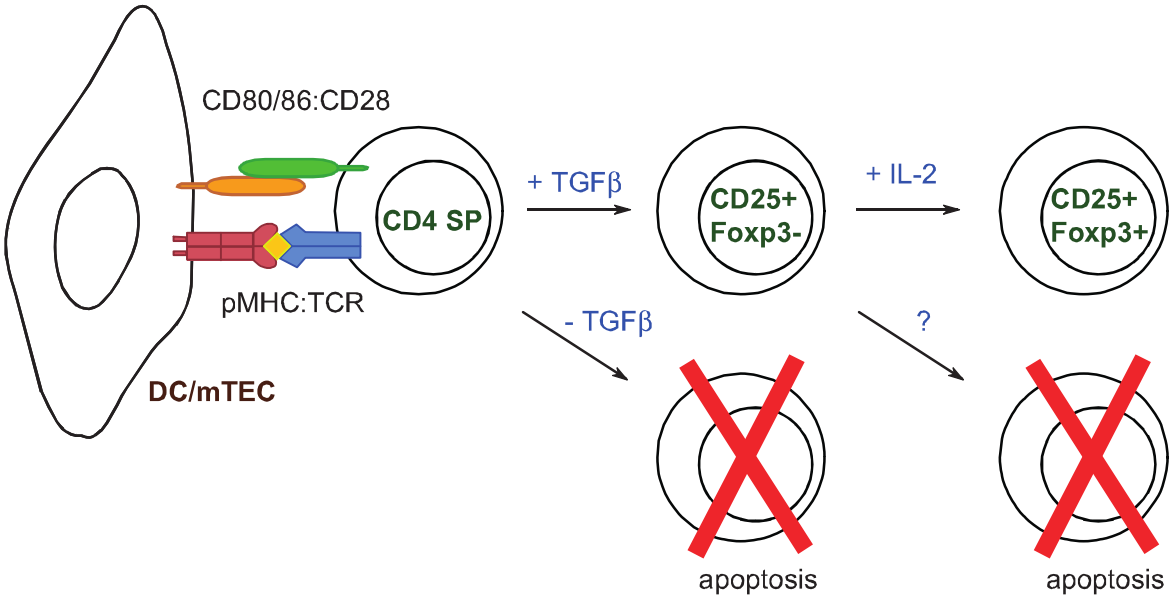

FIG. 13.9. Development of Regulatory T (T_{reg}) Cells. Surface receptors and cytokines (*blue*) important at each step in the two-step model of T_{reg} development are shown.[248,249] The efficiency of T_{reg} selection and the time and location of initial major histocompatibility complex contact on T_{reg} maturation are not known; although T_{reg} cells are found mainly in the medulla, the cortex has also been shown to fully support T_{reg} development.[282] Additionally, the question of whether and how much negative selection and T_{reg} developmental pathways overlap also remains unanswered.

immediate thymic precursor to the fully mature Foxp3+ T_{reg} cells. CD28 expression, CD28-enhanced Lck recruitment, and downstream signaling in the CD4 SP cell are important for this first step because deletion of CD28 or its ligands B7-1 and B7-2 leads to a severe reduction in T_{reg} numbers and proportion.[254–256] The NF-κB subunit c-Rel, which is activated by TCR and CD28 signaling, binds directly to the Foxp3 promoter and a conserved enhancer element, CNS3 (conserved noncoding DNA sequence 3), implying a direct transcriptional role.[257–261] c-Rel also appears to promote the earliest steps in T_{reg} development even before Foxp3 is detectably turned on.[262] The survival of cells that have received the TCR-CD28 signal also depends on TGFβRII-mediated signaling, which rescues these intermediates from Bim-mediated apoptosis.[221]

At this stage, these cells require a second signal through cytokines like IL-2, IL-7, and IL-15 that signal through the γc chain–containing receptors, with IL-2 playing the major role in the final maturation to Foxp3+ T_{reg} stage (see Fig. 13.9). Stat5b, a major transcription factor activated by signaling of the γc cytokine receptor chain, binds directly to the promoter and CNS2 of *Foxp3*, and mice expressing a transgenic, constitutively active version of Stat5 show a dramatic increase in T_{reg} numbers and percentage. This cytokine-dependent final maturation step is thought to be largely independent of TCR stimulation.[263,264] Once induced, Foxp3 suppresses alternate lineage genes and permanently establishes a gene expression pattern characteristic of T_{reg} cells. Although Foxp3 is not required for commitment to the Treg lineage, its continuous expression is important for T_{reg} function as a loss of its expression in mature T_{reg} cells leads to a loss of their suppressor function and their ability to produce effector cytokines including IFNγ, IL-17, and IL-4 etc. is restored[265–267] Thus akin to the role of Th-POK in conventional CD4+ T cells, Foxp3 acts as a dominant factor in the maintenance of the regulatory T-cell lineage.

"AGONIST SELECTION" OF INNATE-TYPE LYMPHOCYTES

Distinctive Features of Innate-Type Lymphocytes

The precedent of T_{reg} selection shows that strong TCRαβ interactions with agonist ligands can promote something other than death in the thymus, parallel to the TCRγδ lineage maturation programs that are triggered by strong agonist-mediated TCR signaling. However, the special conditions of DP cell physiology do not apply to DN2-DN3 thymocytes becoming TCRγδ cells nor to medullary CD4 SP cells becoming T_{reg} cells. Therefore, it has still been a surprise that a major class of T cells, the "innate-type" cells that include NKT cells, are also positively selected by strong agonist ligand and selected apparently from the DP population itself.

The distinguishing features of iNKT cells are a pairing of canonical effector function with canonical TCR rearrangement and recognition specificity.[268] These cells use a limited set of possible Vβ rearrangements—Vβ2, Vβ8.2, or Vβ7 (Vβ11 in humans)—together with an invariant pairing of Vα14 (Vα24 in humans) with Jα18. From the start, they appear to observe a different set of rules from most TCRαβ cells. Although their positively selecting ligands are nonclassical class I MHC rather than class II MHC, they predominantly become CD4+ or revert to a CD4– CD8– phenotype rather than becoming CD8 SP. They acquire a

chronically activated phenotype marked by CD122 (IL2Rβ) expression, CD44 expression, and NK receptor expression in a stepwise process from the DP stage, starting with the first cells that acquire the canonical receptors. Even within the thymus, these cells are spontaneously triggered to express cytokines that normally require multiple rounds of activation to be induced, IL-4, and IFNγ, and they can express them simultaneously, combining Th2- and Th1-like functions.

Promyelocytic Leukemia Zinc Finger and the Id-Driven Maturation Pathway

DP cells need to survive in a DP state for a longer-than-average amount of time in order to acquire the ability to express the invariant iNKT TCR.[269] The particular TCRα segments used in the canonical receptor are among the last ones that most DP cells use for rearrangement, because of the 5′ position of Vα14 and the 3′ position of Jα18. In any mutant that shortens DP lifespan (eg, loss of RORγt, HEB, BclXL), the iNKT TCRα chain does not have a chance to be made.[268,270] Once the receptor is expressed, however, and if its ligand is present in the thymic environment, the cells embark on their new program. In the process, they will make use of the signature transcription factors of Th1, Th2, and NK cells, as well as a canonical regulatory factor of their own, the zinc finger-BTB domain factor PLZF (or Zbtb16). This is the crucial regulator that confers on them an activated CD44-high phenotype as well as their distinctive "ready-to-go" priming for effector cytokine production,[271,272] the same factor that is also required by the "innate-like" Vγ1.1/Vδ6.3 γδ cells.

DP cells, upon expressing the invariant iNKT receptor, embark on a pathway first resembling standard positive selection to a CD4 SP lineage, including use of Runx1, GATA-3, and the transcription factor TOX. In the process, they downregulate CD24 normally, but activate a "survival-associated" pathway module including Egr2, Myc, and NF-κB activation, which drives a burst of proliferation (unlike the conventional positive selection program). The proliferation and emergence of the NKT cells is dependent on IL-15 and CD122 (IL-2/IL-15Rβ), unlike the conventional SP cells that primarily express IL-7 receptors. Following proliferation, the cells turn on PLZF expression, which endows them with effector function. After this, the cells can turn on T-bet, the NK-, CD8-, and Th1-associated factor that promotes IFNγ expression and enables them to acquire their distinctive NK receptor–positive phenotype. With the completion of the iNKT program, the cells leave the thymus, turning on Id2 that maintains their homeostasis in the periphery.

Special Selection Mechanism: Hematopoietic Antigen-Presenting Cells and Distinctive Signaling

The iNKT pathway offers a striking view of how use of distinctive signaling mediators to interpret TCR signaling can instructively determine the developmental outcome. The difference between positive selection of iNKT cells and other conventional TCRαβ cells starts with the APCs they interact with and also includes the channeling of TCR signals through a distinctive pathway. Whereas conventional positive selection is mediated by cTECs presenting peptides on classical class I and class II MHC antigens, NKT cell selection is based on recognition of glycolipids presented on nonclassical class I MHC antigens that are only expressed by hematopoietic cells, not thymic epithelial cells. In general, the main APCs for NKT cell selection are other DP thymocytes themselves. The homotypic cell-cell interaction that this entails also makes it possible for the cells to use a special kind of coreceptor, receptors of the SLAM family (mostly Slamf1 = CD150, or Slamf6 = Ly108), to mediate homotypic adhesion between the future iNKT cell and its developmentally similar APC. The engagement of SLAM family receptors recruits an adaptor that is not used in conventional positive selection, SLAM-associated protein (Sh2d1a), which efficiently couples TCR signaling with Fyn tyrosine kinase activation and protein kinase C . This is a fast track to NF-κB activation,[273] which may even occur preferentially with respect to conventional Ras/MAP kinase pathway activation.[274,275] The potentially altered quantitative balance between signaling pathways is a likely origin for the distinctive program that iNKT selection activates.

iNKT cells are only the best-studied representative of a number of distinct agonist-selected innate-like T-cell lineages. It appears that other subsets share many aspects of the iNKT selection pathway, including use of PLZF and a dependence on SAP and Fyn that separates all of them from most conventional T cells.[273,276] Thus, this is an alternate modality for positive selection that violates the simple picture that fate might be determined only by TCR avidity and developmental stage of contact.

CONCLUSION

The remarkable diversity of mature T-cell types and their potential for long proliferative lifespans offers a challenge to understanding their development in terms of a single commitment pathway. T-lineage commitment is a process that instills a profoundly durable and renewable cell type identity via stable expression of multiple T-cell genes, but enables the cell to keep exploring different variants of function. Past views of T-cell development centered on the need to develop a diverse and self-tolerant recognition repertoire, but increasing appreciation for the sophistication of innate immunity casts the T-cell agenda in a different light. Signaling does not need to be dependent on a single receptor in a plus/minus way, nor is the TCR unique in its ability to transmit quantitatively graded signals; innate immune cells use integrative signaling to adapt to various circumstances very effectively without clonally unique receptors. T-cell development itself produces many lineages that use stereotyped, practically invariant TCRs, and as these cells must consistently be positively selected by agonist in the thymus, it follows that the receptors that evolution has conserved for use by these cells must be self-reactive ones. Seen in this light, many T cells like innate cells must be important for the functions they link to their TCR specificities and not only for the diversity of non-self–structures that they can tell apart.

This chapter has touched on a number of cases where parts of the mechanism that endow T cells with a function to match their specificity can be discerned. However, these are mostly at the frontiers of current understanding. Simple knockout approaches can be foiled by the way a key signaling molecule or transcription factor is often shared between different developmental contexts where it may play different roles. Stage-specific analysis is crucial to reveal the regulatory combinations that are actually guiding each developmental decision. New tools will be needed to dissect the operation of signaling pathways with quantitative, temporal resolution, in order to understand their different outputs.[277,278] But the outlines of the answers are coming into view.

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CHAPTER 14

Peripheral T Lymphocyte Responses and Function

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INTRODUCTION

This chapter focuses on the processes by which conventional thymus-derived (T)-lymphocytes that express αβ T-cell antigen receptors (TCRs) become activated by their cognate peptide (p): major histocompatibility complex (MHC) ligand. It will cover how TCR ligation by foreign antigen-derived p:MHC ligands causes rare naïve T cells from the preimmune repertoire to proliferate in the secondary lymphoid organs (sometimes referred to by immunologists as the periphery) to become effector cells capable of producing microbicidal cytokines and eliminating foreign antigens from nonlymphoid tissues. It will also describe how the innate immune response and signals from costimulatory and cytokine receptors shape the functional and migratory properties of effector T cells, and ends with a discussion of how effector cells give rise to diverse memory cell subsets. An emphasis is placed on the dynamics and anatomy of naïve to effector to memory-cell conversion.

The chapter will focus on conventional T cells that express αβ TCRs. The reader is directed to other chapters in the book for details concerning natural killer T cells, γδ T cells, and regulatory T cells.

NAÏVE T CELLS

To understand effector T-cell generation, it is important to consider the naïve T cells from which they are derived. The capacity of the host to make effector and memory cells in response to new foreign proteins depends on the existence of naïve T cells that express αβ TCRs specific for MHC-bound peptides derived from that protein.[1] Naïve T cells with this particular p:MHC specificity are rare members of a vast repertoire also containing naïve T cells specific for other p:MHC ligands. Each naïve T cell expresses a randomly generated TCR, which is produced regardless of whether the relevant foreign antigen is in the host's body. Thus, naïve T cells are defined as T cells that have not yet been stimulated by a p:MHC ligand for which their TCR has a high affinity.

Generation

A naïve T cell is the end result of a complex developmental process that occurs in the thymus.[2] To complete development, each thymocyte must produce an αβ TCR by randomly recombining one of multiple variable (V), diversity (D), and joining (J) deoxyribonucleic acid segments at the *Tcrb* locus and one of multiple V and J segments at the *Tcra* locus.[3] Once an in-frame rearrangement at a locus has occurred on one chromosome, further rearrangement on the other chromosome is suppressed, although not completely. These processes lead to the situation where most developing thymocytes express a single unique type of αβ TCR.

Thymocytes that successfully express a TCR must then pass positive and negative selection based on the p:MHC specificity of that TCR.[4] Positive selection occurs just after completion of TCR gene rearrangement at the time when thymocytes first express TCRs and coexpress both cluster of differentiation (CD)4 and CD8 coreceptors. To pass positive selection, a thymocyte must receive a TCR signal transduced by low affinity binding to a self p:MHC ligand on radio-resistant thymic epithelial cells in the cortex. It was thought that this process was inefficient due to the improbability of VDJ segment rearrangement by chance producing TCRs with any affinity for a self p:MHC ligand.[5] However, recent work from Marrack and colleagues has shown that all TCRs have germ-line encoded complementarity determining region (CDR) 1 and CDR2 domains within their V segments that bind MHC molecules.[6] This binding has been postulated to cause most thymocytes to undergo negative selection,[7] which is a desirable outcome for the host because these cells could cause autoimmunity if allowed to mature. In this model, the only thymocytes that receive the weak positive selection signal are those that produce a CDR3 through VDJ recombination that partially impairs CDR1 and 2 binding to MHC and allows weak binding to a self-p:MHC complex. Cells that undergo positive selection by recognition of a self-p:MHCI ligand lose CD4 and retain CD8, whereas cells that undergo positive selection by recognition of a self-p:MHCII ligand lose CD8 and retain CD4, in a process controlled by ThPOK and Runx3 transcription factors.[8] In contrast, thymocytes that undergo negative selection either die by apoptosis or differentiate into Foxp3+ regulatory T cells.[9]

Recirculation

CD4+ and CD8+ T cells that complete positive selection exit the thymus and enter the secondary lymphoid organs (lymph nodes, spleen, and mucosal lymphoid organs). Just before leaving the thymus, these cells turn on the KLF2 transcription factor, which promotes expression of several gene products that control thymus exit, such as S1P1, and subsequent circulation through secondary lymphoid organs, such as CD62L and CC chemokine receptor (CCR) 7.[10] T cells also express low levels of CD44 and CD45RO and high levels of CD45RB and CD45RA as they leave the thymus. Over the first 2 weeks after leaving the thymus in adults,

T cells increase the levels of expression of CD28 and the interleukin (IL)-7 receptor α chain, and become better able to proliferate and differentiate if confronted with the relevant foreign p:MHC ligand.[11,12] After completing this maturation process, T cells express a $CD44^{low}$ CD45RO− $CD45RB^{high}$ CD45RA+ CCR7+ CD62L+ $CD28^{high}$ IL-$7R\alpha^{high}$ phenotype, which is used to identify naïve T cells.

Naïve T cells spend their lives ($T_{1/2}$ of 2 to 5 years in humans,[13] and 50 to 100 days in mice[14]) recirculating through secondary lymphoid organs. Expression of trafficking molecules that bind ligands that are only expressed on the specialized blood vessels and sinuses of these organs explain this behavior. For example, the extravasation of naïve T cells through the high endothelial venules (HEVs) of lymph nodes and mucosal lymphoid organs depends on CD62L and CCR7, the ligands for which are only expressed on HEVs.[15] After passing through the HEVs, naïve T cells enter the T-cell–rich paracortical areas of the lymph nodes and mucosal lymphoid organs and are constrained in these regions by sensing CCL19 and CCL21 with CCR7.[16] Similarly, naïve T cells migrate from the marginal zone sinuses of the spleen into the T-cell–rich periarteriolar lymphoid sheathes (PALSs) via an unknown G-protein–coupled receptor-dependent mechanism. Naïve T cells again migrate within the PALSs through CCR7 sensing of CCL19 and CCL21. In all secondary lymphoid organs, naïve T cells are excluded from the B-cell–rich follicles due to lack of expression of CXC chemokine receptor (CXCR) 5, which binds to CXCL13 produced in follicles and guides cells to this location.

Survival

The T-cell areas within lymph nodes contain a network of thin collagen tubes called conduits, which carry lymph-borne antigens and chemokines from the subcapsular sinus, where lymph enters the lymph nodes through afferent lymphatic vessels, to sinuses surrounding HEVs.[17] The conduits are wrapped with fibroblastic reticular cells, which produce IL-7. Naïve T cells migrate along the conduits, placing themselves in a good position to receive IL-7, on which they depend for survival.[18]

To survive for a normal lifespan, naïve T cells must also receive TCR signals through weak recognition of self p:MHC ligands, perhaps the ones that caused the T cells to undergo positive selection in the thymus.[1] Dendritic cells are probably the important antigen-presenting cell (APC) for this process because they are constantly in contact with T cells in the secondary lymphoid organs, and expression of MHCII molecules under the control of the dendritic cell–specific CD11c (*Itgax*) promoter is sufficient to maintain the survival of naïve CD4+ T cells. On the other hand, mice lacking dendritic cells by *Itgax* promoter–directed expression of diphtheria toxin have normal numbers of naïve T cells.[19] This result does not rule out a role for dendritic cells in naïve T-cell maintenance, however, because it is possible that the naïve T cells die at a higher than normal rate without dendritic cells but are also produced at a higher rate. Studies on the turnover rate of naïve T cells in dendritic cell–deficient mice will be needed to address this question.

Self p:MHC ligand presentation by dendritic cells has different consequences for naïve T cells than foreign p:MHC ligand presentation. For example, self p:MHC ligand presentation results in only a subset of the signals that emanate from the TCR when bound by a high affinity foreign p:MHC ligand, including partial phosphorylation of the TCR-associated CD3-zeta chain.[20] In addition, although signals through the TCR and IL-7 receptor are required for the survival of naïve T cells,[21] these signals do not cause the T cells to proliferate in hosts containing normal numbers of T cells due to competition for IL-7. The Tsc1[22,23] and Foxp1[24] transcription factors, the second of which regulates IL-7Rα levels, enforce the quiescent survival of naïve T cells in this situation. In contrast, naïve T cells proliferate when the number of T cells is very low and IL-7 becomes more available, for example, early in life or after radiation or chemotherapy. This "homeostatic" proliferation also depends on IL-7 and low-affinity TCR recognition of self p:MHC complexes,[21] but differs from proliferation in response to foreign p:MHC ligands by being independent of the CD28 costimulatory receptor.[25] Thus, the same signaling events that cause naïve T cells to survive in interphase in T-cell–sufficient hosts cause these cells to proliferate in T-cell–deficient hosts, but using a program different from that engaged during the T-cell response to high affinity TCR ligands.

Survival and proliferation could both contribute to control of the number of naïve T cells in normal hosts. In adults, new naïve T cells are exported from the thymus into the secondary lymphoid organs that are already full of other T cells. Naïve T cells survive in interphase under these conditions. However, in neonates[26] and perhaps the very aged, new naïve T cells enter relatively lymphopenic secondary lymphoid organs and undergo homeostatic proliferation to fill the space. Notably, this proliferation causes the T cells to lose many of the markers that define the naïve phenotype and express surface molecules that are characteristic of memory cells.[21] Thus, although cells with the naïve phenotype dominate the preimmune repertoire, it can contain some cells with a memory cell phenotype.

Abundance

An adult mouse has about 10^8 αβ TCR+ T cells in the secondary lymphoid organs and another 5×10^6 in the blood.[1] The naïve T-cell population is split about 60:40 in favor of CD4+ T cells over CD8+ T cells. About 70% of the cells in both subsets are naïve phenotype cells, at least in young mice housed under specific pathogen-free conditions. Thus, an adult mouse has about 7×10^7 naïve phenotype T cells in its body, which extrapolates to about 3×10^{11} naïve phenotype T cells in an adult human. Because the number of potential TCR amino acid sequences that could be produced by VDJ recombination ($> 10^{15}$)[27] is greater than the number of naïve cells in the body, it is possible that each naïve T cell has a different TCR. Thus, it is possible that each of the 7×10^7 naïve T cells in a mouse has a TCR capable of binding to one of 7×10^7 different foreign p:MHC ligands. This would mean that the frequency of naïve cells expressing TCRs specific for a single p:MHC ligand could be as low as $1/7 \times 10^7$ naïve T cells.

The actual number of naïve T cells specific for single foreign p:MHC-specific ligands has been measured by flow cytometry following labeling with fluorochrome-labeled pMHC tetramers and enrichment with anti-fluorochrome antibody-coated magnetic beads. C57BL/6 mice contain about 200 naïve CD4+ T cells specific for an immunogenic peptide called 2W bound to I-A^b but only about 20 cells specific for peptide 427–441 from the FliC protein of *Salmonella typhimurium* bound to I-A^b.[28] Thus, the frequencies of cells capable of binding to these foreign p:MHCII ligands ranges from about 1:200,000 to 1:2,000,000 of the naïve phenotype CD4+ T cells in mice that were never exposed to these peptides. Similar analyses have been done for foreign p:MHCI-specific CD8+ murine T-cell populations.[29–31] The number of naïve CD8+ T cells in C57BL/6 mice ranged from 15 lymphocytic choriomeningitis virus (LCMV) $L_{338–346}$:D^b-specific cells to 1,100 vaccinia virus B8R:K^b-specific cells per mouse.[31]

An important conclusion from these studies is that naïve populations specific for different foreign p:MHC ligands vary in size in a predictable fashion. There is evidence that size differences between foreign pMHC-specific populations in the preimmune repertoire are caused by negative selection.[32] In other words, naïve foreign pMHC-specific populations can be small because their TCRs by chance bind with high affinity to self p:MHC complexes. Another possibility is that peptides that are recognized by larger naïve populations have structural features that are conducive to recognition by a more diverse set of TCRs (eg, amino acids with prominent side chains).[32,33]

Another important conclusion from these experiments is that the size of naïve p:MHC-specific populations can predict the magnitude of the effector cell response after certain forms of antigen administration. For example, the presence of about 200 2W:I-A^b- and 20 FliC:I-A^b-specific naïve CD4+ T cells correlates with the 100,000 2W:I-A^b- and 15,000 FliC:I-A^b-specific effector T cells induced by injection of the relevant peptides.[28] Similarly, the size of naïve LCMV p:MHCI-specific T-cell populations plays a role the magnitude of the primary CD8+ T-cell response to the virus. LCMV infection of B6 mice activates CD8+ T cells specific for at least 28 different p:MHCI.[31] However, about one-third of the total response is directed against three p:MHCI complexes. The feature of these dominant p:MHCI ligands that correlates best with their potent immunogenicity is the large size of their naïve populations.[31] Therefore, although antigen abundance, efficiency of peptide generation by antigen processing, and MHC binding affinity are important factors in immunodominance,[34] so is naïve T-cell population size.

GENERAL ASPECTS OF EFFECTOR T-CELL FORMATION

Unlike the presentation of low-affinity self p:MHC ligands that maintains the survival of naïve T cells in interphase under nonlymphopenic conditions, the presentation of high-affinity foreign p:MHC ligands induces the specific naïve T cells to produce lymphokines, proliferate, and differentiate into effector T cells that aid in elimination of the antigen from the body. These more dramatic biological effects occur because the responding T cells receive much stronger or more durable signals through the TCR upon recognition of high-affinity foreign p:MHC ligands than they do when recognizing low-affinity self p:MHC ligands.[35] In addition, foreign antigens naturally enter the body during infection or tissue damage, which triggers dendritic cells and other innate immune cells to stabilize foreign p:MHC ligands and induce costimulatory receptors and cytokines.[36,37] The nature of the signals from the TCR, costimulatory receptors, and cytokine receptors then influences the type of effector cells that naïve T cells differentiate into. These three signals and how they vary depending on the nature of the antigen will be described.

Dendritic Cells as Initiating Antigen-Presenting Cells

Arguably, the most important of the three signals is transduced by the TCR pursuant to bind to a foreign p:MHC ligand on an APC. It is clear from ablation experiments that dendritic cells are the only APCs capable of initiating the TCR signal in CD8+ and CD4+ T cells in the spleen.[38] Dendritic cells are also important for initiating APCs for CD4+ T cells in lymph nodes, although other MHCII+ cells can do this job.[38] It has also become clear that many dendritic cell subsets exist.[38,39] A brief synopsis is presented here to give the reader a sense of which dendritic cell types function as APCs for different antigens. Refer to Chapter 16 on dendritic cells for more detailed information on their biology. Dendritic cells are defined in the mouse as cells that express the CD11c integrin, have large amounts of MHCI and MHCII molecules, and reside in or have the capacity to migrate to the T-cell zones of secondary lymphoid organs.[40] The spleen and lymph nodes contain two types of dendritic cells that develop from monocyte-dendritic cell precursors in these organs. One type expresses the myeloid marker CD11b and is often referred to as the myeloid dendritic cell. These dendritic cells are found mainly in the red pulp or marginal zones of the spleen and outer T-cell–rich regions of the lymph nodes. A second type depends on the Batf3 transcription factor, expresses CD8α, and is referred to as the CD8α+ dendritic cell.[41] These dendritic cells are located primarily in the PALSs of the spleen and the T-cell–rich regions of the lymph nodes, and are the major IL-12–producing cells in these locations. Myeloid and CD8α+ dendritic cells turnover rapidly with the latter population having only a 3-day half-life.

Another type of dendritic cell is called the plasmacytoid dendritic cell because of its morphology.[42] These cells develop in the bone marrow and then seed the spleen and lymph nodes from the blood. Plasmacytoid dendritic cells in mice express low amounts of CD11c and the B220 and Gr-1 molecules normally expressed by B cells and granulocytes, and are the most potent producers of type 1 interferons (IFNs)[43] when stimulated through pattern recognition receptors (PRRs).[44] These cells therefore play a key role in inducing an antiviral state in many cell types.

The lymph nodes contain several additional migratory CD11c+ dendritic populations that move to this location

from tissues through afferent lymphatic vessels.[39] All lymph nodes contain CD11b+ dendritic cells that also express F4/80 and SIRPα, distinguishing them from myeloid dendritic cells and other CD103+ dendritic cells that often express an intracellular protein called langerin. In the case of the skin, these two types of dendritic cells migrate to lymph nodes from the dermis and are called dermal dendritic cells and dermal langerin-positive dendritic cells. The skin-draining lymph nodes also contain epidermal Langerhans cells, which express langerin and high levels of CD11b and EpCAM. Epidermal Langerhans cells are generated from a local radio-resistant precursor in the skin and are more long-lived than myeloid and CD8α+ dendritic cells.

The dendritic cell type that is most important for the presentation of p:MHC ligands depends on the nature of the antigen. Resident CD8α+ dendritic cells and CD103+ dendritic cell migrants are critical for p:MHCI ligand production from exogenous antigens because these are the only cells in the body that are capable of a process called cross presentation.[41,45] These dendritic cells express receptors such as CD36 that mediate uptake of apoptotic cells. After taking up apoptotic or other extracellular material, these dendritic cells have the unique capacity to move proteins from the phagosome directly into the cytoplasm. Once in the cytosol, the translocated proteins can be cleaved by the proteosome into peptides, which are then pumped by the transporter associated with antigen processing into the endoplasmic reticulum where binding to MHCI occurs. Other cells of the body cannot translocate ingested proteins into the cytosol and thus are only capable of producing p:MHCI complexes from proteins that are translated in their cytosols (eg, their own proteins or proteins from cytosolic microbes that infect them). Thus, the cross presentation pathway is important for initiating the CD8+ T-cell response to viruses that do not infect dendritic cells but kill their host cells. In contrast, any dendritic cell including plasmacytoid dendritic cells can likely serve as an initiating APC for CD8+ T cells specific for MHCI-binding peptides derived from proteins from viruses that directly infect it.

Early activation of CD4+ T cells specific for p:MHCII ligands derived from soluble antigens can occur in two waves in lymph nodes.[46] Soluble antigens (eg, toxins secreted by bacteria in a subcutaneous infection site) rapidly flow through lymphatic vessels to the draining lymph nodes and into the conduit network. Resident dendritic cells associate with the conduits, then take up the antigen, perhaps from small gaps between the fibroblastic reticular cells that encircle these tubes.[47] These dendritic cells process the antigen, produce p:MHCII complexes, and display them for recognition by naïve CD4+ T cells. This process occurs within several hours of antigen deposition in the skin and results in CD69 induction and proliferation in the T cells.

The initial wave of p:MHCII presentation is followed by a second wave mediated by dermal dendritic cells that take up the antigen at the site of deposition and then migrate to the draining lymph node.[46] These dendritic cells arrive in the T-cell areas 12 to 24 hours after antigen enters the tissue. p:MHCII presentation by these dendritic cells prolongs induction of the IL-2 receptor and is required for T-cell acquisition of the capacity to cause a later delayed-type hypersensitivity reaction.

Other types of antigens are accessed and processed by different dendritic cells. Given their location in the spleen, myeloid dendritic cells probably play a key role as initiating APCs for CD4+ T cells in the case of antigens that are present in the blood.[48] Dendritic cells that migrate from the relevant tissue (eg, submucosal dendritic cells during vaginal infection are likely the only cells capable of producing p:MHCII ligands from microbes that are too large to enter the conduits.[43] For unknown reasons, dermal Langerin-positive dendritic cells are the most important APCs for induction of contact hypersensitivity-causing CD8+ T cells by protein-modifying chemicals applied to the skin surface.[49] Surprisingly, epidermal Langerhans cells are not required for contact hypersensitivity, although the sensitizing chemicals are applied to the skin surface. Rather, p:MHCII presentation by Langerhans cells inhibits the induction of delayed-type hypersensitivity by suppressing the priming of CD4+ Th1 cells and promoting the priming of Th17 cells.[50]

T-Cell Antigen Receptor Signaling

Once dendritic cells displaying foreign p:MHC ligands appear in the T-cell area of a secondary lymphoid organ, they can be recognized by naïve T cells expressing complementary TCRs. In vitro experiments have shown that high-affinity TCR ligation by p:MHC ligands causes the TCR to concentrate in a stable central supramolecular activating cluster (cSMAC) structure at the point of contact between the T cell and the APC.[51] cSMAC formation is often preceded by the formation of TCR microclusters at the periphery of the T cell-APC contact zone, which are capable of the transducing signals.[52] TCR clustering then activates protein tyrosine kinases such as lck, which stimulate signaling cascades that trigger protein kinase c theta, elevate intracellular calcium, convert ras into its active form, and activate the extracellular signal-regulated kinases (ERK1 and ERK2) and stress-activated protein kinases (jun kinase and p38 mitogen-activated protein kinase).[53] These pathways culminate in the nuclear translocation and binding of transcription factors such as NFAT and NF-κB to deoxyribonucleic acid sequences that regulate lymphokine gene expression.[54]

Very little is known about early TCR signaling events in naïve T cells in vivo because the assays used to measure most of these events rely on cell lines and in vitro culture methods. Interestingly, in vivo imaging of naïve T cells showed rapid, p:MHC-dependent TCR internalization that was not contingent on prolonged contacts between T cells and APCs or cSMACs.[55] These transient interactions must be sufficient for TCR signaling, however, because intracellular staining with antibodies that recognize the active forms of the c-jun transcription factor and the p38 mitogen–activated protein kinase showed that both of these molecules are phosphorylated in p:MHC-specific naïve T cells in the spleen within minutes of intravenous injection of the relevant peptide.[56]

This rapid response is likely explained by the fact that the majority of naïve T cells are constantly contacting dendritic cells in the T-cell areas.[56]

Proliferation and Costimulation

Initial TCR binding to p:MHC ligands on dendritic cells causes naïve T cells to begin proliferating in vivo about 2 days later.[57] In vitro experiments indicate that this cell division is driven by production of IL-2[58] and induction of the IL-2 receptor alpha chain (CD25).[59] Surprisingly, however, p:MHC-driven proliferation of naïve T cells is minimally dependent on IL-2 in vivo.[60,61] Therefore, other signals or growth factors must be capable of driving T-cell proliferation in vivo, although IL-2 may contribute. The role of IL-2 as a T-cell growth factor may be difficult to reveal because IL-2 is also required to maintain regulatory T cells[62] and can promote death of activated T cells.[60] Thus, a reduction in IL-2–dependent effector T-cell proliferation could be masked by a removal of regulatory T-cell–mediated suppression and activation-induced cell death. In addition, although IL-2 is not essential for initial p:MHCI ligand-driven T-cell proliferation, it does appear to be essential for memory cell differentiation.[63]

The initial proliferation of naïve T cells is followed by an exponential increase in the number of p:MHC-specific T cells over the next several days.[64,65] Depending on the stimulus, the number of p:MHC-specific T cells reaches its highest level in the relevant secondary lymphoid organs, 5 to 7 days after antigen enters the body. As mentioned previously, naïve mice contain about 200 CD8+ T cells specific for a given p:MHC I complex. Because p:MHCI-specific CD8+ T cells can increase to 10^7 cells at the peak of the primary response,[64,65] it follows that CD8+ T cells can expand 500,000-fold in vivo. Although naïve CD4+ T cells are also capable of dramatic clonal expansion when stimulated appropriately, their burst size appears to be less than CD8-positive T cells.[64] For both CD4+ and CD8+ T cells, the amount of cell division is inversely proportional to the number of naïve precursors,[14,66,67] indicating that in vivo proliferation is limited by competition between p:MHC-specific T cells.

In vivo T-cell proliferation is regulated by signals from the costimulatory CD28 molecule, which is triggered by binding to CD80 and CD86 on APCs.[68] The proliferation of antigen-stimulated CD4+ or CD8+ T cells is greatly reduced in mice in which CD28 cannot interact with its ligands. CD40 ligand (CD154) deficiency also affects T-cell expansion,[69] which may be related to the fact that CD40 signaling in APC induces CD80 and CD86.[70] Although costimulatory signals enhance TCR-driven IL-2 production, the in vivo significance of this effect for T-cell proliferation is unclear, as described above. Although it has been proposed that CD28 acts by promoting TCR aggregation in the cSMAC,[71] enhancing lymphokine messenger ribonucleic acid production[72] and stability,[73] and/or promoting T-cell survival by augmenting Bcl-X_L production,[74] the bulk of recent evidence indicates that it acts by NF-κB signaling.[75] Members of the TNF receptor family, such as OX40, CD27, and 4-1BB are induced in T cells by CD28 signaling several days into the primary response.[76,77] These molecules bind ligands of the TNF family on the surface of APC and transduce signals that sustain the proliferation or survival of p:MHC-stimulated T cells.

Enhancement of costimulatory signals may underlie the observation that in vivo T-cell proliferation is also influenced by inflammation at the time of initial p:MHC presentation. This effect is observed in the case of soluble antigens, where the magnitude of T-cell proliferation is several-fold greater if antigen is administered with a microbial substance[78,79] containing a pathogen-associated molecular pattern[43] such as lipopolysaccharide, which is recognized by a PRR such as toll-like receptor 4.[80] PRR signaling stimulates tissue macrophages to produce tumor necrosis factor–α,[81] which in turn stimulates dendritic cells to migrate from nonlymphoid tissues into the T-cell areas and express higher levels of CD80 and CD86.[82] In addition, these signals result in a maturation process that changes the antigen processing and presentation potential of all dendritic cell types.[83] When inflammation is not present, dendritic cells efficiently engulf extracellular fluid and produce p:MHC complexes from the ingested proteins. However, these p:MHC molecules turn over rapidly on the dendritic cell surface and are presented in the context of low amounts of CD80 and CD86. These factors are thought to be part of the explanation for why p:MHCII presentation by dendritic cells in uninflamed secondary lymphoid organs leads to poor T-cell priming and can result in tolerance.[84] In contrast, inflammatory signals cause dendritic cells to reduce antigen uptake and processing, stabilize p:MHCII molecules, and induce expression of CD80 and CD86. Therefore, inflammatory signals enhance proliferation by driving more dendritic cells into the T-cell areas to present p:MHC ligands and by increasing the costimulatory capacity of these dendritic cells.

PRR signaling in dendritic cells or macrophages can also trigger the production of proinflammatory cytokines, which enhance effector cell proliferation. For example, IL-1 from several sources enhances the proliferation of CD4+ T cells through an early indirect effect on APCs,[85] and a later more potent direct effect on the T cells themselves.[86] PRR signaling causes CD8α+ dendritic cells to produce IL-12[41] and plasmacytoid dendritic cells to produce type 1 IFNs,[42] both of which enhance the proliferation of CD8+ effector T cells.[87]

EFFECTOR T-CELL DIFFERENTIATION

The proliferation of p:MHC-specific effector T cells during the primary response is linked with the acquisition of functions that affect the elimination of antigen such as microbicidal cytokine production and cytolysis. The functional properties that effector cells acquire are influenced by the presence of inflammatory cytokines and costimulatory ligands on APCs present at the time of initial p:MHC presentation in secondary lymphoid organs.

CD4+ T Cells

Many variations on this theme exist in the case of CD4+ T cells. Effector cells that are generated in the presence of IL-12, IL-4, IL-6 and TGF-β, or IL-6 and IL-21 become

IFN-γ–producing Th1 cells, IL-4–producing Th2 cells, IL-17–producing Th17 cells, or IL-21–producing follicular helper cells (Tfh), respectively. These effector T cells play specialized roles in the elimination of intracellular microbes, worms, and extracellular microbes. Polarization of Th cells is covered in detail elsewhere in this volume.

Signaling through the IL-2 receptor plays an essential and early role in the formation of Th1, Th2, and Th17 effector cells. IL-2 receptor alpha chain (CD25) expression is induced in naïve T cells by TCR signaling, which then pairs with IL-2/IL-15 receptor β (CD122) and IL-2 receptor γ (CD132) chains, to produce the high-affinity IL-2 receptor.[88] Under Th1 priming conditions, the IL-2–bound receptor recruits Jak1 and Jak3 that phosphorylate the STAT5 transcription factor, which enters the nucleus and enhances expression of the IL-12 receptor β2 chain and the T-bet and Blimp-1 transcription factors.[89] IL-12 receptor signaling through STAT4 then enhances T-bet expression and enforces the Th1 fate,[90] while Blimp-1 suppresses another transcription factor Bcl6 that is needed for other fates.[91] Under Th2 priming conditions, IL-2 receptor signaling through STAT5 regulates the Th2 cytokine gene cluster and expression of the IL-4 receptor α-chain.[89] In contrast, IL-2 receptor signaling inhibits Th17 differentiation by suppressing components of the IL-6 receptor.[89]

A variation on this theme was recently described for CD4+ T cells during acute systemic *Listeria monocytogenes* or LCMV infections.[92–94] In this case, CD25 is induced by TCR signaling in microbe p:MHCII-specific CD4+ T cells 1 day into the infection. However, by day 3, about half of the responding T cells lose CD25 expression and begin to express CXCR5, while half retain CD25 and do not express CXCR5.[92,94] The mechanism underlying this bifurcation is unknown, although asymmetric division of CD25+ mother cells into CD25+ and CD25– daughter cells is a possibility.[95] Another possibility is that very strong TCR signaling favors CD25 expression.[96] In any case, the CD25+ cells go on to form T-bet^{high} Th1 effector cells in the T cell areas, red pulp, and probably nonlymphoid organs where they could produce IFN-γ in response to p:MHCII presentation by macrophages. It is likely that early CD25+ effector cells become Th2 cells during infections that promote IL-4 production by innate immune cells.[97]

The CD25– CXCR5+ cells formed early during acute infection yield two T-bet^{low} effector cell populations,[92,93] both expressing the B-cell–follicle guiding CXCR5 receptor but only one expressing the Tfh marker PD-1.[98] Both of these CXCR5+ effector cell populations depend on the Bcl6 transcription factor for their formation.[92] The CXCR5+ PD-1– and CXCR5+ PD-1+ effector cell populations probably differentiate depending on quantitative differences in signals from the TCR and the inducible costimulator (ICOS) receptor.[68,94,99,100] Following stimulation by p:MHCII ligands on dendritic cells, the naïve T cells that quickly lose CD25 and express Bcl6 migrate toward the follicles under the guidance of CXCR5 to interact with, and provide helper signals to, antigen-specific B cells that display the relevant p:MHCII complexes and ICOS ligand.[101–103] In return, the T cells receive signals from the TCR and ICOS. If these signals are relatively weak, then Bcl6 expression may not be maintained and the cells acquire a CCR7+ CXCR5^{intermediate} P-selectin ligand (PSGL-1)+ PD-1– phenotype and live in the outer T-cell area. As discussed in more detail below, some of these effector cells yield multipotent central memory cells.

In contrast, if early CD25– CXCR5+ effector cells receive strong TCR and ICOS signals when interacting with B cells, perhaps because of expression of TCRs with very high affinity for the inducing p:MHCII ligand,[91,104] then they become CCR7^{low} CXCR5^{high} PSGL-1– PD-1+ Tfh cells. The cytokines IL-6 and IL-21 are also required for this process,[105,106] with IL-21 probably coming from the developing Tfh cells themselves. The CCR7^{low} CXCR5^{high} phenotype causes Tfh cells to localize in germinal centers, which are rich sources of CXCR5 but not CCR7 ligands.[16] Here, Tfh cells produce IL-21 and CD40 ligand that promote germinal center B-cell differentiation, antibody isotype switching, and plasma cell formation.[98]

CD8+ T Cells

As in the case of CD4+ T cells, IL-2 receptor signaling is not essential for the initial proliferation of p:MHCI-stimulated naïve CD8+ T cells.[63] However, IL-2 receptor signaling plays a key role in effector cell differentiation and memory cell formation. Naïve CD8+ T cells that are stimulated by p:MHCI ligands on dendritic cells in secondary lymphoid organs during acute LCMV infection rapidly express CD25 to produce high-affinity IL-2 receptors.[96] However, as in the case of CD4+ T cells, a subset of the CD8+ T cells sustains CD25 expression longer than the other cells in the population. The CD25^{low} cells preferentially upregulate the IL-7 receptor and the central memory T-cell marker CD62L and produce long-lived memory cells. In contrast, the CD25^{high} cells proliferate more rapidly and become apoptosis-prone terminally differentiated effector cells, although it is likely that some of these cells produce effector memory cells. Thus, for both CD4+ and CD8+ T cells, a lack of IL-2 receptor signaling early in the primary response produces effector cell precursors of central memory cells, whereas IL-2 receptor signaling promotes highly differentiated effector cells and like effector memory cells.

Again like CD4+ T cells, costimulatory signals and cytokines produced by innate immune cells are needed for optimal differentiation of CD8+ effector cells. Naïve CD8+ T cells that are stimulated by p:MHCI ligands also require CD28 signals and either IL-12 or type I IFN (IFNα/β) for maximal proliferation and development of cytolytic activity.[87] The effects of IL-12 and type 1 IFN are mediated directly on the CD8+ T cells. When either IL-12 or type 1 IFN are delivered with antigen, the responding CD8+ T cells form a memory cell population, while immunization with antigen alone induces some clonal expansion, but very few cells survive long-term and those that do are anergic. Thus, IL-12 and type 1 IFN provide a "third signal" that is necessary, along with TCR and CD28 signals, for development of optimal effector functions and formation of a responsive memory population. Dendritic cells activated by PRRs, or CD40 signals pursuant to interaction with CD40 ligand (CD154)+ CD4+ T cells, produce IL-12 and type I IFNs, and are thus equipped to provide all three signals needed by a naïve CD8+ T cell to become a cytotoxic effector cell.

IL-12 and type I IFN are largely redundant in their capacity to support a productive CD8+ effector T-cell response, and which cytokine is critical depends on the infection. Studies using CD8+ T cells lacking receptors for one or both of the cytokines have shown that type I IFN is essential for response to LCMV,[107] while IL-12 signaling plays the major role in responses to vaccinia virus and *Listeria monocytogenes*.[108] In a minor histocompatibility antigen transplant model, rapid graft rejection depends on CD4+ T cells stimulating dendritic cells in a CD40-dependent manner to produce IL-12 needed for generation of cytotoxic CD8+ effector T cells.[109] Thus, it appears that one way in which CD4+ T cells can provide help for CD8+ T-cell responses is by stimulating dendritic cells to produce third signal cytokines.

CD8+ effector T cells differ from their naïve precursors regarding surface markers, function, and trafficking properties. CD8+ effector T cells that are generated in secondary lymphoid organs during microbial infections express slightly lower levels of CD8 and more surface O-glycans than naïve cells,[110] and in the human some CD8+ effector T cells lose CD27 and CD28 but retain CD45RA.[111] Unlike naïve cells, these cells express perforin and granzymes,[110] which are required for efficient cytolytic function. Expression of perforin and granzymes contributes to the defining feature of cytotoxic T cells,[110] that is, the ability to directly kill target cells that display the appropriate p:MHC I complexes. Interestingly, although large numbers of antigen-specific CD8+ T cells accumulate in mice injected with heat-killed *Listeria monocytogenes* bacteria, these T cells do not acquire cytolytic function.[112] This situation may come about because heat-killed *Listeria monocytogenes* bacteria are poor inducers of IL-12 by innate immune cells compared to live organisms.

EFFECTOR T-CELL FUNCTION IN NONLYMPHOID ORGANS

Some CD4+ effector T cells migrate into nonlymphoid tissues at the peak of the primary response.[113] Separate homing pathways to the intestines and skin have been characterized in detail,[114] although other pathways probably exist. Injection of antigen and cholera toxin into the skin induces two populations of CD4+ effector T cells in the draining lymph nodes that do or do not express the fucosylated form of P-selectin glycoprotein-1 (fPSGL-1), which binds to CD62P on inflamed blood vessels.[115] The fPSGL-1− cells are potent helpers of antibody production by B cells and are likely one of the CXCR5+ effector cell populations described above. In contrast, the fPSGL-1+ cells are poor helpers of antibody production by B cells but are capable of IFN-γ production and are likely similar to the T-bet^{high} Th1 effector cells induced during acute infections. After transfer into naïve recipients, these cells migrate to the skin as evidenced by a capacity to cause delayed-type hypersensitivity reactions. Injection of antigen into the skin with complete Freund's adjuvant also induces Th1 effector cells in draining lymph nodes, which migrate to the skin injection site in a CD62E- and CD62P-dependent fashion.[116] Interestingly, in this case and another involving lung migration,[117] the CD4+ T cells in the lymph nodes that migrate to nonlymphoid tissues are those that undergo the most cell divisions in the secondary lymphoid organs. Thus, only the most-divided subset of Th1 effector cells in lymph nodes acquire the appropriate trafficking receptors and have the capacity to migrate to nonlymphoid organs.

The induction of skin-homing capacity is controlled by several factors produced by dendritic cells that migrate from the skin and present p:MHCII ligands to naïve T cells in the draining lymph nodes. One of these factors is IL-12, which induces the expression of enzymes that fucosylate PSGL-1 and convert it into the CD62P-binding form.[118] Dendritic cells from the skin also produce 1,25 dihydroxy-vitamin D3, which induces the putative skin-homing chemokine receptor CCR10 on T cells.[119] It should be noted, however, that regulation of CCR4, which is probably the most important skin-homing chemokine receptor on T cells,[120] is not understood in this context.

The generation of gut-homing CD4+ effector T cells is also controlled by factors produced by dendritic cells at the time of T-cell priming. Presentation of p:MHCII ligands to CD4+ T cells in mucosal lymphoid organs induces effector cells that express large amounts of α4β7 integrin, which facilitates T-cell migration into tissues containing mucosal addressin cell adhesion molecule-1+ blood vessels such as the intestines.[15] CD103+ dendritic cells in the mucosal lymphoid organs produce all-trans retinoic acid, which enhances the expression of α4β7 integrin and CCR9 on CD4+ T cells.[121]

Mycobacterium tuberculosis (TB) infection by inhalation provides another example of CD4+ effector T-cell migration to nonlymphoid tissue.[122] Inhaled TB organisms are taken up in the lungs by CD11b+ dendritic cells, which carry the bacteria to the mediastinal lymph nodes and present TB p:MHCII ligands to naïve CD4+ T cells expressing relevant TCRs. This presentation occurs in an IL-12–rich context, and Th1 effector cells are produced, many of which traffic back to the lungs. The molecules that control this migration are unknown although CCL27, MIP-1α, IP-10, CCR4, CCR5, and CXCR3 may be involved. Once in the lungs, effector T cells produce IFN-γ in response to TB p:MHCII presentation by infected dendritic cells and macrophages, which triggers the microbicidal functions of these cells. During this process, the lung effector T cells start out as PD-1^{high} CD69^{high} cells that convert to KLRG1^{high} CD69^{low} short-lived, terminally differentiated cells as they become activated by local TB p:MHCII presentation. Because TB organisms are not completely eliminated by this process, it goes on for protracted periods of time as new effector cells migrate to the lungs from the draining lymph nodes, eventually damaging the lung tissue. Thus, TB p:MHCII-specific effector T cells and their effects on infected phagocytes knock the TB organisms down but not out, and in the process protect the host in the relative short-term and damage it in the long-term.

CD8+ effector T cells also migrate out of the T-cell areas and into many nonlymphoid tissues, particularly inflamed sites of antigen deposition (eg, the lungs during influenza infection[123,124] and the gut during vesicular stomatitis virus infection[125]). Strong or prolonged IL-2 receptor signaling is an important factor in the generation of highly differentiated nonlymphoid tissue-homing effector CD8+ T cells.[96] The migratory capacities of effector CD8-positive T cells correlate with loss of receptors involved in lymph node

migration (CCR7 and CD62L) and acquisition of receptors such as α4β7 integrin,[126] following instructions from dendritic cells as described above for CD4-positive T cells. The migration of CD8+ effector T cells with cytotoxic potential into nonlymphoid organs is an effective way of eliminating cells that display p:MHC I complexes from all parts of the body.

Several CD8+ effector T-cell–specific homing phenomenon are worth mentioning. One is that CD8+ T-cell recruitment to nonlymphoid tissue depends on prior entry of CD4+ T cells in certain situations. Recognition of p:MHCI complexes on dendritic cells in the secondary lymphoid organs induces the expression of CXCR3 on effector CD8+ T cells, which facilitates entry into nonlymphoid tissues through blood vessels displaying CXCL9 and CXCL10.[127] During certain viral infections, CD4+ effector T cells first enter the relevant nonlymphoid tissue and are stimulated by their p:MHCII ligands to produce IFN-γ, causing local epithelial cells to secrete CXCL9 and CXCL10, which then recruit the CD8+ effector T cells.[128]

Another special homing property of some CD8+ effector T cells relates to the capacity to enter a nonlymphoid tissue and never leave.[129] Viral infections in the skin, brain, and intestinal mucosa result in expansion of viral p:MHCI-specific CD8+ effector T cells in the draining lymph nodes and then migration of some effector cells to the site of infection. Some of these cells can be found at this site long after the infection is cleared. Parabiosis experiments indicate that these T cells are not constantly leaving and entering the site from the blood as expected, but rather remain in the original site without leaving. These resident CD8+ T cells express CD103, which may tether them in the site by binding to E-cadherin on local epithelial cells. Tissue-resident CD8+ effector T cells also express CD69, a marker of acute activation, even under conditions where local relevant p:MHCI complexes cannot be detected. Thus, CD69 expression may be driven in these cells by cytokine receptor rather than TCR signaling. It should be noted that not all CD8+ effector T cells in nonlymphoid tissues are noncirculating residents as evidenced by the presence of p:MHCI-experienced CD8+ T cells in efferent lymphatic vessels, which carry cells from tissues to the blood.[130] The factors that determine whether naïve CD8+ T cells will become nonrecirculating CD103+ tissue-resident effector cells or effectors cells that recirculate through nonlymphoid tissues are not known.

GENERATION OF MEMORY T CELLS FROM EFFECTOR CELLS

Another key function of effector T cells is the production of memory cells.[131] The number of antigen-derived p:MHC-specific effector T cells in the body peaks about a week into the primary response, and then falls rapidly over a 2-week contraction period to about 10% of the peak value.[132] This decline must be due to cell death because the total number of cells in all parts of the body declines shortly after the peak.[113] The T cells that survive the contraction phase can persist stably for the life of the host even when the antigen is cleared from the body due to the expression and function of IL-7 and IL-15 receptors.[133] These long-lived cells are known as memory cells, which are capable of rapid secondary responses that can produce protective immunity to a later challenge from a microbe.[134] Memory cells can be distinguished from effector cells in that most memory cells are not blasts, are not in the cell cycle, and many are not directly cytolytic or producing lymphokines.[135]

Memory cells are heterogenous, however, and exist in at least two subsets: effector memory (Tem) cells and central memory (Tcm) cells.[136] Tem cells express homing receptors that facilitate migration to nonlymphoid sites of inflammation[113] and produce microbicidal cytokines such as IFN-γ within several hours of TCR stimulation. In many ways, these cells resemble effector cells (eg, Th1 or Th2 cells), with the exceptions that they are no longer blasts and express IL-7 receptors. Tcm cells do not produce any of the prototypic effector cell lineage cytokines immediately after stimulation through the TCR, although they secrete IL-2 and proliferate extensively and acquire effector lymphokine production later. These cells express CD62L and CCR7, which are involved in migration through lymph nodes and mucosal lymphoid organs and positioning in the T-cell areas of these organs,[16] and IL-7 receptors, which are critical for their survival.[133]

The Tem and Tcm model fits well for CD8+ memory T cells. A subset of human CD8+ memory cells lacks the naïve cell marker CD45RA but expresses CCR7.[111,137] These memory cells also express CD62L and lack perforin and thus would not be expected to be directly cytotoxic. Mice contain a comparable $CD44^{high}$ memory cell population after clearance of acute viral infection.[125] These similar populations in mice and humans fit the description of Tcm cells. In mice, the CD62L+ Tcm cells undergo slow IL-15–dependent, MHCI-independent homeostatic proliferation, which is thought to account for their numerical stability.[138]

Mice and humans also contain CD45RA– CCR7– CD62L– CD8+ memory T cells. These cells express high levels of β1 and β7 integrins, fPSGL-1, and CCR5, which are predicted to facilitate migration into nonlymphoid tissues,[139] and produce IFN-γ rapidly after TCR stimulation.[125,137] These cells therefore fit the description of Tem cells. In mice, CD8+ Tem cells undergo much less IL-15–dependent, MHCI-independent homeostatic proliferation than Tcm cells,[140] and convert into Tcm cells over long periods of time in some[141] but not all[142] situations. Murine CD8+ Tem cells can recirculate through nonlymphoid organs or reside permanently in nonlymphoid organs, as described above.

Humans also contain a CD45RA+ CCR7– CD8+ T-cell subset that contains especially high levels of perforin and direct ex vivo cytotoxic activity.[111,137] These cells are likely effector cells that were recently stimulated by p:MHCI ligands.

Memory CD4+ T-cell populations also contain Tcm and Tem subsets. The microbial p:MHCII-specific CD4+ T cells that survive in secondary lymphoid organs long after clearance of *Listeria monocytogenes* or LCMV infections consist of two subsets.[92,93] One subset has the characteristics of Tem cells including a $T\text{-}bet^{high}$ CCR7– phenotype, expression of nonlymphoid tissue homing receptors, and immediate IFN-γ production after stimulation with the relevant p:MHCII ligand. These cells correspond to human CD45RA– CCR7– CD4+ Tem cells, which express low or

variable levels of CD62L and high levels of fPSGL-1, and/or β1 and β7 integrins and produce IFN-γ or IL-4 rapidly when stimulated with anti-CD3 antibody in vitro.[137] In the mouse, CD4+ Tem cells are also found in liver, lungs, and gut long after intravenous injection of antigen plus adjuvant.[113] Because CD4+ memory T cells are constantly coming out of tissues and into afferent lymphatic vessels,[130,143] it is likely that CD4+ Tem cells are not fixed in nonlymphoid organs but recirculate through these sites.

The other microbial p:MHCII-specific CD4+ T-cell population that survives after clearance of acute infections in mice has the characteristics of Tcm cells. This population has a T-bet^{low} CCR7+ CXCR5+ Ly6C^{-} PD-1− Tfh-like phenotype and produces IL-2 but not IFN-γ immediately after stimulation with the relevant p:MHCII ligand.[92,93] These cells have been found to be potent helpers of antibody production by B cells.[143–145] In humans, this population can give rise to Tfh cells when stimulated in vitro.[146] In mice, these cells also generate Th1 cells and themselves during secondary immune responses and thus have the capacity of Tcm cells to generate diverse effector cells.[92]

Evidence suggests that Tem and Tcm memory cells derive from like effector cells. IFN-γ-producing CD62L− CD4+ Tem cells that survive after clearance of acute LCMV infection are derived from similar IFN-γ–producing CD62L− effector cells present at the peak of infection.[147,148] Similarly, the T-bet^{low} CXCR5+ CCR7+ Tfh-like Tcm cells that survive after clearance of *Listeria monocytogenes* infection are derived from similar T-bet^{low} CXCR5+ CCR7+ effector cells present at the peak of infection.[92]

Even though the various types of memory cells are derived from effector cells, they must be subsets of their effector cell precursor populations because they are 10 times smaller than those populations. In other words, even though Th1 effector memory cells are derived from similar Th1 effector cells, the former cells are 10 times less abundant than the latter cells. Thus, to understand how memory cells emerge at the end of the primary response, it is useful to consider the factors that cause 90% of the effector cells to die. Effector cells produce reactive oxygen species, which can cause cell death by damaging mitochondrial membranes. Bcl-2 family proteins regulate this type of death. Naïve and memory T cells express anti-apoptotic Bcl-2 molecules in their mitochondria, which inhibit the formation of apoptosis-inducing complexes consisting of Bim and Bak. In mice overexpressing Bcl-2 or lacking Bim, a greater fraction of the effector cell population survive contraction and differentiate into memory cells.[149] Thus, it is conceivable that under normal circumstances, the effector cells that are destined to become memory cells produce less reactive oxygen species or protect themselves better than other effector cells in the population.

Another possibility relates to expression of the IL-7 receptor. The survival of naïve T cells depends on IL-7 receptor signaling, which maintains Bcl-2 expression and limits Bim expression.[149] TCR-driven activation causes effector cells to lose the IL-7 receptor, leading to the fatal situation where Bcl-2 is reduced and Bim is increased. It has been reported that a subset of CD8+ effector cells retains IL-7 receptor expression and survives the contraction phase to become memory cells,[150] although some evidence against this idea has accumulated.[151,152] Thus, it is possible that 10% of the effector cells present at the peak of the T-cell response retain the IL-7 receptor or some other survival factor, which allows these cells to become memory cells.

Another possibility is that metabolic rate determines whether an effector cell will survive to become a memory cell. Quiescent naïve and memory T cells produce adenosine-5′-triphosphate by mitochondrial oxidative phosphorylation.[153] In contrast, activation causes effector cells to shift to glycolytic adenosine-5′-triphosphate production by a pathway that depends on mammalian target of rapamycin. Inhibition of mammalian target of rapamycin with rapamycin early during the primary response promotes the formation of CD8+ memory T cells by inhibiting T-bet expression in favor of another transcription factor, eomesodermin.[154–156] This result fits with a model in which most effector cells are killed by products of their own high rate of glycolysis through a T-bet–dependent program, which when blocked allows a return to oxidative phosphorylation and memory cell formation. In this model, memory cells arise from effector cells with the lowest glycolytic rates in the population. Again, increased glycolytic rate and the favoring of effector cell death over memory cell formation could involve IL-2 receptor signaling.

Tumor necrosis factor receptor family proteins also promote CD4+ T memory cell survival by inducing antiapoptotic factors by TRAF-dependent triggering of NFκB.[77] For example, effector T cells that do not express CD27 survive poorly in vivo.[157,158] Other work indicates that OX40 is dispensable for effector cell generation but required for memory cell formation.[159]

Recent work indicates that Id transcription factors also regulate effector-memory cell conversion. Id2 is required for the maximal generation of CD8+ effector T cells and perhaps the formation of Tem cells.[160–162] Conversely, Id3 appears to be critical for CD8+ Tcm cell formation.[162]

CONCLUSION AND MODELS

What follows is an attempt to unify the information presented in this chapter into a theoretical sequence of events that occur after naïve CD4+ or CD8+ T cells encounter the relevant p:MHC ligands during skin or mucosal infections, respectively. Because these processes are not completely understood, certain aspects of what follows are speculative.

Naïve T-cell populations that are specific for single p:MHC epitopes exist at a frequency of about 1:200,000 in the preimmune repertoire.[1] These T cells, like other naïve T cells, spend their life spans (~2 months in mice, ~2 years in humans) in a series of 1-day stops in the T-cell areas of different secondary lymphoid organs with intervening trips through the blood. While in the T-cell area, these naïve T cells receive survival signals through the IL-7 receptor as it binds to IL-7 made by stromal cells and through the TCR as it binds to the relevant selecting self p:MHC ligand on the surface of an APC, probably a dendritic cell.

Consider first the case of the CD4+ T-cell response to a bacterial infection in the dermis of the skin (Fig. 14.1). In this case, local dermal dendritic cells take up the microbe,

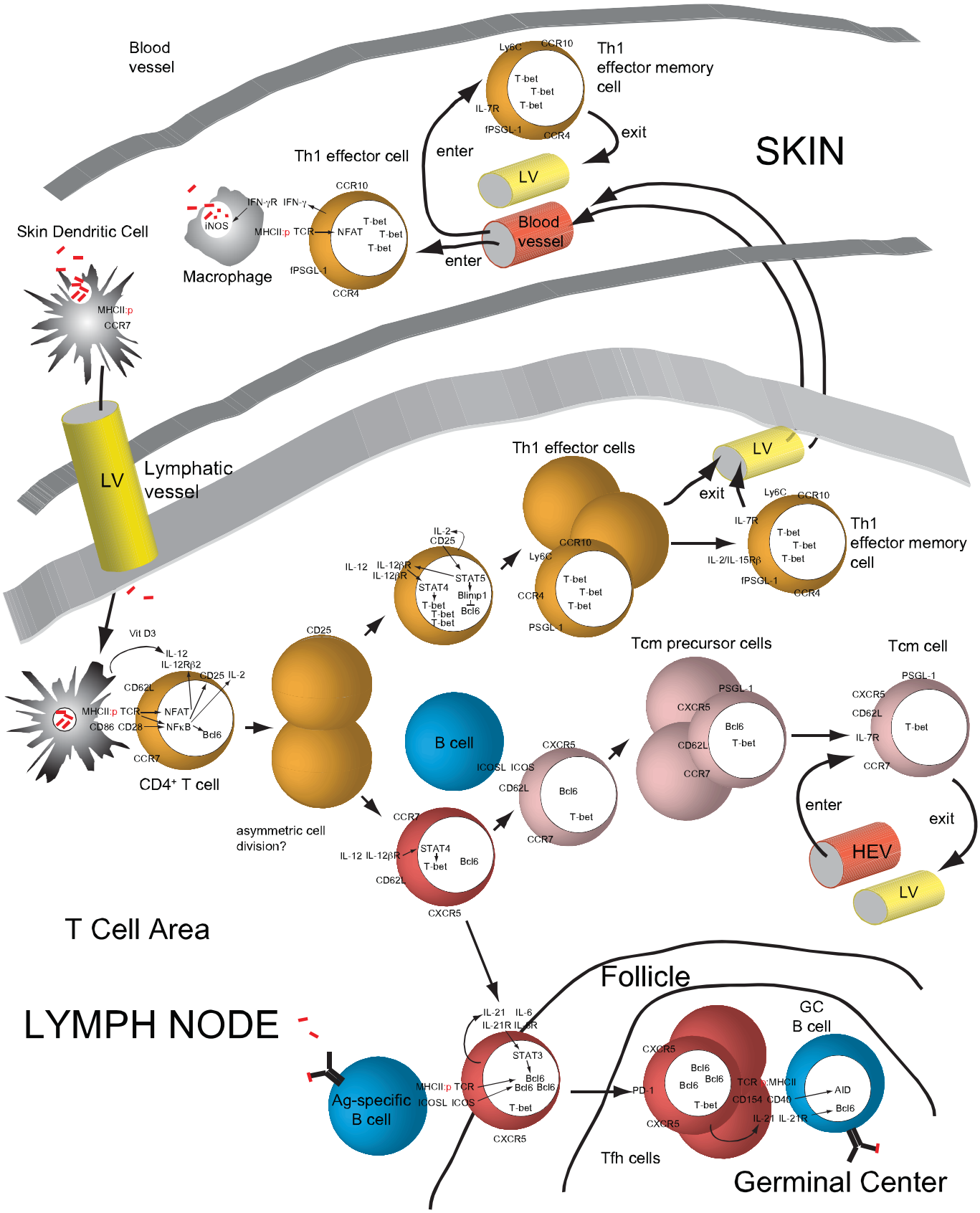

FIG. 14.1. CD4+ T-Cell Response to Bacterial Infection of the Skin. See the text for description. Bacterial antigen is indicated with red rectangles. Surface receptors are shown attached to the cell surface. Transcription factors are shown in the nucleus. Signals are shown with the *smallest arrows*. Sequential cell fates are shown with the *next largest arrows*. Migration routes are shown with the *largest arrows*. LV, lymphatic vessel; R, receptor.

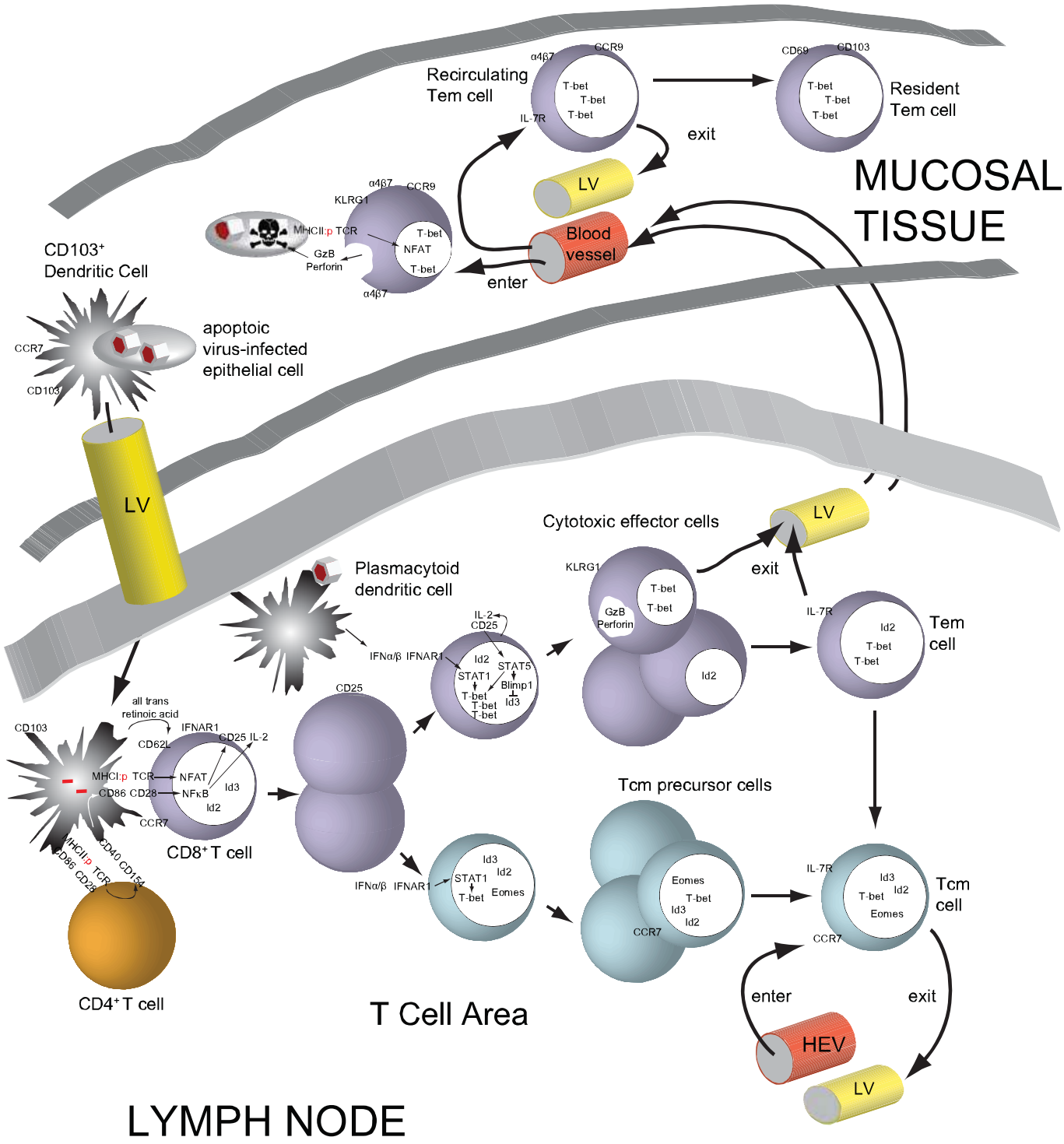

FIG. 14.2. CD8+ T-Cell Response to Viral Infection of a Mucosal Tissue. See the text for description. Virus particles are indicated with hexagons. Surface receptors are shown attached to the cell surface. Transcription factors are shown in the nucleus. Signals are shown with the *smallest arrows*. Sequential cell fates are shown with the *next largest arrows*. A virally infected cell killed by a cytotoxic clust of differentiation 8–positive T cell is indicated with a *skull and cross bones*. Migration routes are shown with the *largest arrows*. LV, lymphatic vessel; R, receptor.

produce microbe p:MHCII complexes,[45] upregulate CD28 ligands,[163] begin producing IL-12[164] and vitamin D3,[119] and migrate through an afferent lymphatic vessel to the draining lymph node. Naïve T cells interact with these dendritic cells[165] and those CD4+ T cells that express bacterial p:MHCII-specific TCRs receive TCR and CD28 signals[56] and induce the translocation of NFAT and NF-κB to the nucleus,[54] which in turn drive expression of CD25, IL-2, Bcl6, and the IL-12 receptor β2 chain, and loss of the IL-7 receptor.[61,89,166] The T cells then divide in response to IL-2 and unknown growth factors,[57] perhaps asymmetrically,[95] to produce CD25+ and CD25− effector cell progeny.[92,94] The CD25+ cells activate STAT5 through IL-2 receptor signaling,[167] which further increases expression of the IL-12 receptor β2 chain,[89] which combines with the IL-12 receptor β1 chain, binds IL-12, and activates STAT4[168] to amplify T-bet expression.[169] IL-2 receptor signaling and STAT5 activation also stimulates Blimp-1 expression,[170] which represses Bcl6,[171] thereby enforcing the Th1 fate and preventing Bcl6-dependent cell fates. The Th1 effector cells also express CCR10, CCR4, and fucosylated PSGL-1 due to activation in the presence of IL-12 and vitamin D3.[114,118] Some of these effector cells then leave the lymph nodes through efferent lymphatic vessels, enter the bloodstream, and migrate into the infection site through skin blood vessels displaying CD62P, CCL17, and CCL27.[116,119,172] After entering this site, the Th1 effector cells interact with infected macrophages displaying bacterial p:MHCII ligands.[173] TCR signaling then causes the T cells to secrete IFN-γ, which triggers production of inducible nitric oxide synthase and nitric oxide as well as killing of intracellular bacteria.[173] Some of the Th1 effector cells in the lymph nodes survive the contraction phase, re-express the IL-7 receptor, and become Th1 effector memory cells,[147] which recirculate through the skin.

The early CD25− progeny do not experience IL-2 receptor signaling and STAT5 activation, do not upregulate the IL-12 receptor β2 chain, and are left with a low amount of T-bet. Blimp-1 is also not turned on, allowing the cells to retain Bcl6 and express CXCR5. The CXCR5+ T cells then migrate toward the follicles.[103] If they interact with an antigen-specific B cell, then they receive TCR and ICOS ligand signals[98] and produce IL-21, which binds the IL-21 receptor and activates STAT3.[174] The combination of TCR, ICOS, and STAT3 signals then amplifies Bcl6 expression, stimulating the cells to express PD-1 and more CXCR5, and to commit to the Tfh lineage. The Tfh cells then migrate into germinal centers, interact with antigen-specific germinal center B cells, induce expression of CD154, which engages CD40 on the B cells and stimulates them to express activation induced cytidine deaminase, and undergo antibody isotype switching and somatic hypermutation.[98] If the early CXCR5+ T cells interact with B cells that are not specific for bacterial antigens, then they receive an ICOS signal that maintains their survival.[92] However, without additional TCR signaling, the T cells do not further upregulate CXCR5, do not lose CCR7 or CD62L, and some of these cell become Tcm cells, eventually losing Bcl6.[92] These Tcm cells recirculate through secondary lymphoid organs like naïve cells.

Now consider the CD8+ T-cell response to viral infection of a mucosal tissue (Fig. 14.2). Local CD103+ dendritic cells with cross-presentation capacity take up apoptotic infected cells,[41] produce viral p:MHCI and p:MHCII complexes, upregulate CD28 ligands, produce all-trans retinoic acid,[121] and migrate through an afferent lymphatic vessel to the draining lymph node. Plasmacytoid dendritic cells are recruited to the lymph node and produce type I IFNs in response to PRR recognition of viral pathogen-associated molecular patterns.[42] Interaction with CD154+ virus p:MHCII-specific CD4+ T cells increases the activation state of the CD103+ dendritic cells through CD40 signaling.[175,176] Naïve virus p:MHCI-specific CD8+ T cells interact with these dendritic cells and produce IL-2 and induce expression of CD25. Unknown T-cell growth factors and perhaps IL-2 then stimulate the CD8+ T cells to divide to produce progeny that express small or large amounts of CD25.[96] The CD25^{high} cells experience IL-2 receptor signaling and STAT5 activation, which stimulates Blimp-1 expression, which cooperates with STAT1 activation from the type I IFN receptor to upregulate T-bet.[177] T-bet and Blimp-1 drive the cells to become terminally differentiated KLRG1+ cytotoxic effector cells loaded with granzyme B and perforin-containing granules.[170,178] Blimp-1 represses another transcription factor Id3, which is required for Tcm cell formation,[162] thereby reinforcing effector cell formation. The effector cells also express α4β7 integrin and CCR9 due to activation in the presence of all-trans retinoic acid,[121] and migrate to the site of infection through mucosal blood vessels displaying mucosal addressin cell adhesion molecule-1 and CCL25.[179] After extravasating into this site, the cytotoxic effector cells interact with infected cells displaying virus p:MHCI ligands. TCR signaling then causes the T cells to expel granzyme B and perforin, which enter the target cell and kill it.[180] Some of the KLRG1+ effector cells in the lymph node survive the contraction phase, perhaps through the action of Id2,[161] and then either become CD103− Tem cells that recirculate through the mucosal tissue or CD103+ Tem cells that enter the tissue and never leave.[129] The recirculating Tem cells may convert to Tcm cells over long periods of time.[141]

The early CD25− progeny do not experience IL-2 receptor signaling and STAT5 activation and do not turn on Blimp-1.[178] Without Blimp-1, the cells retain Id3 expression[162] and are prevented from becoming terminally differentiated Tbet^{high} cytotoxic effector cells. Instead, type I IFN receptor signaling facilitates expression of the Eomes transcription factor,[156] retention of CCR7 expression, and survival as Tcm cells, which recirculate through the secondary lymphoid organs like naïve cells.

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SECTION V

The Intersection of Innate and Adaptive Immunity

CHAPTER 15

The Innate Immune System

Luke A. J. O'Neill

INTRODUCTION

Every organism has to contend with infection by microbes. Infectious agents such as bacteria, viruses, and fungi threaten health and viability by competing for nutrients or more dangerously by killing the infected host as a consequence of infection or in order to spread to another host. This threat occurs largely because microorganisms divide at a faster rate than the host and because of the production of toxins that the microorganism uses for its own viability. The job of the immune system is firstly to sense the danger posed and secondly to mount an appropriate response. This response can be to tolerate the microorganism or to resist it such that at best sterilizing immunity occurs.

The immune system is traditionally split into two categories: innate and acquired (or adaptive) immunity. The term "innate" refers to the feature that the sensors involved are encoded by genes that do not undergo any rearrangement to generate variants. Adaptive immunity, on the other hand, refers to host defense proteins encoded by genes that undergo rearrangement to generate great diversity. This rearrangement process is not driven by the infectious agent, but happens by genetic programming. Antigens then lead to an expansion of antigen-specific B cells or T cells that help clear the infection and importantly persist in the form of memory cells. This means that when the host is re-infected, there are substantially greater numbers of the specific B- and T-cell populations that lead to clearance of the infectious agent. There are therefore insufficient numbers of microbes to trigger substantial systemic symptoms.

It is estimated that more than 99% of life on earth only has innate immunity.[1] What advantage would the evolution of adaptive immunity bestow on an organism? The answer lies in part in the nature of innate immunity. As will be discussed, the effector mechanisms here involve the inflammatory process, which when out of control can harm the host. In mammals, a "sickness behavior" also occurs, which includes processes such as fever, aches and pains, and drowsiness, which are thought to allow the infected host to rest and recuperate.[2] They also present a disadvantage, however, because they leave the host vulnerable to predators and decrease reproductive potential. Adaptive immunity occurs later in the cycle of infection, typically becoming evident several days after infection, and probably only after innate immune defenses have been breached. It is needed for sterilizing immunity. The fact that memory is a key feature of adaptive immunity, however (which is evident because of the expansion of specific B and T cells), means that upon re-infection, the innate response is limited because the pathogen is more rapidly cleared and there are therefore fewer microbial products present to activate innate immunity. In addition, there is evidence that adaptive immune cells actually inhibit the innate immune response, which in the first infection acts in a negative feedback manner.[3] In subsequent infections, the memory cells probably inhibit innate immunity from the start of the infection. In essence, therefore, it appears that adaptive immunity evolved to promote sterilizing immunity and also to prevent innate immunity happening upon reexposure to pathogen, given its potential disadvantages in terms of systemic inflammation. Figure 15.1 illustrates this principal.

Previously thought to be crude and nonspecific, innate immunity is now known to involve a range of receptor families that recognize diverse microbial products. Importantly, these receptors program gene expression changes in target cells. The products of these genes drive inflammation and are also required for adaptive immunity. These proteins include cytokines that promote expansion of B and T cells. Other innate immune proteins have direct antimicrobial effects or act as opsonins, coating bacteria and promoting phagocytosis. Innate immunity has therefore gone from being viewed as a process lacking sophistication, to one determining for both inflammation and adaptive immunity.[4]

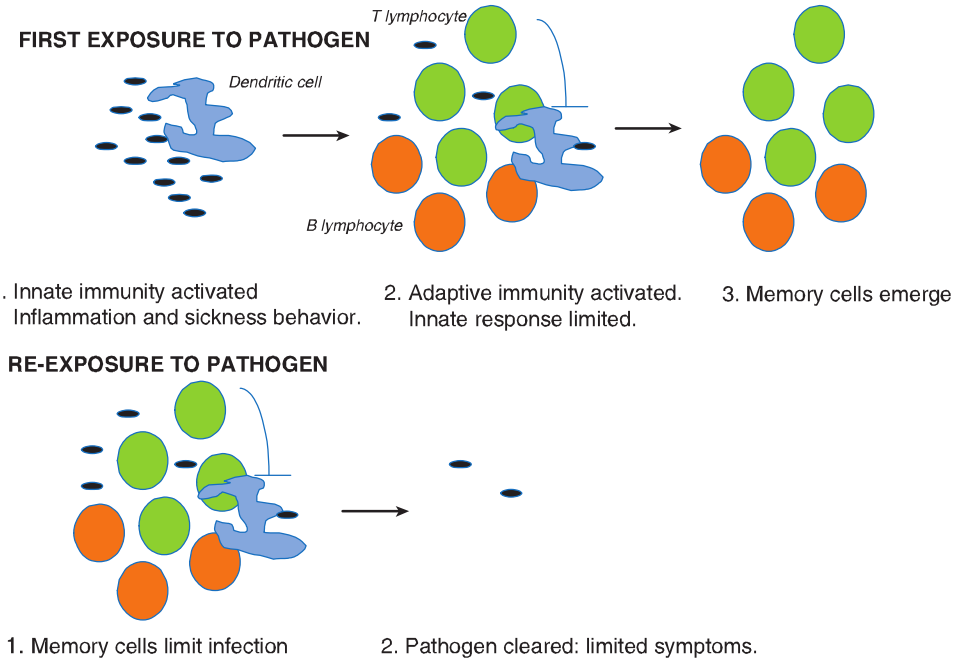

FIG. 15.1. The Relationship between Innate and Adaptive Immunity. A: Upon exposure to a pathogen, the innate immune response is triggered mainly by phagocytes and dendritic cells. Inflammation ensues, which leads to sickness behavior and host defense. The adaptive immune system is then triggered leading to effector T- and B-lymphocytes, some of which become memory cells. Adaptive immunity also has a negative feedback effect on innate immunity, preventing damage. **B:** When an organism is exposed to the same pathogen again, the memory cells can handle the infection, limiting innate immunity such that there are only minor symptoms. The evolution of adaptive immunity therefore provides an advantage to the host: adaptive immunity evolved to clear pathogens and limit innate immunity.

THE FRONTLINE OF INNATE IMMUNITY

The Skin

Innate immunity at its simplest involves barriers that prevent entry of microorganisms in the first place. In mammals, the skin is an obvious barrier that when broken in injury allows pathogens to enter. Many pathogens use insects that can deliver them across the skin into the bloodstream. A good example here is *Plasmodium falciparum*, which causes malaria. It is transmitted by mosquitoes feeding on blood. If the skin is broken, pathogens can enter. The importance of the integrity of the skin barrier can be seen in atopic dermatitis, an inflammatory skin disease. Mutations in the gene encoding fillaggrin, which is important for skin integrity, associate with atopic dermatitis.[5] The altered fillaggrin is less able to maintain the skin barrier, and the dermatitis is likely to be caused by microorganisms penetrating the skin or environmental irritants that provoke allergy.

The Epithelial Barrier

Equally important is the epithelial barrier that lines surfaces inside the body in the respiratory, gastrointestinal, and urogenital tracts. Microbes gain access to these surfaces via processes such as inhalation and digestion. The epithelial cells that make up the epithelium form tight junctions that keep the microorganisms out.[6] Specialized structures called cilia also expel microbes, and damage to cilia, for example caused by cigarette smoke, promotes respiratory tract infections.[7]

DIRECT-ACTING ANTIMICROBIAL FACTORS

The epithelia also produce a range of chemicals that limit microbial growth. Mucus is a key substance that traps microbes and is expelled. The importance of mucus can be seen in cystic fibrosis, where unusually thick mucus is made in lungs that limits expulsion and gives rise to growth of bacteria in the airways.[8] This chronic infection in turn leads to the lung pathology in this disease. In the stomach, acid production by the epithelium leads to low pH that limits growth of microbes. Enzymes that digest microbial proteins are also produced, including pepsin in the gut. A phospholipid-based substance termed pulmonary surfactant is made in the lungs that acts to trap microbes and can also directly lyse bacteria. Similarly, in skin the production of fatty acids in sebum limits bacterial growth. Lysozyme is a prominent enzyme in tears and saliva that breaks down peptidoglycan in the cell walls of gram-positive bacteria. Lysozyme is in fact a prototype of an innate immune protein because it does not affect the host cells, as they lack peptidoglycan. It is made by epithelial cells, but also phagocytes and Paneth cells in the crypts in the small intestine.

Antimicrobial Peptides

Another important example of innate immune proteins produced at barriers is antimicrobial peptides.[9] Epithelial cells are a major source of these peptides, but phagocytes can also make them. They are an ancient form of defense because they are found in most species, including insects and plants. There are three classes: defensins, catelicidins, and histatins. Defensins are 30 to 40 amino acid peptides that usually have three disulphide bonds. Their key property is amphipathicity (they contain both polar and nonpolar properties), and it is this property that is thought to underly their specificity for the membranes of bacteria, fungi, and certain viruses. Their hydrophobic part inserts into these membranes, which are more positively charged than host cell membranes. A pore is formed, rendering the membrane leaky and leading to lysis.

There are three subfamilies of defensins-: α, β, and θ, which have different specificities in terms of target microorganisms.[10] They are produced as propeptides that require processing by proteases. Neutrophils produce α-defensins and store them in so-called primary granules from which they are secreted upon neutrophil activation. Paneth cells in the gut produce a class of α-defensins called cryptidins, which are processed by trypsin prior to secretion into the gut., β-defensins are made in the urogenital and respiratory tracts, skin, and tongue. Keratinocytes in skin are also a key producer of β-defensins.

Catelicidins lack the disulphide bonds in the α-defensins and are produced by epithelial cells, phagocytes, and keratinocytes. They are also produced as propeptides and in neutrophils are processed by elastases in secondary granules and then secreted in response to microbial stimulation. Histatins are produced in the parotid, sublingual, and submandibular glands in the oral cavity. They are histidine-rich and target fungal membranes.

Phospholipase A2

Phospholipase A2 (PLA2) is another antimicrobial protein found in primary granules of neutrophils and similar to defensins, is secreted during degranulation in response to bacterial activation. It is also secreted by epithelial cells and Paneth cells. PLA2 can directly kill gram-positive bacteria by hydrolysing phospholipids in the microbial membrane.[11] The means by which it distinguishes host membranes from bacterial membranes is because its effect on phospholipids is strongly potentiated by another protein termed bactericidal permeability increasing (BPI), which binds to lipopolysaccharide (LPS) in gram-negative bacterial membranes.[12] As will be discussed subsequently, LPS is one of most potent innate immune activators known; it is sensed by a protein termed toll-like receptor-4 (TLR4). LPS does not occur in host membranes, and so BPI only facilitates the effect of PLA2 on bacterial membranes. BPI disrupts the integrity of the bacterial membrane and presumably allows PLA2 to gain access to phospholipids, where it cleaves fatty acids from the sn-2 position, leading to lysis. Similar to PLA2, BPI is found in primary granules in neutrophils.

Lactoferrin

Lactoferrin is an 80 kDa protein that belongs to the transferrin family and therefore acts to sequester iron. This effect is thought to limit the availability of iron to bacteria, altering their metabolism and thereby compromising their ability to replicate.[13] Lactoferrin is found in the secondary granules of neutrophils and occurs at a high level in breast milk, its cellular origin there being epithelial cells. Lactoferrin can also be processed to generate an antimicrobial peptide called lactoferricin that, similar to other such peptides, can lyse bacterial membranes.

The Microbiota

A final front-line mechanism to keep microbes at bay is commensal bacteria in the healthy intestine. Commensal bacteria live in our intestines and serve several functions, including providing vitamin B_{12} and other nutrients. However, they also maintain epithelial barrier integrity, most likely because they are sensed by innate immune receptors that will be described in subsequent sections, and these receptors seal the epithelial barrier against infection.[14] However, importantly they also compete with any pathogenic bacteria in the intestines limiting their growth. Similarly, on skin and in the urogenital tract, there are competing microbes.

THE INITIAL RESPONSE ONCE BARRIERS ARE BREACHED

What happens when skin or epithelial barriers are breached? Pathogens have mechanisms to get across the barriers, or if the barrier is damaged by trauma, pathogens will traverse the broken barrier. Two important cascades are triggered in blood that will generate inflammatory factors or act to wall off pathogens and prevent them from spreading. The first cascade to be characterized in detail is the complement cascade, described in detail elsewhere in this volume. Complement generates the membrane attack complex that will lyse bacteria. Complement factors such as C1q also bind to pathogen surfaces and act as opsonins, promoting uptake by neutrophils and phagocytes. The complement cascade also generates chemotactic factors such as C5a, which attracts neutrophils to the site of infection. Coagulation is the other cascade that is triggered, largely by bacteria. This will form clots that prevent bacteria from spreading.

The Neutrophil

A key cell type, however, that is engaged early in infection is the neutrophil, again described in detail elsewhere. Chemotactic peptides present in bacteria, termed fMLP, act as chemoattractants for neutrophils. Both C5a and fMLP also trigger an important process in the neutrophil termed the respiratory burst.[15] This involves an enzyme complex termed the nicotinamide adenine dinucleotide phosphate-oxidase. This is also present in macrophages and is activated either by the G-protein coupled receptors that bind fMLP or C5a, or by the process of phagocytosis. It is a multicomponent enzyme complex that contains three cytosolic subunits: p40phox, p47phox, and p67phox. It also contains a membrane-associated flavocytochrome complex comprising p22phox and p91phox. The subunits form the enzyme complex following activation of the low molecular weight G protein Rac. The nicotinamide adenine dinucleotide phosphate-oxidase generates reactive oxygen species by transferring an electron from its flavin adenine dinucleotide cofactor to molecular oxygen forming the superoxide anion and other oxygen radicals. This all occurs in the so-called phagolysosome, which comprises a fusion between the phagosome that contains the ingested bacteria with the lysosome. The oxidizing environment will be antibacterial, but in addition, will lead to acidification of the phagolysosome due to potassium and hydrogen ions being drawn in to neutralize the charged superoxide ion. The acidification, however, has been shown to lead to dissociation of proteolytic

enzymes such as elastase from the proteoglycan matrix in the phagolysosome.[16] These proteases will also have a direct effect on bacteria.

In addition, superoxide is a substrate for myeloperoxidase, which is stored in primary granules and generates hypochlorous acid and chloramines, as well as other reactive oxygen species that are bactericidal and fungicidal. A final oxygen radical generated in neutrophils is nitric oxide. It is produced by the enzyme nitric oxide synthase, which is induced by cytokines such as interferon-γ. Nitric oxide is also microbicidal.

THE CONCEPT OF PATTERN RECOGNITION: HISTORICAL PERSPECTIVE

Much of the forgoing components of innate immunity had been worked out at least in broad terms by the mid-1990s. However, the issue of the exact recognition mechanism during innate immunity by the host was poorly understood. In 1989, Janeway made the point that the continued focus on adaptive immunity, which was evident at the time, would lead to an asymptote being approached in our understanding of immunity.[17] He speculated that receptors must exist for microbial structures that were known to be proinflammatory, such as LPS from gram-negative bacteria. Receptors such as mannan-binding lectin (MBL), which binds mannose in microbial membranes (and which does not exist in host membranes) and CD14 (which binds LPS), had already been described at that stage, but importantly these proteins did not signal because they possessed no intracellular domains. MBL was shown to be an opsonin, and CD14 had no clear function. Janeway therefore proposed that pattern recognition receptors (PRRs) must exist that recognize microbial "patterns" such as LPS, termed pathogen-associated molecular patterns (PAMPs), and trigger activation of cells such as dendritic cells, which are prodigious producers of cytokines and also present antigen to T cells. At around the same time, Matzinger was proposing that the job of the immune system is to sense "danger."[18] The danger can be in the form of a microbe or in the form of tissue injury. Again, the idea that specific receptors would exist to recognize products of damaged tissue was proposed. In essence, what was being hypothesized was the existence of receptors that would drive the production of cytokines, as several cytokines had been found that could provoke inflammation and also adaptive immunity. LPS was a powerful inducer of such cytokines and yet no receptor had been convincingly described for LPS that would activate transcription factors such as NF-kB, which was known to be required for increased transcription of genes such as that encoding the potent proinflammatory cytokine tumor necrosis factor (TNF).

How did Janeway's insight that PRRs and PAMPs would form the core of innate immunity lead to the discovery of the TLRs, which became the prototypical PRRs? Five important findings were made.

1) The receptor for the proinflammatory cytokine interleukin (IL)-1, which is a key mediator of many aspects of inflammation and which, similar to LPS, could activate NFκB, was shown to have a signalling domain similar to a *Drosphila melanogaster* protein Toll.[19] The Toll protein had been discovered as being important in the generation of dorsal-ventral polarity in the developing *Drosophila* embryo. At first glance, this seemed odd. What was a receptor important for inflammation doing with a signaling domain highly similar to a protein in fruit fly development? Toll drives a transcription factor termed dorsal, however, which is highly similar to NFκB so it seemed as if a similar "machine" was being used in two different contexts: one for inflammation in the case of IL-1 and the other for development in the fruit fly.
2) In 1994, a protein termed N protein was described in tobacco by Whitham and colleagues, which provided resistance to tobacco mosaic virus.[20] This protein also had the domain found in the type I IL-1 receptor and Toll, which was then named the Toll-IL-1 receptor–resistance (TIR) domain. Clearly, the TIR domain was therefore important for innate immunity in very diverse species.
3) This was confirmed when in 1996, Lemaitre and colleagues reported that Toll also has a role in innate immunity in *Drosophila*, being activated in response to fungi and driving the antifungal peptide drosomycin.[21]
4) Mammalian proteins even more similar to Toll than the IL-1 receptor, and termed TLRs, were then reported[22] and one, termed Toll (subsequently renamed TLR4) when overexpressed in dendritic cells, was shown to drive costimulation of T cells, as reported by Medzhitov and colleagues.[23]
5) In 1999, Poltorak and colleagues reported that the lack of responsiveness of a strain of mouse termed C3H/HeJ to LPS was due to a mutation in the gene encoding TLR4, such that a proline in the signaling domain was converted to a histidine.[24] This rendered TLR4 unable to signal, identifying TLR4 as the long-sought signaling receptor for LPS. It was therefore clear that TLRs were ideal examples of the PRRs proposed by Janeway. Subsequently, 10 TLRs were described in human, although other species have very many more, notably the sea urchin, which has over 150.[25] TLRs were shown to recognize a diverse range of PAMPs and also in some cases products of damaged tissue, termed danger-associated molecular patterns (DAMPs). Complex signaling pathways were elucidated and links to disease established. The discovery of TLRs also galvanized researchers to seek other PRR types, and there are now several families, notably the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), which sense viral ribonucleic acid (RNA), the nucleotide oligomerization domain (NOD)-like receptors (NLRs), which sense bacteria and DAMPs, and the C-type lectin receptors, which sense fungi.[26] Figure 15.2 illustrates the main families of PRRs. A level of complexity in innate immunity was therefore revealed that has provided great insight into the overall workings of the immune systems of many organisms.

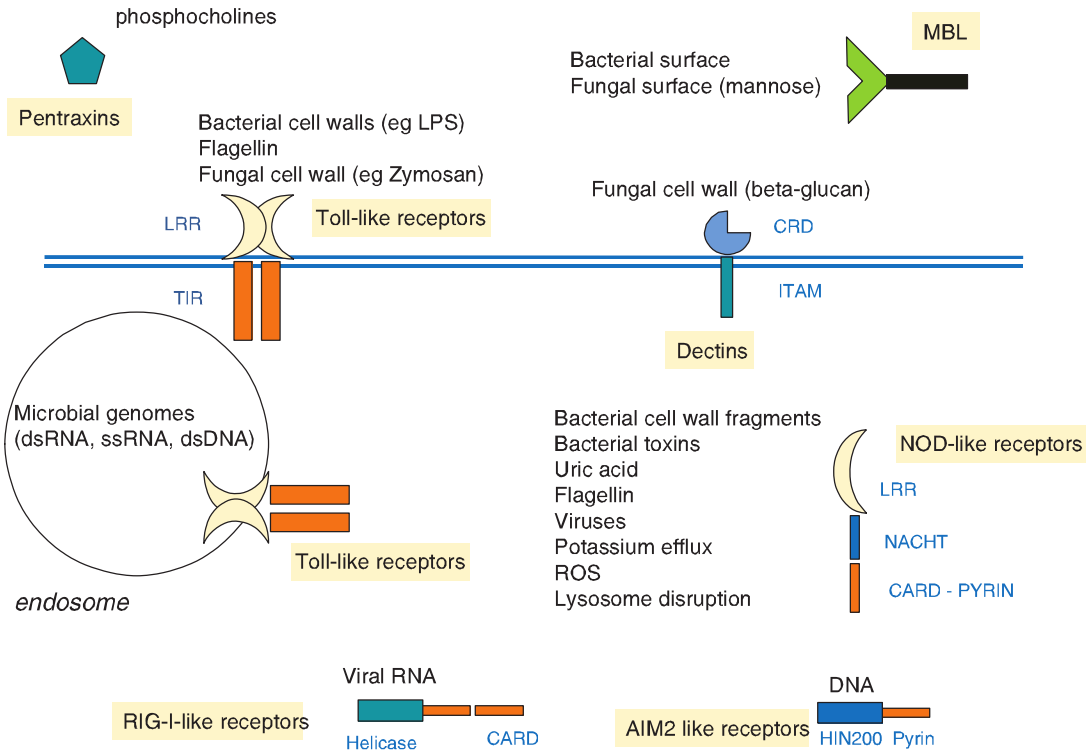

FIG. 15.2. Innate Immune Sensors. Several classes of innate immune sensors have been found. Soluble factors such as pentraxins and mannoan-binding lectins bind pathogen structures and promote phagocytosis. Toll-like receptors (TLRs) sense bacteria, fungi, and protozoans from the plasma membrane, whereas endosomal TLRs sense microbially derived nucleic acids. C-type lectin receptors sense fungal cell wall components and mycobacteria. In the cytosol nucleotide oligomerization domain–like receptors sense microbial products and also, in the case, of Nlrp3 particles such as uric acid and IAPP. Retinoic acid-inducible gene I–like receptor sense viral ribonucleic acid, whereas AIM-2-like receptors sense microbial deoxyribonucleic acid. All lead to signaling pathways culminating in the induction of immune and inflammatory genes.

Toll-Like Receptors

TLRs are type I transmembrane receptors that have leucine-rich repeats (LRRs) and a TIR domain. LRRs are found in many proteins, and that they occur in TLRs is of interest given their capacity to interact with different kinds of ligands. Also of note is that in agnathans, the LRR is used to build a repertoire of variable lymphocyte receptors, which are analogous to the antibody repertoire in human.[27] The LRR domain is therefore of evolutionary interest in that it is used for TLRs but also generation of diversity in the lamprey and the hagfish. The immunoglobulin domain serves this purpose in vertebrates and in fact is fused to the TIR domain in the type I IL-1 receptor. Figure 15.3 illustrates the relationship between these domains.

The 10 TLRs in human have different ligand specificities, and several ligand/TLR structures have been solved.[28] Broadly speaking, TLRs can be split into two families. TLRs 1, 2, 4, 5, and 6 are expressed in the plasma membrane, and their job is to sense bacterial, fungal, and protozoal products. TLRs 3, 7, and 9 are all expressed in endosomal membranes with their LRRs in the lumen and their TIR domains pointing into the cytosol. Their role is to sense viral nucleic acids. There are also differences in relation to signaling,[29] with all of the TLRs with the exception of TLR3 signalling via MyD88, to NF-κB and mitogen-activated protein (MAP) kinases such as p38. TLR2 and especially TLR4 require a bridging adapter termed Mal to recruit MyD88. TLR3 signals via Trif to NF-κB and another transcription factor IRF3. TLR4 can also signal via Trif and does so via the second bridging adapter Tram.

Toll-Like Receptors 2, 1, and 6

TLR2 recognizes multiple ligands. From bacteria, these include lipopeptides, lipoproteins, lipoteichoic acid, and mycobacterial lipoarabinomannan. From yeast, TLR2 recognizes the cell wall structure zymosan. TLR2 also recognizes glycosylphosphoinositol from the parasite *Tryanosoma cruzi*. Alone amongst the TLRs, TLR2 can heterodimerize with other TLRs, and this provides a degree of specificity in ligand recognition.[30] The TLR1/TLR2 dimer binds triacylated lipopeptides whereas TLR2/TLR6 recognizes diacylated lipopepides. In the case of TLR2/6, one acyl chain fits into a hydrophobic pocket in one TLR, whereas the second acyl chain fits into the other TLR.[31] This allows the TLRs to dimerize, an important event for signaling. There is also a coreceptor for TLR2/6 in the form of CD36, which appears to deliver the

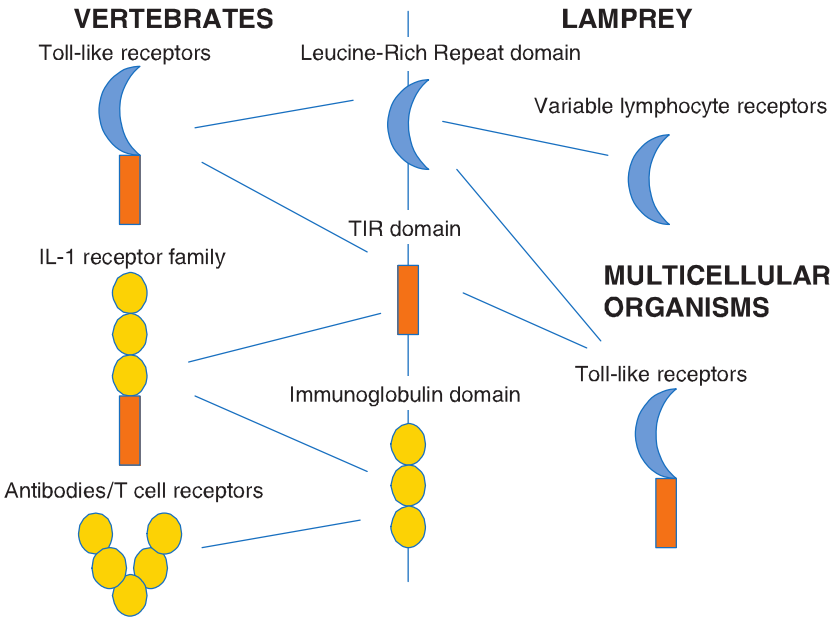

FIG. 15.3. Protein Domains in Innate and Adaptive Immunity. Three protein domains occur in several immune system proteins. The leucine-rich repeat occurs in toll-like receptors in vertebrates and all multicellular organisms combing with the toll-IL-1 receptor–resistance (TIR) domain. The leucine-rich repeat also forms the basis of the repertoire of variable lymphocyte receptors in the hagfish. The immunoglobulin domain only occurs in vertebrates and is found in antibodies and T-cell receptors. However, it also combines with the TIR domain to form interleukin-1 receptor family members.

lipopeptide to the TLR dimer.[32] TLR2 is expressed on macrophage, dendritic cells, B cells, and also on regulatory T cells.

Toll-Like Receptor 4

TLR4 is the receptor for LPS and is the most studied TLR. It acts as a homodimer but on its own is unable to recognize LPS. Similar to TLR2 and CD36, a coreceptor is needed, termed MD2.[33] LPS is a hexacylated lipid, and five of the acyl chains are buried in MD2, whereas the sixth is on the surface and interacts with TLR4.[34] However, two other proteins are needed to deliver LPS to MD2. LBP occurs in serum and binds LPS monomers, transferring them to CD14.[35] CD14 can occur as a soluble protein or as a glycerophosphatidylinositol anchored protein.[36] The job of CD14 appears to be to concentrate LPS and deliver it to MD2. In B cells, there are two other accessory molecules: RP105 and MD1.[37] These appear to serve an analogous function to CD14 and MD2. Recognition of LPS is therefore highly complex. This is presumably because once TLR4 is activated, there can be lethal consequences in the form of septic shock; therefore, several proteins are needed for LPS recognition. Similar to TLR2, a dimerization event occurs upon ligand binding that allows the intracellular TIR domains to come into juxtaposition and trigger signaling pathways. TLR4 has been shown to recognize other ligands, notably F protein from respiratory syncytial virus,[38] although the basis for this recognition is not known. Another example is the metal nickel. In contact dermatitis, there is an inflammatory reaction to nickel, and it has been shown that nickel coordinates two key histidines on different TLR4 proteins, causing a cross-linking event that activates TLR4 and drives the inflammatory process in skin.[39] TLR4 is expressed on multiple cell types, notably on macrophages and dendritic cells but also on neutrophils and in the endothelium.

Toll-Like Receptor 3

TLR3 is the receptor for double-stranded RNA, found in viruses or more commonly in the form of the viral RNA analogue polyIC. PolyIC had been known for some time to be a potent inducer of the antiviral type I interferons. Similar to LPS, therefore, the discovery of TLR3 as the receptor for polyIC provided a molecular explanation for how polyIC induces this response. TLR3 is expressed on macrophages and dendritic cells and importantly on CD8+ dendritic cells, which are potent type I interferon producers.

Toll-Like Receptor 5

TLR5 is the receptor for bacterial flagella.[40] Flagellin is a key protein in flagella, which occur in multiple bacteria species. Flagellin is in many ways a prototypical PAMP because it does not occur in humans and can bear few mutations in bacteria. TLR5 is therefore required for host defence against flagellated bacteria such as *Legionella* and *Salmonella*.

Toll-Like Receptor 7

TLR7 recognizes single-stranded RNA from viruses. It is highly expressed in plasmacytoid dendritic cells, where when activated it induces type I interferons. TLR7 was first described as the receptor for a small molecule termed imiquimod, which is used to treat genital warts. This effect is mediated by type I interferons. TLR7's expression on plasmocytoid dendritic cells reveals a key function in antiviral immunity.

Toll-Like Receptor 9

TLR9 is the deoxyribonucleic acid (DNA) sensing TLR that, similar to TLR3 and TLR7, is found in endosomes. The type of DNA recognized is of interest as it binds to hypomethylated CpG-rich DNA, commonly found in bacteria and viruses. TLR9 is also expressed in plasmacytoid dendritic cells as well as in macrophages and in the epithelium. Once activated, again by dimerization, TLR9 induces a strong proinflammatory signal, increasing TNF production as well as type I interferons. A protein termed Unc93b is required to traffic TLR9 from the Golgi to intracellular vesicles. The CpG-rich DNA

from pathogens is taken up by cells in an endosome, which fuses with the TLR9-expressing endosome. In this way, the receptor can interact with its ligand. TLR9 has no known co-receptors, but it has been shown that it requires cleavage by cathepsins for full activation.[41]

The Recognition of Danger-Associated Molecular Patterns by Toll-Like Receptors

TLRs have also been implicated in the recognition of products of damaged tissues.[42] This is an important area as the inflammatory process can occur under conditions where there is apparently no infection. DAMPs include a range of factors released by damaged cells and include high-mobility group protein 1, heat shock proteins such as hsp90, products of damaged connective tissue such as hyaluronic acid fragments, versican and tenascin-C, the acute-phase reactant serum amyloid A, pyrrole-based lipids such as CEP, and coagulation products such as fibrinogen. This area has been somewhat controversial, as in vitro there is a risk of LPS contamination. However, the consensus has emerged that at least some of these are indeed sensed by TLRs although what the mechanism might be is not fully understood. Many of these are sensed by TLR4, notably high-mobility group protein-1, hsps, hyaluronan fragments, and tenascin-C. TLR2 has been shown to sense versican and CEP. Importantly, these factors are only produced by damaged cells, as would occur during tissue injury. There may be coreceptors involved here, which explains the recognition process, because it is difficult to imagine how a single TLR can recognize multiple ligands. A clearer example is the sensing of host nucleic acids in the case of cell damage. Necrotic cells have been shown to release double-stranded RNA, which is sensed by TLR3. TLR7 can sense host RNA, and TLR9 can sense host DNA in the context of immune complexes. This has particular relevance to autoimmune diseases such as systemic lupus erythematosus (SLE).[43]

Toll-Like Receptor Signaling Pathways

The key output from TLR signaling is cytokines, and TLRs filled the missing gap in our understanding of what actually induces cytokines. Because they bind directly to microbial ligands, TLRs bring us close to the initiation of host defense.

The type of cytokine being induced is dependent on the TLR. TLR4 is the most prodigious activator of macrophages known, with many genes induced by TLR4. Other TLRs are less prolific but all share the feature of driving inflammation. Cytokines such as IL-6, TNF, and the chemokine IL-8 are typical responses for plasma membrane receptors. For the endosomal TLRs, type I interferons are the typical readout. What gives rise to signaling specificity? As stated previously, TLRs use different adapter proteins. All have a TIR domain, and upon TLR dimerization, adapter TIR domains are likely to be recruited to the interface formed between two TLRs. This then launches the signaling pathways.

Broadly speaking, TLR signaling pathways can be divided into two: MyD88-dependent and MyD88-independent. All TLRs, with the exception of TLR3, signal via MyD88. IL-1 receptor family members, including the receptor for IL-18, also signal via MyD88.[44] The MyD88 dependent pathway involves the IL-1 receptor–associated kinases (IRAKs). There are four of these: IRAK-1, IRAK-2, IRAK-4, and IRAK-M. They interact with MyD88 via their death domains, because along with the TIR domain, MyD88 has a death domain. The structure of this multicomponent signaling machine, termed the MyDDosome, has been solved.[45,46] Strikingly, it has a stoichiometry of 14. SIx MyD88 subunits assemble and bring four IRAK-4s to the complex. These sit on top of the assembled MyD88 subunits in a helical structure. Four IRAK-2s or IRAK-4s are then recruited. All of the death domain interfaces in this complex have been solved, and it is predicted to have a high degree of cooperativity. The so-called MyDDosome launches signaling pathways activated by TLRs.

Once assembled, IRAK-4 phosphorylates IRAK-1, and this event appears to be key for signaling. The IRAKs then dissociate from the complex, and IRAK-1 and/or IRAK-2 then interact with the ubiquitin ligase Traf6, a member of the Traf family. Once activated, Traf-6 signals in association with two noncanonical E3 ubiquitin ligases, Ubc13 and Uev1A.[47] This results in polyubiquitination of Traf6. This is a type of ubiquitination termed K63-linked ubiquitination. This covalently modifies Traf-6 causing oligomerization and downstream activation. This activation involves the IkappaB kinase (IKK) complex for the NF-κB pathway. The main form of NF-κB in cells is a p50/p65 dimer. It is, however, kept in check by an inhibitory protein I-κB. Traf6 activates the kinase TAK-1, which in turn activates the IKK complex leading to I-κB degradation (via K48-linked polyubiquitination). This releases the p50/p65 dimer, exposing a nuclear localization sequence and allowing NF-κB to bind its consensus site in target genes. In addition, TAK-1 can activate MAP kinase cascades, notably via MKK3/6, which leads to p38 MAP kinase activation, and MKK7, which leads to activation of Jun N-terminal kinase . Both of these MAP kinases can activate transcription factors, such as ATF3 in the case of p38 and Jun in the case of Jun N-terminal kinase. This in turn will modulate inflammatory gene expression.[48]

MyD88 is also involved in activation of IRF7 by TLR7 and TLR9.[49] This is an important transcription factor for the induction of interferon–alpha, a key antiviral cytokine but also a cytokine implicated in the pathogenesis of SLE.

The importance of the MyD88 pathway can be seen in MyD88-deficient mice, which are unable to mount an effective host defense response to multiple pathogens, including a wide range of bacteria, as well as fungi, protozoans, and certain viruses.[50] In addition, MyD88-deficient mice are resistant to inflammation in a number of disease models including models of SLE and arthritis. However, in the case of humans, the situation is not as clear-cut. This is because of reports of MyD88 deficiency in human.[51] People with this deficiency show higher mortality in childhood but susceptibility to a restricted set of pathogens, the main defect in host defense being against pyogenic infections such as *Streptococci*. This is also the case in subjects with IRAK-4 deficiencies.[52] Once people with these deficiencies reach adulthood, however, no immunodeficiencies are evident. It is likely, therefore, that the MyD88 pathway is somewhat redundant in children and is not required in adults probably

because of the emergence of adaptive immunity. This supports the concept raised at the start of this chapter, that once memory is established, innate immunity is not as important.

The second TIR domain adapter to be found was Mal (also termed TIRAP).[53,54] This protein is required for MyD88-dependent signaling specifically by TLR2 and especially TLR4. It appears to act as a bridging adapter for MyD88 to be recruited to the receptor TIR domains and has a PIP2 binding domain which localizes it to the plasma membrane.[55] It is also a substrate for IRAK-1 and IRAK-4, and upon phosphorylation undergoes ubiquitination and degradation,[56] providing a negative feedback loop in TLR signaling.

The MyD88-independent signalling pathway involves the third adapter, Trif.[57] This adapter is used by TLR3 to signal and activates NF-κB as well as another transcription factor IRF3 via activation of the kinase TBK-1.[58] TLR4 can also activate Trif, but this happens in endosomes. The fourth adapter, Tram, mediates this effect, acting as a bridge for Trif in a manner similar to Mal acting as a bridge for MyD88.[59] Tram has a myristoylation motif that localizes it to the plasma membrane.[60] However, upon activation of TLR4, Tram undergoes phosphorylation by protein kinase C-epsilon.[61] This leads to dissociation of Tram from the plasma membrane, and it is likely that a TLR/Tram complex localizes to the endosome, where Trif is then recruited, again leading to TBK-1 activation and induction of type I interferons. The TLR4 signal is terminated by a splice variant of Tram termed TAG, which displaces the Trif/Tram complex.[62] It is this dual pathway, Mal/MyD88 and Tram/Trif, occurring at different subcellular localizations, that probably make LPS such a powerful innate immune activator, as a wide range of genes can be induced. Figure 15.4 illustrates the TLR4 signaling pathway.

The final TIR domain adaptor to be found is SARM. This is a large protein with a TIR domain and SAM and ARM domain. SARM is a negative regulator of Trif-dependent signaling, acting as another modulator of TLR4 action.[63] It is of interest that there are several mechanisms to inhibit TLR signaling, including microRNAs, decoy receptors, ubiquitin regulators (eg, A20), and splice variants of MyD88 and IRAK2 that are inhibitory.[64] This tells us that TLRs, and in particular TLR4, must be kept under control otherwise the host can be damaged or even succumb.

Viruses have also been shown to target TLRs. The best examples are two vaccinia virus proteins A46R and A52R.[65] A46R has been shown to block recruitment of TIR domain–containing adapters, particularly Mal and Tram.[66] A52R inhibits activation of the IRAKs.[67] Deletion of A52R leads to attenuation of the virus, confirming its importance for viral evasion.[68]

Conservation of the Toll-Like Receptor System Across Nature

Every multicellular organism has proteins with TIR domains, attesting to their evolutionary importance. A particular case is in plants, which have a number of TLRs.[69] As mentioned previously, N protein from tobacco has a TIR domain and is required for antiviral immunity. Similarly, L protein from flax has a TIR domain and is required for resistance to flax rust. Plants also have IRAK-like kinases activated by these receptors, notably BAK1 and BIK1 in Arabidopsis. The receptor for flagellin in Arabidopsis is sensed by a leucine rich repeat protein termed FLS-2. This receptor has an IRAK-like kinase domain actually attached and also activates BAK-1 and BIK-1. FLS-2 activates MAP kinases such as MEKK1, which probably lead to activation of the AtWRKY family of transcription factors. In addition, ubiquitin ligases termed PUBs ubiquitinate FLS-2, downregulating it.[70] The parallel in human is Triad3a, which ubiquinates TLR4 leading to its degradation.[71] These conserved elements confirm the importance of the TLR pathways across evolutionary time. Table 15.1 illustrates the conservation between mammals, insects, and plants.

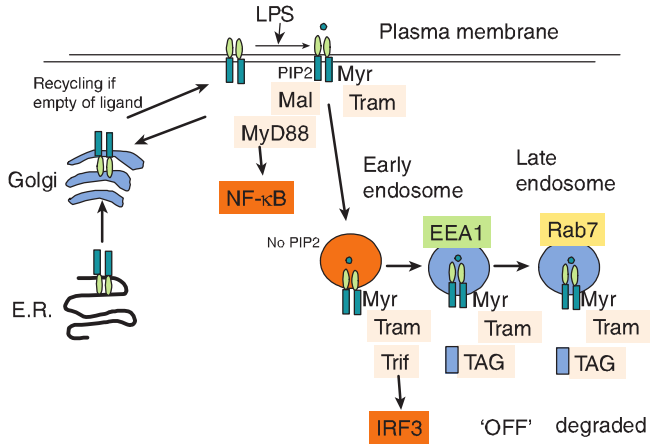

FIG. 15.4. Subcellular Toll-Like Receptor 4 Signaling. The signaling pathway, activated by lipopolysaccharide (LPS) via toll-like receptor-4 (TLR4), is highly complex. In resting cells, TLR4 recycles from the endoplasmic reticulum to the plasma membrane. An LPS/MD2 complex binds TLR4, stabilizing it transiently at the plasma membrane. The activated TLR4 dimer recruits the adapters Mal and MyD88, which then lead to activation of NF-κB, which induces multiple immune and inflammatory genes, such as tumor necrosis factor, interleukin-1beta, and interleukin-6. Mal is retained in the membrane by binding the phospholipid PIP2. Th adapter Tram is also membrane-localized via a myristate in its N-terminus. Tram then undergoes phosphorylation by protein kinase C-epsilon. TLR4 then internalizes in combination with Tram and localizes to an early endosome where it encounters Trif. This adapter activates TBK-1 and promotes IRF3 activation, which regulates the expression of type I interferons and other gene products. The endsome containing TLR4 then matures to a late endosome where TAG is engaged. This protein displaces Trif from Tram, and TLR4 is ultimately degraded.

Therapeutic Targeting of Toll-Like Receptors

TLRs are being targeted clinically, either in the form of new vaccine adjuvants or with inhibitors that would be anti-inflammatory.[72] The best example of an adjuvant approach is imiquimod, a compound found to be antiviral by activating TLR7. This induces type I interferons, and imiquimod is used to treat genital warts. Another example of an adjuvant approach is the use of monophosphoryl lipid A in vaccines for such pathogens as human papilloma virus. This acts via TLR4 but is not as toxic as LPS. In terms of anti-inflammatory

TABLE 15.1 Conservation of Innate Immunity across Species

	Human	Fruit Fly	Plant
Receptor (TIR domain)	Toll-like receptors	Toll	Tobacco: N protein Flax: L6
Adapter protein (TIR domain)	MyD88, Mal, Trif, Tram	dMyD88	
Non-RD kinase	IRAK-1	Pelle	Rice: XA21
RD kinase	IRAK-4		Arabidopsis: BIK1
Ankyrin repeat	IkappaB NF-κB	Cactus Relish	Rice: NH

RD, Arginine-Aspartic acid; TIR, toll-IL-1 receptor–resistance.
Notes: Each receptor senses microbial products and activates the adapters and kinases indicated. Human and insects have the NF-κB family transcription factors on these pathways. Plants lack NF-κB but have other transcription factors such as the WRKY family.

approaches, eritoran, an antagonist toward TLR4, has been tested in septic shock, but with only limited success. As referred to previously, TLR4 has also been implicated in the pathogenesis of contact dermatitis. It is also implicated in rheumatoid arthritis and inflammatory bowel disease. TLR2 has been shown to be important for ischemia/reperfusion injury and may prove interesting as a target to limit this process in transplantation.[73] There is promise that targeting TLR7 and/or TLR9 might be effective in SLE, as in this disease TLR7 senses host ribonucleoprotein complexes (known to be implicated in SLE) while TLR9 senses DNA/chromatin complexes (again implicated in SLE). Whether inhibiting TLRs will be prove to be to immunosuppressant remains to be seen, although given the redundancy in the TLR system in relation of host defense, this may not pose a problem, particularly if a given TLR has a nonredundant role in inflammatory disease.

NOD-Like Receptors

The second major family of PRRs are the NLRs.[74] Nod-like receptors undergo oligomerization in response to activation. They also have LRRs, similar to TLRs. There are several subfamilies that can be defined by additional domains. The main members are summarized in Table 15.2. Of particular interest are the NLRC family, which have antibacterial NOD1 and NOD2 as members and are defined by the caspase recruitment domain (CARD). The Nlrp subfamily contains a pyrin domain, and the prototypical member is Nlrp3, which is a key component of the inflammasome multiprotein complex that regulates caspase-1 and drives production of the proinflammatory cytokine IL-1beta. The NAIP subfamily contains a Bir domin. All NLRs are intracellular proteins, implying that ligands have to be endocytosed in order to activate them.

Nucleotide Oligomerization Domain 1 and Nucleotide Oligomerization Domain 2

NOD1 and NOD2 sense breakdown products of peptidoglyan, which is a major component of the cell wall of gram-positive and gram-negative bacteria. The finding that NOD2 mutations strongly associate with Crohn disease was an important finding. It supported the hypothesis that aberrant sensing of intestinal microbes is important for the pathogenesis of inflammatory bowel disease.[75] NOD2 detects muramyl dipeptide, whereas NOD1 detects *meso*-diaminopimelic acid. This latter component is more common in gram-negative bacteria. Peptidoglycan is constantly remodeled during bacterial growth, and the component parts are produced constantly, making them ideal PAMPs. How the ligands get into the cytosol is not wholly clear, however. Transport systems such as pannexin, PepT1, and PepT2 have been shown to carry muramyl peptides across the plasma membrane, and there is also evidence of endocytosis.[76] NOD1 and NOD2 signaling leads to NF-κB and MAP kinase activation in a manner similar to TLRs. There are no adapters, however, and they recruit RIP2 kinase to activate these downstream pathways. NOD1 and NOD2 agonists can synergise with TLRs, which is likely to be important in vivo.[77]

Role of Nucleotide Oligomerization Domain 1 in Antimicrobial Responses

The first pathogen shown to be sensed by NOD1 in vivo was *Helicobacter pylori*; NOD1-deficient mice were demonstrated to be more susceptible.[78] Key outputs from NOD1 in this model are chemokines, antimicrobial peptides, and type I interferons. NOD1 has also been shown to be important in the host defense response to other pathogens,

TABLE 15.2 Notable Nucleotide Oligomerization Domain–Like Receptor Family Members

Subfamily	N-terminus	Function
NLRB (NAIP)	BIR	Binds flagellin
NLRC		
NOD1	CARD	Binds ieDAP
NOD2	CARD	Binds MDP
NLRC4	CARD	Binds flagellin/NAIP
NLRP		
NLRP1	Pyrin domain	Binds anthrax lethal toxin
NLRP3	Pyrin domain	Mutiple activators (eg, uric acid, IAPP, cholesterol crystals)

BIR, Baculo Virus Repeat; CARD, caspase recruitment domain; IAPP, islet amyloid polypeptide; ieDAP, Diaminopimelic acid; MDP, Muramyl Dipeptide; NAIP, Neuronal Apoptosis Inhibitory Protein; NLRB, Nod-like receptor with BIR domain; NLRP, Nod-like receptor with Pyrin domain; NOD, nucleotide oligomerization domain.

including *Clostridium difficile*, *Legionella pneumophilia*, and *Listeria monocytogenes*.[79] In all cases, impaired recruitment of neutrophils was a feature of the NOD1-deficient mice, highlighting again the importance of neutrophils in antibacterial host defense. NOD1-deficient mice have also been shown to be impaired in their response to the intracellular parasite *Trypanosome cruzi*.[80] Although the *T. cruzi* ligand is not known, macrophages from the deficient mice have an impaired ability to kill intracellular parasites.

Role of Nucleotide Oligomerization Domain 2 in the Antimicrobial Response

Similar to NOD1, NOD2 has been shown to be important for host defense against multiple bacteria. One interesting aspect, however, was that NOD2 was only shown to be required for host defense against *L. monocytogenes* when given by the oral route.[81] Intravenous and intraperitoneal infections were handled normally in NOD2-deficient mice. This suggests that other PRRs can compensate for the lack of NOD2 depending on the route of infection. There is also redundancy in relation to NOD1 in the response to *Salmonella typhiurium* infection. NOD1- or NOD2-deficient mice are normal in their response, but the double knockout is impaired, with decreased inflammation and enhanced colonization of the intestine.[82]

Autophagy, Adaptive Immunity, and Nucleotide Oligomerization Domain 1 and Nucleotide Oligomerization Domain 2

Another interesting aspect of NOD1 and NOD2 biology concerns a process termed autophagy. This is a process whereby an organelle called the autophagosome digests material in the cytosol of cells. Autophagosomes are related to lysosomes and were originally shown to be important in development and in starvation where in effect a cell will digest some of its own contents to provide nutrients. Autophagy has also been found to be important in antimicrobial immunity. It is an important part of intracellular innate immunity and may in fact be the means by which cells such as macrophages clear bacteria in a process that was originally thought to involve the lysosome. Autophagosomes can be viewed as a subtype of lysosome. These insights came from the uncovering of proteins required for the formation of the autophagosome, notably ATG16, which is recruited to membranes and allows formation of the autophagosomal double membrane structure. NOD1 and NOD2 activation have been shown to lead to recruitment of ATG16L to the plasma membrane sites where *S. typhimurium* and *Shigella flexneri* intrude.[83] This leads to degradation of bacteria in autophagosomes and importantly to the processing of bacterial antigens for presentation on major histocompatibility complex class II molecules. This is a classic example of the link between innate and adaptive immunity. Also of interest is the observation of mutations in ATG16L in Crohn disease and the observation that the mutant NOD2 also in Crohn disease is less able to drive ATG16L recruitment.[84] This could mean that impaired antigen presentation to T cells (eg, regulatory T cells that produce IL-10) might be important in Crohn disease pathogenesis.

There is additional evidence that NOD1 and NOD2 promote adaptive immunity. This was first observed when it was realized that muramyl dipeptide was a key component in Freund's adjuvant, a standard adjuvant used in experimental immunology to drive antibody production. This is probably due to NOD2 inducing important T-cell–activating cytokines, but there is also evidence for NOD2 in T cells themselves where it promotes interferon-gamma production.

Inflammasomes

There are 14 members in the Nlrp branch of the NLRs, and those with a known function are components in inflammasomes. The term inflammasome comes from the fact that the regulation of caspase-1 was shown to be due to assembly of a multiprotein complex involving Nlrps and also the scaffold protein Asc.[85] This complex is needed for the activation of caspase-1, the enzyme that processes the pro-forms of IL-1beta and IL-18. Given the importance of these cytokines in inflammation and inflammatory diseases, much attention has focused on inflammasome regulation.

Nlrp3

The Nlrp3 inflammasome has been characterized in detail. Nlrp3 has a carboxy-terminal LRR domain, a central NOD domain, and an amino terminal pyrin domain. The pyrin domain mainly interacts with the key scaffold protein Asc. It is triggered by agents phagocytosed by macrophages, including bacteria and viruses, but also particles such as uric acid, silica, asbestos and cholesterol crystals, and amyloid-containing proteins such as beta-amyloid and islet amyloid polypeptide (IAPP).[86] Uric acid, cholesterol crystals, beta-amyloid, and IAPP all constitute DAMPs. Activation mechanisms are not fully worked out, although potassium efflux is a common event that is required for oligomerization of the Nlrp3/Asc complex. Roles for reactive oxygen species and cathepsins have also been suggested.[87,88] CARD domains in Asc and caspase interact, whereas the pyrin domain in Nlrp3 interacts with the pyrin domain in Asc, allowing for the complex to assemble. This is needed to activate caspase-1. Extracellular adenosine triphosphate is also able to activate the Nlrp3 inflammasome. Also of note is the fact that the system must be primed by other innate receptors, notably the TLRs, which induce Nlrp3 and also pro-IL-1beta expression.

The effect of uric acid on Nlrp3 is of interest because uric acid may be a common danger signal from damaged cells, as well as having a clear role in gout.[89,90] Similarly, the inflammation associated with silica and asbestos is likely to be due Nlrp3 activation.[91] The effect of cholesterol crystals, which form in atherosclerotic plaques, on Nlrp3 implicates the inflammasome in heart disease.[92] Similarly, the effect of beta-amyloid suggests a role in Alzheimer disease.[93] In the case of IAPP, a role in type 2 diabetes is suggested.[94] IL-1 has been implicated in insulin resistance and beta cell loss in type 2 diabetes, but s there is no infection, what drives IL-1beta is not clear. Hyperlipidemia is now known to activate the Nlrp3 inflammasome in adipose tissue.[95] This will

lead to IL-1beta release, which causes insulin resistance. The beta cells in the pancreas respond by secreting insulin, but also IAPP, which modulates insulin action. However, IAPP can form amyloid, and macrophages in the pancreas take up IAPP; the Nlrp3 inflammasome is activated leading to IL-1beta production. These findings support the clinical targeting of IL-1beta in several diseases notably type 2 diabetes and atherosclerosis.[96]

Another important aspect of the Nlrp3 inflammasome concerns genetic mutations. Activating mutations in Nlrp3 lead to autoinflammatory diseases such as Muckle Wells disease and familial Mediteranean fever.[97] Autoinflammatory diseases such as these are prototypical genetic diseases of innate immunity because there are only inflammatory symptoms with no roles for T and B cells and autoimmunity. The diseases are systemic, involving skin inflammation, arthropathy, and amyloidosis. They are treatable with anti-IL-1beta blocking agents, attesting to the potency of this proinflammatory cytokine.

Nlrp3 in Host Defense

Several bacteria have been shown to activate the Nlrp3 inflammasome. A common feature is pore-forming toxins that cause potassium efflux. *Listeria* produces a toxin called listeriolysin that is required for Nlrp3 activation.[98] Secretion of pneumolysin by *S. pneumoniae* or streptolysin O from *S. pyogenes* both result in activation of the Nlrp3 inflammasome.[99]

Nlrp3 is also activated by viruses. Influenza is a potent Nlrp3 activator, and Nlrp3-deficient mice are less able to survive influenza infection.[100] Viral RNA was shown to prime the inflammasome via TLR7, whereas activation of Nlrp3 was mediated by the influenza-encoded M2 ion channel, which led to potassium efflux.[101] Other viruses such as the DNA viruses vaccinia and varicella zoster, and RNA viruses such as encephalomyocarditis virus and vesicular stomatits virus all activate the Nlrp3 inflammasome.[102] It is therefore likely that the Nlrp3 inflammasome is key for the fever response to all these viruses, as IL-1beta is a key pyrogen during infection.

The Nlrp3 inflammasome can also be activated by fungi, notably *Candida albicans* and *Saccharomyces cerevisiase*.[103] Again, there is interplay with TLRs, as zymosan (a PAMP in the cell wall of yeast) acting via TLR2 primes the Nlrp3 inflammasome for activation. *Aspergillus fumigatus* can also activate the inflammasome.[104]

Finally, parasites can also activate the Nlrp3 inflammasome. *Plasmodium falciparum*, the causative agent of malaria, secretes a crystal called hemazoin, which activates Nlrp3.[105] Malaria severity is less in Nlrp3-deficient mice. Schistosomes have also been shown to activate Nlrp3.

Overall, therefore, fever, a common response to infection, is likely to involve the Nlrp3 inflammasome in many cases.

IPAF

Another inflammasome complex comprises the NLR IPAF, also known as NLRC4. IPAF contains an N-terminal CARD, a central NOD domain, and a C-terminal LRR. It responds to bacterial flagellin, which activates caspase-1 via IPAF.[106] Bacteria such as *Salmonella* are sensed by IPAF. The interaction between IPAF and caspase-1 again involves CARD domains. More generally, IPAF is activated mainly by gram-negative bacteria that contain bacterial type III or type IV secretion systems. These include *Salmonella*, *Legionella*, *Shigella*, *Pseudomonas*, and *Yersinia*.[107] Several of these strains when deficient in flagellin do not activate caspase-1. Flagellin added extracellularly does not activate the IPAF inflammasome and is likely to enter the cytosol via the type III or type IV secretion systems. IPAF has a coreceptor that is another NLR family member NAIP5. This protein interacts directly with flagellin and is the actual sensor, the signaling occurring via IPAF.[108] Similar to NODs and TLRs, there is also evidence for an interaction between IPAF and TLR5. In a model of immunization with ovalbumin and flagellin, it was shown that IPAF- or TLR5-deficient mice are normal in the humoral response, but a double knockout mouse was deficient in its response.[109] This implies that both IPAF and TLR5 are acting in a redundant manner in this system. More generally, it is no surprise that innate systems would be redundant in terms of host defense, as a lack of redundancy would leave the host open to manipulation by pathogens.

Nlrp1

The Nlrp1 inflammasome was the first to be characterized biochemically, but its activators are not fully understood. It is expressed in many cell types, including granulocytes, monocytes, dendritic cells, B cells, T cells, and neurons. It also interacts with Asc, and there is evidence for both caspase-1 and caspase-5 in the Nlrp1 inflammasome. Anthrax toxin has been shown to be sensed by Nlrp1, and in mice the Nlrp1b isoform has been shown to be a key determinant of susceptibility to infection by *Bacillus anthracis*.[110] A role for Nlrp1 in antiviral immunity is suggested from the observation that Kaposi sarcoma herpes virus encodes a protein called Orf63 that blocks Nlrp1-dependent responses and is required for reactivation and generation of progeny virus.[111] Finally, mutations in Nlrp1 are associated with the skin disease vitiligo.[112]

AIM2

The final inflammasome to be characterized contains AIM2, which also couples to caspase-1 via Asc. AIM2 is a member of the PYHIN family of proteins, which contain a pyrin domain and HIN200 domain, which binds DNA.[113] The pyrin domain in AIM2 allows it to interact with Asc. It is activated directly by DNA and has been shown to be activated by DNA from bacteria such as *Listeria monocytogenes* and *Francisella tularensis*, and viruses such as vaccinia virus and cytomegalovirus.[114] Responses to these pathogens in vivo are all impaired in AIM2-deficient mice.

Retinoic Acid-Inducible Gene I–Like Receptors

RLRs are a family of RNA helicases that contain a DExD/H box as the defining protein domain.[115] They occur in the cytosol of most cell types and sense viral RNA. The key output from RLRs is type I interferons. There are three RLRs: RIG-I, melanoma differentiation associated factor 5 (Mda5), and laboratory of genetics and physiology 2.

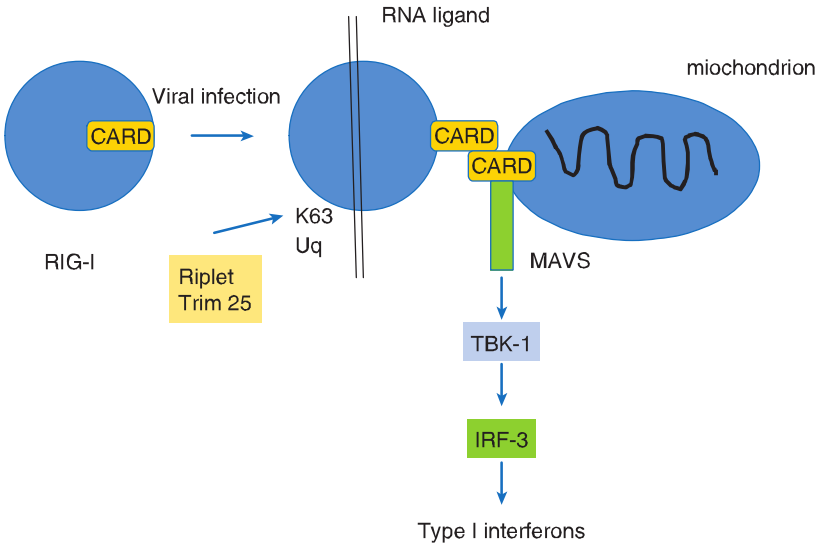

FIG. 15.5. Retinoic Acid-inducible Gene I (RIG-I) Signaling. In response to viruses such as influenza, RIG-I is activated by viral ribonucleic acid. This leads to a dramatic conformational change, such that the caspase recruitment domain (CARD) domains (*orange*) move out from the protein structure and engage with the CARD domain in MAVS, which is in the mitochondrial outer membrane. RIG-I also undergoes K63-linked ubiquitination by Riplet and TRIM25, an event required for activation. MAVS then activates TBK-1, which phosphorylates IRF3, leading to induction of type I interferons and an antiviral response.

Figure 15.5 illustrates RIG-I signaling. RIG-I and Mda5 sense a wide range of viruses and have shared structural features. They both have two CARD domains, a DEAD box Helicase domain, a repressor domain, and a C-terminal domain. Structural insights have revealed that upon binding RNA, a major conformational change occurs releasing the repressor domain and shifting the two CARDs out of the protein to engage with the downstream signaling molecule MAVS.[116–118] Laboratory of genetics and physiology 2 lacks the CARDs and is thought to function as a regulator of RIG-I and Mda5 signaling.

Viruses detected by RLRs include Sendai, respiratory syncytial virus, measles, rabies, influenza, Ebola, hepatitis C, myxoma, West Nile virus, dengue, and vaccinia. RIG-I preferentially recognizes RNA sequences that contain 5′triphosphorylated ends, which serves to distinguish them from host RNA sequences.[119] Polyuridine motifs in the RNA are also important for recognition, and such motifs occur in hepatitis C, Ebola, and influenza virus.

Retinoic Acid-Inducible Gene I–Like Receptor Signaling

The key process in the RLR signaling mechanism is the recruitment of the adapter protein MAVS (also known as IPS-1, Visa, and Cardif). MAVS is a membrane-bound CARD-containing protein that occurs in the outer mitochondrial membrane and also on the membrane of peroxisomes.[120] Activation of RIG-I and Mda5 leads to a translocation to these membranes via the interaction with MAVS. The transcription factors IRF3 and IRF7 are activated by MAVS via the kinases TBK-1 and IKK-epsilon.[121] These in turn lead to induction of type I interferons and other anti-viral genes, with the type I interferons then activating subsequent antiviral responses via the JAK/STAT signaling pathway. RIG-I can also interact with Asc and activate caspase-1, placing RIG-I as another inflammasome component.[122] Another key protein that interacts with RIG-I is STING. This protein was found to be involved in DNA-dependent signaling but also appears to be involved in RNA-dependent signaling via RIG-I.[123]

An excellent example of immune evasion by viruses is the targeting of MAVS by the hepatitis C protease NS4.[124] This leads to MAVS degradation and impaired RIG-I signaling. NS4 is currently the focus of targeting by a protease inhibitor, which could have utility in the treatment of hepatitis C.[125]

RIG-I signaling is a tightly controlled process as overactivation could kill the host; in fact, this may be the reason for the lethal effects of influenza and other viruses. Ubiquitination is a key process here. K63-linked ubiquitination of RIG-I by the ubiquintin ligase Riplet is required for RIG-I signaling.[126] TRIM25 also causes K63-linked ubiquitination, and this has been shown to enhance the interaction with MAVS.[127] RNF125, on the other hand, causes K48-linked ubiquination of RIG-I, leading to its degradation in the proteosome.[128] The deubiquitinase CYLD removes the K63-linked ubiquitin from RIG-I, inhibiting RIG-I activity.[129] Reversible ubiquitination is therefore a key control mechanism for RIG-I.

Cooperation between Retinoic Acid-Inducible Gene I–Like Receptors and Other Pattern Recognition Receptors in Antiviral Immunity

As is the case with NLRs and TLRs, RLRs and other PRRs also cooperate in host defense, in this case against viruses. A good example is the response to West Nile virus. This virus is transmitted by mosquitoes; upon injection into skin, RIG-I triggers an interferon response from resident Langerhans cells and skin fibroblasts.[130] Some virus will then disseminate to the draining lymph node where it infects resident macrophages and dendritic cells. The TLRs are then engaged, particularly TLR7, and induce cytokines to promote adaptive immunity.[131] Some virus will escape to the spleen, and RIG-I will again be engaged here. The situation for TLR3 is more complex because TLR3-deficient mice have improved survival rates.[132] SARM knockout mice, on the other hand, have increased lethality,[133] consistent with the inhibitory effect of SARM on TLR3 signaling.

Retinoic Acid-Inducible Gene I–Like Receptors and Adaptive Immunity

As with other PRRs, RLRs have also been shown to be key for adaptive immunity via the induction of cytokines, in this case type I interferons. Interferons are key regulators of T cells, promoting their survival and clonal expansion after antigen presentation. Interferons also promote the activity of cytotoxic T cells and promote antibody production in B cells. They also promote major histocompatibility complex class I expression as well as costimulatory molecules on dendritic cells.

PKR, 2′-5′-Oligoadenylate Synthase, and RNaseL

Prior to the RLRs, PKR and 2′-5′-oligoadenylate synthase were known to sense viral RNA. PKR is a serine/threonine protein kinase that contains three double-stranded RNA binding domains in the N terminus and a kinase domain in the C terminus.[134] It is activated by double-stranded RNA and therefore acts as an intracellular PRR. A key substrate for PKR is the translation initiation factor eIF-2alpha.[135] This directly restricts both cellular and viral protein synthesis. PKR can also induce apoptosis in virally infected cells.

Oligoadenylate synthase (OAS) is another double-stranded RNA sensor.[136] It is highly induced by Type I interferons. Oligoadenylate produced by OAS activates RNAseL.[137] This enzyme degrades viral RNA but can also degrade ribosomal RNA, limiting translation of host and viral mRNAs. Of particular note is the observation that RNAseL can also generate ligands for RIG-I from viral RNA.[138] This indicates that the OAS/RNAseL axis can serve to amplify antiviral responses triggered by double-stranded RNA. There is also evidence that RNAseL can also promote Mda5 activation. Based on the nature of RNAseL-generated products, both RIG-I and Mda5 can therefore recognize fragments of RNA that are specific cleavage products from viral RNA.

Cytosolic Sensors of Dexoyribonucleci Acid that Induce Type I Interferons

The process of DNA sensing in the cytosol that leads to type I interferons is not as well understood as RNA sensing. As mentioned previously, TLR9 can sense hypomethylated CpG motifs in DNA, but this occurs in endosomes. Intracellular delivery of mammalian or bacterial DNA can, however, induce type I interferons in a TLR9-independent manner. The pathway was shown to involve IRF3 and TBK-1, as with RLRs. Three mechanisms have been proposed. The first is DNA-dependent activator of IFN-regulatory factor (DAI) that interacts with DNA and enhances type I interferons.[139] The precise role of DAI, however, is not clear because DAI-deficient mice have a normal response to infection with DNA viruses.[140] The second mechanism involves RNA polymerase III. This host enzyme has been shown to convert viral DNA into RNA, which can then be detected by RIG-I.[141] This process has been shown to occur during infection with Epstein-Barr virus, herpes virus. and the bacterium *Legionella pneumoniea*. The third process involves DNA sensing with IFI16.[142] This protein was identified as the product of an interferon-inducible gene and was then shown to bind a 70-base-pair double-stranded DNA sequence from vaccinia. Of particular interest is the structure of IFI16 because, similar to AIM2, it also senses DNA but activates caspase-1. IFI16 has a pyrin domain and two HIN200 domains, placing it in the PYHIN family. AIM2 and IFI16 are therefore part of the AIM2-like receptor family.

IFI16 signals via a protein termed STING, which is found in the endoplasmic reticulum membrane.[143] DAI has also been found to signal via STING. The function of STING is to interact with TBK-1 and promote IRF3 activation. STING is in fact essential for TBK-1 activation. STING-deficient mice have impaired interferon induction in response to bacterial, viral, and mammalian DNA, as well as to several DNA viruses, including herpes simplex virus-1, human cytomegalovirus, vaccinia virus and baculovirus, and *Plasmodium falciparum*.[144,145] STING is also required for the immune response to DNA vaccines, presumably because of the essential role it plays in the induction of interferons.

STING also plays a role in the induction of interferon by *Francisella tularensis* and *Listeria monocytogenes*. Cyclic dinucleotides produced by *Listeria* have been shown to be directly sensed by STING, implying that it is actually a PRR for these dinucleotides.[146]

There is also evidence for viral evasion of DNA-sensing pathways. Cytomegalovirus encodes a protein termed M45 that blocks DAI signaling.[147] Another cytomegalovirus protein is pUL83, which inhibits IFI16.[148] The pox virus protein M13L has been shown to target AIM2.[149]

C-Type Lectin Receptors

The term C-type lectin was first used to describe a family of calcium (hence "C")-dependent carbohydrate-binding (lectin) proteins.[150] The carbohydrate binding region was in a carbohydrate recognition domain and the more general term C-type lectin domain is used to describe this family of receptors. Dectin-1 is the best characterized member. It recognizes β-1,3-linked glucans, which occur in the cell wall of fungi, some bacteria, and plants.[151] It binds a range of fungal pathogens, including *Candida*, *Aspergillu*, *Coccidiodes*, *Pneumocystis*, and also bacterial pathogens such as *Mycobacteria*.[151] Two related proteins are CLEC-2 and DNGR-1.[152,153] CLEC-2 has been shown to bind HIV, whereas DNGR-1 has been shown to bind an unknown ligand sequestered in living cells and exposed upon cell death. All three of these C-type lectin receptors (CLRs) signal via a tyrosine kinase termed Syk.[154] This protein couples to CARD9, which in turn can activate NF-κB and promote inflammatory gene expression. It has also been shown to activate Nlrp3 and promote IL-1beta production,[155] These CLRs can also promote phagocytosis. Similar to other PRRs, there are also synergies. Dectin-1 and TLR2 when both activated strongly promote the production of proinflammatory cytokines.[156]

DC-SIGN is another CLR but instead of signaling via Syk, it activates Raf-1 and promotes MAP kinase activation.[157] It binds to mannose and fucose and has been shown to bind HIV, measles virus, severe acute respiratory syndrome, dengue virus, and mycobacteria. Raf-1 is able to modulate the

NF-κB pathway by regulating phosphorylation of the p65 subunit of NF-κB.

Phagocytic Receptors

The PRRs clearly form a major class of innate immune receptors. What distinguishes them from other innate recognition systems is their ability to change gene expression in target cells, the complexity of the gene expression profiles ultimately giving rise to the specific effector mechanisms required to clear the provoking pathogen. Another group of pathogen sensors, however, are proteins that recognize the pathogen but whose function it is to promote phagocytosis.

Scavenger receptors. Scavenger receptors (SRs) are in the plasma membrane and are defined by their ability to bind modified low-density lipoprotein.[158] Six classes are known but most is known about two: SR-A and macrophage receptor with collagenous structure. Both contain a collagenous region and an SR cysteine-rich domain. Both are trimers. They have a role in low-density lipoprotein uptake, but also recognize LPS and lipoteichoic acid from bacteria. SR-A–deficient mice are more susceptible to infection by *Listeria monocytogenes*, herpes simplex, and malaria.[159] They are also more susceptible to LPS-induced sepsis, implying a role in LPS clearance. Macrophage receptor with collagenous structure can bind gram-positive and gram-negative bacteria and promote their phagocytosis.[160]

Macrophage mannose receptor. The macrophage mannose receptor (MR) is a protein found in the plasma membrane of macrophages and contains cysteine-rich and fibronectin type 2 domains, followed by eight carbohydrate recognition domains of the C-type lectin family.[161] MR recognizes mannose-rich carbohydrates from bacteria but can also promote phagocytosis of high-mannose glycoproteins from the host. MR has been shown to be required for phagocytosis of multiple bacteria, fungi, and protozoans. There is evidence that MR can complex with TLRs and promote TLR signaling,[162] yet another example of cooperation between PRRs.

Secreted pattern recognition molecules. There are also a range of secreted pattern recognition molecules that have various functions, although a unifying feature is opsonization. They are secreted into the circulation during infection and can reach high concentrations, effectively acting as acute phase proteins. Three classes have been particularly well-studied.

Mannan-binding lectin. MBL is the best characterized of the collectin family, which have a C-type lectin domain.[163] It binds to the terminal mannose and fucose residents on microbial carbohydrates and importantly can recognize the spatial arrangement of these sugars, which differs according to the host forms. It recognizes a wide range of pathogens, including *Staphylococci*, *Streptococci*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Mycobacteria*, *Candida*, influenza, human immunodeficiency virus, and trypanosomes. As can be seen from this list, MBL can therefore recognize all classes of pathogens. The main function of MBL is to activate the lectin pathway of complement. It is associated with two serine proteases, MASP-1 and MASP-2.[164] These proteases, when activated by ligand-bound MBL, cleave the C2 and C4 complement proteins initiating the complement cascade. MBL can also act as an opsonin, binding to C1qRp. MBL deficiency has been found in humans and leads to increased susceptibility to various infections.[165]

Pentraxins. C-reactive protein (CRP) and serum amyloid P both belong to the pentraxin family.[166] They are acute phase protein in human and mouse, respectively. They are produced mainly by hepatocytes in response to cytokines such as IL-1 and IL-6. They bind to bacterial surfaces and recognize phosphorylcholine. They promote opsonization and can activate the classical complement pathway via C1q. CRP is a classical diagnostic indicator of inflammation. Another pentraxin is PTX3, which plays an important role in host defense against bacteria and fungi.[167] It is expressed on multiple cell types, including dendritic cells, macrophages, and neutrophils. It is strongly induced by TLRs. Similar to CRP and serum amyloid P, it binds directly to bacterial and fungal membranes and promotes phagocytosis.

Peptidoglycan recognition proteins. Peptidoglycan recognition proteins are a family of PRRs that bind peptidoglycan.[168] They are conserved across evolution and were first described in *Drosophila*. They all contain a highly conserved peptidoglycan domain, and some also have transmembrane domains. Most are secreted, however. Four have been described in humans. Their functions have yet to be fully elucidated, although they can clearly bind peptidoglycan. There is some evidence that they might compete with other peptidoglyan recognition PRRs, such as TLR2, possibly acting to limit inflammation, although this has to be demonstrated.

CONCLUSION

Clearly our knowledge of innate immune processes has greatly improved in the past few years. Multiple mechanisms exist that keep pathogens at bay, and several receptor classes can drive innate defence mechanisms via the production of effector cytokines and other mediators. The innate immune system provides an important link to the adaptive response, mainly via the activation of dendritic cells. Other cell types that participate include mast cells, basophils, and natural killer cells, which are discussed at length elsewhere. Innate immunity therefore provides us with a first line of defense, but via the inflammatory response and the activation of adaptive immunity orchestrates the major mechanisms that lead to restoration of the host after infection.

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CHAPTER 16

Dendritic Cells

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INTRODUCTION

Immunology interfaces with medicine at many points, the most prominent being infectious diseases, cancer, transplantation, allergy, and autoimmunity. Antigen-specific immunity is critical for vaccination and for protective immunity against pathogens and tumors. Moreover, when control over immune responses is lost, the result is autoimmune disease. T cells are an essential element that regulates the balance in immunity by killing infected cells, helping antibody formation, and suppressing autoimmune responses. However, T cells are incapable of recognizing native antigens. Instead, they recognize processed peptides presented by major histocompatibility complex (MHC) molecules. Dendritic cells (DCs) are professional antigen-presenting cells that inform the fight against invasive pathogens while enforcing tolerance to self- and harmless environmental antigens. They capture pathogens and receive signals from pathogens that influence the outcome of immune responses. On the basis of these signals, DCs orchestrate antigen-specific T-cell differentiation toward Th1, Th2, Th17, or Tfh pathways. Alternatively, they can silence self-reactive T cells by inducing deletion, anergy, or active regulation (via regulatory T [T_{reg}] cells). This chapter will focus on the discovery, function, and development of DCs, and the mechanisms by which they link innate immunity to adaptive immunity.

DISCOVERY

DCs were discovered as part of an effort to understand the initiation of immunity. The mouse as an experimental animal was critical because of a system, developed by Mishell and Dutton,[1] in which mouse spleen cell suspensions could be stimulated to generate antibody responses in culture. One of the early observations was that lymphocytes alone were not sufficient to induce antibody-forming cell responses and that an adherent accessory cell was required. The search for the accessory cell led to the discovery of DCs. In this section, we will review the early methods used to identify and study DCs.

Identification and Isolation

DCs were discovered by Steinman and Cohn when they examined spleen adherent cells by phase contrast microscopy.[2] They then employed physical techniques to fractionate spleen cells and purify DCs. These cells were found to adhere to plastic or glass, had low buoyant density, and did not bind to erythrocytes coated with antibody. Sequential steps of density centrifugation in bovine serum albumin gradients and adherence to glass were originally used to purify DCs.[3]

At the time, macrophages were thought to be the key accessory cell because they composed a major population of adherent cells and also because their role in innate immunity had been long appreciated. Moreover, purified antigen loaded macrophages induced immune responses when reinjected into their hosts.[4] However, macrophages failed to show robust activity in induction of antibody responses in vitro, and they rapidly degraded ingested antigens suggesting that they would be unable to present it to lymphocytes.[5–7]

Dendritic Cells Initiate T-Cell Responses

In addition to their morphology, the cell surface composition of the newly discovered DCs proved to be distinct. DCs were found to express high levels of MHC antigens.[8,9] At the time, the precise function of the MHC in antigen presentation was not known, but it was already clear that the MHC was a key genetic determinant of immune responses and that it encoded many of the antigens involved in graft rejection.

The mixed leukocyte reaction (MLR) was considered an in vitro model system to study graft rejection and was used by Steinman and Cohn to examine the function of DCs.[10] They found that DCs were nearly two orders of magnitude more potent than unfractionated spleen cells, B cells, or macrophages in stimulating allogeneic T cells in the MLR.[10,11] On the basis of these experiments, they speculated that DCs were the accessory cells that present antigen to T cells to initiate immune responses.[12] However, their conclusions were not widely accepted by immunologists because unlike traditional immune responses, the MLR did not require priming.[13]

Proof that DCs are antigen-presenting cells came from experiments performed by Nussenzweig and Steinman who cocultured DCs with responding T cells and hapten-modified thymocytes. They showed that DCs induced MHC-restricted cytotoxic T cells specific to the hapten and that macrophages and other purified populations of lymphoid cells were nearly inactive as accessory cells.[14]

Monoclonal Antibodies to Dendritic Cells

Although the morphologic and functional differences between DCs and other leukocytes were striking, few immunology laboratories were equipped to perform the purification procedures required to study DCs until monoclonal antibodies to DCs became available. A series of

mouse DC-restricted monoclonal antibodies were produced by Steinman and others starting in 1980s, including 33D1 (specific for the cell surface receptor DCIR2/Clec4a4),[15] NLDC-145 (specific for the adsorptive endocytosis receptor, DEC205/cluster of differentiation [CD]205),[16] and N418 (specific for the CD11c integrin).[17] These monoclonal antibodies were used to establish the unique functions of DCs within heterogeneous mixtures of leukocytes and to identify DCs in situ. For example, 33D1 was used to selectively deplete DCs from mouse spleen suspensions and shows that this depleted MLR stimulating activity[12] and accessory function in the Mishell-Dutton system.[18]

Dendritic Cell Subsets

Shortman and colleagues first showed that DCs are heterogeneous and suggested the existence of DC subsets.[19,20] These subsets differ in their anatomic distribution, cell surface marker expression, and function. In the mouse, three major groups of DCs exist in the steady state: plasmacytoid DCs (pDCs), conventional DCs (cDCs), and migratory DCs (mDCs). pDCs are important mediators of antiviral immunity through their ability to produce large amounts of type I interferons (IFNs) upon viral infection (see following discussion). cDCs are composed of two major subsets, namely CD8α+ and CD8α–.[21] mDCs are present in nonlymphoid tissues such as the liver, gut, skin, lung, and aorta, and they are composed of two main subsets CD103+ and CD103–.[22] These nonlymphoid tissue DCs are referred to as mDCs because they transit from tissue to lymphoid organs.[21,23]

The two major subsets of mouse spleen cDCs have some overlapping functions. Both can process and present antigens to T cells and also secrete cytokines such as interleukin (IL)-12, which can influence the ultimate polarization of the T-cell response to pathogens. However, the two subsets also have unique functions in vivo and are not redundant.[24–26]

Some of the differences in cDC subset function may be accounted for differential expression of toll-like receptors (TLRs) or other mediators of cDC activation. For example, spleen CD8α+ cDCs express TLR3 (recognizing double-stranded ribonucleic acid [RNA]) but lack TLR5 (recognizing flagellin) and TLR7 (recognizing single-stranded RNA), whereas CD8α– cDCs express TLR5 and TLR7 but have low levels of TLR3.[27] Comparative analysis of messenger RNA expression profiles between the two DC subsets revealed that the two are as different from each other as are T- and B-lymphocytes.[28] This is in part reflected in the antigen processing capacity of the two cDCs types. CD8α+ cDCs are specialized for MHCI cross-presentation, and enriched in Tap1, Tap2, calreticulin, calnexin, Sec61, ERp57, ERAAP, as well as cystatin B and C, all of which are involved in MHCI presentation or inhibition of enzymes that process peptides for MHCII presentation. In contrast, CD8α– cDCs, which are biased for MHCII presentation, were enriched in cathepsins C, H, and Z, asparagine endopeptidase, GILT, and H2-Mbeta 1, all of which are implicated in the MHCII antigen-processing pathway.[28] The immunologic consequences of this specialization can be seen after immunization with antigens targeted to one or the other cDC in vivo. Antigens targeted to CD8α+ and CD8α– DCs by chimeric monoclonal antibodies that bind endocytic receptors Dec205/CD205 or DCIR2/Clec4a4 are biased to strong CD8 or CD4 T-cell responses, respectively.[28]

In conclusion, the two major subsets of DCs have distinct microanatomic locations, gene expression profiles, and functions. However the CD8α+ cDC in lymphoid tissue and CD103+ subset in nonlymphoid tissue DCs subsets are far better characterized than the CD8α– cDC or CD103– subset, which are heterogeneous and difficult to distinguish from activated monocytes.

ANATOMIC DISTRIBUTION

Lymphoid Organs

Peripheral lymphoid organs (ie, spleen, lymph nodes, and mucosal associated lymphoid tissues) are the sites where primary immune responses develop, including activation of helper, killer, and antibody-forming cells. Initiation of immune responses is facilitated by the highly organized structure of the peripheral lymphoid organs, with lymphocytes segregated into B- and T-cell areas. In addition to lymphocytes, the T-cell area also contains large interdigitating cells that were initially thought to be macrophages. However, cytologic and functional studies of spleen and lymph nodes in rat and mice revealed that interdigitating cells lack phagosomes and lysosomes, and are poorly phagocytic.[29–31] These features distinguished interdigitating cells from macrophages and suggested that they might be related to the cDCs isolated from mouse spleen.

Mouse interdigitating cells were later proven to be cDCs based on immunohistochemistry using DC-specific monoclonal antibodies. In human lymphoid organs, interdigitating cells in the T-cell area express CD83, a member of immunoglobulin superfamily. This marker is not conserved in the mouse where the interdigitating cells in the T-cell area of lymph nodes express CD11c and DEC205/CD205. In contrast, the two cDC subsets in the mouse spleen segregate into distinct microanatomic compartments. CD8α+ cDCs in spleen T-cell areas resemble those in the lymph nodes in that they express DEC-205, whereas those in the bridging channels and red pulp express DCIR2.[28]

In the T-cell area, cDCs form a dense network of cells that are primarily sessile but constantly protrude and retract large membrane folds into their environment.[32] T cells actively migrate through the DC network in search of antigen, and when they find a cDC presenting cognate antigen, they pause for a prolonged interaction.[33] The large DC membrane protrusions are thought to increase the surface area for antigen presentation and facilitate antigen detection by migrating T cells.[34]

Nonlymphoid Organs

In addition to the lymphoid organs, DCs are also found in most nonlymphoid organs and in all epithelial surfaces that contact the environment. In organs such as heart, lung, kidney, the dermal layer of skin, and meninges and choroid

TABLE 16.1 Cell Surface Comparison between Dendritic Cells and Macrophage

	DC	Macrophage
MHC II	+	–/lo
FcR (CD16/CD32)	–	+
CD11b	–/lo	+
F4/80	–	+
CD115	–/lo	+

CD, cluster of differentiation; DC, dendritic cell; MHC, major histocompatibility complex.

plexus in the brain, they are found in interstitial spaces that are drained by lymphatics.[35–37] Like their lymphoid counterparts, interstitial DCs can be distinguished from macrophages by abundant expression of MHCII and low levels of lysosomal hydrolases. In comparison to macrophages, which are also abundant in the interstitial spaces, interstitial DCs are less phagocytic and more sensitive to radiation. Tissue macrophages and DCs can be distinguished by immunohistology; the former typically express F4/80 and lysosomal hydrolase, whereas the latter express MHCII and CD11c. The standard markers for distinguishing DCs and macrophages in interstitial tissues are listed in Table 16.1. Importantly, although these markers help to differentiate macrophage and cDCs in most tissues, they are not sufficient in defining cell lineages unless used in combination with functional and developmental analysis. This is because CD11c and MHCII can also be expressed by lung macrophages and induced on monocytes during inflammation.[38]

Afferent Lymphatics

Afferent lymphatics in rats, rabbits, guinea pigs, sheep, and humans contain cells with motile processes that were named veiled cells because of their large membrane protrusions.[34,39–41] These cells were found to be poorly phagocytic, express high levels of MHCII, and are potent stimulators of the MLR in vitro. Lymphadenectomy and thoracic duct cannulation in larger animals such as rats and sheep made it possible to collect these cells from afferent lymph. Using this technique, Huang et al. revealed that veiled cells are constantly carrying intestinal epithelial contents via lymphatics to the cDC network in the T-cell area of the mesenteric lymph nodes.[42] Therefore, in the healthy individual, these veiled cells continually carry and present self- and harmless antigen to the adaptive immune system, thereby contributing to the induction of peripheral tolerance (see following discussion); during infection, they carry pathogen-derived antigens and induce specific T-cell activation. Thus veiled cells correspond to mDCs.

Dendritic Cells in the Skin and Other Body Surfaces

Several types of DCs, monocytes, and macrophages exist in the skin and at other body surfaces. Professional antigen-presenting cells in the skin include Langerhans cells (LCs) in the epidermis, and CD103+ and CD103– DCs in the dermis.

DENDRITIC CELL DEVELOPMENT

Origin of Conventional Dendritic Cells in the Lymphoid Organs

DCs, like all other leukocytes, develop from bone-marrow–derived hematopoietic stem cells. Monocytes, macrophages, granulocytes, megakaryocytes, and erythrocytes differentiate from a common myeloid progenitor (CMP) whereas B, T, and natural killer (NK) cells differentiate from a common lymphoid progenitor.[43–45] Although early cell transfer and genetic experiments were interpreted to indicate that DCs originated from lymphoid and myeloid progenitors,[46–49] subsequent work showed that all DCs are derived from myeloid progenitors.[50]

Relationship between Dendritic Cells and Monocytes

In the steady state, DCs and monocytes can readily be distinguished based on cell surface marker expression. For example, DCs are CD11c+CD115–/loMHCII+ and monocytes are CD11b+CD115+F4/80+ (see Table 16.1). In contrast, under inflammatory conditions or in tissues such as lung and intestine, where they can be exposed to pathogenic and nonpathogenic microbes, monocytes develop many of the characteristic features associated with DCs, and the distinction becomes far more difficult. Moreover, the question of how DCs are related to monocytes and macrophages during development was a vexing problem that was only resolved very recently.

The idea that monocytes might be the direct precursors of DCs in vivo was suggested by elegant experiments with human CD14+ monocytes.[51] Monocytes undergoing reverse transmigration, which simulates their entry into lymphatic vessels from tissues, lose expression of monocyte markers CD14 and CD64 and upregulate human leukocyte antigen-DR and CD54.[51] Phagocytosis further stimulates expression of CD80, CD86, human leukocyte antigen-DR, DC-LAMP, and CD83, all of which are expressed by DCs.[51] Consistent with these cell surface changes, activated monocytes stimulate allogeneic T-cell proliferation in MLRs.

Similarly, in mice, microspheres injected intracutaneously are phagocytosed by CD11b+F4/80+ monocytes that can then be found in the draining lymph nodes 3 to 4 days after the injection.[52] These cells display high expression of MHCII, MHCI, CD86, and stimulate allogeneic T-cell proliferation; however, unlike DCs, they express low levels of CD11c and no CD8α. Therefore, monocytes in the skin did not appear to become classical cDCs upon migration to lymph nodes; nevertheless, these activated monocytes shared many of the features associated with DCs. Finally, activated monocytes that appear in the spleen during *Listeria monocytogenes* infection expressed many of the cell surface features of cDCs.[53] These cells produce tumor necrosis factor (TNF) and inducible nitric oxide synthase (iNOS) and were named TNF/iNOS–producing DCs (Tip-DCs).[53] Tip-DC deficiency led to lethal *L. monocytogenes* infection; however, this effect was independent of the development of adaptive CD8 T-cell immunity.[53]

The idea that DCs develop from monocytes was tested by several laboratories in direct transfer experiments in

mice without clear success.[54–57] Indeed, it was always the nonmonocyte fraction of the bone marrow that produced cDCs.[54,56] The only exception to this rule occurred after inflammation induced by Freund's Complete Adjuvant (CFA), when monocytes differentiated into CD11c+CD11b+Mac3+ cells, which again are phenotypically distinct from cDCs.[56]

The inability of monocytes to produce cDCs in steady-state lymphoid organs was further confirmed in a series of elegant genetic studies using reporter mice carrying Rosa26-Stop^{flox}EGFP.[58] In these mice, a lox-flanked Stop transcription signal must be deleted by Cre expression before GFP can be transcribed. Breeding LysM-Cre and Rosa26-Stop^{flox}EGFP mice results in lysozyme promoter–driven Cre expression in monocytes and neutrophils, as well as deletion of the Stop sequence from Rosa26-Stop^{flox}EGFP in these cells. As a result, monocytes and their progeny are irreversibly marked with enhanced green fluorescent protein expression. Classical DCs in the peripheral lymphoid organs remained enhanced green fluorescent protein negative in these mice; therefore, they cannot be derived from monocytes.[58]

In conclusion, experiments in humans and mice show that monocytes can develop some of the features of DCs under conditions of inflammation in vivo, or when cultured with cytokines in vitro, but they are not precursors of cDCs.

Dendritic Cell Progenitors in the Bone Marrow

If monocytes do not produce DCs, the question remains: what is the origin of DCs and where does commitment to this lineage occur? The first clue in solving this problem came from the identification of a common precursor for monocytes, macrophagesm and classical DCs (macrophage-DC progenitors [MDPs][54]). MDP are Lin−CX3CR1+CD11b−CD115+cKit+CD135+ and account for 0.5% of all bone marrow mononuclear cells in mice.[54,59] When cultured with granulocyte-macrophage–colony stimulating factor (GM-CSF) in vitro or adoptively transferred into mice, these cells produced macrophages and DCs, but not neutrophil granulocytes, B- or T-lymphocytes, or NK cells.[54,59,60] Therefore, MDPs are more restricted than CMPs, from which they are derived.

A DC-restricted progenitor that produces cDCs and pDCs but not monocytes in vitro[61] or in vivo[62] was then identified based on reduced cKit (CD117) and residual Flt3 expression and named common-DC progenitor (CDP; Lin−CD115+Flt3+CD117lo). The CDP is downstream of CMP and MDP because adoptive transfer of either CMP or MDP gives rise to CDP and monocytes.[60] More importantly, these experiments showed that the split between the monocyte and DC lineages occurs in the bone marrow between the MDP and CDP stages of development.[60]

Migratory Pre-Dendritic Cells

cDC precursors must migrate from the bone marrow to the lymphoid organs through the blood. However, MDPs and CDPs are restricted to the bone marrow.[60] The identity of the DC precursor was suggested by the finding that low-density CD11c+MHC II−SIRPα^{lo} cells isolated from blood, bone marrow, and periphery had the potential to produce cDCs.[56,63,64] Although this group of cells was heterogeneous, the combination of these markers and persistent expression of Flt3 defined pre-DCs and allowed for their isolation in mice.[60]

Pre-DCs migrate from the bone marrow to the blood and then to peripheral lymphoid organs and nonlymphoid tissues.[60] These cells comprise ~0.5% of all leukocytes in bone marrow, 0.02% in blood, 0.05% in the spleen, and 0.03% in the lymph nodes. Pre-DCs have a short half-life in the blood of < 1 hour[65]; this, together with the small number of these cells in blood and tissues, may explain why previous efforts to identify pre-DCs failed and why human pre-DCs have yet to be identified.

Pre-cDCs isolated from the bone marrow, blood, or spleen give rise to both CD8α+ and CD8α− cDCs in lymphoid and nonlymphoid organs.[60] Thus, the pre-DC is a progenitor with significant plasticity. In conclusion, the DC and monocyte lineages split in the bone marrow, where MDPs give rise to both monocytes and CDP; the latter produce pre-DCs, which migrate from bone marrow through the blood to the periphery to give rise to DCs (Fig. 16.1).

Origin of Nonlymphoid Tissue Dendritic Cells

Skin

The skin contains LCs in the epidermis and CD103+ and CD103− DCs in the dermis.

Epidermis. LCs were discovered in 1868 by Paul Langerhans. Although LCs were initially thought to be of neural crest origin,[66,67] this view was changed with the discovery that LCs are closely related to leukocytes,[68,69] express Fc receptors[70] and MHCII antigens,[71] and are competent to present antigen to primed T cells.[72]

Experiments using bone marrow chimeras and parabiotic mice demonstrated that LCs are long-lived cells that divide in situ in the skin.[73] These cells originate from fetal liver–derived progenitors in an Flt3L-independent manner, and they are only replaced by bone marrow–derived hematopoietic cells during inflammation.[73,74] Thus, the origin of the LCs is different from that of cDCs.

Nevertheless, mouse LCs have been valuable as models for studying the differentiation and migration of antigen-presenting cells.[75–81] LCs in the epithelial layer of the skin can take up particles[82] and contain distinct granules, known as Birbeck granules, which are a type of endocytic organelle enriched in langerin.[83,84] While LCs express Fc receptors and MHCII in situ, the expression of Fc receptors drops and MHCII molecules redistribute from intracellular compartments to the cell surface in cultured LCs that become more highly immunostimulatory.[75,76,85] Cells that resemble LCs are also found in other stratified squamous epithelia such as the vagina, cervix, anus, pharynx, and esophagus.

LCs show many of the phenotypic features of cDCs, including morphology, ability to redistribute large amounts of MHCII from the endocytic system to the cell surface, and capacity to stimulate allogeneic T-cell proliferation in vitro after activation.[75,78,86–88] Importantly, however, LCs differ from DCs in that they are resistant to irradiation, they are self-renewing in situ and not of pre-DC origin in the steady

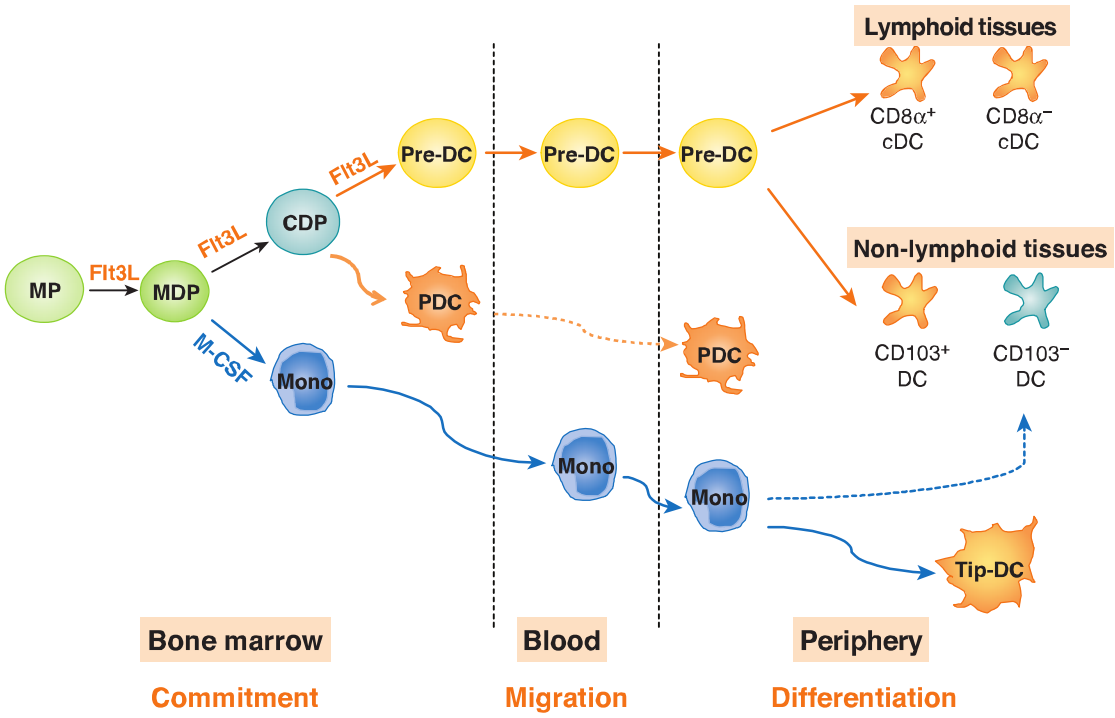

FIG. 16.1. Dendritic Cell and Monocyte Origin and Development.

state, and finally, like monocytes and macrophages, their development is dependent on macrophage-colony stimulating factor (M-CSF) and not on Flt3L.[89] LC precursors, which are CX3CR1+CD45+, colonize the dermis during late embryonic development.[90,91] In mice, these precursors complete their differentiation into LCs by postnatal d2, whereupon they undergo a burst of cell division between postnatal d2 and d7, which accounts for the dramatic increase in LCs during this developmental window. The LC precursor in skin appears to resemble the MDP.[90] Recently, fate-mapping analysis revealed that LCs derive from primitive macrophages during yolk sac stage of embryogenesis, and not from postnatal hematopoietic progenitors.[91] These two lineages can be differentiated by their dependence of Myb gene. The absence of Myb impaired HSC development, but did not affect development of the yolk sac macrophage progenitor that gives rise to LCs, microglia and liver Kupffer cells. This finding places the LC in its own differentiation pathway distinctive from cDC and monocyte.[91]

Dermis. The dermal layer of the skin contains two subsets of DCs: CD103+ and CD103− DCs. CD103+ dermal DCs are langerin+CD11blo, whereas CD103− dermal DCs are langerin−CD11b+.[92–95] Although both LCs and CD103+ dermal DCs express langerin, CD103+ dermal DCs are more closely related to spleen CD8α+ cDCs than to LCs.[25,37,96] CD103+ dermal DCs, like spleen cDCs, are M-CSF–independent and Flt3-dependent.[37] Unlike LCs that can self-renew in situ, dermal CD103+ DCs are continually replenished from the same bloodborne pre-DCs that give rise to lymphoid organ DCs.[95] In addition, like CD8α+ splenic cDCs, development of CD103+ dermal DCs requires expression of the IFN regulatory factor (IRF) 8, Id2, and Batf3 transcription factors (see following discussion). Finally, both dermal CD103+ dermal DCs and CD8α+ cDCs specialize in antigen cross-presentation.[28,96] The CD103− dermal DC compartment appears to be a heterogenous mixture of cells, some of which are Flt3 dependent and of pre-DC origin and others M-CSF–dependent and of monocyte origin.[37]

Skin-draining lymph nodes contain subpopulations of DCs that resemble CD103+ and CD103− dermal DCs.[92,95] Their migration from the skin to the draining lymph nodes is dependent on CCR7 but independent of CD62L. In the steady state, these cells account for 20% of lymph node DCs while the remainder of the DCs are derived from blood pre-DCs.[60] Inflammation dramatically enhances the migration of dermal DCs to the lymph node.[52,58]

Gut, Kidney, Lung, and Liver

Similar to the skin, CD103+ and CD103− DCs are found in nearly all mouse nonlymphoid tissues.[37,97] However, the origin of these cells was not established until very recently. CD103+ nonlymphoid tissue DCs express Flt3, a marker expressed on most cells in the DC lineage but not on monocytes. Furthermore, mice deficient in Flt3L or its receptor, Flt3, showed significant decreases in CD103+ nonlymphoid tissue DCs but normal numbers of monocytes.[37,98] Finally, adoptive transfer of pre-DCs into naïve recipients, or mice in which CD11c cells had been depleted, resulted in reconstitution of the CD103+ nonlymphoid tissue DC compartment in the liver, lung, kidney, and intestine.[37,57] In contrast, adoptive transfer of 50 times more monocytes failed to show detectable DC progeny in naïve mice.[37,57] Thus, in the steady state, CD103+ DCs in all tissues tested are pre-DC

and not monocyte-derived. Only after depletion of endogenous CD11c+ cells were monocytes able to produce some CD103− DCs.[37,57] The idea that all CD103+ nonlymphoid tissue DCs are derived from pre-DCs and not from monocytes is further supported by genetic experiments that show a common requirement for Batf3, Id2, and IRF8 for lymphoid tissue CD8α+ DCs, and CD103+ nonlymphoid tissue DCs, but not monocytes or macrophages (see following discussion).[24,37,99,100]

In conclusion, in addition to DCs in the lymphoid organs, pre-cDCs also contribute to CD103+ DC development in nonlymphoid tissues. CD103− DCs appear to be more heterogenous; some of these cells are derived from pre-DCs while others may be of monocyte origin.

Plasmacytoid Dendritic Cells

pDCs were described by pathologists as a unique population of cells in the peripheral lymphoid organs that resemble plasma cells and monocytes and were referred to as plasmacytoid monocytes. In 1990s, Svensson et al. described a population of cells in human blood that produces large amounts of type I IFN upon exposure to herpes simplex virus, and they named these cells IFN-producing cells.[101] Both of these populations correspond to the pDCs described by Liu and colleagues.[102–104]

pDCs were first purified from human tonsil and blood on the basis of being CD4+CD3−CD11c−. They were then shown to develop DC-like morphology upon culture with IL-3 and anti-CD40. Liu and colleagues discovered that purified pDCs produced 100 to 1,000 times more type I IFN than the other blood cell types following viral activation.[104] Subsequently, pDCs were identified and isolated from lymphoid tissues in mice, and later in rats, monkeys, and pigs. In humans, the pDCs express CD4, MHCII, CD68, CD123, and blood DC antigen 2 (BDCA2), but do not express other lineage marker such as CD3 (T cell), CD14 (monocyte), CD19 (B cell), CD16 and CD56 (NK cell), or CD11c, BDCA1, and BDCA3 (cDC). They are identified as CD4+, CD11c−, and Lin− (CD3, CD14, CD16, CD19, CD56) cells.[105] In mice, pDCs express CD11c (lower levels than cDCs), Gr1, B220, and PDCA1; low levels of MHCII; do not express CD11b or CD19;and they are frequently isolated by virtue of CD11c and B220 expression.[106]

pDCs are closely related to cDCs; they originate from the same progenitor, the CDP. Development of pDCs is also critically dependent on Flt3L. Unlike cDCs, however, pDCs do not express high levels of MHC II in the steady state and therefore, their primary function is not antigen presentation to CD4 T cells. Instead, pDCs focus on sensing infection and producing type I IFN. Depletion of pDC blocks antiviral IFN responses but also results in loss of T_{reg} homeostasis, impaired differentiation of Th cells, and initiation of CD8 T-cell responses (see following discussion).[107,108]

Growth Factors for Dendritic Cells and Monocytes

GM-CSF, M-CSF and Flt3L are essential growth factors for myeloid cell development in vitro and in vivo. During the early stages in myeloid development, shared progenitors of lymphocytes, polymorphonuclear leukocytes, monocytes, and DCs express one or more of the receptors for these growth factors. For example, Flt3 is expressed very early in hematopoiesis on shared progenitors of lymphocytes and all myeloid cells.[109] However, with the exception of pre-DCs, Flt3 expression is lost by nearly all leukocytes by the time they enter circulation.[60] Conversely, M-CSF receptor expression is retained by cells in the monocyte lineage but lost on pre-DCs and DCs.[60]

As might be predicted from their patterns of expression, M-CSF and Flt3L are essential for normal development of the monocyte and DC lineages, respectively. For example, mice deficient in M-CSF or its receptor show deficiencies in monocyte, macrophage, and LC development but have normal spleen and lymph node DCs.[110] In contrast, DC development is impaired in mice deficient in either Flt3L or Flt3, but monocyte development is normal.[59,111] Consistent with these observations, administration or overexpression of Flt3L results in selective expansion of DC populations, including fully differentiated DCs in lymphoid organs.[59,112]

Dendritic Cell Cultures

In the early 1990s, Banchereau and Schuler and their colleagues established in vitro culture systems to produce human monocyte–derived DCs (moDCs), and Inaba and Steinman for the mouse equivalent.[76,113,114] These culture systems are widely used today for basic and clinical studies. Bone marrow cells from mice or monocytes from humans are cultured for 6 days in medium containing GM-CSF to produce cells with dendritic morphology that exhibit modest phagocytic activity, express CD11c and MHCII, and stimulate the MLR. Differentiation to moDCs is further stimulated by addition of lipopolysaccharide, which induces a series of changes that were originally described as maturation. Notably, monocytes do not give rise to DCs in the lymphoid organs in vivo (see previous discussion), and GM-CSF is entirely dispensable for DC development in vivo[115]; thus, the moDCs generated in GM-CSF culture are not entirely representative of the cDCs found in the lymphoid tissues. moDCs may be more closely related to activated monocytes than to steady-state DCs in the lymphoid organs.

Authentic cDCs can be produced in in vitro cultures of bone marrow cells supplemented with Flt3L, which is an essential growth factor for DCs in vivo.[116] Flt3L cultures produce all of the known subsets of DCs, including cells with features of pDCs, CD8α+/CD103+ cDCs, and CD8α− cDCs.[116]

Transcriptional Regulation of Dendritic Cell Development

A number of different transcription factors are known to regulate DC development, but to date none of these are cDC specific. For example, signal transducer and activator of transcription (STAT)3 is required for cDC development in part because this transcription factor is downstream of Flt3.[117] Binding of Flt3L to its receptor on the surface of DC precursors or cDCs induces activation of the phosphoinositide 3-kinase–Akt–mammalian target of rapamycin signaling

pathway; this leads to activation of STAT3.[118] Activation of STAT3 is also regulated by the transcriptional repressor, Gfi-1, deletion of which leads to reduction of lymphoid DCs and increase of LCs.[119] PU.1, an E-twenty-six family transcription factor, is a third transcription factor that is downstream of Flt3 signaling.[120] Although PU.1 deficiency is lethal, mice reconstituted with PU.1-deficient hematopoietic cells show a number of hematopoietic defects, including strong reduction in CD8α+ and CD8α– cDCs.

The Interferon Regulatory Factors

IRF2, 4, and 8 are prominent transcription factors that regulate DC diversification. IRF2–/– mice exhibit reduced CD8α– cDCs, IRF4–/– mice display reduced CD8α– cDCs and slightly reduced pDCs, and IRF8–/– mice have reduced CD8α+ cDCs, pDCs, and LCs.[100,121–123]

NF-kB/Rel

Members of the NF-kB/Rel family also contribute to DC development. CD8α– cDC numbers are dramatically reduced in RelB–/– mice.[124] TRAF6 belongs to TNF receptor–associated factor family and acts upstream of the NF-kB cascade. Consistent with a role for NF-kB in cDC development, TRAF6–/– mice also show a defect in the CD8α– cDC compartment.[125]

Basic Helix-Loop-Helix Transcription Factors

Basic helix-loop-helix factors (E12, E47, HEB, E2-2) interact with helix-loop-helix Id (inhibitors of deoxyribonucleic acid [DNA] binding) proteins to control DC development. Loss of Id2 leads to a severe reduction of CD8α+ cDCs and absence of LCs. Another helix-loop-helix family member, E2-2, plays a crucial role in guiding pDC development and maintaining stability of the pDC lineage. Deletion of E2-2 in developing precursors blocked pDC development, whereas deletion in more mature cells reversed the development, suggesting differentiation plasticity that needs to be constantly reenforced.[126,127] Similar to mice, E2-2 haploinsufficient humans with Pitt-Hopkins syndrome show a pDC defect.[126]

Batf3

Batf3 is expressed at high levels in DCs and is specifically required for development of CD8α+ cDCs and CD103+ nonlymphoid tissue DCs.[24,128] Batf3–/– mice failed to mount antitumor and antiviral immune responses, demonstrating a key role of CD8α+ cDCs and CD103+ nonlymphoid tissue DCs in priming CD8 T cells.

Notch

Terminal DC differentiation appears to depend on signaling through Notch receptors and their transcriptional effector RBP-J. Inactivation of RBP-J in DCs results in severe reduction and functional impairment of splenic CD8α– cDCs.[129] In addition, Notch2 deletion leads to the loss of CD103+ nonlymphoid tissue DCs in the intestinal lamina propria and to a corresponding decrease of IL-17–producing CD4 T cells in the intestine.[130]

In conclusion, DC differentiation is dependent on a number of interacting transcription factors (Table 16.2). Together, they turn on expression of lineage-specific genes and suppress alternative developmental programs.

Human Dendritic Cell Development

Research on human DCs is difficult because it is largely limited to peripheral blood, whereas in the mouse, most of the work has been done on DCs residing in lymphoid or nonlymphoid tissues. As a result, human DC research has relied on peripheral blood monocytes cultured in the presence of cytokines, which generate moDCs and not cDCs.

TABLE 16.2 Transcription Factors Determine Dendritic Cell Development and Homeostasis in Vivo

		Classical DC		Tissue DC		
	Plasmacytoid DC	CD8α+	CD8α–	CD103+	CD103–	LC
PU.1–/–		– – –	– – – –			
Irf8–/–	– – – –	– – – –	n.a.	– – – –		– –
Irf2–/–		n.a.	– – –			–
Irf4–/–	–	n.a.	– – –			
RelB–/–		n.a.	– – – – –			n.a.
Gfi1–/–	– –	– –	– –			+ +
ID2–/–	n.a.	– – – –	n.a.	– – – –		– – – – –
E2-2–/–	– – – –	n.a.	n.a.			
Stat3–/–		– – – –	– – – –			
Stat5–/–	– – –	– – –	– – –			
Ikaros–/–		– – – –	– – – – –			
Xbp1–/–	– – –	– –	– –			
Batf3–/–	n.a.	– – – – –	n.a.	– – – – –	n.a.	
Runx3–/–		+ +	–			– – – – –
RBP-J–/–		n.a.	– – – –			
Notch2–/–		–	– – – –	– – – –	n.a.	

–, decrease; +, increase; CD, cluster of differentiation; DC, dendritic cell; LC, Langerhans cell; n.a., not altered.

Despite these obstacles, three different subsets of DCs have been found in human blood. These subsets are referred to as BDCA1, 2, and 3 based on their expression of cell surface markers.[131] BCDA1+ DCs resemble mouse CD8α– cDCs, BDCA2+CD11c– DCs are equivalent to mouse pDCs, and BDCA3+ DCs are equivalent to mouse CD8α+ cDCs (Fig. 16.2). These interspecies associations were initially based on similarities in gene expression between human and mouse DC subsets,[132] and were recently confirmed in functional experiments.[133–137] Moreover, human DCs can be obtained from cultures of human cord blood supplemented with Flt3L and GM-CSF.[134]

The human equivalents of the bone marrow–derived DC precursors, MDP, CDP, and pre-DC remain to be isolated. However, recent clinical studies revealed genetically defined syndromes associated with DC deficiency, which may shed some light on human DC development. Three different genetic lesions have been associated with the triad of DC deficiency, monocytopenia, and opportunistic infections. GATA2 mutation leads to a loss of DC, monocytes, B cells, and NK cells.[138,139] These patients have normal LCs and macrophages, suggesting that LCs and tissue macrophage have different origin from DCs and monocytes in humans. Mutations in IRF8 are associated with disseminated bacille Calmette-Guérin infection.[140] Two different mutations in IRF8 have been identified. The K108E variant is associated with an autosomal recessive severe immunodeficiency with a complete absence of circulating monocytes and DCs; the T80A variant is an autosomal dominant, milder immunodeficiency and is associated with selective depletion of circulating DCs. Loss of DCs due to IRF8 mutation also leads to myeloproliferation in humans and in mice due to increased serum Flt3L, which induces expansion of myeloid progenitors. In humans, as in mice, loss of DCs is associated with loss of T_{reg} cells, confirming the existence of a homeostatic feedback loop these two cell types[141] (see following discussion).

DENDRITIC CELL HOMEOSTASIS

Lymphoid Organs

Based on their rapid labeling kinetics and loss of label in pulse chase experiments with BrdU, it was proposed that DCs have a very short half-life.[136,137] However, these early experiments are difficult to interpret in view of the more recent finding that DCs in lymphoid and nonlymphoid tissues divide in situ.[37,65,142]

The half-life of cDCs in tissues was measured in parabionts and shown to vary from 5 to 7 days in the spleen, lymph nodes, liver, and kidney, and as many as 25 days in the lung.[37,65] cDC homeostasis in all organs is maintained through a dynamic balance of three parameters: continuous input of pre-DCs from the blood, limited cDC division in situ, and cell death. Fitting BrdU incorporation and parabiosis separation data into mathematical equations produced a numerical estimate of the rate of DC precursor input (~4,300/hour) and DC death (~9,600/hour) in the mouse spleen.[65]

Regulation of Dendritic Cell Division

cDCs in mouse lymphoid and nonlymphoid tissues divide in situ for 10 to 14 days before being replaced by pre-DCs.[65] In the steady state, their division is regulated by

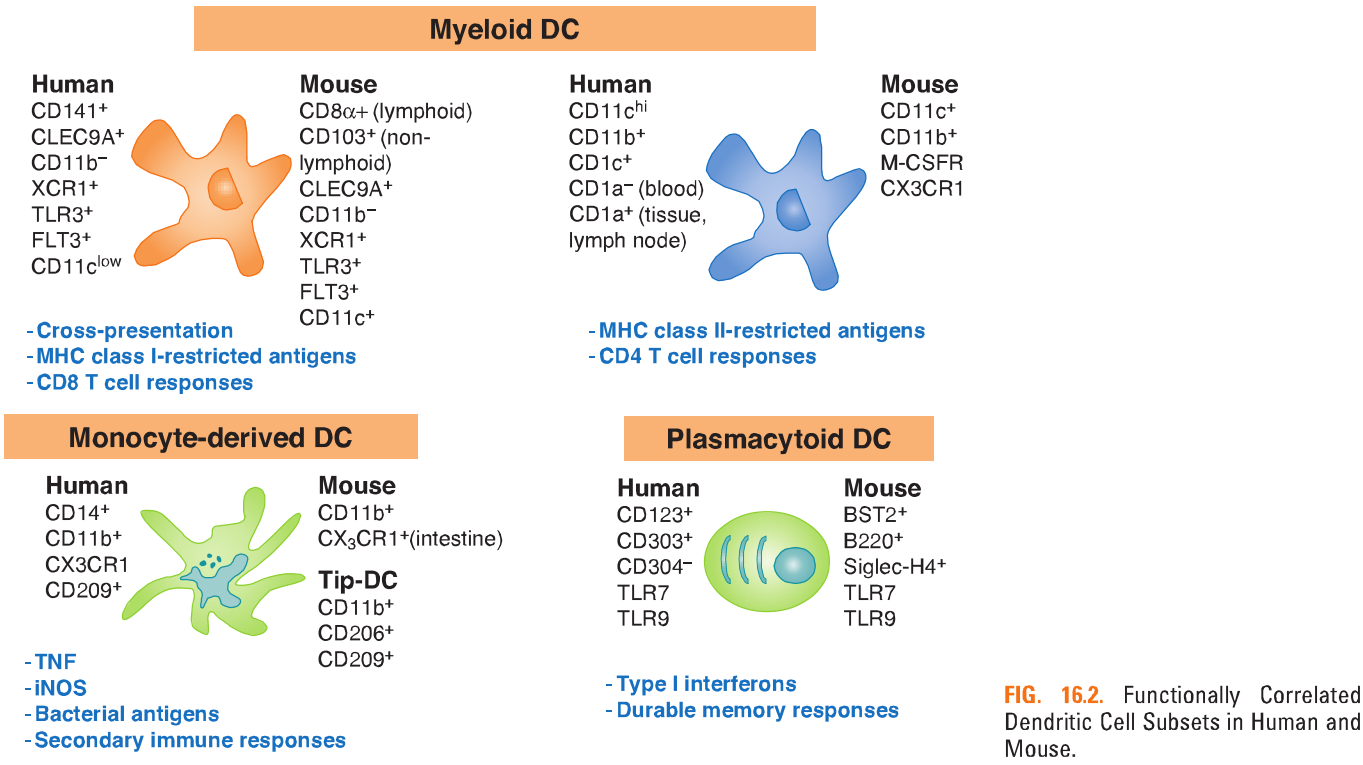

FIG. 16.2. Functionally Correlated Dendritic Cell Subsets in Human and Mouse.

lymphotoxin-beta and Flt3L. Flt3L impacts nearly all stages of cDC development in lymphoid and nonlymphoid tissues, and is essential for their development.[59,112,143] In contrast, the effects of lymphotoxin-beta appear to be limited to CD4+ spleen DCs.[142] How the levels of circulating Flt3L are regulated is not known, but there appears to be a feedback loop between tissue cDCs and Flt3L that results in upregulation of Flt3L in the serum in response to cDC depletion,[144] loss of T_{reg}s,[60,145,146] or TLR ligand injection.[147] In all instances, increased Flt3L production leads to increased export of DC progenitors from the bone marrow and DC division in the periphery.

Conversely, increasing the number of DCs by Flt3L injection leads to an increase in the number of T_{reg} cells. Thus, the feedback loop between DCs and T_{reg}s is mediated by Flt3L, and that maintains the physiologic numbers of these two cell types in the steady state (Fig. 16.3).[146] Alterations in this mechanism lead to immune imbalance and can alter the course of autoimmune disease in mice.[146,148] For example, increasing the number of DCs in diabetes prone nonobese diabetic mice leads to increased numbers of T_{reg}s and a delay in disease onset. Similarly, Flt3L injection can protect mice from inflammatory bowel disease by increasing the number of cDCs and thereby the number of T_{reg}s. Thus, the Flt3L-mediated homeostatic feedback loop between T_{reg}s and DCs has clinical implication for vaccine design as well as the control of autoimmunity. Finally, this mechanism is entirely consistent with the proposed role of DCs in maintaining tolerance and regulating immunologic responses in vivo.[149]

Dendritic Cell Migration

DC migration from peripheral tissues into lymphoid organs is key to their sentinel and antigen trafficking functions. Upon microbial contact or stimulation by inflammatory cytokines, nonlymphoid tissue resident cDCs traffic through afferent lymphatics to the T-cell areas of the lymph node where they can participate in the initiation of immune responses. Migration is dependent on CCR7, which is upregulated by stimuli such as TLR ligands that activate DCs.

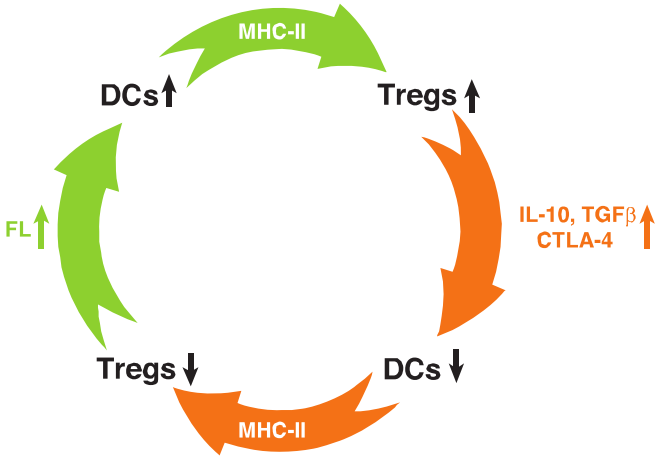

FIG. 16.3. Homeostatic Feedback Loop between Dendritic Cells and Regulatory T Cells.

Even in the absence of invading pathogens, some DCs are always migrating from tissues to lymph nodes. Studying afferent lymph is difficult in mice, but in rats, for example, DCs move along liver sinusoids in hepatic lymphatics to celiac lymph nodes,[150,151] and DCs from intestine migrate to mesenteric lymph nodes.[42,152] These cells are not found in the efferent lymph, indicating that most of the migrating DCs die after their arrival in lymphoid tissues. The DCs that migrate in the steady state might have several functions: to replenish immature populations, to transport self- or environmental antigens, or to be on patrol to identify invaders.

DC migration is a regulated process, controlled at the level of chemokine production and chemokine receptor expression and function.[153] Some immature DCs can express a repertoire of receptors (eg, CCR1, CCR2, CCR5, CCR6, and CXCR4) that bind inflammatory chemokines (eg, CCL5, CCL2, CCL3, CCL4, CCL20, and CXCL12). DC activation due to inflammatory signals is usually associated with downregulation of chemokine inflammatory receptors and the de novo expression of CCR7, the receptor for CCL19 and CCL21. In mice homozygous for an autosomal recessive mutation in CCL21 (paucity of lymph node T cells or plt/plt mice), naive T cells fail to home to secondary lymphoid organs. DCs in these mice also fail to accumulate in the spleen and in the T-cell areas of lymph nodes.[154] Similarly, CCR7−/− mice show defective secondary lymphoid organ architecture, defective homing of DCs and lymphocytes, and defective entry of DCs into lymphatic vessels at peripheral sites both in the steady state and inflammation conditions.[155] CCL19 and CCL21 also increase the maturation and proinflammatory differentiation of cDCs.[156] Therefore, chemokine/chemokine receptor interactions not only orchestrate the DC migration but also influence their immunogenic potential.

There are many other examples in which specific chemokines control the traffic of select populations of DCs (Table 16.3). In skin exposed to ultraviolet light, LCs disappear and are replaced in 2 weeks. The recruitment of LC precursors from blood is dependent on their expression of CCR2.[73] During murine listeriosis, CCR2 is also required for TNF-inducible nitric oxide synthase–producing DCs to migrate into the spleen.[53] Migration of pDCs into inflamed lymph nodes and their redistribution during inflammation depend on CXCR3 and CCR7. CCR6 is used by cDCs to populate epithelial surfaces during inflammation.[157–159] In the steady state, CXCL14 is important for LC progenitors to establish themselves in the skin.[160] All these findings indicate that the differential expression of chemokine receptors by DCs (and their subsets) at different stages of their life history determines their location in vivo.

ANTIGEN PRESENTATION

Antigen Capture

Macropinocytosis

DCs in culture continuously form 0.25 to 1.0 um pinocytic vesicles. These vesicles allow DCs to sample a large volume of extracellular fluid and soluble proteins that are present at low concentrations. Expression of aquaporin 3 and 7 on DCs may also contribute to macropinocytosis.[161]

TABLE 16.3 Animal Models Demonstrate Dendritic Cell Migration Depending on Chemokine Receptor

DC migration	
CCR2–/–, CCR5–/–	The recruitment of new Langerhans cells is dependent on their expression of the CCR2 chemokine receptor and on the secretion of CCR2-binding chemokines by inflamed skin.[73] Recruitment of TNF/inducible nitric oxide synthase–producing DC subset in spleens of *Listeria monocytogenes*–infected mice is dependent on CCR2 and independent of CCR5.[53,246]
CCR6–/–	CCR6 mediates DC localization, lymphocyte homeostasis, and immune responses in mucosal tissue. In CCR6–/– mice, DCs expressing CD11c and CD11b are absent from the subepithelial dome region of Peyer patches.[247]
CCR7–/–, plt/plt (CCL19 and CCL21–/–)	CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs.[155] Plt/plt mice lacking CCR7 ligands, CCL19 and CCL21, have defects in lymphocyte homing and DC localization.[154] CCR7 also governs skin DC migration under inflammatory and steady-state conditions.[248] CCR7 ligands, CCL19 and CCL21, induce a proinflammatory differentiation program in DCs.[156]
CCR8–/–	CCR7 and CCR8 pathways are used by monocyte-derived DCs during mobilization from skin to lymph nodes.[249]
CXCR3–/–	Plasmacytoid DCs migrate to inflamed lymph nodes, produce interferon-α, and help lymph node DCs to induce antiviral CTLs.[250,251]

CTL, cytotoxic T-lymphocyte; DC, dendritic cell; plt, paucity of lymph node T cells; TNF, tumor necrosis factor

Receptor-Mediated Phagocytosis

Phagocytosis is triggered by the attachment of extracellular particles to surface receptors, which mediate particle uptake. Multiple receptors on cDCs enhance recognition and ingestion of particulates including pathogens and dying cells. Importantly, many of the receptors also recognize molecular patterns on pathogens and as a result, activate DCs. In addition, DCs also express Fc receptors that mediate ingestion of opsonized particles and delivery to intracellular compartments that facilitate TAP-1–dependent cross-presentation to CD8 T cells. DCs express both activating and inhibitory forms of Fc receptors.[162] The inhibitory receptors help maintain DCs in an immature tolerogenic state. DCs that lack the FcγRIIB inhibitory receptor are more readily activated because the immunoreceptor-based tyrosine activation motif–associated FcγRIIA receptor is no longer subject to inhibition.[163] These observations have been extended in mice and humans through the identification of monoclonal antibodies that selectively block inhibitory receptors.[164,165]

Other than TLRs, DCs express many different types of pattern recognition receptors including those of the specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN) family, and multiple C-type lectins such as macrophage mannose receptor (MMR), DEC205, and DCIR2. MMR binds a range of bacteria, yeasts, and viruses through interactions between a mannose-type carbohydrate recognition domain and pathogen-associated high mannose structures. Notably, DCs deficient in any single C-type lectin receptor, such as MMR, DEC205, or DCIR2, develop normal adaptive immune response to dying cells, or pathogens, suggesting that this important class of receptors is redundant.

Dendritic Cell Maturation

DCs exist in two functionally distinct and phenotypically different stages. In the steady state, DCs in most tissues are equipped to capture and present antigens to T cells, but the outcome of antigen presentation by steady-state DCs is tolerance and not immunity. Steady-state DCs express high levels of pattern recognition and activation receptors, allowing them to sense changes in the environment, including pathogens and inflammatory cytokines. These signals induce extensive differentiation to a mature or activated state characterized by increased levels of cell surface MHC and coactivator expression, as well as cytokine secretion. In the activated state, DCs initiate potent and specifically polarized T-cell immune responses. Thus, pathogen sensing by DCs is an important mechanism that links innate pathogen recognition to the adaptive immune response.

Toll-Like Receptor Signaling

Microbes and viruses induce DC activation in part by engaging TLRs. DCs express nearly all known TLRs and also express additional cytoplasmic receptors that recognize pathogen patterns such as melanoma differentiation associated protein 5 (MDA-5), RIG-I, and DDX41, and nucleotide oligomerization domain–like receptors, although expression of specific receptors is restricted to distinct DC subsets.[166–168] In many cases, a single microbe will trigger several different TLRs expressed by DCs; therefore, a single TLR may be redundant for inducing immunity to many, but not all, pathogens. Thus, in humans, loss of TLR3 results in susceptibility to herpes simplex virus-1 infection but not to other microbes that also contain the TLR3 ligand.[169] Pathogen recognition by TLR ligation induces rapid DC activation and upregulation of MHC and costimulatory molecules, and also triggers secretion of inflammatory cytokines such as IL-1α, IL-6, TNFα, IL-18, and IL-12, which are critical in driving T-cell differentiation.[170–172]

Cytokines, T, and Natural Killer T Cell Signals

DCs can also sense proinflammatory cytokines such as TNFα and IL-1β, and direct cellular contact with activated T or NKT cells by ligation of CD40, a TNF receptor family molecule that is highly expressed on the surface of DCs. Many of these receptors, including TLRs, IL-1R, and TNF receptors, activate DCs through the NF-κB pathway.

Antigen Processing

DCs process ingested antigens for presentation on both MHC class I and MHC class II. Although DCs are excellent MHCII antigen-presenting cells, they are not unique in this respect. Other cells, including activated B cells and monocytes, can also process and present antigens for presentation by MHCII efficiently. For example, in germinal centers, activated B cells are the key antigen-presenting cell.

Macrophages endocytose antigens and rapidly digest them. In contrast, DCs sequester and preserve the captured antigen for later presentation. Preservation of antigens is critical for immunogenicity and depends on two DC specializations. The first is low lysosomal protease activity. Macrophages contain high levels of lysosomal proteases, including cathepsin S, cathepsin L, cathepsin K, and asparagine endopeptidase. These proteases enable rapid degradation of internalized proteins to single amino acids. Compared to macrophages, DCs express low amounts of proteases, resulting in a limited capacity for lysosomal degradation.[173] This low-level proteolytic capacity appears to be crucial as the peptides loaded on MHC and recognized by T cells consist of peptides between 8 to 17 amino acids. Consistent with this idea, mutations in amino acids in antigens that increase their resistance to lysosomal proteolysis increase immunogenicity by decreasing the rate of proteolysis.[173]

A second feature of DC lysosomes that facilitates antigen presentation is that DC lysosomes are less acidic compared to those in professional phagocytes. In macrophages and neutrophils, the low pH of the phagosome and endosome activates lysosomal proteases delivered to these endocytic vesicles. In DCs, the pH in these compartments is less acidic because the assembly of V-ATPase, an adenosine triphosphate–dependent vacuolar proton pump, appears to be incomplete in the lysosome of steady-state DCs, leading to alkalinization of the endocytic compartment.[174] In addition, DCs show efficient recruitment of Nox2 to endosomes and phagosomes. Nox2 produces reactive oxygen species, which consumes protons and thereby reduces the acidification of the endocytic compartment. NOX2-defective DCs exhibit a higher level acidification of the phagosome and increased proteolysis and decreased antigen presentation.[175] Furthermore, Nox2-deficient patients, who suffer from chronic granulomatous disease, show impaired cross-presentation.[176] Together, the lower levels of proteolytic activity and decreased acidity in endocytic compartment lead to a decrease in the rate of antigen digestion and increased availability of partially processed peptides for loading on MHC. This unique feature of DCs may also help preserve the antigen during the migration of nonlymphoid tissue DCs from the site of antigen capture to the lymph nodes.

Antigens are actively taken up by immature DCs and targeted to MHCII-positive lysosomes. However, these antigens are sequestered in an intact form and are not efficiently utilized for formation of MHCII-peptide complexes. Immature DCs do form stable MHCII dimers, but their presence does not result in immunogenic complexes. DC activation by microbial products or proinflammatory cytokines results in redistribution of MHCII from intracellular compartments to the plasma membrane. In addition, DC activation reduces lysosomal cystatin C, which leads to increased activation of cathepsin S and more efficient processing of Ii.[85]

DENDRITIC CELLS LINK INNATE AND ADAPTIVE IMMUNITY

Plasmacytoid Dendritic Cells

Nucleic acid–sensing pDCs are activated when they sense pathogen or self-derived nucleic acids and are involved in antiviral immunity and autoimmune diseases. Whereas monocytes express TLR4 (lipopolysaccharide sensor) and cDCs express TLR3 (double-stranded RNA sensor), pDCs selectively express TLR7 (single-stranded RNA sensor) and TLR9 (DNA sensor). pDCs endocytose viruses and sense their nucleic acids using TLR7 and TLR9, both of which reside in the endosomal compartment. TLR7 detects single-stranded RNA viruses, such as influenza, respiratory syncytial virus, Sendai, and vesicular stomatitis virus, and synthetic single-stranded RNA analogs such as R848/Imiqimod; TLR9 detects DNA viruses, such as herpes simplex virus-1, herpes simplex virus-2, and murine cytomeglavirus, or cytosin-phosphatidyl-guanosin oligodeoxynucleotides. Viruses that enter the cytoplasm of pDCs are detected after autophagy, a conserved cell-autonomous process involving lysosomal degradation of cellular organelles to deliver cytoplasmic RNA to TLR7-containing endosomal compartments. Both TLR7 and TLR9 use MyD88 as the adaptor protein for activation; thus, MyD88-deficient pDCs have defective responses to a wide range of viruses that enter the cytoplasm. Recruitment (binding) of MyD88 to the TLR leads to assembly of a signal-transducing complex that includes IRAK4, TRAF6, Bruton's tyrosine kinase, and IRF7.[177] This signaling complex is critical for IFN production by pDCs. In most cells, production of IFNα depends on binding of IFNβ to interferon-α/β receptor (IFNAR), which induces IRF7 expression. In pDCs, however, constitutive high-level expression of IRF7 facilitates a rapid IFNα response that is independent of IFNAR signaling.[105]

IFN production by pDCs is rapid, starting 4 hours after exposure to TLR ligands and peaking after 24 hours when pDCs become refractory. Type I IFN produced from activated pDCs not only directly inhibits viral replication, but also activates NK, B cells, and cDCs.[178] For example, human immunodeficiency virus (HIV) or herpes simplex virus infection stimulates pDCs to produce type I IFN and CD40L, which in turn activates cDCs and facilitates antigen presentation and development of antiviral immunity. Similarly, pDC nucleic acid sensing and cDC activation occurs in autoimmune diseases. In patients with systemic lupus erythematosus, pDCs are continuously activated by circulating immune complexes composed of self-DNA or RNA leading to high circulating levels of IFNα.[178]

Given the potential pathologic consequences of high-level IFNα production by pDCs, it is not surprising that this response is highly regulated. pDCs express an array of negative regulators to downregulate IFNα production. In humans, two surface molecules, BDCA2 and immunoglobulin-like

transcript 7 (also known as LILRA4), were found to suppress TLR-induced IFN production from pDCs. Both receptors mediate their inhibitory effects through cytoplasmic immunoreceptor-based tyrosine activation motifs, and both BDCA2 and immunoglobulin-like transcript 7 associate with the γ-chain of the high-affinity Fc receptor for immunoglobulin E (FcεRIγ).[179] Other immunoreceptor-based tyrosine activation motif–mediated inhibitors of pDC IFN production include NKp44 and sialic acid binding Ig-like lectin (Siglec)-H, which recruits DAP12.[180]

Interactions with Innate Lymphocytes

DCs interact with innate lymphocytes (eg, NK, NKT, and γδ T cells) in ways that enhance the functions of both cell types. DCs produce IL-12, IL-15, IL-2, and IFNα/β that affect different facets of NK cell function, whereas the innate lymphocytes induce DC activation.[181,182] Injection of activated cDCs leads to the recruitment of NK cells into the draining lymph nodes while exposure of cDCs to TLR stimuli induces IL-2 and IL-12 production, which in turn activate NK cells to produce IFNγ. DCs also present different glycolipids on CD1d molecules to the invariant T-cell receptor on NKT cells. These include endogenous lysosomal glycosphingolipids (eg, iGb3),[183] microbial lipids,[184,185] or synthetic glycolipids (eg, alpha-GalCer)[186,187] to activate NKT cells in vivo.

Type I IFN appears to be one of the essential mediators of DC activation in vivo. For example, antigen targeted to cDCs can elicit strong Th1 immunity in the presence of PolyI:C. PolyI:C binds to TLR3 on the cell surface and intracellular sensor MDA-5. However, PolyI:C does not directly stimulate DC maturation because deficiency of TLR3 or MDA-5 on DCs does not abrogate immunogenicity.[188] Instead, PolyI:C stimulates nonhematopoietic cells to produce large amounts of type I IFN.[188] High levels of systemic type I IFN stimulate functional maturation of cDCs, which consequently induce a robust Th1 response. Thus, microbial stimuli–induced DC maturation in vivo engages a cascade of cellular and molecular mechanisms, which may amplify the environmental signals to facilitate optimal immunity.

Initiating Adaptive T-Cell Immunity

In the steady state, DCs can endocytose a diverse array of antigens through multiple receptors on their cell surface. However, steady-state DCs typically express relatively low levels of surface MHC class I and II products and only low levels of costimulatory molecules (eg, CD80, CD86). Upon receipt of an activation stimulus, DCs undergo extensive differentiation, and they migrate in increased numbers to secondary lymphoid tissues.

DC–T-cell interactions in the lymph nodes have now been studied in the living state with two-photon microscopy.[33,189–192] Naïve antigen-specific T cells arrest on antigen-presenting DCs, and this stable interaction lasts for at least 18 hours. In the steady state, this stable DC–T-cell interaction leads to tolerance while an activation signal such as TLR ligation allow DCs to initiate immunity. cDC activation results in redistribution of MHCII from intracellular compartments to the plasma membrane; the upregulation of costimulatory molecules such as CD40, CD80, and CD86; and change in profiles of cytokine and chemokines such as TNFα and IL-12. All these changes likely contribute to the initiation of T-cell immunity.

The communication between DCs and T cells is a dialogue in which the DCs also respond to T cells. For example, CD40 and TRANCE/RANK receptor on DCs are ligated by the corresponding TNF family member expressed on activated and memory T cells (ie, CD40L and RANK-L).[193–195] This leads to increased DC survival and in the case of CD40 ligation, upregulation of CD80 and CD86, secretion of IL-12, and release of chemokines such as IL-8 and MIP-1α and β.

Controlling the Quality of the T-Cell Response

DCs are involved in critical T-cell fate decisions such as clonal selection, tolerance versus immunity, Th1 versus Th2, and even memory. In the presence of mature DCs producing IL-12 or IFNs (as might occur when DCs are ligated by CD40L or infected with viruses), CD4 T cells differentiate along a Th1 pathway for IFNγ production. The latter in turn activates the antimicrobial activity of macrophages and promotes killer T-cell differentiation. However, in the presence of exogenous IL-4, DCs induce T cells to differentiate into Th2 cells, which secrete IL-4, IL-5, and IL-13. These cytokines help B cells to make antibodies of the IgG1 and IgE isotypes, activate eosinophils, and stimulate fibrosis. A new and striking pathway that was first discovered with human monocyte–derived DCs involves the epithelial–derived cytokine thymic stromal lymphopoietin (TSLP). This cytokine stimulates DCs to induce "inflammatory Th2 cells" that produce TNFα (rather than IL-10) in addition to IL-4, IL-5, and IL-13.[196] Recent studies have shown that TSLP receptor knockout mice exhibit strong Th1 responses, with high levels of IL-12, IFNγ, and IgG2a, but low production of IL-4, IL-5, IL-10, IL-13, and immunoglobulin E.[197] DCs that are activated with either CD40L or TSLP are similar in appearance, being rich in MHCII and CD86 co-stimulatory molecules. However, they differ significantly in cytokine and chemokine production, and the functional consequences for T cells vary.[196]

Cross-Presentation by Conventional Dendritic Cells

CD8 T cells are critical for protective immunity against intracellular pathogens and malignant tumor cells. When autologous T cells are incubated with influenza-infected DCs, strong proliferative and cytotoxic T-lymphocyte (CTL) responses develop within a week. Thus, DCs present endogenous antigens on MHCI to prime CD8+ T cells. Interestingly, DCs can also present exogenous antigen on MHCI, an unconventional presentation path called "cross-presentation." The earliest experiment demonstrating the capacity of DCs in cross-presentation was done in vitro, when trinitrophenyl-modified cells were taken up by DCs. DCs can present trinitrophenyl, an exogenous antigen, and induce antitrinitrophenyl CTLs in vitro in a MHC-restricted manner.[14] Later, DCs were found to take up exogenous influenza

antigen in the form of nonreplicative virus or apoptotic cells, and present them on MHCI to prime CD8 T cells.[198] In the absence of DCs, mice are unable to process several different antigens through the exogenous pathway, indicating that DCs were a major cell type for cross presentation to CD8 T cells in vivo.[199] DCs cross present exogenous antigen taken up as soluble form or via multiple receptors, including FcR, CD91, Lox-1, DEC205/CD205, and DNGR1. CD8+ DCs in mice and their functional equivalent BDCA3+ DCs in humans excel in cross-presenting exogenous antigens.[24,134,200] Exogenous presentation or cross-presentation is essential for protective immunity against viruses and tumors.

DENDRITIC CELLS CONTROL TOLERANCE

The mechanisms used by DCs to initiate immunity against pathogens pose a major risk for the development of autoimmunity, allergy, and chronic inflammatory disease. For example, infection frequently induces some cell death; therefore, DCs at the site of infection capture pathogens and dying cells, and the two are processed and presented similarly.[201,202] Likewise, at body surfaces, maturing DCs are also capturing environmental antigens to which the body must remain unresponsive. How do maturing DCs focus the immune response on antigens derived from the pathogen and avoid inducing immunity to self- and nonpathogenic environmental antigens?

Thymic Dendritic Cells Contribute to Central Tolerance

Self-reactive thymocytes are deleted by antigen-presenting cells during negative selection. The thymus contains two major populations of antigen-presenting cells that express MHCII, namely, medullary thymic epithelial cells and cDCs. Both cell types are required for efficient negative selection. Medullary thymic epithelial cells express a panoply of self-antigens under the control of the autoimmune regulator "AIRE."[203] Self-antigens for negative selection in the thymus also include antigens expressed by DCs, and antigens that enter the thymus through the bloodstream and are captured by DCs. In the absence of antigen presentation by DCs, negative selection of CD4+ thymocytes is impaired.[204] In addition to negative selection, thymic cDCs also support the development of Foxp3+ T_{reg}s.[205] Thus cDCs contribute to central tolerance in the thymus by more than one mechanism.

Dendritic Cells Mediate Peripheral Tolerance

DCs also mediate tolerance in the periphery. Because central tolerance alone is incomplete, the immune system must continually establish tolerance to harmless or "noninfectious" antigen in the environment. Therefore, the effective control of self-reactive T cells depends on peripheral tolerance. For example, mice and humans deficient in T_{reg}s, which suppress autoreactive T cells in the periphery, succumb to autoimmunity at an early age. DCs constantly carry innocuous antigens from the periphery (eg, from the skin, airways, stomach, intestine, and pancreas) and present them to T cells in lymphoid organs.[206] A critical observation was that bone-marrow–derived cells in the pancreatic lymph nodes present peptides derived from insulin-producing β cells from pancreatic islets, leading to tolerance.[207,208] Although DCs were not implicated directly, a direct role for DCs in maintaining tolerance was established by Hawiger and colleagues by targeting antigens directly to DCs.[209] Furthermore, CD8α+ cDCs are particularly efficient in capturing dying cells[201] and inducing tolerance.[202] Likewise, when DCs capture innocuous proteins from the airway, profound tolerance develops even though the T cells can initially proliferate extensively to the antigen-capturing DCs in the draining lymph nodes.[210]

Deletion and Anergy

Mechanisms for peripheral tolerance can be intrinsic (deletion and anergy) or extrinsic (through suppressive T_{reg} cells). Interestingly, the former requires expression of B7 family members on the steady state DCs (eg, PD-L1 and CD86), which then ligate PD-1 and CTL antigen-4 on the T cells to be tolerized.

Regulatory T cells

Autoreactive T cells can remain quiescent in the presence of T_{reg} cells. There are two types of T_{reg}s: naturally occurring T_{reg}s derived from thymus (natural T_{reg}s) and T_{reg}s induced from Foxp3−CD4+ T cells in the periphery (induced T_{reg}s).[211]

The groups of Belkaid and Powrie found that a subset of gut DCs expressing CD103, the αEβ7 integrin, are specialized at inducing Foxp3+ T_{reg}s and maintaining oral tolerance.[212] CD103+ DCs from mesenteric lymph nodes or gut-associated lymphoid tissue produce transforming growth factor-β, a cytokine critical for induction of Foxp3+ T_{reg}s.[212] Additionally, CD103+ gut DCs use retinal dehydrogenase to metabolize vitamin A to bioactive retinoic acid, which acts as a cofactor for transforming growth factor-β to induce Foxp3+ T_{reg}s.[212,213] Similarly, skin DCs use vitamin D3 to induce T_{reg}s.[214,215] Therefore, DCs are able to employ environmental signals, vitamin A in the gut, and vitamin D3 in the skin to induce tolerance to harmless foreign antigens. The CD8+ and CD103+ DC subset appears to be specialized at T_{reg} induction. When antigens were targeted to CD8α+ DEC-205+ or CD8α− DCIR2+ DC subsets in vivo, only CD8α+ DEC-205+ DCs were able to induce Foxp3+ T_{reg}s from Foxp3− CD4+ T cells.[216]

DCs also maintain the homeostasis of T_{reg}s. Loss of DCs leads to a loss of T_{reg} cells, and the remaining T_{reg} cells exhibit decreased Foxp3 expression. The DC-dependent loss in T_{reg} cells leads to an increase in the number of T cells producing inflammatory cytokines, such as IFNβ and IL-17.[217] Conversely, increasing the number of DCs leads to increased T_{reg}-cell division and accumulation by a mechanism that requires MHCII expression on DCs.[146] Activation of β-catenin is essential for DC to control T_{reg} and peripheral tolerance. DCs lacking β-catenin show decreased production of immunosuppressive cytokines and a decreased ability to support the differentiation of naïve T cells into T_{reg}s in vitro.[218]

In summary, DCs have the capacity to induce tolerance by several mechanisms.

DENDRITIC CELLS IN CLINICAL IMMUNOLOGY

This section focuses on diseases in which DCs play a pathogenic role or can be targets for therapy.

Dendritic Cells in Transplantation

DCs play a key role in the outcome of organ and hematopoietic transplantation. In organ transplantation, both donor and recipient DCs contribute to graft rejection. Donor DCs in grafted organs migrate to lymphoid organs where they stimulate alloreactive T cells in the recipient to induce organ rejection[219,220]; recipient DCs can also capture antigens from the graft and elicit organ rejection. For example, in hematopoietic transplantation, recipient DCs initiate T-cell–induced graft-versus-host reactions.[74,221]

Dendritic Cells in Autoimmune Disease

Several human autoimmune diseases appear to involve DCs. Abundant DCs are found in the synovial exudates of rheumatoid arthritis, and TNF-α produced by DCs contributes to the severity of the disease. Similarly, in psoriasis, DCs infiltrate lesional skin, produce TNF-α, and polarize T cells toward Th1/Th17[222,223]; additionally, pDCs in psoriatic lesion become activated to produce type I IFN, which also contributes to the inflammatory response.

In systemic lupus erythematosus, two subsets of DCs contribute to the onset and severity of the disease; pDCs in patients with systemic lupus erythematosus overproduce IFNα, which activates cDCs and interferes with their ability to maintain peripheral tolerance.[178] Finally, DCs have been implicated in mouse models of type I diabetes by carrying self-antigens from the pancreas to draining lymph nodes where diabetogenic T-cell responses are initiated. In addition, DCs cultured from nonobese diabetic mice show an activated phenotype with increased IL-12 and costimulatory molecule expression.[224]

Dendritic Cells in Viral Infections

DCs mediate antiviral immunity by priming T-cell responses. However, a number of viruses have evolved strategies to subvert DCs, and thereby, the immune system.

The interaction between HIV and DCs is a fascinating example of viral immune subversion. DCs carry HIV from peripheral tissues into draining lymph nodes where the virus is transmitted to CD4 T cells. HIV binds to DCs by CCR4, CXCR5, or DC-SIGN, but productive infection is restricted by SAMHD1, a protein encoded by an Aicardi-Goutières syndrome susceptibility gene.[225] Transmission to CD4 T cells is dependent on DC-SIGN, a C-type lectin pathogen-recognition receptor expressed on the surface of DCs that retains the attached virus in an infectious state. In the lymphoid organs, close interaction between DCs and CD4+ T cells facilitate HIV transmission.[226,227]

Interestingly, DC-SIGN also serves as receptor for several other viruses including hepatitis C virus, Ebola virus, cytomegalovirus, dengue virus, and the severe acute respiratory syndrome coronavirus.[228–230] Dengue is a mosquito-borne flavivirus that causes a disease that can be associated with hemorrhagic fever. Dengue virus targets DCs directly through DC-SIGN but also enters DCs as a passenger in immune complexes that are taken up by Fc receptors.[231,232] When infection occurs through antibody enhancement mediated by Fc receptor, the infected DCs are involved in induction of the T-cell cytokines that mediate the vascular leak syndrome associated with the infection.

Dendritic Cells in Cancer

Tumors can suppress immunity in part through their effects on DCs. DC differentiation and activation can be suppressed by cancer-derived cytokines, such as IL-6, vascular endothelial growth factor, and IL-10.[233] In contrast to their normal counterparts that activate immune responses, DCs derived from tumors induce Foxp3+ T_{regs}[234] and IL-13–producing CD4+ T cells,[235] and suppress proliferation of CTLs[236] and NKT cells.

Dendritic Cell–Targeted Vaccines

DC-based vaccines are currently being used in the clinic, and DC-targeted vaccines are being tested.

DC-based immune therapy is currently available for treating prostatic cancer, but it is far from optimized and is not curative. Monocyte-derived DCs are generated ex vivo, loaded with tumor cells or tumor antigens, and reinjected into the patient.[237] Scientific and practical problems exist with this approach, including limited responses possibly due to inefficient migration of monocyte-derived DCs from injection site to the draining lymphoid organs and inefficient antigen presentation.

DC-targeted vaccines are based on the idea that antigens delivered specifically to DCs in conjunction with the appropriate adjuvants will produce strong and lasting immunity.[209] DC-targeted vaccines require that antigens be delivered specifically to endocytic receptors on DCs together with the appropriate stimuli to induce DC activation. For example, antigens have been incorporated into antireceptor monoclonal antibodies, which are then injected into the vaccine recipient.[209,238,239] This paradigm was established using antibodies to DEC205/CD205, which is abundant on DCs and delivers antigens to both MHC class I and II antigen-processing compartments.[240] In mice, antigens targeted to DEC205/CD205 on activated DCs induce strong immunity against tumors and a number of intracellular pathogens, including malaria and *Leishmania*.[238,241,242] Importantly, these responses are broad, often generating immunity against multiple epitopes[243] in mice of several MHC haplotypes and the responses elicit protection from mucosal infections.[244] Other potential DC targets include LOX-1/OLR1, MMR/CD206, DCIR/CLEC4A, DC-SIGN/CD209, DNGR1, langerin (CD207), and CD40.[28,245]

In conclusion, DCs play important roles in a number of different diseases. Moreover, they are excellent targets in designing new approaches to prevention and treatment of these diseases.

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CHAPTER 17

Natural Killer Cells

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INTRODUCTION

Natural killer (NK) cells were initially described because they spontaneously kill certain tumor targets.[1–4] However, they are now recognized to play important roles in early innate immune responses, especially to viral infections. They interact with other innate immune components and modulate the subsequent adaptive immune response. These effects are due to NK cell responses to proinflammatory cytokines or susceptible targets, which stimulate NK cells to secrete other cytokines and/or kill targets. In this chapter, we will consider all of these issues in detail by describing how they differ from other lymphocytes, their functions, unique target recognition strategies, tolerance, development, and role in immune responses and human diseases. There will be an emphasis on their target recognition receptors because their discovery made it possible to understand NK cell biology more precisely.

GENERAL DESCRIPTION

Developmental studies have provided strong evidence that NK cells belong to the lymphocyte lineage (discussed in detail in the following). Morphologically, NK cells are typically large lymphocytes containing azurophilic granules.[5] However, the large granular lymphocyte morphology is not invariably associated with NK cells because small, agranular lymphocytes may display natural killing,[6] activated cytotoxic T-lymphocytes (CTLs) can display this morphology,[7] and human large granular lymphocyte leukemias contain NK- and T-cell variants.[8] Among lymphocytes, NK cells more closely resemble T cells than B cells. Thus, it is useful to compare and contrast these two lymphocyte populations as well as consider another enigmatic cell termed the "lymphokine-activated killer" (LAK) cell.

Natural Killer Cells versus T Cells

NK cells are most often confused with T cells because they may have similar morphologies, express several cell surface molecules in common,[9,10] and share functional capabilities. While this confusion was frequent before the molecular description of the T-cell receptor (TCR)/cluster of differentiation (CD)3 complex, their similarities remain a potential source of uncertainty. However, mature NK cells are clearly not T cells by several criteria.[11] Conventional NK cells do not require a thymus for development and are normal in athymic nude mice (though this is not the case for the newly described "thymic" NK cell subset, discussed subsequently). NK cells do not express the TCR on the cell surface, do not produce mature transcripts for TCR chains, and do not rearrange TCR genes.[12,13] Mice with the *scid* mutation or deficiencies in *Rag1* or *Rag2* lack TCR gene rearrangements and mature T cells but possess NK cells with apparently normal function.[14–17] Several CD3 components may be found in the cytoplasm of NK cells, particularly immature NK cells, but they are not displayed on the cell surface[18] with the exception of CD3ζ. But CD3ζ is expressed in association with FcγRIII (CD16) and other NK cell activation receptors instead of the TCR/CD3 complex.[19,20] Whereas mice lacking CD3ζ lack most T cells, NK cell number and function are minimally affected.[21] On the other hand, NK cells are completely absent in mice with only partial defects in T-cell subsets, such as in mice lacking components of the IL-15R (see following discussion). NK cells do not require the presence of major histocompatibility complex (MHC) class I (MHC-I) molecules on their targets for lysis in an important functional distinction with CD8+ MHC-I–restricted T cells. Instead, NK cells kill more efficiently when their targets lack MHC-I expression. Thus, NK cells can be clearly distinguished from T cells, even from so-called CD3+ NKT cells that express NK cell markers (see following discussion).

Natural Killer Cells, Lymphokine-Activated Killer Cells, and Interleukin-15

Another area of overlap between NK and T cells concerns cytokine responses. When mouse splenocytes or human peripheral mononuclear cells are exposed to high concentrations of interleukin (IL)-2 (800 to 1000 U/mL), robust lymphocyte proliferation ensues (ie, LAK cells are generated).[22–25] Although most are CD3– NK cells, TCR/CD3+ T cells are also produced. To distinguish NK cells within this population, they are sometimes called "$CD3^-$ LAK" cells or "IL-2–activated NK cells."

In a related phenomenon, NK cells are activated when mice are injected with polyinosinic-polycytidylic acid (poly-lic) or other agents that trigger through Toll-like receptors (TLRs), often on plasmacytoid dendritic cells (DCs).[26] NK cells can also be activated in vitro with interferon (IFN)α/β, IFNγ, or low concentrations of IL-2 that are insufficient to induce proliferation.[3,4,27]

NK cells activated in these various ways, with or without proliferation, display enhanced killing of typical NK-sensitive targets. They also kill a broader panel of targets, including those that are generally resistant to freshly isolated NK cells, such as the murine P815 mastocytoma cells and

freshly explanted tumors. Many agents that enhance killing by NK cells may also activate T cells, such that even T-cell clones may display promiscuous killing of targets that is no longer MHC-restricted.[28] This phenomenon was a frequent source of confusion between NK cells and T cells during the initial characterization of both cell types.

Why cytokine activation leads to enhanced killing is incompletely understood. Interestingly, mouse NK cells constitutively express messenger ribonucleic acid (mRNA) for cytotoxicity components, perforin, and granzymes, but no protein.[29] Cytokine activation enhances expression of mRNA for perforin and granzymes,[30] and translation into expressed proteins that contributes to more lytic capacity,[29,31] but this effect may not explain the capacity of LAK cells to kill a broader panel of targets. Activated NK cells express additional receptors that may deliver stimulatory signals,[32,33] but their contribution to the LAK phenomenon remains unclear. In mice, cytokine activation of NK cells results in expression of an alternatively spliced form of a receptor termed NKG2D (see subsequent discussion), which may play a role,[34,35] and IL-15 contributes to LAK-like activity of CTLs, although how much this applies to conventional NK cells is not understood.[36] Enhanced killing may also be due to effects on adhesion molecules.[37,38] Nonetheless, it remains unclear how each of these factors contribute to the LAK phenomenon of enhanced and broader killing capacity as compared to resting NK cells.

It seems unlikely that high concentrations of IL-2 can be achieved, even locally, to stimulate NK cells in vivo. Furthermore, NK cells tend to be early responders in immune responses whereas the prime reservoir of large amounts of IL-2 is the activated T cell that produces it somewhat later. Although naïve T cells can make IL-2 soon after stimulation[39,40] and DCs can produce IL-2 to enhance NK lytic activity,[41] whether the resultant IL-2 concentrations are sufficient to generate LAK cells in vivo is unclear.

Interestingly, NK cells are apparently normal in mice with a targeted mutation in the IL-2 gene or the IL-2Rα chain,[42,43] indicating that IL-2 itself is not required for normal NK cell development. Paradoxically, NK cells are deficient in mice with a mutation in either IL-2Rβ or IL-2Rγ.[44–46] The discrepancy in NK cell dependence on IL-2 versus IL-2 receptor (IL-2R), as well as the LAK cell phenomenon, may be best understood by comparing the components of the IL-2 and IL-15 receptors.

In brief, the high affinity IL-2R is a heterotrimeric receptor complex comprised of α (p55), β (p75), and γ (p64) chains.[47] Though individual components may bind IL-2 with low affinity, only the intermediate-affinity βγ receptor (K_d ~1 nM) and the high-affinity αβγ receptor (K_d ~10 pM) are capable of signaling. Resting NK cells constitutively express IL-2Rβγ[47,48] and upon activation, may induce IL-2Rα and further upregulate IL-2Rγ chain expression.[47] In contrast, resting T cells generally do not express any functional IL-2 receptors, and most naïve T cells do not respond to high concentrations of IL-2.[49] The IL-2Rγ chain is also termed the common γ subunit (γc) because it is a required component of the multimeric receptor complexes for other cytokines, including IL-15,[50] that is particularly relevant to NK cells. IL-15 does not bind to IL-2Rα but instead utilizes a unique IL-15Rα chain to form a high-affinity complex with IL-2Rβγ.[51,52] The IL-15Rα chain does not directly signal. Its distribution is widespread on numerous cell and tissue lineages including NK cells.

IL-15 has a number of effects on NK cell biology. It is required for NK cell development; mice lacking IL-15 or any component of the trimeric IL-15R complex lack NK cells.[44–46,53,54] Not surprisingly, mice deficient in other components of the IL-15R complex and its signaling pathway (IL-2Rβ, Jak3, and STAT5α/β) exhibit similar defects in NK cell development.[44,55–57] Depending on its relative concentration, IL-15 has an antiapoptotic or proproliferative effect.[58,59] When NK cells are transferred to NK cell–deficient mice, they undergo "homeostatic" proliferation,[60,61] akin to T-cell homeostatic proliferation.[62] Like memory CD8+ T-cell homeostasis, NK-cell homeostasis is IL-15–dependent,[60,61] to a more or less degree.[63] Finally, LAK cells can be generated with IL-15.[64] These studies strongly suggest that LAK cells are generated because high-dose IL-2 acts through the IL-2Rβγ that is normally expressed with IL-15Rα as components of the constitutively expressed trimeric IL-15R complex on resting NK cells. Thus, the LAK cell phenomenon is related to the role of IL-15 and its receptor in NK cell biology.

Interestingly, IL-15 is expressed at very low levels and is difficult to detect in vivo.[65] The IL-15Rα chain can present IL-15 in *trans* to NK cells that can respond through IL-2/15Rβγ alone.[66] For example, IL-15Rα–deficient NK cells develop in bone marrow (BM) chimeric mice in which IL-15Rα–deficient BM was used to reconstitute IL-15Rα–sufficient animals,[67] indicating that IL-15Rα on another cell can allow IL-15Rα–deficient NK-cell development. In certain inflammatory situations in vivo, DC presentation of IL-15 in *trans* can enhance NK cell responses (also known as priming).[31,68] In DCs expressing both IL-15 and IL-15Ra, the IL-15Ra chain provides a chaperone function to stabilize receptor-cytokine complexes on the cell surface.[69] Taken together, *trans* presentation of IL-15 may be physiologically important to NK cell function.

SELECTIVE NATURAL KILLER CELL SURFACE MARKERS

The constitutive expression on NK cells of IL-15R complex with IL-2Rβ has practical usefulness because anti-IL-2Rβ (CD122) is sometimes used to identify naïve CD3− NK cells or deplete them in mice,[70] but anti-CD122 is less useful during an ongoing immune response and CD122 is expressed on regulatory T cells. Anti-IL-15Rα antibodies have not been widely used. Other markers have proven to be more useful for analysis of NK cells.

In the mouse, the NK1.1 molecule is an especially important marker on NK cells in C57BL strains.[11] NK1.1 is an activation receptor encoded by *Nkrp1c* (*Klrb1c*),[71] a member of the *Nkrp1* gene family (see following discussion). In FACS sorting experiments, the NK1.1+ fraction contained all of the natural killing activity in the spleen.[72] In vivo administration of the anti-NK1.1 mAb PK136[73] completely abrogated natural killing but did not affect adaptive immune responses[74]

(mAb PK136 is available from the American Type Culture Collection [ATCC], Manassas, VA [HB-191] and is an IgG2a isotype [ATCC, and data not shown], not IgG2b as originally described[73]). While mAb PK136 is very efficient at NK-cell depletion and is widely used for this purpose, unfortunately it recognizes an epitope on NK1.1 that is confined to C57BL/6, C57BL/10, and a few other strains.[73] Moreover, in Swiss, NIH and SJL/J mice, mAb PK136 recognizes another NKRP1 family member, NKRP1B.[75,76] However, there are now available NK1.1+ congenic strains, such as BALB.B6-*Cmv1*^{r} (catalogued as C.B6-*Klra8*$^{Cmv1\text{-}r}$/UwaJ, stock number 002936 at The Jackson Laboratory, Bar Harbor, ME) in which the C57BL/6 allele of NK1.1 has been genetically bred onto the BALB/c background that otherwise lacks the NK1.1 epitope.[77] Similarly, the NK1.1 allele has been introgressed onto the nonobese diabetic (NOD) background.[78]

A subpopulation of T cells expresses NK1.1, described in detail in another chapter 18. These "natural killer T (NKT) cells" express the TCR/CD3 complex and typically are restricted by the nonclassical MHC-I molecule, CD1, which presents glycolipid antigens to NKT cells. NKT cells respond early during the course of an immune response and may potently activate conventional NK cells.[79] Nonetheless, NKT cells can be distinguished from conventional NK cells by expression of the CD3 complex (ie, conventional NK cells are NK1.1+ CD3−).

The NKG2D (Klrk1) activation receptor is expressed on all NK cells in human and all strains of mice examined.[80] In humans, NKG2D is also expressed on all γδTCR+ and CD8+ T cells, whereas in mice, NKG2D is expressed on most NKT and γδTCR+ T cells but not on resting CD8+ T cells.[81–83] However, essentially all activated mouse CD8+ T cells express NKG2D. In both humans and mice, CD4+ T cells do not express NKG2D, but it is found on a subset CD4+CD28− T cells in patients with rheumatoid arthritis.[84] Blocking anti-NKG2D mAbs and NKG2D-deficient mice have been described.[80,85–87] Regardless, conventional NK cells are NKG2D+ CD3−.

The NKp46 (Ncr1) activation receptor appears to be expressed on all CD3− NK cells in humans and all strains of mice. However, recent reports indicate expression of NKp46 on immune cells in the gut that may be developmentally distinct from conventional NK cells.[88–92] Moreover, depleting anti-NKp46 mAbs have not been described, limiting its usefulness for in vivo functional experiments. Nonetheless, recently developed mice may allow other approaches, such as a mouse where a green fluorescent protein (GFP) cassette was inserted into *Nkp46* and two different transgenic (Tg) mice with a *Nkp46* promoter contruct for expression of Cre or diptheria toxin receptor.[93–95]

The mAb DX5 recognizes a molecule that is coexpressed on most NK1.1+ CD3− cells and on small populations of splenocytes in NK1.1− strains, consistent with identification of NK cells in all strains. However, mAb DX5 recognizes the α2 integrin that is widely expressed on other leukocytes, not just NK cells,[96,97] and its expression is regulated.[98] Nevertheless, the DX5 epitope has been used to identify NK cells in mouse strains that do not express NK1.1, but it has been largely supplanted by other nonpolymorphic markers such as NKp46.

The glycolipid determinant asialo-GM$_1$ is expressed by most if not all murine NK cells and a subpopulation of T cells.[99–101] Although the functional significance of this molecule is unknown, polyclonal rabbit anti-asialo-GM$_1$ (Wako Chemicals USA, Richmond, VA) has been used to effectively deplete NK cells. In more recent studies, the anti-NK1.1 mAb PK136 has become the reagent of choice for NK-cell depletion because of an available defined mAb and its more restricted reactivity with NK cells.[11,72–74] However, anti-asialo-GM$_1$ remains in use for NK-cell depletion when anti-NK1.1 cannot be employed.[73]

In addition to NKG2D and NKp46, human NK cells selectively express CD56. Although it is also found on neural tissues and some tumors, CD56 is generally not expressed by other hematopoietic cells or lymphocytes.[102–104] This 140 kDa molecule is derived from alternative splicing of the gene encoding neural cell adhesion molecule (NCAM) involved in nervous system development and cell-cell interactions.[105,106] CD56 may be involved in adhesion between NK cells and their targets,[107] but this function is controversial. Curiously, mouse CD56 is not expressed on hematopoietic cells,[108] indicating that its role on NK cells is not conserved. Nevertheless, CD56 is particularly useful as a pan-NK–cell marker in humans.

Human NK cells can be functionally divided according to the level of CD56 expressed.[103,109] Most human peripheral blood NK cells are CD56^{dim}, a phenotype associated with more cytotoxicity and less cytokine production than a smaller subset of NK cells that express CD56 at higher levels (CD56^{bright}). These cells also tend to differentially express receptors involved in target recognition as well as CD16. The CD56^{bright} cells may undergo a maturation process to become CD56^{dim} cells[110] and may be related to a subset of NK cells identified in mice, termed "thymic" NK cells.[111]

Other molecules selectively expressed on NK cells are better discussed below under the general topic of NK cell receptors because they are molecularly defined and their ligands are known.

A MOLECULAR DEFINITION OF NATURAL KILLER CELLS?

A precise molecular definition of NK cells has been elusive. There are no known molecules that are exclusively expressed on NK cells and are responsible for critical functions only displayed by NK cells. The NK cell is therefore still defined by function to the exclusion of other cells, a concept first articulated 25 years ago.[11]

The defining functional feature of NK cells remains their intrinsic ability to perform natural killing (ie, they spontaneously lyse certain tumor cells in a perforin-dependent manner). Unlike other lymphocytes, NK cells do not express surface immunoglobulin or the TCR/CD3 complex, and generally do not require MHC-I expression on targets for lysis. Therefore, a current working definition is that an NK cell is a sIg$^-$, TCR/CD3$^-$ lymphocyte that can mediate perforin-dependent natural killing against targets that may lack MHC-I expression.

It is noteworthy that T cells were historically defined by an awkward functional definition (thymus-derived, sIg$^-$ lymphocytes responsible for cell mediated immunity).[112]

With the molecular definition of the TCR and coexpressed CD3 molecules, immunologists can now define a T cell as a cell expressing the TCR/CD3 complex.[113] The availability of molecular probes and mAbs directed against this complex provides precise definition even in pathologic tissue sections, without the need for functional analysis (cell mediated immunity, thymus dependence). Similarly, a molecular definition should permit unequivocal identification of NK cells to define their role in normal immune responses and pathologic settings.

Presumably, such a definition will require further knowledge of the molecular basis for NK-cell function, such as the receptors involved in natural killing. On the other hand, one difficulty is that the function that is most attributed to NK cells, natural killing, can be displayed by other cells, such as cytokine-treated T cells. Moreover, NK cells can utilize more than one receptor for target recognition, and individual NK cells can simultaneously express several of these receptors. Thus, there is, as yet, no consensus on the elusive "NK-cell receptor" analogous to the TCR, and the general sentiment in the field is that there is unlikely to be such a receptor.

In the absence of a precise definition, most investigators currently consider the following phenotypes to be surrogate markers of bona fide NK cells. Mouse NK cells are typically NK1.1+ (in appropriate strains), FcγRIII+ (CD16), CD122+, and CD3−. Human NK cells are generally CD56+ and CD3−. In general, mouse and human NK cells also express NKG2D and NKp46, with caveats as elaborated previously.

Note that these markers are generally correlated with cells having natural killing capacity but the markers themselves are not required for natural killing. It should be emphasized that these phenotypes can lead to some confusion due to expression of other molecules on NK cells that are used to help define other immune cells. For example, NK cells express B220 (CD45R) that is often used as a B-cell–specific marker; CD19 is more reliable to distinguish B cells from NK cells.[114] Similarly, NK cells express CD11b, first described as Mac-1 on macrophages.[98] Moreover, NK cells express CD11c, a marker used to define certain DC populations, leading to publications describing a novel type of DC, termed killer DCs.[115,116] However, detailed investigation suggests that these cells are developmentally unrelated to DCs and are actually activated NK cells.[117–119] Therefore, markers associated with NK-cell function have been extremely useful in shaping our current concepts of NK cell biology and elaborate their effector functions, but caution may be necessary to avoid confusion with other immune cells.

EFFECTOR FUNCTIONS OF NATURAL KILLER CELLS

Cytotoxicity

A hallmark of NK-cell effector function is target killing, mediated by a process termed granule exocytosis that can be initiated by exposure to susceptible targets or cross-linking of specific activation receptors. Like CTLs, NK cells possess preformed cytoplasmic granules that resemble secretory lysosomes with properties of both secretory granules and lysosomes.[120] Granule formation is affected by Lyst, the molecule defective in humans with Chediak-Higashi syndrome[121,122] in which enlarged lysosomes are observed apparently due to decreased lysosome fission.[123] Normal granules contain perforin and granzymes (granule enzymes). Perforin, a pore-forming protein, is rendered inactive by association with calreticulin and serglycin, and is activated by a cysteine protease.[124] Granzymes are first produced as inactive proenzymes that are activated by N-terminal cleavage by dipeptidyl peptidase I, also known as cathepsin C. However, granzymes are rendered inactive by the acidic pH of the granules. Upon activation by a sensitive target, NK (and T) cells are triggered to rapidly polarize the granules and reposition the microtubule organizing center toward the target in a dynein-dependent manner.[125] The granule membrane ultimately fuses with the plasma membrane, and externalizes, releasing granule contents. Calcium-dependent polymerization of perforin results in "perforation" of the target cell plasma membrane, and granzyme entry by an as yet incompletely understood process. A recent study suggests that perforin induces a plasma membrane repair process that results in endocytosis of perforin and granzymes into enlarged endosomes, called "gigantosomes."[126] Perforin pores in the gigantosome membrane then allow delivery of granzymes that mediate cleavage of caspases and Bid, ultimately leading to target cell apoptosis.[127]

Recently, many details of the granule exocytosis pathway have come from studies of the heterogeneous human disorder, hemophagocytotic lymphohistiocytosis (HLH).[128,129] In particular, genetic studies of heritable HLH, termed familial HLH (FHL), led to identification of the first described FHL mutation in the perforin gene (*PRF1*), responsible for FHL2. Studies of patients with FHL without *PRF1* mutations led to discovery of other genes whose products (MUNC13-4, syntaxin 11, MUNC18-2) affect granule exocytosis by cytotoxic lymphocytes. Fusion of the cytolytic granule with the plasma membrane requires vesicular RAB27a, a member of the small GTPase superfamily. Defects in RAB27a are associated with the human disorder Griscelli syndrome, type 2. Mice have been described with defects in granule exocytosis components including LYST (*beige*), perforin (*Pfn1*−/−), Unc13d (equivalent to MUNC13-4, also known as *Jinx*), and Rab27a (*ashen*). As highlighted by the names of the mutant mice, many mutations of molecules in the granule exocytosis pathway are associated with skin pigment changes because these molecules also affect melanosomes in melanocytes.[130]

Human T and NK cells also express another pore-forming molecule, granulysin, that is related to a family of saposin-like proteins.[131] Based on crystallographic studies, these molecules appear to be active against bacteria, fungi, and tumor cells by charge association with target membranes and subsequent disruption, leading to target cell lysis.[132] Granulysin is contained in cytolytic granules containing the other cytolytic proteins, such as granzymes.[133] In a perforin-dependent manner, granulysin causes target apoptosis but is not expressed in mouse cytotoxic lymphocytes.[134]

NK-cell cytotoxcity can be demonstrated in several related ways. Natural killing refers to the process by which NK cells kill certain tumor targets without need for prior host sensitization with the target. Natural killing was first

assessed with a simple in vitro assay for target membrane integrity that is still used today, the standard ^{51}Cr-release assay.[135] The prototypical NK-sensitive tumor target for mouse NK cells is YAC-1 (TIB-160 from ATCC), a thymoma derived from Moloney virus–infected A strain mice, whereas the standard human target is K-562 (CCL-243 from ATCC), an erythroleukemic cell line derived from a human patient with chronic myelogenous leukemia in blast crisis.[136] Maximal killing by enriched, IL-2–activated NK cells usually occurs with effector:target (E:T) ratios of <10:1 whereas unfractionated, freshly isolated peripheral blood or splenocyte preparations usually require E:T ratios of >100:1. Even at high E:T ratios, not all targets are killed, with percentage-specific cytotoxicity typically ranging from ~10% with fresh NK cells to ~80% with activated NK cells. Note that perforin-dependent leakage of ^{51}Cr from the targets is mostly complete within about an hour; 4-hour assays are standard. Longer periods may reflect other apoptotic processes, such as Fas-induced apoptosis.

While ^{51}Cr release is still the gold standard, there are also numerous nonradioactive tests for perforin-dependent killing, including release of intracellular enzymes or use of fluorochromes for target labeling.[137–139] The release of granule components, including granzymes, into the supernatant can be determined by conversion of an appropriate substrate, such as granzyme A-mediated cleavage of alpha-N-benzyloxy-carbonyl-L-lysinethiobenzyl ester (also known as BLT-esterase activity).[140]

A particularly useful new flow cytometric assay exploits the orientation of lysosomal-associated membrane protein-1 (LAMP-1, CD107a) on the luminal side of cytotoxic granules in unactivated NK cells. During granule exocytosis, the granule fuses with the plasma membrane, resulting in externalization of the granule membrane and exposing CD107a on the external surface of the plasma membrane as an indicator of NK-cell activation.[141–143] By contrast to other methods, the CD107a assay provides the opportunity for measuring NK-cell responses at the single cell level, isolating triggered NK cells,[144] and possibly simultaneously assessing other NK-cell functions.

Activated NK cells and CTLs also induce perforin-independent target cell killing by expressing Fas ligand (tumor necrosis factor [TNF] superfamily 6) that binds Fas (TNF receptor superfamily 6, TNFRSF6) on the target, triggering apoptosis.[145–150] Similarly, other TNF superfamily members, such as TNF-related apoptosis-inducing ligand (TRAIL TNFSF10), can be involved in related processes.[151] However, mice deficient in TNF family members or their receptors may manifest significant alterations in lymphoid organogenesis and splenic architecture, and NK cell number and function,[152–154] such that the relative contributions of these pathways to NK-cell function are incompletely understood. Moreover, NK cells from mice deficient in perforin, granzymes, or molecules involved in granule formation or exocytosis demonstrate profound defects in natural killing in vitro.[155–158] Similar defects have been found with NK cells derived from patients with deficiencies in granule exocytosis.[128] Thus, the available data strongly suggest that granule exocytosis is the predominant mechanism for natural killing.

In addition to natural killing, cytotoxicity by NK cells can be triggered by deliberate cross-linking of activation receptors (discussed in greater detail in the "Activation Receptors" section). Plant lectins can also trigger target killing.[159] In general for all stimuli, cytotoxicity occurs via granule exocytosis and can be measured with the same assays for natural killing.

Cytokine Production

When exposed to NK-sensitive targets or cross-linking of receptors, NK cells also produce cytokines, including IFNγ, TNFα, and granulocyte-macrophage colony stimulating factor (GM-CSF).[160–162] They can also be similarly triggered to produce chemokines, such as RANTES, lymphotactin, MIP-1α, and MIP-1β.[163] Moreover, NK cells produce cytokines in response to other cytokines. For example, in response to IL-12, NK cells produce IFNγ.[164] Similarly, NK cells respond to type I IFNs (IFNα/β) produced by DCs stimulated by in vivo administration of poly-I:C and other ligands for TLR and nucleic acid sensors.[165] Cytokine-stimulated responses may obscure detection of specific activation by activation receptors in vivo.[163,166]

While cytokine production can be indirectly measured with RT-PCR for mRNA, it should be noted that resting NK cells typically already express abundant levels of cytokine mRNA even though the proteins are not synthesized,[167] as described previously for granule components in mouse NK cells.[29] Enzyme-linked immunosorbent assays (ELISA) of tissue culture supernatants are often used, but recent studies have utilized intracellular staining of cytokines, such as IFN, for analysis of individual NK-cell responses that may be more informative, akin to use of the CD107a degranulation assay.[168]

In immune responses, NK-cell production of cytokines should occur relatively early and may thereby influence the subsequent adaptive immune response. Moreover, their responses to cytokines are regulated by complex interacting pathways.[169] A fuller description of NK-cell cytokine responses and production is provided in the following sections on NK cell responses during infections and interactions with DCs.

NATURAL KILLER CELL RECOGNITION OF TARGETS

Molecular dissection of NK-cell recognition of their targets opened new frontiers in NK-cell biology because it not only explained target recognition but it led to identification of receptors that are selectively expressed on NK cells. In addition to providing molecular tools for detailed studies of NK cell function, this analysis yielded several surprises. In contrast to CTL recognition: 1) NK cell receptors are germline encoded and are not strictly "clonotypic" as defined in terms of clonotypic TCRs (unique receptor only expressed by the rare effector clone and its progeny); 2) individual NK cells express both inhibitory and activation receptors for target recognition, and often simultaneously express several different receptors of each type; 3) the receptors are often promiscuous and may have overlapping ligand specificities; and 4) NK-cell receptors specifically bind MHC-I molecules but they are functionally and structurally distinct from other

receptors that bind MHC-I (ie, TCR and CD8). In the following sections, we will discuss NK-cell receptors involved in target recognition by first considering the relationship between target susceptibility to natural killing and expression of MHC-I.

Target Cell Major Histocompatibility Complex-I and Natural Killer Cells: The "Missing-Self" Hypothesis

Whereas initial studies suggested that natural killing was "non-MHC-restricted,"[11] substantial progress in understanding NK-cell recognition began with ascertaining the role of MHC-I molecules in natural killing (Fig. 17.1). Kärre and colleagues discovered that MHC-I–deficient tumors remained susceptible to in vivo rejection, apparently by NK cells.[170] Conversely, target cell expression of MHC-I molecules appeared to have a protective effect against NK-cell–mediated lysis in vitro. A number of methods, such as IFNγ treatment, to upregulate MHC-I correlated with target protection but other effects could not be excluded.[171] There was significant variability in capacity of specific MHC-I molecules to protect targets[172,173]; in vitro culture conditions could influence NK-cell specificities,[174] and the specificities of individual human NK cell clones were not easily assignable to specific MHC-I alleles.[175] Thus, the MHC-I effect on natural killing was controversial for some time.

Several groups, however, observed that MHC-I–expressing parental targets were resistant to natural killing, whereas mutants selected for absence of MHC-I expression became susceptible. The parental (resistant) phenotype could be restored by reconstitution of MHC-I expression by transfected expression of molecules to correct the defect, be it β_2-microglobulin (β2m)[176] or transporter associated with processing (TAP).[177,178] Studies utilizing mice with a targeted mutation in the β2m gene added substantial support to the MHC-I protective effect, as normal expression of MHC-I heavy chains requires β2m.[179,180] β2m-deficient lymphoblasts were susceptible to lysis by normal NK cells. Moreover, β2m−/− BM transplanted into otherwise syngeneic normal hosts was rejected by recipient NK cells.[180,181] These results resembled hybrid resistance whereby NK cells in irradiated F_1 hybrid mice reject parental BM transplants.[182] Hybrid resistance is regulated by parental determinants that are genetically linked to the MHC-I region, H-2D.[183] Thus, in several distinct NK-cell recognition systems, the target cell expression of certain MHC-I molecules correlated with resistance to

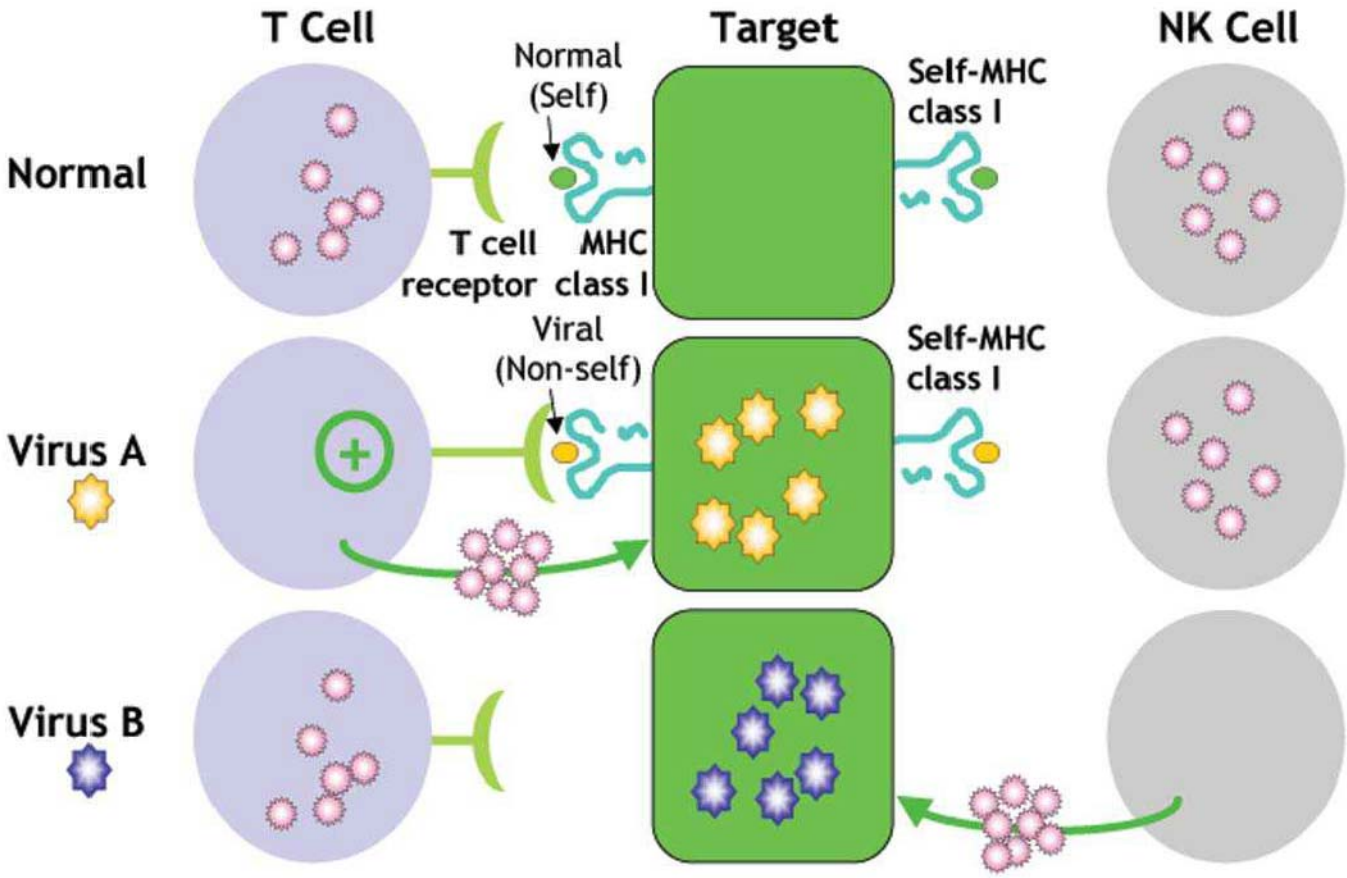

FIG. 17.1. Major Histocompatibility Complex (MHC)-I Expression on Targets is Inversely Related to Natural Killing. Targets expressing MHC-I are more resistant to lysis by natural killer (NK) cells than targets lacking MHC-I expression. This is the exact opposite of the requirements for MHC-I–restricted cytotoxic T-lymphocytes that recognize foreign peptides presented by MHC-I. As depicted, T cells can recognize virus-infected cells, but some viruses may evade T cells by downregulating MHC-I. These infected cells then become more susceptible to NK cells, which generally tend not to discriminate between self- and viral-peptides, though there are some peptide contributions to NK recognition as described in the text.

natural killing whereas absence of MHC-I was associated with susceptibility to NK cells.

NK cells, therefore, have a different relationship to target cell MHC-I molecules than MHC-I–restricted CTLs (see Fig. 17.1). Strictly speaking, NK cell lysis is "non-MHC–restricted,"[11] at least as far as MHC restriction is precisely defined for T cells having a requirement for specific self-MHC molecules presenting a given peptide antigen.[184] However, the term "non-MHC–restricted" (and its synonyms) is now somewhat outdated because it implies, when viewed in a broader sense, that MHC plays no role in NK-cell cytotoxicity. Avoidance of these terms will minimize confusion concerning the relationship of target cell MHC-I molecules with NK-cell specificity.

As initially observed and discussed by Kärre in the "missing-self" hypothesis, the relationship between target expression of MHC-I and resistance to natural killing highlights a fundamental distinction between NK and T cells[185] (see Fig. 17.1). Whereas T cells are triggered by detection of "foreign" epitopes, Kärre proposed that NK cells are equipped to detect the absence of "self" epitopes. The [missing-self] hypothesis suggests that NK cells survey tissues for expression of MHC-I molecules that are normally ubiquitously expressed and that somehow prevent NK-cell activity. If MHC-I molecules are downregulated or mutated, NK cells can then lyse the target. The generally opposite requirements of NK and T cells for target cell MHC-I expression may be physiologically important. Several pathogens, including herpes viruses, possess mechanisms that prevent the normal expression of MHC-I molecules on infected cells, providing means to avoid MHC-I–restricted T cells.[186] Moreover, tumorigenesis is frequently associated with alterations in MHC molecules, either mutation in structural genes or decreased expression, again leading to escape from T-cell surveillance.[187–189] In either case, however, the MHC-I–deficient cells should become more susceptible to natural killing. The host, therefore, is endowed with two components (T and NK cells) with opposing requirements for self–MHC-I expression. This fail-safe system should eliminate pathologic processes that might otherwise evade immune responses by any alteration of MHC-I expression (either increased to avoid NK cells or decreased to avoid T cells). The missing-self hypothesis thus provided a tentative physiologic explanation for MHC-I–associated resistance, creating a framework for initial attempts to define NK-cell recognition of their targets.

Current Principles of Target Recognition by Natural Killer Cells

The MHC-I–associated resistance to natural killing inspired a panoply of models and their variants to explain not only resistance but also natural killing.[172] The *target interference* or *masking* model predicted that a single NK-cell receptor activates natural killing when it engages its putative target cell ligand.[185] MHC-I molecules mask the putative target cell ligand and block its recognition by the NK-cell receptor. This hypothesis was initially favored because it was the simplest.[190] Moreover, it made the most sense if one considered that NK cells should have only one defined receptor analogous to the TCR. The *effector inhibition* or *inhibitory receptor* model suggested that NK cells are inhibited from natural killing by an NK-cell receptor that binds MHC-I on the target and delivers negative signals overriding a default pathway of activation.[185]

Although the target interference model has not been refuted, it is now known that NK cells express inhibitory receptors that physically bind MHC-I in a manner that may be influenced by MHC-bound peptides[191] (Fig. 17.2).

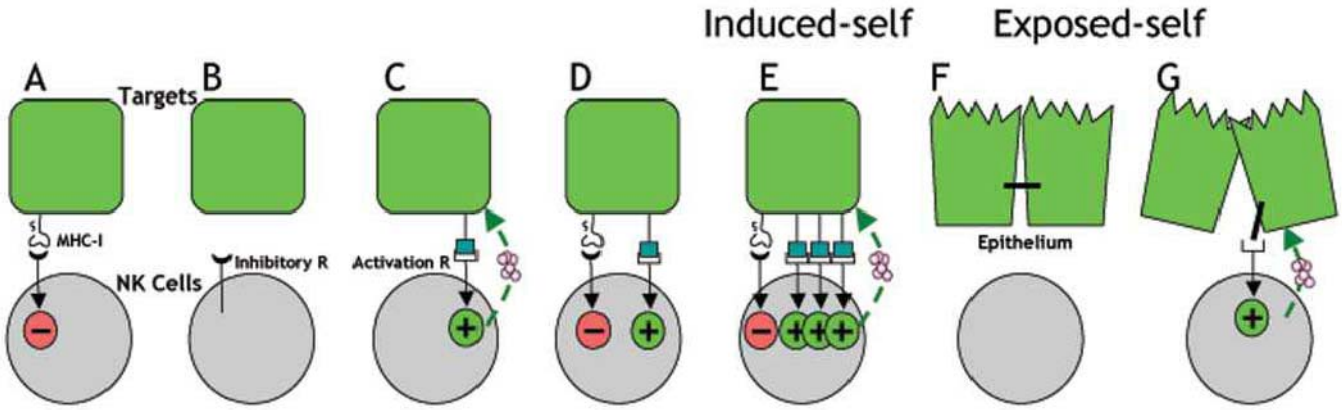

FIG. 17.2. Current Principles of Target Recognition by Natural Killer (NK) Cells. Pictured are several scenarios of interactions between ligands on targets (*top row*) and receptors on NK cells (*bottom row*). Successful activation of NK cells is shown by the *dashed upward line*. **A:** Targets expressing majoe histcompatibility complex (MHC)-I are resistant to lysis by NK cells because of MHC-I–specific inhibitory receptors. **B:** The absence of MHC-I (or lack of receptors specific for target MHC-I, not depicted) does not automatically result in target killing. **C:** Activation receptor engagement is required to trigger target killing in absence of MHC-I. **D:** In the situation where both inhibitory and activation receptors are engaged, the inhibitory receptor effect often dominates and no killing occurs. **E:** In the induced-self model, induced expression of NKG2D ligands can overcome the inhibitory influence of MHC-I, resulting in NK cell activation. **F:** Normal epithelium masks ligands for NK-cell activation receptors at the tight junctions. **G:** Under pathologic situations, the epithelial architecture may be disrupted, leading to ligand exposure. (Not depicted are inhibitory ligands at the epithelial tight junctions that may inhibit NK cells when they transmigrate through epithelial barriers.) NK cells can also recognize pathogen encoded ligands on infected cells (not shown, but similar to *C* or *E*).

All known inhibitory receptors contain cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) consisting of V/I/L/SxYxxL/V (single amino acid code where x is any amino acid).[192] Ligand engagement leads to phosphorylation of the ITIM, which leads to inhibition, traditionally thought to be due to recruitment and activation of intracellular phosphatases, although recent evidence suggests more complexity.

MHC-I inhibitory receptors on NK cells have either of two general structures[193]: 1) C-type lectin-like receptors that are disulfide-linked dimers with type II transmembrane topology (extracellular carboxyl termini). These receptors are encoded in the NK gene complex (NKC) and were first described in mice. 2) Immunoglobulin (Ig)-superfamily receptors that have type I transmembrane orientation. These molecules are encoded in a different genetic region, termed the leukocyte receptor complex (LRC), and were first described in humans. Although ongoing studies indicate that both structural types of receptors are expressed on mouse and human NK cells, the lectin-like receptors (Ly49 receptors) are the major MHC-specific inhibitory receptors in mouse whereas the Ig-like receptors (killer Ig–like receptors [KIRs]) predominate in human.

The absence of MHC-I does not always result in killing, indicating that release from inhibition does not result in activation by default (Fig. 17.2B). Instead, it was suggested that NK cells express two functionally different receptors for target cell ligands.[194,195] In this *two receptor model*, one receptor triggers activation upon ligand binding (Fig. 17.2C) whereas the MHC-I–specific receptor inhibits activation by negative signaling. In many circumstances, the inhibitory receptor effect dominates over the activation receptor (Fig. 17.2D), but the outcome usually reflects the integration of signals from both types of receptors, which can be affected by ligand expression or affinities (not shown).

Many, but not all, NK-cell activation receptors are encoded in the NKC and LRC, having similar structural properties as their inhibitory receptor counterparts except for absence of cytoplasmic ITIMs. The activation receptors typically do not have signaling motifs in their cytoplasmic domains but contain charged transmembrane residues that facilitate association with reciprocally charged residues in the transmembrane domains of signaling chains having immunoreceptor tyrosine-based activation motifs (ITAMs) analogous to ITAMs in TCR and B-cell receptor (BCR) complexes (D/ExxYxxL/Ix_{6-8}YxxL/I). NK cells express three ITAM-containing signaling chains: CD3ζ, FcεRIγ, and DAP12 (DNAX associated protein of 12 kDa, also known as killer activating receptor–associated protein, KARAP; Ly83; tyrosine kinase binding protein, Tyrobp). NK cells also express DAP10 (hematopoietic cell signal transducer, Hcst) that lacks ITAMs and instead contains a motif for recruitment of phosphatidylinositol 3-kinase (PI3K) and Grb2. The signaling chains typically provide two major functions: facilitate cell surface expression of the associated activation receptor, and transduce signals.

To date, the ligands for activation receptors fall into several major groups. One group is encoded by the host and is expressed normally. Presumably, NK-cell attack against cells expressing these ligands is limited by inhibitory receptors (Fig. 17.2D). Another group of ligands is characterized by their relatively low expression on normal tissues and induced expression under "stress" conditions (Fig. 17.2E). Other ligands become exposed when tissue architecture is altered (Fig. 17.2F,G). Because the ligands are encoded in the normal host genome, they would be recognized by the NK cell as indicators of pathologic conditions, either as "induced-self" or "exposed-self," respectively. Another group of ligands is found on infected cells and is encoded by the pathogen (not shown but similar to Fig. 17.2C or E).

Finally, many other NK-cell receptors have been discovered that do not fall neatly into the categories described here. Some appear to have similar inhibitory function as the MHC-specific inhibitory receptors but bind non-MHC ligands, strongly suggesting MHC-independent self-recognition. The function of these and other receptors remains under intense investigation.

NATURAL KILLER CELL RECEPTORS

In the following sections, we will describe the major receptors on NK cells in detail by first discussing the MHC-specific inhibitory receptors that helped elucidate NK recognition paradigms before delving into MHC-independent inhibitory receptors, activation receptors, and other receptors found on NK cells. Given the large number of receptors now identified (Table 17.1), this section will primarily discuss work on the receptors that have been studied most extensively. This summary will illustrate the experimental approaches that led to identification of these receptors, their features, and outline general principles applicable for study of other receptors that will not be discussed in detail due to space constraints. Nonetheless, description of the major activation receptors and their ligands help illustrate and provide molecular handles on the various functions of NK cells.

Inhibitory Natural Killer-Cell Receptors Specific for Major Histocompatibility Complex–I Molecules

The mouse and human MHC-specific inhibitory receptors are remarkably different in protein structure. Each will be discussed separately.

Mouse Ly49

The Ly49A receptor was the first inhibitory MHC-I–specific NK-cell receptor to be described in molecular terms.[191,196] Ly49A was originally identified as a molecule of unknown function on a T-cell tumor.[197,198] It is a disulfide-linked homodimer (44 kDa subunits) with type II membrane orientation, and C-type lectin superfamily homology.[199,200] Previously termed Ly49, it is now appreciated that Ly49A (Klra1) belongs to a family of highly related molecules.[195,201–203] Indeed, genetic analysis revealed that the genes for Ly49A and NK1.1 are linked in the NKC (Fig. 17.3), leading to studies indicating that Ly49A is constitutively expressed on a distinct subpopulation (20%) of NK cells in C57BL/6 mice.[203]

TABLE 17.1 The Panoply of Receptors Expressed by Natural Killer Cells[a]

Receptor	H	M	Inhibitory (I) or Activation (A)	Other Names	Ligand
Ly49A		X	I	Ly49, Ly-49, Klra1	$H2D^d$, D^k, D^p, alleles in $H2^r$, and $H2^q$
Ly49C		X	I	Klra3	$H2K^b$, numerous
Ly49E		X	I	Klra5	Urokinase plasminogen activator
Ly49G2		X	I	LGL-1, Klra7	$H2D^d$
$Ly49I^{129}$		X	I	Klra9	m157
Ly49Q		X	I	Klra17	$H2K^b$
KIR2DL1	X		I	CD158a, p58.1, EB6	HLA-C2 (HLA-Cw2, Cw4, -Cw5, -Cw6)
KIR2DL2/KIR2DL3	X		I	CD158b, p58.2, GL183	HLA-C1 (HLA-Cw1, -Cw3, -Cw7, -Cw8)
KIR2DL4	X		I	CD158d	HLA-G
KIR3DL1	X		I	CD185e1, NKB1, p70, NKAT3	Bw4 (HLA-A and B)
KIR3DL2	X		I	CD158k, p140, NKAT4	HLA-A3, -A11
Lilrb4		X	I	gp49	$\alpha v\beta 3$ integrin
CD94/NKG2A	X		I	Kp43	HLA-E
		X	I		Qa-1
LILRB1	X		I	CD85j, ILT2, LIR1	Folded HLA, UL18
LILRB2	X		I	ILT4, LIR2	Folded, free HLA
LAIR-1	X		I		Collagen
Siglec-7	X		I	P75, AIRM1	Carbohydrates
Siglec-10	X		I		Carbohydrates?
Siglec-E		X	I		?
PILRa	X		I		CD99
PILRb	X		I		CD99
FcγRIII	X	X	A	CD16	Fc of IgG
Ly49D		X	A		Chinese hamster MHC-I, $H2D^d$
Ly49H		X	A		m157
$Ly49P^{MA/My}$		X	A		M04 + $H2D^k$
KIR2DS1	X		A	CD158h, p50.1	HLA-Cw7
KIR2DS2	X		A	CD158j, NKAT5, p50.2, clone 49	
KIR2DS3	X		A	NKAT7	
KIR2DS4	X		A	CD158i, NKAT8, clone 39	HLA-Cw4
KIR2DS5	X		A	CD158g, NKAT9	
KIR3DS1	X		A	CD158e2	
CD94/NKG2C	X		A		HLA-E
CD94/NKG2C		X	A		Qa-1
NKG2D	X		A, costimulation	KLRK1	MICA, MICB, ULBP/RAET1
NKG2D		X	A, costimulation	Klrk1	H60, RAE1, MULT1
Nkrp1c		X	A	NK1.1	?
Nkrp1b(d)		X	I		Clrb
Nkrp1f		X	A?		Clrg
NKRP1A	X		I		LLT1
NKp80	X		A		AICL
NKp65	X		A	KLRF2	CLEC2A
2B4	X	X	A,I	SLAMF4	CD48
CD2	X	X	A		CD48
NTBA	X	X	A	SLAMF6, Ly108	NTBA
Ly9	X	X	A	SLAMF3	Ly9
CD84	X	X	A	SLAMF5	CD84
CRACC	X	X	A	SLAMF7	CRACC
NKp46	X	X	A		Hemagglutinin
NKp44	X		A		?

(continued)

TABLE 17.1 The Panoply of Receptors Expressed by Natural Killer Cells[a] *(Cont.)*

Receptor	H	M	Inhibitory (I) or Activation (A)	Other Names	Ligand
NKp30	X		A		B7-H6
CD69	X	X	A		?
Ly6		X	A		?
Gp42		Rat	A		?
Klrg1		X	I		Caherins
CEACAM1	X		I	CD66a	CEA
CD226	X	X	A	DNAM-1	necl-5 (CD155, PVR), nectin-2 (CD112, PVRL2)
CD96	X		A	Tactile	necl-5
CRTAM	X		A		necl-2
TIGIT	X		I		PVR, PVRL2

AICL, activation-induced C-type lectin; AIRM1, adhesion inhibitory receptor 1; CRTAM, class I-restricted T-cell–associated molecule; LGL, large granular lymphocyte; NK, natural killer; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain.
[a]These are the major receptors discovered on NK cells in humans and mice, listed in order of appearance in the text.

Multiple lines of evidence indicate that Ly49A is an inhibitory receptor specific for MHC-I, particularly $H2D^d$: 1) Functional analysis: the Ly49A+ NK-cell subset were equivalent to Ly49A– NK cells in killing several targets, but they could not lyse a large panel of targets that were readily lysed by Ly49A– NK cells. This phenotype was related to MHC-I expression of certain H2 haplotypes on target cells.[191,204,205] Transfected expression of $H\text{-}2D^d$ selectively rendered a susceptible target resistant to natural killing by Ly49A+ NK cells. Moreover, killing through disparate stimuli by Ly49A+ NK cells was also inhibited. 2) Cell binding: Ly49A+ tumor cells bound specifically to immobilized MHC-I molecules[206] and to $H\text{-}2D^d$-transfectants.[207] 3) Antibody blocking: $F(ab')_2$ fragments of mAb directed against either Ly49A or the $\alpha1/\alpha2$ (but not the $\alpha3$ domain) of $H\text{-}2D^d$ reversed resistance in killing experiments (permitted lysis) and blocked the cell binding assay.[191,204,206,207] 4) In vivo expression: the apparent level of Ly49A expressed per NK cell was downregulated in MHC congenic and Tg mice expressing $H\text{-}2D^d$.[208–210] This was not due to negative selection because the percentage of Ly49A+ NK cells was unchanged. 5) Gene transfer: primary NK cells and T cells expressing a Ly49A transgene and a Ly49A-transfected NK cell line were specifically inhibited by $H\text{-}2D^d$.[211,212] 6) Inhibition by Ly49A is ITIM-dependent, based on gene transfer of mutant Ly49A molecules.[212] 7) $H2D^d$ tetramers bind Ly49A transfectants.[213] 8) Ly49A tetramers bind $H2D^d$ on transfected cells.[214,215] 9) Biophysical studies: recombinant Ly49A binds recombinant $H2D^d$ in surface plasmon resonance (SPR) studies with K_D = ~2.0 μM.[216]

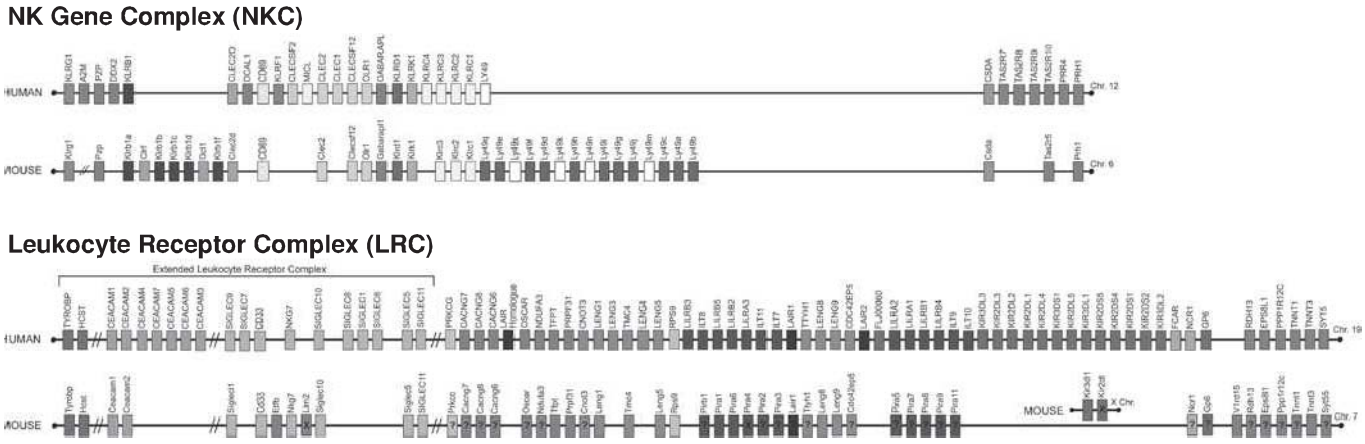

FIG. 17.3. The Genomic Organization of the Natural Killer (NK) Gene Complex and Leukocyte Receptor Complex in Humans and Mice. The figures are not drawn to scale with precise gene locations being modified as new sequence information becomes available (genome.ucsc.edu/ and www.ebi.ac.uk/ipd/kir/). The *grey shading* is coordinated to represent related genes. *Question marks* indicate genes whose precise location is not known. An "*X*" indicates genes not homologous to other aligned genes. *Double slashes* represent large genomic distances. Note that most but not all genes are expressed on NK cells. Many remain orphan genes because the functions of their gene products have not been determined. Modified from Kelley et al.[312]

10) Crystallography: the structure of Ly49A complexed with $H2D^d$ was determined.[217] 11) Less extensive studies also indicate that Ly49A recognizes $H\text{-}2D^k$, $H2D^p$, and alleles in $H2^r$ and $H2^q$.[191,208,213,218,219] Therefore, Ly49A is an MHC-I–specific receptor for $H\text{-}2D^d$, $H\text{-}2D^k$, and $H2D^p$, and alleles in $H2^r$ and $H2^q$.

The nature of the Ly49A interaction with MHC-I, however, is fundamentally different from TCR/MHC-I interactions because the former appears to be relatively independent of the specific peptide bound by $H2D^d$.[220,221] However, bound peptides are required for appropriately folded MHC-I molecules that can be recognized. Despite its structural homology to C-type lectins that are carbohydrate-binding proteins,[222,223] Ly49A does not have the residues for coordinate binding to calcium that is required for lectin binding. Moreover, Ly49A binding to its MHC ligands is not carbohydrate-dependent based on functional, SPR, and crystallographic analyses.[216,224] In the crystallographic structure of Ly49A complexed to $H2D^d$ (2.3Å resolution), the lectin-like structure of Ly49A was confirmed[217] (Fig. 17.4). Two interaction sites were seen between the lectin-like domain of Ly49A and $H2D^d$: site 1 involved the "left" side of the peptide-binding cleft of $H2D^d$ and a wedge-like site 2 involved the undersurface of the peptide-binding cleft. The residues in Ly49A involved in binding either ligand site are overlapping. Mutational analysis revealed that Ly49A binds site 2 where it contacts α1, α2, and α3 of $H2D^d$ and β2m.[214,216,225] This site is near Asn80, an Asn-linked glycosylation site conserved in all MHC-I molecules, leaving open the issue of whether carbohydrates could affect the interaction, such as affinities or kinetic parameters, but this has not been studied in depth. These studies also provide a structural explanation for species-specific β2m requirements as revealed by functional studies.[226] Thus, Ly49A recognizes site 2 in the MHC molecule in terms of *trans* recognition between the NK-cell receptor and target cell MHC-I molecule.

Most but not all other Ly49 receptors are MHC-I–specific inhibitory receptors. First noted by Southern blot analysis and cDNA cloning, genome sequence analysis revealed 15 complete Ly49 genes in C57BL/6 mice.[199–202,227] There is evidence for alternative splicing and alternative transcriptional start sites for the Ly49 genes, though their importance has not been elucidated.[202,228,229] Notably, Ly49C has broad specificity for H2 alleles, as revealed by tetramer staining, and is the only known inhibitory NK-cell receptor specific for an $H2^b$ haplotype allele ($H2K^b$) in C57BL/6 mice, notwithstanding unconfirmed reports that Ly49I also binds $H2K^b$.[168,213,230] Ly49C is recognized by two mAbs: 5E6, which also binds Ly49I, and 4LO3311, which has exquisite specificity for Ly49C.[231–233] X-ray crystallographic studies have indicated that Ly49C binds $H2K^b$ in a manner similar to Ly49A interaction with $H2D^d$ (see Fig. 17.4).[234,235] Only a site 2 interaction was seen with the contact residues, showing a similar but distinct topology to the Ly49A-$H2D^d$ interaction. Interestingly, however, peptides bound to $H2K^b$ clearly affect functional interactions with Ly49C[236] and affinities as measured by SPR.[234] However, Ly49C does not directly engage the peptide, indicating long-range effects. Finally, both Ly49A and Ly49C

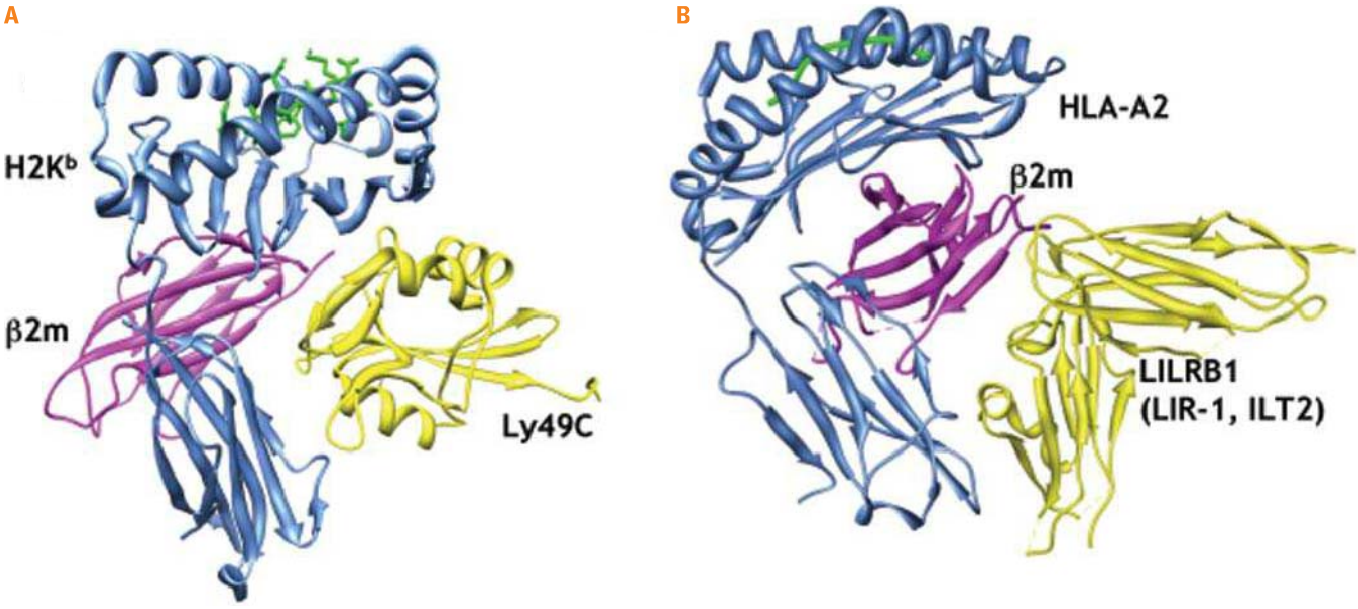

FIG. 17.4. Crystal Structures of Natural Killer (NK)-Cell Receptors in Complex with Their Ligands. **A:** Mouse Ly49C bound to $H2K^b$ at site 2 (PDB ID = 3C8K).[193,235] Ly49A interaction with site 2 of $H2D^d$ is very similar.[170] Site 1 of Ly49A-$H2D^d$ interaction is approximately located where the $H2K^b$ label is placed. **B:** Human LILRB1 (LIR1, ILT2) bound to human leukocyte antigen-A2 (PDB ID = 1P7Q).[266] Figures were produced using the University of California, San Francisco Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (www.cgl.ucsf.edu/chimera).[1030] The structures are viewed from the side with the NK cell positioned at the top of the figure and the target cell surface at the bottom. The major histocompatibility complex molecules are oriented similarly.

appear to undergo conformational changes upon ligand binding, and a Ly49 dimer can engage two MHC-I molecules.[235,237] Thus, Ly49 receptors bind their MHC ligands in a structurally related manner.

Recent studies suggest that Ly49 molecules also can bind MHC in a *cis* interaction between receptor and ligand on the NK cell itself.[168,238] For example, an MHC ligand for Ly49A or Ly49C on the same cell prevents binding of MHC tetramer.[168,238] If the cells are briefly exposed to mild acidic conditions, MHC-I expression is lost (due to disruption of the noncovalently linked MHC-I heterotrimer), and Ly49A binding to cognate MHC-tetramers is restored. This *cis* interaction is dependent on site 2 residues in the MHC molecule. These findings may help explain the observation that the presence of self-MHC ligands leads to downregulation of Ly49 expression, as previously noted on primary NK cells in MHC congenic mice.[208–210] *Cis* interactions may also explain functional differences in NK cells in MHC-congenic mice or NK cells that do or do not express MHC ligands in Tg mice that are mosaic for MHC expression.[239–241] Finally, there are biophysical data supporting a role for the relatively long, flexible stalk region of Ly49 receptors in allowing either *trans* or *cis* interactions.[242] At the moment, the physiologic importance of *cis* interactions is incompletely understood but may be relevant to NK-cell tolerance and education, as discussed below.

Although they have not been studied as extensively as Ly49A and Ly49C, other inhibitory Ly49 receptors and their ligands have been identified[202,230] and characterized with other MHC allele specificities, as detailed in a recent review.[243] Moreover, they are structurally related.[235,244] Individual NK cells may express multiple Ly49 receptors simultaneously,[210,233,245] often (but not always) two or more, suggesting that individual NK cells may be inhibited by more than one MHC-I molecule.

Ontogenetic studies demonstrate that the total repertoire of Ly49 expression does not reach adult levels until sometime after 3 weeks of age, concomitant with attainment of full NK-cell cytolytic activity.[246] Thereafter, the expression of Ly49 receptors is generally thought to be fixed and stable on an individual NK cell. Ly49E is expressed only on fetal NK cells, but NK cells in mice deficient in Ly49E are otherwise normal.[247] In adult mice, developmental studies indicate that Ly49 receptors are first expressed on immature NK cells in the BM, before a phase of constitutive proliferation[98] that appears to be modestly affected by MHC haplotype.[168] There are only modest effects on the final "repertoire" of MHC-specific receptors expressed by splenic NK cells in different MHC environments.[210,248]

The expression of inhibitory Ly49 receptors appears to occur in a stochastic manner. There is evidence for monoallelic expression of Ly49 receptors (expression from one chromosome), initially described as "allelic exclusion," a term that has fallen out of favor because it has a specific meaning and mechanism for TCRs and BCRs.[249] At least some Ly49 genes possess bidirectional, overlapping promoters directed in opposite orientations.[250] Transcription factors driving transcription in one direction prevent binding of other factors driving transcription in the opposite direction. Directionality and monoallelic expression may also be controlled by DNA methylation.[251] A "probabilistic" model has been proposed to explain these findings that may also explain the stochastic expression of Ly49 genes and their stable expression. However, recent studies of *Ly49* indicate highly variable transcriptional start sites, suggesting that the probabilistic model may not be correct.[229] Other data indicate that TCF-1 but not LEF-1 in the T-cell factor/lymphoid enhancer family of DNA-binding proteins affects some but not all Ly49 receptor expression.[252–254] Thus, the elements controlling Ly49 gene expression are incompletely understood.

Analysis of the Ly49 receptors thus far is largely based on examination of the C57BL/6 alleles, but the Ly49 receptors display extensive polymorphism. The Ly49 family is encoded in the NKC located on mouse chromosome 6 with the syntenic human region being chromosome 12p13.2[195,203,255,256] (see Fig. 17.3). While the NKC also contains genes for other lectin-like receptors, the Ly49 genes are clustered with the exception of *Ly49b*. Corresponding to restriction fragment length polymorphic (RFLP) variants originally detected with the Ly49A cDNA,[203] there is significant allelic polymorphism of the Ly49 cluster between inbred mouse strains with differences in gene number as well as alleles for the *Ly49* genes.[227,257–259] In contrast to C57BL/6J mice, genomic sequence analysis shows 8 putative *Ly49* genes in BALB/c mice, 19 in 129 mice (of which at least 9 appear to be pseudogenes) and 22 in NOD mice. Array-based comparative genomic hybridization analysis of 21 mouse strains compared to the reference C57BL/6J strain indicated that these mice could be grouped into five clusters that correspond to or are predictive of restriction fragment length polymorphic patterns on Southern blot analysis.[203,260] There are also multiple alleles for individual *Ly49* family members.[227,249,257,258,261] Thus, there is significant polymorphism of the *Ly49* molecules at both the haplotype (gene numbers) and individual gene (alleles) levels, not unexpected because the Ly49 molecules bind highly polymorphic MHC-I molecules.

The MHC-I specificities have generally been well characterized for only a few Ly49 alleles. Interestingly, mAbs specific for one Ly49 allele may bind another molecule with a different function or specificity in another mouse strain,[262–264] similar to what was recognized for mAb reactivity with different MHC alleles.[265] Thus, the polymorphisms also raise practical issues when studying Ly49 molecules in different mouse strains.

Finally, it should be noted that Ly49 receptors may be expressed by other cells; some are selectively expressed on non-NK cells and some may have specificities for non-MHC ligands. NKT and other T-cell subsets may express Ly49 receptors but they have not been thoroughly studied.[266,267] Ly49B and Ly49Q are not expressed on NK cells; rather, they are expressed on myeloid cells.[268,269] Interestingly, Ly49Q recognizes $H2K^b$ and positively regulates TLR signaling.[270,271] On the other hand, Ly49E appears to recognize urokinase plasminogen activator, though physical binding has not been established.[272] Interestingly, Ly49B, Ly49E, and Ly49Q are predicted to be distinct in fine structure from the

known MHC-I–specific Ly49 receptors on NK cells.[235] Thus, while Ly49 receptors are predominantly NK-cell inhibitory receptors for MHC class I, they may have other roles that are less well understood; still other Ly49 receptors have activation function, as discussed subsequently.

Human Killer Immunoglobulin-like Receptors

In contrast to mouse NK cells, human NK cells can be cloned by limiting dilution in the presence of irradiated feeder cells, phytohemagglutinin, and IL-2, leading to establishment of short-term NK-cell clones that have differences in target killing and surface molecules. mAbs were isolated that reacted specifically with these clones; reactivity correlated with the capacity of the clones to kill certain tumors, and the mAbs affected cytotoxicity. This general approach led to the identification of the human NK-cell receptors.

A series of studies[273–275] showed that the mAbs GL183 and EB6 identify serologically distinct 55 kDa or 58 kDa molecules, initially termed p58. These molecules had several features: 1) selective expression on overlapping NK cell subsets; 2) expression on NK cell clones correlated with expression of certain human leukocyte antigen (HLA) class I alleles on resistant targets; 3) a target susceptible to a given NK-cell clone bearing p58 molecules reactive with either mAb was made resistant by transfection of cDNAs encoding certain HLA-C molecules; 4) the otherwise resistant, HLA-C–transfected targets could be lysed in the presence of the appropriate anti-p58 mAbs. The mAb effect occurred with $F(ab')_2$ fragments, suggesting that the interaction between p58 and an HLA class I molecule on the target cell inhibits the NK cell. Thus, the p58 molecules displayed features consistent with a role as inhibitory human NK-cell receptors specific for MHC-I, analogous to the mouse Ly49A receptor that was being studied in parallel, as described previously.

Other studies noted that NK-cell specificity was skewed when the NK cells were grown in the presence of cells bearing allo-MHC determinants.[276,277] This specificity correlated with reactivities that mapped to paired residues at position 77 and 80 in the .α1 domain of HLA-C. All known HLA-C molecules could be divided into two groups, one with Asn77-Lys80 (HLA-Cw2, -Cw4, -Cw5, -Cw6) and the other with Ser77-Asn80 (HLA-Cw1, -Cw3, -Cw7, -Cw8). Indeed, transfection analysis showed that p58 specificity for HLA-C molecules was related to expression of the EB6 epitope for the former (specificity 1, now termed HLA-C1), whereas the latter was related to the GL183 epitope (specificity 2, HLA-C2) on the NK-cell clones.[276,278,279] Thus, human NK-cell receptors showed promiscuous specificity that was dependent on residues 77 and 80 in HLA-C.

The NKB1 (p70) molecule was serologically similar to p58 molecules with regard to subset expression, and correlation of expression on NK-cell clones to specificity for HLA class I.[280,281] In contrast to p58 molecules, however, NKB1 had a distinct M_r (70 kDa) and specificity for HLA-B. The NKB1+ clones were specifically inhibited by targets expressing transfected HLA-Bw4 molecules, and the anti-NKB1 mAb reversed the inhibition. Analysis of informative HLA-B alleles showed that this specificity was conferred by a region in the α1 domain overlapping the area on HLA-C recognized by p58 molecules.[282] Finally, HLA-A3, -A11–specific receptors have similar properties to p58 and NKB1 except that they appear to be disulfide-linked dimers termed p140,[283] whereas others have found that a monomeric HLA-A3–specific receptor resembles NKB1.[284] Thus, representative alleles of all classical HLA class I loci are capable of inhibiting NK cells through p58/NKB1/p140 receptors although HLA-B and -C alleles dominate human NK-cell specificities, and it is not yet known if there are receptors reactive with each HLA allele.

When the cDNAs for the p58 and NKB1 molecules were cloned, they were surprisingly found to encode type I integral membrane proteins with Ig-like domains[285–287] unlike the lectin-like Ly49 family of type II receptors. The Ig-like receptors are now collectively known as killer Iq-like receptors (KIRs) or CD158.[288,289] The KIR nomenclature is based on whether the receptor has two or three Ig-like external domains (KIR2D or KIR3D, respectively), and possession of a long (L) or short (S) cytoplasmic domain. In general, the L forms are inhibitory because they contain ITIMs, whereas the S forms appear to be activation receptors (see following discussion). Each distinct receptor is also designated by a number. The KIR2DL1 (CD158a, p58.1) molecule bears the original EB6 epitope and is specific for HLA-C (Lys80, specificity 2), whereas KIR2DL2 (CD158b1, p58.2) and KIR2DL3 (CD158b2, p58) have the GL183 epitope and are specific for HLA-C (Asn80, specificity 1). (As detailed in the following, structural analysis supports grouping of HLA-C alleles into two mutually exclusive groups, HLA-C1 and HLA-C2, based on direct interaction of KIR2DL2/3 and KIR2DL1, respectively, with residue 80 of HLA-C, validating original functional groupings but simplifying HLA-C groupings to just residue 80.[276,277]) KIR2DL4 (CD158d, p49) reportedly binds HLA-G[290] but displays both inhibitory and activation functions.[291–293] KIR3DL1 (CD158e1, NKB1, NKAT3) is specific for HLA-A and HLA-B molecules with the Bw4 epitope.[282] KIR3DL2 (CD158k, p140, NKAT4) has HLA-A3 and HLA-A11 specificity.[283,284]

There is unequivocal evidence that the KIR2DL and KIR3DL molecules are inhibitory HLA class I–specific receptors. In addition to the data with NK cell clones and mAbs mentioned previously, the following have been described: 1) KIR bind directly to HLA class I: soluble KIR2DL-Fc fusion proteins bind cells expressing the appropriate transfected HLA class I alleles.[294,295] In addition, a soluble KIR2DL molecule containing only the extracellular domain binds specifically to its HLA-C ligand in solution.[296] 2) Gene transfer of KIR: KIR2DL specificity and inhibitory function were transferred when KIR2DL cDNAs were transiently expressed with vaccinia constructs in human NK-cell clones.[294] Similarly, Tg expression of KIR2DL2 in mice conferred inhibition of rejection of BM expressing Tg HLA-Cw3.[297] 3) SPR measurements indicate that the KIRs bind their HLA ligands with $K_d = \sim 10$ μM.[298–301] Binding is affected by peptide bound by HLA molecule.[300,301] Through histidine-rich domains, the KIRs bind Zn++, which affects KIR multimerization and binding kinetics to HLA ligands.[302,303] 4) Crystallographic studies dem-

onstrate KIR2DL1 (2.8 Å resolution) and KIR2DL2 (3.0 Å resolution) interactions with their cognate HLA ligands.[299,304] Thus, KIR molecules are clearly MHC-I–specific inhibitory receptors on NK cells.

Interestingly, structural studies indicate that KIR molecules bind HLA class I molecules in a manner analogous to recognition of MHC by TCRs (Fig. 17.5). In particular, both KIR2DL1 and KIR2DL2 use surface loops near their interdomain hinge regions to bind their cognate HLA-C ligands (Cw4 and Cw3, respectively) with a footprint overlying the "right" side of the peptide-binding cleft (when viewed from the "top" in standard depictions of MHC-I molecules).[299,304] The receptors bind both α1 and α2 helices with interactions between KIR2DL1 and Lys80 of HLA-Cw4 and between KIR2DL2 and Asn80 of HLA-Cw3. These interactions with residue 80 of the HLA-C molecules were lost when the reciprocal residues were swapped, accounting for the previously described HLA-C groupings and KIR specificities in functional studies[276,277] and mutational analysis indicating that residue 80 is more significant for KIR interaction than residue 77.[305–308] Although neither KIR2DL molecule has extensive contacts with peptides bound to HLA-C, KIR2DL interactions with HLA-C imposes physical constraints on the p8 position of the peptide. This may account for observed peptide preferences in functional studies and antagonism of certain peptides on KIR inhibitory function.[309,310] A recent structure (1.8 Å resolution) of KIR3DL1 complexed to HLA-B*5701 revealed a similar recognition strategy.[301] Thus, KIRs and Ly49s bind their MHC ligands in markedly different ways, despite their analogous functions as MHC-specific inhibitory receptors.

Also unlike the Ly49s, the KIRs are encoded in the LRC on human chromosome 19q13.4 that encodes many other Ig-like receptors (see Fig. 17.3)[311]; the KIR genes are clustered toward the telomeric end of the LRC.[312] Interestingly, the mouse LRC on chromosome 7qA1 does not include genes for KIR-like molecules that instead are encoded on the X chromosome.[313,314] On the other hand, like the Ly49s, the KIRs display remarkable polymorphism with at least 11 genes[315–317] (see www.ebi.ac.uk/ipd/kir/index.html for updated database).

The human *KIR* locus also demonstrates considerable haplotype diversity with at least 27 different haplotypes,[315,316,318] recently defined at the sequence level.[317] While there has not been a consensus definition, two major types of haplotypes have been described.[289] Group B contains one or more of the following: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1*, whereas group A haplotypes have none of these genes. Reflecting extensive allelic polymorphism of individual genes, a large number of different KIR genotypes have been described, and they are distributed differently in the various ethnic populations.

As with the Ly49s, only a few KIR alleles have been well characterized with respect to HLA class I specificities. Nonetheless, these genetic variants have not only provided clues to new receptors and ligand specificities but also valuable links to the role of NK cells and their receptors in

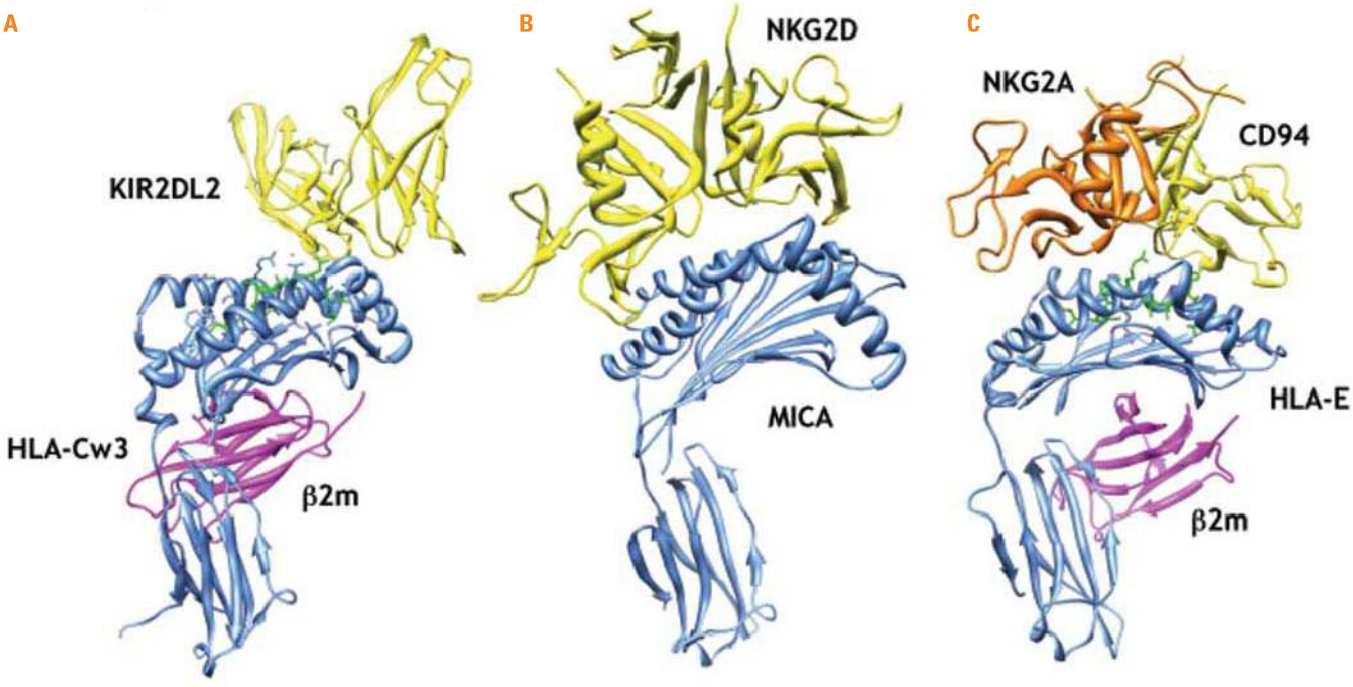

FIG. 17.5. Additional Structures of Natural Killer–Cell Receptors in Complex with Their Ligands. **A:** Human KIR2DL2 with HLA-Cw3 (PDB ID = 1EFX).[236] Two KIR molecules are apparent in the crystal structure with only one molecule (killer Ig–like receptor [KIR] A) contacting the human leukocyte antigen (HLA) molecule. In this view, KIR B molecule is not shown. **B:** Human NKG2D with MICA (PDB ID = 1HYR).[412] **C:** Human CD94/NKG2A with HLA-E (PDB ID=3CDG).[382] The figures were produced and oriented as described in Figure 17.4.

disease pathogenesis (discussed in the subsequent clinical section), and more broadly, human evolution.

Convergent Evolution of Major Histocompatibility Complex–Specific Natural Killer-Cell Receptors

Despite the controversy surrounding the initial cloning of mouse Ly49s and human KIRs and leukocyte Ig-like receptors (LILRs), additional data have provided new interpretations of the distinctly different receptors used by mouse and human NK cells, respectively, to recognize MHC-I. Detailed genome sequence information is available on the NKC and LRC in mice, humans, and other species. In the mouse, MHC-specific NK-cell receptors with Ig-like domains have not yet been described, although there is conservation of several genes in the broader LRC on mouse chromosome 7[312] (see Fig. 17.3). Activated mouse NK cells do express gp49b (Lilrb4), an Ig-like inhibitory receptor also expressed on other leukocytes, including mast cells.[319–322] However, it is not expressed by resting NK cells and is not specific for MHC-I. Instead, it binds the integrin αvβ3 and appears more important for responses of other cells, such as neutrophil, eosinophil, and DCs.[323–328] Mouse *Kir3dl1* is on the X chromosome (see Fig. 17.3) and expressed in NK and T cells, but its function and ligand remain unknown.[313,314] The Ly49 locus in humans consists only of *LY49L* (*KLRA1*) that is a pseudogene because of a point mutation that gives rise to a splicing abnormality.[329] Thus, current data indicate that mouse NK cells do not express functional KIR orthologues while human NK cells do not express functional Ly49 orthologues.

One reason for this discrepancy may be that the corresponding orthologue is present in the genome but has not been identified. Indeed, genomic sequencing has revealed a multitude of candidate orphan receptors in the genome and specifically the LRC and NKC that have yet to be studied carefully.[311,330] In that regard, identification and functional analyses of NK cell receptors led to the recognition that they are encoded in genomic regions containing gene clusters for related receptors that are expressed on other leukocytes, not just NK cells. Dissection of the expression and function of these receptors is a rich area of research that is beyond the scope of this chapter.

The alternative and currently favored view for the discrepancy is that mice and humans independently evolved analogous receptors to serve the same function. While both human and mouse NK cells express a conserved relatively nonpolymorphic lectin-like receptor, CD94/NKG2, it does not possess many of the features that are shared by mouse Ly49 and human KIRs:

1) both Ly49s and KIRs are constitutively and selectively expressed on naïve, unstimulated NK cells (with exceptions for rare populations of T cells);
2) both bind MHC-I molecules with intermediate affinity (K_D = ~2-10 μM);
3) binding to MHC is promiscuous;
4) MHC-bound peptides have only a modest effect, if at all, on recognition;
5) both use ITIMs to inhibit NK-cell activation;
6) they are expressed in a stochastic fashion on overlapping subsets of NK cells;
7) a single NK cell simultaneously expresses one or more of either type of inhibitory receptor (each may be functional);
8) once they are expressed, their expression appears to be stable;
9) both are germ-line encoded by small families of genes that are clustered in the genome;
10) both display impressive polymorphism, in terms of gene number and alleles for each gene;
11) both are related to molecules that lack ITIMs and instead are activation receptors; and
12) both are involved in NK-cell education by self-MHC-I (see following discussion).

Thus, the mouse Ly49 receptors and human KIRs are analogous receptors in an apparently striking example of convergent evolution,[331] whereby each species came up with a different genetic solution to provide extremely important functions for species reproduction and survival.

In other species, *Ly49* and *KIR* genes have been analyzed primarily with respect to sequence and gene number.[332] For example, the *LY49L* gene in baboons appears to be functional but the putative polypeptide lacks an ITIM.[333] In rats, the *Ly49* cluster appears to have markedly expanded with at least 25 genes,[334,335] demonstrating one of the most rapid rates of gene expansion.[336] Dog, cat, and pig appear to have only one *Ly49*, whereas horse represents the only known nonrodent mammal with several *Ly49* genes.[337] The chicken genome has several lectin-like receptor genes that are genetically linked to the MHC.[338–340] On the other hand, multiple KIR genes have been described in primates and cattle.[341–344] Rhesus macaques have a profound plasticity of KIRs with multiple genotypes and haplotypes[345] encoding receptors that bind MHC-I.[346,347] In rat, a KIR-like sequence has been reported,[314] and dogs and cats lack functional KIRs.[348] Interestingly, pigs and marine carnivores each possess a single Ly49 and KIR gene, but it is not clear if these are functional.[348] Finally, several species have no readily identifiable Ly49 or KIR genes[332] (eg, teleost fish instead possess a large number of novel immune-type receptors with sequence homology to mammalian LRC-encoded receptors[349]). Perhaps these species have had alternative convergent evolutionary stratetgies to preserve inhibitory MHC-I–specific receptors.

Additional studies of the Ly49-like and KIR-like molecules as well as potential new orthologues in other species will be of interest to evolutionary biologists for several reasons including prior description of NK-like cells in lower vertebrates and MHC genes that are coevolving.[332,350] Moreover, in primitive chordates, NK-like, missing-self–like recognition affects histocompatiblity reactions[351] but the molecular determinants of histocompatibility involves molecules unrelated to mammalian Ly49, KIR, or even MHC itself.[352–354] Thus, evolutionary studies of NK-cell receptors and their ligands may provide unique insight into missing-self and other histocompatiblity reactions.

While we have focused thus far on mouse Ly49 and human KIR as the major MHC-I–specific receptors, there are other well-described NK-cell receptors belonging to

either the lectin-like receptor or Ig-like receptor superfamilies. These receptors also have specificities for MHC-I molecules.

Human and Mouse CD94/NKG2

The analysis of CD94 (Klrd1) and NKG2 (Klrc, excluding NKG2D [Klrk1]) family of molecules was especially challenging and required insightful investigations. Identified by subtractive hybridization, the human NKG2 molecules are type II integral membrane proteins with external C-type lectin domains[355] encoded in the NKC[356] (see Fig. 17.3). Initial attempts to express NKG2 molecules on the cell surface were thwarted. Meanwhile, mAb reactivity suggested that CD94 was variably expressed on human NK cells as a disulfide-linked dimer (70 kDa NR, 43 kDa R),[357] and both activation and inhibition functions for CD94 were described.[358–361] Surprisingly, cDNA cloning revealed that CD94 has a short seven amino acid cytoplasmic domain, suggesting that it cannot signal on its own.[360] Furthermore, anti-CD94 immunoprecipitates were not detectable from radiolabeled CD94 transfectants, despite easily detectable expression on FACS analysis with the same mAbs.[362,363] These apparent discrepancies were resolved when it became clear that CD94 heterodimerizes with NKG2 molecules[362]; NKG2A is the 43 kDa molecule previously identified as Kp43 with anti-CD94 mAbs.[364,365] While CD94 may be expressed as a homodimer, the NKG2 partner provides the signaling motif, whether activation or inhibition.[364,366] (NKG2B is an alternatively spliced form of NKG2A. The rest of the NKG2 family is discussed in the following.)

The ligand specificity for CD94/NKG2 receptors was also initially thought to be promiscuous as interactions with many classical (class Ia) and nonclassical (class Ib) HLA molecules had been described.[359,362,364,367–370] However, human CD94/NKG2 receptors directly recognize HLA-E, a MHC-Ib molecule homologous to mouse Qa-1.[371–373] HLA-E (and Qa-1) is widely expressed with limited polymorphism.[374–376] While HLA-E heavy chain is expressed with β2m and a peptide occupying its peptide-binding cleft, its peptide repertoire is largely derived from the leader sequences of MHC-Ia molecules, as previously noted for mouse Qa-1.[377,378] HLA-E (or Qa-1) expression thus requires normal production of HLA-E (or Qa-1) and synthesis of certain MHC-Ia molecules. Mouse CD94/NKG2 recognizes Qa-1 that shares many features with HLA-E.[379,380] These findings need to be considered in the context of the prevailing view at the time that mouse and human NK cells use structurally different receptors to recognize MHC-I molecules.[381] Clearly, the CD94/NKG2 receptors and their ligands are conserved in humans and mice.

The crystal structure of human CD94/NKG2A bound to HLA-E was resolved to 2.5Å and 4.4Å resolution.[382,383] Remarkably, CD94/NKG2A interfaces with HLA-E in a manner analogous to TCR recognition of peptide-loaded MHC-I,[384] including TCR binding to HLA-E itself.[385] Both innate and adaptive receptors for HLA-E lay across the peptide-binding cleft, though peptide itself plays a relatively minor role in binding CD94/NKG2A.[382,383] Strikingly, this binding is distinct from that of Ly49 receptors to their classical MHC-I ligands (see previous discussion). On the other hand, this binding interface is similar to binding of another NKC-encoded, lectin-like receptor, the NKG2D activation receptor, to its MHC-like ligands (see following discussion). Thus, NKC-encoded, lectin-like receptors surprisingly use different strategies to contact their ligands, even though these ligands have structurally related MHC-I folds.

Despite its conservation between mice and humans, the role of CD94/NKG2 receptors in NK-cell function is still incompletely understood. For example, viruses encode peptides that bind and enhance expression of HLA-E, providing a CD94/NKG2A-dependent mechanism to avoid NK-cell attack.[386,387] By contrast, human NK cells expressing CD94/NKG2C (an activation receptor) expand in response to cytomegalovirus (CMV)-infected targets.[388] When a large number of human NK-cell clones were obtained from two normal individuals, CD94/NKG2 seemed to account for the majority of self-MHC–specific receptors on clones from one individual whereas KIRs dominated the self-specific receptors on clones from the other individual, suggesting that some individuals may depend on CD94/NKG2 for self-tolerance.[389] Qa-1 and HLA-E can present peptides derived from other molecules, including the signal sequence of heat shock protein 60 (Hsp60) that is induced by a number of stimuli,[390,391] a multidrug resistance transporter,[392] or blastocyst MHC expressed in embryonic tissues.[393] This may result in loss or gain of recognition by the inhibitory CD94/NKG2A receptor, suggesting intrinsic mechanisms to perturb inhibition by CD94/NKG2A in certain circumstances.

Yet, CD94 appears to be dispensable in certain strains of mice, such as DBA/2J, that do not appear to have any untoward NK-cell phenotype.[394] This finding has been recapitulated in studies of a CD94 knockout mouse on the 129 strain background.[395] Interestingly, CD94 knockout mice are more susceptible to ectromelia virus,[396] as detailed below.

CD94/NKG2 molecules may be important in T-cell function. CD94/NKG2A is rapidly induced on antigen-specific CD8+ T cells during polyoma virus and other infections,[397–399] and CD8+ T cells expressing CD94/NKG2A preferentially proliferate during persistent infection, suggesting that CD94/NKG2 receptors may play a role in memory T-cell responses,[400] and that TCR specificity is correlated with CD94/NKG2A expression by human CTL.[401] Indeed, CD94/NKG2A inhibits antigen-specific cytotoxicity in polyoma virus responses, although this effect is pathogen-dependent. CD94/NKG2A has been studied with other viral infections, including herpes simplex virus,[402] murine CMV,[403] gHV68, and influenza,[404] for example. Although not all studies revealed functional consequences, the recurring theme is the appearance of CD94/NKG2A on previously activated CD8+ T cells. Thus, CD94/NKG2 receptors may regulate T-cell responses.

Other Human Immunoglobulin-like Receptors Specific for Major Histocompatibility Complex-I Molecules

The human LILR family is encoded in the LRC (see Fig. 17.3), just centromeric to the *KIR* genes. There are two general forms of these receptors, subfamily A that appears to be activation receptors, and subfamily B that has the ITIMs

characteristic of inhibitory receptors. The best characterized members, LILRB1 (also known as CD85j, Ig-like transcript 2 [ILT2], or leukocyte IG-like receptor 1 [LILR1]) is broadly expressed whereas LILRB2 (CD85d, ILT4, or LIR2) is not expressed on NK cells but is expressed by myelomonocytic cells, including DCs and monocytes. Both recognize HLA class I molecules[405,406] with LILRB1 exclusively binding folded HLA class I molecules with β2m, whereas LILRB2 can bind both folded and free HLA class I heavy chains.[407] Interestingly, a human CMV protein, UL18, binds LILRB1 with 1000-fold higher affinity than HLA molecules, implicating a role for LILRB1 in host defense.[408] The ligands and functions of other LILRBs (LILRB3 [CD85a, ILT5, LIR3], LILRB4 [CD85k, ILT3, LIR5], LILRB5 [CD85c, LIR8], and LILRB6 [CD85b]) are as yet unknown but they may not be able to bind HLA due to structural constraints.[409]

LILRB1 has four Ig-like domains and binds a conserved region in the α3 domain of most, if not all, classical and nonclassical HLA class I molecules (HLA-A, -B, -C, -E, -F, and -G).[408] Interestingly, the crystal structure of LILRB1 bound to HLA-A2 (3.4Å resolution) reveals that it binds MHC molecules under the peptide-binding domain where it contacts α3 and β2m, more akin to Ly49 engagement of MHC than KIR[409] (see Figs. 17.4 and 17.5). Even though LILRB1 and LILRB2 have differing capacities to bind free HLA molecules, LILRB2 has an HLA class I binding site that overlaps with but is distinct from that for LILRB1.[410] LILRB1 binds UL18 in a manner structurally similar to HLA class I binding.[411]

Interestingly, LILR genes demonstrate allelic polymorphisms,[412] though less so than the adjacent KIR cluster.[413] Nonetheless, polymorphisms in LILRB1 may affect receptor expression.[414] Moreover, polymorphisms in LILR genes are associated with certain autoimmune diseases, such as rheumatoid arthritis.[415]

Major Histocompatibility Complex–Independent Natural Killer-Cell Inhibitory Receptors

As already mentioned, NK cells also express inhibitory receptors for non-MHC ligands, such as mouse gp49b, which binds the αvβ3 integrin,[323] and there is a growing list of other such molecules.[416] Some of these receptors will be discussed in a more appropriate context in the following sections. Most of these receptors contain cytoplasmic ITIMs so their inhibitory function can be predicted even if not directly tested, though some caution is required because the motifs may be involved in other signaling processes.

Human and mouse NK cells express the ITIM-bearing leukocyte-associated Ig-like receptor 1 (LAIR-1, CD305), which is an Ig-like molecule broadly expressed by most leukocytes and encoded in the LRC.[417,418] Initial reports indicating that LAIR-1 binds epithelial cellular adhesion molecule were irreproducible.[419,420] Instead, LAIR-1 binds multiple forms of collagen,[421] which has been validated in crystallographic and biochemical studies.[422] Interestingly, LAIR-1 mediates inhibition that is independent of Src homology 2 (SH2)-domain–containing phosphatases, and instead recruits C-terminal Src kinase (Csk),[423] suggesting that Csk may be involved in inhibitory signaling. Although the in vivo context for functional interaction awaits further characterization, it is reminiscent of the broader reactivity of the Siglecs.

The CD33-related sialic acid binding Ig-like lectins (CD33rSiglecs) are type I receptors with varying numbers of Ig-like domains expressed on a broad array of cells and encoded in the "extended" LRC[312,424,425] (see Fig. 17.3). Despite having sialic acid recognition in common, the Siglecs appear to show differences in carbohydrate recognition, depending on the specific glycan context.[424] Human NK cells express Siglec-7 (p75, adhesion inhibitory receptor 1 [AIRM1]) and Siglec-10, whereas some mouse NK cells express a related Siglec-E.[425] Siglec-7 has been most extensively studied. As expected, its cytoplasmic ITIM can recruit SHP-1 and inhibit NK-cell functions.[426,427] Moreover, expression of its ligand on targets inhibits NK cells in a Siglec-dependent manner.[428] However, the effects appear to be modulated by *cis* interactions between the Siglec receptor and its carbohydrate ligands on the NK cell itself,[424,428,429] reminiscent of *cis* interactions between Ly49 and MHC ligands, as previously discussed.

The Ig-like receptors, termed paired Ig-like receptor (PILR)α and β, are not encoded in the LRC, rather on human chromosome 7 (mouse chromosome 5).[430,431] Mouse PILRα is an ITIM-containing inhibitory receptor, whereas PILRβ is an activation receptor that couples to DAP12.[430,432] Both receptors are expressed on NK (and other immune) cells and recognize CD99. Interestingly, sialylated O-linked glycans on CD99 are involved in recognition by PILRs.[433]

Thus, NK cells (and other leukocytes) express multiple inhibitory receptors that are capable of MHC-independent recognition. How they participate in NK-cell responses and contribute to MHC-dependent effects are beginning to be elucidated, and some appear to play a specific role in the context of activation receptors, as discussed in the following.

Natural Killer-Cell Activation Receptors

NK cells clearly kill MHC-I–deficient targets more efficiently than MHC-I–sufficient targets. However, this enhanced killing does not occur simply because a nonspecific default pathway is unleashed when MHC-I is absent. Instead, it is clear that susceptible targets express ligands for NK-cell activation receptors. In general, these receptors and their ligands were defined following description of the inhibitory receptors.

Approaches to Identification of Activation Receptors

Initial progress in elucidating NK-cell activation receptors was difficult. The approaches that yielded the molecular definition of the TCR, such as subtractive hybridization, mutagenesis of T-cell tumors, and anticlonotypic mAbs,[434–436] were of limited success.[437,438] Unlike the working paradigm of MHC restriction that guided the molecular identification of the TCR, the principles guiding NK-cell activation by targets were unclear. Breakthroughs in identifying NK-cell activation receptors thus required other approaches.

Some activation receptors were recognized because they were first identified on other cells, such as FcγRIII. Other

activation receptors were identified by genetic means, such as cDNA clones for molecules resembling the inhibitory receptors but lacking cytoplasmic ITIMs. Others were identified by a genetic positional cloning approach. Specific stimulation of NK cells through mAbs proved useful for the initial identification of candidate activation receptors and to validate the activation function of receptors identified by other means.

NK cells can be stimulated to mediate antibody-dependent cellular cytotoxicity (ADCC) through the FcγRIII (CD16) receptor that binds the Fc portion of the IgG coating a target. In a related way, anti-FcγRIII can also trigger through CD16 in a process termed "redirected lysis" or "reverse ADCC" because the antibody binds in the opposite orientation to ADCC. A few mAbs against other NK-cell surface molecules also can activate in the redirected lysis assay, highlighting a relatively unique functional property of the recognized molecules (and mAbs), because activation does not occur when most NK-cell surface molecules are cross-linked. First, popularized for analysis of anti-TCR antibodies,[439] redirected lysis occurs when IgG reacts specifically with the NK-cell receptor, and its Fc portion binds a target cell Fc receptor (FcγR) that apparently provides bridging and cross-linking effects.[439] Target lysis does not occur if FcγR binding on the target is prevented with FcγR-deficient targets, $F(ab')_2$ fragments of the anti–NK-cell receptor antibody, or anti-target cell FcγR Ab blockade. In the latter case, Fc regions must be removed to prevent inadvertent triggering of conventional ADCC via CD16 on the NK cell. Thus, the redirected lysis assay is a helpful experimental tool.

Gene transfer studies have been helpful adjuncts to study NK-cell receptors. NK cells are difficult to transfect, and there are few useful tumors with the notable exception of RNK-16, a rat NK tumor line.[440] Viral vectors, such as vaccinia virus, have been useful for gene transfer with the caveat that functional experiments have to be performed within a small time frame before untoward effects occur.[441] Recent use of lentiviral vectors also show promise for gene transfer into primary NK cells.[442]

Recent studies have exploited reporter cell assay systems similar to those used to identify TCR ligands.[443] ITAM-mediated signaling leads to inducible, nuclear factor of activated T cells (NFAT)–dependent expression of a reporter molecule, such as β-galactosidase or GFP. Even an inhibitory receptor can be used to activate the reporter cell by fusion of its extracellular domain to a suitable transmembrane and cytoplasmic domain containing ITAMs. Such reporter cells can then be used to detect ligands.[444–446]

In the following sections, we will describe NK-cell activation receptors with an emphasis on those with known ligands.

Activation Receptors Related to Major Histocompatibility Complex–Specific Inhibitory Receptors

Despite initial characterization as inhibitory receptors for MHC-I, the Ly49 family contains other members (ie, Ly49D and Ly49H in C57BL/6 mice) without cytoplasmic ITIMs. Instead, they are activation receptors containing charged transmembrane residues for association with DAP12 that facilitates expression and provides signal transduction capacity.[447–450] Although Ly49H will be considered below in the context of viral infection, Ly49D has no known role in viral defense. A positional cloning approach indicated that Ly49D is the product of the *Chok* locus, which controls NK-cell specificity for killing of a xenogeneic target, Chinese hamster ovary cells,[159,451] due to recognition of a Chinese hamster MHC-I molecule.[452] Interestingly, when Ly49D was transfected into RNK-16 cells, Ly49D can recognize $H2D^d$,[440] but $H2D^d$ tetramers do not bind Ly49D for unclear reasons, although potentially reflecting lower avidity.[213,262] Several other Ly49 receptors have been identified in non-C57BL/6 mouse strains that have properties of activation receptors (charged transmembrane residues, no ITIMs) but they have been less well characterized,[257,258] except for Ly49P in MA/My mice, which is involved in controlling viral infection that is related to MHC-I recognition[453] (see subsequent details). Thus, some members of the Ly49 family are activation receptors with apparent specificity for MHC-I, potentially with less avidity.

Molecular cloning of the KIR family also led to the identification of two domain receptors (also known as p50) or three domain Ig-like receptors with short cytoplasmic domains lacking the ITIM.[454,455] These molecules are now known as the KIR2DS or KIR3DS, respectively, with numbers for specific molecules (ie, KIR2DS1 [CD158h, p50.1], KIR2DS2 [CD185j, NKAT5, p50.2, clone 49], KIR2DS3 [NKAT7], KIR2DS4 [CD158i, NKAT8, clone 39], KIR2DS5 [CD158g, NKAT9], and KIR3DS1 [CD158e2]). Expression of these molecules may be difficult to determine because mAbs for KIR2DL molecules cross-react with KIR2DS molecules.[456] KIR2DS molecules can associate with DAP12 and activate NK cells in the redirected lysis assay.[454,457] KIR2DS and KIR3DS molecules can recognize HLA class I molecules with specificities similar to corresponding inhibitory KIRs, apparently with lower avidity.[458–461] However, KIR2DS4 and HLA-C alleles and KIR3DS1 and HLA-B alleles influence their respective interactions.[462,463] Thus, further analysis is needed to support the hypothesis that activating forms of KIRs bind HLA alleles less well than the inhibitory receptors as a potential explanation for dominance of inhibition over activation (see Fig. 17.2D).

It remains possible that the activating forms of the Ly49s and KIRs have other ligands and perhaps their MHC specificities are instead somehow related to their physiologically relevant ligand. Indeed, this has been demonstrated for Ly49H, an activation receptor that recognizes a virus-encoded ligand with an MHC-I–like fold.[444,446,464] Moreover, KIR2DS4 may recognize a non-MHC ligand.[465] Thus, further analysis is required for understanding the role of Ly49 and KIR activation receptors in MHC-I recognition and with respect to their inhibitory counterparts.

In addition to NKG2A, the NKG2 family also contains NKG2C, NKG2E, and NKG2F, which are products of different genes.[355,466] (NKG2B is an alternatively spliced isoform of NKG2A, whereas NKG2H is an alternatively spliced isoform of NKG2E.) NKG2C and NKG2E lack cytoplasmic ITIMs and contain charged transmembrane residues for association with DAP12.[467] While the role of NKG2F is unknown

because it lacks an external domain and remains inside the cell, associated with DAP12 but not with CD94,[468] NKG2C and NKG2E form functional heterodimers with CD94.[364] Like CD94/NKG2A receptors, these heterodimers recognize HLA-E or Qa-1, but unlike CD94/NKG2A receptors, they activate NK cells.[469,470] Interestingly, the inhibitory form binds with higher affinity to HLA-E than the activating form.[382,383,471,472] There also appears to be some peptide preference between the different functional forms[382,383,473] that may be relevant in certain physiologic situations. Thus, the CD94/NKG2 receptors may discriminate between subtle differences in their MHC-Ib ligands.

FcγRIII (CD16)

Frequently overlooked but perhaps the first molecularly defined activation receptor on NK cells is FcγRIII (CD16), through which NK cells mediate ADCC against IgG-coated targets.[474,475] Unlike other Fcγ receptor–bearing effector cells, NK cells are generally thought to express only one of the known Fcγ receptors that binds IgG with low affinity,[476] although others suggest that human NK cells may express FcγRII isoforms.[477] There are two human FcγRIII isoforms with identical extracellular domains.[476] Human NK cells express only FcγRIIIA, which is a transmembrane molecule, whereas FcγRIIIB has a glycosylphosphatidyl-inositol (GPI) linkage and is expressed by neutrophils. In mice, only the transmembrane isoform (FcγRIII) is present[478] and displays 95% sequence conservation with muFcγRII. The recently described FcγRIV is a newly recognized orthologue of human CD16A but is not expressed on mouse NK cells.[479,480] There are species differences in CD16 binding to mouse IgG isotypes; mouse IgG3 mAbs bind human CD16 the most efficiently (3 > 2a > 2b >> 1), whereas they bind mouse CD16 with the lowest affinity (2b > 2a > 1 >> 3).[476] In the laboratory, a rabbit antimouse Ig polyclonal Ab, whose Fc portion binds strongly to both human and mouse CD16, could be added to facilitate Fc receptor binding.

The transmembrane FcγRIII molecules are physically associated with FcεRIγ and less commonly with CD3ζ.[481] FcγRIII can also associate with γζ heterodimers. The associated chains are required for optimal cell surface expression of FcγRIII and for signal transduction. After cross-linking, FcγRIII activates biochemical events that are reminiscent of T-cell activation, leading to granule exocytosis and cytokine production.[161,482–485] In vivo, ADCC may be useful in host defense against pathogens or infected cells if Abs are bound to their surface, triggering not only killing but also cytokine production and other NK-cell responses. Although NK cells are generally thought to participate early in a primary immune response (see subsequent discussion), the delay required for isotype switching to IgG production suggests that CD16 cross-linking on NK cells plays a role in secondary immune responses in vivo.

Regardless, ADCC is remarkably similar to natural killing and was important for the initial establishment of the concept of NK-cell activation receptors.[161] Yet, CD16 is not required for NK-cell target recognition because human CD16−CD3− lymphocytes can still mediate natural killing.[486] Moreover, CD3ζ is phosphorylated upon CD16 ligation but not when NK cells are exposed to NK-sensitive targets. Deficiency of γ chain abrogated ADCC but not natural killing.[474] Thus, CD16 is not involved in natural killing.

CD16-related artifacts must be considered when studying NK cells. Flow cytometry experiments may be flawed if CD16 binding is not taken into account. To eliminate this possibility, $F(ab')_2$ fragments should be used. Alternatively, blockade of FcγRIII binding may be sufficient with protein A or G (that bind Fc region on Ig) or anti-FcγRIII mAbs, such as unlabeled mAb 2.4G2 (ATCC HB 197) that reacts with both mouse FcγRII and FcγRIII.[478] Similarly, as discussed previously, antibody blockade experiments should be done with caution if the antibody specifically reacts with the target because ADCC may be stimulated.

NKG2D

NKG2D (KLRK1) was first cloned from human NK cells as a cDNA related to NKG2A and C.[355] However, NKG2D is distinct from other NKG2 molecules for several reasons. There is only limited sequence homology between NKG2D and other NKG2 molecules (28% amino acid identity for the lectin-like domain) whereas other NKG2 molecules are closely related to each other (70% identity). Rather than heterodimerizing with CD94, NKG2D is expressed as a disulfide-linked homodimer on all NK cells in humans and mice. In humans, NKG2D is also expressed on all γδTCR+ and CD8+ T cells, whereas in mice, NKG2D is expressed on most NKT and γδTCR+ T cells but not on resting CD8+ T cells.[81–83] However, essentially all activated mouse CD8+ T cells express NKG2D. In both humans and mice, CD4+ T cells do not express NKG2D, but it is found on a subset of CD4+ T cells in patients with rheumatoid arthritis.[84] Finally, NKG2D has functional properties and ligand specificities that distinguish it from the other NKG2 molecules, indicating that NKG2D should not be considered as a member of the NKG2 family.

NKG2D does not have any known cytoplasmic motif and was first shown in humans to preferentially associate with a signaling chain termed DAP10, encoded by a gene localized 130 bp away from the gene for DAP12.[487] DAP10 does not have any ITAMs; instead, it contains a YxxM motif for recruitment of PI3K.[487] This motif is similar to that found in CD28, and functional studies indicate that NKG2D can act as a costimulatory molecule on T cells.[488–490] Moreover, DAP10 has a site for recruitment of Grb2.[491] Thus, NKG2D may provide qualitatively different signals, resulting in different cytokine production, for example,[490] than activation receptors associated with ITAM-signaling chains.

Other studies have suggested that NKG2D functions as a primary activation (triggers alone) rather than costimulatory receptor (does not stimulate unless it synergizes with another receptor) on NK cells.[80,492] Such studies need to be reconsidered in light of several factors. 1) Many studies of NKG2D function use targets that are poorly killed by NK cells. When these targets are transfected with NKG2D ligands, killing is enhanced in an NKG2D-dependent manner. However, such studies do not distinguish whether NKG2D functions as a primary activation receptor or as a costimulatory receptor (analogous to CD28 requirement for

full activation of T cells) as the same experimental outcome is anticipated in either case. 2) When cross-linking is done with immobilized mAbs alone and CD16 coengagement on NK cells is avoided, NKG2D functions as a costimulatory receptor on mouse IL-2-activated NK cells.[80] 3) In mice but not humans, there are two alternatively spliced isoforms of NKG2D.[34,493] A long form (NKG2D-L) contains a 13 amino acid extension at the amino terminus (cytoplasmic domain) as compared to the short form (NKG2D-S). Resting NK cells predominantly express NKG2D-L that preferentially associates with DAP10. However, activation of NK cells with cytokines causes a transient increase in NKG2D-S that associates with ITAM-containing DAP12 as well as DAP10. However, others have found that NKG2D-L can associate with DAP12, albeit to a lesser degree, and that both isoforms are present in resting NK cells.[494] Regardless, in the absence of DAP10, mouse NKG2D can associate with DAP12,[35] allowing it to signal akin to a primary activation receptor. Thus, mouse NKG2D is an unusual example of a receptor with the same extracellular domain but with potentially different functional outcomes (primary activation versus costimulation), depending on its associated partner chain.

There is remarkable plasticity for NKG2D in that it can bind many apparently disparate ligands that are only superficially related to each other by sequence alignment, and human NKG2D can bind mouse ligands and vice versa. Based on soluble MICA binding, the first human NKG2D ligands were found to be MICA and MICB (MHC-I chain-related, A and B) encoded on chromosome 6p21.3, centromeric to HLA-B locus.[81] Subsequently binding studies of UL16 from human CMV (HCMV) identified the ULBP (UL16 binding protein) family that in turn binds NKG2D.[495] The UL16-binding proteins (ULBPs) are encoded by genes in the retinoic acid expressed transcript (*RAET1*) gene family (official HUGO nomenclature) encoded on chromosome 6q24.2-q25.3 and discovered by genomic mining. Whereas *RAET1F*, *RAET1J*, *RAET1K*, and *RAET1M* are pseudogenes, other *RAET1* genes give rise to ULBP1 (RAET1I), ULBP2 (RAET1H), ULBP3 (RAET1N), ULBP4 (RAET1E), ULBP5 (RAET1G), and ULBP6 (RAET1L), some of which have been extensively studied as ligands for NKG2D.[495–498] MICA, MICB, ULBP4, and ULBP5 are considered to be transmembrane proteins whereas the other RAET1 proteins are GPI-linked.[499]

Mouse NKG2D ligands were discovered with soluble NKG2D that was used for expression cloning.[82,83] Two types of ligands were originally defined: the minor histocompatiblity antigen H60 and members of the retinoic acid early inducible gene-1 (RAE-1) family. It is now clear that the mouse NKG2D ligands are the H-60 family, consisting of H60a, b, and c, the RAE-1 family, consisting of RAE-1α, β, γ, δ, and ε (also known as Raet1a-e), and murine ULBP-like transcript (MULT1).[82,83,500–502] There are strain-specific differences in ligand expression. For example, BALB/c mice express H60a, RAE-1α, β, and γ, whereas C57BL/6 mice do not express these molecules and express RAE-1δ and ε.[503] In addition, there are allelic forms of NKG2D ligands (eg, 80 different alleles of MICA and 33 alleles of MICB [hla.alleles.org/classo.html]), but these have not been fully characterized in humans (or mice).

Where studied, all NKG2D ligands have distant amino acid similarity to MHC-I, although they share only about ~25% amino acid identity with each other. Within a family, however, they may be much more closely related (up to 90% identity). None of the NKG2D ligands associates with β2m or binds peptides.[504–507] Many contain only the α1/α2 platforms and many are GPI-linked to the plasma membrane. The ligands display binding to NKG2D in two ways based on affinity.[501,502,504,508,509] For example, RAE1α, β, γ, and δ, and H60b and c demonstrate low-affinity interactions with mouse NKG2D, similar to MICA to human NKG2D (K_D = ~300 to 8700 nM). In contrast, RAE1ε (also described as RAE1B6),[509] H60a and MULT1 show a much higher affinity interaction (K_D = 6 to 30 nM). Thus, closely related molecules within a family may display wide disparity in affinities whereas distantly related molecules can share high affinity with NKG2D.

Structural studies at 2.6 to 3.5 Å resolution indicate that the NKG2D ligands have MHC-I–like folds, though the "peptide-binding cleft" is closed.[504,505,510] NKG2D binds its ligands more analogous to TCR docking on MHC and unlike Ly49 recognition (see Figs. 17.4 and 17.5), despite the relationship of NKG2D and Ly49 receptors as NKC-encoded, lectin-like homodimers that bind MHC-related molecules.[504,510,511] Indeed, NKG2D interactions with its ligands are much more aligned with CD94/NKG2A recognition of HLA-E, as described previously. Moreover, NKG2D uses largely nonoverlapping patches to engage a similar orthogonal footprint on its disparate ligands. Interestingly, the putative immunoevasion molecule, UL16, binds MICB in essentially the same manner as NKG2D is predicted to bind, even though UL16 has a different three-stranded β-sheet structure.[512] Additional structures of NKG2D complexed with other ligands should yield additional insight as to how NKG2D can bind a panoply of distantly related ligands with varying affinities.

NKG2D function has been described in the "induced-self" model (see Fig. 17.2E) because expression of its ligands can be inducible and can override inhibitory influences of MHC-I.[513–515] In many cases, low basal transcriptional levels are markedly upregulated in pathologic conditions. For example, MICA and MICB expression is markedly enhanced on epithelial tissues in inflammatory bowel disease.[516] Whereas this notion was initially thought to be related to heat shock elements in MICA promoter, recent detailed studies indicate that NKG2D ligand transcription in some cells is not affected by heat shock or hypoxia in vitro.[517] Instead, transcription of NKG2D ligands can be induced by several other stimuli. For example, DNA damage from ionizing radiation or chemotherapy agents results in upregulation of human and mouse NKG2D ligand transcripts and concomitant surface protein expression.[517] This process was dependent on activation of the ataxia telangiectasia, mutated (ATM), and ATM- and Rad3 (ATR)-related pathways. Interestingly, several micro-ribonucleic acids (miRNAs) can regulate MICA and MICB expression, and they are decreased following heat shock, allowing upregulation of MICA and MICB.[518] On the other hand, Dicer knockdown also results in a DNA damage response that results

in enhanced MICA and MICB expression.[519] Regardless, NKG2D ligand expression due to genotoxic responses may be relevant to chemotherapy effects in cancer, such as multiple myeloma.[520]

Other external stimuli, such as phorbol ester, retinoic acid, cytokines, and TLR stimulation, can induce NKG2D ligand expression on mouse and human cells.[500,521,522] The phorbol ester effect is regulated by the transcription factor JunB[523] and retinoic acid stimulation was the impetus for original identification of the RAE-1 family.[524] Cytokines can also regulate NKG2D ligands. For example, IFNγ can downregulate transcripts for H60a expression on tumor cells and alter NK-cell susceptibility.[525] For MICA, the IFNγ effect is mediated through miRNAs.[526] Interestingly, TLR-induced primarily MICA and not MICB, through pathways dependent on ATM, ATR, and miRNAs.[522] Viral infection can also induce NKG2D ligands,[527] perhaps not surprisingly because viruses target NKG2D and its ligands (described in the following). For example, murine CMV (MCMV) infection induced RAE-1 mRNA and surface expression within 18 hours. This effect was independent of the DNA damage response but required PI3K activation.[527] However, PI3K activation alone was insufficient to induce ligand expression, suggesting that additional signals are required for NKG2D ligand expression by viral infections and perhaps other stimuli.

For other NKG2D ligands, transcripts are constitutively expressed in a wide variety of tissues[495,501]; posttranslational modifications alter surface protein expression levels. For example, mRNA for MULT1 is widely expressed in normal tissues[501] and MULT1 surface expression is regulated by ubiquitination.[528] MULT1 has a long cytoplasmic domain, unlike most other NKG2D ligands, which are GPI-linked. Under normal circumstances, MULT1 is ubiquitinated on its cytoplasmic Lys residues and is targeted for degradation. However, cellular stress, such as heat shock or ultraviolet irradiation, reduced its ubiquitination and allowed surface expression. In particular, MULT1 expression is regulated by the E3 ubiquitin ligases, membrane-associated RING-CH (MARCH) 4 and membrane-associated MARCH 9.[529] A human virus, Kaposi sarcoma–associated herpesvirus (KSHV), exploits this pathway to avoid NK-cell attack (detailed in the following).

Despite the nearly universal acceptance of the induced-self model for NKG2D function, it is not clear if this hypothesis is applicable to all of its ligands.[530,531] It remains possible that constitutive expression or affinity of some NKG2D ligands may be too low to permit NKG2D activation to override MHC-I–dependent and –independent inhibitory receptors. Continued analysis at the protein level is anticipated, as well as study of NKG2D ligands in specific tissues, exemplied by analysis of H60c in the skin where it is selectively expressed and can also costimulate dendritic epidermal T cells.[532]

On the other hand, chronic exposure to membrane-bound or soluble NKG2D ligands results in downregulation of NKG2D expression and lower functional responsiveness.[85,533–535] Patients with tumors expressing MIC frequently contain soluble MIC in their peripheral blood, presumably as a result of proteolytic cleavage of membrane-expressed ligands,[533,536] though the enzyme required is unclear, and the process apparently requires palmitoylation of MICA.[537] Mice Tg for NKG2D ligands show impotent NKG2D responses,[534,538] a topic better discussed subsequently in the context of NK cell tolerance and education. On the other hand, acute upregulation of an NKG2D ligand alone can rapidly induce immune responses[539] (albeit effects not examined for NK cells). Taken together, these findings may explain why tumors frequently express NKG2D ligands that would otherwise enhance their susceptibility to NK-cell attack, supporting a role for NKG2D in tumor surveillance and also the general concept that NKG2D-mediated activation is balanced between acute, induced-self, and chronic expression of its ligands.

Additional studies support a role for NKG2D in tumor surveillance. For example, mice are more sensitive to developing methylcholanthrene-induced fibrosacromas when NKG2D is neutralized by chronic anti-NKG2D mAb administration[86] or targeted deletion.[540] Moreover, mice lacking γδT cells are more susceptible to carcinogenesis apparently due to an NKG2D-dependent effect.[500] IFNγ is a known host mediator that shapes the tumor phenotypes in a broader process known as "immunoediting,"[541,542] and IFNγ also mediates downregulation of H60 expression on tumors.[525] Thus, NKG2D and its ligands are important in the host response (or lack thereof) to tumors.

A role for NKG2D in antiviral responses is indicated by studies that show that viruses use different strategies to interfere with ligand recognition by NKG2D. This was first noted when the HCMV protein UL16 was found to bind ULBPs.[495] In infected cells, UL16 retains some (MICB, ULBP1, and ULBP2) but not all NKG2D ligands in the endoplasmic reticulum and cis-Golgi, preventing their expression on the cell surface and protecting from NK cell lysis.[543–545] HCMV also encodes UL142, which retains certain alleles of MICA in the cis-Golgi.[546] In mice, MCMV encodes four molecules that downregulate expression of all NKG2D ligands. gp40 from the *m152* open reading frame (ORF) downregulates all five RAE-1 ligands but has no effect on H60 or MULT1.[503,547] H60 is downregulated by the product of the *m155* ORF at a post-Golgi level, perhaps by targeting H60 for proteasomal degradation[548,549] and MULT1 expression is affected by *m145*.[550] The herpes virus Fc receptor (fcr-1), the product of the *m138* ORF, targets RAE-1ε, H60, and MULT1.[551,552] Interestingly, KSHV encodes an E3 ligase, termed K5, that can downregulate MICA and MICB expression.[553] This effect was due to ubiquitinated internalization of MICA, but not degradation, and protected cells from NK-cell cytotoxicity, suggesting that KSHV targets a normal pathway regulating NKG2D ligand expression. HCMV and other herpes viruses (KSHV, Epstein-Barr virus) encode miRNAs that affect MICA and/or MICB expression.[554,555] Non–herpes viruses also target the NKG2D pathway; adenovirus E3/19K sequesters MICA and MICB in the endoplastic reticulum,[556] whereas the orthopoxviruses, cowpox, and monkeypox viruses encode a secreted, high-affinity NKG2D ligand that antagonizes NKG2D recognition and activation.[557] Thus, the multitude of viral strategies that affect NKG2D recognition

strongly suggests that NKG2D plays an important role in the host response to viral infections.

Surprisingly, an NKG2D knockout mouse displayed enhanced resistance to MCMV infections.[87] This mouse also displayed modest changes in NK-cell development with faster NK-cell maturation that were not demonstrated in another NKG2D knockout,[540] even though both were generated directly in C57BL/6-derived embryonic stem cells. A subsequent retargeting of the *Klrk1* locus recapitulated the enhanced resistance to MCMV,[558] which for the moment is at odds with viral neutralization of NKG2D.

Finally, NKG2D may play a role in autoimmune disorders. In autoimmune type I diabetes mellitus (TIDM), MICA polymorphisms are reportedly associated with increased risk,[559] but *MICA* is closely linked with *HLA*, which clearly imparts significant risk so additional analysis of large patient cohorts is needed to segregate the *MICA* effect from that of *HLA* alleles in TIDM or any other HLA-associated autoimmune disease.[560] Nonetheless, NKG2D ligands are expressed in the prediabetic NOD mouse pancreas and anti-NKG2D blockade prevents TIDM.[561] In human patients with inflammatory syndromes, such as rheumatoid arthritis, Wegener granulomatosis, and unstable angina, there is an unusual population of CD4+ CD28– NKG2D+ T cells that is expanded.[562–564] NKG2D can provide a costimulatory signal for T cells, at least for CD4+ CD28– T cells in rheumatoid arthritis and rheumatoid arthritis synovium expresses NKG2D ligands.[84,489] IL-15 induces NKG2D expression on intestinal epithelial T-lymphocytes that then display a LAK cell–like promiscuous killing capacity against enterocytes which upregulate NKG2D ligands from gliadin exposure, suggesting that NKG2D may play a pathogenic role in celiac sprue.[36,565,566] On the other hand, others have postulated that CD4+ NKG2D+ T cells may be normally immunosuppressive as they are inversely correlated with disease activity in patients with systemic lupus erythematosus.[567] Thus, NKG2D may play a pathogenic role, albeit somewhat unclear, in several autoimmune disorders in humans and mice.

Natural Killer Gene Complex–encoded Lectin-like Receptors Recognize Natural Killer Gene Complex–encoded Lectin-like Ligands

First identified by functional studies in the rat, Nkrp1 (Klrb1) molecules belong to a family of lectin-like, disulfide-linked homodimers with type II orientation encoded in the mouse and rat NKC[71,255,330,568–571] (see Fig. 17.3). Expression is relatively selective for NK cells, although Nkrp1 molecules are also expressed by NKT cells in rats and mice.[572,573] There is only a single gene (*NKRP1A*, CD161) in humans that is expressed on a subpopulation of NK cells.[574] The continuing allure of the Nkrp1 family stems from observations that NK1.1 (the most widely known specific serologic marker of NK cells) is encoded by *Nkpr1c* (*Klrb1c*) in C57BL/6 mice,[71] Nkrp1 receptors are conserved, and they have interesting genetics with respect to their ligands.[575]

Initial functional studies indicated that rodent Nkrp1 molecules can activate NK cells through redirected lysis,[32,571,576–578] which can be prevented by inhibitory Ly49 receptor engagement.[140] Mouse Nkrp1c (NK1.1) is functionally associated with FcεRIγ, although its deficiency curiously does not affect NK1.1 expression.[579] An Nkrp1a loss mutant of rat RNK-16 cells failed to kill certain targets; transfection restored killing capacity, suggesting that rat Nkrp1a is an activation receptor specific for target determinants.[580] Thus, Nkrp1 molecules were among the first described NK-cell–specific activation receptors though inhibitory forms were later described.

Inasmuch as Nkrp1 molecules are homologous to C-type lectins, the ligands for Nkrp1 molecules were initially presumed to be carbohydrates but Nkpr1 molecules lack residues for coordinate binding of calcium that is required for authentic C-type lectin recognition of carbohydrates.[222] While the ligands for Nkrp1c have not yet been identified, Nkrp1f recognizes C-type lectin related g (Clrg, also known as Clec2i, Dcl1).[445,581–583] Nkrp1f is presumed to be an activation receptor because it has a charged transmembrane residue and no cytoplasmic signaling motifs. The inhibitory receptor, Nkrp1d, is expressed on all NK cells in C57BL/6 mice and is specific for Clrb (Clec2d; osteoclast inhibitory lectin, Ocil). Thus, the Nkrp1 family is an MHC-independent system that is presumably involved in self-tolerance and is the first example of a lectin-like receptor recognizing a lectin-like ligand unlike MHC-like ligands for other NKC-encoded receptors.

Genome sequence analysis indicates that *Nkrp1d* in C57BL/6 mice is represented by *Nkrp1b* in other strains.[75,76,569,584] The Nkrp1b molecule from SJL and SW mice was discovered to be reactive with the anti-NK1.1 mAb, highlighting the close similarities of these molecules and serologic cross-reactivity. Whereas the older literature suggested that there are also strain differences in transcript expression with markedly lower expression in BALB/c NK cells,[585] more recent data demonstrate abundant expression of Nkrp1 transcripts.[584] Moreover, the BALB/c allele of Nkpr1b also reacts with Clrb, indicating conserved specificity and function.

The Clr molecules belong to an NKC-encoded family with type II orientation and C-type lectin homology.[581] They are most closely related to the CD69 molecule encoded by an adjacent gene. In C57BL/6 mice, seven Clr genes have been identified at the genomic level. RT-PCR analysis indicates that *Clrb* is broadly expressed, whereas *Clrg* and *Clrf* genes are present in restricted and nonoverlapping tissues, including NK cells, and *Clra* and *Clrc* transcripts have not yet been identified. *Clre*, a probable pseudogene, has numerous stop codons in its expected open reading frame. Genomic analysis indicates the existence of a rat *Clr* family,[570] whereas in humans, three genes, *LLT1*,[586] *AICL*,[587] and *DCAL-1*[588] are localized next to CD69 and are related to the Clr family of genes.

Human NKRP1A binds LLT1, a functional homolog of mouse Clr.[589,590] Moreover, activation-induced C-type lectin (AICL CLEC2B) is the ligand for the NK-cell receptor activation receptor, NKp80 (KLRF1), which is also encoded in the NKC but is absent in rodents.[591–593] Finally, CLEC2A (keratinocyte-associated C-type lectin, KACL) is primarily expressed in the skin and is recognized by another NK-cell activation receptor, termed NKp65 (KLRF2).[594] All of these

receptors and their ligands are lectin-like molecules encoded in the human NKC.

The genetics of the *Nkrp1* and *Clr* loci is especially interesting from several viewpoints. 1) These loci are co-mingled in the NKC, from rodents to humans[445,570,575,584,589,590] (see Fig. 17.3). 2) In mice, there is limited allelic polymorphism, with conservation of gene order and content, despite genetic proximity to the highly polymorphic *Ly49* cluster.[445,584,595] For example, compare the conservation of the Nkrp1 gene cluster with polymorphism of the Ly49 cluster in C57BL/6 and 129 strain mice.[227,257,260,445,595] 3) At the individual gene level, *Nkpr1* alleles appear to be much less divergent than the corresponding *Ly49* alleles in the same inbred mouse strains.[257,445,584,595] 4) The *Nkpr1-Clr* interval of the NKC appears to be genetically protected with suppression of recombination.[596,597] Genetic linkage of Nkrp1 and its ligands thus resembles the tight genetic linkage of receptor and ligand genes and recombinational suppression of the self-incompatibility loci in plants to prevent self-fertilization and related mating loci in other species.[598,599] Furthermore, the coevolution of such linked genes for receptors and ligands also represents an interesting issue in evolutionary biology because reciprocal mutations in both receptor and ligand genes are simultaneously needed to generate new specificities.[598] Nevertheless, the genetic pressure to conserve the *Nkrp1-Clr* gene order and coding sequences may therefore reflect a critical role for Nkrp1 and Clr molecules in innate immune cell interactions and functions.[575]

While the in vivo role of Nkrp1 and Clr molecules is incompletely understood, the best evidence for their functional importance comes from studies in rats. Rat CMV encodes RCTL (rat CMV C type lectin-like), which closely resembles rat Clrb and regulates its expression on infected cells.[600] RCTL interacts with the Nkrp1b inhibitory receptor and inhibits NK cell killing of infected cells. An RCTL loss mutant rat CMV is attenuated in vivo in an NK-cell–dependent manner. These studies, indicating that viruses encode decoy ligands for Nkrp1 receptors, support the importance of Nkpr1-Clr interactions in immune responses.

2B4, CD2, and Signaling Lymphocytic Activation Molecule Family of Receptors

The 2B4 (CD244, SLAMF4) molecule was originally identified on mouse NK cells with a mAb that perturbed mouse NK-cell function.[601] 2B4 is a type I integral membrane protein that belongs to the family of Ig-like molecules, including signaling lymphocytic activation molecule (SLAM) (SLAMF4, CD150), NK, T, and B cell antigen (NTBA, also known as SLAMF6 or Ly108), Ly9 (SLAMF3, CD229), CD84 (SLAMF5), and CD2-like receptor activating cytotoxic cells (CRACCs; SLAMF7, CD319).[602] Although these receptors may be broadly expressed, 2B4, NTBA, Ly9, CD84, and CRACC are expressed on NK cells. NTBA, Ly9, CD84, and CRACC are involved in homophilic interactions whereas 2B4 recognizes CD48, a GPI-linked molecule expressed on hematopoietic cells.[603,604] CD48 itself is also recognized by CD2, albeit at a ninefold lower affinity than by 2B4 (K_d = ~16 μM). Anti-CD2 mAbs can stimulate NK cells in the redirected lysis assay[605] and granule exocytosis,[606] but CD2– NK cells can still mediate natural killing.[9] The cytoplasmic domains of SLAM, 2B4, NTBA, Ly9, CD84, and CRACC contain a motif with sequence similarly to the ITIM, termed the immunoreceptor tyrosine-based switch motif (ITSM) consisting of TxYxxV/I consensus sequence.[607] The ITSMs allow interactions with a signaling adapter, SLAM-associated protein (SAP, also known as SH2D1A). The importance of SAP and its associated receptors is highlighted by the X-linked lymphoproliferative (XLP) syndrome, a human immunodeficiency involving abnormal proliferation of T and B cells during Epstein-Barr virus infections due to mutations in SAP.[608]

SAP is related to Ewing sarcoma–associated transcript (EAT2, also known as SH2D1B1) and EAT2-related transducer (ERT, also known as SH2D1B2), which is present in rodents and only expressed in NK cells.[604] SAP and the SAP-related molecules contain a single SH2 domain for interaction with the ITSMs of presumably all SLAM family members except CRACC.[609–613] Instead, CRACC recruits EAT2, which functionally substitutes for SAP.[612] Subsequently, the SAP-related proteins can recruit a wide variety of downstream signaling molecules including Fyn, a Src-family tyrosine kinase, and tyrosine phosphatases, inositol phosphatases, and adaptor molecules.[614]

The complexities of this receptor-ligand signaling pathway are evident from the previous description. Receptors have overlapping ligand specificities; they recruit different adaptors that in turn can recruit downstream signaling molecules, even those with opposing functions. In this regard, functional studies of SLAM family molecules on NK cells have often been ambiguous, often depending on the experimental approach.[602] For example, 2B4 cross-linking with mAb can both stimulate as well as inhibit mouse NK cells,[601] whereas a 2B4-deficient mouse primarily displays effects consistent with 2B4 being primarily an inhibitory receptor.[615] However, the inhibitory effect of 2B4 is more apparent in mice than humans.[604] Some of these outcomes may depend on spatial distribution, 2B4 isoforms, or differential recruitment of SAP or its related molecules.[602] Further analysis should enlighten NK-cell function and signaling because 2B4 and related receptors demonstrate MHC-independent regulation of NK cells, and mediate inhibition and activation in a functionally distinct manner from the MHC-specific inhibitory receptors and ITAM-signaling chain associated receptors, respectively.

Natural Cytotoxicity Receptors

A series of mAbs that redirected lysis of human NK cell clones lacking KIRs led to the identification of the "natural cytotoxicity receptors" (NCRs), NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337).[616–620] These molecules are selectively expressed on NK cells, though NKp44 is expressed only upon activation. Whereas NKp46 is encoded in the LRC (see Fig. 17.3), NKp44 and NKp30 are encoded in the class III region of the MHC on human chromosome 6.[621,622] cDNA cloning revealed that they are type I integral proteins with one (NKp30, NKp44) or two (NKp46) Ig-like extracellular domains.

They contain charged transmembrane residues for association with ITAM-signaling chains (ie, ζγ heterodimers (NKp46, NKp30) or DAP12 (NKp44). In the mouse, only the gene for NKp46 is present in the syntenic region of chromosome 7 with the genes for other NCRs being absent (NKp44) or a pseudogene (NKp30).[623] The NCRs appear to play a role in cytotoxicity against tumors of varying origins because anti-NCR antibodies block target killing.[624] A mouse NKp46-Ig fusion protein binds RMA-S targets and deficiency of NKp46 leads to impaired in vivo clearance of RMA-S cells.[93] Interestingly, human NCRs apparently recognize mouse tumors and vice versa, suggesting conservation of these receptor-ligand pairs across species but identity of their target ligands were elusive,[624] until recently for NKp30 and NKp46.

In functional and structural studies, NKp30 clearly recognizes B7-H6, a member of the CD28 family that also includes CTLA-4 and PD-1.[625,626] B7-H6 is not expressed normally, is found on tumor cells, and sensitizes targets to NKp30-dependent cytotoxicity by NK cells. In a 2.0 Å structure of NKp30 complexed to B7-H6, NKp30 engages its ligand in an antibody-like manner distinct from the way other receptors bind B7 family molecules. Other potential NKp30 ligands include pp65 of HCMV, though it is a tegument protein and not expressed on the plasma membrane of infected cells.[627] HLA-B–associated transcript 3 (BAT3) was also proposed, but it is a nuclear protein that is released with DNA damage or endoplasmic reticulum stress.[628] Finally, NKp30 reportedly binds poxviral hemagglutinin independent of B7-H6 and BAT3.[629] Nonetheless, at least one ligand is now well characterized for NKp30.

Human NKp46 appears to recognize influenza hemagglutinin on infected cells.[629,630] The interaction is dependent on sialic acid residues on oligosaccharides on NKp46 itself,[630] but this specificity is difficult to explain due to the ubiquitous expression of sialylated saccharides. NKp46 and NKp30 may recognize heparan sulfate proteoglycans on their cellular targets, though this is also controversial.[631,632] Nevertheless, NKp46-knockin (GFP)/knockout mice are susceptible to influenza, consistent with a role for NKp46 in defense against influenza,[93] albeit possibly in an indirect manner.[633] These studies may provide important clues to understanding of a receptor (NKp46) conserved in humans and mice.

NKp46 Tg mice have been proposed to be useful for NK-cell analysis.[95] These mice are Tg for a construct consisting of 400 bp from the human NKp46 promoter driving expression of enhanced GFP and the human diptheria toxin receptor. However, NKp46+ cells in the gut may be derived from a non–NK-cell lineage.

Human NK-22 cells were identified in the gut as being CD3− CD56+ NKp44+ cells that produce IL-22 but not IL-17 upon stimulation with IL-23.[88] In the mouse, a similar functional population producing IL-22 was identified among NKp46+ cells and to a lesser degree, among NK1.1+ cells.[89–91,634] Immature human NK cells in secondary lymphoid tissue and the uterus may produce IL-22.[635,636] However, other reports indicate that NK-22 cells are more closely related to lymphoid tissue inducer (LTi) cells.[634,637–639] Thus, there may be heterogeneity among IL-22–producing NKp46+ cells.

Other Activation Receptors

Several other NK-cell–expressed molecules can activate cytolysis. For example, mAbs against mouse CD69 and Ly-6 and rat gp42 can trigger killing.[32,33] CD69 is encoded in the NKC and is structurally related to other NKC-encoded receptors. Interestingly, CD69 expression is upregulated upon stimulation,[322] and its expression is not confined to NK cells.[640] CD69 is functionally active on a large variety of hematopoietic cells when cross-linked by anti-CD69 mAbs. Its ligand is unknown, but it regulates the function of the sphingosine 1-phosphate receptor-1 through a membrane interaction.[641] Ly-6 belongs to a large family of small (15 to 18 kDa) GPI-anchored molecules that can activate lymphocytes when cross-linked.[642,643] Rat gp42 is a GPI-anchored protein with two Ig-like domains that was originally identified on IL-2–activated NK cells and the rat RNK-16 NK cell line.[33] Anti-gp42 can activate RNK-16 but not IL-2-activated NK cells. However, CD69, Ly-6, and gp42 are not expressed on freshly isolated NK cells and are expressed only after activation through other pathways; they are therefore not involved in triggering natural killing by freshly isolated NK cells. Although their physiological role is unknown, their activation potential and enhanced killing by IL-2–activated NK cells suggest that these molecules may contribute to this phenotype.

Receptors Involved in Recognition of Epithelial Tissues

NK cells possess receptors that are specific for ligands expressed at cell-cell junctions in epithelial tissues. Interestingly, ligands, such as cadherins, carcinoembryonic antigen (CEA)-related adhesion molecules (CEACAMs), nectins, and nectin-like proteins (necls), are involved in forming adherens junctions and are engaged in homotypic or heterotypic interactions, and thus, are not normally exposed.[644] KLRG1 (also known as mast cell–associated function antigen, MAFA) is a lectin-like receptor with a single cytoplasmic ITIM.[645–647] Unlike human *KLRG1*, mouse *Klrg1* resides relatively distant and centromeric from the rest of the NKC, consistent with gene duplication and chromosomal inversion events. First discovered on rat mast cells, it has a broader distribution in human and mouse, including NK and T cells but not mast cells. Expression of Klrg1 on NK cells is downregulated in MHC-I–deficient mice, unlike the Ly49s that are modestly upregulated, but no MHC binding has been observed for Klrg1.[648] Instead, mouse Klrg1 binds three of the seven "classical" cadherins, E-, N-, and R-cadherin, leading to inhibition of NK lysis.[649,650] Biophysical studies show the K_D = 120 mM for mouse KLRG1-mouse E-caherin.[651] The structure of KLRG1 at 1.8 Å resolution revealed a typical lectin-like domain for KLRG1, which binds E-cadherin at a site distinct from where the integrin $a_E b_7$ (CD103) binds E-cadherin. A KLRG1-deficient mouse demonstrates that it is dispensable for NK- and T-cell differentiation and antiviral responses, but it has not been

exhaustively studied, especially for epithelial responses.[652] Human CEACAM1 (CD66a) contains a cytoplasmic ITIM and can directly bind CEA, thereby potentially regulating NK-cell activities in human MHC-I–deficient patients and during pregnancy.[653–655] Mouse NK cells also express CEACAM1.[656] Inasmuch as CEACAM1 binds homophilic and heterophilic ligands expressed on epithelial and other cells,[657] NK cells thus express several inhibitory receptors for ligands normally expressed on epithelial cells.

NK cells also express cell-cell adhesion Ig-like receptors for molecules located at the adherens junction (ie, nectins and necls).[658,659] Specifically, they express CD226 (DNAM-1, DNAX accessory molecule-1) that recognizes necl-5 (CD155, poliovirus receptor, PVR) and nectin-2 (CD112, PVRL2).[660] NK cells also express CD96 (Tactile, T-cell activation, increased late expression) that binds necl-5 but not nectin-2, and class I-restricted T-cell–associated molecule (CRTAM), a receptor that recognizes necl-2.[661–663] DNAM-1 associates with lymphocyte function–associated antigen (LFA-1) and regulates its capacity to bind its ligand, ICAM-1.[660,664] In addition, DNAM-1 can recruit actin-binding proteins to form an adhesive complex with other cells. DNAM-1–deficient mice have defective NK and T cells, and fail to control tumors, both transferred and de novo carcinogen-induced.[665,666] Although these receptors can activate Src and other downstream pathways,[659] they are thought to function on NK cells as adhesion receptors that enhance cytotoxicity, rather than as primary activation receptors. Conversely, NK cells also express T-cell immunoglobulin and ITIM domain (TIGIT), which recognizes PVR and PVRL2 but not PVRL3, and inhibits NK-cell killing.[667,668]

These studies suggest that these receptors may affect different NK-cell functions as related to epithelial tissues.[644,649] For example, the inhibitory receptors may regulate NK-cell transmigration across an epithelial barrier or prevent NK-cell attack against normal tissues.[649,669] While the activation receptors could also affect NK-cell transmigration,[649,669,670] they also could be poised to attack cells that have disordered cell-cell junctions, such as tumors that typically lose cadherin expression and expose nectins and necls, potentially making them more susceptible to NK attack, consistent with an "exposed-self" model for NK-cell activation (see Fig. 17.2F, G). On the other hand, tumors may become resistant to NK cells by altering expression of these molecules. Current evidence provides some support to these complex scenarios that will require further investigation.[644]

Accessory Molecules

The role of accessory molecules in NK-cell activation has been difficult to address without knowledge of the "NK-cell (activation) receptor." Nevertheless, NK-cell cytotoxicity is critically dependent on classical accessory and adhesion molecules, such as LFA-1.[671,672] Insect cells expressing ICAM-1, a ligand for LFA-1, can be killed by human NK cells through an LFA-1–dependent process, suggesting that LFA-1 alone is sufficient to trigger killing.[673] LFA-1 cross-linking does not lead to granule polarization, suggesting that it may be insufficient for triggering the killing process by itself.[143] This issue is further complicated by the capacity of receptors like DNAM-1 to modulate LFA-1 association and function on NK cells.[660] Moreover, NK cells express multiple receptors capable of binding target ligands, with each receptor potentially affecting different downstream signaling pathways. When pairs of these receptors (eg, CD16, NKp46, NKG2D, 2B4, DNAM-1, or CD2) are cross-linked, synergistic NK-cell activation in terms of cytokine production and target killing can be seen for some but not all pairs.[674] However, this approach has not been used for the panoply of NK-cell receptors. Thus, how "accessory" molecules or other receptors contribute to each activation receptor function on NK cells remains to be systematically determined.

Ligands Recognized by Both Activation and Inhibitory Receptors

Evident from the previous discussion is the general concept that NK cells usually express both activation and inhibitory receptors simultaneously. Moreover, some of these receptors with opposing functions bind essentially identical ligands. For example, both activation and inhibitory receptors in the Ly49 and KIR families reportedly bind MHC-I molecules, and CD94/NKG2A and CD94/NKG2C bind HLA-E/Qa1. In general, where studied, the inhibitory receptors tend to bind ligands with higher affinities than their corresponding activation receptor counterpart, perhaps accounting for the common observation that inhibition tends to dominate over activation. However, the overall relevance of paired activation and inhibitory receptors to NK-cell immune responses remains to be elaborated.

SIGNAL TRANSDUCTION IN NATURAL KILLER CELLS

NK cells receive two basic types of external stimuli from cytokines and chemokines, and from target cell recognition. In general, their cytokine and chemokine responses are related to those found in other cells responding to the same pathways. However, as can be appreciated from the previous description of individual target recognition receptors, the pathways leading to target killing are complicated because some receptors are coupled to ITAM-containing signaling chains (CD3ζ, FcεRIγ, or DAP12). Indeed, some receptors use more than one of these signaling chains, which are generally thought to be equivalent but not dissected in detail. Other receptors, such as NKG2D (through DAP10), have Tyr motifs for recruitment of PI3K and Grb2. Still others (2B4 and related receptors) have ITSMs. Moreover, integrin signaling also affects NK-cell activation by targets, and it was reported that NKG2D signaling through DAP10 can affect IL15 responses and vice versa,[675] which highlights the possible cross-talk of signaling pathways more broadly in immune cells.[676] Finally, because individual NK cells can express (and use) multiple different receptors simultaneously, and some but not all work synergistically,[674] the outcomes of these activation pathways may reflect profound complexity, even without consideration of inhibitory receptor signaling events.[677]

The study of NK-cell signal transduction also comes with some caveats. Most studies have been performed on bulk populations of NK cells, NK cell clones, or in vitro adapted cell lines that may not be representative of pathways triggered in individual NK cells which may express only some but not all signaling molecules detected in the entire NK-cell population. When NK cells are triggered by target cells, there may be unknown stimulation through other receptors because unknown target ligands may inadvertently trigger or modulate functions of other NK-cell receptors. Finally, there may be species (human versus mouse) differences in signaling. Despite these complexities, themes have emerged in understanding of NK-cell signal transduction.

For the signal transduction pathways stimulated by NK-cell activation receptors coupled to ITAM-containing signaling chains, downstream events resemble those found in TCR and BCR signaling.[678] Activation receptor cross-linking leads to ITAM phosphorylation by Src family tyrosine kinases, recruitment and activation of Syk family tyrosine kinases, and subsequent downstream activation events. Inasmuch as TCR and BCR signaling is covered in detail elsewhere, we will highlight here notable differences with TCR and BCR signaling. 1) Individual NK cells may simultaneously express multiple activation receptors, each of which may associate with different ITAM-signaling chains that could be phosphorylated by different Src family tyrosine kinases and/or lead to different downstream signaling events. 2) NK cells express multiple Src kinases, including Lck, Fyn, Src, Yes, Lyn, and Fgr,[679] with redundancies in their contributions to ITAM phosphorylation. For example, Fyn is activated following Ly49D (DAP12) cross-linking but NK cells deficient in Fyn, Lck, or both still kill in a Ly49D-dependent manner.[680] 3) NK cells express both Syk family tyrosine kinases, ZAP-70, and Syk itself, whereas T and B cells express either, respectively, but not both. Deficiency of either ZAP-70, Syk, or both has only minimal effects on NK cell killing,[681] suggesting other pathways for transmitting NK activation signals. 4) NK cells express many adapter molecules found in T and B cells, but their contributions to NK signal transduction are less well defined. For example, SLP-76 and linker for activation of T cells are dispensable for NK-cell signaling, although they are both required for T-cell signaling.[682,683] 5) Different isoforms of signal transduction molecules are responsible for NK-cell activation. For example, mouse NK cells utilize phospholipase C-γ2 (PLCγ2) as a critical signaling mediator[684] more like B cells than T cells.[685] 6) In contrast to T and B cells, NK-cell signaling components are generally not required for normal NK-cell development. For example, NK cells are not deficient in number or maturation state in PLCγ2-deficient mice, unlike B cells, which require PLCγ2 for development.[684,685] Taken together, these findings highlight differences between NK-cell and T- and B-cell signaling.

Illustrating some of the aforementioned issues are studies on the Vav family of guanine nucleotide exchange factors, Vav1, Vav2, and Vav3, which are all expressed in mouse NK cells.[686] Vav2 and Vav3 are required for FcRγ and DAP12 signaling, whereas Vav1 is required for DAP10 signal transduction. Additionally, NK-cell number and differentiation are not apparently affected by Vav deficiencies even when they are required for NK-cell activation. In contrast, deficiencies in Vav family members lead to T- and B-cell development defects.[687] These observations also indicate the redundancies in NK-cell receptor signaling.

On the other hand, Vav1 appears to be central to human NK-cell activation by targets[688,689] even though the role of other Vav isoforms in human NK cells is less clear. Vav1 is also involved in macromolecular complexes with c-Cbl that are formed during NK-cell activation.[690] Interestingly, c-Cbl provides an inhibitory influence on NK-cell activation.[689] During NKG2D and 2B4 synergistic signaling, strong Vav1 signals override the c-Cbl effect to allow NK-cell activation, again placing Vav1 as a central player in human NK-cell activation.

Recruitment and activation of PI3K appears to be critical to NK-cell activation for target killing.[691,692] How PI3K is activated depends on the receptor and its proximal signal transduction events.[677] In the case of NKG2D, the YxxM motif in DAP10 is phosphorylated and recruits PI3K directly,[693] which appears to be sufficient to activate NK-cell killing in the absence of DAP12 or Syk family tyrosine kinases.[694] The pathway may be more complex in human NK cells where evidence suggests that DAP10 recruits a Grb2-Vav1 intermediate for activation.[491] For other activation receptors, Syk activation is upstream of PI3K[695] and may directly recruit PI3K.[696] PI3K in turn activates a mitogen activated protein kinase (MEK) for stimulation of extracellular signal-regulated kinase (ERK).[691] Ultimately, actin polymerization occurs, granules become polarized toward the target, and exocytosis occurs, resulting in target apoptosis, as discussed previously. Interestingly, in human NK cells, engagement of LFA-1 by ICAM-1 on insect cells was sufficient to induce granule polarization but not degranulation,[143,674] suggesting additional complexity regarding the signals required for polarization versus degranulation.

High-resolution microscopy has enabled visualization of events following NK-cell activation. Like CTLs, NK cells form discrete protein clusters at the site of contact with their targets, termed the immunological synapse,[697,698] though obviously the TCR is not involved. Following target contact, the NK immunological synapse (NKIS) is formed at the NK-target interface in discrete stages whereby receptor signaling, filamentous (F)-actin rearrangement, and polarization of granules occur.[699,700] CD2, F-actin, LFA-1, and Mac-1 accumulate in the peripheral supramolecular activation complex (pSMAC), whereas granules (perforin) and the activation receptor accumulate centrally (cSMAC).[701] Other peripheral supramolecular activation complex and centrally supramolecular activation complex components and patterns are found depending on whether or not the contacts lead to cytolysis.[698,702] With this basic outline of microscopic events, molecules required for NKIS formation and granule polarization are being dissected.[125,692,703–705]

Mechanism of Inhibition by Natural Killer–Cell Receptors

The now widely accepted view is that the NK-cell inhibitory receptor mechanism consists of ligand binding and receptor cross-linking followed by ITIM phosphorylation, and preferential recruitment of SHP-1, and that this mechanism

operates with both structural types of inhibitory receptors. A typical human KIR molecule has two ITIMs separated by ~24 residues. In contrast, Ly49A has only one ITIM per chain, but Ly49 molecules are normally expressed as homodimers. CD94 has a minimal cytoplasmic tail, but NKG2A has two ITIMs. Each ITIM can be phosphorylated upon receptor cross-linking (though difficult to visualize) or tyrosine phosphatase inhibition,[212,706–710] presumably by a Src family tyrosine kinase as in B cells,[711] although redundant expression and function of these kinases have precluded identification of a single kinase required for ITIM phosphorylation in NK cells. ITIM phosphorylation results in recruitment and activation of the intracellular tyrosine phosphatase, SH2-containing protein tyrosine phosphatase (SHP)-1 (also known as hematopoietic cell phosphatase (HCP), protein tyrosine phosphatase 1C (PTPIC), and protein-tyrosine phosphatase, nonreceptor-type, 6(PTPN6)).[212,706–708,712] Two ITIMs are required for sequential binding of the two SH2 domains in SHP-1.[710,713] The NK-cell MHC-specific inhibitory receptors preferentially recruit SHP-1 rather than SH2-containing inositol polyphosphate 5-phosphatase (SHIP), though they may occasionally bind SHP-2.[706,714,715] Inhibitory receptor engagement results in early KIR and SHP-1 recruitment to areas surrounded by LFA-1.[716] Moreover, early events in NK-cell activation are blocked by inhibitory receptors, such as formation of the NKIS.[716–718] On the other hand, KIR phosphorylation occurs within clusters at the NKIS, which may serve to focus inhibition on downstream targets,[719] such as Vav1, which is recruited into the mature synapse during NKG2D/DAP10 signaling.[704] Indeed, the downstream target of the ITIM-recruited tyrosine phosphatase appears to be Vav1 in human NK cells activated by targets.[688] Thus, the general consensus has long been that the inhibitory receptors recruit tyrosine phosphatases that dephosphorylate molecules in the activation receptor cascade, such as Vav1.

On the other hand, related ITIM-containing inhibitory receptors bind other effector molecules, such as Csk by LAIR-1 and LILRB1,[423,720] suppressor of cytokine signaling 3 (SOCS3) by Siglecs,[721] and SHIP by KLRG1[722] and FcγRIIB.[723] Even a KIR ITIM may recruit other molecules such as β-arrestin[724] and PI3K.[725] Morever, a SHIP-deficient mouse demonstrates abnormalities in NK-cell function and Ly49 receptor expression,[726] even though Ly49 receptors recruit SHP-1. The functional significance of these signaling effector molecules in NK-cell inhibition and the specificity for ITIM recruitment of selective molecules are poorly understood and is potentially related to the complexity of inhibitory receptor signaling.

Emerging data suggest that inhibitory receptor signaling may not be as simple as phosphatase recruitment to dephosphorylate proximal molecules in the activation pathway that then prevents all downstream signaling events. For example, inhibitory receptors efficiently blocked granule polarization but inefficiently prevented degranulation.[727] During ongoing NK-cell activation, KIR2DL2 engagement by photoactivated HLA-C ligand disrupted the NKIS but had little effect on ongoing calcium flux.[728] Thus, some but not all of the signaling events downstream of NK-cell activation receptors may be effectively regulated by the inhibitory receptors, directing future studies to selected aspects of NK-cell signal transduction.

Inhibitory signaling also induces other events. Normal human NK-cell activation by susceptible targets led to Tyr phosphorylation of Vav1 and its association with c-Cbl, which was also Tyr phosphorylated.[690] In turn, c-Cbl was associated with a signaling complex consisting of an adaptor protein (Crk, either CrkII or CrkL, depending on the cell), a scaffold protein (p130CAS, 130 kDa Crk-associated substrate), and C3G (Crk SH3 domain-binding guanine-nucleotide exchange factor). These c-Cbl complexes have the potential to signal a wide variety of cellular pathways including Rap1 GTPase activation, actin reorganization, cell adhesion, and mitogen-activated protein kinase activation,[729] which appear to be critical for NK killing. By contrast, ligand engagement of either KIR or CD94/NKG2A blocked the formation of Cbl-Crk-p130CAS-C3G complexes.[690] Moreover, ligand engagement of either KIR or CD94/NKG2A induced the association of the tyrosine kinase c-Abl with Crk, and Crk phosphorylation that was shown to be required for active dissociation of Crk from Cbl and for inhibition. Thus, these studies showed that inhibitory signaling ironically involved Tyr phosphorylation and disruption of macromolecular signaling complexes, which in turn, suggest that NK-cell activation (and inhibition) may be subject to more qualitative controls than previously recognized.

Another possible explanation for inhibitory signaling is related to the inhibitory influence that c-Cbl has on NK-cell activation[689] and other immune cells, such as T and B cells.[730] Although Cbl family molecules exhibit E3 ubiquitin ligase activity, and may exert their negative effects on lymphocyte signaling by enhancing degradation of signaling components,[731] there are as yet little data supporting this mechanistic role for Cbl in NK-cell activation.[689,690] Nonetheless, current information suggests additional aspects of NK-cell inhibitory receptor signaling are yet to be discovered.

Finally, Ly49 receptors on NK cells can acquire their cognate MHC-I ligands from surrounding cells, resulting in display of both molecules on the NK cells.[732,733] Broadly speaking, this effect is probably related to trogocytosis, a poorly understood phenomenon of intercellular transfer of surface molecules.[734] How much this contributes to *cis* effects on inhibitory receptor function and its physiological relevance require further evaluation.

NATURAL KILLER–CELL TOLERANCE

NK cells display potent effector functions that must be controlled to prevent inadvertent damage to normal tissues (ie, NK cells must demonstrate tolerance to self). Although the missing-self hypothesis provides a rationale and starting point for dissection of NK-cell tolerance, it has a number of caveats that need to be considered before delving into current understanding of this aspect of NK-cell biology.

Influence of Host Major Histocompatibility Complex–I Environment

The missing-self hypothesis implies that there should be overt NK-cell autoreactivity in MHC-I–deficient hosts, but this was not observed in humans or mice.[179–181,735–738] Instead,

NK cells from MHC-I–deficient mice demonstrate poor killing of MHC-I–deficient targets or rejection of MHC-I–deficient BM, even though they appear normal in number, tissue distribution, and expression of activation receptors. Another very difficult issue is related to hybrid resistance whereby host NK cells in an F_1 hybrid animal can reject BM grafts from either inbred parent.[739,740] It was difficult to understand how NK cells discriminate between cells expressing the full complement of self-MHC alleles versus those otherwise normal cells expressing only some self-MHC molecules. NK-cell–mediated rejection of MHC-I–sufficient BM grafts also depends on which host MHC-I allele is present.[741,742] Thus, NK cells are regulated by host MHC environment, not just by the target cell in effector responses.

The MHC-specific inhibitory receptors were likely to be involved in self-tolerance because they explain the influence of MHC expression on NK-cell effector functions against target cells. However, how these receptors are related to self-tolerance in vivo was a challenging issue. Several hypotheses were proposed. An individual NK cell can simultaneously express multiple inhibitory receptors but some may not recognize self-MHC. The "at least one receptor" model suggests tolerance is achieved as long as each NK cell expresses at least one receptor with self-MHC specificity.[389,743] There are changes in the repertoire of MHC-I–specific inhibitory receptors, depending on the MHC haplotype, but these differences are modest, regardless of whether the receptor is self-specific or not.[210,248,389,743,744] As determined by antibody reactivity, the expression level of an NK receptor on an individual NK cell decreases when the host expresses the MHC ligand for that receptor.[208,209,745] Functional analyses of NK cells with "downregulated" receptor expression suggest that such NK cells are more sensitive to small changes in MHC ligand expression on the target, as explained by the "receptor calibration" model.[744,746] However, these studies involved in vitro conditions that could alter their intrinsic functional capacities. Thus, several hypotheses based on the MHC-specific inhibitory receptors were proposed to account for NK-cell self-tolerance.

Also unexplained was the observation that NK cells were found in wild-type mice expressing none of the known self-MHC–specific inhibitory receptors. These cells were functionally "hyporesponsive," showing defective antiactivation receptor cross-linking and killing of NK-sensitive targets,[747] but it was possible that other, yet to be defined receptors were playing a role. Another issue was the appropriate pairing of polymorphic inhibitory receptors with their highly polymorphic cognate MHC ligands.[260,317] The genes for the receptors and their ligands are located on different chromosomes (ie, the receptor and ligand genes segregate independently). Thus, there must be mechanisms to provide NK cells with the appropriate inhibitory receptors with specificity for self-MHC because the appropriate pairs are not inherited together, unlike the closely linked *Nkpr1-Clr* gene pairs.[575]

The Licensing Hypothesis

Studies with target cell-free stimulation and single cell assays of NK-cell responsiveness revealed insight into NK-cell tolerance.[168,747,748] Freshly explanted, resting murine NK cells from MHC-I–deficient mice were functionally defective in triggering by immobilized antiactivation receptor antibodies.[168] Conversely, in wild-type mice, functional competence correlated with expression of a Ly49 inhibitory receptor for self-MHC-I.[168] CD94/NKG2 receptors appeared not to be relevant,[168] as confirmed by studies of a CD94-deficient mouse.[395] Particularly informative were studies with a single chain trimer MHC-I molecule, consisting of antigenic peptide-linker-β2m-linker-H2K^b as a single polypeptide that binds only Ly49C.[168] In Tg mice expressing only this MHC-I molecule, only Ly49C+ NK cells were functionally competent. Although crystallographic studies revealed two potential sites on MHC-I that could be recognized by Ly49 receptors, studies of Tg mice bearing mutant MHC molecules indicate that the Ly49 receptors recognize the same site on their MHC ligands (site 2) for both education and effector inhibition.[749] Thus, NK cells use their MHC-specific receptors interacting with self-MHC-I to become competent to be triggered through their activation receptors by (Fig. 17.6), an education process termed "licensing."

Despite initial confusion over the term licensing,[750] most investigators now concur that licensing or education by self-MHC–specific receptors results in fuctionally competent NK cells.[751] Moreover, human NK cells are also subjected to a similar process involving KIR and self-HLA ligands[752,753] that may also explain clinical studies relating KIR and HLA alleles and disease and even human immunodeficiency virus (HIV) control.[754,755] Such KIR-HLA relationships frequently involve pairs with high affinities and resolution of chronic infections[756] that are difficult to explain when only considering effector inhibitory function of the KIRs. Thus, licensing may be clinically relevant and may be applicable to other clinical uses of NK cells (see following discussion) because it is a second function of the NK-cell receptors for MHC.

Licensing leads to appropriate pairing of inhibitory receptors with self-MHC and strongly suggests that there are two types of self-tolerant NK cells (see Fig. 17.6).[750,757] Regardless of the MHC-I environment, licensed NK cells are tolerant because they have inhibitory receptors for self-MHC, the same receptors involved in licensing. Unlicensed NK cells are also tolerant because they are not functionally competent and have no need for inhibition by self-MHC under steady-state conditions. In hosts heterozygous for MHC alleles, each MHC allele could potentially license different NK-cell populations. This aspect of licensing is relevant to hybrid resistance because an (A × B)F_1 hybrid animal should have NK cells that are separately licensed on different MHC alleles.[750] F_1 hybrid NK cells that were licensed by MHC alleles from parent A should be inhibited by A alleles but not B alleles, and thus reject BM from parent B. The converse should also be true. In the F_1 animal itself, NK cells are licensed by either parental allele so all NK cells should be inhibited by normal tissues that codominantly express both MHC alleles. Licensing potentially explains how NK cells distinguish cells expressing the full complement of MHC-I from those expressing only some alleles.

Although initial studies on licensing were focused on only a few receptors and MHC alleles to demonstrate convincing effects, subsequent studies have demonstrated that

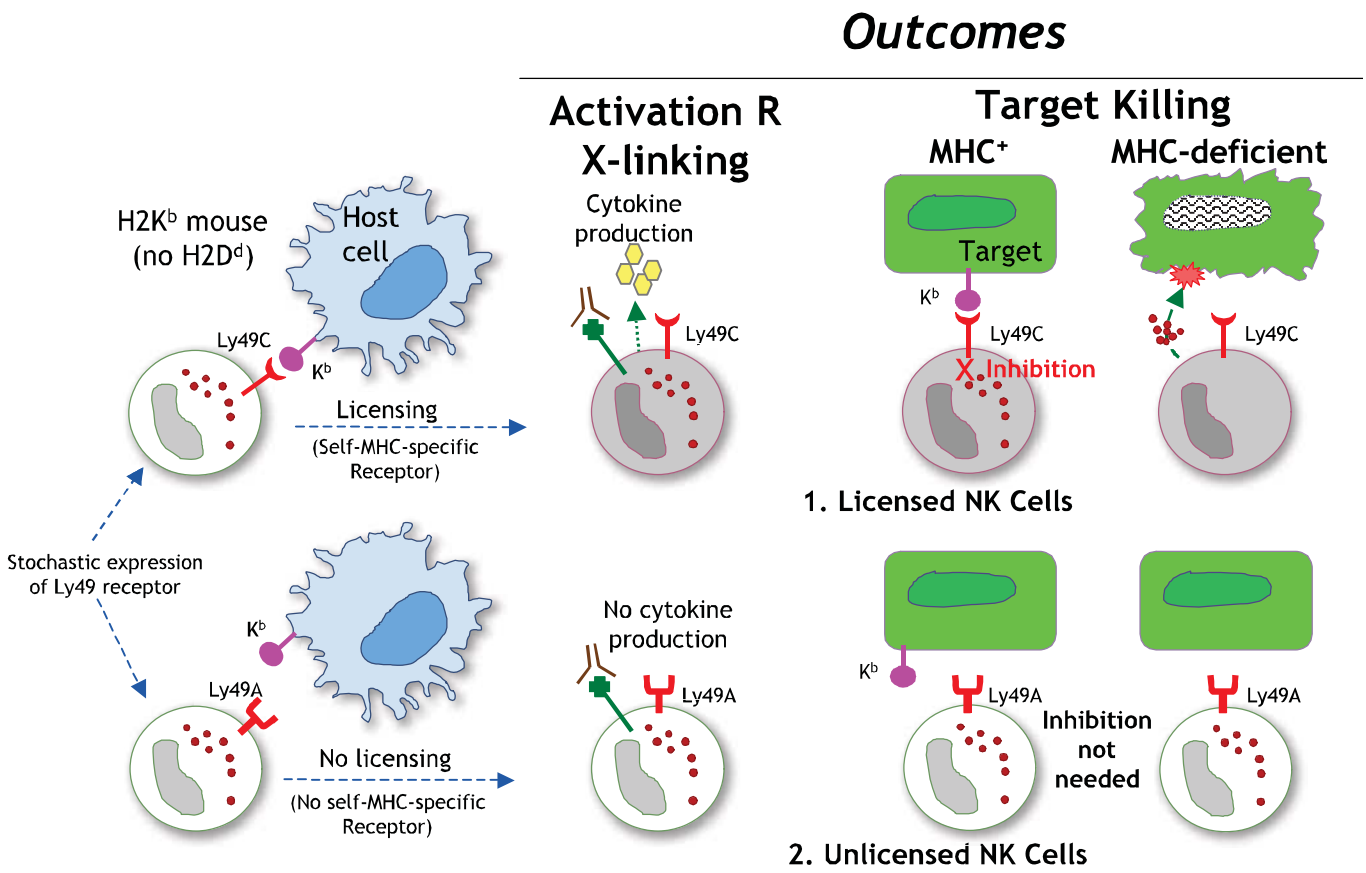

FIG. 17.6. Licensing of Natural Killer (NK) Cells by Host Major Histocompatibility Complex (MHC)-I. Depicted is the situation in a mouse expressing only H2K^b that is a ligand for Ly49C but not Ly49A. Cells expressing Ly49C therefore have a self-specific receptor, whereas those expressing Ly49A do not. Ly49C engagement by self-MHC-I results in a licensed NK cell that has functional competence to be triggered through its activation receptors. These cells can be inhibited by self-MHC through the same receptor that conferred licensing. Ly49A+ NK cells remain self-tolerant because they are unlicensed. For clarity, only one MHC allele and cells expressing one Ly49 receptor are shown. Activation receptors are also not shown for the target killing depiction. Normally, the host has multiple MHC alleles, each of which could license different NK cells, and individual NK cells express multiple Ly49s simultaneously. Each NK cell is likely to be licensed separately, potentially by different MHC alleles, and through different Ly49s. Modified from Elliott and Yokoyama.[750]

the licensing status of an individual NK cell is dependent on the Ly49 receptor, MHC allele, and the number of expressed self-MHC–specific Ly49 receptors.[219,758] For example, different MHC alleles appear to be more potent in licensing Ly49A+ NK cells, in a hierarchical manner.[219] The same hierarchy of MHC alleles was seen with Ly49A downregulation and inhibition of Ly49A+ NK cells in effector responses. An MHC allele was more potent at inhibition as compared to licensing, providing a margin of safety against NK-cell autoreactivity.[219,759] Interestingly, MHC heterozygosity was comparable to homozygosity with capacity of an MHC allele to license.[219] Studies of mice bearing one, two, or more MHC alleles also show similar effects.[758] These results have prompted a refinement of the licensing hypothesis (ie, the "tuning" or "rheostat" model) whereby the strength of inhibitory receptor engagement by self-MHC determines the degree to which an NK cell is capable of being activated.[750,760]

Where and with whom licensing occurs is currently unclear. While original studies suggested that licensing occurs during development in the BM,[168] adoptive transfer of otherwise unlicensed NK cells from an MHC-deficient host into an MHC-sufficient environment results in licensed donor NK cells.[761,762] The converse was also observed (ie, loss of function when wild-type NK cells were transferred into an MHC-deficient environment). These studies add to the discussion of whether an NK cell interacts with self-MHC in *trans* (on another cell) on in *cis* (on the same NK cell).[763] What is difficult to exclude is the possibility that NK cells may take up MHC-I from other cells via their MHC-specific receptors,[732,733] but the transfer studies indicate that the NK cell itself does not need to synthesize MHC-I. Thus, licensing may be a dynamic process in which NK cells constantly test the same site on their MHC-I ligands to simultaneously maintain functional competency and effector inhibition.

Although licensing is a positive outcome (ie, the acquisition of functional competence), it requires the ITIM of the self-specific Ly49 receptor to deliver signals that ultimately result in a licensed NK cell[168] (see Fig. 17.6). The nature of such signals is not yet known, and at least two qualitatively different models are being considered. The MHC-specific receptor could confer licensing in a positive way by delivery of signals that directly induce a differentiation process and

license NK cells, akin to a stimulatory receptor and also known as the "arming" hypothesis[764] In the second model, also known as the "disarming" hypothesis,[764] the MHC-specific receptors decrease the effect of a putative, as yet uncharacterized, self-specific activation receptor. If unimpeded by self-MHC-I, NK cells become hyporesponsive due to overstimulation through the activation receptor. This model is somewhat akin to T-cell anergy and places the self-MHC–specific receptor in a familiar light as an inhibitory receptor. Both models are similar to those previously proposed,[765] but engagement of the MHC-specific receptor with self-MHC is now known to be a required key step[168] and is common to both models. Also, the models are not mutually exclusive and either could be affected by coreceptors or adhesion molecules. Regardless, licensing may be explained by either the arming or disarming hypothesis.[750]

For the arming hypothesis, it is difficult to reconcile the involvement of the ITIM in the self-MHC specific receptor in licensing,[168] though recent analysis shows that inhibitory receptor signaling is more complex than previously recognized.[689,690] Recent studies also suggest that there are few differences in genes expressed by licensed verus unlicensed NK cells.[766] Instead, in licensed NK cells, the activation receptors appeared to be localized in nanodomains in the plasma membrane, whereas in unlicensed cells, the activation receptors were apparently confined to the actin meshwork.[766] Future studies will be needed to validate this correlation and how the self-MHC–specific receptors mediate these changes.

For the disarming model, studies on mice constitutively expressing activation receptor ligands by transgensis or retroviral transduction of hematopoietic stem cells have been informative.[534,538,767,768] In support of the disarming hypothesis, continuous engagement of NKG2D or Ly49H resulted in hypofunctional NK cells, akin to anergy. Indeed, m157 Tg mice could not resist MCMV infections normally, and the Ly49H+ NK-cell subset was selectively hyporesponsive. However, the anergic phenotype was unaffected by expression of a self-MHC–specific receptor.[767] Although it remains possible that receptor-ligand interactions that normally govern NK-cell self-tolerance were not well represented in these Tg mice, the current data nonetheless do not support the disarming hypothesis as an explanation for licensing and suggest that "anergy" may be a distinct tolerance mechanism for NK cells, perhaps analogous to separable tolerance mechanisms for T and B cells.[769]

In addition to bone marrow rejection, licensed NK cells may provide primary protection in viral infections and may enhance CD8+ T-cell responses.[770–772] On the other hand, unlicensed NK cells may gain functional competence by exposure to cytokines.[168] During MCMV infection, unlicensed NK cells (ie, cells without self-MHC–specific receptors) appear to control viral replication in both MHC-deficient and -sufficient mice.[773–775] Thus, the role of self-tolerant NK cells in immune responses requires additional study.

NATURAL KILLER–CELL DEVELOPMENT

Early evidence indicated that the complete phenotypic and functional maturation of NK cells occurs in the BM and requires an intact microenvironment because BM ablation or congenital BM defects lead to abnormal NK cells.[776–780] Indeed, certain aspects of NK-cell development are regulated by direct interactions between developing NK cells and stromal elements, such as interactions between membrane lymphotoxin-α (LTα)–expressing NK-cell precursors (NKPs) and LTα–responsive stromal cells that are necessary for normal development.[154,781] In vitro, NK cells can be generated from early hematopoietic cells in a cytokine cocktail consisting of stem cell factor (c-kit ligand), IL-7, flt-3 ligand, and IL-15.[782–789] Direct contact with stromal cells appears to be required for acquisition of Ly49 receptors by developing NK cells. Moreover, the Tyro3 family of receptors (Tyro3, Mer, Axl) on NK cells and their ligands (Gas6, protein S) on stromal cells are apparently necessary for expression of NK-cell receptors and functional differentiation in vitro and in vivo.[790,791] Thus, NK-cell development requires certain cytokines and direct stromal cell contact.

Although the general topic of NK-cell development is currently an active area of investigation, and is thus subject to future modifications, several major themes have emerged that will be summarized here. The earliest step involves the commitment of hematopoietic stem cells in the BM to the common lymphoid progenitor that can give rise to NK, T, and B cells, but not to myeloid cell lineages.[792,793] Mice deficient in various transcription factors, including Ikaros and PU.1, display severe defects in the development of all lymphoid cells including NK cells, while myeloid and erythroid lineages are less affected.[794–798] Thus, NK-cell development appears to share a common pathway with other lymphocytes, providing evidence that NK cells belong to the lymphocyte lineage.

Commitment to the NK-cell lineage from the common lymphoid progenitor (CLP) appears to involve an intermediary cell with T- and NK-cell potential (T/NK progenitor), perhaps reflecting the close resemblance of T- and NK-cell effector functions. Consistent with this bipotentiality, CD3ε and FcεRIγ Tg mice exhibit selective defects in both NK- and T-cell development.[799,800] Moreover, deficiencies in several genes, including transcription factors and IL-15,[54,801] affect development of NK-cell and certain T-cell subpopulations, particularly NKT cells and memory T cells. T/NK progenitor populations, including immature thymocytes, can give rise to T and/or NK cells but not to other lineages, depending on culture conditions, such as Notch signaling.[802–808] Interestingly, the transcription factor Bcl11b is required to maintain T-cell fate by apparently repressing NK-cell fate. Specifically, Bcl11b is required for T-cell specification,[809–812] and its deletion, in committed T-cell precursors or even mature T cells, results in loss of T-cell phenotype and instead acquisition of NK cell-like phenotype,[809] highlighting the developmental pathways shared by these cell lineages.

NK cells also have a developmental relationship to Lymphoid Tissue inducer (LTi) cells. In addition to phenotypic similarities as previously discussed for NK-22 cells, LTi and NK cells also share a relatively unique feature in expressing surface LTα, which influences development of lymphoid tissues and NK cells, respectively.[154,781] Moreover, deficiencies of either Ikaros or Id2 result in defects in LTi and NK cells, though other cells are also affected.[813,814] Interestingly,

mice lacking the thymocyte selection-associated high mobility group box protein DNA-binding protein lack both LTi and CD122+ NK cells.[815] The NK-cell defect is NK cell–intrinsic, and Id2 overexpression could not compensate for thymocyte selection-associated high mobility group box protein (TOX)–deficiency.

Until recently, the acquisition of IL-2/15Rβ subunit (CD122) was considered to mark the earliest identifiable committed NKP.[805,816] However, an earlier progenitor population (pre-NKP) has been identified that has not yet expressed CD122.[817] Upon adoptive transfer, these lineage-negative (lin–) cKit– Flk2– CD27+ CD244+ IL-7Rα+ CD122– cells from the BM could give rise to NK cells in the BM, blood, and spleen but no other cell lineages. Thus, IL-15 is not required for commitment to the NK cell lineage, even though it is required for subsequent NK-cell developmental stages as illustrated by the persistent expression of CD122.[44,53–57]

CD122 expression is regulated by the combined action of the T-box transcription factors, T-bet (Tbx21) and Eomesodermin (Eomes) that have redundant function in NK-cell development.[801,818] Specifically, *Tbx21*–/– *Eomes*+/– mice have marked decreases in CD122 expression and NK cells, whereas *Tbx21*–/– *Eomes*+/+ mice manifest no defect in CD122 expression and more modest defects in NK-cell number. Moreover, Eomes targets the promoter of *Cd122*. IL-15 appears to regulate the basic leucine zipper (bZip) transcription factor E4BP4 (E4 binding protein 4,[819] also known as nuclear factor IL-3[820]) in NK-cell development. *E4bp4*–/– mice display profound NK-cell deficiency.[821–823] Gene transduction of *E4bp4*–/– hematopoietic stem cells with *E4bp4* rescued NK-cell development in vitro even in the absence of IL-15, suggesting that E4BP4 acts downstream of IL-15 signaling. *E4bp4*–/– mice show no alterations in other hematopoietic cells except for a defect in CD8a+ cell development.[824] This latter finding may be relevant to phenotypic overlap between NK cells and DCs.[115–119] Regardless, Eomes affects NK-cell development by promoting CD122 expression, whereas E4BP4 affects commitment to the NK-cell lineage by apparently acting downstream of IL-15.

Committed NKPs next differentiate into mature NK cells in a series of putative developmental intermediate stages that occur in the BM.[98,816] Originally defined by correlating phenotypic markers, including NK-cell receptors, integrins, and other molecules with apparently distinct developmental stages,[825,826] these stages have been confirmed by studies following serial acquisition of markers during NK-cell differentiation in vitro.[789] Regardless, these stages of committed NKPs are undergoing constant refinement as new rate-limiting steps are being uncovered, such as in studies of mice lacking specific transcription factors or as new markers and tools are developed.[827] Nonetheless, a few highlights should be noted.

NK-cell receptors involved in target recognition are expressed at immature stages, with NK1.1 and CD94/NKG2 being among the first, followed by NKp46, and NKG2D then the Ly49s.[827] Following Ly49 acquisition, developing NK cells undergo spontaneous proliferation at an immature stage.[98] Thereafter, as NK cells acquire high-level expression of Mac-1 (αMβ1) and CD43, proliferation markedly decreases unless challenged by pathogens such as viruses that can stimulate mature NK-cell proliferation.[98,166] NK cells then gain functional activities associated with splenic NK cells, indicating that Mac-1 expression correlates with a late maturation stage. A selective NK-cell deficiency in a Tg mouse is manifested by a failure to become mature, functional Mac-1^{hi} cells,[828,829] consistent with this differentiation step. Finally, while CD27 is expressed from the very earliest stages of NK-cell development,[817] its diminished expression within the Mac-1+ subset is associated with a more responsive capacity.[830] Thus, Mac-1 and CD27 expression are now commonly used to ascertain NK-cell maturation in the mouse.

In addition to the transcription factors mentioned previously, several other transcription factors, including Ets-1, Id2, IRF-1, MEF, and GATA-3, have been implicated in NK-cell development and function.[813,831–836] The need for Id2 in NK-cell development can be overcome by deletion of the gene for the E protein, E2A.[814] IRF-1–deficient mice lack NK cells, and this defect can be overcome by the addition of IL-15, suggesting that stromal cells produce IL-15 in an IRF-1–dependent manner.[813] Expression of Bcl2 restored the CD8+ T-cell deficiency but not the NK-cell deficiency in IRF-1–deficient mice.[837] IRF-2–deficient mice manifest a late defect in NK-cell maturation with normal constitutive proliferation but increased apoptosis thereafter.[838,839] In contrast to the other transcription factors, GATA-3 does not affect NK cell number; rather, it controls their IFNγ production and their homing to the liver.[836] Similarly, CCAAT/enhancer binding protein (C/EBP) γ-deficient NK cells display defective effector functions.[840] The relationships of the transcription factors to each other are just beginning to be elaborated in terms of regulating NK-cell development and function.

As for mouse NK cells, human NK-cell developmental stages have been identified by correlating markers on in vivo subsets.[841–844] In general, these stages have been recapitulated by in vitro development of functional human NK cells from hemapoietic stem cells from umbilical cord blood or differentiated embryonic stem cells.[845,846] As in the mouse, a combination of cytokines and stromal cells is necessary for in vitro differentiation.

These studies suggest that human NK cells differentiate in secondary lymphoid tissues as revealed by studies of CD56^{bright} and CD56^{dim} NK cells.[110,847] CD56^{bright} NK cells preferentially express CD94/NKG2 receptors (versus KIRs), are CD16–, and are better cytokine producers and less efficient killers than the CD56^{dim} subset that tend to be KIR+ and CD16+.[109] A CD34+ CD45RA+ hematopoietic precursor cell (HPC) was identified that expressed the integrin $\alpha_4\beta_7$ at high levels.[847] This population was found at low levels (< 1% of HPCs) in the BM but was markedly enriched in lymph node (LN; > 95% of HPCs), and could be differentiated into CD56^{bright} NK cells in vitro. On the other hand, in nonreactive LNs, NK cells tend to be CD56^{bright} whereas in reactive LNs, they tend to be CD56^{dim}, the predominant population in peripheral blood.[110] CD56^{bright} cells acquire the CD56^{dim} phenotype when stimulated in vitro with proinflammatory

cytokines, such as IL-12, or with IL-15 in *trans* in a humanized mouse model.[848] Taken together, these data suggest that human NK cells differentiate in secondary lymphoid tissues in response to inflammation by first becoming $CD56^{bright}$ then completing maturation to the $CD56^{dim}$ phenotype.

Although the maturation of mouse NK cells in secondary lymphoid tissues has not been studied carefully, there is an abundance of NK cells with an immature phenotype in organs, such as the liver.[98] On the other hand, recent studies also provide evidence for thymic maturation of a subpopulation of mouse NK cells, termed "thymic" NK cells[111] These cells are characteristically CD127+ $CD69^{high}$ $Ly49^{low}$ $CD11b^{low}$ in contrast to conventional resting splenic NK cells that are CD127− CD69− $Ly49^{hi}$ $CD11b^{hi}$. Thymic NK cells were enriched in LNs and are absent in GATA-3-deficient and in athymic nude mice, indicating that these cells require GATA-3 and an intact thymus for their development. Indeed, thymic NK cells can develop in vivo and in vitro from double negative (CD4− CD8−) 1 (DNI) subsets of immature thymocytes.[789] Interestingly, mouse thymic NK cells bear some phenotypic markers that tend to be found on human $CD56^{bright}$ NK cells.[111] The contribution of secondary lymphoid tissues to NK-cell development, the role of thymic NK cells in immune responses, and the relationship of these mouse and human NK-cell subsets remain to be clarified.

ROLE OF NATURAL KILLER CELLS IN IMMUNE RESPONSES

Mature NK cells in the periphery are involved in rapid innate defense. However, they constitute only a small population of cells (about 2.5% of splenic leukocytes in C57BL/6 mice). How can this small population quickly respond with enough of a critical mass to effect significant innate defense? One mechanism involves the expression of multiple activation receptors by individual NK cells.[449] By contrast to clonally distributed TCRs endowing the individual T cell with the ability to respond only to one antigen, an individual NK cell appears capable of responding to multiple activation receptor ligands. Furthermore, the naïve T-cell population contains only rare cells with a TCR for the relevant antigen, whereas large percentages of the NK-cell population express any given activation receptor in an overlapping fashion. This multiple activation receptor expression on sizeable subpopulations would allow a substantial number of NK cells to quickly respond to a given specific insult. Another mechanism is related to their constitutive expression of cytokine receptors that permit many NK cells to be stimulated by proinflammatory cytokines produced early in the course of an immune response. Finally, NK cells appear poised to respond rapidly. They constitutively express mRNA for effector molecules, such as granzymes and cytokines, but no protein due to translational control, and can rapidly produce these molecules upon stimulation.[29] Thus, large numbers of NK cells can rapidly respond to a particular stimulus through their activation or cytokine receptors.

Where do NK cells respond? In the mouse, mature conventional NK cells are found primarily in the spleen, blood, and liver. Interestingly, NK cells are localized to the red pulp of the spleen.[849,850] In the liver, they are in the sinusoidal regions rather than the parenchyma, and few NK cells are present in other solid organs. In viral infections, NK cells infiltrate the liver parenchyma in the vicinity of infected foci.[849,851] Several chemokines, including MIP1α, have been implicated in NK-cell localization to liver parenchyma during immune responses,[852] for example, but the NK cell "chemokine code" needs to be clarified.[853] Surprisingly, there are relatively few NK cells in naïve lymph nodes,[849,854] and the thymic NK-cell phenotype is overrepresented among the few resident NK cells,[111] although NK cells can be recruited to draining LNs.[855,856] However, human NK cells are relatively abundant in LNs where they appear to mature, as discussed previously. Study of human NK-cell responses in solid tissues has been limited for obvious reasons. Nonetheless, NK cells seem best suited for surveying the blood for transformed or infected cells and pathogens during acute immune responses but can be recruited to pathologic sites as needed.

As early innate immune responders, NK cells have the capacity to shape the adaptive immune response. For example, early NK-cell control of viral infection essentially limits antigen load and presentation by DCs thereby limiting CD4+ T-cell responses.[857] By contrast, early control also reduces activation of plasmacytoid DCs, thereby lessening the detrimental effects of type I IFNs, preserves the conventional DC compartment, and accelerates CD8+ T-cell responses.[858] Early IFNγ production by NK cells can prime and polarize T-helper responses.[855,859–861] In leishmania infections, later production of IL-10 by NK cells has an inhibitory effect on T cells.[862] In addition, NK-cell lysis of targets also enhances T-cell responses.[863] Thus, NK cells have the capacity to enhance, suppress, and polarize adaptive immune responses.

Natural Killer Cells and Tumor Surveillance

An abundant early literature supported a role for NK cells in resisting tumor growth and metastasis.[3] Most prior work utilized experimental protocols involving adoptive transfer of tumor cells into mice where NK cells may eliminate > 90% of tumor cells within the first 24 hours.[864] Several long-term assays of in vivo tumor clearance are available, such as survival or lesion size, but T-cell responses need to be excluded even in syngeneic hosts due to the possibility of tumor-specific peptides. Clearance of intravenously administered radiolabeled tumor cells can be measured with radioactivity of the lung as an index of tumor burden. Because this lung clearance assay can be performed as early as 4 hours after tumor inoculation, it is relatively confined to innate NK-cell responses in the unimmunized host.[15] In these assays, NK-deficient mice are unable to clear adoptively transferred tumors.[828]

On the other hand, few studies are available on control of primary tumor formation by NK cells (ie, tumor surveillance).[865] Probably the most systematic studies in a single experimental model involve fibrosarcoma development after subcutaneous methylcholanthrene challenge.[866–868] In brief, NK cells collaborate with NKT cells in preventing

fibrosarcomas through mechanisms involving perforin, TRAIL, and IFNγ. There is also evidence for involvement of NKG2D[86] and its ligands with respect to the immunoediting of tumors by NK cells.[525,542]

In humans, epidemiological data indicates a strong correlation between high cytotoxic activity of peripheral blood lymphocytes and reduced cancer risk.[869] In addition, ongoing studies of BM transplantation for leukemia demonstrate a relationship between KIR, HLA alleles, and reduced risk of relapse from leukemia in some circumstances.[870,871] Thus, current data support the initial concept that NK cells are involved in tumor immunosurveillance.

Natural Killer Cells in Host Defense Against Pathogens

While NK cells reportedly respond to a wide variety of microorganisms including viruses, bacteria, parasites, and fungi,[872,873] the role of NK cells in infections is probably best illustrated by human patients with selective NK-cell deficiencies.[874,875] Although the molecular basis for most human NK-cell deficiencies is unknown, several points can be gleaned. 1) NK-cell deficiency is associated with a propensity for severe or recurrent virus infections, particularly herpes viruses. 2) Difficulty with tumors is not a common feature, except for virus-related lesions. 3) The disorder is rare, perhaps because patients succumb to overwhelming infection before the syndrome is recognized. 4) Defective NK cells can be found in other genetic and acquired immunodeficiency disorders that affect other immune components. For example, NK cell activity is significantly diminished in AIDS.[876,877] NK-cell infection with herpesvirus 6 induces de novo expression of CD4 rendering susceptibility to HIV-1 infection,[878] perhaps accounting, in part, for increased susceptibility of patients with acquired immunodeficiency syndrome to opportunistic infections, such as severe CMV.[879] Immature NK-cell number and function in the developing fetus may be clinically relevant to the classic "TORCH" syndrome, birth defects associated with maternal toxoplasma, rubella, CMV, and herpes virus infections.[880] Thus, NK cells appear to be especially important in controlling infections, especially from herpes viruses.

In mouse models, detailed evaluation of NK-cell responses against *Listeria* and viral infections have been especially revealing.[881,882] In vivo antibody depletion of NK cells results in marked viral replication in internal organs (spleen, liver), and lethality with MCMV, vaccinia virus, or mouse hepatitis virus.[883–885] A similar phenotype was observed in Tg mice lacking NK cells.[886,887] Interestingly, if depleting antibody was given to wild-type mice later in the infection, there was no untoward effect.[884] Thus, NK cells are significant in early, innate immunity to infections.

Natural Killer–Cell Cytokine Responses and Production during Infection

During infection, NK cells can respond to several different cytokines resulting in production of other cytokines. In listeriosis, the classic model for T-cell–dependent resistance, *scid* mice achieve acute control of infection despite absence of T cells,[888] due to early NK-cell production of IFNγ.[889] However, NK cells do not appear to respond directly to *Listeria*. Rather, macrophages produce IL-12 that then stimulate NK-cell secretion of IFNγ and infection control.[164,890] Furthermore, TNFα can synergize with IL-12 to induce NK-cell production of IFNγ, whereas IL-10 is antagonistic.[164] The increased susceptibility of mice lacking IL-12 receptor, IFNγ, or the IFNγ receptor signaling pathway[891–894] are consistent with macrophage production of IL-12 that stimulates NK cells to secrete IFNγ in listeriosis.

While a similar IL-12-IFNγ pathway is also operational in MCMV infections,[886,895,896] IL-18 also contributes somewhat to NK cell control of MCMV.[897] However, IL-12 appears to be more critical than IL-18 because uniform lethality was observed in IL-12p35−/−mice challenged with MCMV while all IL-18−/− mice survive.[897] On the other hand, neutralization of IL-18 is a common feature of orthopox-viruses.[898] For example, ectromelia virus (mousepox) contains an ORF for an IL-18 binding protein (IL18BP) that effectively neutralizes the effects of IL-18 on NK cells.[899] In addition to augmented IFNγ production, IL-18 enhances perforin-dependent cytotoxicity.[900–902]

Importantly, not all viral infections are controlled by NK cells. For example, NK-cell depletion has little effect on lymphocytic choriomeningitis virus (LCMV) infections.[883,896,903] Yet, during infections, even with LCMV, cytotoxicity of NK cells is enhanced and proliferation ensues. These events constitute systemic effects directly or indirectly mediated by cytokines, such as IL-12, IL-18, and IFNα/β.[904] However, IFNγ production is not seen in LCMV infections.[895,905] This apparent paradox is due to an inhibitory effect of IFNα/β on IL-12–dependent IFNγ production.[905] Inhibition by IFNα/β is mediated through the STAT1 signaling pathway. In the absence of STAT1, IL-12 responsiveness is restored and IFNα/β induces IFNγ production. These studies indicate that the NK-cell cytokine response to infection varies with the pathogen even though many responses may appear to be similar.

A challenging issue is the role of IL-15 in NK cell responses in vivo[906] because IL-15 and IL-15Rα-deficient mice lack NK cells.[53,54] Nevertheless, NK cells can be stimulated during infection by IL-15 and control viral replication during in vitro cultures.[906–910] Moreover, IL-15 can provide protection to herpes simplex viral infections.[910] IFNα/β can stimulate IL-15 production that can drive NK-cell proliferation.[911] Interestingly, however, NK-cell proliferation can occur in an IFNα/β- or IL-15–independent manner.[912,913]

NK-cell responses to cytokines are also regulated by other innate lymphocytes. For example, in TCRδ−/− mice, *Listeria* infection is enhanced compared to TCRβ−/− mice and is associated with diminished production of IFNγ by NK cells, suggesting that γδT cells regulate NK-cell responses.[914] Similarly, NKT cells can regulate NK cells because administration of α-galactosylceramide, a potent ligand for the TCR on NKT cells, results in nearly concomitant activation of NK cells[79] that may be exploited as a potential cancer immunotherapy.[915] The mechanism behind the NK and NKT cell cross-talk involves IFNγ, apparently produced initially by an activated macrophage.[916] Inasmuch as NKT cells can

recognize glycolipid antigens in mycobacteria,[917,918] these studies indicate a potential physiologically important mechanism and therapeutic intervention for NK-cell activation in innate control of these organisms.[919]

Natural Killer–Cell Activation Receptors in Infection

There are several observations from studies of viral evasion indicating the importance of NK-cell activation receptors in infection. Because viruses have evolved numerous strategies to downregulate MHC-I molecules on infected cells to avoid MHC-I–restricted cytotoxic T-lymphocytes,[186] virally infected cells should have enhanced susceptibility to NK-cell lysis. However, viruses also encode proteins that evade NK cells.[920] In many cases, viral interference of NK cells is related to enhanced function of inhibitory MHC-I–specific NK-cell receptors. For example, murine and rat CMV contain ORFs *m144* and *r144*, respectively, that encode molecules with sequence and structural homology to MHC-I and enhance in vivo virulence presumably by interacting with as yet unidentified NK-cell inhibitory receptors.[921–925] MCMV also encodes m157 that can bind the inhibitory receptor Ly49I in 129 strain mice (see following discussion), as well as other MHC-I–like molecules of unknown function.[444,446,464,926] HCMV encodes an MHC-I–like molecule (UL18) that binds LIR1 (ILT2), an Ig-like inhibitory receptor on NK cells,[405,411,927] though its functional significance is somewhat unclear.[928] HCMV also encodes a peptide that binds and enhances expression of HLA-E that in turn binds CD94/NKG2A, a lectin-like NK-cell inhibitory receptor.[386,929–931] Another example is the selective downregulation of MHC-I by HIV-1.[932,933] In this case, the virus downregulates HLA-A and B but not HLA-C or E; the former HLA molecules tend to be restricting elements for MHC-I–restricted CTLs, whereas the latter are selectively recognized by human KIRs and CD94/NKG2A. Therefore, viruses have evolved mechanisms to selectively engage inhibitory receptors that presumably prevent the action of NK-cell activation receptors.

Viruses can also directly block triggering of NK-cell activation receptors. For example, both HCMV and MCMV encode multiple proteins that block NKG2D ligand expression by intracellular retention with functional consequences in vitro and in vivo,[920] as previously detailed. HIV-1 Nef also blocks NKG2D ligand expression.[934] Cowpox and monkeypox viruses use a different strategy; they encode a high-affinity, soluble antagonist that binds NKG2D and blocks its ligand recognition.[557] HCMV, Epstein-Barr virus, and KSHV use yet another strategy by encoding miRNAs that target MICB mRNA.[554,555] More generally, KSHV also avoids NK-cell activation through K5 that downregulates expression of ICAM-1 and B7-2, ligands for NK cell coreceptors involved in target-induced stimulation.[935] Thus, viruses use multiple strategies to specifically thwart NK-cell responses through their activation receptors.

The viral evasion strategies implicate NK-cell activation receptors that specifically recognize infected cells; several have been identified. Human NKp46 binds hemagglutinin of influenza virus and hemagglutinin-neuraminidase of parainfluenza virus, suggesting it may be involved in resistance to these viruses.[630] However, this interaction is dependent on sialic acid residues that are widely expressed, and the in vivo significance of these findings is difficult to assess in humans. Nevertheless, susceptibility of NKp46-deficient mice to influenza infections corroborates these findings.[93]

The autosomal dominant *Cmv1* gene in the NKC is responsible for resistance of certain mouse strains to MCMV[596,597,936,937]; MCMV-resistant C57BL/6 mice are susceptible when depleted of NK cells.[938] Extensive genetic and immunological evidence established that *Ly49h* is responsible for genetic resistance to MCMV.[449,938–942] A DAP12-signaling mutant mouse could not resist MCMV,[943] consistent with in vitro signaling studies showing that Ly49H signals through DAP12.[448,939] Thus, Ly49H is an NK-cell activation receptor responsible for genetic resistance to MCMV.

The ligand for Ly49H is encoded by the *m157* ORF in MCMV.[444,446] m157 and 11 other putative MCMV molecules have predicted MHC-I folds, now validated by crystallography.[464,926] Interestingly, in mice lacking adaptive immunity, m157 mutant MCMV clones emerge during MCMV infection, indicating selection pressure from Ly49H+ NK cells result in escape mutant viruses.[944] The reasons for maintenance of *m157* in the MCMV genome are still under investigation, but several observations suggest *m157* may be advantageous to the virus under certain circumstances. 1) m157 binds to the Ly49I inhibitory receptor in 129 mice.[446] 2) *m157*-deletion viruses cause a modest decrease in viral titers in mice lacking both Ly49I and Ly49H, suggesting yet another immune evasion role.[945] 3) A unique aspect of herpes virus biology is the capacity to become latent. If the virus kills the host during the acute viral replicative phase during which Ly49H mediates its control, then there will be no latent phase. 4) Host-virus coevolution often results in attenuated viruses,[946] and other MCMV proteins have positive effects on host responses.[947] Indeed, as one aspect of this coevolution, natural variants of m157 show differential binding activities to a broader range of inhibitory receptors in different mouse strains, including inhibitory Ly49C from B6 mice.[948,949] Thus, studies of m157 reflect the coevolution of the host and virus.

Interestingly, other Ly49 activation receptors also recognize MCMV-encoded proteins. In MA/My mice, the Ly49P activation receptor is responsible for resistance in the context of $H2^k$.[453,950–952] This receptor-ligand interaction is more complex than Ly49H-m157 because Ly49P appears to recognize the cell surface combination of $H2D^k$ in association with m04/gp34 encoded by MCMV. However, there appears to be another molecule required for Ly49P recognition as m04 expression on $H2D^k$-expressing cells was insufficient for recognition; infection with *Δm04* MCMV was required.[453] Recent studies demonstrate that other Ly49 activation receptors from different mouse strains (BALB/c, NOD/LtJ, PWK/Pas) can also recognize MCMV-infected cells in an m04-H2 allele-dependent manner, providing in vivo control.[953] Taken together, these remarkable studies indicate NK cells recognize MCMV in an "MHC-restricted" manner that is fundamentally different than MHC-restriction as defined for T-cell biology.

Loci for resistance to other pathogens, such as ectromelia virus and herpes simplex virus, have also been genetically mapped to the NKC.[954,955] These loci are termed *Rmp1* and *Rhs1*, respectively, and C57BL/6 mice are resistant. *Rmp1* was initially mapped using DBA/2 mice that lack a functional *Cd94* gene.[394,954] Recent studies of a CD94-deficient mouse show ectromelia virus susceptibility, suggesting that *Rmp1* is encoded by *Cd94*.[396] CD94/NKG2E appears to recognize ectromelia virus–infected cells via Qa-1 and synergizing with NKG2D. Thus, NKC-encoded NK-cell activation receptors mediate NK-cell–dependent resistance to viruses.

In addition to target killing, NK-cell activation receptors also trigger cytokine production in vitro. For example, Ly49H+ NK cells can be selectively activated to produce IFNγ and chemokines within 6 to 8 hours after coculture in vitro with MCMV-infected macrophages or m157 transfectants,[444,956] and m157 itself is expressed soon after infection.[957] However, during early MCMV infection in vivo, IFNγ production was not confined to the Ly49H+ NK-cell subset in C57BL/6 mice, indicating early relative "nonspecific" stimulation of NK cells.[166] Moreover, infection stimulates NK-cell proliferation[958,959] but early (days 1 to 2 postinfection) in vivo NK-cell proliferation was nonselective with respect to Ly49H expression. This proliferation resembled the cytokine-driven "bystander proliferation" observed in T cells in response to viral infections or stimulation with IFNα/β,[960] suggesting that the initial phase of viral-induced NK-cell proliferation represents a nonspecific response to proinflammatory cytokines (IL-12, IFNα/β) and proliferative cytokines such as IL-15.[912] The nonselective proliferation phase was followed by a period of preferential proliferation of Ly49H+ NK cells peaking at days 4 to 6 of MCMV infection.[166] The selective phase of NK-cell proliferation reflected the augmentation of pro-proliferative cytokine stimulation by Ly49H signaling mediated via DAP12.[59] Other activation receptors also trigger specific proliferation in MCMV infection, such as Ly49L+ NK cells in BALB/c mice.[953] However, in infection with vaccinia virus, which lacks a ligand for Ly49H, the initial phase of nonspecific NK-cell proliferation was found but the later specific proliferation of Ly49H+ NK cells was absent.[166] Thus, initial virus-specific NK-cell responses may be masked by generic cytokine responses and only later become detectable.

In humans, how NK cells participate in viral infections has been challenging to understand. However, an unprecedented opportunity to study human NK-cell responses during an epidemic of *Puumala hantavirus* revealed rapid and sustained expansion of NK cells.[961] Most cells expressed CD94/NKG2C and a self-HLA–specific KIR, consistent with expansion of licensed NK cells. Interestingly, expansion of CD94/NKG2C+ NK cells occurs in HCMV+ individuals but not with Epstein-Barr virus or herpes simplex virus infections,[962] though this could not explain the finding in the hantavirus-infected individuals. Regardless, children infected with HCMV show a similar expansion of CD94/NKG2C+ NK cells; in one case of a T-cell–deficient infant, the expanded NK-cell population was associated with resolution of infection, suggesting that human NK cells can control HCMV.[963,964] Moreover, expansion of CD94/NKG2C+ NK cells also occurs in the setting of acute Chikungunya virus infection.[965] In vitro studies revealed that human NK cells can respond to HCMV-infected fibroblasts by producing cytokines and degranulating. These responses were enhanced by IL-12 and type I IFNs, and there was evidence for stimulation through NKp46 and DNAM-1. However, there was no apparent involvement of the CD94/NKG2C activation receptor,[966] despite expansion of CD94/NKG2C+ NK cells with prolonged culture.[388] Thus, the factors driving human NK-cell expansion and control of viral infection are not completely understood.

MEMORY-LIKE NATURAL KILLER RESPONSES

Although innate immunity is typified by the absence of memory responses, several recent studies suggest that NK cells have memory-like responses, more typical of adaptive immunity, apparently blurring the distinction between innate and adaptive immune responses.[751] In classic contact hypersensitivity (CHS) assays in vivo, NK cells appear to mediate hapten-specific responses in a T- and B-cell–independent manner.[967,968] Adoptive transfer of Ly49C/I+ liver NK cells from sensitized C57BL/6 mice transferred CHS. In addition, hapten-specificity was long lived, as NK-dependent responses lasted at least 4 weeks. Subsequent studies indicated that exposure to virus and viral particles, specifically influenza virus, vesicular stomatitis virus, HIV-1, and MCMV, can also induce virus-specific memory-like NK cells.[969,970] Upon adoptive transfer, NK cells can then protect against lethal challenge with the sensitizing virus. While MCMV protection apparently occurred with sensitized splenic NK cells, protection from other viral challenges required liver NK cells expressing CXCR6. Thus, emerging studies suggest that NK cells can display memory-like responses.

The role and basis for NK-cell memory-like responses will require detailed investigation. It is not yet clear if this aspect of NK-cell function is relevant to "vaccination" and how much it contributes to immune protection when immune T and B cells are present. It is unclear how NK cells can demonstrate "antigen" specificity in the absence of the somatic rearrangement mechanism underlying T- and B-cell antigen receptor gene recombination because NK memory-like responses were seen in *Rag2*–/– mice.[967] Moreover, cytokine stimulation alone, without "antigen" stimulation, can induce NK cells to have more robust responses later.[971] Finally, there appear to be significant differences in the inflammatory characteristics and histological features of CHS related to NK-cell responses,[972] suggesting that there may be aspects to be considered that differ from conventional concepts of T-cell–mediated CHS.[968] Thus, although mechanistic details should be forthcoming, this topic has generated intense interest as indicated by review articles which now outnumber the primary literature by about three to one, because the concept of NK-cell memory breaks traditional views on innate and adaptive immunity, and potentially opens the door for examination of related processes in other innate immune components.

NATURAL KILLER CELL AND DENDRITIC CELL INTERACTIONS

Given that both NK cells and DCs, when separately studied, are critical early responders in host immune defense, it was not surprising that these cells could communicate in a bidirectional manner.[973] Since the initial description of this "cross-talk," innumerable studies have been published indicating that NK cells can respond to signals derived from the various DC subsets and vice versa, in a variety of different immune responses in mice and humans.

Data support both cell contact-independent and -dependent pathways. The contact-independent pathways are perhaps better understood. For example, a variety of stimuli, such as certain TLR ligands,[26,974,975] can activate DCs to produce cytokines. Indeed, activated DCs can produce cytokines, including IFNα/β, IL-12, IL-15, IL-18, and apparently IL-2,[26,975–979] that can stimulate NK-cell production of other cytokines, such as IFNγ. Moreover, these cytokines enhance NK-cell cytotoxicity by increasing protein expression of perforin and granzymes[29] as well as perforin-independent cytotoxic pathways. The cytokines can also enhance responses mediated by ITAM-signaling chain–associated receptors.[59] The in vivo relevance of TLR-induced, DC-produced cytokines is evident in studies showing that TLR9-dependent activation of plasmacytoid DCs in MCMV infections is critical for appropriate Ly49H-dependent NK-cell control of infection.[974]

Direct NK-DC cell-cell contact can also enhance NK-cell activation.[973] Several molecules have been implicated in this process, including cytokine-induced DC expression of ligands for human or mouse NKG2D.[980,981] Human DCs can also activate resting NK cells via the NKp30 receptor.[982] Other studies indicate that mouse NK cells can be activated by DCs in a TREM-2–dependent manner through the DAP12 signaling pathway.[983] DCs can also present IL-15 in *trans* that promotes NK-cell development and primes NK cells to respond to bacterial and viral pathogens.[31,984] This process may also be relevant to human NK-cell differentiation.[848]

On the other hand, NK-DC interactions can induce DC maturation.[985] For example, MHC-I–deficient targets can activate NK cells that then induce DC maturation.[986] NK-DC interactions also enhance TH1 polarization apparently through NK-cell production of IFNγ.[855] During MCMV infections, recognition of virus-infected cells by NK cells is critical for maintenance of DC subsets in the spleen.[987] While NK-DC cell contact is also subject to inhibitory effects and even killing of DCs by NK cells,[988,989] the capacity of NK-DC interactions to induce T-cell responses[855,981,986,990] has led to interest in exploiting these interactions for therapeutic vaccines.[991]

A hallmark of DC maturation is migration to a draining LN. Interestingly, however, deliberate introduction of DCs into the lymphatics leads to robust recruitment of activated NK cells.[855] Real-time imaging indicates that NK cells contact DCs in the superficial regions of the LNs where they are less motile and their interactions with DCs are more extensive than T-cell motility and contacts with DCs.[992] *Leishmania* infection led to NK-cell secretion of IFNγ and migration to the paracortex where CD4 T-cell activation occurred, indicating dynamic interactions in the LN not previously appreciated. In humans, DCs in LNs may be involved in differentiation of $CD56^{bright}$ NK cells into $CD56^{dim}$ NK cells.[110]

NATURAL KILLER CELLS AND MATERNAL-FETAL INTERACTIONS

One enigmatic area of continuing interest concerns NK cells in maternal-fetal interactions.[993–995] Several observations are related: 1) Initially described as granulated metrial gland cells, maternal uterine NK (uNK) cells accumulate in the uterus, near the fetal trophoblast layer, in all mammalian species examined thus far. 2) At the time of implantation, uNK cells are the most abundant leukocyte present. 3) In humans, uNK cells closely resemble $CD56^{bright}$ subpopulation, but there are phenotypic differences, including expression of activation markers, and different repertoire of MHC-specific receptors. 4) In some mice lacking NK cells, reproductive defects have been described.[996,997] 5) Trophoblasts can stimulate mouse uNK cells.[998,999] 6) There are significant differences in the anatomy of the maternal-fetal interface that may limit extrapolation of experimental results from one species to another,[994] although recent studies suggest that rodent uNK cells may interact with trophoblast tissues in a manner similar to human uNK cells.[1000,1001] Thus, uNK cells may provide important clues to understanding maternal-fetal interactions.

Interestingly, counterintuitive insight has come from epidemiological data regarding KIR and HLA genotype associations with reproductive failure.[1002,1003] The role for HLA-G in these disorders is unclear, though early studies suggested that human trophoblasts express the nonclassical MHC-I molecule, HLA-G,[1004] but not other MHC-I or -II molecules; HLA-G may protect the fetus from uNK cell attack.[1005] However, HLA-G is relatively nonpolymorphic, so this may not be able to explain alloantigenicity of the fetus. By contrast, recent studies, using newly available staining reagents, indicate that trophoblast cells predominantly express HLA-C.[1003] Furthermore, protection from reproductive failure is associated with maternal KIR haplotypes encoding the KIR2DS1 activation receptor that is specific for HLA-C2, whereas unopposed inhibitory receptors are associated with increased risk. In addition, protection increased when the fetus carried HLA-C2. The implication of this work is that uNK cells sense fetal tissues via their activation and inhibitory receptors to guide the dynamic process of placental remodeling,[1006] and suggest a mechanism for selection of KIR and HLA genotypes in different populations.

Indeed, uNK cells appear to regulate the placental vasculature. Mice lacking NK cells show defects in placental blood vascular remodeling that appears to be partially IFNγ-dependent.[1007] In addition, human uNK cells produce angiogenic factors, particularly early in pregnancy.[1008] Recent studies in mice and rats recapitulate some of the findings observed in humans,[1000,1001,1003] providing new experimental approaches to better understand the role of uNK cells in maternal-fetal tolerance.

NATURAL KILLER CELLS IN HUMAN DISEASE

Throughout this chapter, we have used human disorders involving NK cells to help illustrate their biology. Whereas NK-cell dysfunction has been associated with a large number of other diseases, it is not yet clear if they are clearly involved in a pathogenic manner. However, KIR and HLA genotypes have been linked with an increasing number of human ailments,[754] providing strong evidence implicating a pathogenetic role for NK cells in these diseases. For example, hepatitis C virus can cause a chronic, persistent infection, although some patients resolve the infection. Interestingly, patients who are homozygous for a KIR gene (KIR2DL3) are more likely to clear hepatitis C virus if they are also homozygous for the HLA-C alleles recognized by KIR2DL3.[756] In papillomavirus infections that can go on to produce cervical carcinomas, resistance to developing neoplasia is similarly associated with genotypes encoding certain KIR-HLA receptor-ligand pairs.[1009] In HIV-1 infections, certain KIR and HLA alleles are associated with reduced risk of infection and slower progression.[1010–1013] These associations are even more compelling given a recent report indicating that the KIR genotypes are associated with amino acid polymorphisms in HIV-1 itself, suggesting that NK cells through their KIRs selected specific HIV-1 genotypes.[1014] These sequences enhance binding of inhibitory KIRs to HIV-1–infected CD4+ T cells and reduce the antiviral activity of NK cells, providing a putative mechanism to explain the association of specific KIR and HLA genotypes with HIV-1 progression. Thus, while the means by which other KIR-HLA combinations modify disease progression is a topic under current investigation, the emerging data nonetheless demonstrate the importance of NK cells and KIR specificities in human disorders and treatments.

Studies also suggest that KIR specificities may be clinically useful to guide treatment. For example, in BM transplantation for leukemia, donor NK cells may help provide as in anti-leukemia effect against residual malignant cells in the recipient if there is a mismatch between the donor and recipient HLA alleles.[870] While this observation was not uniformly observed in other transplant centers,[871,1015,1016] improvements in determing KIR polymorphisms have guided donor selection strategies, leading to superior outcomes in BM transplant therapy for leukemia and neuroblastoma.[1017,1018] Thus, KIR genotyping is clinically useful.

Dysfunctional NK cells are found in patients with FHL who typically demonstrate HLH, as described previously. NK-cell dysfunction is also found in systemic onset juvenile idiopathic arthritis and macrophage activation syndrome associated with that disease.[1019] However, it is not yet clear if NK dysfunction is a cause or effect of systemic illness.

Early studies used LAK cells for adoptive immunotherapy of cancers refractory to conventional therapy.[1020] Several cases of complete remission were reported, but the treatment required intravenous administration of high doses of IL-2 that has significant toxicity. Improved understanding of NK-cell biology and their receptors and specificities has renewed interest in using NK cells for adoptive immunotherapy.[1021,1022] In addition, clinical use of IL-15 may allow for more specific NK-cell activation while sparing the toxicity seen with IL-2.[1023] However, these approaches are still in their infancy and will require controlled studies.

Finally, NK-cell proliferative disorders are associated with both chronic viral infections (Epstein-Barr virus) and autoimmune phenomenon.[8,1024,1025] NK-cell malignancies are particularly aggressive and can appear as lethal midline granuloma due to the propensity of NK lymphomas to present in the sinus and nasopharyngeal passages where they are especially destructive.[1026] NK cells are also associated with hydroa vacciniforme and hypersensitivity to mosquito bites.[1027]

CONCLUSION

Detailed studies have moved NK cells from the fringes of immunology into the mainstream. Along the way, there have been innumerable challenges, some from "mistaken notions" based on existing paradigms for other immune cells, because NK cells have novel mechanisms of participating in immune defense and tolerance.[1028] Nonetheless, as immunologists and physicians gain more appreciation for the molecular basis for NK-cell biology, the future holds promise for insight into a number of enigmatic and unusual human diseases as well as common disorders, such as hepatitis C virus and HIV infections, and exploitation of NK cells for immunotherapy.

Finally, the elucidation of KIR genotypes and their role in infection and pregnancy support a broader view. The net outcome of selection for certain KIR and HLA genotypes may reflect a short-term survival advantage in terms of infection and a longer-term survival advantage as determined by reproductive influences. Similar processes must also be ongoing in other mammalian species and support the basis for convergent evolution of the NK-cell inhibitory receptors for MHC-I. Thus, "NK cells are centrally involved in both immunity and reproduction."[1029]

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CHAPTER 18

CD1d–Restricted Natural Killer T Cells

Albert Bendelac

INTRODUCTION AND DEFINITION

Natural killer T (NKT) cells are cluster of differentiation (CD)1d-restricted T cells that use semi-invariant αβ T-cell receptors (TCRs) and reside as long-lived effector cells in lymphoid tissues and in the microvasculature of organs such as the lung and the liver. They promptly release a broad range of cytokines and chemokines in response to microbial and self-lipid antigens presented by CD1d glycoproteins, exerting a protective or pathogenic role in infection, inflammation, allergy, and cancer. The NKT lineage is best defined by expression of *Zbtb16*, which encodes promyelocytic leukemia zinc factor (PLZF), a master transcription factor that directs acquisition of innate-like effector properties during thymic development.

The NKT lineage is a member of the larger family of so-called innate-like lymphocytes, which includes B1 B cells, marginal zone B cells, intraepithelial γδ T-cell sublineages, the CD8αα TCRαβ population of intestinal lymphocytes, and the MR1-restricted mucosal associated invariant T cells.[1] Like NKT cells, these lineages express semi-invariant TCRs or B-cell receptors encoding specificity for conserved microbial and self-ligands. Their "canonical" antigen receptors are sufficient to instruct lineage differentiation during development in the thymus or bone marrow, in a process matching antigen specificity with specialized effector functions and homing to dedicated tissue environments. These stereotypical properties, which are reminiscent of truly innate lymphoid cells, such as natural killer (NK) cells, represent a distinct host defense strategy, perhaps corresponding to an early phase of evolution of adaptive T- and B-cell immunity.

CANONICAL NATURAL KILLER T T-CELL RECEPTORS

In mice, the vast majority of CD1d-restricted NKT cells express the semi-invariant Vα14-Jα18 TCRα chain paired with β chains made of Vβ8, Vβ7, or Vβ2 joined to variable DβJβ segments, whereas the homologous Vα24-Jα18 chain associated with Vβ11 is used in humans[2] (Table 18.1). The "canonical" sequence of the TCR alpha chain is entirely encoded in the genome, although alterations due to nucleotide trimming and N additions can be tolerated if they preserve the antigenic specificity of the TCR. These canonical rearrangements arise randomly and at very low frequency in both fetal and adult life, but massive thymic expansion post-TCR expression ensures a high frequency of NKT cells among recent thymic emigrants.[3]

Other semi-invariant TCRs have been identified among mouse CD1d-restricted NKT cells, including Vα10-Jα50/Vβ8,[4] Vα3.2-Jα9/Vβ8, and Vα8/Vβ8,[5] but their combined frequency is modest compared to the dominant Vα14-Jα18 TCR. Nevertheless, these cells appear to adopt a similar NKT effector phenotype, likely because they follow the same thymic developmental pathway.

ANTIGENIC LIGANDS OF NATURAL KILLER T CELLS

Cluster of Differentiation 1d

CD1d is one of five members of the mammalian family of lipid-presenting, β2-microglobulin–associated, major histocompatibility complex (MHC)-like CD1 molecules, and the only one conserved in mouse.[6,7] The mouse CD1 locus contains *cd1d1* encoding a functional surface glycoprotein and a duplicated gene *cd1d2*, which is generally poorly expressed and, in the C57 background, is inactivated by a frameshift mutation preventing surface expression.[8] CD1d is found constitutively on the cell surface of most antigen-presenting cells, including dendritic cells (DCs), macrophages, and B cells, with particularly high levels on marginal zone B cells.[9] Of relevance to NKT-cell development, CD1d is also prominently but transiently displayed on cortical thymocytes. It is also expressed by endothelial cells and hepatocytes.

CD1d is assembled in the endoplasmic reticulum by association with β2-microglobulin before reaching the cell surface and undergoing extensive rounds of internalization and recycling between the late endosome/lysosome and the plasma membrane[10–12] (Fig. 18.1). The rapid rate of internalization depends upon a tyrosine motif encoded in its cytoplasmic tail, which binds adaptor protein (AP)-2 and AP-3 in mouse, and AP-2 in human. This intense recycling accounts for the steady state accumulation of CD1d in the late endosome/lysosome for mouse or late endosome for human. While a diversity of exogenous and endogenous lipids can load CD1d in various compartments,[13–15] the late endosome/lysosome environment is optimized for lipid antigen acquisition due to the presence of efficient glycolipid processing enzymes and lipid transfer proteins such as saposins,[16–18] Gm2 activator,[16] Niemann-Pick type C2 protein,[19] and, in humans, CD1e.[20] In this acidic compartment, short or polyunsaturated lipids loaded at the cell surface are quickly removed and replaced by long and saturated lipids that bind more stably to CD1d but require lysosomal transfer proteins for loading.[21] Unlike MHC class II, CD1d expression and its presentation of lipid antigens at the cell surface are

TABLE 18.1 Semi-invariant T-Cell Receptors of Cluster of Differentiation 1d–Restricted Natural Killer T Cells

Human			
Canonical hVα24	TRAV10 TRAJ18	GTG GTG AGC G C GAC AGA GGC TCA ACC GTG GTG AGC GAC AGA GGC TCA ACC V V S D R G S T	Variable hVβ11
Mouse			
Canonical mVα14	TRAV11 TRAJ18	GTG GTG GGC G TA GAT AGA GGT TCA GCC GTG GTG GGC GAT AGA GGT TCA GCC V V G D R G S A	Variable mVβ8, mVβ7, mVβ2,

independent of toll-like receptor (TLR) signaling and DC maturation.[22] Thus, immature DCs can readily present purified lipid antigens to NKT cells and become activated upon CD40 engagement by NKT cells.

Lipid Ligands of Natural Killer T cells

bial α-glycosylated Lipid Ligands

The first universal NKT ligand emerged from studies of marine sponge extracts that prolonged survival of mice bearing B16 melanoma.[23] The active principle of the sponge *Agelas mauritianus* was an alpha-branched galactosylceramide, which after slight modification led to the synthesis of an extremely potent variant named KRN7000, commonly referred to as αGalCer[24,25] (Fig. 18.2). Over 95% of mouse and human NKT cells recognize αGalCer, irrespective of their variable CDR3 β sequence.[26,27] Furthermore, the mouse CD1d-αGalCer tetramers stain the NKT cells of both human and nonhuman primates,[28,29] attesting to the high degree of conservation of this recognition system.

The marine sponge lipid turned out to be closely related to microbial lipids found in some gram-negative lipopolysaccharide (LPS)-negative bacteria. Notably, *Sphingomonas*, a member of the class of α-proteobacteria and a ubiquitous bacterium found in terrestrial and marine environments (including as a bacterial symbiont of sponges), uses alpha-branched glycuronylceramides as a substitute for LPS in the outer membrane of its cell wall.[30] It can infect dendritic cells and macrophages to activate NKT cells.[31,32] Other structurally related α-glycosylated lipids, such as α-galactosyldiacylglycerol, found in *Borrelia burgdorferi*, the agent of Lyme disease,[33] and α-glucosyldiacylglycerol, found in *Streptococcus pneumoniae*,[34] also activate mouse and human NKT cells. Such α-branched glycolipids have not been reported in vertebrates, suggesting that they represent a pathogen signature that the canonical NKT TCR may have evolved to recognize.

Endogenous β-glycosylated Lipid Ligands

Vα14 NKT cells are spontaneously autoreactive to CD1d-expressing cells.[35] For example, NKT hybridomas or clones

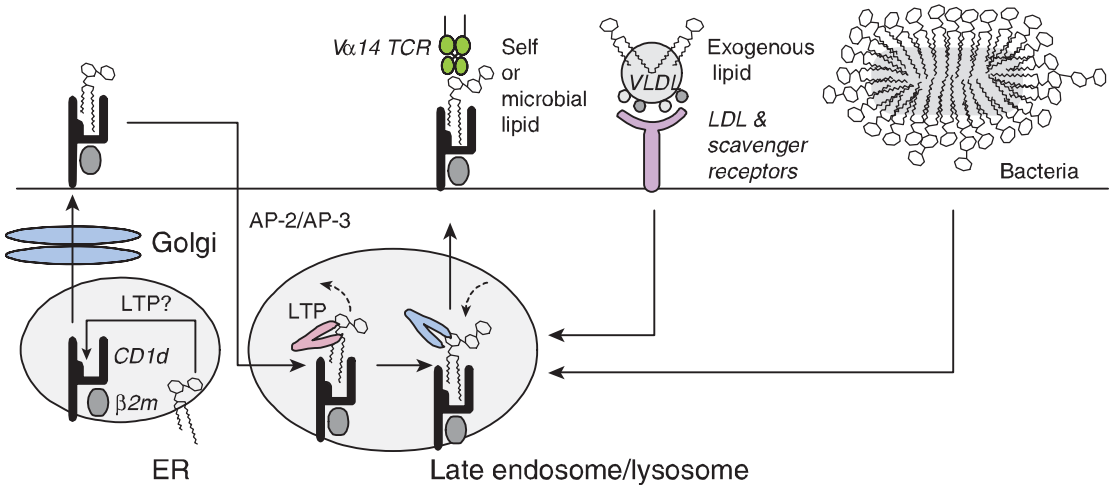

FIG. 18.1. Presentation of Lipid Antigens by Cluster of Differentiation (CD)1d. CD1d acquires lipid antigens after biosynthesis in the endoplastic reticulum and Golgi prior to reaching the cell surface. Multiple rounds of AP-2/AP-3 clathrin-mediated internalization to the late endosome/lysosome and recycling to the plasma membrane allow lipid exchange. Lysosomal degradation pathways provide lipid antigens for exchange in a process assisted by lysosomal lipid transfer proteins such as saposins, Gm2 activator, NPC-2, and CD1e. Exogenous lipids bound to lipoproteins enter the cell through scavenger receptors and low-density lipoprotein receptor. Microbial organisms access the lysosome after phagocytosis.

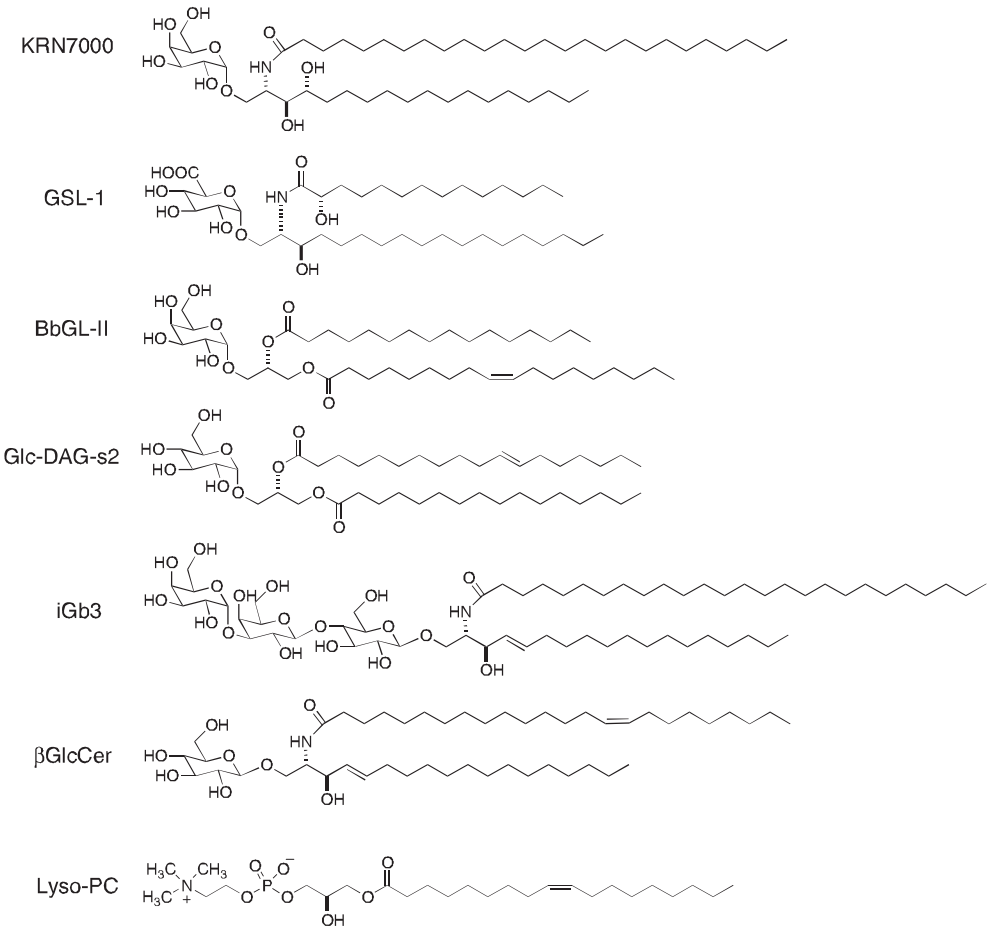

FIG. 18.2. Self- and Foreign-Lipid Antigens Recognized by Natural Killer T Cells. From top to bottom, KRN7000, or reference α-GalCer, synthetic version of the original marine sponge antigen; *Sphingomonas* GSL-1; *Borrelia burgdorferi* BbGL-II, α-galactosyldiacylglycerol; *Streptococcus pneumoniae*, α-glucosyldiacylglycerol; isoglobotrihexosylceramide iGb3; β-glucosylceramide; and lyso-phophatidylcholine.

can be activated to secrete modest amounts of cytokines when cultured in the presence of fresh thymocytes, DCs, and various tumor cell lines.[8,35–38] This autoreactivity and the agonist ligands involved appear to be central not only for NKT-cell thymic development,[36] but also for their response to various microbial infections, especially when the pathogens themselves do not express NKT ligands.[31,39] The identification of self-ligands is therefore of major relevance to key aspects of the biology of NKT cells.

In the mouse system, recognition of self-ligands by Vα14 NKT cells is largely dependent upon CD1d endosomal trafficking,[40,41] suggesting the recognition of endosomal/lysosomal lipid antigens. This may not be the case for their human counterpart, Vα24 NKT cells.[42] The search for a lysosomally-loaded self-ligand recognized by NKT cells led to the identification of a trihexosylceramide, iGb3, which is synthesized in the Golgi as an intermediate in the biosynthetic pathway of iGb4 and is also generated in the lysosome after degradation of iGb4 by β-hexosaminidase AB.[43] iGb3 is further degraded into LacCer by α-galactosidase A (Fig. 18.3). Mice lacking β-hexosaminidase B lacked 95% of their NKT cells, a defect associated with the impaired ability of their thymocytes to stimulate autoreactive NKT hybridomas. Conversely, unlike αGalCer, iGb3 is recognized with a Vβ7 > Vβ8 > Vβ2 hierarchy of affinities that mirrors the relative selection of NKT thymic precursors.[44] Furthermore, DCs lacking α-galactosidase A exhibited greater spontaneous stimulation of NKT cells,[45] consistent with the demonstrated accumulation of iGb3.[46–48]

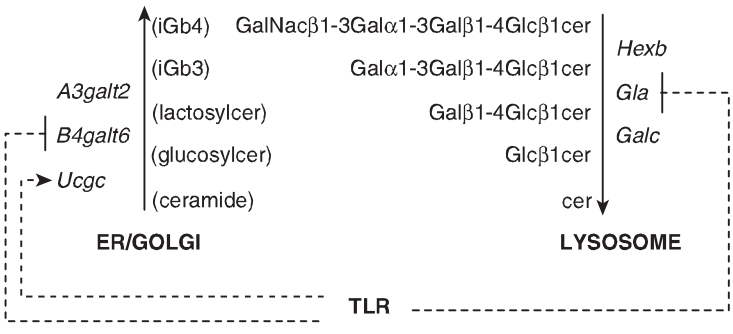

FIG. 18.3. Toll-Like Receptor (TLR)-Mediated Regulation of the Synthesis and Degradation of Natural Killer T Cell Glycosphingolipid Antigens. TLR signaling downregulates *B4galt6* encoding lactosylceramide synthase and *Gla* encoding α-galactosidase A, but upregulates Ucgc encoding β-glucosylceramide synthase. These changes result in the net accumulation of β-glucosylceramide and iGb3.

Among several genes encoding Galα1-3Gal transferases, *a3galt2* is considered more specialized for iGb3 synthesis. As *a3galt2* null mutant mice did not show NKT cell defects[49] and the human homologous gene appeared dysfunctional,[50] iGb3 may not be the sole endogenous glycosylceramide recognized by mouse and human NKT cells. Other reports have suggested that β-galactosylsylceramide[51] and β-glucosylceramide[52] might also function as natural ligands of NKT cells, but these claims have not yet been widely replicated. While the respective importance of all these candidate ligands remains to be further studied in physiological and pathological conditions, it is possible that NKT cells might recognize multiple endogenous β-glycosylceramides as weak agonists.

Importantly, several enzymes involved in the synthesis and degradation of β-glycosylceramides appear to be coordinately regulated in conditions associated with NKT-cell activation[45,52,53] (see Fig. 18.3). For example, TLR signaling was shown to specifically downregulate α-galactosidase A in the degradation pathway,[45] leading to increased iGb3.[46] In the biosynthetic pathway, TLR signaling upregulated β-glucosylceramide synthase while downregulating lactosylceramide synthase, thus increasing β-glucosylceramide.[52] Collectively, these findings suggest that microbial organisms lacking NKT ligands can nevertheless activate NKT cells through TLR-mediated accumulation of endogenous glycosphingolipid ligands.

Lysophosphatidylcholine was also reported to stimulate a fraction of human but not mouse NKT cells,[54,55] suggesting that upregulation of this ligand in multiple myeloma and other inflammatory conditions might also contribute to NKT-cell activation.

Structural Basis of Lipid Recognition by Natural Killer T cells

The lipid-binding pocket of CD1d is particularly well adapted to bind self- and microbial glycosphingolipids, with the acyl chain in the A' hydrophobic pocket and the sphingosine chain in the F' hydrophobic channel.[56–58] The hydroxyl groups of the sphingosine emerge from the groove to establish hydrogen bonds with the α1 helix Arg79 and Asp80, while the galactose is stabilized though hydrogen bonds between its 2- and 3-hydroxyl groups and Asp153 of the α2 helix. Thus, the protruding sugar is solidly anchored in a position parallel to the plane of the α-helices, explaining the exquisite stimulatory properties of several carbohydrate hydroxyl groups. In contrast, the trisaccharide chain of iGb3 protrudes in orthogonal orientation to the plane of the α-helices, with the proximal glucose forming hydrogen bonds with the α2 helix, in particular Asp153 and Thr156, whereas the position of the ceramide backbone is similar to αGalCer (Fig. 18.4).

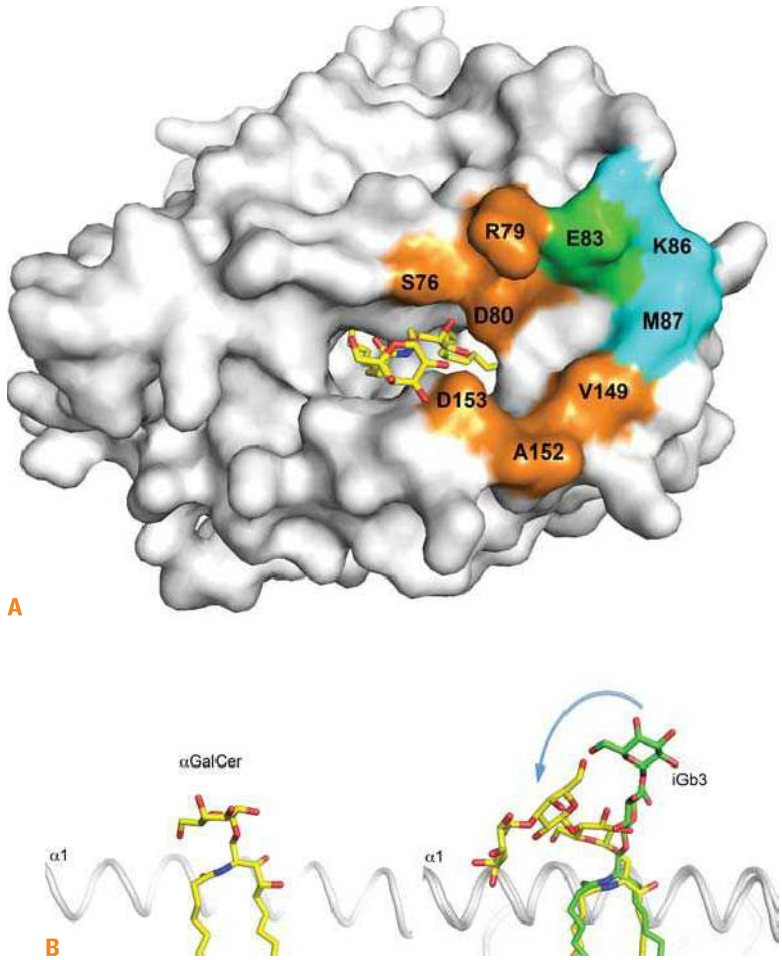

FIG. 18.4. Natural Killer T (NKT) T-Cell Receptor (TCR) Recognition of αGalCer and iGb3. **A:** Conserved footprint of the mouse NKT TCR on the surface of cluster of differentiation (CD)1d-αGalCer and CD1d-iGb3 (the lipid shown is αGalCer): *orange*, CDR3α; *cyan*, CDR2β; *green*, CDR3α and CDR2β. **B:** Binding mode of αGalCer and iGb3 to CD1d and TCR. *Left*, αGalCer (same structure before and after TCR binding); *right*, iGb3 before (*green sticks*) and after (*yellow sticks*) TCR binding. Note that the distal sugar is not represented before TCR binding. Courtesy of Dirk Zajonc (La Jolla Institute of Allergy and Immunology, San Diego, CA).

How could such highly dissimilar structures be recognized by the same TCR? In the ternary structure of the human TCR-CD1d-αGalCer complex, the TCR docked parallel to and at the extreme end (F' pocket) of the CD1d binding groove, enabling a lock-and-key type of interaction with the solidly anchored, rigid αGalCer antigen. The docking was parallel to the long axis of the binding groove, contrasting with the diagonal footprints observed for MHC-restricted TCRs.[59] The CDR3α loop encoded by the conserved germline Jα18 segment provided a majority of contacts, straddling the antigen binding cleft and engaging electrostatic interactions with α-GalCer, the α1 and the α2 helices of CD1d. CDR1α interacted solely with αGalCer, and CDR2β formed a stretch of interactions with the α1 helix. Consistent with the great diversity of CDR3β usage, this loop contact was limited to a single van der Waals contact with Gln150 of the α2 helix.

The ternary structure of the TCR-CD1d-iGb3 complex was recently elucidated. Unsurprisingly, given the conservation of Jα18 and CD1d in both structures, the overall footprint of the TCR on the CD1d-glycolipid surface was unchanged compared with CD1d-αGalCer. Strikingly, however, the TCR appeared to simply "squash" the trisaccharide headgroup of iGb3 over the α2 helix of CD1d, with the proximal β-linked glucose molded into an orientation similar to the α-linked galactose of αGalCer (see Fig. 18.4). Each of the three sugars made stabilizing polar and van der Waals interactions with CD1d residues. The proximal and second sugars also contacted the TCR CDR2α loop, which is not involved in CD1d-αGalCer recognition. The importance of the distal sugar is reflected by the lack of detectable stimulation by lactosylceramide where the third sugar is absent. These conformational changes imply a highly dynamic interaction process during the association phase. The energy penalty incurred for binding self-ligands could be overcome in specific conditions that strengthen the immune synapse. For example, this may occur during TLR-induced inflammation, which upregulates lymphocyte function–associated antigen (LFA-1) and intercellular adhesion molecule (ICAM-1) integrin interactions, or during thymic development, which involves homophilic engagement of signaling lymphocytic activation molecule (SLAM) family receptors.

Additional crystallographic studies further illustrated the difference between self- and foreign-antigen recognition. The α-glycosyldiacylglycerol antigens of *Borrelia burgdorferi*[33,60,61] and *Streptococcus pneumoniae*[34] adopted configurations relatively similar to αGalCer, although some induced fit was observed for both CD1d and the glycolipid upon TCR binding. In the case of *Streptococcus pneumoniae*, an unusual sn2 alkyl chain, vaccenic acid (with an unsaturation at C7), was important for favorable positioning of the glucose. After insertion of a stretch of hydrophobic aminoacids in the CDR3β loop in order to enhance binding to CD1d, other self-lipids such as β-GalCer, Galα1-4GlcCer (lactosylceramide), and phosphatidylinositol could be recognized.[62] Although somewhat contrived, this system revealed a similar bending of the β-linkage between the proximal sugar and the ceramide backbone as seen for iGb3, raising the possibility that several β-linked self glycolipids might serve as autoantigens.

DEVELOPMENT AND HOMEOSTASIS OF NATURAL KILLER T CELLS

Development

The major determinant of NKT cell development is the semi-invariant TCR, which, upon binding to CD1d ligands expressed by cortical thymocytes, provides the signals required for induction of the lineage-specific transcription factor PLZF. Therefore, insights into the peculiar nature and context of TCR engagement and its downstream signaling have considerably advanced our understanding of the molecular mechanisms of NKT-cell development.

T-Cell Receptor Expression and Positive Selection

The use of CD1d-αGalCer tetramers specific for the canonical mVα14-Jα18/hVα24-Jα18 TCRs has revealed a sequence of selection, expansion, and differentiation events preceding the terminally differentiated NK1.1+ stage.[3,63,64] As illustrated in Figure 18.5, NKT cells originate from mainstream thymocyte precursors that transit through the pre-TCRα+TCRβ+ stage to reach the CD4+CD8+ double positive (DP) stage, where stochastic Vα-Jα rearrangements lead to expression of canonical Vα14-Jα18/Vβ8, Vβ7 or Vβ2 TCRs. These rearrangements involve distal gene segments that require prolonged cell survival mediated by Bcl-xL, whose expression depends on HEB-induced RORγt.[65–67] All Vβ families can pair with the Vα14-Jα18 chain, but only the biased set of Vβs confers specificity for the endogenous ligands. Although Vβ8 predominates among mature NKT cells, the most enriched Vβ family relative to preselection frequency is Vβ7, followed by Vβ8 and Vβ2.[44] This is consistent with the observation that endogenous ligands displayed on the surface of thymocytes activate the Vα14-Jα18 TCRs with a Vβ7 > Vβ8 > Vβ2 hierarchy of affinity, and that in mice expressing very low levels of CD1d, NKT cells showed increased representation of Vβ7.[44,68–71] Notably, this hierarchy of affinity parallels that of iGb3 but is different from αGalCer, which favors Vβ8. Thus, the naturally selected Vβ repertoire seems to precisely reflect the affinities of the preselected repertoire for endogenous ligands, suggesting that negative selection plays little role in shaping the Vβ repertoire. However, this does not imply that NKT cells are impervious to negative selection because transgenic overexpression of CD1d decreased the relative frequency of the high affinity Vβ7+ cells and exposure to αGalCer-induced deletion of NKT thymocytes.[69]

Developmental Stages

The earliest signaled NKT precursors are rare CD4+CD8+ DP cells that express the semi-invariant TCR. These cells become CD4+ single positive (SP) cells expressing high levels of CD24, a marker of immature cortical thymocytes, as well as CD69, a marker of positive selection.[64] This is the so-called stage 0 of NKT development. As $CD24^{high}CD4+$ cells mature into the next $CD24^{low}CD4+$ stage 1, they undergo several rounds of cell division.[3] Cell cycle may be initiated as early as stage 0, as suggested from the analysis of cyclin D2- or c-Myc-deficient mice.[72,73] The lineage expansion following positive selection ensures the high frequency that is critical for innate immunity. During this phase, a fraction of CD4+

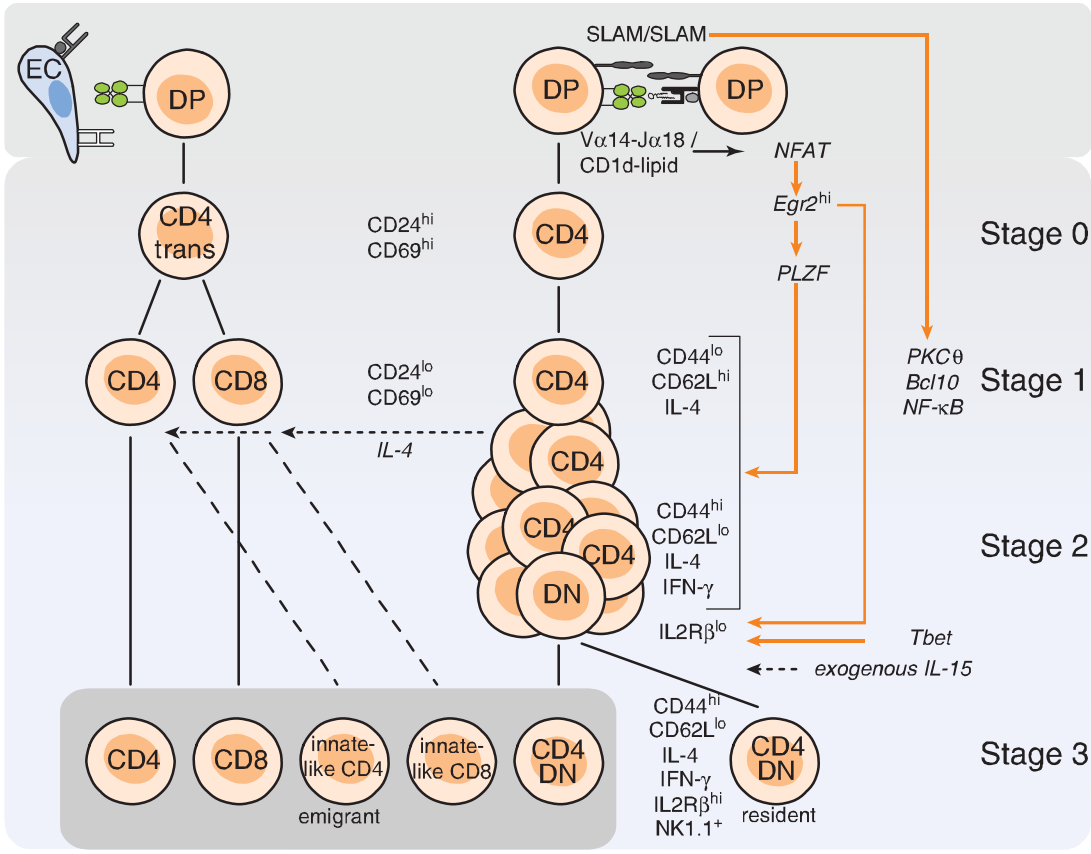

FIG. 18.5. Development of Natural Killer T (NKT) Cells. Thymic stages of NKT-cell development are aligned with corresponding stages of cluster of differentiation (CD)4 and CD8 T-cell development. Stage 0 NKT cells correspond to the transitional CD4 cells. Note the cross-talk between NKT cells and CD4 and CD8 T cells, which is mediated by interleukin-4, and results in the differentiation of "innate-like" CD4 and CD8 T cells in Balb/C mice. DP, CD4+CD8+ double positive; DN, CD4-CD8- double negative; EC, epithelial cell.

cells downregulates CD4 to become CD4-CD8- double negative (DN) T cells,[64] acquiring a more restricted Th1 cytokine and chemokine profile and an enhanced ability to reject tumors.[74–76] Dividing cells first activate their interleukin (IL)-4 locus (stage 1) then interferon (IFN)-γ (independently of both stat6 and stat4) as they upregulate CD44 and downregulate CD62L (stage 2).[3,63,77] Thus, unlike other αβT cells, postselection NKT lineage cells undergo a sequence of events that is reminiscent of the antigen-driven activation, expansion, and effector differentiation of mature T cells.[78]

NKT lineage cells emigrate from the thymus after upregulation of the S1P1 receptor,[79] as dividing effector-type cells. They represent up to 5% of recent thymic emigrants in the mouse spleen.[3,80] Within a couple of days after emigration, they express NK lineage receptors, including the activating NK1.1 and NKG2D receptors and the inhibitory CD94/NKG2A, Ly49A, C/I, and G2 receptors. Acquisition of this terminal differentiation program is associated with cessation of division and with upregulation of IL2Rβ, which is necessary for IL-15 signaling. Intriguingly, a fraction of NKT thymocytes downregulate the S1P1R and remain as permanent residents in the thymic medulla, where they undergo the same terminal maturation program.[79] These thymic residents appear to be absent in humans.[81]

Recent thymic NKT emigrants and their immediate precursors, stage 2 NKT thymocytes, express neuropilin 1 (Nrp-1), a transmembrane receptor for vascular endothelium growth factor and semaphorin family members.[82] Nrp-1 represents a convenient marker of recent thymic NKT emigrants, as it remains expressed for a few days and is downregulated after terminal maturation to stage 3.

Homotypic Thymocyte-Thymocyte Interactions

The thymic cell types involved in presenting NKT ligands have been thoroughly investigated. Unlike MHC class I or class II, CD1d is prominently expressed on cortical thymocytes, which is consistent with the ability of cortical thymocytes to stimulate NKT hybridomas.[36] Bone marrow chimera experiments demonstrated that NKT-cell development required CD1d expression by cortical thymocytes but not radioresistant stromal cells.[83–85] This is radically different from conventional T-cell development that is driven by MHC expression on thymic epithelial cells. Transgenic experiments using promoters for Lck, MHC class I, or MHC class II to redirect CD1d to various cell compartments in CD1d-deficient hosts suggested that expression on cortical thymocytes was necessary and sufficient for lineage development.[70,86–88] This conclusion was recently confirmed after

conditional deletion of CD1d using Cd4-Cre.[89] Mixed chimera experiments using CD1d knockout pLck-CD1d transgenic TCR Cα knockout bone marrow (where thymocyte development is arrested at the DP stage) as the sole source of CD1d expression demonstrated that expression of CD1d solely on cortical thymocytes was sufficient for the full differentiation of NKT cells.[87] Intriguingly, however, the transition to the NK1.1 positive stage was partially impaired after emigration to peripheral tissues, although it was preserved for the cells remaining as residents in the thymus. Terminal differentiation was fully restored if CD1d was re-expressed on MHC class II expressing cells or if the cells were transferred into wild-type recipients.[80,87]

Homophilic Signaling Lymphocytic Activation Molecule-f Family Interactions

A major pathway that is selectively recruited by the thymocyte-thymocyte interactions involves homophilic binding of SLAM family receptors, mainly Slamf1 (SLAM) and Slamf6 (Ly108), which are expressed by cortical thymocytes, but not by thymic epithelial cells.[90] These partially redundant receptors signal through the adaptor SLAM-associated protein (SAP) and the kinase Fyn, explaining earlier reports of developmental arrest in SAP- or Fyn-deficient NKT precursors.[91–95] SAP- and Fyn-deficient NKT thymocytes were blocked at stage 0 and could not be rescued by expression of Bcl-2 or Bcl-xL. They showed lower induction of CD69,[90] suggesting defective signaling by the TCR, perhaps due to a role of Slamf-SAP-Fyn signaling in stabilizing the immune synapse.[96] Individual ablation of Slamf1 or Slamf6 resulted in a modest twofold defect in the expansion of the NKT lineage between stages 1 and 2. However, in "pseudo-double mutant" mixed chimeras where Slamf1/CD1d double deficient NKT precursors were forced to see their ligands on Slamf6-deficient thymocytes, a > 10-fold reduction of NKT cells was observed, demonstrating a requirement of Slamf receptors at the time of TCR engagement by CD1d ligands.[90] Notably, the Slamf locus exhibits considerable polymorphism, which may underlie some of the reported variations in NKT-cell frequencies in different mouse strains and in humans. Genetic studies have provided support for this hypothesis by identifying the lack of expression of Slamf1 by cortical thymocytes in the nonobese diabetic (NOD) strain, which is spontaneously NKT deficient.[97]

Signaling in the Natural Killer T-Cell Microenvironment

Engagement of the semi-invariant TCR activates the same Ras/MAP kinase and calcineurin pathways as reported for MHC-restricted TCRs during positive selection.[98,99] Notably, NKT thymocytes show elevated and sustained expression of the early growth response (Egr) factors 1 and 2 compared with the weak and transient expression observed in MHC-restricted thymocytes.[100] Egr1 is thought to be downstream of the Ras/MAP kinase pathway, whereas Egr2 is mainly induced by the calcineurin/nuclear factor of activated T cells (NFAT) pathway. Egr1 and Egr2 mediate the survival of MHC-restricted T-cell precursors after positive selection through induction of Bcl2 and Bcl-xL.[101–103] In the case of NKT cells, however, sustained Egr elevation has specific lineage-determining consequences. Egr2 directly binds to the promoters of NKT lineage–specific genes such as *Zbtb16*, encoding PLZF, and *Il2rb*, encoding the β chain of the IL-15 receptor, and it is required for their induction. This suggests a direct connection between the peculiar signaling emanating from the TCR synapse and these NKT lineage checkpoints.[100] The sustained elevated Egr levels likely result from the TCR recognition of agonist ligands[36,43] or from the Slamf-SAP–mediated stabilization of the immune synapse.[96] As some *Zbtb16* messenger ribonucleic acid induction was detected in SAP-deficient NKT precursors, SAP may not be absolutely required for PLZF induction.[104]

TCR and Slamf-SAP-Fyn signaling both involve the canonical NF-κB pathway through PKCθ and Bcl-10.[105,106] Mice lacking these downstream signaling components showed partial defects in NKT-cell development,[107,108] as further detailed in the following.

Thus, NKT-cell development is tightly dependent on the signaling elicited through the TCR and Slamf-SAP-Fyn pathways, the specific contributions of which remain to be dissected. Notably, redirecting the expression of MHC class II proteins on cortical thymocytes through ectopic expression of the transcription factor CIITA led to the differentiation of "innate-like" CD4+ thymocytes in a SAP-dependent manner. These cells resembled stage 2 NKT cells and expressed PLZF, reinforcing the notion that homotypic thymocyte-thymocyte interactions, Slamf-SAP-Fyn signaling, and PLZF define a dedicated thymic pathway for the production of innate-like effector T cells.[109–112] In that context, the reciprocal expression patterns of CD1 and Slamf receptors by thymocytes and of MHC proteins by epithelial cells may well serve the primary purpose of creating different niches for different thymic lineages.[90]

Cluster of Differentiation 4 and Cluster of Differentiation 8 Coreceptor Expression

NKT cells originate from the same pool of DP precursors as MHC-restricted T cells and emerge from thymic selection as CD4 SP cells expressing the CD4 lineage factor ThPOK/c-Krox in a Gata-3–dependent manner.[113,114] A fraction goes on to downregulate CD4 and acquire the DN phenotype, but they still stably express ThPOK, which is essential to downregulate CD8. In humans, some NKT cells can express CD8αα homodimers. ThPOK-deficient NKT cells did not express CD4, and some of them also failed to repress CD8, but otherwise they appeared to develop normally.[113] Their functional properties, however, have not been fully assessed. CD8α-deficient mice showed a modest but significant bias toward the selection of high-affinity Vβ7 TCRs compared with littermate controls.[83] A similar bias was observed after anti-CD8 antibody treatment, suggesting a minor role of CD8 as a coreceptor for CD1d. Transgenic expression of CD8α resulted in the disappearance of NKT thymocytes, suggesting a role in negative selection,[83] a conclusion subsequently challenged when the transgenic model was found to have impaired Vα-Jα rearrangements.[114]

Thus, while NKT-cell development does not appear to rely on CD4 or CD8 coreceptors, the induction of ThPOK and CD4 in this lineage may simply reflect the path of high

affinity TCRs whose signaling is coreceptor independent.[115] However, the subsequent CD4 downregulation in up to 50% to 70% of NKT cells, despite persistent ThPOK expression, remains to be explained.

Cluster of Differentiation 28-B7 Interactions

A modest decrease in the thymic expansion of NKT cells was reported in mice lacking CD28 or B7,[116,117] but the nature of the B7-expressing cell type (epithelial cells or DCs) has not been determined.

Transcriptional Control of Natural Killer T-Cell Development: The Central Role of Promyelocytic Leukemia Zinc Factor

The transcription factor PLZF encoded by *Zbtb16* was identified as the signature master transcription factor of the NKT-cell lineage.[104,118] PLZF is a member of the BTB-ZF family of transcription factors, which also includes Bcl6 and ThPOK. It is composed of a bric-a-brac, tramtrack, and broad (BTB) homodimerizing domain and nine Kruppel-type Cys2His2 zinc fingers. The BTB domain binds a corepressor complex made of histone deacetylases, N-Cor, and SMRT, whereas deoxyribonucleic acid binding is mediated by the zinc fingers. PLZF is induced in NKT precursors just after TCR signaling, with high amounts detected on nearly half of stage 0 and all of stage 1 and 2 cells, and lower levels found in stage 3 cells.

Mice lacking PLZF through either the *luxoid* mutation, which induces a frameshift leading to a truncated BTB protein without zinc fingers, or deletion of exon 2 encoding the BTB domain, exhibited a block in NKT-cell development,[104,118] as well as defects in spermatogonial cell maintenance and osteoblast differentiation.[119,120] PLZF-deficient NKT precursors identified by CD1d-αGalCer tetramers were blocked at stage 1, unable to acquire effector characteristics. Instead, they maintained a naïve phenotype and function, recirculating between blood, lymph node, and spleen, rather than homing to liver and lung, and producing IL-2 instead of IL-4 and IFNγ upon stimulation. Intriguingly, however, they incorporated BrdU at the same high rate as wild type, but without showing expansion, suggesting aborted division or increased cell death.

Ectopic expression of PLZF under the CD4 or Lck promoter induced a typical stage 2 NKT program in CD4 T cells, with downregulation of CD62L, upregulation of CD44 and LFA-1, dual secretion of IL-4 and IFNγ, and homing to liver and lung.[104,121–123] Notably, the full effector conversion depended on expression of PLZF at high levels comparable to stage 1 and 2 NKT thymocytes, which was only achieved in the CD4 promoter transgenic model.[124] In these mice, nearly 100% of CD4 cells acquired the effector phenotype at the CD4 SP stage. This effector conversion was also observed in MHC class II–restricted TCR/RAG knockout transgenic models. Thus, while the induction of PLZF requires the signaling environment of NKT cells, the NKT effector program can be transferred by PLZF alone. The molecular basis of the extensive gene reprogramming induced by PLZF, in particular the direct target genes and the biochemical mechanisms of gene activation or repression, are currently being studied.

Other Transcription Factors

The induction of Tbet represents an essential step at the transition between stage 2 and stage 3,[125–127] coinciding with the relative decrease in PLZF expression. Both Tbet and Egr2 contribute to *Il2rb* induction,[100] allowing responsiveness to IL-15. While the pathways leading to Tbet induction independently of Stat1 or Stat4 remain unclear, a role for Ets1 or MEF has been suggested.[127,128]

Mice lacking Gata-3 exhibited severe but complex NKT-cell defects[129] consisting of a considerable reduction of most of their thymic and peripheral NKT cells, with the strange exception of their stage 3 thymocytes which accumulated normally. Gata-3 is required for ThPOK induction, explaining the loss of CD4 by residual NKT cells.[113]

Mice lacking TGFβRII had severe NKT-cell developmental defects, particularly in a competitive chimera setting.[130] Mutant NKT cells had defective expression of the IL-7 receptor and increased apoptosis at stage 1, but a normal rate of cell division at stage 2. Tbet and IL2Rβ were expressed by a larger than normal fraction of stage 2 cells. These effects were mediated by different branches of TGFβ signaling, including Tif-1γ, Smad4, and the Smad4/Tif-1–independent pathway.

Complex and somewhat conflicting results have been reported regarding the role of different NF-κB factors. Mice lacking IKKβ or expressing a degradation resistant form of I-κBα generally exhibited severe cell-intrinsic defects at stage 1 and 2, due in part to apoptosis. These effects appeared to be mediated by the partly redundant functions of NF-κB1, RelA, and c-Rel.[131–134] The upstream activator of NF-κB may include signaling through TCR, SLAM/SAP/Fyn, or CD28/B7. In contrast, ablation of RelB in the alternate pathway impaired early NKT cells development nonspecifically by disrupting the thymic stroma.

While early reports suggested some cell-intrinsic role for lymphotoxin signaling in NKT-cell development,[135] more recent analysis indicated that the major attrition in lymphotoxin-deficient animals occurred during the transition from the thymus to the periphery, possibly related to a defect in NKT thymocyte emigration in the absence of interaction between LTβR on thymic stromal cells and lymphotoxin on bone marrow–derived cells.[136]

cMyc is induced as early as stage 0, when NKT cells enter S phase. Its conditional ablation resulted in developmental arrest at stage 0,[72] similar to cyclin D2-deficient mice (unpublished data), suggesting that entry into cell cycle occurred at stage 0, one stage earlier than previously thought.[64]

Micro-Ribonucleic Acids

Conditional ablation of *Dicer* in thymocytes led to a massive loss of NKT thymocytes but the micro-ribonucleic acids involved have eluded identification.[137–140]

Other Promyelocytic Leukemia Zinc Factor–Positive Lineages

γδT cells expressing the semi-invariant Vγ1Vδ6 receptor were previously shown to express the NK1.1 marker, home to the liver, and produce IL-4, like Vα14-Jα18 NKT cells.[141]

These cells were not only dependent on SAP for their differentiation, but also expressed PLZF.[142–144] In OP-9 supported thymocyte cultures, PLZF could be induced after cross-linking with Vγ-specific TCR antibodies. Likewise, TCR αβ thymocytes upregulated *Zbtb16* messenger ribonucleic acid after injection of anti-TCRβ antibodies in vivo.[100] These findings support the notion that agonist signaling induces PLZF to generate innate-like αβ and γδ lineages. MR-1–specific Mucosal-associated invariant T cells were also reported to express PLZF[104] and home to liver and gut.[145] Together with experiments showing that CD4 T cells selected by MHC class II expressed on thymocytes also acquired PLZF in a SAP-dependent manner,[109–112] these observations reveal an authentic cellular and molecular pathway dedicated to the generation of innate-like lymphocytes.

Deficiencies or mutations affecting certain genes associated with TCR signaling produced a phenotype characterized by a cell-intrinsic expansion of the PLZF-expressing thymic lineages, including Vγ1Vδ6 T cells and Vα14-Jα18 NKT cells. This expansion was associated with a bystander conversion of MHC-restricted thymocytes, mainly CD8 T cells, into innate-like CD62L^{lo} CD44^{hi} CD122^{hi} eomesodermin^{hi} effectors, caused by IL-4 secreted by the PLZF-expressing cells. This complex phenotype was observed in mice lacking the Tec kinases Itk or Rlk,[146–148] or bearing the Y145F mutation in the scaffold protein SLP76, which impairs binding of these kinases after TCR signaling.[143] Mice lacking the inhibitor of E proteins Id3, which can be induced downstream of the TCR-activated MAP kinases, showed a strikingly similar phenotype,[143,149,150] as did mice after conditional ablation of CREB binding protein,[151] a general transcriptional coactivator, and KLF2, a transcription factor that controls CD62L and S1P1 receptor upregulation after positive selection.[148,152]

The convergent impact of these different genes suggests the existence of a negative regulatory pathway that physiologically contains the expansion and function of innate-like T cells. Notably, wild-type BALB/c mice spontaneously exhibited a similar, albeit milder, phenotype as mutant C57BL/6 mice, including a relative expansion of PLZF-expressing NKT thymocytes and a bystander induction of CD44, CD122, eomesodermin, and the rapid production of cytokines by CD8 SP thymocytes.[148] This phenotype was dependent on *klf13*.[153] Thus, there is a natural crosstalk between innate-like and conventional T cells during thymic development, whose variations may have a profound impact on the immune system.

Homeostasis

NKT cells are produced at a similar rate from fetal to adult life, and they persist indefinitely after adult thymectomy. The mechanisms ensuring their long life in peripheral tissues are detailed in the following.

Interleukin-15

Like NK cells and CD8 memory cells, mature NKT cells critically depend on IL-15 for their terminal maturation as well as their survival and homeostatic renewal.[154–156] Cell-type–specific ablation of IL-15Rα, which is essential to present IL-15 at the surface of IL-15–producing cells, revealed that the sole source of IL-15 for development was a nonhemopoietic radiation-insensitive stromal cell type, most likely a medullary epithelial cell.[157] In contrast, both radiation-resistant and hemopoietic cells served as partly redundant sources of IL-15 for peripheral NKT cells.

CXCL16

CXCR6 signaling, in response to CXCL16 produced by endothelial cells, is critical for the survival of liver NKT cells in basal conditions, but not for their specific accumulation or their crawling behavior in the liver sinusoids.[158]

Cluster of Differentiation 1d Ligands

In mice lacking CD1d in peripheral tissues, the full terminal maturation of NKT cells after thymic export is partially altered but the overall frequency, tissue distribution, survival, and functional properties of NKT cells are not significantly impaired.[80,87] Thus, peripheral NKT cells do not require contact with self-antigens for homeostasis.

Microbiota

NKT cells did not exhibit major disturbances in their development or function in germ-free mice.[78,159,160] However, mice lacking NKT cells were reported to harbor changes in the composition of their microbiota.[160]

TISSUE DISTRIBUTION AND RECIRCULATION

In C57BL/6 mice, NKT cells represent ~0.2% of the T-cell population in blood and peripheral lymph nodes; ~2.5% of T cells in spleen, mesenteric, and pancreatic lymph nodes; 5% in the lung; and up to 40% in the liver. Furthermore, parabiotic experiments have shown that, unlike most lymphocytes, NKT cells do not recirculate and are lifelong residents of lymphoid tissues, lung, and liver.[161]

Tissue and Intravascular Residents

This tissue residency is particularly remarkable in the liver and lung where NKT cells are mainly found within the capillary microvasculature.[158,161] Intravital imaging using confocal microscopy of the liver of CXCR6^{GFP} mice, where a majority of brightly fluorescent liver lymphocytes are Vα14 NKT cells, demonstrated their presence inside the sinusoid capillaries, adhering to the luminal side of endothelial cells and crawling, without directional bias, at a speed of ~10μm/min (Fig. 18.6). While other effector T cells and monocytes can adopt a similar crawling behavior transiently, before they extravasate to enter inflamed tissues, NKT cells are constitutively and permanently crawling in steady-state conditions.

The expression of high amount of CXCR6 by NKT cells matches the production of the corresponding chemokine ligand CXCL16 by endothelial cells lining the sinusoids. CXCR6 appeared to be important for the survival of liver NKT cells[158] as well as NK cells,[162] but it was not required for liver accumulation. Furthermore, the adhesion and crawling processes were unaltered by pertussis toxin treatment, suggesting independence from $G\alpha_i$ protein signaling.[163]

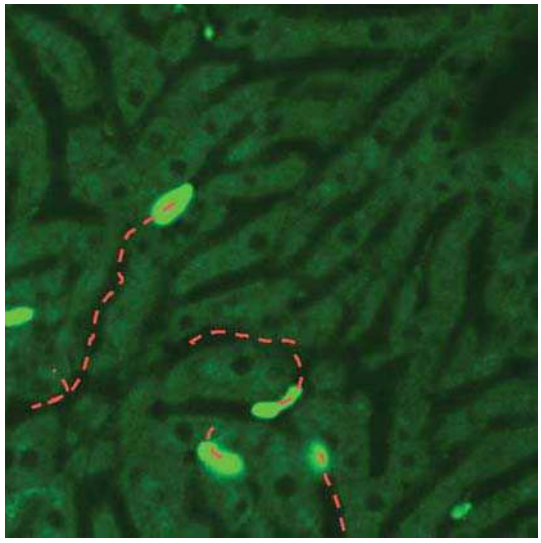

FIG. 18.6. Intravascular Patrolling by Liver Natural Killer T (NKT) Cells. Fluorescent NKT cells in CXCR6-GFP "knock-in" mice are crawling on the luminal side of sinusoid endothelial cells. *Dashed lines* show their path over a period of 10 mn in a live imaging study. (Courtesy of Frederic Geissmann, King's College, London, UK, and Dan Littman, New York University, New York, NY, USA)

Thus, the constitutive activation of LFA-1 was neither a consequence of TCR signaling, as NKT cells normally accumulated in the liver of mice lacking CD1d outside of the thymus,[80,87] nor of chemokine-induced $G\alpha_i$ protein signaling. Notably, transgenic expression of PLZF in MHC class II–restricted CD4 T cells was sufficient to induce their liver retention, indicating that the adhesion and crawling behavior are an integral component of the PLZF-induced functional program.[161]

Organ-specific Natural Killer T-Cell Sublineages

DN NKT cells expressing Rorγt and producing IL-17 and IL-22 but not IL-4 or IFN-γ selectively resided in skin and draining axillary and inguinal lymph nodes.[164,165] While they shared the usual $CD44^{hi}CD62L^{lo}$ effector phenotype of other NKT cells, they did not express the stage 3 markers Tbet, IL2Rβ, CD69, or NK receptors and instead displayed a $CD103^{hi}$ CCR6+ CD121a+ phenotype. The identification of a minor cell population in the thymus with a similar phenotype suggested that they constituted a separate developmental sublineage.[166] Intriguingly, their expression of Nrp-1, a marker of recent thymic emigrants and their rapid disappearance after adult thymectomy, suggested that, unlike most NKT cells, they represented a short-lived population.[82]

A subset of NKT cells expressing the IL-25 receptor and biased toward production of IL-4, IL-3, and IL-9 was found in the thymus, spleen, and lung, but was poorly represented in the liver and bone marrow.[167] Whether these cells constitute a tissue-specific sublineage that differentiates in the thymus or in the periphery remains to be determined.

NATURAL KILLER T-CELL FUNCTIONS

Functional Properties

DC activation and the explosive release of cytokines and chemokines by NKT cells have well-documented functional consequences in a variety of pathological or therapeutic conditions involving lymphoid tissues and body organs. Important factors biasing the Th1, Th2, or Th17 outcome of NKT-cell activation in vivo have recently emerged. They include the structure of the lipid antigen, the antigen-presenting cell type, the tissue, and the coexposure to TLR ligands. These will be discussed in two separate contexts: the administration of synthetic NKT ligands, which function as potent vaccine adjuvants, and the activation of NKT cells in the context of microbial infection.

Vaccine Adjuvant Properties

Dendritic Cell–based Network of Activation

Several studies have characterized a cascade of activation events following the exogenous administration of NKT ligands such as αGalCer (Fig. 18.7). The central feature was a cross-activation between NKT cells and DCs initiated upon the presentation of αGalCer by resting DCs to NKT cells. Activated NKT cells upregulated CD40L, Th1 and Th2 cytokines, and chemokines; CD40 crosslinking induced DCs to upregulate CD40, B7.1, and B7.2 and IL-12p40, which in turn enhanced NKT-cell activation and cytokine production.[168,169] DCs upregulated MHC class I– and class II–mediated antigen presentation, particularly cross-priming, and secreted CCL17,[170] a chemoattractant for naïve CCR4+ CD8 T cells. IL-12 released by DCs promoted prolonged IFN-γ production by NK cells and activated their cytolytic properties.[171,172] Thus, αGalCer promotes robust CD4 and CD8 T-cell–mediated adaptive immune responses against coadministered nonreplicating protein antigens.[173–175] Furthermore, although TLR signaling is not involved in the innate response to αGalCer, the combination of TLR and NKT ligands was synergistic.[176]

Th1 and Th2 Variants

Different synthetic agonists showed biased Th1 versus Th2 outcomes mainly due to their differential ability to induce IL-12 from DCs and to transactivate NK cells. Variants of the original αGalCer (KRN7000) with short or unsaturated lipid chains induced similar Th2 but decreased Th1 cytokines.[177–179] While early reports pointed to the lower TCR affinity of a variant with truncated phytosphingosine chain (psC9:0, called "OCH"),[180] several other Th2 variants, including acylC8:0 or acylC20:2, demonstrated identical interactions based on affinity measurements and crystal structures.[56,181,182] All these Th2 variants had increased solubility in water and could be rapidly loaded onto CD1d molecules at the cell surface. In contrast, αGalCer was almost exclusively loaded in the lysosome due to a requirement for lipid transfer proteins. Whereas αGalCer remained stably associated with CD1d, the short or unsaturated variants were rapidly ejected upon recycling to the acidic endosome.[21] Interestingly, αGalCer was found associated with surface CD1d molecules on lipid rafts, whereas the short or unsaturated variants were loaded to CD1d molecules outside of these rafts, perhaps as a consequence of their different loading compartments.[182] However,

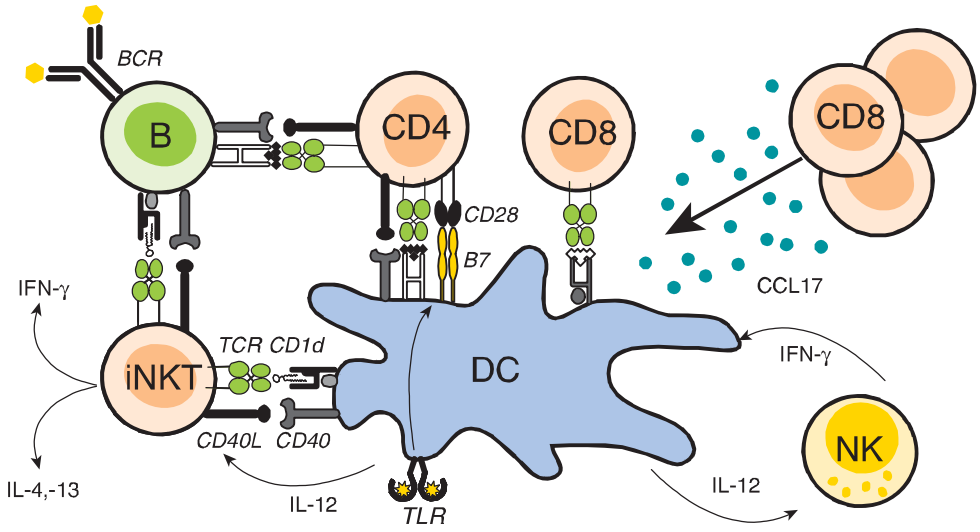

FIG. 18.7. Cellular and Molecular Network Activated by the Natural Killer T (NKT) Ligand αGalCer. Cross-activation between NKT cells and dendritic cells (DCs) lead to cluster of differentiation (CD)40L-CD40 interactions, activating the DC to present peptide antigens to CD4 and also to CD8 cells (cross-priming). NKT cells promptly release Th1 and Th2 cytokines. Naïve CD8 T cells are chemoattracted through CCL17 secreted by DCs. NK cells are "transactivated" by DCs and interleukin-12 to release interferon-γ in a prolonged manner. B cells seeing antigens can receive cognate help from NKT cells and/or from CD4 cells.

this is unlikely to explain the Th2 bias, as the variants induced the same balance of cytokines as αGalCer when tested in vitro with purified antigen-presenting cells. A more likely explanation for the Th2 bias was suggested by in vivo studies with mice carrying a floxed CD1d allele. Indeed, while αGalCer presentation was mostly limited to lysosomally active DCs and macrophages, which produced IL-12, the Th2 variants were broadly presented by all CD1d-expressing cell types, most of which did not produce IL-12.[89] Thus, structural differences in lipids resulted in the involvement of different antigen-presenting cell types, altering the cellular network and the outcome of NKT-cell activation (Fig. 18.8).

Anergy

A limitation to the adjuvant properties of NKT ligands is the rapid NKT TCR downregulation and apoptosis, followed by a long-lasting depletion and a state of anergy.[183–185] Anergy could be partially avoided by CD28 costimulation or PD1 blockade,[186,187] or by the administration of αGalCer-pulsed DCs, or αGalCer conjugated to nanoparticles.[188–190] The mechanism of NKT-cell anergy remains obscure.

Natural Killer T-Cell Help to B Cells

NKT-cell activation by agonist ligands promoted antibody production, affinity maturation, and switching against associated antigens. Help could be indirect, through CD40L-CD40 mediated activation of the DCs presenting peptide antigen to CD4 T cells, which in turn provided MHC II–restricted help to B cells recognizing a linked antigen. Alternatively, direct NKT-B cell cognate interactions have been demonstrated in vitro and in vivo.[191–197] The relevance of this direct cognate help was illustrated by infection with *Sphingomonas*, which carries abundant glycosphingolipid ligands in its cell wall.[198] The production of immunoglobulin (Ig)G antibodies against the bacterial membrane appended enzyme pyruvate dehydrogenase complex-E2 depended on CD1d expression by B cells, as evidenced in mixed bone marrow chimeras where CD1d expression was limited to one Ig allotype–marked, B-cell compartment. NKT cells engaged in cognate and prolonged interactions with B cells, after acquiring a stereotypical Bcl-6 dependent follicular helper program with induction of CXCR5, PD1, and IL-21, and migration to the germinal center.[196,197] Intriguingly, immunization with αGalCer-nitrophenol (NP) induced faster but less sustained germinal center formation with fewer long-lived plasma cells and inferior memory compared with keyhole limpet hemocyanin-NP or ovalbumin-loaded (OVA)-NP,[196,197] suggesting that NKT cell help to B cells was intrinsically inferior to conventional T-cell help. However, other variables might have contributed to these differences, including the monovalency of NP in the case of αGalCer, differences in bioavailability, cellular targeting, half-life, and processing of the different immunogen preparations. It is also possible that the rapid induction of NKT-cell anergy after exposure to αGalCer might limit the interactions required for effective B-cell help.

Splenic marginal zone B cells constitutively express approximately eight times more CD1d on their surface than other B-cell subsets.[9] They responded to antigen in an accelerated manner with increased sensitivity conferred by the complement receptor CD21, which lowers the B-cell receptor signaling threshold to complement-coated antigen.[199] These characteristics have long suggested a special function in CD1d-mediated B-cell responses. In support of such

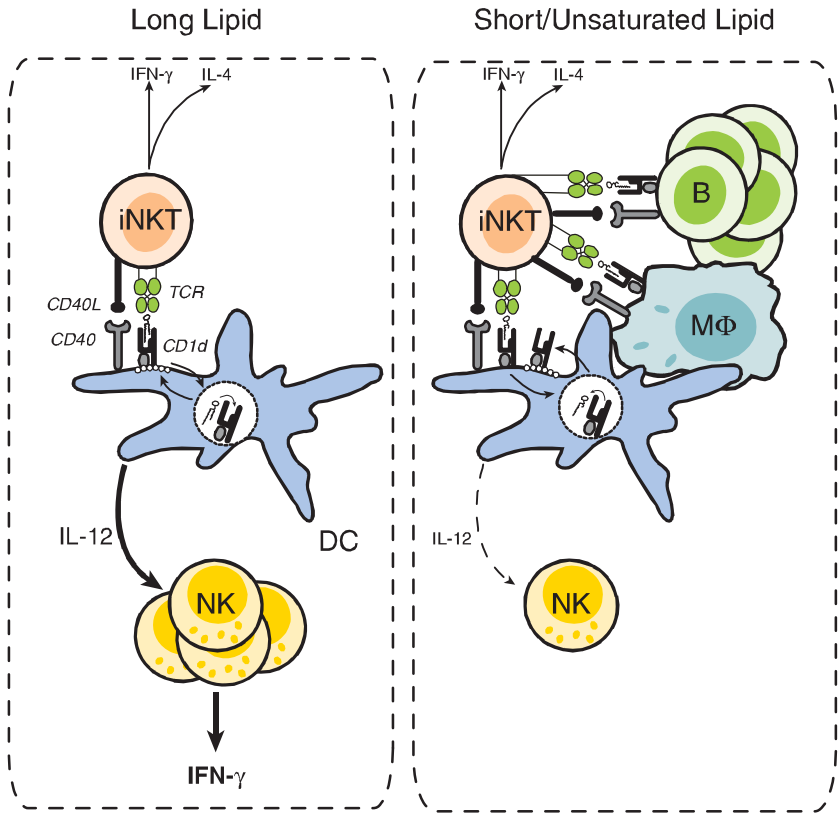

FIG. 18.8. Th1 and Th2 Agonist Ligands of Natural Killer T (NKT) Cells. *Left*, long and saturated αGalCer variants (including KRN7000) load cluster of differentiation (CD)1d in the lysosomal compartment due to their requirement for lipid transfer proteins. The main antigen-presenting cell is a dendritic cell (DC), which produces lots of interleukin (IL)-12 and transactivates NK cells. *Right*, short or polyunsaturated lipids load CD1d directly at the cell surface, but are rapidly ejected upon recycling to the lysosome due to displacement by longer, hydrophobic peptides at acidic pH. These short or polyunsaturated lipids are promiscuously presented by all CD1d expressing cells, limiting DC interactions, interleukin-12 production, and natural killer transactivation. CD1d complexed with long and saturated variants sit in lipid rafts, whereas CD1d complexed with short or polyunsaturated variants are excluded from lipid rafts.

NKT-MZB interactions in vivo, depletion of marginal zone B cells impaired the IgM anti-NP antibody response after systemic administration of αGalCer-NP.[200]

Natural Killer T-Cell Help to Macrophages and Myeloid Suppressor Cells

Subcapsular CD169+ macrophages efficiently captured and presented αGalCer to NKT cells in lymph nodes after footpad injection of 200 nm silica beads coated with the lipid antigen.[201] This selective capture further emphasizes the importance of the formulation of αGalCer in targeting different antigen-presenting cell types. Notably, agonist-mediated NKT-cell interaction with myeloid suppressor cells could reverse their suppressive properties. Furthermore, natural interactions between NKT cells and myeloid suppressor cells have been suggested in transplanted cancers and influenza infection.[202–204]

Microbial Infections

The recognition of either microbial α-linked glycolipid antigens or self-β-linked antigens induced by TLR signaling ensures the recruitment of NKT cells in most microbial infections (Fig. 18.9). Which of these modes of activation predominates for individual microorganisms is somewhat controversial, as TLR signaling induces IL-12, which is a potent amplifier of IFN-γ secretion, and NKT cells upregulate their IL-12 receptor upon TCR signaling.

Direct Microbial Lipid Recognition

Glycosphingolipids closely related to αGalCer were found in the cell wall of *Sphingomonas*,[30,205] a prominent gram-negative LPS-negative member of α-proteobacteria, an abundant class of bacteria. These glycosphingolipids included the dominant α-branched glucuronyl and galacturonyl ceramides (GSL-1) and the less abundant di- (GSL-2), tri- (GSL-3), and tetraglycosylated (GSL-4) species. Although they form structures that are reminiscent of LPS, their synthesis pathway and their role in the microbial cell wall are not well understood. GSL-1 activates large proportions of mouse and human NKT cells,[31,32,206,207] but it is unclear at present whether the more complex GSL-2, -3, and -4 can be recognized by NKT cells or even whether they can be efficiently processed into GSL-1 by host antigen-presenting cells. During infection, *Sphingomonas* was phagocytosed by macrophages and DCs, and elicited an activation cascade similar to exogenous αGalCer. NKT-deficient mice had 15- to 1000-fold more residual bacteria than their wild-type littermates in the first few days after infection.[31,32] High doses of *Sphingomonas* induced a lethal toxic shock in wild-type but not NKT-deficient littermates. These observations have led to the hypothesis that NKT cells and their canonical TCR specificity might have evolved to meet the challenges of these gram-negative LPS-negative bacteria. While *Sphingomonas* can cause severe infection, particularly in immunocompromised hosts, other more deadly members of the class of α-proteobacteria may have provided stronger evolutionary pressures on the NKT-cell system. Particularly interesting is the case of *Ehrlichia*, a tick-borne pathogen and member of the *Rickettsiales*, which is of widespread significance for mammals, including wild and domesticated ruminants, dogs, and humans in some regions of the world such as Africa and east Asia. *Ehrlichia muris*

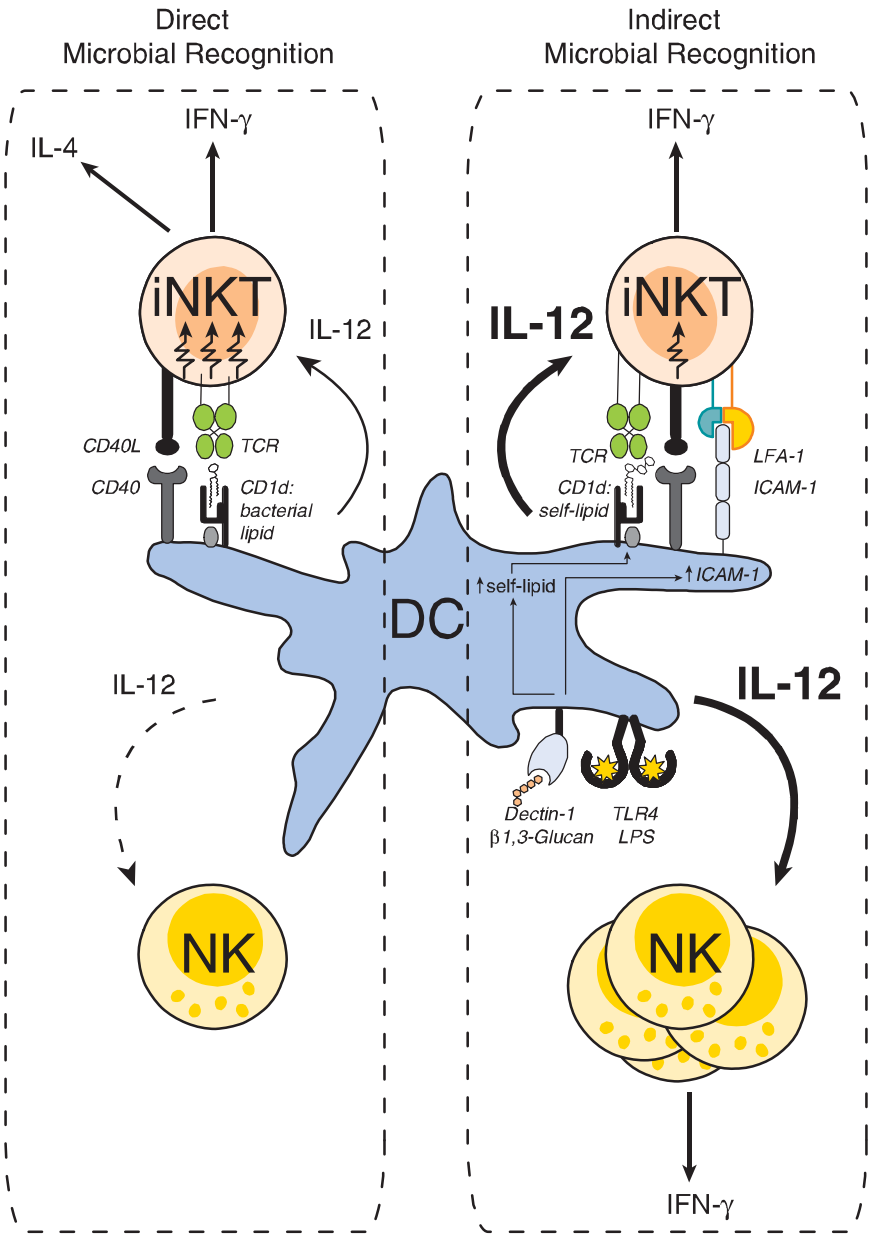

FIG. 18.9. Dual Recognition of Self- and Microbial Ligands in Microbial Infections. *Left,* microbial organisms expressing agonist ligands can elicit direct activation of natural killer T (NKT) cells. *Right,* microbial organisms lacking NKT ligands activate NKT cells through the enhanced recognition of self NKT ligands. These self ligands are upregulated by toll-like receptor– or dectin-1–mediated signals, and their recognition is enhanced by upregulation of intercellular adhesion molecule at the immune synapse. Interleukin-12 secreted by dendritic cells amplifies interferon-γ produced by NKT cells and by natural killer cells.

activated NKT cells independently of MyD88, and its clearance was profoundly impaired in CD1d- or Jα18-deficient animals.[31] However, the cell wall composition of *Ehrlichia*, a gram-negative, LPS-negative obligate intracellular bacterium, has not been elucidated.

Other bacteria such as *Streptococcus*[34,208] and *Borrelia*[33] expressed α-linked diacylglycerol lipids that bound CD1d and could directly engage the mouse and human NKT TCR, resulting in accelerated microbial clearance. *Borrelia* was normally cleared by NKT-deficient mice, except at later time points in the joints.[163,209] Intravital microscopy of the liver response to an intravenous inoculation of *Borrelia burgdorferi* showed rapid microbial uptake by stationary Kupffer cells followed by the attraction and arrest of NKT cells in the sinusoids. Stable, CD1d-mediated contacts between Kupffer cells and NKT cells depended on the secretion of CXCL9 by Kupffer cells and the pertussis toxin–sensitive response of CXCR3-expressing NKT cells.

Self-Lipid Recognition

Many bacteria that do not harbor NKT ligands nevertheless induce massive amounts of IFN-γ in a NKT- and CD1d-dependent manner.[31,39] This secretion of IFN-γ is considerably amplified through TLR-induced IL-12 released by DCs and macrophages and the transactivation of NK cells (see Fig. 18.9). TLR signaling regulates several glycosphingolipid enzymes, leading to the accumulation of stimulatory glycosphingolipids (see Fig. 18.3), and activation of LFA-1/ICAM-1 interactions further facilitate NKT-cell recognition of these low-affinity ligands. However, contrary to an early report,[210] NKT cells do not usually constitute the predominant cell-type producing IFN-γ in response to IL-12

in vivo,[39,211] which explains why they generally do not appear to play an essential role in many bacterial infections. Nevertheless, bacterial clearance and neutrophils were reportedly decreased in the lungs of CD1d-deficient mice after infection with *Pseudomonas aeruginosa*.[212] This may not be the case at other sites of infection.[213] Different conclusions were also reported regarding the importance of NKT cells versus NK cells in lethal LPS-induced toxic shock.[214,215]

Fungal and Parasitic Infections

The production of IgG antibodies to the malaria circumsporozoite antigen, a key component of protective immune responses in humans, was suggested to depend on NKT-cell recognition of malarial glycosylphosphatidylinositol antigens in a mouse model.[216] However, additional experiments failed to detect a CD1d-dependent component to this antibody response and glycosylphosphatidylinositols have not been identified as NKT-cell antigens in other reports.[217,218] In the context of helminth infection, DCs pulsed with *Schistosoma mansoni* eggs activated NKT cells to secrete Th1 and Th2 cytokines in vitro in a β-hexosaminidase B-dependent but MyD88-independent manner, suggesting recognition of the self-ligand iGb3 in the absence of TLR signaling.[219]

Intratracheal infection by the fungal pathogen *Aspergillus fumigatus* indirectly stimulated NKT-cell production of IFN-γ through activation of the dectin-1/MyD88 pathway by the major cell wall polysaccharides, β-1,3 glucans. Clearance of the pathogen was impaired in NKT-deficient mice.

Viral Infections

Viruses can activate TLR signaling through the adaptors MyD88 and TRIF, which in turn activate autoreactive NKT cells. Relatively modest defects in viral clearance have been reported in CD1d-deficient mice infected with encephalomyocarditis virus[220] or coxsackie B3,[221] but these defects were not observed in Jα18-deficient mice, ruling out a specific role of Vα14 NKT cells. Influenza virus clearance was normal or modestly impaired in mice lacking NKT cells.[222,223] Infections with lymphocytic choriomeningitis virus, mouse cytomegalovirus, vaccinia virus, and coronavirus were unaffected. In humans, a profibrotic role of Vα24 NKT cells was suggested in hepatitis C[224] and non-Vα24 CD1d-restricted T cells were found in the liver.[225] Although a specific role of Vα14 NKT cells in herpes simplex virus infection is controversial,[226,227] studies have suggested that viral invasion may be associated with countermeasures against CD1d or NKT cells. For example, herpes simplex virus-1 drastically and specifically impaired CD1d recycling from lysosome to plasma membrane, an essential pathway for glycolipid antigen presentation to NKT cells.[228] Kaposi sarcoma–associated herpes virus encodes two modulators of immune recognition MIR1 and MIR2 that downregulated CD1d along with other immunologically relevant molecules such as MHC class I, CD86, and ICAM-1 through ubiquitination of lysine residues in their cytoplasmic tail.[229] The lethal outcome of infections with Epstein-Barr virus in patients with X-linked lymphoproliferative immunodeficiency syndrome due to SAP mutations was hypothesized to result from the absence of NKT cells.[93] Which of these effects or associations reflect specific viral evasion/immune defense strategies and the nature of the putative NKT ligands involved in these infectious conditions remain to be determined.

Autoimmunity, Inflammation, and Allergy

Asthma

Studies in the OVA/Alum model of asthma in mouse and a report that humans with asthma harbored high percentages of Vα24 NKT cells in their bronchoalveolar lavage suggested a broad role of NKT cells in various forms of airway allergic inflammation. However, these studies have not been widely confirmed.[230]

Other studies have focused on the presence of airborne NKT ligands in natural environments, for example in house dust or in air samples where *Sphingomonas* is one of the predominant microbial species identified.[231,232] Airway exposure to these NKT ligands resulted in the rapid CCL17-induced recruitment of resident intravascular NKT cells into the lung with formation of eosinophilic granulomas. Notably, a single airway exposure to protein antigen associated with NKT ligands led to massive recall allergic airway inflammation upon airborne challenge with protein alone. Likewise, coadministration of house dust extracts containing unidentified NKT ligands promoted allergic sensitization to airborne OVA in an NKT-dependent manner.

Thus, emerging studies indicate that natural exposure to airborne NKT ligands in the environment may constitute a previously unappreciated cause of allergic sensitization.

Diabetes and Autoimmune Diseases

Many reports have suggested a role of NKT cells, usually of regulatory nature, in autoimmune diseases such as type I diabetes, arthritis, and lupus, and in atherosclerosis and inflammatory bowel disease.[233] These reports mostly relied on comparisons between wild-type versus Jα18- or CD1d-deficient mice but did not offer precise mechanistic insights into the putative function of NKT cells and their mode of recruitment. Some of these studies did not use littermates as wild-type controls and have not been widely reproduced. Furthermore, the recent discovery that NKT thymocytes impact the function of MHC-restricted T cells in a bystander manner, through the secretion of IL-4,[148] suggests that alternative, indirect mechanisms might contribute to these observations.

Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is an enigmatic disease characterized by the presence of antimitochondrial antibodies, liver lymphocytic infiltrates, and the chronic destruction of the biliary epithelium leading to cirrhosis.[234] The autoantibodies recognize an epitope of the mitochondrial pyruvate dehydrogenase complex-E2 enzyme that is particularly well conserved in *Novosphingobium aromaticivorans*, a strain of *Sphingomonas*. Further, patients with PBC, including those

lacking antimitochondrial antibodies, were specifically seropositive against *Sphingomonas*, which was detected by polymerase chain reaction in stool samples of 25% of diseased or healthy individuals, suggesting that PBC may be induced by aberrant host reactivity to this bacterium.[235] Patients with PBC also showed an enrichment of Vα24 NKT cells in liver biopsies and a depletion in blood.[236] In light of the recent finding that *Sphingomonas* cell wall glycolipids specifically activate NKT cells, these studies suggested that NKT cells might play a key role in the pathogenesis of PBC by promoting aberrant responses to *Sphingomonas*. An experimental model of infection induced a chronic liver disease with PBC-like granulomas and antimicrobial autoantibodies, which could be transferred by lymphocytes. These provocative findings support the hypothesis that PBC might be the consequence of cryptic episodes of bacterial infections, perhaps triggered by commensals containing NKT ligands, leading to NKT cell–assisted breakdown of tolerance to shared mitochondrial/bacterial antigens.

Cancer

A role of Jα18 and non-Jα18 CD1d-restricted NKT cells in cancer surveillance has been inferred from reports that CD1d- and Jα18-deficient mice seemed to differ from wild-type mice in their susceptibility to a variety of spontaneous and transplanted cancers.[237] Vα14 NKT cells were suggested to exert protection against spontaneous sarcomas. These included fibrosarcomas in mice injected intramuscularly with methylcholantrene,[238] osteosarcomas and hemopoietic tumors in p53+/- mice,[239] and carcinomas in transgenic adenocarcinoma of the mouse prostate mice.[240] Some transplanted cancers seemed aggravated in their progression by the presence of non-Vα14 NKT cells, apparently because of their secretion of IL-13 and interaction with myeloid suppressor cells.[202,203] These cancer studies have commonly concluded that endogenous ligands might be induced and presented by the tumor themselves or by antigen-presenting cells. The crosstalk between myeloid-derived cells and NKT cells resulted in suppression, or on the contrary, to exacerbation of immune responses against cancer. In most cases, however, the nature of these putative ligands has escaped precise identification. One exception lies with the proposed role of lysophosphatidylcholine as a tumor antigen in myeloma patients.[54]

A serious challenge to the interpretation of these studies is their near exclusive reliance on comparisons between wild-type and NKT-deficient mice and the lack of direct evidence of NKT cell involvement in the antitumor response. Moreover, some studies did not use proper littermate controls, or could not be independently reproduced. For example, the longstanding but isolated report that methylcholantrene-induced sarcomas were naturally controlled by Vα14 NKT cells[238] could not be confirmed in recent double-blind studies comparing large groups of CD1d- or Jα18-deficient mice and their littermate controls.[241] In addition, some of the reported results may be explained by an indirect rather than a direct function of NKT cells, for example due to their cross-talk with conventional T cells.[148]

CONCLUSION

Recent Advances

In the past 4 years, pivotal discoveries have advanced our understanding of the biology of NKT cells, particularly regarding their dual recognition of self- and foreign-lipid antigens; their role in infectious and allergic diseases; their specialized effector functions within microenvironments; their expression of a lineage-specific master transcription factor, PLZF; and their cross-talk with MHC-restricted T cells.

Remaining Challenges

The nature and the hierarchy of self- and foreign antigens recognized by NKT cells and their proposed role in a variety of diseases remain a work in progress. Are there other, perhaps more broadly relevant NKT ligands and what controls their expression?

Which class of microorganisms might explain the evolution of this elaborate lineage? Might NKT cells protect against diseases that have recently disappeared, at least in Western societies? In that regard, typhus-like disease after infection by tick-borne *Ehrlichia* may be comparable to malarial disease in its devastating impact in endemic regions.

What are the signaling events underlying NKT-cell differentiation, particularly the signals inducing PLZF, the molecular mechanisms of PLZF function, and the acquisition of NK receptors? Are there master transcription factors, similar to PLZF, that control other innate-like lineages such as CD8αα TCRαβ IELs and B1 B cells?

Finally, will NKT agonists demonstrate efficiency and safety as vaccine adjuvants to combat infections and cancer in humans?

Other Cluster of Differentiation 1–restricted T Cells?

Although there is strong evidence that the majority of CD1d-restricted αβ T cells in mice belong to the NKT lineage,[5] little is known about the human populations of αβT cells restricted by CD1d or other CD1 isotypes such as CD1a, b, and c.

Convergent studies reviewed in this chapter suggested that thymic selection by ligands predominantly expressed on cortical thymocytes or other hemopoietic cells was a key determinant of innate-like lineage decision. As CD1a, b, c, and d are all predominantly expressed on cortical thymocytes, they might mainly select PLZF-expressing T cells. This hypothesis remains to be directly tested in fresh human T-cell populations. Notably, humanized mice expressing a CD1b-restricted human TCR together with human CD1b under its own regulatory elements developed effector-like T cells that expressed PLZF.[242] Other insights have come from studies of a small subpopulation of human αGalCer-specific T cells that did not express Vα24, but used the remaining conserved elements of the NKT TCR, Jα18 and Vβ11. These cells expressed intermediate amounts of PLZF and kept a naïve $CD62L^{hi}$ $CD45RO^{hi}$ phenotype.[124] This natural example suggests that PLZF expression may be a reliable marker of T cells selected by CD1 molecules, but that different levels of expression may be imparted depending on as yet unknown factors such as perhaps, the intensity of TCR

signaling. Other studies based on the large scale cloning of fresh T cells have suggested that 3% to 10% of the cord blood T-cell population may be CD1-restricted and naïve-like, with a larger proportion of memory-type cells appearing in adults.[243] These important studies could not, however, determine whether the CD1-restricted T cells were selected by CD1 molecules or were merely cross-reactive cells primarily selected by MHC.

Most fresh CD1-restricted T cells identified so far in mice and humans have shown a CD4+ or CD4-CD8β- DN phenotype, with occasional expression of CD8α but rarely CD8β.[124,243] This coreceptor expression pattern parallels the one found in mouse and human CD1d-restricted NKT cells where it is associated with expression of ThPOK/cKrox, suggesting that most CD1-restricted T cells might also express this transcription factor.

These observations provide a background for studies of fresh, unimmunized CD1-restricted T cells in humans, which are now possible through the use of tetramers. Together with studies of "humanized" mice expressing human CD1-restricted TCRs and CD1 molecules, a more definitive characterization of the different populations of CD1-restricted T cells should emerge. Will they demonstrate the heavy contribution of germline TCR segments and the basal autoreactivity that is a hallmark of NKT cells? Will they display adaptive or innate-like features? Forthcoming answers to these fundamental questions will not only be crucial for clinical applications, but will also shed light on the evolutionary logics of lipid antigen recognition.

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CHAPTER 19

Macrophages and Phagocytosis

Siamon Gordon

INTRODUCTION

Macrophages (Mϕ) represent a family of mononuclear leukocytes that are widely distributed throughout the body within and outside of lymphohemopoietic organs. They vary considerably in lifespan and phenotype, depending on their origin and local microenvironment. Mature Mϕ are highly phagocytic, relatively long-lived cells that are adaptable in their biosynthetic responses to antigens and microbial stimuli. The functions of Mϕ within tissues are homeostatic, regulating the local and systemic milieu through diverse plasma membrane receptors and varied secretory products. They react to, and themselves generate, signals that influence growth, differentiation, and death of other cells, recognizing and engulfing senescent and abnormal cells. These activities contribute substantially to recognition and defense functions against invading microorganisms, foreign particulates, and other immunogens. Innate immune functions of Mϕ complement their contributions to acquired humoral and cellular immunity, in which they regulate activation of T- and B-lymphocytes; this is achieved in part through their specialized derivatives, dendritic cells (DCs) of myeloid origin. Mϕ, with or without DCs, process and present antigen; produce chemokines and cytokines such as interleukin (IL)-1, IL-6, IL-12, IL-18, IL-23, tumor necrosis factor (TNF)-α, IL-10, and TGFβ; and phagocytose apoptotic and necrotic cells. Acting directly or under the influence of other immune cells, Mϕ capture extra- and intracellular pathogens, eliminate invaders, and deliver them to appropriate subcompartments of lymphoid organs. As key regulators of the specific as well as the natural immune response, Mϕ boost as well as limit induction and effector mechanisms of the specific immune response by positive and negative feedback.

The properties and roles of DCs, especially in antigen presentation, are described in detail elsewhere in this volume. Here, we focus on other members of the Mϕ lineage, consider their interrelationship, and outline specialized properties that underlie their roles in the execution and regulation of immune responses. A number of texts and presentations deal with the history and broad aspects of Mϕ immunobiology.[1–8]

SOME LANDMARKS IN THE STUDY OF MACROPHAGES

Our understanding of Mϕ developed in parallel with the growth of immunology as an experimental science. Metchnikoff, a comparative developmental zoologist, is widely credited for his recognition of phagocytosis and leukocyte recruitment as a fundamental host defense mechanisms of primitive, as well as highly developed multicellular organisms.[3,4,7] The Nobel awards of 2011 to Bruce Beutler,[9] Jules Hoffman,[10] and Ralph Steinman[11] reflect the paradigm shift of immune recognition from lymphocytes to innate antigen-presenting cells (APCs). Metchnikoff already clearly stated the link between capture of infectious microorganisms by the spleen and subsequent appearance of reactive substances (antibodies) in the blood, although mistakenly ascribing their production to the phagocytes themselves. The importance of systemic clearance of particles by Mϕ, especially Kupffer cells in liver and other endothelial cells, was enshrined in the term reticuloendothelial system. Although it was rejected by influential investigators in the field in favor of the term mononuclear phagocyte system, the appreciation that sinuslining Mϕ in liver and elsewhere share common properties with selected endothelial cells is worth preserving.[3] Earlier studies by Florey and his students, including Gowans, established that circulating monocytes give rise to tissue Mϕ. Van Furth and his colleagues investigated the life history of Mϕ by kinetic labeling methods; subsequently, the development of membrane antigen markers facilitated a more precise definition of specialized Mϕ subpopulations in tissues such as brain. The appearance and potential importance of Mϕ during development also became evident as a result of sensitive immunocytochemical methods. Morphologic and functional studies by Humphrey and many others drew attention to striking diversity among Mϕ-like cells in secondary lymphoid organs, especially within the marginal zone of the spleen, where complex particulates and polysaccharides are captured from the circulation.

The era of modern cell biology impinged on Mϕ studies following the studies of Cohn,[12] Hirsch, and their colleagues. Their work touched on many aspects of cell structure and function, including phagocytosis (the zipper mechanism of Silverstein), fluid- and receptor-mediated endocytosis, secretion, and antimicrobial resistance. Isolation and in vitro culture systems became available for cells from mice and humans, especially after the identification of specific growth and differentiation factors such as colony-stimulating factor-1 (CSF-1). It is perhaps fitting that the earliest known natural knockout (ko) affecting Mϕ, a natural mutation in the op gene in the osteopetrotic mouse, should involve CSF-1.[13] Cell lines retaining some but not all features of

mature Mϕ have been useful for many biochemical and cellular studies. Macrophages and dendritic cells can be derived from embryonic stem cells and induced pluripotent cells by growth in appropriate culture conditions and transfection of selected transcription factors.

The role of Mϕ as antigen-processing cells able to initiate adaptive immune responses had false trails ("immunogenic ribonucleic acid [RNA]" was thought to be involved at one time) and encompassed early genetic strategies (Mϕ of mice selected for high antisheep erythrocyte antibody responses by Biozzi and colleagues displayed enhanced degradative properties; adherent cells from defined guinea pig strains were shown to play an important role in major histocompatibility complex [MHC] Ia-restricted anti-insulin responses). For many years, the APC functions of adherent cells were highly controversial as promoted by Unanue, who concentrated on intracellular processing by Mϕ, and Steinman, who discovered the specialized role of "DCs" in antigen presentation to naive T-lymphocytes. The importance of Mϕ as effector cells in immunity to intracellular pathogens such as *Mycobacterium tuberculosis* was recognized early by Lurie and Dannenberg. Mackaness used *Listeria monocytogenes* and bacille Calmette-Guérin (BCG) infection in experimental models and developed the concept of Mϕ activation as an antigen-dependent but immunologically nonspecific enhancement of antimicrobial resistance. The subsequent delineation of T-lymphocyte subsets and characterization of interferon (IFN)-γ as the major cytokine involved in macrophage activation, including MHC II induction, merged with increasing knowledge of the role of reactive oxygen and, later, nitrogen metabolites as cytotoxic agents. The role of virus-infected Mϕ as MHC I–restricted targets for antigen-specific CD8+ killer cells was part of the initial characterization of this phenomenon by Zinkernagel and Doherty. D'Arcy Hart was an early investigator of the intracellular interactions between Mϕ and invaders of the vacuolar system, especially mycobacteria, which survive within Mϕ by inhibiting acidification and phagosomelysosome fusion, thus evading host resistance mechanisms. Mouse breeding studies by several groups defined a common genetic locus involved in resistance to BCG, *Leishmania*, and *Salmonella* organisms. The host phenotype was shown to depend on expression in Mϕ and, many years later, the gene (termed N-ramp for natural resistance-associated membrane protein) was identified by positional cloning by Skamene, Gros, and their colleagues. Positional cloning by Beutler and associates led to the identification of the gene responsible for lipopolysaccharide (LPS) resistance in particular mouse strains. Together with studies by Hoffmann and his colleagues on the toll pathway in *Drosophila*, this work resulted in an explosion of interest in the identification of mammalian toll-like receptors (TLRs) and their role in innate immunity to infection. At the same time, it became apparent that some malignant tumors contain macrophage populations that may favor their growth.

This brief survey concludes with the identification of Mϕ as key target cells for infection, dissemination, and persistence of human immunodeficiency virus (HIV), tropic for Mϕ by virtue of their expression of cluster of differentiation (CD)4, chemokine coreceptors, and DC-SIGN, a C-type lectin also expressed by DCs. Although Mϕ had been implicated by earlier workers such as Mims as important in antiviral resistance generally, their role in this regard was neglected before the emergence of HIV as a major pathogen.

Many molecules have been identified as important in Mϕ functions in immunity and serve as valuable markers to study their properties in mice and humans. These include Fc and complement receptors, which are important in opsonic phagocytosis, killing, and immunoregulation; scavenger receptors originally implicated in foam cell formation and atherogenesis by Brown and Goldstein; nonopsonic lectin receptors, such as the mannose receptor (MR) and β-glucan receptor (dectin-1) and secretory products such as lysozyme, neutral proteinases, TNFα, chemokines, and many other cytokines. A range of membrane antigens expressed by human and rodent mononuclear phagocytes has been characterized and reagents made available for further study of Mϕ in normal and diseased states. Recently, the role of deoxyribonucleic acid (DNA)-binding transcription factors including members of the NF-κB and ETS (Pu-1) families has received increased attention in the study of differential gene expression by Mϕ. Gene inactivation has confirmed the important role of many of these molecules within the intact host, and use has been made of cell-specific or conditional ko to uncover the role of Mϕ in immunologic processes. Naturally occurring inborn errors in humans such as the leukocyte adhesion deficiency syndrome and chronic granulomatous disease have contributed to the analysis of important leukocyte functions, including those of Mϕ, in host resistance to infection. Mutations in a monocyte-expressed gene (nucleotide oligomerization domain [NOD]-2), involved in cytosolic sensing of microbial products and NF-κB activation, have been implicated in a subset of individuals with an enhanced susceptibility to Crohn disease. The validity of murine ko models for human genetic deficiencies has been confirmed for key molecules involved in Mϕ activation, such as IFNγ and IL-12. *N*-ethyl *N*-nitrosourea mutagenesis has begun to reveal new macrophage innate immune functions, as has increasing application of system biology tools for microarray, proteomic, epigenetic, and microRNA analysis.

PROPERTIES OF MACROPHAGES AND THEIR RELATION TO IMMUNE FUNCTIONS

Introduction

Mϕ participate in the production, mobilization, activation, and regulation of all immune effector cells. They interact reciprocally with other cells while their own properties are modified to perform specialized immunologic functions. As a result of cell surface and auto- and paracrine interactions, Mϕ display marked heterogeneity in phenotype,[14,15] a source of interest and considerable confusion to the investigator. Increasing knowledge of cellular and molecular properties of Mϕ bears strongly on our understanding of their role in the immune response. These will be reviewed

briefly, with emphasis on functional significance, and attention will be drawn to unresolved and controversial issues.

Growth and Differentiation: Life History and Turnover

In contrast to T- and B-lymphocytes, monocytes from blood give rise to terminally differentiated Mϕ that cannot recirculate or reinitiate DNA replication except in a limited way. Unlike other myeloid granulocytic cells, Mϕ can be long lived and retain the ability to synthesize RNA and protein to a marked extent, even when in a relatively quiescent state as "resident" cells. These are distributed throughout the tissues of the body and constitute a possible alarm-response system, but they also mediate homeostatic and poorly understood trophic functions. Following inflammatory and immune stimuli, many more monocytes can be recruited to local sites and give rise to "elicited" or "immunologically activated" Mϕ with altered surface, secretory, and cytotoxic properties. The origins of Mϕ from precursors are well known: from yolk sac (and possibly earlier paraaortic progenitors), migrating to fetal liver, then spleen and bone marrow, before and after birth.[16] Yolk sac precursor cells may contribute to the establishment of selected tissue macrophages such as Langerhans cells in the adult.[17] In the fetus, mature Mϕ proliferate actively during tissue remodeling in developing organs. In the normal adult, tissue Mϕ do not self-renew extensively except in specialized microenvironments such as epidermis,[18] nervous system, or lung; after TH2-type parasitic infection, there can be considerable further replication at local sites of inflammation.[19] Growth and differentiation are tightly regulated by specific growth factors and their receptors (eg, IL-3, CSF-1, granulocyte-macrophage [GM]-CSF/IL-34, IL-4, IL-13) and inhibitors (eg, IFNα/β, transforming growth factor [TGF]-β, leukemia inhibitory factor), which vary considerably in their potency and selectivity. These processes are modulated by interactions with adjacent stromal and other cells (eg, through c-kit/ligand and Flt-3/ligand interactions). The growth-response of the target cell to an extrinsic stimulus decreases progressively and markedly (from 10^8 or more to 10^0) during differentiation from stem cell to committed precursor to monoblast, monocyte, and Mϕ, yet even the most terminally differentiated Mϕ such as microglial cells can be "reactivated" to a limited extent by local stimuli. Elicited/activated Mϕ respond more vigorously than resident Mϕ to growth stimuli in vivo and in vitro, but the molecular basis for their enhanced proliferation is unknown.

Although this general picture of blood monocyte-to-tissue Mϕ differentiation has been accepted for some time as a result of parabiosis, adoptive transfer, and irradiation-reconstitution experiments, recent studies in mouse and man have demonstrated monocyte heterogeneity and distinct properties,[20–23] with a subpopulation remaining within the vasculature, to perform a patrolling function. Our understanding of DCs and osteoclast differentiation is still compatible with a relatively simple model (Fig. 19.1) in which major Mϕ populations in mouse tissues can be characterized by selected antigen markers such as F4/80 (Emrl,

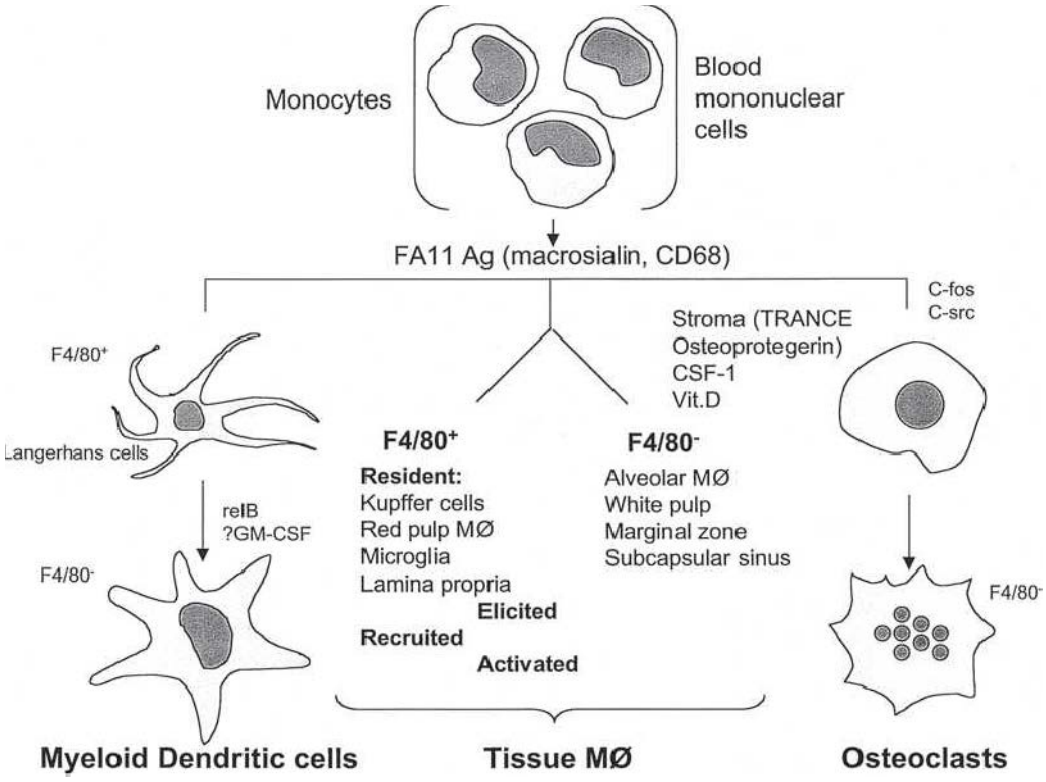


FIG. 19.1. Differentiation of mononuclear phagocytes based on antigen markers FA-11 (macrosialin, murine CD68) and F4/80. See text for detailed discussion.

a member of a family of EGF-TM7 molecules) and macrosialin (CD68), a pan-Mϕ endosomal glycoprotein related to the lysosome-associated membrane protein (LAMP) family. The DCs of myeloid origin (see elsewhere in this volume) share many properties with monocyte/macrophages,[24] but are specialized to capture, process, and present antigens to naïve lymphocytes. Circulating precursors of DCs and and macrophages are normally present in the mononuclear fraction of blood in small numbers[25]; studies in the mouse may not reflect the origin and differentiation of precursor cells in humans.[26] Monocytes that have crossed the endothelium may be induced to "reverse migrate" into the circulation by selected stimuli in tissues.[27] Finally, the mouse spleen has been shown to serve as a reservoir of monocyte/Mϕ for recruitment to sites of inflammation.[28]

Circulating mononuclear precursors for osteoclasts are less defined and differentiate into mononucleate cells, recruited in response to sphingosine-1-phosphate to bone, for example,[29] where they fuse to form multinucleate bone-resorbing osteoclasts.[30] Local stromal cells, growth factors such as CSF-1, steroids (vitamin D metabolites), and hormones (eg, calcitonin, for which osteoclasts express receptors) all contribute to local maturation. Osteoprotegerin, a naturally occurring secreted protein with homology to members of the TNF-receptor family, interacts with TRANCE, a TNF-related protein, to regulate osteoclast differentiation and activation in vitro and in vivo.

Use of antigen markers such as CD34 on progenitors, CD14 and CD16 on monocytes, and chemokine receptors and multichannel fluorescein-activated cell sorter analysis have made it possible to isolate leukocyte subpopulations and study their progeny and differential responses in different mouse tissues and models of disease.[31] The mononuclear fraction of blood may contain precursors of other tissue cells, including mesenchymal stem cells able to synthesize matrix proteins such as collagen, and some endothelial cells. Perhaps the mysterious follicular dendritic cells (FDCs) with mixed hemopoietic and mesenchymal properties fall in this category.

The large-scale production of immature and mature DC-like cells from bulk monocytes in cytokine-supplemented culture systems (IL-4, GM-CSF, TNFα) has revolutionized the study of these specialized APCs. Individually, the same cytokines give rise to Mϕ-like cells, and early during in vitro differentiation, the cellular phenotype is reversible. Later, when mature DCs with high MHC II, APC function, and other characteristic markers are formed, differentiation is irreversible. This process is independent of cell division, although earlier progenitors in bone marrow and GM-CSF–mobilized blood mononuclear cells can be stimulated to multiply, as well as differentiate, in vitro. These examples of terminal differentiation observed with DCs and osteoclasts may extend to other specialized, more obvious Mϕ-like cells. Mature Mϕ can be derived by growth and differentiation in steroid-supplemented media in Dexter-type long-term bone marrow cultures that contain stromal fibroblasts and hemopoietic elements. These Mϕ express adhesion molecules responsible for divalent cation-dependent cluster formation with erythroblasts (EbR). This receptor, possibly related to V-CAM, cannot be induced on terminally differentiated peritoneal Mϕ if these are placed in the same culture system. This contrasts sharply with the ready adaptation of many tissue Mϕ to conventional cell culture conditions, when the cells often adopt a common, standard phenotype. Irreversible stages of Mϕ differentiation may therefore occur in specialized microenvironments in vitro or in vivo.

Little is known about determinants of Mϕ longevity and turnover. Growth factors such as CSF-1 enhance Mϕ survival and prevent induction of an apoptotic program. The expression of Fas-L and Fas on Mϕ has been less studied than on lymphocytes; they and other members of the TNF and its receptor family may play a major role in determining Mϕ survival, especially in induced populations, where cell turnover is markedly enhanced. Tissue Mϕ vary greatly in their lifespan, from days to months. Apart from inflammatory and microbial stimuli, local and systemic environmental factors such as salt loading and hormones, including estrogen, are known to influence Mϕ turnover.

Tissue Distribution and Phenotypic Heterogeneity of Resident Macrophages in Lymphoid and Nonlymphoid Organs

The use of the F4/80 plasma membrane antigen made it possible to detect mature Mϕ in developing and adult murine tissues and define their anatomic relationship to other cells in endothelium, epithelium, and connective tissue, as well as the nervous system.[32,33] Subsequently, other membrane antigens,[34] macrosialin, sialoadhesin, and others were identified as useful markers for Mϕ in situ (Table 19.1). Mϕ subpopulations in different tissues display considerable heterogeneity in expressing these and selected receptor antigens (eg, complement receptor [CR]3 and class A scavenger receptor [SR-A]), drawing attention to unknown mechanisms of homing, emigration, and local adaptation to particular microenvironments. From the viewpoint of immune responses, a few aspects deserve comment.

Fetal Liver and Bone Marrow

Mature Mϕ form an integral part of the hemopoietic microenvironment and play a key role in the production, differentiation, and destruction of all hemopoietic cells. The fetal liver is a major site of definitive erythropoiesis from midgestation.[16] The bone marrow becomes active in the production of hemopoietic cells from shortly before birth, and Mϕ are a prominent component of the hemopoietic stroma throughout adult life. Mature "stromal" Mϕ in fetal liver and adult bone marrow express nonphagocytic adhesion molecules such as sialoadhesin (Sn), an immunoglobulin (Ig)-superfamily sialic acid-binding lectin (Table 19.1), and the EbR referred to previously, which is also involved in adhesion of developing myeloid and possibly lymphoid cells (Fig. 19.2). VLA-4 has been implicated as a ligand for EbR. Ligands for Sn include CD43 on developing granulocytes and on lymphocyte subpopulations. Sn clusters at sites of contact between stromal Mϕ and myeloid but not erythroid cells. Chemokines are able to induce polarized expression of adhesion molecules such as intercellular adhesion molecules and CD43 in leukocytes, but the significance

TABLE 19.1 Selected Differentiation Antigens Used to Study Murine Macrophage Heterogeneity

Ab	Ag	Structure	Ligands	Cellular Expression	Function	Comment
F4/80	F4/80 (EMR1)	EGF-TM7	?	Mature Mϕ, absent T areas	Peripheral tolerance	Useful marker development, CNS
FA-11	Macrosialin (CD68)	Mucin-LAMP	OX-LDL	Pan-Mϕ, DC	Late endosomal	Glycoforms regulated by inflammation and phagocytosis
5C6	CR3 (CD11b, CD18)	β2-integrin	iC3b, ICAM	Monocytes, microglia, PMN, NK cells	Phagocytosis, adhesion	Important in inflammatory recruitment, PMN apoptosis
2F8	SR-A (I, II)	Collagenous, type II glycoprotein Isoforms differ, cysteine-rich domain	Polyanions, LTA, LPS, bacterial proteins Modified proteins β-amyloid apolipoprotein A, E		Adhesion, endocytosis	Protects host against LPS-induced shock
				Mϕ, sinusoidal endothelium	Phagocytosis of apoptotic cells and bacteria	Promotes atherosclerosis
SER-4	Sn (Siglec-1)	Ig superfamily	Sialyl glycoconjugates (eg, CD43)	Subsets tissue Mϕ	Lectin	Strongly expressed
3D6						Marginal zone metallophils in spleen and subcapsular sinus of lymph nodes

CNS, central nervous system; DC, dendritic cell; ICAM, intercellular adhesion molecule; Ig, immunoglobulin; LAMP, lysosome-associated membrane protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; Mϕ, macrophages; NK, natural killer; OX-LDL, oxidised low density lipoprotein; PMN, polymorphonuclear neutrophil; Sn, sialoadhesin; SR-A, type A scavenger receptor.

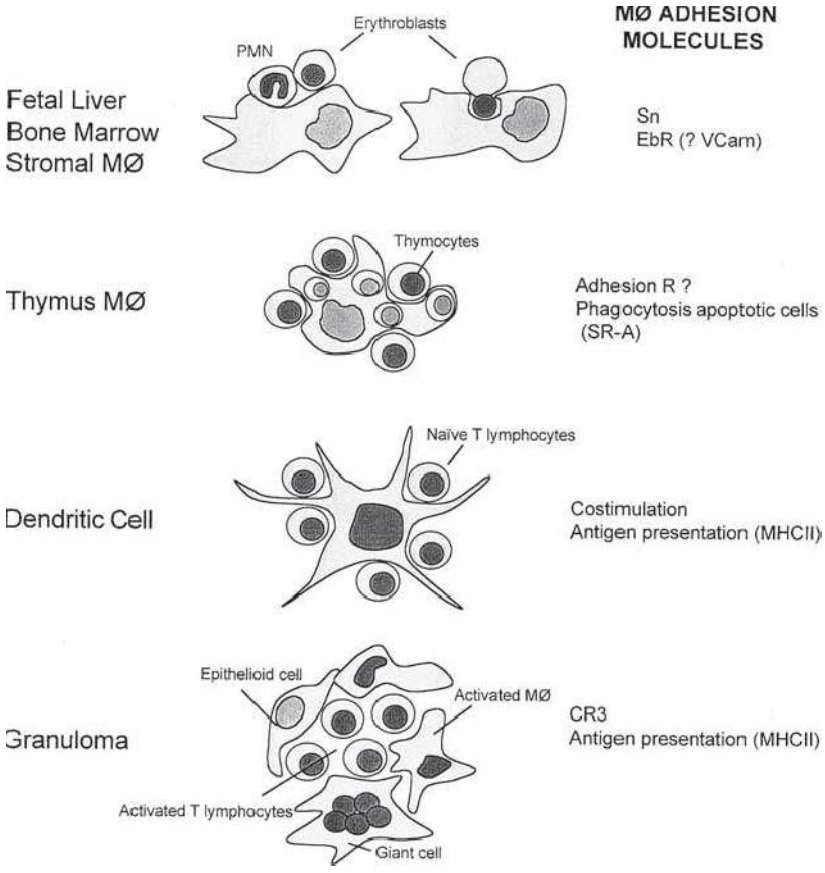


FIG. 19.2. Associations of tissue macrophages with other hemopoietic cells to illustrate variations on a common theme. See text for details.

of altered ligand distribution for interactions between Mϕ and bound hemopoietic cells is unknown. Adhesion of immature cells to stromal Mϕ may play a role in regulating their intermediate stages of development before release into the bloodstream, whereas fibroblasts in the stroma associate with earlier progenitors, as well as with Mϕ. Discarded nuclei of mammalian erythroid cells are rapidly engulfed by stromal Mϕ, but the receptors involved in their binding and phagocytosis are unknown. Mϕ also phagocytose apoptotic hemopoietic cells generated in bone marrow, including large numbers of myeloid and B cells. We know little about the plasma membrane molecules and cytokine signals operating within this complex milieu, but it is clear that stromal Mϕ constitute a neglected constituent within the hemopoietic microenvironment.

Thymus

Apart from their remarkable capacity to remove apoptotic thymocytes, the possible role of Mϕ in positive and negative selection of thymocytes has been almost totally overlooked; more attention has been given to local DCs and their specialized properties. Mature Mϕ with unusual features are also present in cortex and medulla. Clusters of viable thymocytes and Mϕ can be isolated from the thymus of young animals by collagenase digestion and adherence to a substratum (see Fig. 19.2). The nonphagocytic adhesion receptors responsible for cluster formation are more highly expressed by thymic than other Mϕ, but their nature is unknown (N. Platt, unpublished observations). These Mϕ also express MHC class II antigens and other receptors such as the SR-A (see subsequent discussion), which contributes to phagocytosis of apoptotic thymocytes in vitro, but is redundant in vivo; other markers, such as the F4/80 antigen, are poorly expressed in situ but can be readily detected after cell isolation. A striking difference between thymic and several other tissue Mϕ subpopulations is their independence of CSF-1; the CSF-1–deficient *op/op* mouse lacks osteoclasts and some Mϕ populations, including monocytes, peritoneal cells, and Kupffer cells, but contains normal numbers of thymic Mϕ, as well as DCs and selected Mϕ in other sites. A second ligand for the CSF-1 receptor Fms, IL-34, may account for CSF-1 independence.[35] Factors involved in constitutive recruitment of thymic Mϕ are unknown; following death of thymocytes induced by ionizing radiation or glucocorticoids, intensely phagocytic Mϕ appear in large numbers; it is not known what proportion arises locally and by recruitment.

Spleen

From the viewpoint of the Mϕ, the spleen is perhaps the most complex organ in the body.[36,37] It contributes to hemopoiesis, which persists postnatally in some species or can be induced by increased demand, can serve as a reservoir as noted previously, and contributes to the turnover of all blood elements at the end of their natural lifespan. The spleen filters a substantial proportion of total cardiac output, captures particulate and other antigenic materials from the bloodstream, and plays an important role in natural and acquired humoral and cellular immunity. The organ is rich in subpopulations of Mϕ that differ in microanatomic localization, phenotype, life history, and functions (Fig. 19.3). Mϕ are central to antigen capture, degradation, transport, and presentation to T- and B-lymphocytes, and contribute substantially to antimicrobial resistance. Recent work has unveiled an unexpected role in facilitating activation of other lymphocyte subsets, such as invariant natural killer T cells[37]; CD 169+ macrophages also activate CD8 T cells in response to dead cell–associated antigens in lymph nodes and by transferring antigen to DCs in the spleen. Because other hemopoietic and secondary lymphoid organs can replace many of these functions after maturation of the immune system, the unique properties of the spleen have been mainly recognized in the immature host and in immune responses to complex polysaccharides. Splenectomy in the adult renders the host susceptible to infection by pathogenic bacteria such as pneumococci that contain saccharide-rich capsular antigens; the marginal zone of the spleen in particular may play an essential role in this aspect of host resistance.

The properties of Mϕ in the unstimulated mature mouse spleen are different according to their localization in red or white pulp and the marginal zone. Mϕ are intimately associated with the specialized vasculature. Species differences in splenic anatomy and phenotype are well recognized, although Mϕ display broadly common features in humans and rodents. Subpopulations of Mϕ, DCs, and cells with mixed phenotypes have been characterized by in situ analysis by antigen markers, liposome or diphtheria toxin-depletion studies, various immunization and infection protocols, and cytokine and receptor gene ko models in the mouse. The results raise questions about the dynamics and molecular basis of cell production, recruitment, differentiation, emigration, and death within each distinct splenic compartment. Cell isolation methods are still primitive in correlating in vitro properties with those of Mϕ subpopulations in vivo and remain an important challenge. Detailed aspects of splenic architecture, DC origin and function, and T- and B-lymphocyte induction and differentiation are described elsewhere in this volume. Here, some features of Mϕ in the normal and immunoreactive organ are highlighted.

Marginal Zone Macrophages

The marginal zone of spleen consists of a complex mixture of resident cells (reticular and other fibroblasts, endothelium), Mϕ, DCs, and lymphoid cells, including subpopulations of B-lymphocytes. It constitutes an important interface with the circulation that delivers cells, particulates, or soluble molecules directly into the marginal sinus or via the red pulp. Resident Mϕ are present as specialized metallophilic cells in the inner marginal zone, and other Mϕ are found in the outer zone; the latter may be more phagocytic. Sn is strongly expressed by the marginal metallophils, compared with only weak expression in red pulp and virtual absence in the white pulp. Sn+ cells appear in this zone 2 to 4 weeks postnatally in the mouse as the white pulp forms. Liposomes containing clodronate, a cytotoxic drug, can be delivered systemically and deplete Sn+ cells and other Mϕ; regeneration of different Mϕ subpopulations in spleen occurs at different times, and this procedure has been used to correlate their reappearance with distinct immunologic functions. Marginal

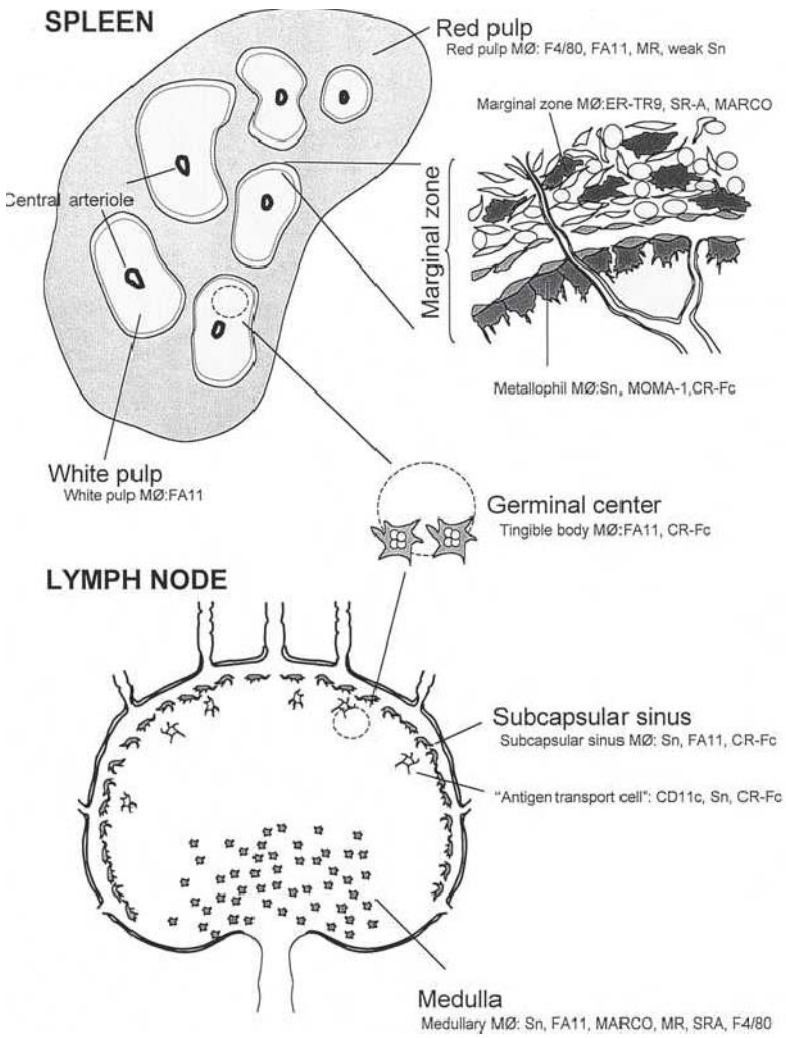


FIG. 19.3. Microheterogeneity of macrophages in spleen, resting, and antigen-stimulated lymph nodes. See text for markers and details.

zone Mϕ lack F4/80 but may express an undefined ligand for F4/80 on circulating activated DCs, which mediates peripheral tolerance to anterior chamber or gut-derived antigens.[38] Marginal zone Mϕ express phagocytic receptors, such as SR-A, which is more widely present on tissue Mϕ, as well as MARCO, a distinct collagenous scavenger receptor, which is almost exclusively present on these Mϕ in the normal mouse. The structures and possible role of these pattern recognition receptors in uptake of microbes are discussed subsequently. In vivo studies have shown that an Mϕ lectin, the MR, may be involved in transfer of mannosylated ligands to the site of an immune response in the white pulp.[39] The MR contains a highly conserved cysteine-rich domain, not involved in mannosyl recognition, that reacts strongly with ligands on a subset of marginal metallophilic Mϕ, sulfated glycoforms of Sn, and CD45, among others; this has been demonstrated with a chimeric probe of the cysteine-rich domain of the MR and human Fc (CR-Fc) and by immunochemical analysis of tissue sections and affinity chromatography of spleen ligands. After immunization, this probe additionally labels undefined cells in the FDC network of germinal centers, as well as tingible body Mϕ. It is possible that marginal zone Mϕ can be induced to migrate into white pulp as described after LPS injection; alternatively, they may shed complexes of soluble MR-glycoprotein ligand for transfer to other CR-Fc+ cells, which may be resident or newly recruited mononuclear cells. Finally, the marginal metallophilic Mϕ population depends on CSF-1 for its appearance and on members of the TNF receptor family, as shown with *op/op* and experimentally produced ko mice.

White Pulp Macrophages

The F4/80 antigen is strikingly absent on murine white pulp Mϕ, which do express FA-11 (macrosialin), the murine homolog of CD68. Actively phagocytic Mϕ express this intracellular glycoprotein in abundance compared with DCs. After uptake of a foreign particle (eg, sheep erythrocytes or an infectious agent, such as BCG or *Plasmodium yoelii*), white pulp Mϕ become more prominent, although it is not known whether there is migration of cells into the white pulp or transfer of phagocytosed material and reactivation of previous resident Mϕ. Tingible body Mϕ appear to be involved in uptake and digestion of apoptotic B-lymphocytes.

Red Pulp Macrophages

These express F4/80 antigen and MR strongly and in the mouse include stromal-type Mϕ involved in hemopoiesis. Extensive phagocytosis of senescent erythrocytes results in accumulation of bile pigments and ferritin, and play an important role in iron turnover[40] and tolerance.[41] The role of various phagocytic receptors in clearance of host cells and pathogens by red pulp Mϕ requires further study.

There is no evidence that Mϕ, other than interdigitating DCs, associate directly with CD4+ T-lymphocytes in the normal spleen. Following infection by BCG, for example, or by other microorganisms such as *Salmonella*, there is massive recruitment and local production of Mϕ, many of which associate with T-lymphocytes. Newly formed granulomata often appear first in the marginal zone (focal accumulations of activated Mϕ and activated T cells). As infections spread into the white and red pulp, the granulomata become confluent and less localized, obscuring and/or disrupting the underlying architecture of the spleen. The possible role of activated Mϕ in T-cell apoptosis and clearance in spleen has not been defined.

Lymph Nodes

F4/80 antigen is relatively poorly expressed in lymph node (see Fig. 19.3), but many macrosialin (CD68)+ cells are present. The subcapsular sinus is analogous to the marginal zone and contains strongly Sn+ cells; this is the site where afferent lymph enters, containing antigen and migrating DCs derived from skin and mucosal surfaces. The medulla contains Sn+, CD68+ Mϕ, which also express high levels of SR-A. As in the spleen marginal zone, subcapsular sinus Mϕ are strongly labeled by the CR-Fc probe. Following primary or secondary immunization, the staining pattern moves deeper into the cortex and eventually becomes concentrated in germinal centers. The kinetics of this process strongly suggests a transport process by Mϕ-related cells resembling antigen transport cells described previously. CR-Fc+ cells can be isolated by digestion of lymph nodes and form clusters with CR-Fc– lymphocytes. Adoptive transfer has shown that fluorescein-activated cell sorter–isolated CR-Fc+ cells resemble DCs in their ability to home to T-cell areas and to present antigen to naive T and B cells. Overall, there is considerable heterogeneity in the population of migratory APCs involved in antigen capture, transport, and delivery to T and B cells, and it may turn out that specialized tissue Mϕ as well as myeloid-type DCs can migrate in response to immunologic stimuli, especially TLR ligands.[42]

Peyer Patch

Although less studied, the Mϕ in Peyer patch resemble the CD68+, F4/80– cells described in spleen and white pulp and in other T-cell–rich areas. They are well placed to interact with gut-derived antigens and pathogens taken up via specialized epithelial M cells in the dome, and deliver antigens to afferent lymphatics, as myeloid DCs. These cells are distinct from abundant F4/80+ cells in the lamina propria found all the way down the gastrointestinal tract and may play a role in the induction of mucosal immunity. Recent studies have described heterogeneous populations of resident and recruited macrophages and DC in the mouse intestine.[43] The role of the microbiome[44] has received a great deal of attention in regard to innate cell phenotype and epithelial integrity in the gut.

Nonlymphoid Organs

Regional F4/80+ and CD68+ Mϕ are well described in liver (Kupffer cells), dermis, neuroendocrine and reproductive organs, and serosal cavities, where they are able to react to systemic and local stimuli. In the lung, alveolar Mϕ are strongly CD68+ but only weakly F4/80+ and are distinct from interstitial Mϕ and intraepithelial DCs. In the lamina propria of the intestine, Mϕ display a downregulated phenotype, ascribed to TGFβ of local origin.[45] In addition, resident Mϕ are found throughout connective tissue and within the interstitium of organs, including heart, kidney, and pancreas. These cells vary greatly depending on their local microenvironment; for example, in the central nervous system, microglia within the neuropil differ strikingly from Mϕ in the meninges or choroid plexus.[46] Perivascular Mϕ in the brain can be distinguished from resident microglia by their expression of endocytic receptors (eg, the SR-A and MR, and of MHC I and II antigens). Microglia are highly ramified, terminally differentiated cells of monocytic origin; many Mϕ markers are downregulated. Their phenotype is influenced by the blood–brain barrier, normally absent in circumventricular organs, and disrupted by inflammatory stimuli. Microglia can be reactivated by local LPS and neurocytotoxins; they are then difficult to distinguish from newly recruited monocytes, which acquire microglial features once they enter the parenchyma of the brain. Resting microglia are unusual among many tissue Mϕ in that they constitutively express high levels of CR3 and respond to CR3 ligands, such as mAb, by induced DNA synthesis and apoptosis. In other sites, such as lung and liver, CR3 expression is a feature of recent myeloid recruitment, including monocytes. Resident Kupffer cells lack constitutive CR3 but express a novel CR implicated in clearance function.

Resident tissue macrophages in human tissues express CD68 antigen, but their phenotypic diversity and microheterogeneity in different organs remain poorly defined. Access to skin biopsies, bronchoalveolar lavage, and placenta, for example, provides material for further analysis.

Enhanced Recruitment of Monocytes by Inflammatory and Immune Stimuli: Activation in Vivo

In response to local tissue and vascular changes, partly induced by resident Mϕ during (re)activation by inflammatory, infectious, and immunologic stimuli, monocytes are recruited from marrow pools and blood in increased numbers; they diapedese and differentiate into Mϕ with altered effector functions as they enter the tissues.[47] These Mϕ are classified as "elicited" when cells are generated in the absence of IFNγ and as "immunologically activated" after exposure to IFNγ. Enhanced recruitment can also involve that of other myeloid or lymphoid cells; selectivity of the cellular response depends on the nature of the evoking stimulus (immunogenic or not), the chemokines produced,

and the receptors expressed by different leukocytes. Mϕ and other cells produce a range of different chemokines and express multiple seven-transmembrane, G protein-coupled chemokine receptors. The chemokines can also act in the marrow compartment, especially if anchored to matrix and glycosaminoglycans, may display other growth regulatory functions, and can control egress. Locally bound or soluble chemokines induce the surface expression and activity of adhesion molecules on circulating white cells, as well as directing their migration through and beyond endothelium. Feedback mechanisms from periphery to central stores and within the marrow stroma may depend on cytokines and growth factors such as macrophage inflammatory protein-1α and GM-CSF, which inhibit or enhance monocyte production, respectively. The adhesion molecules involved in recruitment of monocytes, originally defined by studies in humans with inborn errors and by use of inhibitory antibodies in experimental animal models,[48] overlap with those of polymorphonuclear neutrophils and lymphocytes and include L-selectin, β_2-integrins, especially CR3, CD31, an Ig-superfamily molecule, and CD99; additional monocyte adhesion molecules for activated endothelium include CD44, vascular cell adhesion molecules, β_1-integrins, and newly described receptors such as EMR2 and CD97, members of the EGF-TM7 family.[33] The mechanisms of constitutive entry of monocytes into developing and adult tissues, in the absence of an inflammatory stimulus, are unknown.

By contrast with the uncertain precursors of resident Mϕ and DC populations, distinct monocyte subsets have been implicated in the enhanced mobilization and turnover in response to inflammatory, infectious, and metabolic stimuli, as noted previously (Fig. 19.4). Differential expression of the fractalkine receptor, CCR2, and other chemokine receptors, together with antigen markers (Gr-1 in mouse and CD14 in human), have made it possible to define monocyte heterogeneity. Although such subsets seem to be conserved across several species, their properties may reflect stages of cell activation along a continuous spectrum rather than true differentiation.

The migration and differentiation of newly recruited monocytes once they have left the circulation are poorly understood. They are able to enter all tissues, undergoing alterations in membrane molecules and secretory potential under the influence of cytokines and surface interactions with endothelial cells, leukocytes, and other local cells. Phenotypic changes mentioned in the following section have been characterized by a range of in vitro and in vivo studies. Well-studied examples include murine peritoneal Mϕ—resident, elicited by thioglycollate broth or biogel polyacrylamide beads, and immunologically activated by BCG infection. The latter provides a useful model of granuloma formation in solid organs but does not fully mimic the human counterpart associated with *M. tuberculosis* infection. Granuloma Mϕ vary in their turnover and immune effector functions and display considerable heterogeneity; lesions contain recently recruited monocytes, mature, epithelioid Mϕ (described as secretory cells), and Langhans giant cells. Interactions with T-lymphocytes, other myeloid cells, DCs, fibroblasts, and microorganisms yield a dynamic assembly of cells as the granuloma evolves, heals, and resolves (see Fig. 19.2). Apoptosis and necrosis of Mϕ and other cells contribute to the balance of continued recruitment and local proliferation. The emigration of Mϕ rather than DCs from sites of inflammation is less evident, although it has become clear that elicited Mϕ within the peritoneal cavity, for example, migrate actively to draining lymph nodes.[49]

Gene ko models have confirmed the role of molecules previously implicated in recruitment, activation, and granuloma formation. These include the adhesion molecules listed previously, their ligands, such as intercellular adhesion molecule-1, and key cytokines such as IFNγ, IL-12, IL-23, and TNFα, as well as their receptors. Antimicrobial resistance and Mϕ cytotoxicity resulting from production of reactive oxygen and nitrogen metabolites are now accessible to study

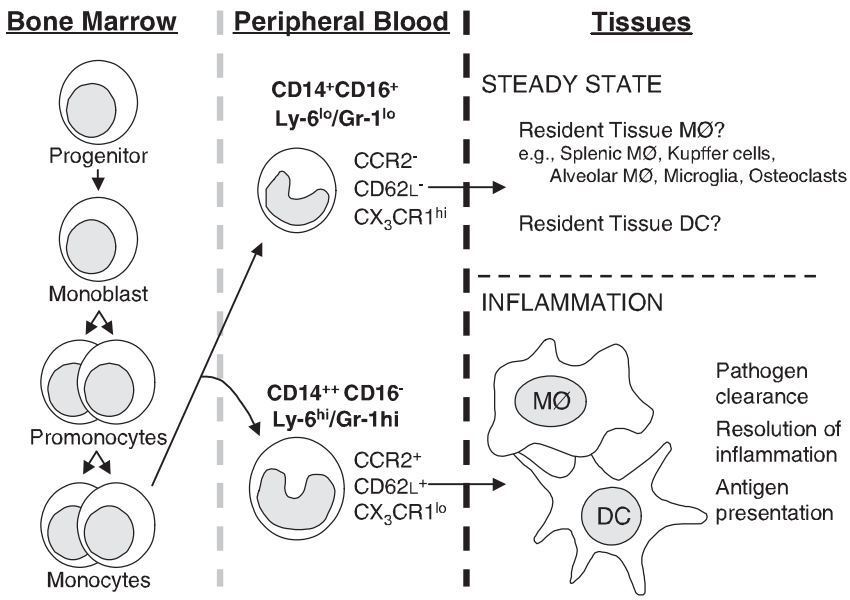


FIG. 19.4. Distinct monocyte subsets give rise to inflammatory and resident macrophages and dendritic cells. The origin of different resident subpopulations is not clear. See text for further details.

in knockouts of the phagocyte oxidase and inducible nitric oxide synthase. kos of membrane molecules of immunologic interest expressed by Mϕ and other cells include MHC class II and I, CD4, and CD40L, other accessory molecules such as B7–1 and B7–2, and the Mϕ-restricted intracellular molecule N-ramp.

The study of inborn errors in humans[50,51] and of disease models in genetically modified mice has brought insight into essential, nonredundant contributions of molecules that regulate Mϕ activation in vivo, including immunopathology syndromes such as septic shock and autoimmunity. Examples include myeloid antigens such as TREM-1/2,[52] associated with DAP-12, receptor-ligand pairs such as CD200/CD200 receptor,[53] and suppressors of cytokine signaling proteins.[54,55] TNFα is essential for host resistance to infection[56] and also contributes to immunopathology. Highly effective anti-TNFα therapy for chronic inflammatory diseases such as rheumatoid arthritis can result in reactivation of latent tuberculosis.

The potential for innate,Th1- and Th2-type regulation of Mϕ demonstrated in vitro, and discussed subsequently, can result in highly complex, often coexistent, heterogeneity of Mϕ phenotype in situ (see Fig. 19.4). Although almost all granuloma Mϕ express lysozyme,[57] only subpopulations express cytokines such as IL-1β, IL-6, and TNFα. Pro- and anti-inflammatory cytokines, IL-12, IL-18, IL-10, and TGFβ, produced by Mϕ themselves and other cells, modulate the phenotype of Mϕ in vivo.

Intravital imaging has provided insights into the dynamics of interactions of myeloid and lymphoid cells in granuloma formation.[58] Studies of human granulomatous, chronic inflammation provide opportunities to dissect genetic and acquired influences (eg, in NOD-2 deficiency in Blau syndrome[59] and Crohn disease[60]). The mouse resistance protein lrgm1 (LRG-47)[61] has also been implicated in pathogen defense.[62]

Apart from the local interactions outlined, Mϕ regulate systemic host reactions to immune and infectious stimuli by producing circulating cytokines such as IL-6 and arachidonate- and other lipid-derived metabolites, including resolvins, that contribute to the resolution of acute inflammation.[49] These mediators also act on neural and endocrine centers, crossing the blood–brain barrier, or are generated locally by reactive microglia and Mϕ. MicroRNAs have been identified that play a role in resolution.[63] Glucocorticosteroids are powerful immunomodulators[64] and form part of a network that regulates monocyte recruitment and Mϕ functions through circulating mediators such as migration inhibition factor. Mϕ contain potent enzymes involved in steroid biosynthesis and catabolism.[65]

Although the immunologic relevance of Mϕ-induced responses may seem evident, many aspects remain unclear. For example, what is the role and phenotype of monocyte/macrophages in the immune reconstitution inflammatory syndrome, associated with dual acquired immunodeficiency syndrome/tuberculosis infection, recipitated by antiretroviral treatment[66]? Do Mϕ actively suppress or destroy activated T-lymphocytes, thus contributing to regulation of immune responses and peripheral tolerance, or are Mϕ only passive removers of dying cells? Do Mϕ contribute to recruitment, differentiation, and death of DCs at sites of inflammation before their migration to secondary lymphoid organs? Do adjuvant-stimulated Mϕ interact with B-lymphocytes, directing their migration into germinal centers? Are interactions of activated Mϕ with antibody and complement, through different Fc and complement receptors, implicated in fine-tuning humoral responses? Are activated Mϕ themselves cytocidal for infected host cells, and to what extent do they in turn interact with and provide targets for attack by natural killer cells and cytotoxic T-lymphocytes? Study of a range of experimental models and disease processes in vivo should yield new insights, as well as extend and confirm mechanisms already defined in vitro.

Phagocytic Recognition and Intracellular Infection

The initiation and localization of an immune response depends on recognition by Mϕ and other cells of particulate agents or soluble proteins that are foreign or modified-self.[67] Phagocytic and endocytic recognition by Mϕ and DCs depends, in turn, on opsonic (mainly antibody, complement) and nonopsonic pattern recognition receptors that interact with a range of related ligands (Fig. 19.5). Innate and acquired responses are thus interlinked. Different FcR are involved in uptake and destruction of targets as well as in negative regulation of effector functions.[68] CRs[69] are also heterogeneous; CR3 interacts with C3-derived ligands formed by activation of the classical, alternate, or lectin pathways and mediate phagocytosis, cell migration, and cell activation. Other ligands include intercellular adhesion molecules. CR3 functions are modulated by fibronectin, via integrins, other adhesion molecules, and inflammatory stimuli. FcR ligation and cross-linking activates tyrosine kinases such as syk that are essential for phagocytosis; CR3 signaling is less defined and may not trigger a respiratory burst or arachidonate release, unlike FcR, thus favoring pathogen entry. Antibody-mediated uptake targets an organism or soluble antigen to a different, degradative compartment[70] (Fig. 19.6) and usually results in its neutralization and destruction, although enhancement of infection can also occur in Mϕ.[71] Flavivirus infection in the presence of specific antibody can result in the dengue hemorrhagic shock syndrome. Immune complexes, with or without complement, localize antigens to FDCs and other FcR+ CR+ cells. Mϕ themselves are able to produce all components of the complement cascade in significant amounts at local sites, which may be less accessible to circulating proteins made by hepatocytes.

Nonopsonic receptors reacting directly with ligands on microorganisms[72] include CR3, lectins, especially the MR and β-glucan receptor, the scavenger receptors SR-A and MARCO, and the family of TLRs. MRs are present on Mϕ, DCs, and sinusoidal endothelium.[39] They mediate phagocytosis and endocytosis, including macropinocytosis, and structurally resemble another multilectin, Dec 205, present on DCs as well as tissue Mϕ and epithelial cells in thymus; carbohydrate recognition by the latter has not been demonstrated. The MR has eight C-type lectin domains, homologous to the mannose-binding protein, a circulating

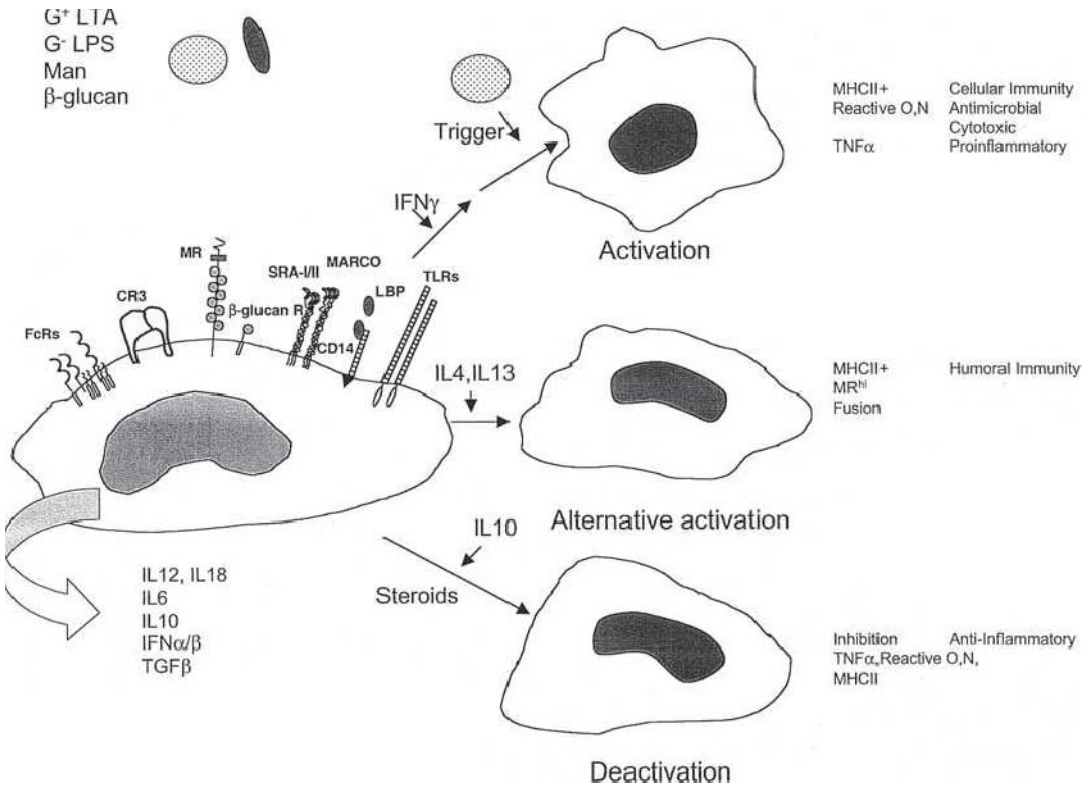


FIG. 19.5. Macrophage activation and the role of microbial stimuli and cytokines. See text for details.

hepatocyte-derived, acute-phase reactant. Mannose-binding protein, also known as mannan-binding lectin, contains a single lectin domain per polypeptide, which oligomerizes like other collectins to achieve multivalent interactions and activate complement via associated serine proteases. MR expression on Mϕ is selectively down- and upregulated by IFNγ and IL-4/13, respectively. A possible role of the cysteine-rich domain in transport of immunogenic glycopeptides within secondary lymphoid organs has been proposed.[73]

The β-glucan receptor, previously reported as dectin-1, is related to C-type lectins and is responsible for phagocytic recognition of unopsonized zymosan and for Mϕ activation (Figs. 19.7 and 19.8).[74] It contains an immunoreceptor tyrosine-based activation motif–like motif in its cytoplasmic domain that is essential for phagocytosis and induced secretory responses (eg, TNFα). It cooperates with TLR2 and synergizes with other TLRs in cell activation, utilizing syk and CARD9,[75] and can promote TH17 differentiation.[76] It is essential for resistance to a range of fungal particles in vivo, as shown by studies with ko mice and in human primary innate immunodeficiency.[77] Dectin-2, a distinct mannan-binding lectin, has also been implicated in TH17 differentiation.[45,78]

SR-A mediates endocytosis of modified proteins (eg, acetylated lipoproteins) and selected polyanions, such as apolipoprotein A1 and E,[79] LPS, and lipoteichoic acid.[72] In addition, it can serve as an adhesion molecule and contributes to phagocytic clearance of apoptotic thymocytes and gram-negative as well as gram-positive bacteria. MARCO, a related collagenous receptor,[72] mediates cell adhesion and phagocytosis of bacteria but is independently regulated, as discussed subsequently. MARCO[80] and Mincle,[81] a lectin-like receptor, contribute to macrophage responses to trehalose dimycolate, a virulence factor of pathogenic *M. tuberculosis*. An initial report of a phosphatidylserine receptor implicated in the recognition of novel lipid ligands expressed on the surface of apoptotic cells was not confirmed. CD36 (thrombospondin receptor), vitronectin receptors, CD91, and CD44 have all been implicated in the uptake of senescent polymorphonuclear neutrophils by Mϕ.[82] Other opsonins for apoptotic cell clearance include milk fat globule protein (lactadherin). A role for Mϕ SR-A in immune induction has not been demonstrated, but studies in SR-A ko mice have revealed an important inhibitory role in limiting TNFα production by immunologically activated Mϕ. Wild-type, BCG-primed mice produce granulomata rich in SR-A+ Mϕ; SR-A ko mice restrict growth of this organism and form normal granulomata containing activated, MHC II+ Mϕ; on additional challenge with LPS, the ko mice die more readily than wild-type animals. TNFα levels in the circulation rise markedly because of unopposed triggering via CD14, a receptor for the LPS-binding protein, and contribute to septic shock, because blocking anti-TNF mAb protects these mice.

The family of TLRs consists of homo- or heterodimeric transmembrane molecules related to the IL-1 receptor, which are involved in innate immunity to microbial constituents and activation of Mϕ responses, and are discussed elsewhere in this volume.[83] Downstream signaling depends on association with other soluble and membrane molecules, as well as with intracellular proteins. MyD88, for example,

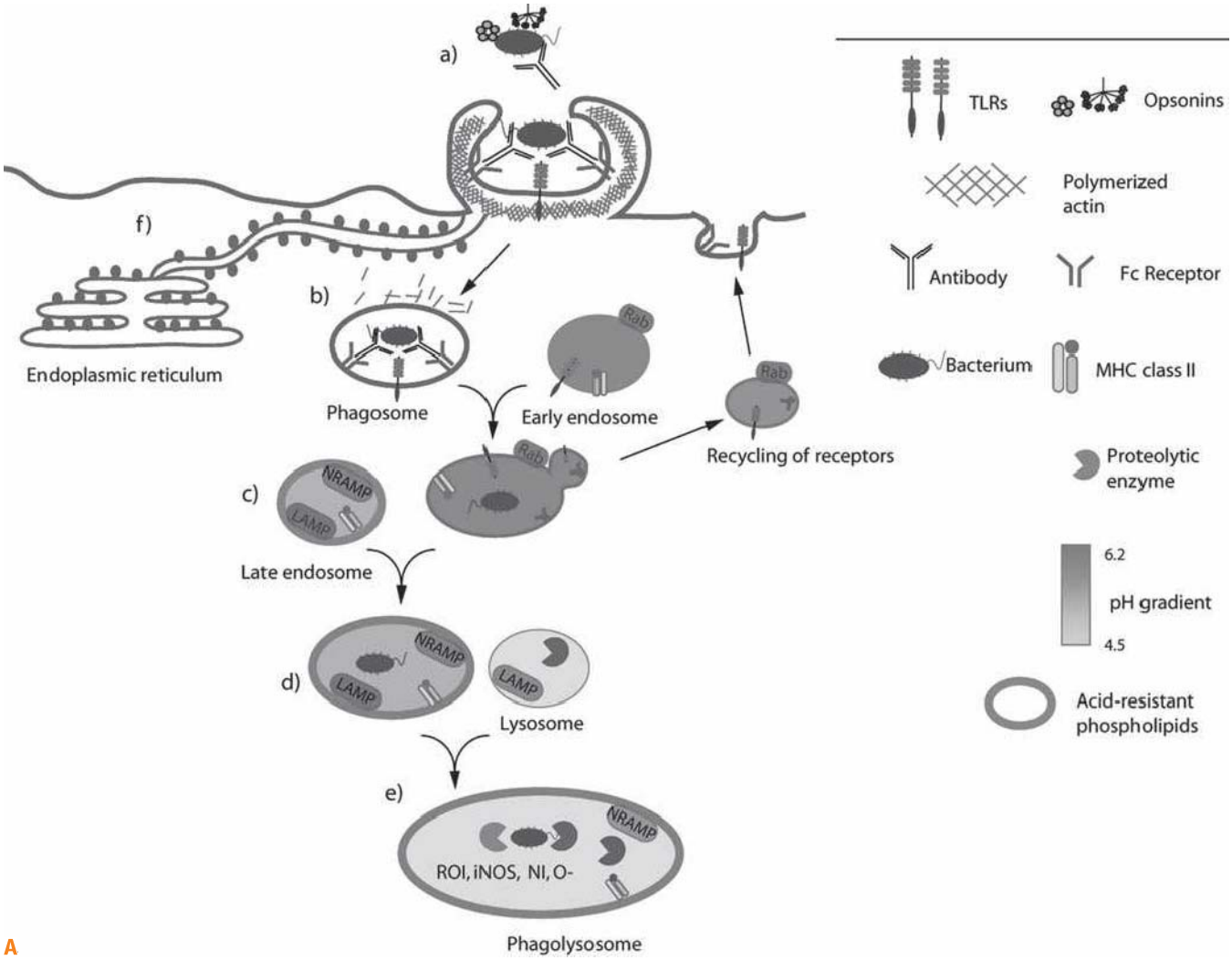


FIG. 19.6. Phagocytic Pathway in Macrophages. **A:** Heterophagy. Fc receptor-mediated phagocytosis. **a:** Microbes are coated with a variety of opsonins, including complement, pentraxin 3, and antibodies. A number of receptors are involved in initial recognition of microbes and induction of proinflammatory signaling (eg, the toll-like receptors [TLRs], and especially TLRs 2, 4, and 5), but these receptors are not phagocytic. Receptors involved in phagocytosis include the complement receptors, Fc receptors, and others. **b:** Fc receptor ligation initiates a signaling cascade that results in actin polymerization and extension of the plasma membrane. Phagocytosis mediated by this method occurs via the "zipper" mechanism (ie, sequential binding between the Fc receptors and their ligands along the length of the microbe). **c:** Fusion with the early endosome results in a slight drop in pH that results in the uncoupling of receptors with their ligands. Receptor recycling is facilitated by the Rab proteins, which also confer the ability to undergo subsequent fusion. The developing phagosome now contains major histocompatibility complex (MHC) class II and TLRs, including those that signal from within the developing endosome (eg, TLR9). **d:** Fusion with the late endosome results in the addition of the lysosome-associated membrane proteins, the accumulation of acid-resistant phospholipids, and a subsequent drop in pH. **e:** On fusion with lysosomes, the low pH results in the activation of a number of proteolytic enzymes. These are necessary for both direct antimicrobial activity and the creation of peptides for presentation via MHC class II. The phagolysosome is a highly oxidative environment, exposing the pathogen to destructive reactive oxygen and reactive nitrogen intermediates. **f:** Under certain circumstances, the phagosome may contain markers specific for the endoplasmic reticulum (ER). The ER may contribute directly to the creation of the phagocytic membrane in some circumstances (eg, phagocytosis of latex beads), or ER-derived vacuoles may contribute MHC class I and other molecules to the endosomes or the developing phagolysosome. *(continued)*

has been implicated in many but not all TLR-induced signaling resulting in transcription factor regulation, cell activation, or apoptosis.

Naturally occurring microbial ligands for these nonopsonic receptors are still poorly defined; individual receptors mediate microbial binding and uptake of microorganisms, although each contributes only part of total binding (see Fig. 19.6A).[72,84] Particle uptake involves multiple membrane receptors, the cytoskeleton, bulk membrane flow, and remodeling, including formation of a phagocytic synapse and signaling pathways[85,86] (see Fig. 19.8[87]). Phagosome formation and maturation resemble endocytic uptake, initiating Mϕ vesicle trafficking and recirculation, fusion with lysosomes, acidification, ion fluxes, and digestion. Table 19.2 lists immunologic and other markers

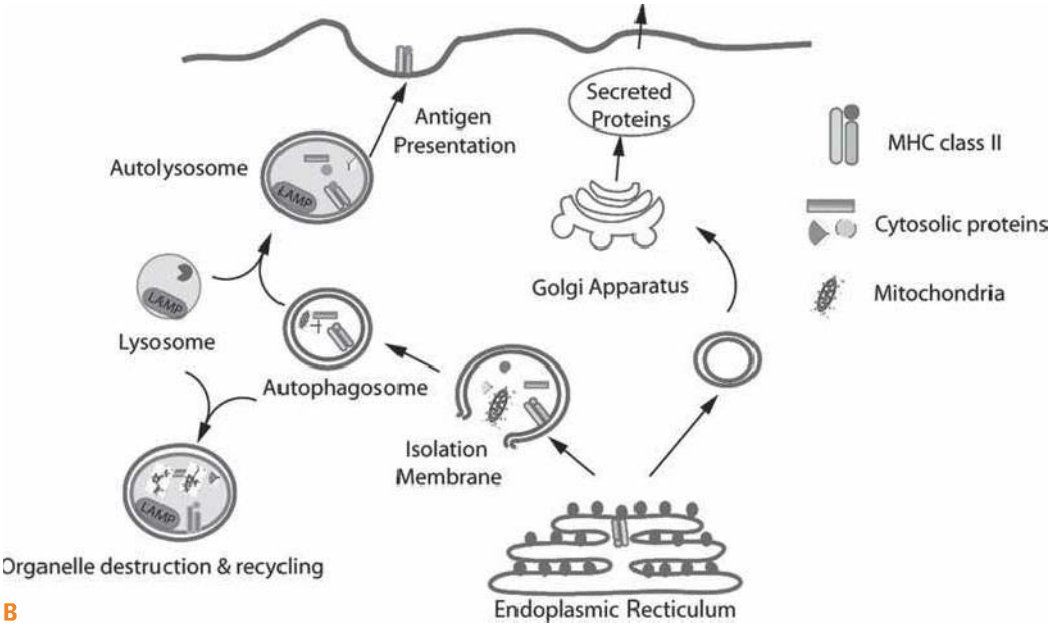


FIG. 19.6. *(continued)* **B:** The autophagy pathway. Autophagy is a homeostatic process that can be further enhanced in macrophages in the presence of interferons or by starvation. Cytosolic proteins and organelles are found in ER-derived cytoplasmic vesicles. These vesicles fuse with lysosomes, and the proteins and organelles are degraded and recycled. Conventional wisdom states that endogenous and cytoplasmic proteins are presented by MHC class I molecules, whereas exogenous peptides are presented by MHC class II molecules; however, it has become clear that peptide presentation is altered considerably on induction of autophagy. The presentation of peptides from intracellular and lysosomal source proteins is increased on MHC-II.

used to identify intracellular compartments.[88] Guanosine triphosphate–binding proteins and complex signaling cascades play an important role in these dynamic events.[62] A key issue that needs to be resolved is how cell and receptor functions are modulated so that microbial phagocytosis or invasion induces inflammatory responses, unlike the uptake of apoptotic cells. The MHC II biosynthesis and subcellular localization and proteolytic processing of peptide antigens in vacuolar and cytosolic compartments of APCs are discussed elsewhere in this volume. Cytokines, especially IL-4/13, IL-10, and IFNγ, influence endocytosis via MR-dependent and -independent pathways and selectively alter vesicle dynamics.

Pathogens vary in using Mϕ plasma membrane molecules for entry and evasion of host defenses, and modify the composition of the resultant phagosome membrane (Fig. 19.9).[89] Mycobacteria, for example, employ a range of mechanisms to evade killing by Mϕ, including delayed maturation of phagosomes and inhibition of fusion with lysosomes and acidification (Figs. 19.9 and 19.10).[90,91] *Listeria monocytogenes* escapes into the cytosol by disruption of the phagosome membrane,[92,93] whereas *Leishmania* multiplies in phagolysosomes.[94] Humoral (antibody, complement) and cellular (IFNγ) mechanisms overcome parasitization of Mϕ by diversion to lysosomes or induce killing via oxygen/nitrogen (O/N)- dependent and other mechanisms.

Entry of microbial constituents such as muramyldipeptide from vacuolar compartments to the cytosol can result in sensing by NOD-like receptors, inflammasome assembly, activation of caspase-1, and processing and release of IL-1 β.[95–97] Nucleic acid recognition results in cytoplasmic and mitochondrial-associated protein signaling responsible for type 1 interferon gene expression (see Fig. 19.9).[98] A novel ligand, c-di-AMP, secreted by cytosolic *Listeria monocytogenes*, activates a host type I IFN response[92] and may serve as a sensing mechanism for many intracellular pathogens (see Fig. 19.10).[99] STING is a direct innate immune sensor of cyclic di-GMP, another cytosolic metabolite of intracellular microbial infection.[100] It has recently become clear that induction of autophagy and apoptosis by intracellular pathogens, including *Mycobacterium tuberculosis*, provides important host-protective responses[101] (see Figs. 19.6B and 19.9A, B). For a review on autophagy in immunity and inflammation, see Levine et al.[102]

Although the "canonical" entry pathway described here and illustrated in Figure 19.6A is used and modified by many pathogens, recent evidence has shown that organisms such as *Legionella pneumophila*[102,103] (see Fig. 19.9C, D) and *Brucella abortus*[104] induce vacuoles with novel membrane components or colonize compartments derived from the Golgi apparatus and the endoplasmic reticulum. The relative contributions of plasma membrane and endoplasmic reticulum (see Fig. 19.6A) to vacuole formation varies considerably, depending on the nature of the phagocytic cargo or invading pathogen.[105] Opsonins such as antibody are able to divert the cargo to lysosomes. IFNγ can induce

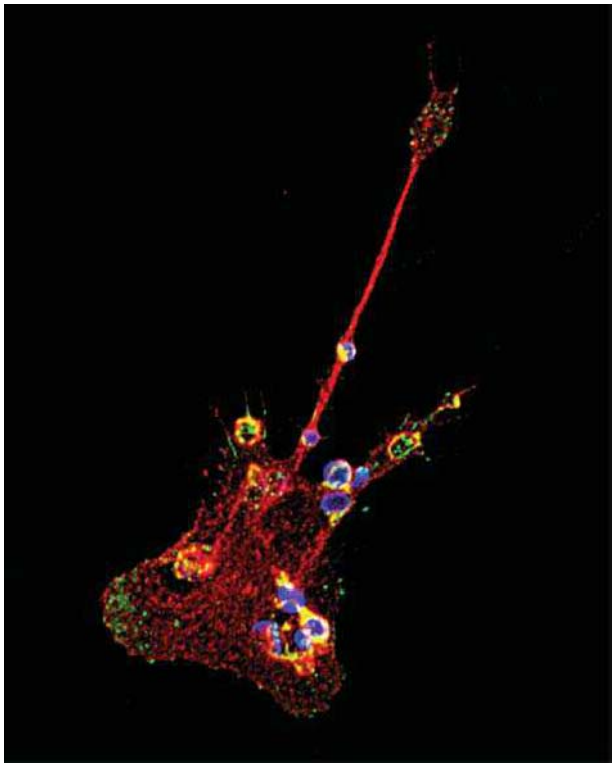


FIG. 19.7. Macrophages Rock! Macrophage engaging zymosan particles. Macrophages are adherent but motile. They use focal adhesions to anchor themselves to the extracellular matrix. Membrane ruffles and lamellipodia form at the leading edge of the cell to enable them to crawl around, and they extend pseudopodia to explore their environment and capture microbes and dead cells for phagocytosis. Consequently, their morphology is highly variable and constantly changing. © Helen Goodridge and David Underhill

TABLE 19.2 Membrane or Content Markers Used to Identify Phagosomes as Resembling a Given Endocytic Compartment or the Endoplasmic Reticulum

Compartment	Markers
Early endosome	Membrane markers: TfR, Rab5, annexins, I, II, and III
	Proteases: immature cathepsin D
Late endosome	Membrane markers: M6PR, Rab7, LAMP1, LAMP2, CD63, CD68
	Hydrolases: acid phosphatase, aryl sulfatase, trimetaphosphatase
	Proteases: cathepsin B, D, H, dipeptidyl peptidase I and II
	Phospholipid: LBPA
Lysosome	Membrane markers: LAMP1, LAMP2, CD63
	Hydrolases: acid phosphatase, aryl sulfatase, trimetaphosphatase
	Proteases: cathepsin B, D, H, dipeptidyl peptidase I and II
ER	Membrane markers: calnexin, calreticulin
	Enzyme: glucose-6-phosphatase

CD, cluster of differentiation; ER, endoplasmic reticulum; LAMP, lysosome-associated membrane protein; LBPA, lysobisphosphatidic acid; M6PR, mannose-6-phosphate receptor; TfR, transferrin receptor. From de Chastellier C. Electron microscopy. In: Cossart P, Boquet P, Normark, et al., eds. *Cellular Microbiology*, 2nd ed. Washington, DC: ASM Press, 2005:451, with permission.

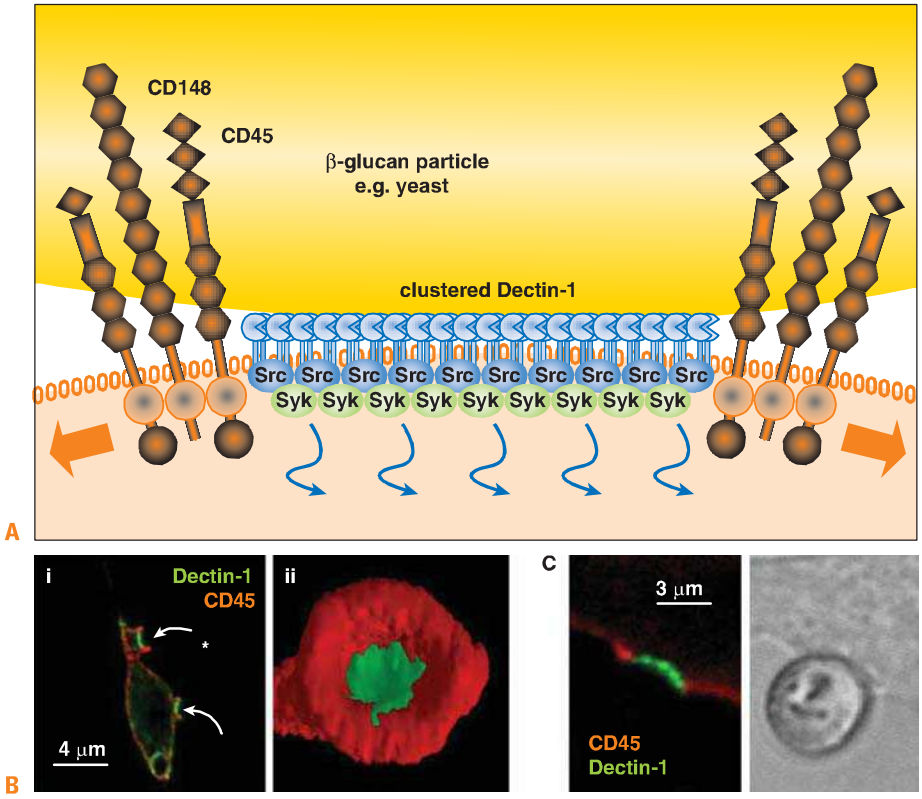
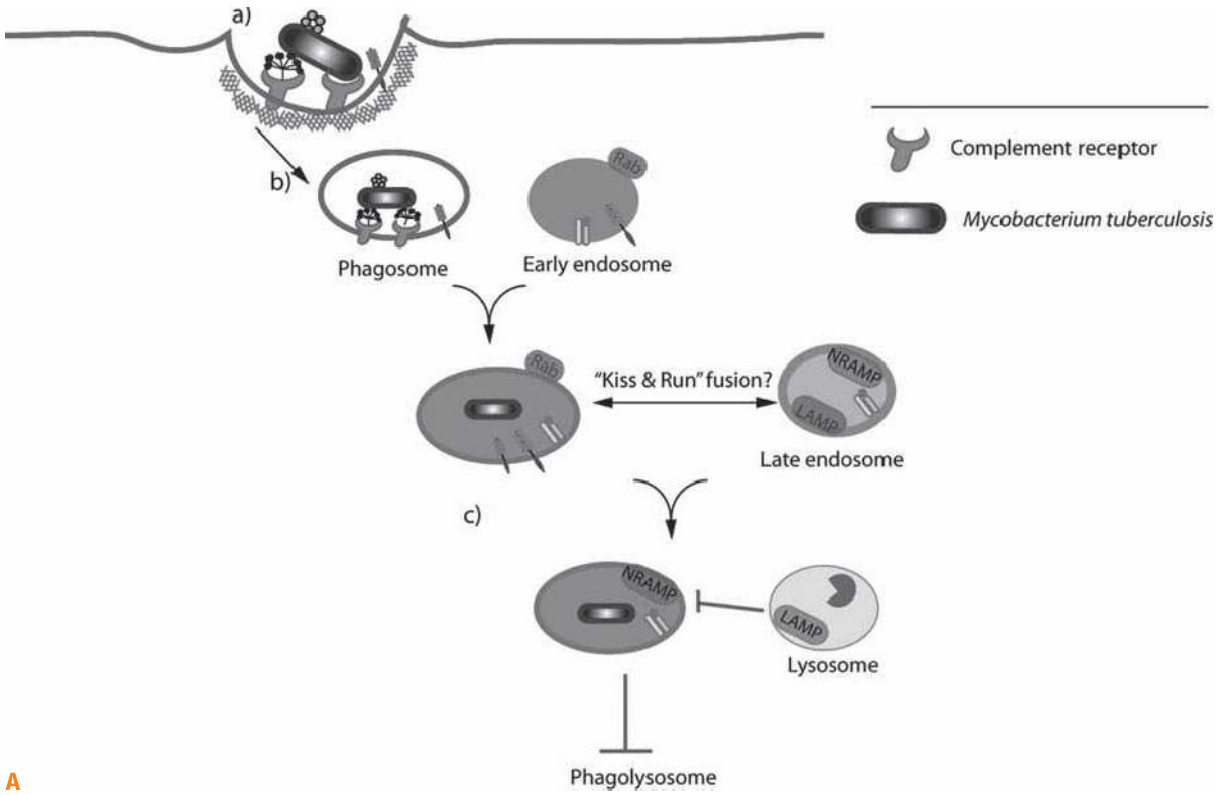


FIG. 19.8. The Dectin-1 Phagocytic Synapse. **A:** To permit sectin-1 signaling, cluster of differentiation (CD)45 and CD148 tyrosine phosphatases (which negatively regulate signal transduction downstream of dectin-1) are excluded from the contact site upon detection of beta-glucan particles by dectin-1 at the macrophage surface.The phagocytic synapse is similar to the immunological synapse that forms between an antigen presenting cell and a T cell. **B:** Confocal imaging of a macrophage encountering zymosan particles (**i**) shows two phagocytic synapses (arrows), and a three-dimensional isosurface model (**ii**) of one of them (*asterisk*). **C:** A phagocytic synapse that formed upon contact of a swollen *Aspergillus fumigatus* conidium with a macrophage. Images from Goodridge et al.,[87] with permission from Nature Publishing Group.



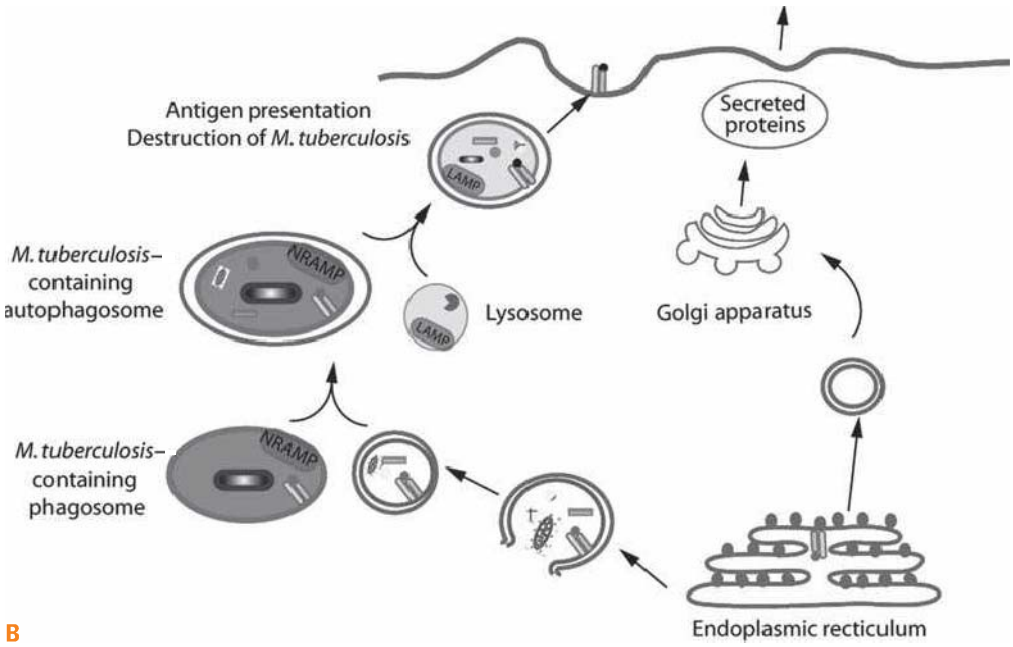
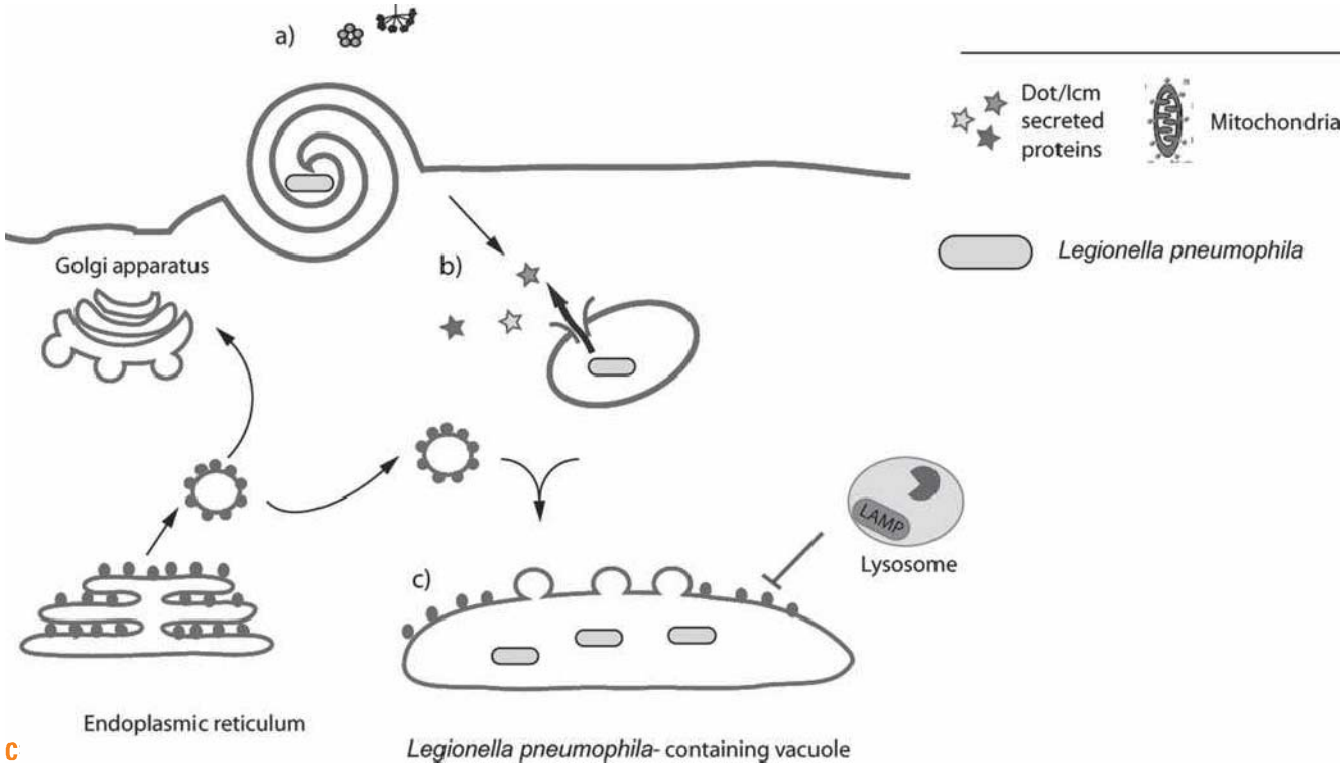


FIG. 19.9. Interactions of Selected Intracellular Pathogens with the Phagocytic Pathway. **A**: *Mycobacterium tuberculosis* evades destruction by subverting normal phagolysosome maturation. **a**: Phagocytosis of *M. tuberculosis* occurs via the complement pathway (although it may not require direct binding of complement to the bacterium) and is characterized by (**b**) "sinking" phagocytosis (ie, very little filopodia formation or actin polymerization). **c**: The *M. tuberculosis*–containing vacuoles contain markers of the early and late endosomes such as Rab5 and Nrampl but are devoid of most lysosomal markers, including the lysosome-associated membrane proteins, and do not undergo normal acidification. It has been proposed that the colocalization of some but not the normal allotment of endosomal markers can be explained by the concept of "kiss and run" fusion. This implies that the early and late endosomes may have transient contact with the *M. tuberculosis*–containing vacuoles, and there may be a selective transfer of markers rather than a complete fusion. The high pH of the *M. tuberculosis*–containing vacuole (pH 6.2) does not allow optimum loading of major histocompatibility complex (MHC) class II molecules, and thus they remain loaded with nonmycobacterial peptides. Elevated pH also inhibits production of inducible nitric oxide synthase, which is required for killing. In the presence of interferon (IFN)-γ, normal acidification may be restored, resulting in destruction of the pathogen. **B**: *M. tuberculosis*–containing phagosomes are targeted to the autophagy pathway on treatment with IFNγ. IFNγ treatment can both restore the normal process of acidification and alter the expression of a number of endoplasmic reticulum proteins, the result of which is the targeting of *M. tuberculosis*–containing phagosomes to the autophagosomes. The fusion between the *M. tuberculosis*–containing phagosomes and lysosomes results in an autolysosome with low pH that destroys *M. tuberculosis* and results in *M. tuberculosis* peptides being presented via MHC class II. *(continued)*



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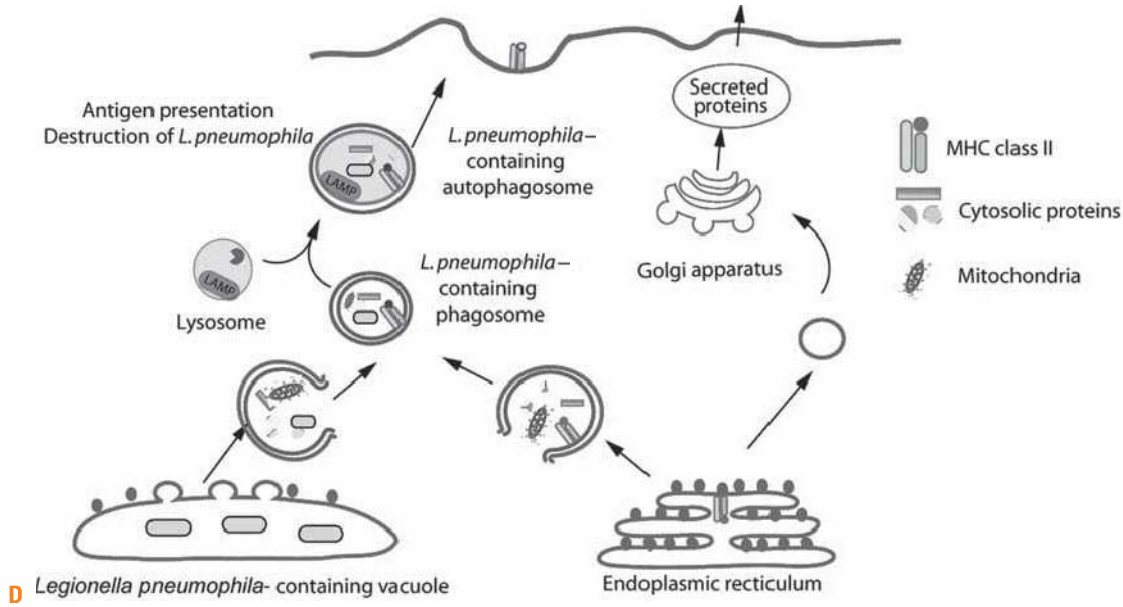

D

FIG. 19.9. *(continued)* **C:** *Legionella pneumophila* survives intracellularly by subverting phagosome maturation at an early stage. **a:** The recognition and uptake of *L. pneumophila* are not well characterized, but phagocysis occurs via a "spiral" mechanism. **b:** Once inside the cell, *L. pneumophila* uses a specialized secretion system (ie, Dot/Icm secretion system) to secrete proteins directly into the cytosol. These proteins alter the morphology of the vacuole in a number of ways, for example, by actively recruiting vesicles in transit from the endoplastic reticulum to the Golgi apparatus and inhibiting the fusion of lysosomes. **c:** The *L. pneumophila*–containing vacuole thus has many similarities to the Golgi apparatus and endoplastic reticulum, and is rich in peptides, the primary carbon source for *L. pneumophila.* **D:** Macrophages enhance autophagy in response to *L. pneumophila.* A consequence of residing in a vacuole that so closely resembles the endoplastic reticulum is that it is subject to autophagy. *L. pneumophila*–containing phagosomes fuse with lysosomes, resulting in destruction of the pathogen and antigen presentation. Factors secreted from *L. pneumophila* cause macrophages to increase the number of autophagosomes, although autophagosomes containing *L. pneumophila* mature more slowly, and thus it is believed that the bacteria encode factors to delay normal progression.

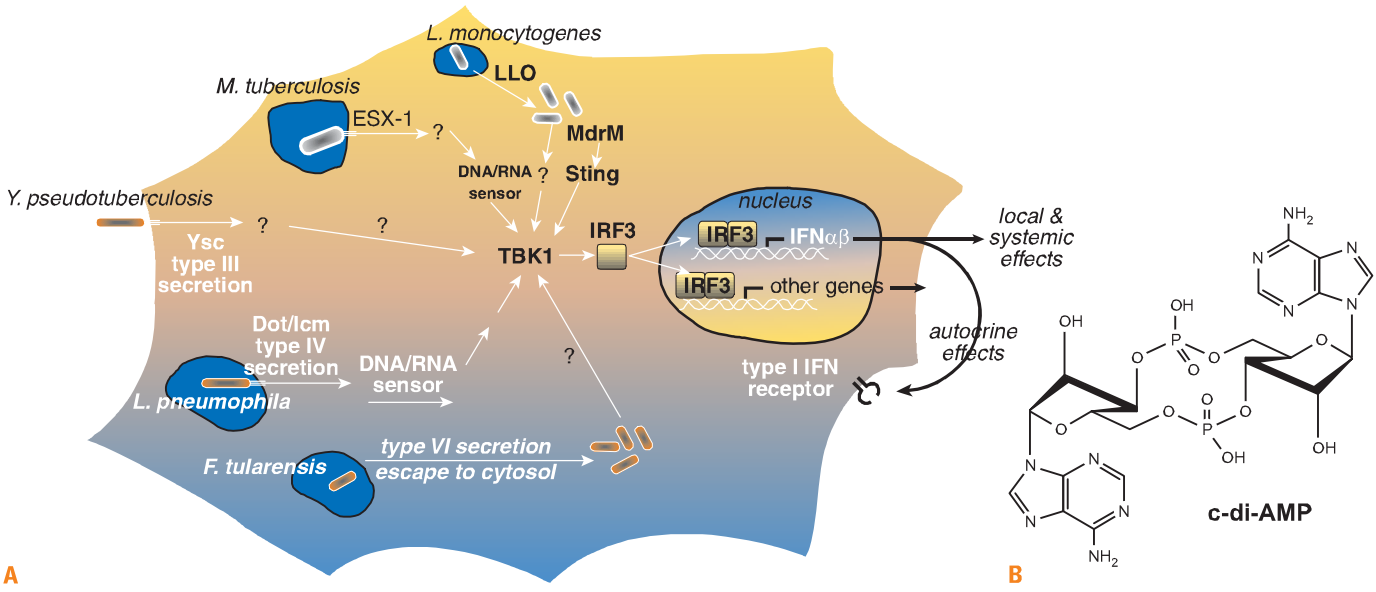

FIG. 19.10. Host Pathways Leading to the Expression of Interferon (IF) Beta and Coregulated Genes. **A:** Virulent, but not avirulent, intracellular pathogens activate a cytosolic surveillance pathway of innate immunity. Induction of IFN beta by all depicted bacterial species is MyD88 and TRIF-independent, indicating that toll-like receptors are not required. Sting and TBK1 are necessary for IRF3 activation.Wild-type *Listeria monocytogenes* enters the cytosol and activates the expression of IFN beta, while LLO-minus mutants that fail to enter the cytosol do not. Mutants in the multidrug efflux pump, MdrM, enter the cytosol, but induce threefold less IFN beta expression. The *L. monocytogenes* ligand is c-di-AMP **(B)**, while the receptor remains unknown. Mycobacterium tuberculosis resides in a phagosome that resembles early endosomes. Mutants in the ESX-1 auxiliary secretion system and phthiocerol dimycocerosate synthesis fail to induce IFN beta expression. Both TBK-1 and IRF3 are required. The infectious spore from *Histoplasma capsulatum* induces the expression of IFN beta, whereas the parasitic yeast form does not. *Legionella pneumophila* resides in a modified vacuolar compartment that has features of the endoplasmic reticulum. The Dot/Icm-type IV secretion system is necessary for intracellular growth and, independently, for induction of IFN beta expression. Both MDA5 and MAVS are required, suggesting that the ligand may be ribonucleic acid. *Francisella tularensis* needs to enter the cytosol to induce expression of IFN beta. The putative type VI secretion system is required for entry into the cytosol and induction of IFN beta. In each case, nucleic acids may be the ligands specifically recognized by cytosolic receptors, leading to the activation of IFN beta and co-regulated genes. Courtesy of D.A. Portnoy and R.E. Vance. For further information see Vance et al.[91] and Woodward et al.[92]

guanosine triphosphate-binding proteins that associate with vacuoles inhabited by a range of intracellular pathogens (eg, *Toxoplasma gondii*), thus marking them for destruction within the macrophage.[62,106]

Clearance of proteinase-inhibitor complexes (eg, by CD91) and of haptoglobin-hemoglobin complexes by the Mϕ-receptor CD163, and protection by hemeoxygenase-1, are essential homeostatic functions of tissue Mϕ, limiting potentially injurious extracellular molecules.[107] Other molecules released by injured infected cells can serve as alarmins[108] and danger signals (eg, heat shock proteins[109] and S100 family members,[110] inducers of inflammatory and immune responses).

Major unsolved questions remain concerning phagocytosis, intracellular infection, and immune responses. How do sterile particulate antigens[111] and microbial agents induce T=cell responses, and what are the relative contributions to this process of Mϕ and DCs, abundant and sparser professional phagocytes, respectively? What is the role of Scavenger and RAGE[72,110] receptors mediating entry of diverse ligands in subsequent adaptive immunity? Does TLR engagement within vacuoles determine the kinetics of phagosome maturation as well as induce local intracellular responses? What determines the balance between total antigen degradation and loading of MHC molecules? What interactions take place between intracellular pathogens and host Mϕ, especially in regard to hypoxia[112] and nutritional requirements of the host and microbe? What is the role of pathogen-derived secretory products in the vacuolar milieu, in recruitment of organelles such as endoplasmic reticulum and mitochondria, and in effects on host cell biosynthesis? What are the intracellular killing mechanisms, and how can organisms survive, or become latent, within Mϕ? To what extent do extracellular pathogens interact with macrophages and DCs[113]? Finally, what receptor-mediated signals induce the secretion of Mϕ molecules such as IL-12, IL-23, and IL-10 that direct and regulate the resultant specific immune response?

Gene Expression and Secretion

Knowledge of Mϕ gene expression and protein synthesis is growing rapidly from the application of gene array and proteomic technologies. After surface and endocytic stimulation, the mature Mϕ is able to secrete a very large range of high- and low-molecular weight products. These include enzymes involved in antimicrobial resistance (lysozyme), neutral proteinases and arachidonate metabolites that

TABLE 19.3 Modulation of Macrophage Phenotype

Category	Stimulus	Selected Marker Changes	Function
Innate activation	Microbial products (eg, LPS, other TLR ligands)	Costimulatory molecule expression, MARCO upregulation	Phagocytosis, adaptive immunity
Classic activation	Interferon γ	MHC II upregulation, proinflammatory cytokine secretion, inducible NO synthase	Cell-mediated immunity (eg, intracellular pathogens)
Alternative activation	IL-4/IL-13	Upregulation of MHC II, arginase, mannose receptor, Ym1, FIZZ1 (resistin-like), production of selected chemokines, macrophage fusion	Parasitic and allergic immunity, repair
Innate and acquired deactivation	Apoptotic cells, IL-10, glucocorticoids, TGFβ, PGE2	Various surface and secretory markers, (eg, anti-TNFα actions)	Anti-inflammatory and altered immunity

IL, interleukin; LPS, lipopolysaccharide; MHC, major histocompatibility complex; NO, nitric oxide; PGE2, prostaglandin E2; TGFβ, transforming growth factor-β; TLR, toll-like receptor; TNFα, tumor necrosis factor-α.

contribute to inflammation and tissue repair, cytokines such as IL-1 and TNFα that modulate the activities of other leukocytes and endothelium, and reactive oxygen and nitrogen intermediates implicated in host defense. Proinflammatory cytokines account for part of the effects of immune adjuvants in promoting, broadening, and sustaining humoral responses. The ability to release these products depends on the prior history of the Mϕ, whether resident, recruited, or activated (primed), its encounters with microbial wall products, including LPS acting via TLRs, or with apoptotic cells, and exposure to cytokines and other immunomodulatory molecules in its immediate environment. Ligation of specific receptors induces various signaling pathways[114] and is able to alter gene expression in the Mϕ selectively. NF-κB,[115] Pu-1 and other transcription factors,[116–119] and IFN regulatory factor families[119,120] contribute to Mϕ-restricted or activation-dependent changes in gene expression, as does epigenetic regulation.[121] Product expression depends further on translational regulation, posttranslational modification such as proteolytic processing, intracellularly or at the cell surface, and coexpression of inhibitors such as IL-10. Messenger RNA turnover varies greatly for different products due to the presence or absence of specific 3′ instability sequences. Many Mϕ products are labile and act close to the cell surface; overproduction results in tissue catabolism and systemic effects associated with widespread infection or chronic inflammation, often as a result of an immunologically driven disease process.

Whereas most bioactivities have been defined in vitro, there is evidence that expression of Mϕ secretory activities may be quite different in situ; lysozyme production is characteristic of all Mϕ in culture but is downregulated on most resident cells in vivo, and its expression by granuloma Mϕ, for example, depends on induction by immune or phagocytic stimuli. 5′ promoter sequences of human lysozyme and CD68 transgenes have been used to target tissue- and Mϕ activation-specific expression of reporter molecules in vivo. The promoters of these and other Mϕ-restricted molecules may, in due course, make it possible to direct Mϕ biosynthetic activities precisely, to boost or inhibit immune responses.

Modulation of Macrophage Activation in Vitro

Our understanding of Mϕ activation derives from studies of induction of MHC II and costimulatory antigens; of effector functions such as proteinase, TNFα, reactive oxygen intermediate and reactive nitrogen intermediate release; of expression of membrane receptors such as MRs; and of resistance to infectious agents, for example, *Mycobacteria*, *Listeria*, *Candida*, and HIV. Generalizations can be made, but it must be remembered that organisms vary considerably in their ability to evade or survive Mϕ restriction mechanisms, and they interact with Mϕ in individual ways. Various inhibitory cell surface molecules (eg, CD200, SIRPα,[122] and TAM receptors[123]) are known to regulate Mϕ activation through interactions with other activating plasma membrane receptors. Receptors including FcRγ use paired immunoreceptor tyrosine-based activation motif and immunoreceptor tyrosine-based inhibitory motif intracellular signaling motifs.

Figure 19.4 and Table 19.3 illustrate various pathways and markers of Mϕ activation that result from microbial, cellular, and cytokine interactions. Knowledge is based mainly on in vitro experiments and in vivo challenge of selected animal models. Innate activation depends on direct stimulation by microbial products, independent of cytokines, although often enhanced by concomitant stimulation (eg, by IFNγ). Newly discovered markers of innate activation of mouse peritoneal macrophages include upregulation of MARCO, SR-A, via a TLR pathway, and of CD200, a more widely expressed IgSF membrane glycoprotein able to inhibit TLR, NOD-like receptors, and inflammasome activation. Induction of MARCO, a phagocytic receptor for a range of bacteria, represents an adaptation of the innate immune response to microbial contact Analysis of the actions of individual cytokines (IFNγ, IL-10, IL-4/13) on defined Mϕ targets (murine peritoneal Mϕ and

human monocyte–derived Mϕ) reveals three characteristic and distinctive in vitro phenotypes across a spectrum of activation.[124,125] IFNγ and its production and amplification via IL-12, IL-23, or IL-18 play a central role in MHC II induction, enhanced antimicrobial resistance, and proinflammatory cytokine production, characteristic of Th1-type (M1-type) responses; conversely, IL-10 suppresses markers of activation while inducing selective expression of other Mϕ genes. A comparable link between Mϕ/APC and the induction of Th2-type responses has proved elusive to identify. IL-4/13 have closely overlapping functions and induce an alternative, M2-type activation phenotype in Mϕ consistent with increased APC function and humoral responses in allergy and parasitic infection as well as giant cell formation (see Table 19.3).[126] It is important to distinguish modulation of Mϕ immunologic properties by IL-4/13 from marked deactivation and inhibition of proinflammatory and cytotoxic functions by IL-10 and glucocorticosteroid. Immune complexes are also able to induce an analogous, regulatory alternative activation pathway, which overlaps with, but differs from IL-4/13– and IL-10–induced phenotypes.[127] By extension, GM-CSF, glucocorticosteroids, TGF-β, and type I IFN all modulate Mϕ gene expression with individual signatures.

The interplay of cytokines derived from Mϕ themselves, from activated T- and B-lymphocytes, and from other cells (eg, natural killer cells, endothelial cells) results in reciprocal positive or negative interactions and time-dependent changes in activating and inhibitory signals. Some predictions from in vitro studies can be extended to the intact host. For example, IFNγ, IL-12, and IL-23 deficiency results in inability to restrict opportunistic organisms in murine models and in humans, and inducible nitric oxide synthase is important for resistance to a range of infectious agents.[128] IL-10 deficiency, on the other hand, results in overactive Th1-dependent inflammation, for example, in gut. IL-4 deficiency by itself has little effect on Mϕ phenotype in vivo because IL-13 mimics many of its actions. These cytokines share a common receptor subunit, and its targeted genetic ablation makes it possible to study Mϕ that lack the ability to respond to both IL-4 and IL-13.

The foregoing analysis is oversimplified. Combinations of cytokines in vitro have different effects on Mϕ than the sum of the parts. For example, the combination of IL-4 and GM-CSF induces differentiation of human monocytes into immature DCs, whereas each alone induces cells with distinctive Mϕ properties. Furthermore, a particular "Th2-type" cytokine such as IL-10 can display radically different effects on antimicrobial (inducible nitric oxide synthase dependent) killing, which is markedly suppressed, and anti-HIV activities of Mϕ, which are enhanced. Whereas IFNγ and IL-4 may have opposing actions on MR expression and phagocytosis of yeast, in combination they synergize to enhance uptake markedly. Other combinations of cytokines, such as IFNαβ and IFNγ, can antagonize each other, presumably by competition for signaling pathways. Selected pathogens such as *Francisella tularensis* are able to modulate macrophage responses to infection from an M1- to an M2-type, thus facilitating their survival.[129] Finally, alternative activation of macrophages has recently been implicated in thermoregulation.[130] A great deal remains to be learned about Mϕ behavior in physiology as well as disease.

CONCLUSION AND SOME REMAINING ISSUES

Mϕ influence and respond to all other cells involved in immunity, during both the afferent and efferent limbs. Many of the molecules that mediate particular functions are now defined, but their role within the Mϕ and in intercellular interactions is often poorly understood. Mϕ developed during the evolution of multicellular organisms before immunologically specific, clonotypic responses of B- and T-lymphocytes emerged. A recent report of a rearranging, TCR-like receptor in macrophages needs confirmation.[131] Mϕ themselves diversified in parallel with T-helper lymphocytes, generating DCs as specialized APCs for naïve T-lymphocytes and yielding a range of effector cell phenotypes in response to diverse activated T cells, both CD4+ and CD8+. Mϕ and their derivatives cluster with differentiating hemopoietic cells in fetal liver and bone marrow, with developing thymocytes, with naïve CD4+ T lymphocytes and antigen during immune induction, and with activated T cells and microbial pathogens in granuloma formation (see Fig. 19.2). In addition, they associate with antigen-stimulated B-lymphocytes during cell expansion, diversification, and apoptosis. A major challenge will be to define the role of specific and accessory surface molecules by which Mϕ discriminate between live and dying cells and to uncover the intrinsic and extrinsic factors that control Mϕ activities within these diverse immune cell interactions.

Our understanding of the multiple roles of Mϕ and DCs in immunoregulation is also evolving as we better appreciate their specializations and adaptations. Central issues in the immunobiology of Mϕ remain interesting topics for further investigation. These include the following:

Mϕ display broad functions in homeostasis, beyond host defense and immunity, which may be special instances of a more general role in preserving host integrity, comparable to that of the central nervous and endocrine systems. Their dispersion, plasticity, and responsiveness raise obvious questions for the biologist. In particular, what are their roles in development, in trophic interactions within different organs, in angiogenesis,[132] repair, and fibrosis[133]?

The Mϕ lie at the heart of the classic immunologic question of recognition of altered or non-self, especially of particulates. What are the actual exogenous and endogenous[134] ligands recognized by the diverse range of plasma membrane receptors capable of direct detection, and what determines whether uptake of a target is immunologically silent or productive? How can this information be harnessed for vaccine development?

The delineation of further subsets of CD4+ T lymphocytes (TH17, regulatory T cells) suggests that it will be useful to define the effects on the Mϕ phenotype of contact-and cytokine-dependent interactions with these cells. It is likely that further distinctive type 2 activation pathways of Mϕ will be discovered by microarray and protein analysis.

Once activated, Mϕ change their ability to recognize and destroy targets, directly or in concert with antibody, complement, and other less-defined opsonins. Can Mϕ directly kill virus-infected and other immunologically activated cells? If so, do they use MHC matching, even in a limited way, and do they contribute to tolerance and, by implication, autoimmunity by failure to perform such a suppressive function?

A special case in which Mϕ are present in large numbers at a site of "failure" to respond immunologically is the fetoplacental unit. CSF-1 is produced locally at high levels; does this deactivate Mϕ or make them switch to perform a trophic role? Do tumors that are rich in Mϕ adopt a similar strategy[135–137]? Catabolism of tryptophan by Mϕ enzymes has been put forward as another mechanism for preventing local destruction of an allogeneic fetus.[138] This is but one example of a growing interest in immunometabolism.[139]

Although Mϕ express a large number of genes involved in household functions and share expression of others with a limited range of cell types, they also express highly restricted molecules responsible for unique functions. Can these be harnessed for Mϕ-specific gene targeting at selected microanatomic sites to deliver functionally precise signals at predetermined times? Techniques are becoming available for at least part of this fantasy, and they should provide new insights into the multiple roles of the Mϕ in immunity.

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CHAPTER 20

Granulocytes and Mast Cells

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INTRODUCTION

Polymorphonuclear leukocytes or granulocytes are hematopoietically derived blood cells that typically act at the frontline of innate defense in host response to foreign microorganisms. Granulocytes contain heterogeneous cytoplasmic granules, which are storage pools for cell-specific intracellular enzymes, preformed receptors, cationic proteins, and other cell-specific molecules. According to their granular staining properties, polymorphonuclear leukocytes are classified into three different populations: neutrophils, eosinophils (Eos), and basophils (Bas). Neutrophil granules stain preferentially with neutral dyes, eosinophilic granules stain with acidic colorants such as eosin, and Ba granules stain with basic dyes.

Mast cells (MCs), similarly to polymorphonuclear leukocytes, represent another crucial effector cell type of the innate immune system that also stores elevated amounts of preformed inflammatory mediators within their cytoplasmic granules. However, while polymorphonuclear leukocytes are mainly peripheral blood–circulating cells, MCs are tissue resident cells distributed throughout the vascularized tissues or serosal cavity. Granulocytes and MCs differ in their functions and roles during the inflammatory process, MCs, Bas, and Eos being, for instance, essential components of allergic inflammation. Interestingly, recent data have revealed that granulocytes and MCs may also play key roles in orchestrating the transition from innate to adaptive immunity. These latter observations have caught the attention of immunologists who are currently reevaluating the importance of granulocytes and MCs, and very intensively working to clarify their multifaceted aspects in immunity.

NEUTROPHILS

Neutrophils are well known to function as the first line of defense against invading pathogens, principally bacteria and fungi, but also viruses.[1] These cells, together with monocytes, macrophages, and dendritic cells (DCs), feature the characteristic properties of the "professional" phagocytes and utilize several effector mechanisms to destroy pathogens, including the generation of massive amounts of reactive oxygen species (ROS) in combination with the discharge of many potent antimicrobial enzymes or factors.[1] Because of their powerful microbicidal equipment, neutrophils are often depicted as harmful cells that can cause damage to the surrounding tissues during acute inflammation (as observed in those inflammatory diseases dominated by neutrophils).[1] Nonetheless, extensive research performed in the last 20 years has recognized neutrophils as highly versatile and sophisticated cells displaying a significant synthetic capacity as well as an important role in linking the innate and adaptive arms of the immune response.[2,3]

Neutrophil Generalities

Neutrophils are the most abundant (40% to 70%) circulating leukocyte type in human blood, normally present at 2.5 to 7.5 $\times$ 10^9 cells/L.[4] Morphologically, these cells can be identified by the peculiar shape of their nucleus, which is polymorphous and usually consists of three to five sausage-shaped masses of chromatin connected by fine threads (Fig. 20.1). Mature neutrophils are terminally differentiated, nondividing cells that develop and mature in the bone marrow from pluripotent CD (cluster of differentiation)34-positive (CD34+) stem cells, under the regulatory effects of several colony-stimulating factors (CSFs), including granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), interleukin (IL)-3, and IL-6.[4] Neutrophils can be cytofluorimetrically identified by their characteristic morphology (high side scatter) and expression pattern of plasma membrane proteins such as CD66b, CD11b, CD15, and CD16, in conjunction with the lack of expression of CD2 and CD19. In humans, circulating polymorphonuclear leukocytes are 10 to 20 μm in diameter, display a half-life previously thought to correspond to 7 to 12 hours, but more recently reevaluated and extended to up to 90 hours,[5] and exist in a dynamic equilibrium with a "marginated" pool that is sequestered within the microvasculature of many organs.[1,4] In the resting uninfected host, the peripheral neutrophil population is maintained within a constant number by several mechanisms. One of them consists in programming neutrophils to spontaneously undergo apoptosis[1,3] to be, in turn, cleared by tissue macrophages located in the bone marrow, spleen, and liver.[6] In this latter context, a feedback loop involving an IL-23/IL-17/G-CSF axis, crucial for the regulation of neutrophil production, has been recently identified in mice.[7] According to this model, the uptake of apoptotic neutrophils by macrophages and DCs would determine a downregulation of their IL-23 production. Consequently, the Th17 subset of proinflammatory T-lymphocytes would be poorly sustained and thus much less IL-17A would be generated. As IL-17A positively regulates fibroblast- and endothelial cell–derived G-CSF, which is essential for controlling both granulopoiesis and neutrophil survival, the final outcome of this circuit—triggered by the massive neutrophil

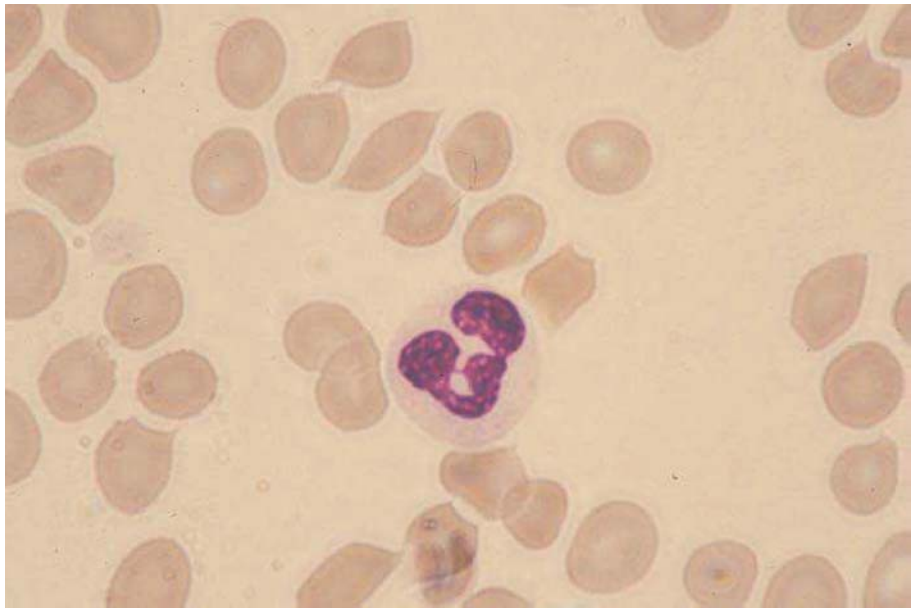

FIG. 20.1. A Polymorphonuclear Neutrophil Circulating in Peripheral Blood. From *Anderson's Atlas of Hematology*, Anderson, Shauna C., PhD. Copyright 2003, Wolters Kluwer Health/Lippincott Williams & Wilkins.

apoptosis at peripheral sites—would consist in a decrease in the levels of neutrophils released from the bone marrow.[7] On the other hand, the number of circulating neutrophils can dramatically increase (even up to 10-fold) under acute inflammatory conditions (eg, during a bacterial infection), from accelerated neutrophil production and release from the bone marrow.[4] Moreover, even the lifespan of neutrophils is significantly extended under inflammatory conditions, as various host- and pathogen-derived mediators such as G-CSF, GM-CSF, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, lipopolysaccharide, and nucleic acids inhibit neutrophil apoptosis and hence prolong their survival.[8]

During an acute inflammatory response, neutrophils are rapidly recruited to the site of injury by a coordinated sequence of events that begin with the elaboration of various mediators able to specifically promote their migration from the intravascular compartment. Mediators derive from numerous sources (tissue macrophages, endothelial cells, activated plasma components) and include vasoactive amines, proinflammatory lipids, small polypeptides, chemotactic factors, and cytokines such as TNFα, IL-1β, or IL-17A (the latter being one of the most abundant products of Th17 cells).[1,3] Chemotactic factors, in particular, are generated in temporally distinct waves and include C5a, leukotriene-B_4 (LTB_4), formyl-Met-Leu-Phe (fMLF), as well as neutrophil specific chemokines, such as CXCL8/IL-8, CXCL1/GROα, CXCL5/epithelial cell neutrophil-activating protein-78, etc.[1,3] Once recruited at an inflammatory site, neutrophils function as mobile arsenals that recognize, phagocytose, and ultimately destroy their targets. If the acute inflammatory response correctly subsides, then neutrophils may actively participate in its resolution (see following discussion). If not, an uncontrolled and continuous release of the proinflammatory cargo (ROS and proteases) by neutrophils recruited at the site of infection/injury may eventually lead to destruction of bystander tissue, and thus to exacerbation of the ongoing inflammation, ultimately provoking the onset of chronic inflammatory/autoimmune diseases.[9]

Neutrophil Microbicidal Mechanisms

To destroy and eliminate invading pathogens, neutrophils essentially utilize two fundamental mechanisms[10]: an oxygen-dependent process that is mediated by the generation of ROS, which include O_2^- (superoxide anion), hydrogen peroxide, singlet oxygen, and other products derived from the metabolism of hydrogen peroxide; and an oxygen-independent process consisting in the release into the phagocytic vacuole of lytic enzymes; and antimicrobial polypeptides stored in their intracellular granules.[10] The oxygen-dependent process, also referred to as the "respiratory burst," is defined as an increase of a mitochondrial independent oxidative metabolism that leads to the generation of O_2^-, which occurs through the activation of the phagocytic NADPH oxidase, an enzymatic system that is unique to phagocytes (neutrophils, monocytes, macrophages, DCs, and also Eos). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a multiprotein complex formed by a flavocytochrome-b_{558} (a heterodimer of gp91*phox* and p22*phox* chains, where *phox* stands for phagocyte oxidase), three cytoplasmic components (namely p40*phox*, p47*phox*, p67*phox*) and either Rac1 or Rac2 from the Rho family of low-molecular-weight GTPases (Fig. 20.2). Upon cell stimulation, the cytosolic components of the complex become phosphorylated and assemble together with the cytochrome and Rac1/2 on the plasma membrane, thus forming the active enzyme that produces superoxide anion radicals, by catalyzing the transfer of electrons from NADPH to molecular oxygen (see Fig. 20.2). O_2^- is converted by superoxide dismutase into hydrogen peroxide, which, in the presence of myeloperoxidase and halogens, is then metabolized into hypochlorous acid. The latter represents one of the neutrophil's

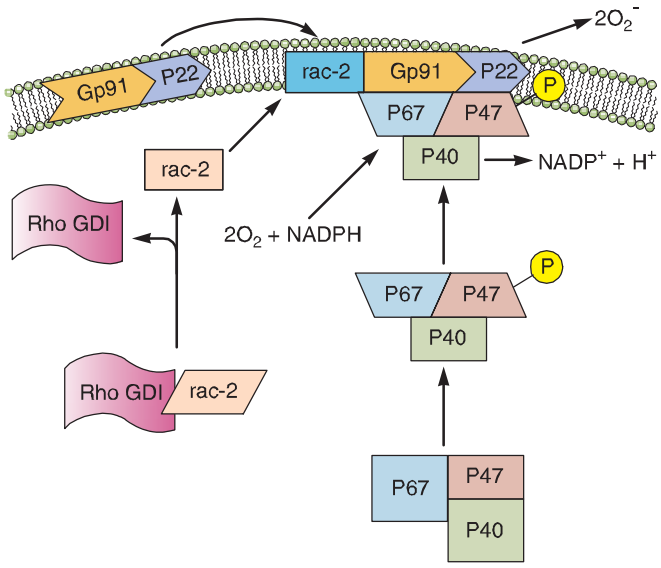

FIG. 20.2. Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase Assembly. In the resting neutrophil, the cytochrome b subunits gp91-phox and p22-phox are tightly bound in the membrane. p47-phox, p67-phox, and rac-s complex are in the cytosol. Upon activation, Rho GDP-dissociation inhibitor (GDI) releases rac-2, and p47-phox becomes phosphorylated. This causes translocation of rac-2, p47-phox, and p67-phox to the membrane and complex formation with the cytochrome components, thereby completing the assembly of the active oxidase. (After Burg ND, Pillinger MH. *Clin Immunol.* 2001;1:7–17.)

major weapons against microbes, as it also synergizes with granule proteins to kill pathogens in the neutrophil phagolysosome. O_2^- can also reacts with other cellular radicals, such as nitric oxide, to form different species of cytotoxic oxidant, such as peroxynitrite. The critical role of NADPH oxidase and its products in host defense is best illustrated by the plight of patients with chronic granulomatous disease, in which mutations in any of the NADPH oxidase complex subunits (gp91*phox*, p22*phox*, p40*phox*, p47*phox*, and p67*phox*) leads to a severe immunodeficiency characterized by defective killing of phagocytosed pathogens for the lack of ROS generation.[10] These infections typically involve microorganisms for which oxidant-mediated killing is particularly critical for effective host defense, such as *Staphylococcus aureus*, *Aspergillus* spp., *Nocardia*, and a variety of gram-negative enteric bacilli.

The release of potent proteolytic enzymes contained in their granules represents the other crucial mechanisms utilized by neutrophils to eliminate pathogens following phagocytosis.[10] Neutrophil granules are subdivided into peroxidase-positive granules (based on the presence of myeloperoxidase, their marker), also called primary or azurophil granules (owing to their affinity for the basic dye azure A), and peroxidase-negative granules that include the specific (secondary) granules, the gelatinase (tertiary) granules, and the secretory vesicles[11] (Fig. 20.3). The different types of granules appear at progressive stage of neutrophil development,[11] with the primary granules, as suggested by the name, being the first ones to appear during hematopoiesis at the promyelocyte stage. As highlighted in Figure 20.3, granules are released in a hierarchical order and under separate control by mature neutrophils, depending on the type of stimulus.[11]

Azurophil or primary granules undergo limited exocytosis in response to stimulation and are packaged with acidic hydrolases and antimicrobial proteins that contribute primarily to the killing and degradation of engulfed microorganisms into the phagolysosome.[11,12] These granules contain myeloperoxidase, an enzyme that catalyzes the formation of hypochlorous acid, hydrolases, lysozyme, matrix metalloproteinases, and three structurally related serprocidins (serine proteases with microbicidal activity): proteinase-3, cathepsin G, and elastase. The latter proteins can degrade a variety of extracellular matrix components, including elastin, fibronectin, laminin, type IV collagen, and vitronectin. Azurophil granules also contain antimicrobial molecules such as bactericidal/permeability-increasing protein (which is important for killing gram-negative bacteria) and α-defensins, a family of small cysteine-rich antibiotic peptides with broad antimicrobial activity against bacteria, fungi, and certain enveloped viruses.[11,12]

Specific (or secondary) granules, which are formed at the myelocytic stage, are smaller and less dense than the azurophil ones, and contain unique constituents, such as collagenase, haptoglobin, vitamin B_{12}-binding protein, as well an extensive array of membrane-associated proteins including cytochromes, signaling molecules, and receptors.[11,12] Secondary granules also contain an arsenal of antimicrobial substances, such as lactoferrin, neutrophil gelatinase-associated lipocalin, lysozyme, hCAP18 (cathelicidin human cationic antimicrobial protein of 18 kDa, the proform of LL-37), and pentraxin-3. The inhibitory effect of antimicrobial proteins include depriving ions essential for microbial survival, degrading structural components of microorganisms (eg, peptidoglycan), and disrupting the integrity of target cell membrane by punching pores in the membrane or by perturbing membrane integrity. Neutrophil-specific granules also contain an important family of soluble proteinases, known as matrix metalloproteinases (MMPs), which include neutrophil collagenase-2 (MMP-8), gelatinase-B (MMP-9), and leukolysin (MMP-25). These proteinases are generally stored as inactive proenzymes and undergo proteolytic activation following granule fusion and interaction with azurophilic granule contents. Neutrophil MMPs disrupt major structural components of bacteria and/or extracellular membranes, and are therefore crucial not only for bacterial killing, but also for neutrophil extravasation and migration.[11,12]

Tertiary granules are produced at the metamyelocyte stage of differentiation and are smaller, lighter, and more easily exocytosed than the other granule classes.[11,12] These granules are indeed important primarily as a reservoir of matrix-degrading enzymes and membrane receptors needed during polymorphonuclear leukocyte extravasation and diapedesis. The primary constituent of tertiary granules is gelatinase, a latent metalloenzyme with the capacity for tissue destruction. Finally, the secretory vesicles are smaller than the other granules, are generated by endocytosis during the late stage

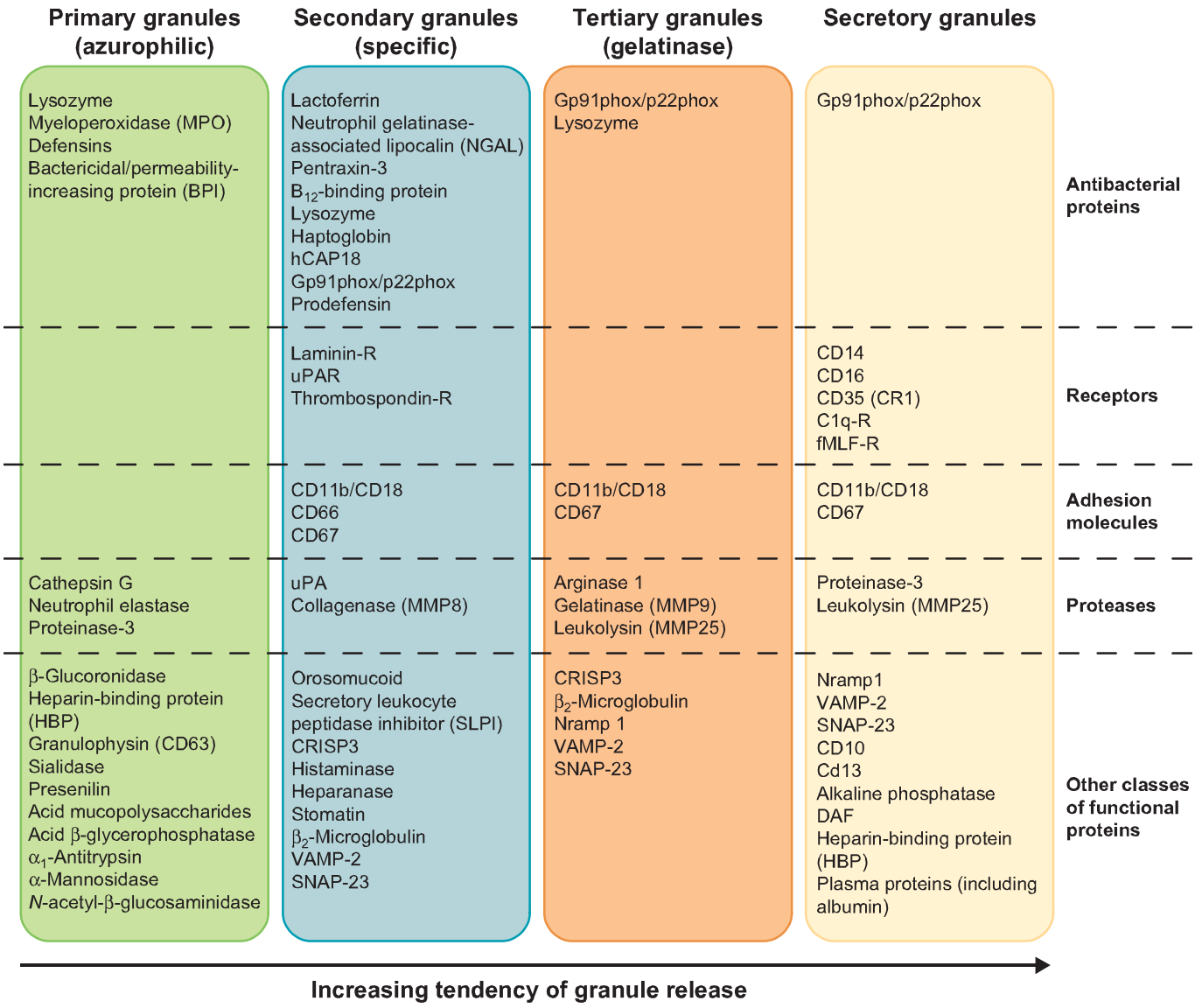

FIG. 20.3. Main Constituents of Neutrophil Granules.

of nuclear neutrophil segmentation in the bone marrow, and are the most readily mobilizable.[11,12] These vesicles are preferentially directed to the plasma membrane, as reflected in the density of vesicle-associated membrane protein (VAMP), a fusogenic protein associated with the granule membrane. Secretory vesicles do not contain toxic substances, but mainly plasma proteins like albumin and receptors (including β_2-integrins, the complement receptor [CR]1, receptors for formylated bacterial peptides [fMLF-R], CD14, the Fc portion of γ-immunoglobulins (Igs) [FcγRIII/CD16], and the metalloprotease leukolysin). Heparin-binding protein (also known as CAP37 or azurocidin), whose release is essential for the polymorphonuclear leukocyte–induced increase in vascular permeability at the initial stage of extravasation, is also stored in the secretory vesicles.

An additional nonphagocytic microbicidial mechanism used by neutrophils to capture and destroy microbes in the extracellular space consists in the ability of neutrophils to form so-called neutrophil extracellular traps (NETs).[13] The latter structures consist of nuclear chromatin decorated with antimicrobial peptides and enzymes (eg, bactericidal/permeability-increasing protein, elastase, pentraxin3, cathepsin G, and many others) that lacks membranes and cytosolic markers.[13] NETtosis, a novel type of neutrophil death mechanism that occurs under settings of extreme neutrophil stimulation (different from necrosis, apoptosis, and also independent from caspase activation), underlies the generation of NETs.[14] Accordingly, the nuclear envelope, granules, and cell membranes gradually dissolve during NETtosis, allowing the nuclear contents to mix and condense in the cytoplasm before being released into the extracellular space.[14] NETs, in turn, bind to various gram-positive and gram-negative bacteria (such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Streptococcus pneumoniae*,

and group A streptococci), as well as to pathogenic fungi (such as *Candida albicans*). Similarly to what happens in the phagolysosome, the high local concentration of antimicrobial peptides and enzymes is responsible for the killing of the pathogens trapped by NETs.[13] The observation that neutrophils from patients with chronic granulomatous disease do not form NETs has suggested, on the one hand, that ROS-mediated signaling/cascades are involved in NET generation, and, on the other hand, that the lack of NETs might contribute to the pathogenesis of chronic granulomatous disease.[3,14] Whatever the case, it is noteworthy to remark that both the oxygen-dependent and -independent effector mechanisms in host defense toward pathogens are also utilized by neutrophils for their cytotoxic and tumoricidal activities.

Neutrophil Receptors

Under inflammatory conditions, neutrophils sense a wide range of extracellular ligands that, through the interaction with specific receptors, subsequently trigger a number of effector functions, including adhesion, migration, phagocytosis, survival, cell activation, gene expression modulation, target cell killing, and mediator production and release.[1,3,15] For instance, agonist-stimulated neutrophils may trigger not only degranulation, but also the release of arachidonic acid and/or other eicosanoids (eg, prostaglandin [PG]E_2), via the activation and/or the upregulation of PLA_2 and COX-2, respectively. Upon appropriate stimulation, neutrophils can also generate LTA_4 through the action of 5-lipoxygenase, as well as LTB_4 by converting LTA_4 via the action of LTA_4 hydrolase.[15] A nonexhaustive list of neutrophil receptors includes 1) receptors for proinflammatory mediators (eg, the anaphylotoxin complement component C5a, LTB_4, platelet-activating factor [PAF], substance P, and fMLF); 2) receptors for cytokines, such as IFNγ, IL-1, IL-4, IL-6, IL-10, IL-13, IL-15, IL-18, TNFα, G-CSF, GM-CSF, and many others; 3) receptors for chemokines, including CXCR1 and CXCR2; 4) receptors/adhesion molecules for the endothelium; 5) receptors for tissue matrix proteins; and 6) opsonin receptors, such as FcγRs and those for the major cleavage fragments of the complement system (see following discussion). Neutrophils also express a variety of pattern recognition receptors (PRRs), including all toll-like receptors (TLRs), with the exception of TLR3, cytoplasmic ribonucleic acid helicases involved in viral ribonucleic acid recognition such as MDA5 and RIG-I, and deoxyribonucleic acid binding cytoplasmic proteins (IFI16 and LRRFIP1).[3]

The Role of Neutrophils in Acute Inflammation

In order to carry out their prototypical defensive role in acute inflammation, bloodstream neutrophils must extravasate. To do so, they attach to activated endothelium, transmigrate through postcapillary venules (diapedesis), and then migrate toward a corresponding chemotactic gradient to the injury site where they recognize their target, engulf, and finally destroy it.

Neutrophil Extravasation

In general, leukocyte recruitment during inflammation, also called extravasation, is a multistep and highly complex phenomenon characterized by a number of predetermined steps occurring in the vessel lumen, known as leukocyte capture (or tethering), rolling, activation, and firm adhesion (arrest)[16] (Fig. 20.4). These latter steps are not discrete phases of inflammation; rather, they simply represent a sequence of events from the perspective of each leukocyte. Moreover, each of these steps appears to be necessary for the effective leukocyte recruitment; blocking any of them can severely reduce leukocyte accumulation in the tissue.[17] Adhesion of blood leukocytes to the endothelium during inflammation requires specific leukocyte–endothelial interactions involving different families of adhesion molecules. The latter include members of the selectin family and their cognate carbohydrate and glycoprotein ligands, which mediate the initial leukocyte deceleration along the vessel wall (a process called "rolling"), as well as members of the integrin family and their cognate Ig superfamily ligands, which mediate the subsequent high-affinity adhesion and arrest of leukocytes to the venules[16,17] (Table 20.1).

Under noninflammatory conditions, neutrophils (as well as other leukocytes) travel primarily through the center of the blood vessel lumen, where the flow is fastest. In response to proinflammatory signals, however, both the neutrophils and the blood vessels undergo a series of changes. As a consequence of vascular dilatation, blood flow first increases and then slows, thus facilitating the interactions between leukocytes and the endothelial cells. The process of capture/tethering and rolling then ensues, in which L-selectins on neutrophils, and P- or E-selectins on endothelia, interact with sialyl-Lewisx moieties or PSGL-1 on their respective cell partners. Although these interactions are reversible and transient, they prepare neutrophils for a tighter binding, integrin-mediated step.[16,17] The most important neutrophil integrins are four, each one composed of an identical β-subunit (β_2, known as CD18) noncovalently linked to different α-subunits: CD11a/CD18 ($\alpha_L\beta_2$, lymphocyte function antigen), CD11b/CD18 ($\alpha_M\beta_2$, CR3), CD11c/CD18 ($\alpha_X\beta_2$, CR4), and CD11d/CD18.[18] When neutrophils encounter their specific chemoattractants (eg, CXCL8), displayed bound to glycosaminoglycans on the vessel wall, neutrophil integrins are converted from an inactive to an active conformation[16,17] (see Fig. 20.4). Activated integrins can interact with their counterreceptors on the surface of the TNFα and/or IL-1β–activated endothelium (eg, intercellular adhesion molecule-1 [ICAM-1] and intercellular adhesion molecule-2), leading to the strong adhesion and arrest of the cells. Neutrophils then flatten and transmigrate between and through the endothelial cells of postcapillary venules into the surrounding tissue (see Fig. 20.4), also secreting a broad range of MMPs that degrade the basement membrane. Transmigration involves homophilic interaction of CD31/PECAM-1 and JAM-A on neutrophils and endothelial cells, where CD31/PECAM-1 and JAM-A act sequentially to mediate neutrophil migration through the venular walls.[17,18] Once in the interstitial compartment, neutrophils migrate along the chemotactic gradient toward the site of

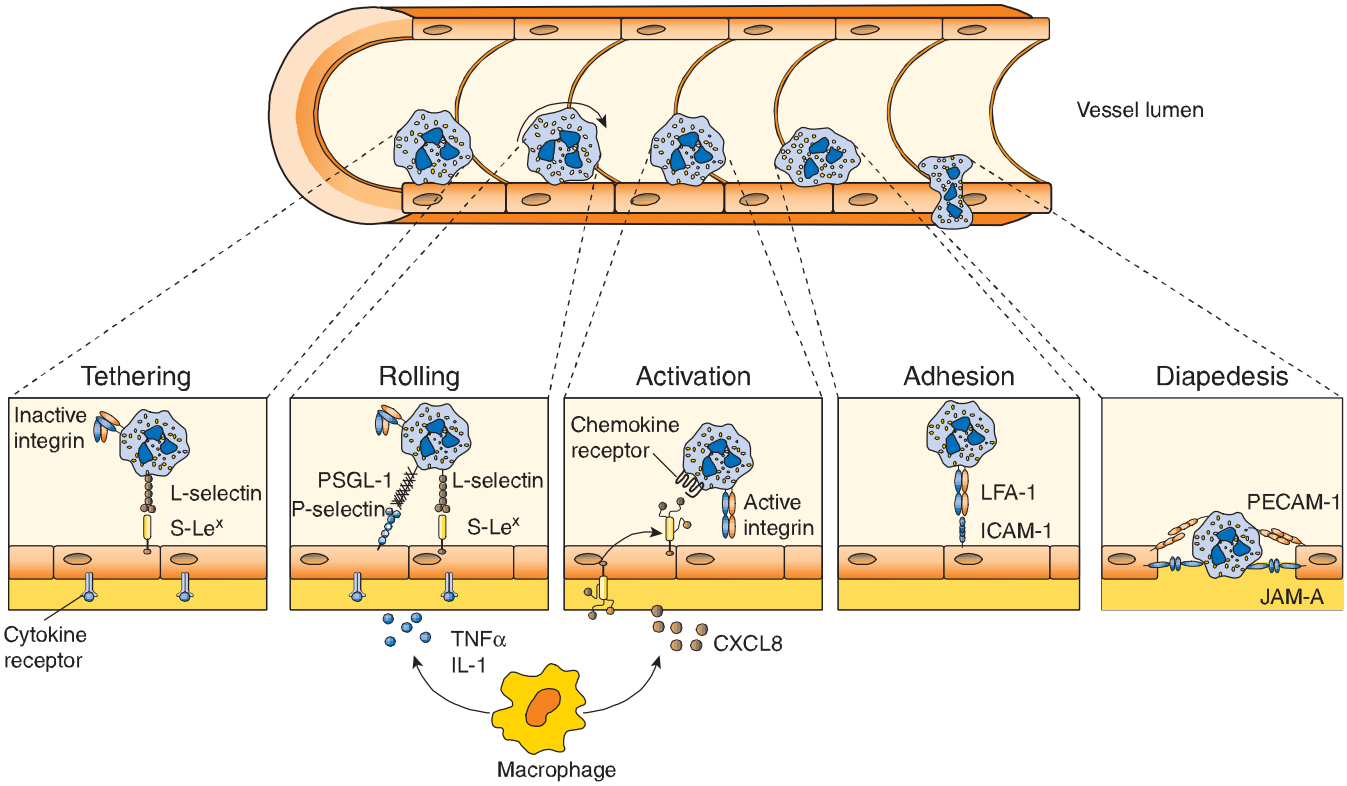

FIG. 20.4. Leukocyte Transmigration. Following an inflammatory stimulus, tissue-resident macrophages and other cells release inflammatory mediators such as tumor necrosis factor α and interleukin-1, which induce the rapid expression of preformed P-selectin (and transcription-dependent E-selectin expression) on the endothelium. The interaction between selectins and their glycoprotein ligands initiates leukocyte tethering and rolling. Activation by chemokines—and other leukocyte activators (eg, leukotriene-B_4 or platelet-activating factor)—presented on endothelial cells causes leukocyte integrin activation, thus resulting in transition from cell rolling to cell firm adhesion, in view of the strength of integrin-mediated binding with endothelial immunoglobulin superfamily members. Leukocytes can then transmigrate through the endothelial monolayer and chemotactically move toward the inflammatory stimulus. Examples of adhesion molecules involved in each step are depicted.

injury or infection and, once arrived, begin to react with the etiopathogenic agent.

The fundamental importance of adhesive glycoproteins in vivo is testified by those individuals affected leukocyte adhesion deficiency (LAD) disease, who display an abnormally high susceptibility to bacterial infections.[19] Several types of LAD disease have been identified. In LAD-I, mutations of the β2 integrin typically eliminate the expression of all four integrin complexes. Because of the multiple defects in adhesion-related functions, patients with LAD-I develop recurrent bacterial and fungal infections, mostly with *Staphylococcus aureus* or gram-negative enteric microbes. Neutrophilia with paucity of neutrophils at inflamed or infected sites is characteristic of LAD-I, while typical clinical features include frequent skin and periodontal infections, delayed separation of the umbilical cord and omphalitis, and deep tissue abscesses. LAD-II is caused by mutations in the membrane transporter for fucose and thus is associated with loss of expression of fucosylated glycans on the cell surface. Fucosylated proteins such as sialyl-Lewis X (CD15s) are ligands for endothelial selectins and are important for the rolling phases of leukocyte extravasation. Patients with LAD-II also have leukocytosis and form pus poorly, although infections tend to be less severe in patients with LAD-II than in patients with LAD-I. Finally, LAD-III has been recently described. LAD-III is an autosomal recessive disorder caused by mutations in the human kindlin-3 gene, which codes for a protein essential for integrin activation. Consequently, LAD-III is characterized by impaired adhesion of leukocytes to the endothelium of inflamed tissues and by severe bleeding. Curiously, a number of LAD-III patients additionally suffer from osteopetrosis.

Neutrophil-Mediated Phagocytosis

Neutrophils play a critical role in host protection as they eliminate microorganisms through phagocytosis (the cellular process of engulfing particles larger than 0.5 μm). While many cells in our body are capable of phagocytosis, neutrophils do it to an extent sufficient to be considered "professional phagocytes" (eg, a single neutrophil can engulf up to 10 to 12 particles [eg, bacteria]).[20] Phagocytosis is triggered either through receptor (eg, mannose receptor or Dectin-1) recognition of certain polysaccharides present on the surface of some yeast cells and/or upon the binding of opsonized microorganisms through, for instance, FcγRs and CRs.[20] Neutrophils constitutively express the low-affinity

TABLE 20.1 Main Adhesion Molecules Involved in Leukocyte-Endothelial Cell Interaction

Adhesion Molecule	Distribution	Ligands and Counterreceptors	Function
E-selectin (CD62E)	Endothelial cells	PSGL-1, ESL-1, CD44[a], CD43[a]	Rolling
P-selectin (CD62P)	Endothelial cells, platelets	PSGL-1, PNAd	Rolling
L-selectin (CD62L)	All leukocytes except effector and memory effector T cells	PNAd, MAdCAM-1, PSGL-1, E-selectin, P-selectin	Rolling
Selectin ligands			
PSGL-1	All leukocytes	All selectins (essential for P-selectin)	Rolling
sLe^x	Myeloid cells, some memory T cells, HEVs	All selectins	Rolling
PNAd	HEV, some sites of chronic inflammation	L-selectin, P-selectin	Rolling
Integrins			
$\alpha_M\beta_{2(MAC\text{-}1;\ CD11b/CD18)}$	Granulocytes, monocytes, some activated T cells	ICAM-1, fibrinogen, C3b, JAM-C	Adhesion, transmigration
$\alpha_L\beta_{2(LFA\text{-}1;\ CD11a/CD18)}$	All leukocytes	ICAM-1, ICAM-2, JAM-A	Adhesion, transmigration
$\alpha_D\beta_{2(CD11d/CD18)}$	Monocytes, macrophages, eosinophils	ICAM-1, VCAM-1	Adhesion
$\alpha_x\beta_{2(p150,95;\ CD11c/CD18)}$	DCs	Fibrinogen, C3b	Adhesion
$\alpha_4\beta_{1(VLA\text{-}4)}$	Most leukocytes	VCAM-1, fibrinogen, JAM-B	Rolling, adhesion
$\alpha_4\beta_{7(LPAM\text{-}1)}$	Lymphocytes, NKCs, mast cells, monocytes	MAdCAM-1, fibronectin, VCAM-1	Rolling, adhesion
Immunoglobulin superfamily			
ICAM-1 (CD54)	Most types of cells	LFA-1 Mac-1, fibrinogen	Adhesion, transmigration
ICAM-1 (CD102)	Endothelial cells, platelets	LFA-1 Mac-1	Adhesion, transmigration
VCAM-1 (CD106)	Endothelial cells	VLA-4, $\alpha_4\beta_7\alpha_D\beta_2$	Rolling, adhesion
MAdCAM-1	HEVs in PP and MLN	$\alpha_4\beta_7$, L-selectin	Rolling
PECAM-1	Endothelial cells, platelets, leukocytes	PECAM-1	Transmigration
JAM-A	Endothelial cells, platelets, most leukocytes	JAM-A	Transmigration
JAM-B	Endothelial cells, HEVs	JAM-B, JAM-C	Transmigration
JAM-C	Endothelial cells, HEVs, platelets, monocytes, DC, some T cells	JAM-C, JAM-B	Transmigration

CD, cluster of differentiation; DC, dentritic cell; ESL, HEV, high endothelial venule; ICAM, intercellular adhesion molecule; JAM, LFA, MAdCAM, mucosal addressin cell adhesion molecule; NKC, PECAM, PNAd, PSGL, sLe^x, VCAM, VLA.
[a]CLA decorated.

FcγRs (FcγRIIA/CD32A and FcγRIIIA/CD16A), and, when exposed to IFNγ or G-CSF, the high-affinity FcγR (FcγRI/CD64) as well.[3] CRs expressed by neutrophils are CR1 (also known as CD35), which binds to complement components C1q, C4b, C3b, and mannan-binding lectin; CR3, which binds to iC3b, intercellular adhesion molecule-1, and some microbes; and CR4, which binds to iC3b. By expressing these latter receptors, neutrophils are able to recognize and bind, in a cooperative manner, IgG-opsonized particles and/or complement-opsonized microbes, and then activate their phagocytosis. During the phagocytic process, the foreign particle is internalized, initially through membrane recruitment to the site of particle contact, and then via membrane extensions outward to surround the particle and form a new vesicle called a cytoplasmic phagosome.[20] (Fig. 20.5). The phagosome then undergoes fusion with neutrophil granules to form a phagolysosome, a protected space in which proteolytic enzymes and other bactericidal components are discharged and pathogen degradation occurs. At the same time, NADPH oxidase assembles on the phagosomal membrane after phagocytosis and starts to generate ROS into the phagolysosome to kill bacteria by oxidizing microbial proteins and lipids. The activity of NADPH oxidase also leads to the acidification of the phagosome, which enhances the effectiveness of pH-sensitive antimicrobial compounds. Thus, neutrophil mechanisms of pathogen destruction within the phagosome are multiple and involve granule fusion, toxic oxygen radical production, activation of latent proteolytic enzymes, and the activity of antibacterial proteins (see Fig. 20.5). Remarkably, an activation of gene transcription and a selected generation of cytokines also occur during phagocytosis, a feature that neutrophils utilize for boosting a more effective innate immune response. For instance, recruited neutrophils that phagocytose

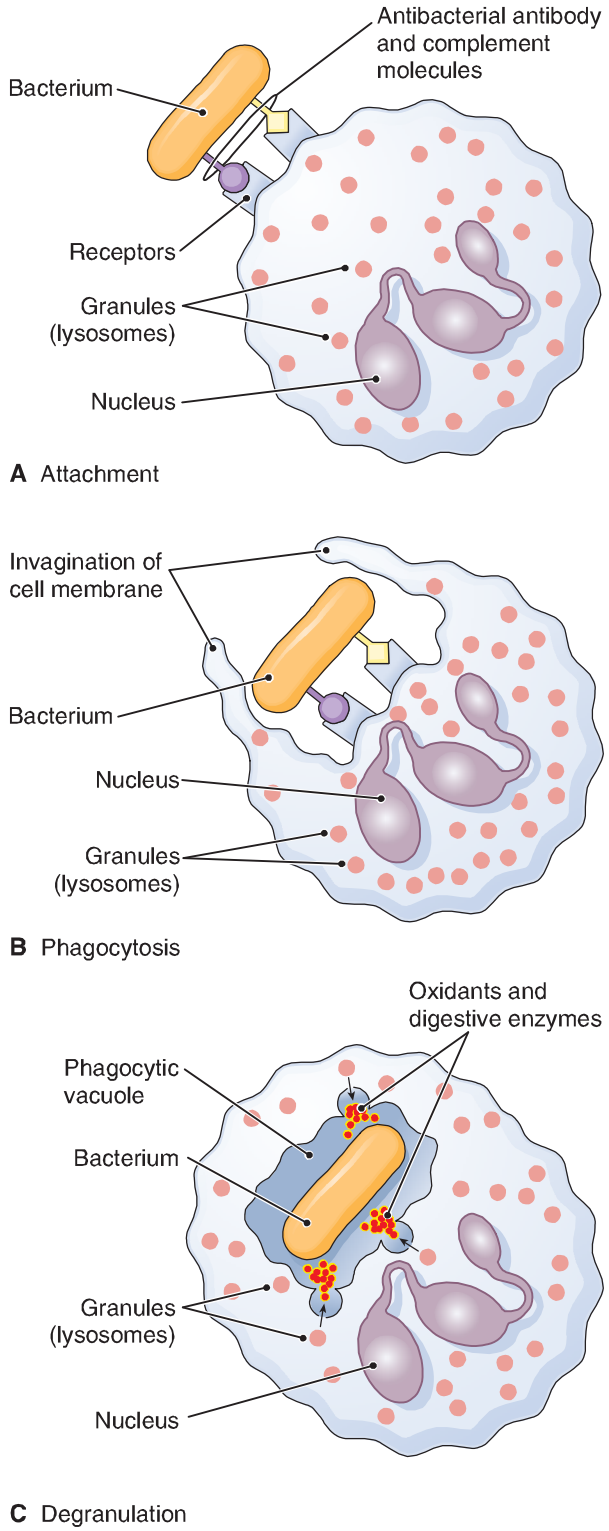

FIG. 20.5. Phagocytosis. The figure shows ingestion, digestion, and destruction of foreign particulate matter (a bacterium, in this example) by a neutrophil. **A:** Cell membrane receptors bind to antibody and complement molecules previously attached to the bacterial surface. **B:** The cell membrane creeps around the bacterium and envelopes it. **C:** The bacterium is trapped in a special space, the phagocytic vacuole, into which lysosomes discharge proteases, which together with oxidants kill it. Then, digestive enzymes dissolve it. (Thomas H. McConnell, *The Nature Of Disease Pathology for the Health Professions*, Philadelphia: Lippincott Williams & Wilkins, 2007.)

a pathogen also respond by producing chemokines, in particular CXCL8, to amplify their own recruitment, but also CCL3, CCL4, and CCL19 that serve to recruit monocytes and DCs.[21]

Role in the Resolution Phase of Inflammation

Locally activated neutrophils not only amplify the inflammatory process, but, surprisingly, actively participate in its resolution phase.[22] To do so, during the late, final phases of a resolving, acute inflammatory reaction neutrophils, for instance, switch their eicosanoid biosynthesis potential from LTB_4 to lipoxins, with profound modifications of their effector functions. In fact, lipoxin A_4 (LXA_4) and LXB_4 stop neutrophil chemotaxis, adhesion, and transmigration through endothelium (by decreasing P-selectin expression), inhibit Eo recruitment, stimulate vasodilation (by inducing synthesis of PGI_2 and PGE_2), inhibit LTC_4- and LTD_4-stimulated vasoconstriction, inhibit LTB_4 inflammatory effects, and inhibit the function of natural killer (NK) cells.[22] Neutrophils also contribute to the biosynthesis of resolvins (such as resolvin E1, resolvin E2, resolvin D1, and resolvin D2) and protectin D1, which all inhibit neutrophil transendothelial migration and tissue infiltration, as well as stimulate resolution and reduce the magnitude of the inflammatory response in vivo.[22] Furthermore, neutrophils might also serve as major producers of anti-inflammatory cytokines such as transforming growth factor (TGF)β and IL-1 receptor antagonist, the latter being an endogenous inhibitor of IL-1β signaling and mediated effects.[21] Finally, neutrophils must be cleared from the inflammatory site as inflammation resolves. Indeed, neutrophils undergo apoptosis and are engulfed by tissue macrophages, which then reprogram into the M2 phenotype and start to generate antiinflammatory cytokines such as TGFβ and IL-10.[6,23]

Novel Neutrophil Effector Functions

The functions described previously for neutrophils in host defense are fundamental for combating infectious diseases. However, more recent discoveries on neutrophils as source of a variety of cytokines[21] have revealed that these cells are not only key components of the inflammatory response, but also crucial effectors of innate and adaptive immune regulatory networks.[23]

Neutrophil-Derived Cytokines and Chemokines

Numerous in vitro and in vivo studies, focusing on novel aspects of the neutrophil biology and function, have recently shed new light on the potential role that neutrophils can exert in the modulation of innate and adaptive immune responses.[23] It is now unequivocal that neutrophils are not, as for a long time thought, terminally differentiated cells "devoid of transcriptional and protein synthesis activity."[23a] In fact, besides the several preformed or rapidly generated inflammatory mediators described previously, neutrophils display the capacity to de novo synthesize and release also several chemokines and cytokines with immunoregulatory properties.[21,23] It is, however, important to mention that, at least in vitro, neutrophils usually produce, on a per-cell

basis, fewer molecules of a given cytokine than mononuclear leukocytes.[21] However, considering that neutrophils clearly predominate over other cell types under inflammatory conditions in vivo, it becomes obvious that the contribution of neutrophil-derived cytokines can be of foremost importance. To date, a wide range of stimuli able to induce characteristic signatures of chemokine and cytokine synthesis by neutrophils have been identified. Among these, cytokines themselves, chemotactic factors (fMLF, LTB_4, PAF, C5a, and CXCL8), phagocytic particles, microorganisms (such as fungi, viruses, and bacteria), and PRR ligands can all induce the synthesis and release of chemokines and cytokines by neutrophils.[21] Considering that neutrophils usually represent the first cell type infiltrating at the site of infections, a stimulus-specific response of neutrophils in terms of cytokine production might direct the evolution of certain types of inflammatory and immune reactions to support the transition from innate to adaptive immunity.

Table 20.2 lists all the cytokines that, to date, have been shown to be released by neutrophils in vitro, either constitutively or following appropriate stimulation, or in vivo. Numerous in vivo observations, in fact, not only have confirmed and reproduced the in vitro findings, but often have clarified their biological meaning and implications. As outlined in Table 20.2, neutrophils can produce proinflammatory, anti-inflammatory, immunoregulatory, angiogenic, and fibrogenic cytokines, chemokines, and ligands belonging to the TNF superfamily. The role of these molecules in mediating various neutrophil-dependent immunoregulatory functions is partially described in the following chapter. For instance, chemokines are particularly represented among the cytokines produced by neutrophils and include those primarily chemotactic for neutrophils themselves, monocytes, DCs, NK cells, and T helper (Th)1 and Th17 cells. It follows that a role for neutrophils in orchestrating the sequential recruitment to, and activation of, distinct leukocyte types in the inflamed tissue is plausible, as already demonstrated to occur in several experimental models.[21,23]

TABLE 20.2 Cytokines Expressed in Resting or Activated Neutrophils

C-X-C Chemokines	**Proinflammatory Cytokines**	**TNF Superfamily Members**
GROα/CXCL1	**TNFβ**	**FasL**
CINC-2α/GROχ/ MIP-2α/**CXCL2**	IL-1α, IL-1β	CD30L
CINC-2β/GROγ/ MIP-2β/CXCL3	**IL-6**(?),IL-7, IL-9	TRAIL
PF4/CXCL4	IL-16(?), **IL-17A/F**(?)	**LIGHT**[a]
ENA-78/CXCL5	IL-18	**Lymphotoxin-β**
GCP-2/CXCL6	MIF	APRIL, **BAFF/BLyS**
IL-8/CXCL8		RANKL
MIG/CXCL9 IP-10/ CXCL10 I-TAC/ CXCL11	**Anti-inflammatory Cytokines**	**Colony Stimulating Factors**
	IL-1ra	G-CSF
C-C Chemokines	IL-4(?), **IL-10**(?)	M-CSF(?)
MCP-1/ CCL2MIP-1α, CCL3	**$TGFβ_1$, $TGβ_2$**	GM-CSF(?)
MIP-1β, CCL4		IL-3(?)
TARC/CCL17	**Immunoregulatory Cytokines**	SCF[a](?)
PARC/CCL18	IFNα, IFNβ, **IFNγ**(?)	**Angiogenic and Fibrogenic Factors**
MIP-3α, CCL19	**IL-12**	**VEGF**
MIP-3β, CCL20	IL-23(?)	BV8/Prokineticin-2
MDC/CCL22		HB-EGF
	Other Cytokines	**FGF-2**
	Oncostatin M	**TGFβ**
	GDF (?)	**HGF**
	NGF, BDNF, NT4	
	PBEF/visfatin/ NAMPT	
	amphiregulin	

?, requires definitive corroboration; GRO, growth regulated oncogene; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; MIP, macrophage inflammatory protein; CINC, Cytokine induced neutrophil chemoattractant; PF4, Platelet factor-4; ENA-78, epithelial-derived neutrophil-activating peptide 78; GCP-2, granulocyte chemotactic protein-2; IL-, interleukin-; MIG, Monokine induced by gamma interferon; I-TAC, Interferon-inducible T-cell alpha chemoattractant; IP-10, Interferon gamma-induced protein 10; MCP, monocyte chemotactic protein; TARC, Thymus and activation regulated chemokine; PARC, pulmonary and activation-regulated chemokine; MDC, Macrophage-derived chemokine; TNF, tumor necrosis factor; MIF, macrophage inhibitory factor; IL-1ra, IL-1 receptor antagonist; TGF, Transforming growth factor; IFN, interferon; GDF, Growth Differentiation factor; NGF, nerve growth factor; BDNF, Brain derived neurotrophic factor; NT4, Neurotrophin-4; PBEF, pre-B-cell colony-enhancing factor; NAMPT, Nicotinamide phosphoribosyltransferase; TRAIL, TNF-related apoptosis-inducing ligand; APRIL, a proliferation-inducing ligand; BAFF/BLyS, B-cell activating factor/B lymphocyte stimulator; RANKL, Receptor activator of nuclear factor kappa-B ligand; G-CSF, macrophage colony stimulating factor; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; SCF, stem cell factor; HB-EGF, heparin binding-like epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor.

Cytokines in **bold** refer to neutrophil studies in animal models that confirm human findings.

[a]Messenger ribonucleic acid only.

Neutrophils in Immunoregulation

There is now wide experimental evidence that neutrophils have the capacity to modulate the migration, maturation, and function of several leukocyte types including DCs, T cells, and B cells.[23] Regarding DCs, it is noteworthy to mention that neutrophils have been shown to produce biologically active CCL20 and CCL19, two structurally related CC-chemokines that have been suggested to play a fundamental role in trafficking of, respectively, immature and mature DCs to mucosal surfaces and lymphoid organs. Likewise, neutrophils release several antimicrobial compounds, such as lactoferrin, LL-37, and cathepsin G, that have been found to act as chemoattractants for immature DCs. In addition, neutrophils can proteolytically activate prochemerin to generate chemerin, one of the few chemokines that attracts both immature DCs and plasmacytoid DCs.[3] Neutrophils can also modulate DC maturation and function either through the release of several mediators or through direct physical interaction between Mac-1 (CD11b/CD18) and DC-specific intercellular adhesion molecule.[24] Neutrophils also act as transport vehicle for pathogens and, in turn, deliver antigens to DCs, thus playing an important role in the activation of T-cell immune responses controlled by DCs.[24]

Concerning the interactions between neutrophils and B cells, of particular interest are the findings that neutrophils produce significant amounts of BLyS/BAFF (B-lymphocyte stimulator/B-cell activating factor) and APRIL (a proliferation-inducing ligand), two related members of the TNF

family that are well known to be essential for B-lymphocyte homeostasis.[25] Therefore, it is plausible to assume a role of neutrophils not only in sustaining B and plasma cell antibody production and survival, but also in promoting B-cell–dependent autoimmune diseases and tumors, as already elegantly demonstrated in the case of B-cell lymphoma.[25]

Cross-talk between neutrophils and T cells has been repeatedly described to occur during infections or other inflammatory responses and diseases. Current evidence now indicates that neutrophils exhibit a significant chemotactic effect toward Th1 or Th17 cell subsets, through the release of CCL2, CXCL9, and CXCL10, or CCL2 and CCL20, respectively.[26] Neutrophils have also a role in directing T-cell polarization, for instance through their capacity to produce the Th1-inducing cytokine, IL-12. The latter has been clearly demonstrated in mouse models, in which strong Th1-dependent T-cell responses that result in pathogen clearance are elicited upon infection with *Candida albicans*, *Helicobacter pylori*, or *Legionella pneumophila*. Strikingly, depletion of neutrophils reverses the Th1 responses into a predominant Th2-response, therefore making the mice susceptible to infection. Besides the neutrophil's ability to modulate T-cell functions through the production of chemokines and cytokines, recent reports suggest that neutrophils travel to the lymph nodes during infections and express both major histocompatibility complex (MHC) II and costimulatory molecules.[27] However, whether neutrophils directly acquire antigen-presenting functions or transmit signals to naïve T cells remains still puzzling.

Neutrophils have also been shown to modulate the maturation, activation, and functions of NK cells, either by themselves or in cooperation with other cell types.[28] In this context, it is worth mentioning that neutrophils, by interacting with specific subsets of peripheral blood myeloid DC (eg, 6-sulpho LacNAc+ DC, also known as slanDC) can strongly potentiate IFNγ release by NK cells.[29] Importantly, the potential pathophysiologic relevance of a cell network among neutrophils, slanDC, and NK cells has been suggested by immunohistochemical studies that have revealed their colocalization in several chronic inflammatory pathologies, such as Crohn disease and psoriasis.[29] Finally, it has also been proposed that mature postmitotic neutrophils can also "transdifferentiate" into much-longer-lived cells with macrophage- or DC-like characteristics, which might constitute a further manner for neutrophils to act as regulatory cells of the adaptive immune response.[3,23]

It is important to mention that despite of all experimental observations in vivo that strongly suggest that neutrophils could potentially act as important players in the orchestration of immune responses, additional reevaluations and validations are required. Indeed, in order to investigate the role of neutrophils in vivo, an antigranulocyte receptor-1 monoclonal antibody, RB6-8C5, has been extensively used to deplete mice of neutrophils.[30] However, RB6-8C5 not only binds to Ly6G, which is present on neutrophils, but also to Ly6C, which is expressed on neutrophils, DCs, and subpopulations of lymphocytes and monocytes. Therefore, it has recently been shown that in vivo administration of RB6-8C5 depletes not only neutrophils but also other granulocyte receptor-1+ (Ly6C+) cells. Luckily, a more specific anti-Ly6G monoclonal antibody (1A8) has been raised; it is now preferentially used to deplete neutrophils in vivo under different experimental settings.[31] Obviously, it will take some time prior to controlling and, eventually, revising all data on neutrophil depletion generated by using RB6-8C5. More importantly, the future availability of conditional knockout mice, selectively targeting, one by one, neutrophil function such as survival, migration, or activation, will help to finally clarify the specific contributions of neutrophils under different inflammatory/immune settings.

Role of Neutrophils in Angiogenesis and Tumor Growth

There is no longer doubt that, in addition to macrophages, neutrophils may positively or negatively influence the angiogenic process and tumor growth.[32] In the former case, it has been observed that neutrophils, via elastase release, may indirectly generate massive amounts of bioactive, angiostatin-like fragments, and thus inhibit fibroblast growth factor (bFGF) plus vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation.[33] However, as described for macrophages, neutrophils can also favor malignant growth and progression, in relation to the type of tumor environment in which they reside, for instance via a remarkable production of proangiogenic molecules such as VEGF and CXCL8.[34] On the other hand, experimental studies of tumor cure and prevention have suggested that, at least in some models, engagement of neutrophil functions can be crucial for the establishment of an effective antitumoral immune response and immune memory reactions.[35] Indeed, neutrophils can produce several cytotoxic mediators for tumor and endothelial cell killing, including TNFα, defensins, proteases (such as elastase and cathepsin G), ROS, nitric oxide, and angiostatic chemokines (CXCL9, CXCL10, and CXCL11).[35] It has been recently found that neutrophils exposed to IFNs express and produce TNF-related apoptosis-inducing ligand,[36] another TNF superfamily member that selectively stimulates tumor cell killing. More recently, in vivo evidence proving that, similarly to M1 and M2 macrophages, neutrophils also polarize from an N1, proinflammatory and antitumoral phenotype, to an N2 anti-inflammatory and protumoral phenotype, has been provided,[37] thus supporting the notion that the tumor environment can profoundly shape the functional status of neutrophils. Furthermore, it is now well established that myelopoiesis can be profoundly modified during inflammation and cancer, releasing altered mature myelocytes and myeloid-derived suppressor cells (MDSCs) that exert immunosuppressive and protumoral activity, mainly by inhibiting T-cell functions.[38] Although mature human neutrophils do not seem to be a major component of such MDSC population, several mouse tumor models have revealed the existence of a granulocytic MDSC population with potent T-cell suppressing activity.[38] Nonetheless, low-density granulocytes (so called because of their abnormal behavior upon density centrifugation) able to inhibit T-cell activation and function have been found in human patients with cancer.[39] Further research is now needed to better understand the origin, phenotype, and relationship to mature

neutrophils of all these immunosuppressive granulocytic populations. Such studies will better clarify the real role of neutrophils in cancer as well as in other inflammatory/autoimmune diseases (such as infections, psoriasis, and lupus), in which the presence of MDSC- or low-density granulocyte–like cells have been also described.[40]

Neutrophils in Diseases

Previous sections have already summarized some of the most common inherited disorders of neutrophil function that impair critical responses for host defense. Dramatic clinical consequences may also be observed as a consequence of acquired neutropenia (eg, a susceptibility to infectious diseases when neutrophil counts fall below 500 cells/μL^4). Neutropenia can be due to depressed production (ie, hereditary neutropenias) or increased peripheral destruction.[4] Apart from the disorders associated with genetic dysfunctions or quantitative alterations of neutrophils, there are other pathologic situations in which neutrophils themselves become the predominant contributors to tissue injury, especially when the mechanisms supposed to control and inactivate their hypothetical beneficial and protective effector functions are deregulated. Examples of such pathologies, tentatively classified according to the major neutrophil-activating event,[1] are 1) diseases caused by ischemia reperfusion injury (ie, myocardial infarction); 2) bacterial infections (endotoxic shock, osteomyelitis, adult respiratory distress syndrome,); 3) cytokine-mediated diseases (rheumatoid arthritis, inflammatory bowel diseases); 4) diseases caused by crystal deposition (gout); 5) antineutrophil cytoplasmic antibody–associated vasculitis (Wegener granulomatosis, pauci-immune necrotizing crescentic glomerulonephritis); and 6) airway diseases (chronic obstructive pulmonary disease, bronchiectasis, bronchiolitis, cystic fibrosis, and even certain forms of asthma are characterized by neutrophil infiltration of the airway wall). One of the key challenges in neutrophil-dominated conditions is how to manipulate neutrophil function to abolish their destructive potential in a way that does not compromise their antibacterial and antifungal capacity. This has been difficult to achieve, as successful treatments in animal models have frequently proven ineffective or limited by side effects when used in human inflammatory diseases.

EOSINOPHILS, BASOPHILS, AND MAST CELLS

Eos, Bas, and MCs are critical effector cells not only in allergic inflammation, but also in innate and adaptive immunity. In addition, they have a crucial role in the surveillance of epithelial tissues, especially of the mucosa of the gastrointestinal, respiratory, and urogenital tracts: MCs as tissue resident sentinel cells, while Eos and Bas principally upon recruitment from the bloodstream. The microbicidal activity of these cells can be induced either after direct microorganism recognition or after activation by complement- or other leukocyte-derived products. Whereas neutrophils destroy internalized microorganisms by delivering cytotoxic intracellular compartments to them, Eos, Bas, and MCs are mainly involved in host defense against large parasites that cannot be internalized by other professional phagocytes. Eos can directly kill the parasites, whereas Bas and MCs preferentially release the contents of their granules into the external milieu upon activation, thus creating an environment hostile to invading organisms. Furthermore, the unique property of Bas and MCs to express the high-affinity Fc receptor for IgE (FcεRI) that, after activation by antigen-specific IgE, induces rapid discharge of potent inflammatory mediators (histamine being one of them), is central to the initiation and propagation of immediate hypersensitivity reactions. Coughing, sneezing, and vomiting, all expulsive responses that typically are caused by basophil- and MC-derived mediators and accompany allergic and pathologic inflammatory diseases, may actually reflect mechanisms that evolved to expel parasites. The properties and roles of Eos, Bas, and MCs in pathologic conditions such as allergy and autoimmunity are described in detail Chapter 45. Herein, we focus on the specialized properties that highlight their role in the innate and acquired immune response.

Eosinophils

Eosinophil Generalities

Eos are end-stage, multifunctional leukocytes involved in protection against parasitic helminths and bacterial and viral infections, that are also implicated in the pathogenesis of numerous Th2-type inflammatory processes and tissue injury.[41] These granulocytic, bilobed, nucleated cells were named as Eos by Paul Ehrlich in 1879, because of their intense staining with eosin, an acid aniline dye (Fig. 20.6). Eos are approximately 12 to 17 μm in diameter and represent 1% to 6 % of the total blood leukocyte population. Besides circulating and massively recruited at sites of Th2-dominated inflammation, Eos also reside in various organs, such as the gastrointestinal tract, mammary glands, and bone marrow.[41,42] Eos derive from bone marrow, CD34+ myeloid-committed progenitors, and upon maturation can be cytofluorimetrically identified by their characteristic morphology/side scatter and expression pattern of plasma membrane proteins such as CD66b, CCR3, IL-5Ra in con-

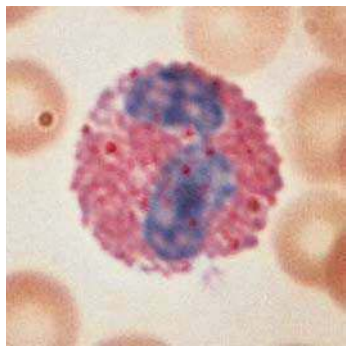

FIG. 20.6. A polymorphonuclear eosinophil circulating in peripheral blood. (Reprinted with permission from Cohen BJ, Wood DL. *Memmler's The Human Body in Health and Disease*. 9th Ed. Philadelphia: Lippincott Williams & Wilkins, 2000.)

junction with the lack of expression of CD16 (to better distinguish them from neutrophils), and other lineage markers (such as CD2, CD14, and CD19).[41–43] Eosinophil differentiation and expansion from progenitor cells mainly occurs in response to three cytokines, namely IL-3, IL-5, and GM-CSF, which provide permissive proliferative and differentiation signals through GATA-1, PU.1, C/EBPα, and C/EBPε transcription factors.[44] IL-5 is, however, the most specific factor for Eo development, differentiation, and release from the bone marrow into the peripheral circulation, especially during helminthic infection or allergic inflammation.[41–43] Also, allergen challenge or the experimental administration of CCL11/eotaxin-1 (acting through the CCR3 receptor) can cause bone marrow release of Eo precursors and mature Eos.[45,46] However, basal levels of Eo development can also occur in the absence of IL-5 as in antigen-induced tissue eosinophilia, as suggested by the presence of tissue Eos in asthmatic patients treated with anti–IL-5–neutralizing antibodies.[45,46] Once released from the bone marrow, Eos enter the circulation (with a half-life of 8 to 18 hours) and then traffic to tissues, at mucosal surfaces and/or sites of allergic inflammation, in response to a variety of different chemoattractants including CCL11, CCL24/eotaxin-2, CCL26/eotaxin-3, CCL5/RANTES, or LTB_4, LTD_2, PAF, and C5a. IL-4 and IL-13 play a central role in promoting Eo trafficking to mucosal tissue by upregulating eotaxins (CCL11 and CCL26) and endothelial expression of vascular cell adhesion molecule (VCAM)-1, the counterreceptor for VLA-4, which is expressed on the surface of Eos.[45,47] In addition, GM-CSF, IL-3, and IL-5, as well as IL-33 and IFNγ, are important for promoting long-term Eo survival and activation in vitro and in tissues.[41–43,46]

Eosinophil Granules

Human Eos have a bilobed nucleus with highly condensed chromatin and contain up to four different populations of secretory organelles: primary granules, secondary/specific/crystalloid granules, small amorphous granules (containing arylsulfatase and acid phosphatase), and secretory vesicles.[48] These four organelles, along with vesiculotubular structures and small vesicles involved in transport and secretion by the activated cell, serve as the major subcellular sites for the eosinophil armamentarium of preformed cytotoxic and inflammatory mediators.[48] Eos also contain lipid bodies, which are non–membrane-bound lipid-rich organelles that are the major site of eicosanoid synthesis as they contain eicosanoid synthetic enzymes including 5-lipoxygenase, leukotriene C_4 synthase, and cyclooxygenase.[41–43,48,49] Eos contain greater numbers of lipid bodies than neutrophils, which even increase during eosinophil activation in vitro or engagement in inflammatory reactions in vivo.[41–43,48,49]

Primary granules are similar to those found in other granulocyte lineages, are formed early in Eo development, and are enriched in Charcot–Leyden crystal protein (which represent less than 5% of total granules).[48,49] Charcot–Leyden crystal/galectin-10 is a hydrophobic protein of unknown function that is produced and released in high levels by activated Eos. Its characteristic hexagonal bipyramidal crystals can be detected as a hallmark of Eo involvement in host allergic and other immune responses, for instance in the stool or sputum of patients with gastrointestinal or respiratory eosinophilia.[48,49] Specific granules have a distinctive ultrastructural appearance with an electron-dense core and contain cationic proteins that give Eos their unique staining properties. The major cationic proteins in the specific granules are major basic protein (MBP, composed of two related cationic proteins, MBP-1 and MBP-2), Eo peroxidase (EPO), Eo cationic protein (the Eo's two ribonucleases RNase2 and RNase3, respectively), and Eo-derived neurotoxin (EDN). MBP is the major component of the crystalloid cores of specific granules and accounts for more than 50% of the Eo granule protein mass. MBP is highly cationic, it lacks enzymatic activity, and its toxicity is mediated by enhanced membrane permeability resulting from interactions of the cationic protein with the plasma membrane. MBP has in vitro activity toward parasites, including schistosomes and other helminths. In patients with asthma, serum and bronchoalveolar lavage fluid MBP concentration correlates with bronchial hyperresponsiveness. EDN and Eo cationic protein demonstrate in vitro toxicity to parasites and single-stranded ribonucleic acid pneumoviruses, including respiratory syncytial virus. EPO is another highly cationic protein that makes up approximately 25% of granule proteins and that catalyzes the oxidation of halides, pseudohalides, and nitric oxide to oxidant products that are toxic to microorganisms and host cells. It has been shown that, depending on the activation stimulus, Eo granule cationic proteins can be selectively secreted or released into tissues by a number of different secretory pathways, ranging from classical granule fusion and exocytosis (eg, in killing parasitic helminths), piecemeal degranulation (eg, vesicular transport from the granules in the absence of classical exocytosis), and cytolytic degranulation (release of intact membrane-bound granules directly into the tissue upon eosinophil apoptosis/cell death).[41–43,48,49] Studies in the past 15 to 20 years of the biochemistry, functions, and localization in tissues of the unique enzymatic and nonenzymatic cationic proteins present in Eo granules have provided compelling evidence supporting a pathologic proinflammatory and effector role for the Eo in directly inducing tissue damage.

Eosinophil Mediators

In addition to containing numerous highly basic and cytotoxic granule proteins that are released upon activation, Eos can also synthesize an arsenal of lipid mediators, ROS, and inflammatory/hematopoietic cytokines that mediate the pathophysiologic role of this granulocyte in health and disease (Table 20.3). Lipid-derived mediators generated by Eos include PGE_2, thromboxane, LTC_4, and PAF, which are all considered as responsible for many of the Eo-triggered proinflammatory activities, namely the increase in leukocyte trafficking, endothelial adhesion, smooth muscle contraction, vascular permeability, and mucus secretion.[41–43] Eo-derived cytokines (including TGFβ, GM-CSF, IL-3, IL-4, IL-5, CXCL8, IL-10, IL-12, IL-13, IL-16, IL-18, TNFα, CCL5, stem cell factor [SCF], NGF, and CCL11) are in part stored as preformed in granules and thus can be rapidly released upon degranulation (similarly to MCs).[41–43,50–52]

TABLE 20.3 Major Eosinophil-Derived Mediators

Class	Mediators	Physiologic Effects
Cationic granule proteins	MBP, ECP, EDN, EPO	Cytotoxic and bactericidal properties, smooth muscle cells contraction, mast cell activation and survival
Lipid mediators	LTD_4, LTE_4, PAF, PGE_1, PGE_2, 15-HETE	Leukocyte trafficking, endothelial adhesion, smooth muscle cells contraction, vascular permeability, inflammation
Cytokines	TNFα, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-11, IL-12, IL-13, IL-16, IL-17, IL-25	Inflammation, immunoregulation
Chemokines	CXCL1, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL5, CCL7, CCL11, CCL13	Leukocyte chemoattraction and tissue infiltration of leukocytes
Growth factors	HB-EGF, LBP, NGF, GM-CSF, TGFα, TGFβ, SCF, VEGF, APRIL	Growth of various cell types

ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; IFN, interferon; IL, interleukin; LTD, leukotriene D; LTE, leukotriene E; MBP, major basic protein; PAF, platelet-activating factor; PGE, prostaglandin E; TNF, tumor necrosis factor.

Interestingly,GM-CSF is among the cytokines produced in greatest quantities by Eos.[53]

Eos are functionally activated via an array of cell surface molecules, including Ig receptors for IgG (FcγRII/CD32) and IgA (FcαRI/CD89), receptors for complement-derived fragments (CR1/CD35, CR3, and CD88), cytokines (IL-3R, IL-5R, GM-CSF, as well as IL-1αR, IL-2R, IL-4R, IFNαR, and TNFRI), chemokines (CCR1 and CCR3), adhesion molecules (VLA4, α4β7), leukotrienes (CysLT1R and CysLT2R; LTB_4-R), prostaglandins (PGD_2 type 2R), PAF, and ligands for TLRs (TLR1, TLR4, TLR7, TLR9, and TLR10, the TLR7/8 representing an important mechanism for host defense against viral infections).[41–43]

Eosinophil Effector Functions

Eos have historically been considered as end-stage effector cells, causing damage to parasitic pathogens during helminthic infections or to host tissues in allergic diseases. However, as outlined in the following, accumulating evidence suggest that Eos can perform various immune regulatory functions, pointing to a more complex role of these cells not only in regulating inflammation and bridging innate and adaptive immunity, but also in maintaining epithelial barrier functions and affecting tissue remodeling.[41–43]

Eosinophils in Host Defense. The earliest recognized Eo effector functions were those associated to their role in host defense against multicellular helminthic parasites, although it is now clear that Eos are also involved in host defense against viral and bacterial infections.[54] Among the several mechanisms through which Eos can exert defensive functions, the best described consist in their release of cytotoxic cationic proteins (such as MBP, Eo cationic protein, EDN, and EPO), production of ROS, antibody- and complement-mediated killing, and the expulsion of extracellular deoxyribonucleic acid traps.[43,54] Despite substantial in vitro data, there is a lack of convincing evidence of the effective role of Eos in host defense in vivo, in particular during helminthic infections. Indeed, the only human Eo-specific condition, known as hereditary EPO deficiency, has not been related to increased susceptibility to helminthic infections. Furthermore, the results from infection studies carried out in mouse models remain unclear and controversial.[55,56] Considering that many of these experiments were performed using human pathogens, which do not naturally infect rodents, and that there are several phenotypic and functional differences between human and murine Eos, the relevance of these data should be reconsidered.[55,56] It is noteworthy to mention that, as described subsequently, some of the mechanisms used by Eos in host defense against pathogens may also produce detrimental effects on the host.[42,54]

Eosinophils in Immunoregulation. Accumulating evidence suggest that Eos can perform discrete immune regulatory functions mainly through production and release of cytokines and other immunomodulatory molecules, or via antigen presentation. For instance, Eos produce cytokines that are able to act on Eos themselves, the so-called autocrine cytokines, including IL-3 and GM-CSF, which function, in part, to prevent apoptosis and prolong Eo survival, once these cells are recruited into sites of tissue inflammation, such as the lung in asthma.[41–43] Eos can regulate their own recruitment, as well as that of DCs and T cells, through the secretion of CCL3, CCL5, and CCL11. Eo-derived cytokines may also influence the functions of other immune cells. For instance, Eos play an important role in the regulation of MC functions, including activation, differentiation, maturation, and survival, mainly via the release of SCF.[50–52] Eos have also been implicated in the regulation of B-cell function, not only under homeostatic conditions, but also during immunization responses with alum.[57] Eos seem indeed able to modulate early stages of B-cell activation and IgM production.[57] The best characterized role of Eos in immune regulation is their ability to initiate Th2-type responses by modulating DC and T-cell recruitment and activation. Eo-derived EDN can recruit and activate DCs and, in turn, skew them to a Th2-promoting phenotype.[58] In several murine models of parasitic infections and airway allergen challenge, Eos have been shown not only to precede Th2 cells arrivals in tissues, but also to be able to migrate to draining lymph nodes and polarize Th2 immune responses through the secretion of cytokines such as IL-4 and IL-25.[42,58] However, human Eos also express indoleamine 2,3-dioxygenase which, through kynurenine production, inhibits Th1 effector functions.[59]

Another important role of Eos in immune regulation is their function as antigen-presenting cells. Eos possess the capacity to internalize, process, and present antigenic peptides within the context of surface expressed MHC II, to provide costimulatory signals to T cells through surface expression of molecules such as CD80, CD86, and CD40, and to physically interact with CD4+ T cells.[60] In humans, although circulating Eos from healthy donors are generally devoid of surface MHC class II expression, they can be induced to express MHC class II and costimulatory molecules upon appropriate cytokine stimulation and after transmigration through endothelial cell monolayers.[42,60] Human Eos also constitutively express a Jagged1, a Notch ligand, suggesting a capacity of Eos to provide a polarization signal to naive CD4+ T cells.[61]

Eosinophil Homeostatic Functions. As mentioned, at baseline conditions Eos are present in tissues such as the gut, mammary gland, uterus, thymus, and bone marrow, via recruitment by the CCL11/CCR3 axis. Although their homeostatic role in all these tissues is not fully understood, Eos seem to be mainly involved in regulating the morphogenesis and maintenance of mucosal organs, as well as the immune homeostasis of the thymus and bone marrow.[41–43] Thymic Eos, for instance, are thought to be involved in the MHC class I–restricted negative selection of double positive thymocytes. In the bone marrow, instead, Eos colocalize with plasma cells, and by secreting a proliferation inducing ligand (APRIL) and IL-6, they play a crucial role in long-term maintenance and survival of the same plasma cells.[41,43,62]

Eosinophils in Tissue Remodeling. A growing body of evidence has proven the ability of Eos to influence tissue remodeling and fibrosis in many Eo-associated diseases, mainly due to their ability of secreting high levels of TGFβ. Human Eos also produce other profibrotic and angiogenic factors, such as osteopontin, VEGF, and MMPs.[50–52] Furthermore, Eos produce and release NGF and promote the extension of neurites in nerve cells.

Eosinophils in Diseases

Eos are currently thought to participate in the pathogenesis of the chronic phases of allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, and hypereosinophilic syndromes.[41–43] Allergic diseases are associated with a mild peripheral blood eosinophilia, although in tissues and in the nasal secretions, sputum, and BAL fluid, Eos can be more significantly elevated. Studies in murine models support a role for Eos in airway remodeling, airway hyperactivity, and mucous production. Elevation of peripheral blood Eos is also observed in drug reactions, helminthic infections, as well as in specific primary immunodeficiency diseases, most notably Omenn syndrome and hyper-IgE syndrome. Eosinopenia is typically seen in acute bacterial or viral infections and with systemic corticosteroid treatment. Finally, the use of Eo-specific antibodies has demonstrated that almost all human or mouse cancers are associated with an important Eo infiltrate at some point in tumor growth.[43]

Mast Cells and Basophils

Mast Cell Generalities

MCs were identified as granular cells in the mesentery of the frog by Dr. Von Recklinghausen in 1863 and were named "Mastzellen" by Dr Paul Ehrlich in 1878. Initial studies focused on their histologic characteristics, distribution, and abundance in health and disease. The discovery of histamine in 1910, slow-reacting substance of anaphylaxis (now leukotrienes) in 1938, and IgE in 1966 provided initial insights into the role of MCs in allergic reactions. MCs are tissue-dwelling inflammatory cells widely distributed throughout the body and are common at perivascular sites and at mucosal surfaces, particularly in those tissues that form interfaces with the external environment, such as skin, conjunctivae, and intestinal and airway mucosa.[63–65] Thus, MCs are strategically placed so as to function in a first line of host defense.[66,67] Morphologically, MCs in tissues appear as round, spindle-shaped, or spider-like cells, with round or oval nuclei, ranging between 7 and 20 μm in diameter, and readily identified using cationic dyes such as toluidine blue or methylene blue.[68] These dyes impart a blue-to-purple change in color, known as "metachromasia," which occurs as a result of their abundant intragranular content of sulfated proteoglycans (eg, heparin and chondroitin sulfates).[68] Of all other hematopoietic cells, only Bas share this staining feature with MCs, along with other properties (see Table 20.4

TABLE 20.4 Major Features of Mast Cells and Basophils

	Mast Cells	Basophils
Origin	Hematopoietic stem cells	Hematopoietic stem cells
Lifespan	Months	Days
Primary location	Tissues	Intravascular circulation
Site of maturation	Connective tissues	Bone marrow
Differentiation factors	Stem cell factor	Interleukin-3
Histamine content	1 to 15 pg/cell	1 to 2 pg/cell
Size	7 to 20 μm	5 to 10 μm
Nucleus	Oval or round	Segmented
Granules	Smaller and more numerous compared with basophils	Larger and fewer compared with mast cells
Peptidoglycans	Heparin and chondroitin sulfates	Predominantly chondroitin sulfates
Chymase content	High	Absent
Tryptase content	High	Low
Lipid mediators	PGD_2, LTB_4, LTC_4, LTD_4, LTE_4, PAF	LTC_4, LTD_4, LTE_4
Activation by		
Substance P	Activation	No effect
Morphine	Activation	No effect
fMLF	No effect	Activation

fMLF, formyl-Met-Leu-Phe; LTB,leukotriene B; LTC, leukotriene C; LTD, leukotriene D; LTE, leukotriene E; PAF, platelet-activating factor; PGD, prostaglandin D.

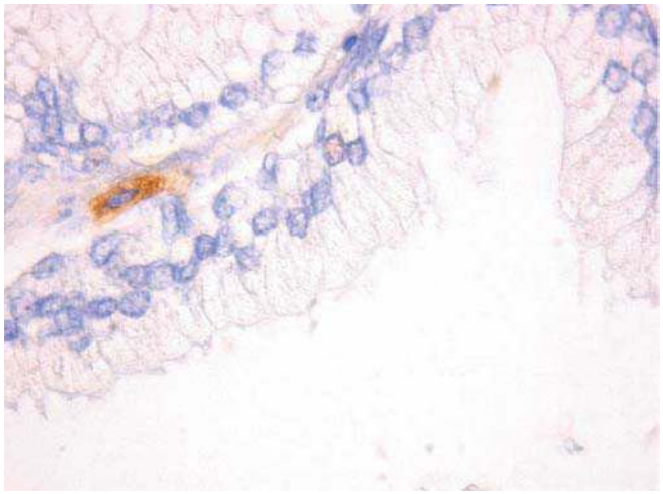

FIG. 20.7. Formalin-fixed, paraffin-embedded human prostate tissue stained with an anti–mast cell tryptase antibody.

for the major similarities and differences between MCs and Bas). MCs can be cytofluorimetrically identified by their characteristic expression pattern of plasma membrane proteins serving as markers, including FcεRI (as Bas and a subpopulation of DCs), CD23, CD117, and CD203, in conjunction with the lack of expression of lineage markers (such as CD2, CD14, and CD19).[69,70] Furthermore, MCs can be identified either by biochemical reagents that detect their intracellular proteases for their enzymatic properties or by immunostaining using antibodies toward tryptase (Fig. 20.7) or chymase (the latter representing the primary method of choice for identifying human MCs in tissues).[71]

Human MCs originate from CD34+/CD117+/CD13+ multipotent hematopoietic progenitors in bone marrow, and then migrate through blood to tissues where they mature.[72,73] Details of their differentiation and phenotypic diversification have not been definitively identified, but are likely included within the CD34+/KIT+ cell subpopulation and are clearly distinct from the basophil lineage (which is KIT/CD117– but IL-3R+).[74] In mice, a hematopoietic stem cell progresses to a multipotent progenitor, a common myeloid progenitor and a granulocyte/monocyte progenitor.[75,76] A monopotent MC progenitor is found in bone marrow and intestine, and a common basophil/MC progenitor is also found in mouse spleen, observations that await study in human. After their homing in the tissues, maturation of the MC precursors is dependent on SCF expressed on the surface of fibroblasts, stromal cells, and endothelial cells.[68,77] Accordingly, in vitro studies using mouse and human MCs confirm that soluble SCF protects MCs from apoptosis and induces their proliferation, chemotaxis, and also some degree of activation and secretion.[68] Consistently, mutant mice lacking KIT- or SCF-mediated functions are often used as experimental tools to implicate a biologic role of MCs in given models.[68] While there are several studies on the mechanisms of localization of MCs to different tissues, there is little information about specific molecules that could regulate MC-progenitor trafficking to tissues, movement, and incremental recruitment upon inflammation.[63,72] Accordingly, while a range of chemokines are active on human and mouse MCs in vitro, no MC-selective chemokine has been identified yet.

MC localization to small intestine is reliant on adhesive interactions controlled by $\alpha_4\beta_7$ integrin, VCAM-1, and mucosal addressin cell adhesion molecule-1 (MAdCAM-1).[63] CXCR2 ligands also play a critical role for the constitutive localization of MC progenitors to the intestine.[63,78] In contrast to the small intestine, MC progenitors are not abundant in normal lung. However, MCs are detected in the bronchial epithelium and airway smooth muscle, associated with pulmonary inflammation and abundant in human asthma.[79] VCAM-1 interactions with both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, but not mucosal addressin cell adhesion molecule-1, are essential for the trafficking of MC progenitors to the lung during antigen-induced pulmonary inflammation.[79] It is interesting to note that in a model of *Aspergillus fumigatus* extract–induced allergic pulmonary inflammation, IgE can influence the number and function of mature MCs, but not MC progenitor recruitment.[80]

On the basis of their location, histochemical staining, content of proteases, and reactivity to selected secretagogues and antiallergic drugs, two major subtypes of MCs have been described in rodents: mucosal-type MCs, which express MC protease-1 and -2; and connective tissue–type MCs, which are positive for MC protease-4, -5, -6, and carboxypeptidase A.[81] In humans, mucosal MCs preferentially express mouse MC protease-1 and -2, whereas connective tissue MCs express MC protease-4, -5, -6, and carboxypeptidase A.[81,82] Human MCs also exhibit heterogeneity and are thus classified by their content of serine proteases as tryptase-only MC, chymase-only MC, or both tryptase- and chymase-positive MC.[81,82] Both in humans and mice, these MC phenotypes are reversible in certain microenvironmental conditions, and transdifferentiation between the phenotypes has been shown.[81,82] Each MC subtype predominates in different locations: tryptase-only MC cells are prominent within the mucosa of the respiratory (nose, lung) and gastrointestinal tracts, and increase upon mucosal inflammation, whereas tryptase- and chymase-positive MC cells are prominent within connective tissues such as the dermis, submucosa of the gastrointestinal tract, heart, conjunctivae muscularis of the uterus, and perivascular tissues.[81] In light of murine studies highlighting a "fine tuning" on the MC functions and effector properties by cytokines or matrix proteins, it seems likely that human MCs will prove to be even more heterogeneous than currently thought. Accordingly, MC phenotype, behavior, and responsiveness may be dramatically altered by cytokines, including IL-4 (which upregulates the expression of FcεRI and is particularly pivotal in regulating functional responses of MCs in mucosal inflammation), IL-5 (which promotes MC proliferation in the presence of SCF), and IFNγ (which induces the expression of the high-affinity activating FcγRI/CD64 and also decreases MC numbers).[68] Furthermore, MCs express an array of adhesion and immune receptors that may assist in the recognition of invading pathogens and in the sampling of different stimuli coming from the microenvironment.[67] This versatility is reflected in the numerous

TABLE 20.5 Major Basophil and/or Mast Cell–Derived Mediators

Class	Mediators	Physiologic Effects
Preformed mediators	Histamine	Vasodilation, angiogenesis, mitogenesis, suppressor of T-cell activation
	5-HT	Leukocyte regulation, vasoconstriction, pain
	Chymase	Tissue damage, pain, angiotensin II synthesis
	Tryptase	Activation of PAR, inflammation, pain, tissue damage, degradation of antigens and peptides
	kininogenases	Synthesis of kinins, pain
	Nitric oxide synthase	Nitric oxide production
	Carboxypeptidase A	Degrades enzymes
	CRH	Inflammation, vasodilation, mast-cell VEGF release
	Endothelin	Sepsis
	Kinins	Inflammation, pain, vasodilation, mast cell trigger
	Somatostatin	Anti-inflammatory effects, mast cell trigger
	VEGF	Neovascularization, vasodilation
	Chondroitin sulfate	Connective tissue component, anti-inflammatory, mast cell inhibitor
	Heparin	Angiogenesis, NGF stabilization, mast cell inhibitor
Lipid mediators	LTB_4, LTC_4, PAF, PGD_2	Leukocyte chemotaxis, vasoconstriction, pain, platelet activation, vasodilation, inflammation, bronchoconstriction
Cytokines	TNFα, TGFβ, IFNα, IFNβ, IFNγ, IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25, SCF, MIF	Inflammation, leucocyte migration/proliferation
Chemokines	CXCL8, CCL3, CCL2, CCL7, CCL13, CCL5, CCL11, CCL19	Chemoattraction and tissue infiltration of leukocytes
Growth factors	CSF, GM-CSF, bFGF, VEGF, NGF, LIF	Growth of various cell types
Antimicrobic products	Nitric oxide, superoxide, antimicrobial peptides	Microbial killing

bFGF, fibroblast growth factor; CRH, corticotropin-releasing hormone; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; LTB, leukotriene B; LTC, leukotriene C; MIF, migration inhibitory factor; NGF, nerve growth factor; PAF, platelet-activating factor; PAR, proteinase activated receptor; PGD, prostaglandin D; SCF; stem cell factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

IgE-dependent and -independent activation pathways that modulate the quality and magnitude of MC responses and cytokine release. MCs are endowed with a range of potent proinflammatory effector molecules that may be selectively released not only through FcεR1 activation, but also in response to a variety of other receptors including C3aR and C5aR, FcγRIIa/CD32a, KIT/CD117/SCF-R, IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, GM-CSFR, IFNγR, CCR3, CCR5, CXCR2, CXCR4, NGFR/TRKA, the type 1 receptor for cys-LTs (CysLT1R), the adenosineA3 receptor, and many TLRs, among others.[68] Depending on the type, property, strength, and combination of the stimuli that they receive, MCs secrete a diverse and wide range of biologically active products (Table 20.5) that can trigger, direct, or suppress the immune response.[68,83,84]

Basophil Generalities

Bas are the rarest of the granulocytes (typically constituting 0.5% to 1.5% of peripheral blood leukocytes), are 5 to 10 μm in diameter, and exhibit a bean-shaped or bilobed condensed nucleus.[85] Compared to MCs, they have little proliferative capacity and possess fewer, but larger (up to 1.2 μm) round metachromatic granules (Fig. 20.8) enriched in histamine (stored at about 1 to 2 pg per cell).[86,87] Bas develop from CD34+ progenitors, differentiate and mature in the bone marrow in response to IL-3, and circulate in the periphery with a half-life thought to be of a few days.[85,88] Although not predominantly tissue-dwelling cells, Bas are able to infiltrate inflamed tissues, particularly at sites of allergic inflammation within several hours after exposure to allergens, and are often accompanied by a simultaneous influx of eosinophilic granulocytes and Th2 lymphocytes.[82,85,89] Bas can be cytofluorimetrically identified by their selective pattern of plasma membrane proteins serving as markers, including FcεRI (like MCs), CD123 (the α chain of the IL-3 receptor), CD11b, and CD13, in conjunction with the absence of other lineage markers (such as CD2, CD14, CD16, CD19, and MHC class II).[89] They also express, and dynamically respond to, ligands for a variety of functional cytokine/chemokine receptors (eg, IL-5R, GM-CSFR, CCR2, and CCR3), complement receptors (CD11b, CD11c, CD35, and CD88), prostaglandin receptors (CRTH2), Ig Fc receptors (FcεRI and FcγRIIb), and TLRs. For example, following FcεRI activation (which typically triggers granule exocytosis and mediator release), a number of basophil cell surface markers, including CD13, CD63, CD107a, CD164, and CD203C, may increase.[85,89,90]

Mast Cell and Basophil Mediators

The mediators produced by MCs and Bas are schematically divided into preformed mediators, newly synthesized lipid mediators, and cytokines/chemokines (see Table 20.5).[82,86,89]

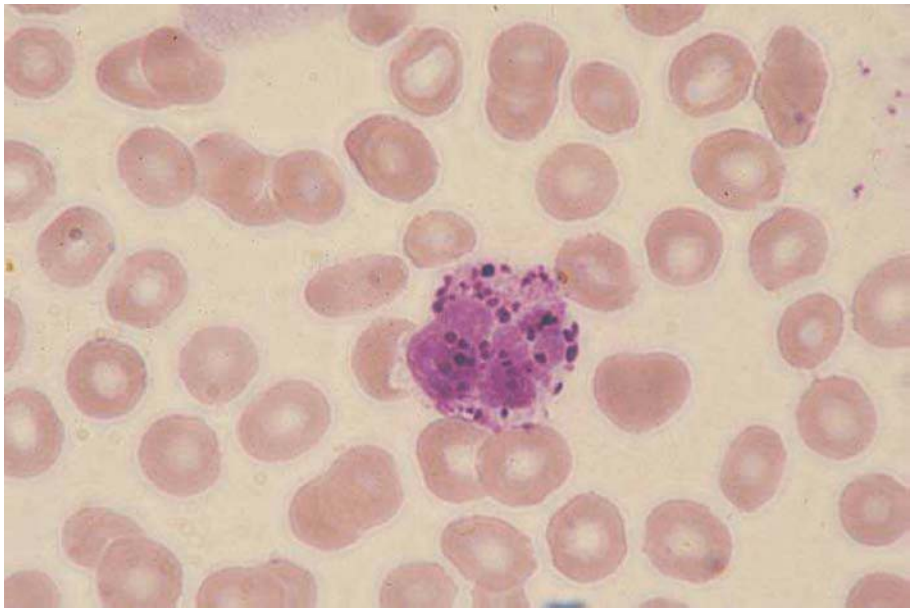

FIG. 20.8. A Polymorphonuclear Basophil Circulating in Peripheral Blood. From *Anderson's Atlas of Hematology,* Anderson, Shauna C., PhD. Copyright 2003, Wolters Kluwer Health/Lippincott Williams & Wilkins.

Such subdivision is not exclusive as TNFα, for instance, occurs as both preformed and as a newly synthesized molecule.[91] Preformed mediators, including histamine or MC neutral proteases (tryptase, chymase, cathepsin G, and carboxypeptidase) are stored in cytoplasmic granules and form complexes with the negatively charged, very abundantly expressed, sulfated proteoglycans.[82,92] Upon MC/Ba activation, the granules fuse with the plasma membrane and, within minutes, release their content into the extracellular environment where the various mediators dissociate from proteoglycans.[68,85] Other than being secreted upon stimulation, some molecules are also constitutively released, as in the case of MC tryptase. Consequently, tryptase levels are considered as a parameter that reflects the MC burden, and, in fact, they significantly increase in systemic mastocytosis.[82,92] Curiously, the function of tryptase in vivo is still unknown, even though, in vitro, it digests fibrinogen, fibronectin, prourokinase, proMMP-3, protease-activated receptor-2, and complement component C3; it activates fibroblasts as well. The functions of MC/Ba-derived proteoglycans and proteases are less well understood, but proteases provide some protection against snake and insect venoms.[82,92] Differently from MCs, humans Bas do not express chymase, appear to contain very low levels of heparin and tryptase (with some exceptions), but contain MBP and Charcot–Leyden crystal (which are typical eosinophil products). Activated Bas have been shown to also contain granzyme B and to directly kill target cells, at least in part by this cytotoxic serine protease.[82,90,93] More importantly, Bas, together with neutrophils, seem to be the exclusive peripheral blood source of histamine.

Newly synthesized lipid mediators by FcεRI-activated MCs are PAF, PGD_2, and LTA_4. The latter is then converted to LTB_4 or conjugated with glutathione to form LTC_4 (the parent compound to the cysteinyl leukotrienes, which also include LTD_4 and LTE_4).[86,92] Activated Bas can produce the same lipid mediators but PGD_2 (as they lack the PGD_2 synthase).[82,86,93] PGD_2 functions as bronchoconstrictor and attracts both Eos and Bas, LTB_4 attract neutrophils and effector T-cells, while cysteinyl leukotrienes attract Eos and work as potent bronchoconstrictors, in addition to promote vascular permeability and to induce mucus production.

MCs produce several cytokines, including IL-3, IL-4, GM-CSF, IL-5, IL-6, IL-10, and IL-13, growth factors, including bFGF, SCF, VEGF, and several chemokines, including CXCL8 and CCL3.[82,86,92] In addition, MCs are considered the only cells storing very abundant levels of preformed TNFα, whose critical role in host defense against bacterial infections has been widely highlighted: in fact, specific experimental animal models lacking MC-derived TNFα result in drastically reduced neutrophil influx and significantly increased mortality.[91] Bas are a major source of GM-CSF and VEGF, and release significant amount of chemokines (CXCL8, CCL3, CCL11) in response to IgE cross-linking. Chief among the cytokines produced by Bas are IL-4 and IL-13, which are released in response to FcεRI or C5a stimulation within the range of 20 to more than 600 pg/million and < 20 to 2000 pg/million cells, respectively.[82,86,93]

Mast Cell and Basophil Effector Functions

MCs and Bas, although derived from distinct progenitors, have been recognized to express partially overlapping functions in many aspects of natural and acquired immunity.[86,87] Traditionally, MCs and Bas, together with Eos, are considered essential components of IgE-mediated classic type 1 hypersensitivity (allergic) reactions and allergic inflammation (see Chapter 45). Nonetheless, MCs and Bas have also the ability to release and synthesize highly bioactive, proinflammatory, and cytotoxic substances, independent of signaling through FcεRI. Therefore, these cells, besides

being important modulators of allergic reaction, can also contribute to leucocyte recruitment, stromal and tissue cell activation, modulation of immune reactions, tissue remodelling, and angiogenesis.[84,89,90,94]

Mast Cells and Basophils in Allergy. Allergen-specific IgE production, with subsequent fixation of IgE to FcεRI receptors on MCs and Bas, followed by rapid degranulation and release of histamine and specific eicosanoids (eg, LTC_4), is central to the initiation and propagation of immediate hypersensitivity reactions.[65,82] It has been widely accepted that MCs contribute significantly to acute inflammatory reactions to antigens/allergens toward which the host bears antibodies of the IgE class.[95] MCs are responsible for virtually all of the increased vascular permeability and tissue swelling early in the IgE-dependent passive cutaneous anaphylactic response.[95,96] If the stimulation is of more persistent or of more severe nature, acute response may undergo transition into a late-phase reaction, which, except for the time scale ranging from a few to several hours from initial antigen challenge, is characterized by new recruitment of leukocytes (Eos, Bas, and Th2 lymphocytes) to the site of inflammation.[65] Bas play a crucial role in sustaining allergic chronic inflammation upon their entering the affected tissue, not only due to the release of stored and newly synthesized mediators, such as histamine and LTC_4, but also via the release of high amounts of the cytokines IL-4 and IL-13.[89,90,97]

Mast Cells and Basophils in Innate Immunity and Host Defense. Recent studies have demonstrated that MCs express many PRRs, including TLRs 1 to 10 and nucleotide oligomerization domain–like Receptors (NLR) (including nucleotide oligomerization domainreceptors and the NLRP3). Expression of various PRRs thus permits MCs to respond to both pathogen-associated molecular and "danger signals" resulting from cell stress or injury. Several MC functions triggered by PRR have been implicated in host defense.[84,94,98] These latter include enhancement of the recruitment or function of granulocytes, phagocytosis-dependent bactericidal activities, and proteolytic degradation of endogenous mediators, which would otherwise be elevated to toxic levels, such as endothelin-1 and neurotensin. Secretion of proteases/enzymes and formation of extracellular traps that contain antimicrobial peptides, histone, deoxyribonucleic acid, and tryptase have also been proposed as potential mechanisms by which MCs exert protective functions.[94,98] In addition to their role in bacterial infections, MCs can promote host resistance to certain parasite and virus infections. However, the mechanisms involved in these functions have not been fully elucidated.[94,98] Differently from MCs, the role of Bas in host defense is less studied, especially in bacterial or viral infections. Some evidence for a role of Bas in host defense against parasitic infections and tick infestations has been reported, but the molecular mechanisms underlying the Ba-mediated protection in these processes remain to be determined.[89,99,100]

Mast Cells and Basophils in Immunoregulation. MCs and Bas manifest immunoregulatory functions in IgE-dependent and -independent inflammatory reactions, mainly by secreting soluble mediators and cytokines or by functioning as antigen-presenting cells. These cells contribute to the initiation of acquired immunity by orchestrating DC migration, maturation, and function and by interacting with T and B cells.[84,89,90,94] MCs have the potential to influence DC functions, mainly through the release of histamine, PGD_2, PGE_2, LTB_4, or cytokines such as TNF, IL-1, IL-16, IL-18, and CCL5. Generally, both in vitro and in vivo evidence indicate that MCs predominantly inhibit IL-12p70 production and induce a Th2-promoting phenotype in DCs.[94,101] MCs can also modulate B-lymphocyte functions by supporting their survival, proliferation, and IgA production, mainly through the expression of CD40L and IL-6.[94,102] Evidence of cell-contact interactions between MCs and B cells in vivo have been found to occur in secondary lymphoid organs and inflamed tissues.[94,103]

MC surface molecules and secreted products can also influence various aspects of the biology of T cells, for example, by polarizing naive T cells to Th1, Th2, Th17, and regulatory T (T_{reg}) cells or by modulating the functions of distinct T-cell subsets.[94,101] Interestingly, MCs through IL-6 secretion and via OX40/OX40L contact have recently been shown to counteract T_{reg}-mediated suppression, thus leading to the establishment of Th17-mediated inflammatory responses.[104] MCs can also induce antigen-specific T-cell proliferation and activation (of both CD8+ and CD4+ T cells) due to their expression of MHC class I and II molecules, of costimulatory molecules of the B7 family (such as ICOS-L, PD-L1, CD80, and CD86), and of members of the TNF/TNF receptor families (such as OXOL and CD40L).[84,94,101] Finally, MCs produce several mediators with anti-inflammatory activities, including TGFβ, IL-4, IL-10, and IL-9, and have been shown to play a crucial role in transplantation engraftment, for instance toward allogeneic skin graft.[94,101,105] Most likely, MCs manifest predominantly proinflammatory effects in the earlier phase of the immune response, while the anti-inflammatory effects of these cells are more pronounced at the later phase of the response to limit ongoing inflammation.[105]

Bas have the ability to regulate the acquired immune response, mainly by potentiating humoral responses and promoting Th2 polarization.[86,89] Bas can enhance B-cell survival and proliferation, and Ig production mainly through the release of IL-4, IL-6, BAFF, and APRIL.[89,106,107] The ability of Bas to polarize Th2 immune responses is dependent on their function as antigen-presenting cells and on IL-4 secretion. These Th2-promoting functions of Bas have been demonstrated in responses to protease allergens, helminthic parasites, or antigen-IgE complex in vivo.[108–110] Recently, the Th2-promoting functions of Bas have been linked to the development of systemic lupus erythematosus in mice deficient of the Src family protein tyrosin kinase Lyn, and in human patients.[111]

Mast Cells and Basophils in Disease

The most striking increase in MCs occurs during parasitic diseases and mastocytosis (the latter being a pathologic excess of MCs, most notably in the skin, bone marrow, gastrointestinal tract, spleen, liver, and lymph nodes, that is

usually caused by gain-of-function mutations of KIT).[68,112] The importance of MCs in IgE-allergic reactions (including rhinitis, urticaria, and asthma) is emphasized by the increased numbers of these cells seen in affected tissues in both mice and humans.[68,95] MC hyperplasia and increased MC products at sites of tissue injury have also been observed in chronic inflammatory and autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis. A pathologic role of MCs in these diseases has been suggested mainly by the utilization of the K/BxN mouse model of antibody-induced arthritis and the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis.[94,112] Moreover, studies in mice indicate that activation of MCs via the NLRP3 can contribute to IL-1β overproduction and chronic urticarial rash in subjects with cryopyrin-associated periodic syndrome, a disorder associated with NLRP3 mutations.[113] There is evidence for MCs in promoting, but also in protecting against, tumor growth. The accumulation of MCs at the periphery of tumors has been observed in both rodent models and in a diverse array of tumors in humans. It is generally thought that MCs promote early angiogenesis events in tumor development through the release of mediators such as VEGF, CXCL8, angiopoietin-1, bFGF, heparin, and proteases.[114] Finally, MCs could also contribute to the pathogenesis of acute or chronic vascular events: accordingly, acute myocardial infarctions show elevations of both histamine and mature β-tryptase, suggesting that MC activation occurs concomitantly with ischemia.[94,112]

As far as Bas, it has remained obscure whether these cells actually play a crucial role or are just redundant with MCs in allergic reactions in vivo.[89,97] Human Bas have been shown to release IL-1β and TNFα upon the crosslinking of surface-bound IgD, but not IgE, suggesting a possible role of these cells in autoinflammatory disorders such as hyper-IgD syndrome.[115] Bas may also contribute to the production of autoantibodies that cause lupus nephritis.[116]

CONCLUSION

In the last few years, our perception of granulocytes and MCs has changed dramatically. In fact, there has been mounting evidence that the function of these cells is not limited to acting as first line of defense against invading pathogens, but is extended to perform additional and unexpected activities in strict collaboration with adaptive immune and other non-immune cells. Thus, cells of innate and adaptive immunity together orchestrate complex functional programs to promote host defense, control the development of self-tolerance, and avoid autoimmunity. In this context, the gene expression pattern and phenotype of the cells discussed in this chapter must rapidly change in a coordinate, time-dependent manner in response to microenvironmental soluble and cellular signals.[117] Granulocytes and MCs, in view of their wide variety of membrane receptor are able to mediate delivery of costimulatory signals, their broad array of molecules involved in cell–extracellular-matrix adhesion and in cell–cell contacts, their extensive assortment of soluble pro- and anti-inflamatory mediators, may profoundly influence the development, intensity, and duration of adaptive immune responses that ultimately serve for host defense, allergy, and autoimmunity. A representative example of this aptitude is the plasticity that MCs manifest as a result of their reciprocal interactions with the T-cell populations exerting regulatory or activatory functions in normal and/or pathologic immune responses.[101,105] In this context, it has been demonstrated that CD4+CD25+ T_{reg} cells establish a cell-cell contact with MCs through the OX40:OX40L axis, which regulates the MC degranulation threshold and contributes to tissue tolerance.[104,118] However, in an inflammatory environment, this MCs/CD4+CD25+ T_{reg} cell interaction induces a Th17 switch by T_{reg} cells.[104,118] On the same line, it is remarkable that, under particular settings, granulocytes and MCs display functions that in the past were ascribed only to immune adaptive cells. For instance, it has been recently demonstrated that neutrophils, among their various immunoregulatory functions,[23] can also display a B-cell–helper function that promotes Ig class switching, Ig somatic hypermutation, and antibody production by activating marginal zone B cells through BAFF, APRIL, and IL-21 secretion.[119] Similarly to neutrophils, MCs are also able to provide costimulatory signals that sustain B-cell expansion and drive the development of IgA-oriented humoral immune responses,[102] thus ascribing a B-cell–helper function that in the past was retained exclusive of T cells.

Novel activities have been described for myeloid cells also in the context of host defense, such as for instance in the case of Bas that, during primary and secondary exposure to parasites, might display different roles.[93,120] Indeed, in a murine model of primary helminthes infection,[121] Bas often accumulate in affected tissues where they produce large quantities of Th2 cytokines that not only regulate the eosinophil recruitment to the lung and the IgE production, but also favor mucosal MC infiltration.[121] However, their major defensive role occurs during the secondary infection, in which worm expulsion is more rapid (by 5 days) than in the primary one (by 10 days postinfection), as revealed by Ba depletion studies that cause an impaired worm expulsion by mice, regardless of the presence or the absence of MCs.[122]

In view of the continuously emerging findings in the field, it is predictable that in the next years there will be the discovery of additional, unsuspected biologic features that granulocytes and MCs possess.

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CHAPTER 21

The Major Histocompatibility Complex and Its Proteins

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INTRODUCTION

The extended family of glycoproteins known as major histocompatibility complex (MHC) and MHC-like molecules is now recognized to comprise a class of receptors, usually expressed at the surface of somatic cells of vertebrates, which confer a wide range of functions in the regulation of the immune response. Their cumbersome moniker, MHC, derives from the early determination that the genes that encode these molecules control one of the fundamental manifestations of an immune response, skin graft rejection, but contemporary immunologists, virologists, and tumor biologists rarely think about these molecules with allogeneic (ie, genetically distinct) skin transplantation in mind. The name MHC is a general label, and specific immunologic functions or molecular structures invoked when a particular MHC is mentioned are dependent on biologic context. In recent years, our understanding of the structure, evolution, and molecular basis of MHC-based interactions has blossomed, and our ability to exploit engineered forms of these molecules has also expanded enormously. Our blueprint for this chapter is to give an overview of MHC-regulated immunologic function and then to summarize the explosive growth in understanding of the molecular structures of MHC molecules and their ligands. Of necessity, we discuss briefly some aspects of the cell biology and cellular maturation of MHC molecules but leave the detailed description of these essential aspects of the classic MHC molecules to the chapter on antigen presentation. Basic aspects of the genetics of MHC molecules not only descriptions of the genetic loci that encode them but also discussion of what we know of the evolution of their encoding genes will be raised.

Functional glycoproteins are molecular machines and their cellular and immunologic activities depend on the shape of these macromolecules, their surface charge and ability to interact with solvent, as well as the flexibility and relationship of their structured domains and unstructured regions. To understand molecules of the MHC in a rational functional context, we consider it crucial to describe their overall structures as well as their structural interactions with bound ligands and their binding to their cognate receptors found on T cells and natural killer (NK) cells. Despite a large and ever increasing database of MHC and MHC-like structures, a number of general questions remain unanswered. We will present some of the unsolved mysteries of the evolution and function of the MHC-like molecules and their viral homologs.

The importance of MHC molecules is underscored by two general characteristics: the enormous degree of polymorphism of genes of the *Mhc* and the identification of a large number of human diseases that are profoundly influenced by either defects of MHC expression or by polymorphic variants. Every student of immunology must be acquainted with the basic biology of MHC-encoded molecules, and the student's comprehension of the regulation of the immune response, encompassing the inflammatory, NK, T-cell, and B-cell responses, will be lacking unless the function and ongoing evolution of the MHC is understood.

We stress that the evolution of the MHC is *ongoing* because for no other set of genetic markers is there such a large database cataloguing genetic polymorphism; as a result, it is apparent that not only are new alleles being identified, but that many of these new alleles have arisen recently. Dramatic examples of the ongoing evolution of MHC molecules are seen not only in new variants of the deoxyribonucleic acid (DNA) sequences observed by those that perform the necessary typing of *Mhc* genotypes for transplantation but also in surveys of the sequences of viruses, particularly the cytomegaloviruses (CMVs), which appear to have purloined host *Mhc* genes for immunoevasion. In sequence gazing, we observe the interplay between host and pathogen, as the pathogenic viruses, exploiting their rapid generation times, develop new variations on the MHC theme for distinct purposes.

The prototypes of MHC molecules are the MHC class I (MHC-I) and class II (MHC-II) molecules, obligate cell-surface heterodimers that bind and display self- or foreign peptides at the cell surface so that T-cell receptors (TCRs) or NK cell receptors can interact with the molecular complexes in an MHC- and peptide-dependent manner.

MHC molecules are crucial for both TCR- and NK-mediated interactions. Our efforts will be to preserve some sense of the historical development of this exciting field of study and also to focus on paradigmatic genetic, structural, and functional features that unify this extensive gene/protein family. Finally, the *Mhc* provides a genetic link from immune responsiveness to autoimmune disease—those well-known strong associations of particular *Mhc* genes to particular human diseases—and we will provide an outline of the molecular basis for such associations.

Mhc Nomenclature—Dialects of *Mhc*

The names of the genes and proteins that are critical to understanding the *Mhc* reflect the historical discovery of their functions and differ among different species. Often confusing to students of immunology, the nomenclature of

the *Mhc* differs for different species and must deal not only with a number of distinct, but usually linked, genetic loci but also with their encoded molecules, many of which are heterodimeric glycoproteins. Because the first genes of the *Mhc* identified were those that encoded cell surface molecules that could be detected by antibodies or by transplantation responses, these are the ones that are referred to as *Mhc* genes. Now we know of more than 400 genes that map to the human or mouse *Mhc*, although technically they are all "*Mhc*" genes, the "MHC" molecules refer specifically to the MHC-I or MHC-II molecules that are related in structure and function. (Genes for several complement components and related molecules also map here and are occasionally referred to as MHC class III.) Other *Mhc*-encoded molecules with distinct structure and function are usually referred to by their specific names. Molecules that exhibit structural similarity with MHC-I molecules, whether they map to the *Mhc* or not, may be called as a group "MHC-Ib" to distinguish them from the "classical" MHC-I molecules, also referred to as "MHC-Ia." Another subset of molecules that exhibit the MHC-I fold but that are expressed by viruses may be called "MHC-Iv" molecules.[1,2]

By convention, genetic loci are indicated by designations in italics, and the encoded protein products or phenotypic descriptions are shown in a standard font. The extended genetic region is referred to as the complex; thus the general term used for all species is the *Mhc* or *MHC*. The mouse *Mhc* is referred to as *H2* (previously called *H-2)* because it was the second genetic locus involved in control of expression of erythrocyte antigens identified by Gorer.[3,4] The *Mhc* in the rat is known as *RT1*, the human locus is known as *HLA* (for human leukocyte antigen), *DLA* for the dog, *GPLA* for the guinea pig, *SLA* for the swine, and *RLA* for the rabbit. For other species, based on a suggestion by Klein,[5] the taxonomic name forms the basis for the designation, contributing the first two letters of the genus and the first two of the species to name the locus. Thus, we have *Patr* for the chimpanzee, *Pan troglodytes*; *Gogo* for the Gorilla, *Gorilla gorilla*; *Mamu* for the Rhesus macaque, *Macaca mulatta*; *Mane* for the pig-tailed macaque, *Macaca nemestrina, Mafa* for the cynomolgous monkey, *Macaca fascicularis*; and *Papa* for the Bonobo, *Pan paniscus*. A single site for curated sequences of many species of MHC molecules is the IPD-MHC Database at www.ebi.ac.uk/ipd/mhc/.[6] The most important and widely studied models are those of the human, the mouse, and the rat. For the mouse and rat *Mhc*, the search engine on the Jackson Laboratory Web site (www.informatics.jax.org/mgihome/nomen) is the most comprehensive.

The naming of new HLA genes and alleles and their quality control are governed by the World Health Organization Nomenclature Committee for Factors of the HLA System. Software conversion tools to assist in gene/protein identification are to be found at the IMGT/HLA database (www.ebi.ac.uk/imgt/hla/dictionary.html). Regular updates on HLA nomenclature featuring new alleles can be found in the journal *Tissue Antigens* and via http://hla.alleles.org/nomenclature/nomenc_updates.html. The standardization of serologically defined HLA antigens has been achieved by the exchange of typing reagents and cells in the International Histocompatibility Workshops. The *HLA Dictionary* is a summary of HLA-A, HLA-B, HLA-C, HLA-DRB1/3/4/5, and HLA-DQB1 alleles and their association with serologically defined HLA-A, -B, -C, -DR, and -DQ antigens, respectively, available free online at the IMGT/HLA database site.[7] As defined by the Nomenclature Committee (http://hla.alleles.org/nomenclature/naming.html), each HLA allele name has a unique number corresponding to up to four sets of digits separated by colons (Table 21.1 and Fig. 21.1). The digits before the first colon describe the type, which often corresponds to the serologic antigen carried by an allotype (a genetically distinguishable form of the molecule within the same species). The next set of digits lists the subtypes, numbers being assigned in the order in which DNA sequences have been determined. Longer names are assigned only when necessary. Precise designation of human genes is by a nomenclature including a number following the locus (eg, HLA-A*01:01 and HLA-DRB1*01:01). Summaries of the hundreds of human alleles that have been identified are in Tables 21.2 and 21.3. Comprehensive databases of human *Mhc* genes are maintained at the IMGT/HLA database (see previous link)[8,9] where, at the time of this writing, more than 7000 HLA class I and class II alleles have been tabulated.

TABLE 21.1 Nomenclature of HLA Loci and Alleles

Nomenclature	Definition
HLA	The HLA region and prefix for HLA gene
HLA-DRB1	Particular HLA genetic locus
*HLA-DRB1*13*	Group of alleles encoding DR13 antigen or with sequence similarity to other DRB1*13 alleles
*HLA-DRB1*13:01*	A specific HLA allele
*HLA-DRB1*13:01:02*	An allele that differs by a synonymous mutation from DRB1*13:01:01 (ie, DNA sequence difference but no amino acid sequence difference)
*HLA-DRB1*13:01:01:02*	An allele that contains a mutation outside the coding region distinguishing it from DRB1*13:01:02
*HLA-A*24:09N*	A "null," unexpressed allele
*HLA-A*30:14L*	An allele encoding reduced or "low" cell surface expression
*HLA-A*24:02:01:02L*	An allele encoding a reduced or "low" cell surface expression, where the mutation is outside the coding region
*HLA-B*44:02:01:02S*	An allele encoding a protein that is expressed as a "secreted" molecule only
*HLA-A*32:11Q*	An allele for which a previous effect on surface expression has not been confirmed and is considered "questionable"

DNA, deoxyribonucleic acid; HLA, human leukocyte antigen.
Source: Adapted from the designations summarized by the *WHO Nomenclature Committee For Factors of the HLA System,* Stephen G.E. Marsh, http://hla.alleles.org. Also available at the IMGT website: www.ebi.ac.uk/imgt/hla/nomenclature/index.html.

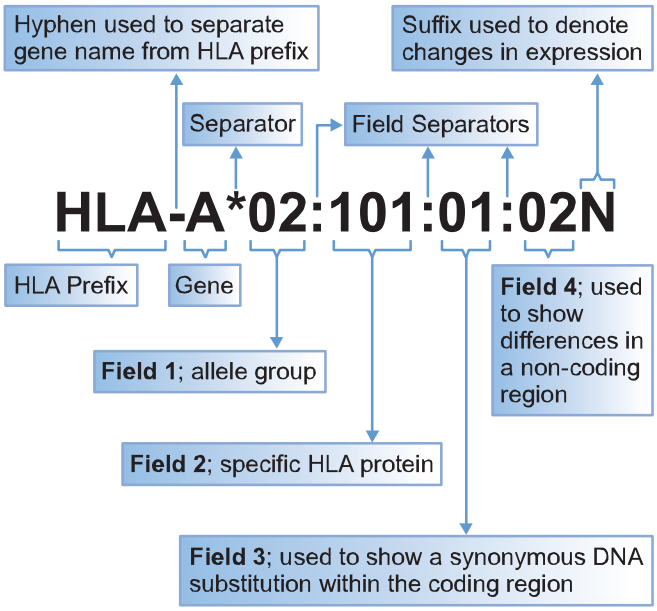

FIG. 21.1. Summary of Current HLA Gene Nomenclature. This illustration explains the nomenclature for HLA alleles as described in http://hla.alleles.org/nomenclature/naming.html.[164]

The commonly used term "haplotype" refers to the linkage of particular alleles at distinct loci that occur as a group on a parental chromosome.[10] The concept of haplotype is important in typing the *HLA* loci in the human where the linked *Mhc* genes (those genes in "linkage disequilibrium") of one chromosome of one parent will generally segregate as a group to the children. Individual haplotypes of the *Mhc* in the mouse are referred to by a lowercase letter superscript as $H2^b$, $H2^d$, or $H2^k$. Thus, the $H2^k$ haplotype refers to the full set of linked genes, $H2\text{-}K^k$, $H2\text{-}IA^k$, $H2\text{-}IE^k$, $H2\text{-}D^k$, and extends to the genes of the *Q* and *T* regions as well.[11,12] (Some haplotype designations, such as $H2^a$, refer to natural recombinants and thus have some of the linked genes from one haplotype and some from another.) A complication that demands the precise use of gene and encoded protein names is that the number of genes in a particular homologous genetic region may differ between strains or between individuals. In the mouse, although some strains have only a single gene at the *D* locus ($H2\text{-}D^b$ for instance), other strains may have as many as five genes in homologous region ($H2\text{-}D^d$, $H2\text{-}D2^d$, $H2\text{-}D3^d$, $H2\text{-}D4^d$, and $H2\text{-}L^d$).[13,14]

Table 21.4 summarizes the haplotypes of common mouse strains. Included in this listing are a number of congenic inbred mouse strains, strains that contain the *Mhc* derived from one strain, and the remaining background genes from another. A regularly updated and comprehensive listing can be obtained at http://jaxmice.jax.org/literature/catalog/mhc_h2_haplotypes.pdf. An ongoing project, the "Collaborative Cross," aims to establish a panel of mouse recombinant inbred lines derived from eight diverse founder mouse strains as a resource for mammalian genetics. These lines, when their breeding is completed, will provide an opportunity for mapping a wide variety of quantitative trait loci with respect to measurable phenotypes and can be expected to play an important role in studies of immune recognition.[15]

The Immunologic Function of MHC Molecules

MHC molecules are a molecular reflection of the health status of either the cell that synthesizes them (for MHC-I molecules) or of the local environment in which the cell resides (for MHC-II). The structure of the MHC molecule depends not only on the amino acid sequence of the two polypeptide chains (α and β for MHC-II; heavy [or α] and β2m [for the light chain] for MHC-I) that form the core of the complex but also of the variable bound peptide that forms an integral part of the trimer. The MHC molecule, governed by the sequence of the encoding structural genes for the *Mhc-I* heavy chain and the *Mhc-II* α and β chains, as well as other genes involved in antigen processing and presentation, must satisfy at least two distinct recognition functions: the binding of peptides or in some cases nonpeptidic molecules and the interaction with either T or NK cells via their respective receptors. The TCR may augment its interaction with the MHC molecule by virtue of interaction with a T cell–expressed coreceptor (cluster of differentiation [CD]8 for MHC-I and CD4 for MHC-II). Some NK receptors may also serve as coreceptors when expressed on T cells.[16] The binding of peptides by an MHC-I or MHC-II molecule is the initial selective event that permits the cell expressing the MHC molecule (the antigen-presenting cell [APC] or when this cell is to be the recipient of a cytolytic signal, the target cell) to sample fragments derived either from its own proteins (for MHC-I–restricted antigen presentation) or from those proteins ingested from the immediate extracellular environment (for the case of MHC-II). The biochemical steps involved in the production of antigen fragments from large molecules are collectively known as "antigen processing," whereas those that concern the binding of antigen fragments by MHC molecules and their display at the cell surface are known as "antigen presentation."

Specifically, MHC-I glycoproteins gather from the cell's biosynthetic pathway fragments of proteins derived from infecting viruses, intracellular parasites, or self-molecules, either expressed normally or in a dysregulated fashion as a result of tumorigenesis, and then display these molecular fragments, in complex with the mature MHC-I molecule, at the cell surface.[17–21] The cell-bound MHC-I/β2m/peptide complex on the APC is exposed to the extracellular milieu and is available for interaction with either T cells or NK cells. The T cell bearing an αβ receptor recognizes the particular MHC/peptide complex by virtue of a specific physical binding interaction. Each T cell is representative of a clonal population and bears a unique TCR encoded by somatically rearranged TCR genetic elements. T cells bearing αβ receptors undergo a complex selective process in the thymus. Only a small proportion of T cells that enter the thymus ultimately reach peripheral lymphoid organs, such as lymph nodes and spleen. A particular TCR can only bind a very limited selection of MHC/peptide complexes. The recognition by T cells is considered "MHC restricted" in that only a limited set of MHC molecules can bind a particular TCR and is also

TABLE 21.2 Listing of HLA Class I Alleles[a]

HLA-A Alleles	HLA-B Alleles	HLA-C Alleles
A*01:01:01:01—A*01:104	B*07:02:01—B*07:144	C*01:02:01—C*01:61
A*02:01:01:01—A*02:336	B*08:01:01—B*08:80	C*02:02:01—C*02:55
A*03:01:01:01—A*03:135	B*14:01:01—B*:14:28	C*03:02:01—C*03:139
A*11:01:01—A*11:112	B*15:01:01:01—B*15:238	C*04:01:01:01—C*04:107
A*23:01:01—A*23:50	B*18:01:01—B*18:68	C*05:01:01:01—C*05:72
A*24:02:01:01—A*24:190	B*27:01—B*27:86	C*06:02:01:01—C*06:69
A*25:01:01—A*25:16	B*35:01:01:01—B*35:186	C*07:01:01—C*-7:220
A*26:01:01—A*26:72	B*37:01:01—B*37:31	C*08:01:01—C*08:56
A*29:01:01:01—A*29:32	B*38:01:01—B*38:37	C*12:02:01—C*12:68
A*30:01:01—A*30:58	B*39:01:01:01—B*39:68	C*14:02:01-C*14:34
A*31:01:02—A*31:59	B*40:01:01—B*40:180	C*15:02:01—C*15:56
A*32:01:01—A*32:37	B*41:01—B*41:19	C*16:01:01—C*16:44
A*33:01:01—A*33:54	B*42:01:01—B*42:16	C*17:01:01:01—C*17:11
A*34:01:01—A*34:09	B*44:02:01:01—B*44:139	C*18:01—C*8:05
A*36:01—A*36:05	B*45:01—B*45:13	
A*43:01	B*46:01:01—B*46:30	
A*66:01—A*66:16	B*47:01:01:01—B*47:08	
A*68:01:01:01—A*68:85	B*48:01:01—B*48:27	
A*74:01—A*74:15	B*49:01:01—B*49:20	
A*80:01—A*80:02	B*50:01:01—B*50:15	
	B*51:01:01—B*51:126	
	B*52:01:01:01—B*52:27	
	B*53:01:01—B*53:27	
	B*54:01:01—B*54:24	
	B*55:01:01—B*55:54	
	B*56:01:01—B*56:32	
	B*57:01:01—B*57:52	
	B*58:01:01—B*48:36	
	B*59:01:01:01—B*59:05	
	B*67:01:01—B*67:03	
	B*73:01—B*73:02	
	B*78:01:01—B*78:07	
	B*81:01—B*81:05	
	B*82:01—B*82:03	
	B*83:01	

[a] This summarizes HLA alleles as of January, 2012, as described by Robinson et al.[8,9] and available at http://www.imgt.org or at www.ncbi.nlm.nih.gov/projects/gv/mhc/main.fcgi?cmd-init. HLA-Bw4 alleles are generally agreed to include B5, B5102, B5103, B13, B17, B27, B37, B38(16), B44(12), B47, B49(21), B51(5), B52(5), B53, B57(17), B58(17), B59, B63(15), B77(15), and A9, A23(9), A24(9), A2403, A25(10), A32(19) (http://hla.alleles.org/antigens/bw46.html). Similarly, HLA-Bw6 alleles include B7, B703, B8, B14, B18, B22, B2708, B35, B39(16), B3901, B3902, B40, B4005, B41, B42, B45(12), B46, B48, B50(21), B54(22), B55(22), B56(22), B60(40), B61(40), B62(15), B64(14), B65(14), B67, B70, B71(70), B72(70), B73, B75(15), B76(15), B78, B81, and B82.

HLA, human leukocyte antigen.

termed "antigen specific" in that a particular T cell identifies a particular peptide. For any given T-cell clone, single amino acid substitutions of either the MHC or the peptide may severely diminish, obliterate, or even augment the functional interaction of the TCR with the MHC-II/peptide complex. The MHC-I system draws its spectrum of peptides from proteins in the cytosol that are degraded by the proteolytic proteasome complex to peptides that are transported from the cytosol to the endoplasmic reticulum (ER) with the aid of the intrinsic membrane peptide transporter, the transporter associated with antigen processing (TAP), are then trimmed at their amino termini[22] and are cooperatively folded as an intrinsic component of the newly synthesized MHC-I molecule.[23] Interactions with the peptide-loading complex (PLC), which includes TAP, the chaperone tapasin,[24] and Erp57,[25] are crucial to MHC-I loading. MHC-I molecules are unique among proteins in that their three-dimensional structure and thermal stability are exquisitely dependent on the heavy/light chain heterodimer being bound by an appropriate peptide.[26,27] In mutant cells that lack the necessary apparatus for generating and transporting peptides to the ER where peptide loading takes place, MHC-I molecules are expressed poorly and are inherently unstable.[28] MHC-II/peptide loading is controlled in part by the multifunctional chaperone/groove protector, Ii, as well as the important catalytic machinery of the endosomes, molecules known as HLA-DM and -DO in the human, and H2-M and H2-O in the mouse.[24] Although there remains some controversy as

TABLE 21.3 Listing of HLA Class II Alleles[a]

HLA-DR Alleles	HLA-DQ Alleles	HLA-DP Alleles	HLA-DM Alleles	HLA-DO Alleles
α chain	α chain	α chain	α chain	α chain
DRA	DQA1	DPA1	DMA	DOA
DRA*01:01:01—DRA*01:02	DQA1*01:01:01—DQA1*01:09	DPA1*01:03:01:01—DPA1*01:10	DMA*01:01:01:01—DMA*01:04	DOA*01:01:01—DOA*01:04N
	DQA1*02:01	DPA1*02:01:01—DPA1*02:04		
	DQA1*03:01:01—DQA1*03:03:02	DPA1*03:01—DPA1*03:03		
	DQA1*04:01:01—DQA1*04:04	DPA1*04:01		
	DQA1*05:01:01:01—DQA1*05:11			
	DQA1*06:01:01—DQA1*06:02			
β chain	β chain	β chain	β chain	β chain
DRB1	DQB1	DPB1	DMB	DOB
DRB1*01:01:01—DRB1*01:45	DQB1*05:01:01:01—DQB1*05:14	DPB1*01:01:01—DPB1:01:03	DMB*01:0101:01—DMB*01:07	DOB*01:01—DOB*01:05
DRB1*03:01:01:01—DRB1*03:77	DQB1*06:01:01—DQB1*06:47	DPB1*02:01:02—DPB1*02:02		
DRB1*04:01:01—DRB1*04:107	DQB1*02:01:01—DQB1*02:06	DPB1*03:01:01—DPB1*03:01:02		
DRB1*07:01:01:01—DRB1*07:22	DQB1*03:01:01:01—DQB1*03:39	DPB1*04:01:01:01—DPB1*04:02:01:02		
DRB1*08:01:01—DRB1*08:49	DQB1*04:01:01—DQB1*04:08	DPB1*05:01:01—DPB1*05:01:02		
DRB1*09:01:02—DRB1*09:17		DPB1*06:01		
DRB1*10:01:01—DRB1*10:04		DPB1*08:01		
DRB1*11:01:01—DRB1*11:121		DPB1*09:01		
DRB1*12:01:01—DRB1*12:35		DPB1*10:01		
DRB1*13:01:01—DRB1*13:135		DPB1*11:01:01—DPB1*11:01:02		
DRB1*14:01:01—DRB1*14:122		DPB1*13:01—DPB1*19:01		
DRB1*15:01:01:02—DRB1*15:69		DPB1*20:01:01—DPB1*20:01:02		
DRB1*16:01:01—DRB1*16:19		DPB1*20:01:01—DPB1*35:01:01		
DRB2		DPB1*35:01:01—DPB1*35:01:02		
DRB2*01:01				
DRB3		DPB1*35:01:01—DPB1*41:01:01		
DRB3*01:01:02:01—DRB3*03:03				
DRB4		DPB1*41:01:01—DPB1*41:01:02		
DRB4*01:01:01:01—DRB4*01:08				
DRB5		DPB1*44:01—DPB1*136:01		
DRB5*01:01:01—DRB5*02:05				
DRB6				
DRB6*01:01—DRB6*02:02				
DRB7				
DRB7*01:01:01—DRB7*01:01:02				
DRB8				
DRB8*01:01				
DRB9				
DRB9*01:01				

[a]This table is based on a listing of alleles maintained by the World Health Organization Nomenclature Committee for Factors of the HLA System, as of January 2012. All new and confirmatory sequences are generally submitted directly to the committee via the IMGT/HLA database using the sequence submission tool provided. The IMGT/HLA database may be accessed at www.ebi.ac.uk/imgt/hla. Serologic assignment of HLA-DR molecules is largely determined by the DRB1 gene product, whereas assignment of DQ molecules reflects serologic contributions from both DQA1 and DQB1 gene products.

HLA, human leukocyte antigen.

TABLE 21.4 Commonly Used Mouse Strains: *H-2* Haplotypes[a]

		H-2 Complex							
Strain	**Haplotype**	***KK***	***Ab***	***Aa***	***Eb***	***Ea***	***D***	***Q***	***T***
Common strains									
129/J	*bc*	*b*	*b*	*b*	*b*	–	*b*	*b*	*f*
AKR/J	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
ASW/Sn	*s*	*s*	*s*	*s*	*s*	–	*s*	*b*	*b*
BALB/c	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*b*	*c*
C3H/HeJ	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
CBA/J	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*b*	*b*
C57BL/6	*b*	*b*	*b*	*b*	*b*	–	*b*	*b*	*b*
C57BL/10	*b*	*b*	*b*	*b*	*b*	–	*b*	*b*	*b*
C57BR	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*a*	*a*
DBA/2J	*d*	*d*	*d*	*d*	*d*	*d*	*d*	*b*	*c*
NOD/LtJ	*g7*	*d*	*g7*	*d*	–	–	*b*		
NON/LtJ	*nb1*	*b*	*nb1*	*?*	*k*	*k*	*b*		
NZB/BINJ	*d2*	*d*	*d*	*d*	*d*	*d*	*d*	*a*	*a*
NZW/LacJ	*z*	*u*	*u*	*u*	*u*	*u*	*z*		
P/J	*p*	*p*	*p*	*p*	*p*	*p*	*p*	*a*	*e*
PL/J	*u*	*u*	*u*	*u*	*u*	*u*	*d*		
RIII	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*c(r)*	*b*
SJL	*s2*	*s*	*s*	*s*	*s*	–	*s*	*a*	*a*
Congenic strains									
B10.BR	*k2*	*k*	*k*	*k*	*k*	*k*	*k*		
B10.D2	*d*	*d*	*d*	*d*	*d*	*d*	*d*		
B10.S	*s*	*s*	*s*	*s*	*s*	–	*s*		
BALB.B	*b*	*b*	*b*	*b*	*b*	–	*b*		
BALB.K	*k*	*k*	*k*	*k*	*k*	*k*	*k*		
C3H.SW	*b*	*b*	*b*	*b*	*b*	–	*b*		
Recombinant strains									
A	*a*	*k*	*k*	*k*	*k*	*k*	*k*		
A.TL	*tl*	*s*	*k*	*k*	*k*	*k*	*d*		
B10.A	*a*	*k*	*k*	*k*	*k*	*k*	*d*		
B10.A(1R)	*h1*	*k*	*k*	*k*	*k*	*k*	*b*		
B10.A(2R)	*h2*	*k*	*k*	*k*	*k*	*k*	*b*		
B10.A(3R)	*l3*	*b*	*b*	*b*	*b/k*	*k*	*d*		
B10.A(4R)	*h4*	*k*	*k*	*k*	*k/b*	–	*b*		
B10.A(5R)	*l5*	*b*	*b*	*b*	*b/k*	*k*	*d*		
B10.T(6R)	*y2*	*q*	*q*	*q*	*q*	–	*d*		
B10.S(7R)	*t2*	*s*	*s*	*s*	*s*	–	*d*		
B10.S(8R)	*as1*	*k*	*k*	*k*	*k/s*	–	*s*		
B10.S(9R)	*t4*	*s*	*s*	*s*	*s/k*	*k*	*d*		
B10.HTT	*t3*	*s*	*s*	*s*	*s/k*	*k*	*d*		

*Adapted from Kruisbeek[561] and www.imgt.org/IMGTrepertoireMHC/Polymorphism/haplotypes/mouse/MHC/Mu_haplotypes.html#polymorphism. A dash indicates abnormal gene expression, although precise mechanism may differ in different strain. Blanks indicate insufficient data for characterization. Additional strains may be identified at http://jax-mice.jax.org/findmice/index.html.

NOD, nonobese diabetic.

to the precise site of interaction of HLA-DM with HLA-DR, evidence supports the view that this interaction catalyzes peptide interchange, leading to the selection of high-affinity antigenic peptides.[29]

MHC-II molecules, in contrast to MHC-I, are expressed on a more limited set of somatic cells—B cells, macrophages, dendritic cells, activated but not resting T cells in the human—and have a somewhat more specific function in peptide selection and presentation. In general, they bind peptides derived from the degradation of proteins ingested by the APC, and they sort their MHC-II molecules into cellular compartments where the degraded peptides are generated and catalytically transferred to the binding site of the MHC-II. The MHC-II antigen-presentation pathway is based on the initial assembly of the MHC-II αβ heterodimer with a dual function molecule, the invariant chain (Ii),

which serves as both a chaperone to direct the αβ heterodimer to an endosomal, acidic, protein processing location where it encounters antigenic peptides and also serves to protect the antigen-binding site of the MHC-II molecule so that it preferentially will be loaded with antigenic peptides in this location.[30–33] The loading of the MHC-II molecule with antigenic peptide, a process dependent on the release of the Ii derived "CLIP" peptide, in part dependent on the MHC-II–like molecule, HLA-DM in the human,[34,35] H2-M in the mouse,[36] then leads to the cell surface expression of MHC-II/peptide complexes. The MHC-II–recognizing T cells then secrete cytokines and may also be induced to proliferate or to undergo programmed cell death. Such MHC-restricted cytokine production that facilitates and augments the recruitment of additional inflammatory cells as well as APCs and antibody-producing cells is a contemporary explanation for what was historically referred to as "T-cell help." Under some physiologic circumstances, particularly during viral infection, antigens incorporated into antigen-presenting dendritic cells via an "outside in" pathway may alternatively enter the MHC-I presentation pathway. This phenomenon is known as "cross-presentation."[37,38]

MHC molecules perform a crucial role in the thymus by shaping the TCR repertoire as T-cell precursors mature into cells that eventually emigrate from the thymus and populate the spleen and other secondary lymphoid organs. In a complex, multistep process termed "thymic education,"[39] developing T cells expressing randomly rearranged TCR αβ receptors are first subjected to positive selection on MHC-I and MHC-II molecules expressed by cells of the thymic cortex in order to select for further maturation of only those cells capable of recognizing peptides in the context of self-MHC molecules. Positive selection may be viewed as setting the minimum threshold for T-cell activation. T cells surviving positive selection then migrate to the thymic medulla where those T cells with higher-than-average affinity for self-MHC-II/peptide complexes, and which therefore pose the threat of autoimmune disease should they be allowed to emigrate to secondary lymphoid organs, are deleted by negative selection. MHC-I and MHC-II molecules thus play a critical role in shaping the peripheral TCR repertoire. Indeed, mice engineered to lack MHC-I or MHC-II expression are also deficient in T cells.[36,40]

Distinct from the recognition of MHC molecules by TCRs, a number of NK-cell receptors, both activating and inhibitory, bind MHC-I molecules, and several NK receptors interact with MHC-I–like molecules encoded by CMVs.[2,41] In general, the NK/MHC-I interaction, as compared to the TCR/MHC interaction, shows considerably less peptide specificity, although the interaction is peptide dependent, and in some cases may exhibit clear-cut peptide preferences.[42–44] The functional purpose of the MHC-I or MHC-I–like molecule in NK-cell recognition appears to be more subtle than that in T-cell recognition. The NK cell is tuned to a balance of inhibitory and activating signals conveyed to it via MHC interaction. In its resting state, the inhibitory signals predominate. MHC-I is a sensor of the biosynthetic and metabolic state of the cell in which it is synthesized. When the level of MHC-I is dysregulated by tumorigenesis or viral infection, this change can be detected by the NK cell. This ability of the NK cell to sense altered levels of MHC-I on target cells is the basis of the "missing self hypothesis," which explains that NK cells detect and lyse those cells defective in MHC-I expression due to the loss of the inhibitory signal that results from engagement of NK receptors by MHC-I.[45–47] The prototype NK receptor in the mouse is the NK inhibitory receptor, Ly49A, a C-type lectin-like molecule that signals its interaction with a normally expressed MHC-I molecule, such as H2-D^d.[48] Distinct clones of NK cells differ in the combinatorial expression of different NK receptors that have different MHC preferences. Thus, in the mouse, each distinct NK clone may express a different combination of NK inhibitory receptors such as Ly49A, Ly49C, Ly49G2, and Ly49I.[49] Because each inhibitory molecule may exhibit slight differences in its MHC-I and/or peptide preference and specificity, this kind of combinatorial expression of NK activity offers a breadth of specificity toward different potential target cells. Recently, it has been shown that developing NK cells within the thymus are also subject to an education process, known as "licensing," which prevents improper activation of their cytolytic function in peripheral tissues.[50–52] NK cells that develop in MHC-I–deficient humans and mice fail to kill target cells that lack MHC-I expression. Such targets are readily killed by NK cells that develop in MHC-I–sufficient thymi. Human NK cells seem to go through a similar selective/educating process, but the relevant NK receptors on human cells are generally of the killer cell immunoreceptor (KIR) inhibitory receptor family.[53]

In addition to showing preference for distinct pathways of antigen presentation, the MHC-I and MHC-II molecules also show preferential restriction to T cells of the CD8- or CD4-bearing lineages. This results from the interaction of CD8 with the nonpolymorphic α3 domain of MHC-I molecules,[54–58] whereas CD4 binds to membrane proximal domains of MHC-II.[59–64] The CD8 and CD4 molecules serve as "coreceptors" on the surface of T lymphocytes, providing both adhesion (increase in avidity) and specific activating signals, mediated through the kinase, lck, which modulate the avidity of the T cell in a time-dependent manner.[65–68] Additional complexities arise from the interaction of CD8 with MHC-I. CD8 is expressed as either the CD8αα homodimer or the CD8αβ heterodimer, which are expressed in a developmentally regulated fashion and seem to have distinct functions.[69] The numerous interactions of MHC molecules with other cellular components as well as with the wide variety of peptides and of various immunologic receptors reflect the robust potential of the MHC structure as a molecular sensor and as a master regulator of immune responses. These molecular interactions then read out in different cell trafficking and signaling functions.

THE MAJOR HISTOCOMPATIBILITY COMPLEX

Mhc Genetics

The *Mhc* is an extended region of the genome that spans some 4 million basepairs (Mb) on the short arm of human chromosome 6 in the region 6p21.3. The *Mhc* in the human may be considered to map from HLA-F to the gene encoding the MHC chaperone tapasin (TAP binding protein), a distance of some 2 Mb, but the "extended" human

Mhc (also known as the xMHC) covers some 7.6 Mb from the HIST1H2AA locus to SYNGAP1.[70–72] In the mouse, the *Mhc* encompasses almost 3.5 Mb on chromosome 17, extending from the *H2-Ke1* gene at basepair position 34056423 to the *H2-M2* gene at basepair position 37620474 (www.imgt.org/IMGTrepertoireMHC/LocusGenes/index.php?repertoire=listIG_TR&species=mouse&group=MHC). Although *Mhc* genes were among the first to be mapped here, it is now clear that a large number of genes with function unrelated to immune recognition also reside in this region. The interested reader is referred to the regularly updated maps and linkages available at various Web sites including www.ebi.ac.uk/imgt/hla, and the MHC haplotype project at www.sanger.ac.uk/HGP/Chr6/.

A schematic of the human *Mhc* is shown in Figure 21.2. For a rough guide, we also provide a simple illustrative map comparing human, mouse, and rat *Mhc* regions (Fig. 21.3) and refer the reader to the National Center for Biotechnology Information homology Web site: www.ncbi.nlm.nih.gov/projects/homology/maps/ and National Center for Biotechnology Information Web viewer for access to detailed maps and sequences.

The human *Mhc* map reveals clusters of genes grouped roughly into an Mhc-II region covering about 1000 kb, an Mhc-III of about 1000 kb, and an *Mhc* class I region spanning 2000 kb (see Fig. 21.2). *HLA-DP* genes (*DPA* encoding the α chain and *DPB* encoding the β chain) are proximal to the centromere on the short arm of the chromosome and are linked to the genes encoding the related HLA-DM molecule (*DMB* and *DMA*). Between these and the *DQ* genes lie *LMP* (for low molecular weight proteins[73–76]) and *TAP*[77–81] genes that encode molecules that are involved in peptide generation in the cytosol and peptide transport across the ER membrane, respectively. Low molecular weight proteins

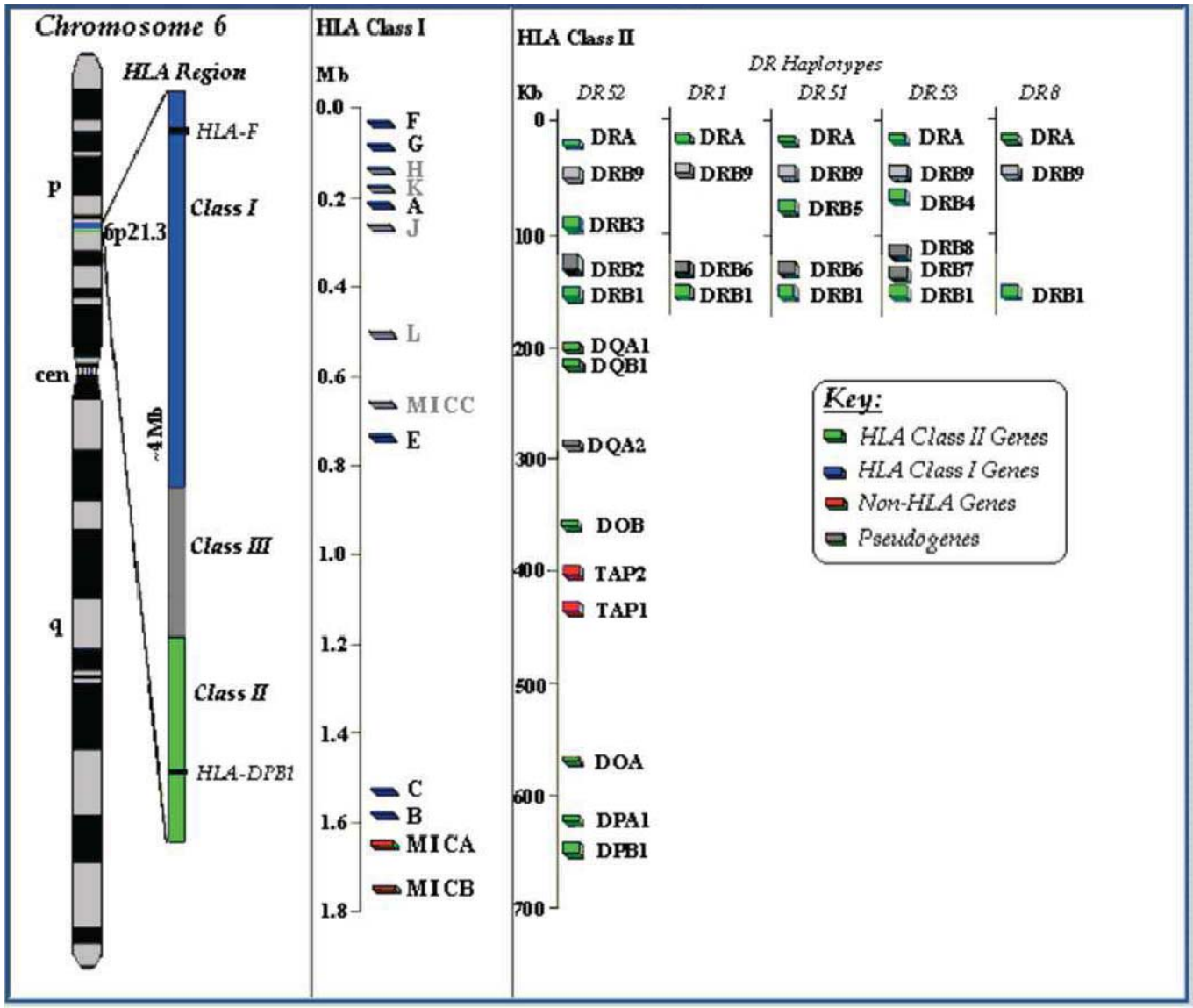

FIG. 21.2. Map of the Human MHC (HLA Region) on the Short Arm of Chromosome 6. A variety of online tools for seeking and manipulating MHC sequences can be found at www.ncbi.nlm.nih.gov/gv/mhc/main.cgi?cmd=init.

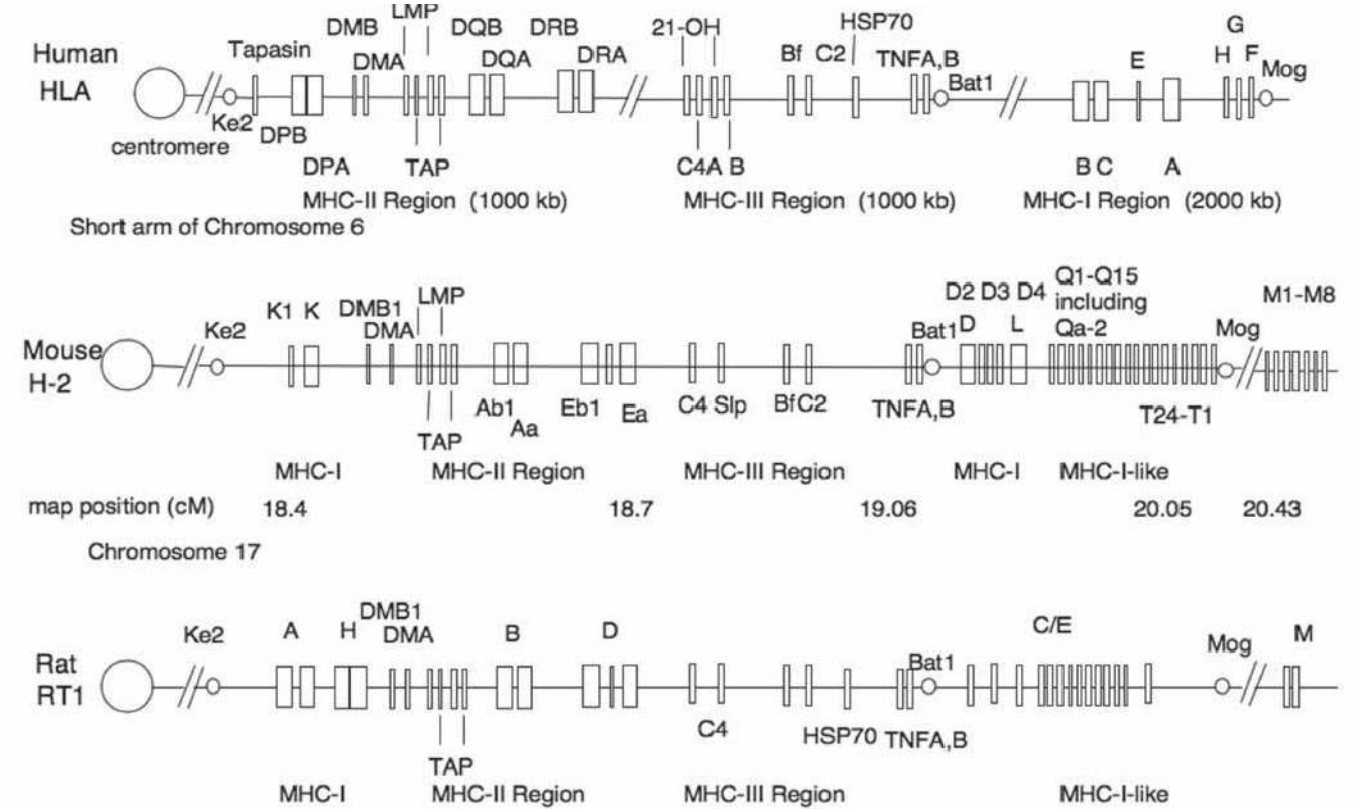

FIG. 21.3. Comparative Maps of Human, Mouse, and Rat *Mhc* Regions. This schematic map is not complete nor drawn to scale and is derived from maps available at www.ncbi.nlm.nih.gov/projects/homology/maps/.

are subunits of the catalytic proteolytic proteasome complex that regulate the specificity of cleavage of proteins, and thus modulate the repertoire of peptides available for MHC-I–restricted antigen presentation.[82–85] The *TAP* genes encode a two-chain intrinsic membrane protein that resides in the ER of all cells and functions as an adenosine triphosphate–dependent transporter that pumps peptides generated in the cytosol into the lumen of the ER.[86,87] The selective transport of cytoplasmically generated peptides by different TAP proteins in the rat demonstrates that the spectrum of MHC/peptide complexes expressed at the cell surface can be significantly altered by differences in the antigen presentation pathway,[88–90] although there is little evidence for this phenomenon in humans.[91] Nevertheless, TAP deficiency syndromes in humans[92–95] emphasize the importance of peptide delivery in the antigen presentation pathway for normal immune function.

The major *Mhc-II* genes of the human are *HLA-DRA* and *HLA-DRB* that encode the chains that form the HLA-DR molecule, a major antigen-presentation element. Genetic mapping of the human DRB region indicates that several alternative arrangements of *DRB* loci explain the varied serotypes and genotypes observed among different individuals (see Fig. 21.2). In particular, note that several HLA-DR "haplotypes," originally defined serologically as DR52, DR1, DR51, DR53, and DR8, differ not only in particular allelic genes at the *DR* locus but also in the number and precise location of several of the *DRB* genes and pseudogenes. The *Mhc-III* region is important in immunologic terms for several reasons—the structural genes for several complement components map here, as well as the structural genes for 21-hydroxylase (CYP21A2),[96,97] an enzyme critical in the biosynthesis of glucocorticoids, a deficiency of which can lead to the genetic disease congenital adrenal hyperplasia. Also located in the *Mhc-III* region are the structural genes for tumor necrosis factor (TNF) A (also known as lymphotoxin α) and B, which are cytokines made by activated T cells.[98–100]

The more distal region of the *Mhc* encodes other MHC molecules. In humans, the cluster of the major *Mhc-I* genes lies here, spanning two Mb, including the genes encoding HLA-B, HLA-C, HLA-E, and HLA-A, as well as HLA-H, HLA-G, and HLA-F. HLA-A, HLA-B, and HLA-C are the major MHC-I molecules of man. (A summary of these is in Table 21.2.) Serologic identification of HLA-C molecules has been difficult and imprecise. However, HLA-C molecules interact directly with NK receptors of the KIR2D family. Direct binding studies have analyzed the kinetics of the interaction of the KIR2 immunoglobulin (Ig)-like NK receptors,[101–103] and three-dimensional structures of KIR2DL2 in complex with HLA-Cw3 and KIR2DL1 complexed with HLA-Cw4 have been published.[104,105] Recently, the structure of a KIR3DL1 molecule in complex with HLA-B*57:01 emphasizes the unique nature of different KIR/MHC interactions.[106] The precise functions of HLA-E, HLA-F, and HLA-G are not yet clear. HLA-E and its murine analog Qa-1 bind hydrophobic leader peptides derived from some MHC-I

molecules, forming a complex recognized by the C-type lectin-like NK receptor CD94/NKG2.[107–111] This implies an important function for HLA-E because these molecules are expressed on placental trophoblast cells and would be expected to bind the inhibitory NK receptor, CD94/NKG2A, preventing NK-mediated rejection of the fetus.[112] HLA-E may serve as a recognition element for some T cells as well, so it also seems capable of a classical role.[113,114] Additional evidence now supports an antigen presentation function of HLA-F and HLA-G,[115,116] and the tissue restricted expression of HLA-G as well as the observation that a soluble form leads to apoptosis of CD8+ T cells suggest that this molecule may be involved in the mother's immunologic tolerance of the fetus.[117] *HLA-H* is a pseudogene mapping to this region.[118] This should not be confused with the more distantly related *HLA-HFE*, an *Mhc-Ib* gene erroneously called *HLA-H* by some authors,[118,119] which controls hereditary hemochromatosis by virtue of the interaction of its encoded protein with the transferrin receptor.[120–125] The observations that extravillous trophoblast cells express an unusual combination of HLA-C, HLA-E, and HLA-G molecules; that uterine NK cells express NK inhibitory receptor (KIR) molecules known to interact with HLA-C allelic products; and that complex genotypes predispose to preeclampsia raise the possibility that important aspects of fetal rejection and clinical infertility may be related to MHC recognition by NK cells in the placenta.[126]

Comparison of the mouse, rat, and human *Mhc* maps (Fig. 21.3) reveals several interesting differences.[5,127–129] The *Mhc* genes proximal to the centromere of the mouse and rat belong to the *Mhc-I* family, rather than to the *Mhc-II* family, as they do in the human. This mapping has suggested that an intrachromosomal recombination event that occurred in some common rodent ancestor relocated some of the *Mhc-I* genes from a more distal location to the proximal site.[130] Inspection of the current human, mouse, and rat maps clearly indicates similarities in the relative locations and organization of *Mhc-II*, *Mhc-III*, and the distal *Mhc-I* genes.[128] Various genetic expansions and contractions[131] are obvious as well. In particular, the mouse *Q* and *T* regions have expanded the pool of *Mhc-I* genes, which are relatively few in the human and the rat. Early studies of congenic mouse strains mapped multiple genes to the *Q* and *T* regions,[132–134] and recent evidence suggests significant differences in the number of genes of this region in different strains. The mouse has some *MHC-Ib* genes that seem to be relatively unique in function. In particular, the *H2-M3* gene, which maps distal to the *Q* and *T* regions, encodes a protein that exhibits a preference for binding peptides that have N-formyl amino terminal modifications. This antigen presentation function may be geared to bacterial, protozoal, and mitochondrial antigens.[135–137] Rat homologues of the mouse *H2-M3* and *H2-M2* genes have also been identified.[138,139]

Mhc Polymorphism

The *Mhc's* function in immune responsiveness is also reflected in its genetic polymorphism. Polymorphism is the presence at any given time of a larger than expected number of genetic variants in a population. As populations change and evolve, we expect that genetic variants should arise but because of the selection exerted on most gene products, relatively few of these genetic variants will persist. A genetic locus that exhibits variant alleles at a frequency of more than 1% is considered "polymorphic."[5] A genetic locus that is relatively invariant is often referred to as "monomorphic," even if more than one allele is known. *HLA* genes exhibit a high degree of polymorphism; a number of different mechanisms may contribute to the generation and maintenance of polymorphism. Among these are the selective advantage of a heterozygous pool of antigen-presenting elements in a given individual that might allow the binding and presentation of antigenic peptides derived from a wide variety of environmental pathogens. Limited polymorphism would make the entire population susceptible to a chance infectious agent for which all individuals would be unable to respond, whereas widespread polymorphism would be expected to allow the APCs of at least a proportion of the population to effectively bind and present antigens derived from invading pathogens.[140] Although such a view was originally based on HLA molecules as presenting elements for pathogen-derived peptide fragments to T cells and their antigen-specific TCRs, studies suggest an additional role for *Mhc-I*–related resistance to viral infection via NK cell–mediated recognition[141,142] and altered antigen presentation pathway.[143] Additionally, the presence of a high degree of polymorphism also implies that there is little or no selective disadvantage to the expression of new variant MHC molecules.

The human *Mhc-I* and *Mhc-II* genes are clearly polymorphic, with more than 5000 alleles at the *Mhc-I* loci and some 2000 alleles at the *Mhc-II* loci known (see Tables 21.2 and 21.3) (www.ebi.ac.uk/imgt/hla/index.html).[8,9] In experimental animals, it is more difficult to demonstrate polymorphism in terms of population genetics, although typing of wild mice has confirmed the existence of natural polymorphism predicted from the analysis of inbred strains and their mutants.[144,145] The polymorphism of *Mhc-I* and *Mhc-II* genes, so evident in man and mouse, has also been documented in analyses of cichlid fishes—animals that diverged from the line leading to mammals at least several hundred million years ago.[146–149]

Genetic Mechanisms for *Mhc* Evolution

As both an extended genetic region and a group of genes with many belonging to the Ig supergene family,[150,151] the *Mhc* has served as a prototype for elucidating mechanisms that contribute to the evolution of a multigene family and that add to the polymorphism that is such a dominant characteristic of the classical MHC molecules.[152] The analysis of mutations in the mouse, mostly those of *Mhc-I* genes, has led to the understanding of the mechanisms that give rise to polymorphism. Both induced and spontaneous mutations affecting skin graft acceptance or rejection have been identified, and many of these have been mapped to the *Mhc*. Gross recombinational events have been documented in the *Mhc*,[153,154] as well as more subtle mutations, many of which are multiple amino acid substitutions in a relatively small part of the protein that seem to derive from nonreciprocal recombinational events. Such recombination that occurs over short sequences

is known as "gene conversion" because of its similarity to the phenomenon that occurs in yeast.[153–161] Convincing evidence for interlocus gene conversion has been documented in for the *Mhc-II* genes of teleost fish[162] and by deep sequencing of *Mhc-I* genes in recently founded bird populations.[163]

Some of the polymorphisms of *Mhc* genes that have been identified clearly reflect point mutations.[164] Structural studies have shown that the profound immunologic effects of mutations of the *H2* genes $H2K^{bm1}$ and $H2K^{bm8}$ result from minimal detectable changes that may affect thermostability.[165] In addition to such mouse mutants, a number of somatic cell variants and mutants, some due to major deletions or regulatory defects, others clearly point mutants of structural genes, have been described.[166]

The *Mhc* and Transplantation

Although the early description of the genes of the *Mhc* was based on identification of loci involved in tumor and allograft rejection, and although these genes clearly play a role in such complex phenomena, a contemporary understanding of the function of *Mhc* genes in immunology requires little understanding of the rules of transplantation. The early history of transplantation is chronicled extensively in several books[5,167] and reflects a developing interest in tumor immunology and congenic mouse strains. The mouse has served as the model system for understanding the genetic underpinnings of skin, tumor, and organ transplantation, so a brief description of some relevant principles is in order. Comprehensive manuals and reviews are available.[168] Propagation of a mouse strain by repeated matings of brothers and sisters leads to the establishment of an inbred strain, a group of animals that is genetically identical at all loci. More complete descriptions of the process by which brother–sister mating leads to homozygosity at all loci are given elsewhere.[5,167]

"Congenic" mouse strains, also known as "congenic resistant" or "CR" strains, are those derived by first crossing two inbred strains that differ in a histocompatibility phenotype such as resistance to a transplantable tumor or ability to reject a skin graft. These are then successively backcrossed to one parental strain, and the resistance phenotype is preserved. Following at least 10 backcross generations (N10), a point at which $(1/2)^9 = 0.002$ of the genes of the selected strain should be present, the new strain is propagated by brother–sister mating. Several relevant inbred and congenic mouse strains are listed in Table 21.4 along with their *H2* designations.

The early rules of transplantation were determined by observation of the ability of either transplantable tumors or allografts (usually from skin) to survive in a particular inbred mouse strain host. The graft rejection phenomenon is an extremely sensitive and specific bioassay that permits the detection of genetic differences as small as a single amino acid in an MHC protein. It has been particularly valuable in assessing spontaneous and induced mutants (see previous discussion) and remains the absolute experimental discriminator of "histocompatibility."

In addition to the *Mhc* genes, we should not overlook the genes that encode minor histocompatibility antigens. In the mouse, these were originally identified as genetic loci responsible for graft rejection after extended periods of time. More recently, several minor histocompatibility loci have been identified as those that encode polymorphic proteins that give rise to peptides presented by MHC molecules,[169–173] and we now can understand the complexities of transplantation tolerance in terms not only of *Mhc* genes but also in terms of numerous proteins that may give rise to variant peptides for T-cell recognition. Not only can peptides derived from polymorphic genes throughout the genome serve as minor histocompatibility antigens but also defective translation products, or peptides resulting from transcription of introns or noncoding strands of DNA may also produce immunologically significant peptides, which, bound by self-MHC molecules may stimulate T cells.[174,175] Whether such defective, newly synthesized proteins serve as a major source of MHC-I–bound peptides remains controversial.[176] Minor antigens that are confined to hematopoietic cells can be recognized as targets by antitumor cytolytic cells and may explain the lower incidence of relapse in hematopoietic stem cell (HSC) transplant recipients who experience graft-versus-host disease (GVHD).[177]

The *Mhc* and Clinical Transplantation

Processed foreign antigen complexed to HLA class I or class II molecules is recognized by a specific clonally distributed TCR for antigen on the surface of T lymphocytes. The T cell bearing an αβ receptor is capable of recognizing the unitary structure of the HLA molecule itself coordinately with the exposed parts of the peptide antigen. Corecognition of HLA and peptide antigen means that TCRs are highly specific and genetically restricted to recognizing HLA molecules of the individual from which they were derived. Thus, a killer (cytotoxic) T cell raised against an influenza virus peptide in an individual expressing HLA-A2 will not recognize influenza-infected HLA-A1. This concept is known as "MHC restriction" and was described by Shevach and Rosenthal for recognition of amino acid polymers,[178] by Zinkernagel and Doherty for recognition of viral antigens,[179,180] and by Shearer et al. for recognition of altered self-ligands.[181] Given that T cells are MHC-restricted, it is difficult to understand why they should ever recognize a foreign HLA type. However, in practice they do, data indicate that such alloreactive T cells arise with remarkably high frequency. It is estimated that between 1/10 to 1/1000 activated clonally distinct T cells are capable of responding to any random allogeneic HLA molecule.[182–185] Given the number of T cells in the human lymphoid system, this represents a striking tendency for T cells that are normally restricted to recognizing self-HLA molecules complexed to foreign peptides to cross-react on allogeneic HLA molecules. This cross-reaction can arise from direct recognition of the allogeneic HLA/peptide complex, which usually depends on the peptide antigen as well as the allogeneic HLA molecule. Alternatively, allorecognition by T cells can occur indirectly.[186,187] In such cases, peptides derived from the allogeneic HLA molecules are presented as nominal antigen after processing by the host cells bearing self-HLA molecules. In the normal course of

events, T-cell alloreactivity is an in vitro curiosity, although it is still not entirely clear why the fetal "allograft" does not stimulate the maternal immune system. However, it is the clinical transplantation of organs and hematopoietic stem cells across HLA compatibility barriers that produces graft rejection or GVHD due to T-cell alloreactivity. Fully allogeneic transplants theoretically expose the recipient immune system to up to 12 non–self-HLA allele products expressed by the allograft. Moreover, the "self-peptides" constitutively presented by allogeneic HLA molecules are likely to be quite distinct from those presented by syngeneic HLA molecules because the polymorphisms of the peptide antigen-binding cleft of the MHC-I molecule that distinguish HLA alleles alter the spectrum of selected peptides. In the presence of appropriate costimuli, allogeneic HLA/peptide complexes probably stimulate powerful T-cell responses because of the high density of unusual determinants and the diversity of new peptide ligands presented by the allogeneic HLA/peptide complexes. Because there are many MHC-linked genes encoding a host of proteins, many lacking known immunologic function, it is likely that polymorphisms in these molecules contribute significantly to the alloresponse (see previous discussion of *Mhc* genetic maps and *Mhc* alleles). Accordingly, many studies have demonstrated an incremental improvement in long-term graft survival with progressively higher levels of HLA matching at HLA-A, -B, and -DR loci. For this reason, HLA matching is essential in allogeneic HSC transplantation and highly desirable in solid-organ transplantation. The degree of HLA matching usually required for renal transplantation is shown in Figure 21.4 and for bone marrow transplantation in Table 21.5. Survival of HSC transplants varies according to the nature of the disease, disease stage, and age of the patient but can be >70% in some cases (see Centre for International Blood and Marrow Transplant Research at www.cibmtr.org/pages/index.aspx). The effect of HLA matching on solid-organ transplantation has been monitored by the Collaborative Transplant Study since 1982 (www.ctstransplant.org/).

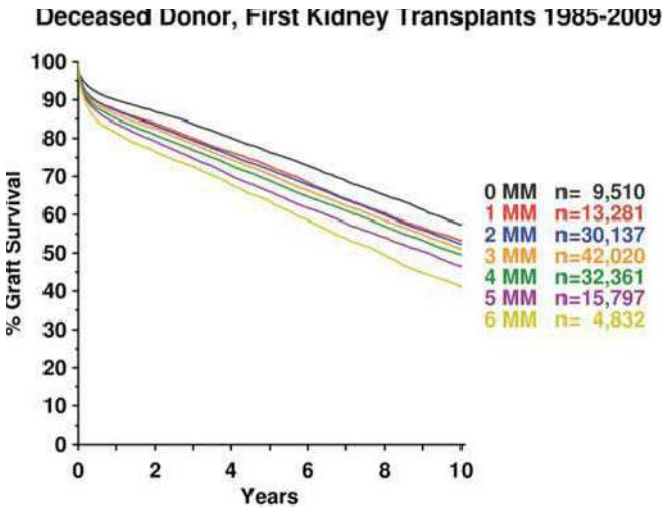

FIG. 21.4. Effect of HLA Matching on First Deceased Donor Kidney Graft Survival. Survival of first kidney transplants based on number of HLA-A, -B, and -C mismatches as a function of time. Courtesy of Collaborative Transplant Study, G. Opelz, University of Heidelberg (www.ctstransplant.org/public/graphics.shtml).

TABLE 21.5 MHC Matching versus Success of Bone Marrow Transplantation

MHC Compatibility	Risk of Graft Rejection (%)	Risk of Acute Graft-versus-Host Disease (%)	Survival (3 Years) (%)
Share two haplotypes (HLA identical sibling)	2	40	50
Share one haplotype plus phenotypically identical	7	40	50
1 HLA mismatch	9	70	50
>2 HLA mismatches	21	80	15
Share zero haplotypes (unrelated)			
"Matched"	3	80	35
"Mismatched"	5	95	35

HLA, human leukocyte antigen; MHC, major histocompatibility complex.
Reprinted from Christiansen FT, Witt CS. Allogeneic bone marrow transplantation. In: Bradley J, McCluskey J, eds. *Clinical Immunology*. Oxford: Oxford University Press; 1997:445, with permission.

In addition to the allogeneic cellular response, the antibody response to HLA molecules and ABO blood groups can also cause rejection of certain grafts, especially where these antibodies are preformed and, therefore, present at the time of organ transplantation. Antibodies to ABO blood-group antigens react with these determinants on vascular endothelium, and, therefore, ABO-incompatible solid organs can be rapidly rejected by humoral mechanisms. In patients who have been transfused or previously transplanted, or in multiparous females, exposure to allogeneic HLA molecules can also result in the production of anti-HLA class I antibodies. These preformed antibodies can lead to acute and hyperacute rejection of grafts expressing the particular HLA molecules recognized by these antibodies. Renal graft survival improves with fewer HLA mismatches. In Figure 21.4, cumulative data for graft survival are plotted as a function of time. Curves represent those groups with the indicated number of mismatches. Therefore, for solid-organ transplants, individuals are not only matched as closely as possible for their HLA types to avert cellular rejection but, it is also necessary to ensure ABO compatibility and to exclude preformed antidonor HLA antibodies in the host. Paradoxically, some patients who have received multiple blood transfusions prior to transplantation appear to develop some form of T-cell tolerance to allogeneic donor HLA alleles; renal graft survival is actually enhanced in these individuals. This is known as the "transfusion effect," and in some centers, pretransplant transfusion and even donor-specific transfusions are routinely carried out.

Transfusion of potential renal transplant recipients carries the risk, however, of inducing undesirable anti-HLA

antibodies in the patient. Testing for anti-HLA antibodies is known as the "crossmatch." In practice, many laboratories crossmatch only for anti-HLA class I antibodies. Crossmatch compatibility to exclude anti-HLA class I antibodies is essential in renal transplantation and is widely practiced in heart/lung transplantation. Crossmatching for liver transplantation is practiced at only some centers, and the evidence that a positive crossmatch predicts allogeneic liver graft rejection has not convinced everyone of its importance in routine matching. Patients awaiting renal transplantation are usually monitored regularly for anti-HLA class I antibodies because the level and specificity of these antibodies can change with time. This monitoring involves regular crossmatching of patient serum against a panel of randomly selected cells bearing different HLA types. The percentage of positively reacting cells is known as the "panel reactivity." When carrying out a crossmatch between a patient's serum and donor cells, many centers test the current as well as "historical peak" serum from the patient. The historical peak is defined as the patient serum sample giving the highest panel reactivity throughout the monitoring period and is thought to be a reflection of previous HLA sensitization. Most centers now prescreen patients on transplant waiting lists for anti-HLA antibodies using MHC-coated beads as a source of antigen and a highly sensitive flow cytometry technology for their detection. In highly sensitized patients with multiple host antidonor antibodies, these unwanted antibodies can sometimes be functionally eliminated using B-cell ablation therapies (anti-CD20 monoclonal antibodies), plasmapharesis, and intravenous gammaglobulin infusions, thus allowing successful transplantation. Similar approaches, coupled with plasmapharesis, are now taken with increasing frequency in transplanting kidneys across the ABO barrier. The role of antibody crossmatching in allogeneic HSC transplantation is less clear, and many centers do not take the class I or class II crossmatch into account when identifying a bone marrow transplant donor. On the other hand, some large centers place considerable importance on a positive crossmatch as a predictor of bone marrow rejection, and it is therefore advisable to crossmatch bone marrow donor–recipient pairs when there is a high risk of rejection (eg, aplastic anemia). Crossmatching is also used to detect anti-HLA antibodies that may cause refractoriness to platelet transfusion with random platelets.

Family Studies in Histocompatibility Testing

The linkage of HLA loci on chromosome 6 means that individuals will usually inherit a set of nonrecombined HLA alleles encoded at linked HLA loci from each parent. This set of genes (the haplotype) is often identifiable in family studies, where all the alleles present on one chromosome cosegregate. In identifying donors for HSC transplantation, testing of family members is essential to determine haplotypes accurately.[188,189] This is because sharing of HLA antigens from different haplotypes is quite common in families, so mismatches within HLA subtypes (ie, allele-level mismatches) are easily overlooked as a result of mistaken haploidentity of siblings or other family members. Because unrecognized HLA mismatching is poorly tolerated in HSC transplantation, high-resolution sequence-based DNA matching (or its equivalent) is required to avoid GVHD.[190–192] The impact of HLA on the outcome of HSC transplants has been studied in unrelated donor–recipient pairs by several groups,[193,194] and guidelines for matching have been published by the U.S. National Marrow Donor Panel.[195] These data show that unrelated matched transplants can be as effective as sibling matched donor–recipient transplants in donor survival. Mismatching beyond a single, or at most two, HLA loci is associated with increased GVHD and inversely with a lower rate of leukemic relapse. Although allogeneic HSC transplantation can be carried out across single-locus mismatches, there is little correlation with the magnitude of a given mismatch (ie, number of polymorphisms between alleles) and the subsequent immune response. Donor–recipient MHC differences of just a single amino acid can provoke strong alloresponses comparable to reactions between vastly different MHC products.[191] An example of haplotyping in a family study is shown in Figure 21.5. Matching for HLA-DP in allogeneic stem cell transplantation appears to improve graft success in both stem cell and renal transplantation,[196,197] but testing for this locus is not routinely carried out clinically for renal transplants and only for HSC when several donors are available and a rational choice of the best donor has to be made.[198,199] HLA-C matching is also important for chronic myeloid leukemia.[200] Typically, an HLA typing laboratory will test for HLA-A, -B, -DRB1, -DRB3, -DRB4, -DRB5, and -DQ loci for HSC transplant matching.[198] Many centers will also insist on HLA-C testing. In the family study shown in Figure 21.5, the mother and father are mismatched at both haplotypes. Among the children, John and Andrew are haploidentical (and therefore phenotypically identical). Jane and Jim share a single haplotype, as do Tom and Jim. Jane's paternal haplotype is a recombinant involving a crossover event between HLA-A and HLA-B. Recombination is observed between HLA-A/B and HLA-B and HLA-DR in about 1% of meiotic events. The implications of this family study are that Andrew and John would be ideal bone marrow donors for each other. However, none of the other siblings would be suitable as a donor for these brothers. Even though there is sharing of a single haplotype between Tom and both Andrew and John, the complete mismatch in the second haplotype would make Tom unsuitable as a donor for HSC transplantation, which requires very close matching of HLA. On the other hand, haplotype mismatching is common in renal transplantation, in which perfect HLA matching is not absolutely required or routinely achievable. However, for renal and other solid organ transplantation, ABO blood group compatibility is usually essential because these determinants are expressed on vascular endothelium where recognition by isohemagglutinins leads to rapid intravascular coagulation and organ failure. When a matched sibling donor does not exist for a patient requiring allogeneic HSC transplantation (70% of cases), searching of the extended family or unrelated bone marrow registries is indicated. The National Marrow Donor

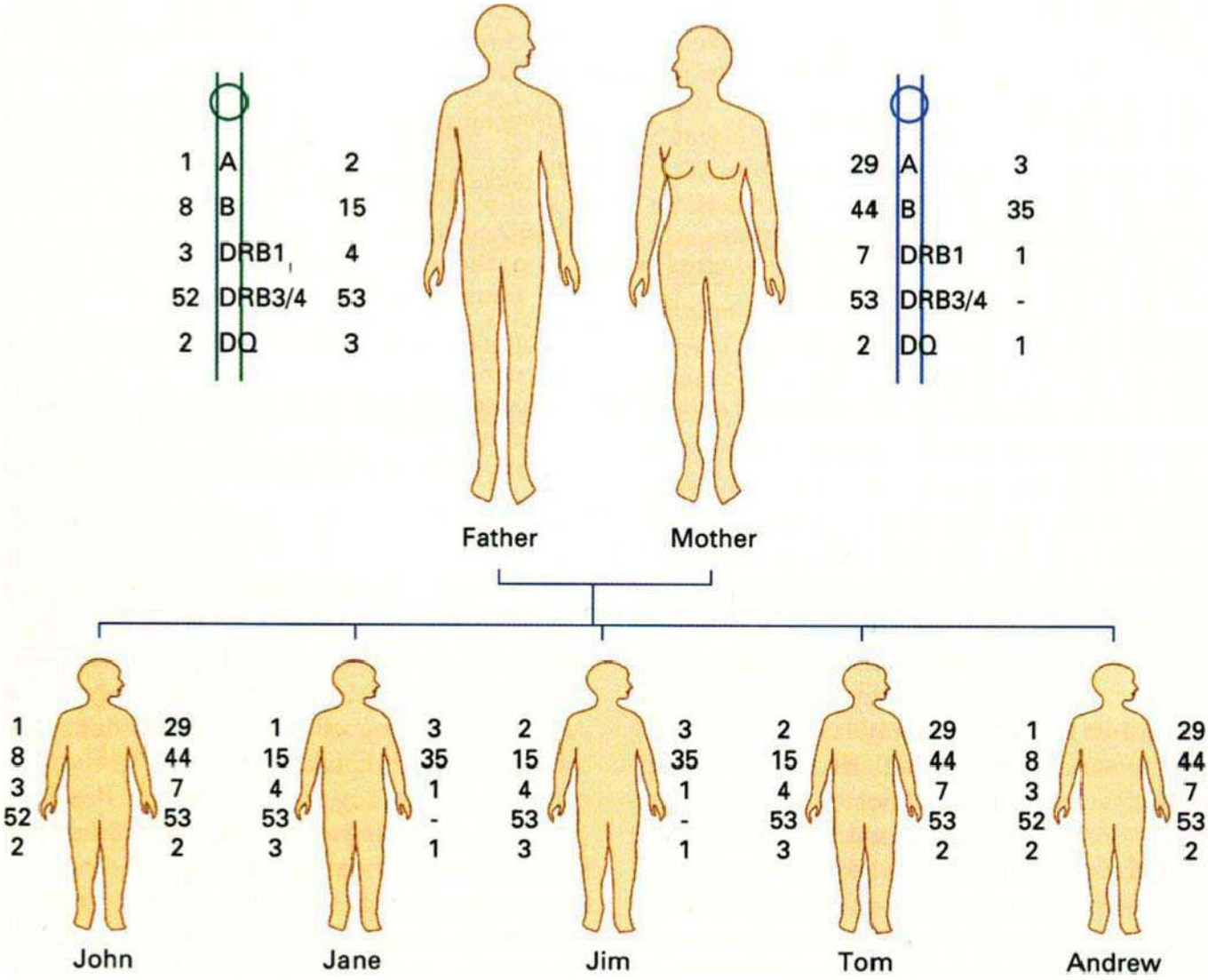

FIG. 21.5. Segregation of Haplotypes in a Family. From McCluskey,[558] with permission.

Panel (www.marrow.org) has several million potential donors suitable for unrelated stem cell transplantation; these donors are used in the United States and worldwide. Bone marrow donor registries also exist in Europe, Australia, Hong Kong, and Japan; these registries often provide donors for patients in other parts of the world. Mobilization of stem cells in the blood following administration of hematopoietic growth factors is now widely used to avoid the need for marrow collections from donors.

In the last decade, many HSC transplant protocols have sought to reduce the pretransplant conditioning of patients. There have also been controversial reports indicating dramatic outcomes of HSC transplantation where deliberate KIR-ligand mismatching leads to donor-versus-recipient NK-cell alloreactivity toward cells of hematopoietic origin.[201–204] The Velardi group in Perugia has championed this approach providing evidence that in the absence of GVHD, a high frequency of donor NK cells in KIR-ligand mismatched transplants can remove recipient's target cells.[202] In particular, HLA haplotype–mismatched hematopoietic transplants can be beneficial in adult acute myeloid leukemia and childhood acute lymphoblastic leukemia leading to the development of adoptive NK-cell transfers as an experimental therapy for graft-versus-leukemia treatment.[205] However, these transplants must be rigorously T-cell depleted, and variable results appear to reflect different transplant protocols, disease parameters, and KIR-mismatching assignment.[203,204,206]

Cord blood banks have also been established around the world. However, cord blood donation of stem cells is often unsuitable for adult transplantation because of limitations in the volume of cord blood collections providing inadequate numbers of stem cells. Many centers will combine different cord bloods that are matched with the recipient as much as feasible to increase the stem cell number. Cord bloods offer the advantage of finding donors faster than adult unrelated registries[197] and theoretically providing banked stem cells from ethnic minority groups that are not well represented in bone marrow donor registries.[198] Cord blood transplants induce less GVHD than bone marrow or peripheral blood stem cell transplants, but posttransplant engraftment is

slower.[196,207] Cord transplants may also tolerate greater levels of HLA mismatching than unrelated bone marrow transplant, but this is dependent on the transfer of sufficient CD34+ stem cells.[208] The results of transplants using bone marrow or peripheral blood stem cells are now considered comparable clinically,[207] but the impact of HLA matching in patients transplanted at early stage in their disease appears to be more marked. Functional tests of HLA compatibility testing for HLA identity at all HLA loci is a daunting task for most laboratories because of the very large number of alleles present in the population. Moreover, in renal transplantation, some mismatches appear to be well tolerated and are associated with long-term graft survival, whereas other mismatches of similar genetic disparity are poorly tolerated and are associated with early rejection.[209,210] Reliable methods for predicting these "taboo" mismatches are not readily available. Similarly, high-resolution HLA typing does not predict all GVHD when selecting suitable unrelated donors for HSC transplantation.[190,211] Therefore, there has been a great deal of interest in developing functional or in vitro cellular tests of overall donor–recipient compatibility. Unfortunately, none of the tests so far developed provides convincing predictability of impending graft rejection or, more importantly, GVHD.

Among the tests used historically for assessing functional compatibility are the mixed lymphocyte reactivity (MLR) test and allogeneic T-helper or cytotoxic T-lymphocyte precursor studies. The MLR or MLC (C = culture) involves measuring T-cell proliferation of host T cells in response to donor lymphocytes and vice versa. In a one-way MLR, the stimulating lymphocytes are irradiated to prevent their proliferation; in the two-way MLR, both stimulator and responder cells are allowed to proliferate. In MLR studies, it is necessary to include controls showing that responder cells can all respond and that stimulator cells can all stimulate across an appropriate barrier such as third-party donor cells. Responses can vary widely, and individual laboratories use their own cutoff values to define negative (ie, nonreactive) and positive (ie, reactive) MLR results.[211] Unfortunately, known HLA mismatches can be present in a negative MLR, and a positive MLR can be obtained between phenotypically HLA-identical transplant pairs. Because the MLR is biased toward measuring HLA class II discrepancies and is notoriously irreproducible,[211] most laboratories have abandoned this test in favor of implementation of high-resolution polymerase chain reaction–based HLA class II typing. Measurement of allogeneic cytotoxic T-lymphocyte or helper T–cell precursor frequencies is carried out at specialized bone marrow transplant centers but is not universally accepted as being predictive of GVHD.[212,213] The test is labor intensive and requires a skilled technician for reproducibility. Precursor frequencies are estimated by limiting dilution analysis or ELISpot of donor-versus-host lymphocytes (ie, T cells expected to cause GVHD). High precursor frequencies (up to 1 in 10^4 cells) are thought to be associated with a greater risk for acute GVHD.[214] It is possible that precursor studies detect major and minor incompatibilities, and so, theoretically, they might give a broad measure of the transplant barrier, but technical improvements will be required before this test is widely adopted in clinical practice.[215]

The *Mhc* and Disease

In addition to the control of transplant acceptance or rejection and immune responsiveness, the *Mhc* in the human plays an important role in the etiology of a number of diseases, many of which are autoimmune in nature.[123,216–218] Several human diseases are associated with the *Mhc-III* genes because some of the structural genes for enzymes involved in the adrenal steroid biosynthetic pathway (ie, 21-hydroxylase, CYP21A2) map to this region. As many as 200 diseases have well-established genetic linkages to the *Mhc*,[217,219–222] the most important of which are summarized in Tables 21.6 and 21.7. Recent genome-wide association studies confirm the importance of the HLA-DRB1 locus in rheumatoid arthritis and type 1 diabetes,[71,223–225] and fine mapping analysis suggests that a total of five amino acid polymorphisms (three in HLA-DRB1, one in HLA-B, and one in HLA-DPB1), all located in peptide-binding grooves, almost completely explain the MHC association with the risk of rhuematoid arthritis.[226] The precise mechanisms underlying the association of most of these diseases with the particular *Mhc* haplotypes are unknown, but several models have been proposed, including the cross-reactivity of antimicrobial antibodies and the molecular mimicry of

TABLE 21.6 Some HLA Disease Associations

Disease	MHC-I	Strength of Association
Ankylosing spondylitis	HLA-B27	+++
Reiter disease	HLA-B27	++
Psoriasis	HLA-C*06	+
Abacavir drug hypersensitivity	HLA-B*57:01	+++
Behcet disease	HLA-B*51	+
Birdshot retinopathy	HLA-A*29	+++
	MHC-II	
Narcolepsy	HLA-DQB1*06:02	++
Insulin-dependent diabetes mellitus	HLA-DQ8	++
	HLA-DQ2	+
	HLA-DR2	–
Rheumatoid arthritis	HLA-DR4	+
Celiac disease	HLA-DQ2	+++
	HLA-DQ8	+
Multiple sclerosis	HLA-DR2	+

HLA, human leukocyte antigen; MHC, major histocompatibility complex
+++ = very strong association that is clinically useful as a diagnostic tool
++ = strong association with likely primary involvement in disease pathogenesis
+ = clear association with likely role in disease pathogenesis
– = negative or protective influence on disease probability
For a more detailed summary of MHC and disease associations see Shiina et al.[220]

TABLE 21.7 HLA Disease Associations and Relative Risk[a]

Disease	Antigen	Estimated or Relative Risk
Graves disease or myasthenia gravis	B*08:01 DRB1*03:01 DQA1*05:01 DQB1*02:01	4 or 2.5
Multiple sclerosis	DRB1*15:01 DQB1*06:02	4
Multiple sclerosis	DQA1*01:02	4
Psoriasis	C*06:02	5
Celiac disease	DQA1*02:01/DQB* 02:02 (DQ2.2)	1
	DQA1*05:01/DQB1* 02:01 (DQ2.5)	7
SLE	DRB1*15:01	2
Type 1 diabetes or SLE	DRB1*03:0101	4.5

[a] These examples were taken from single nucleotide polymorphism associations reported by de Bakker et al.,[71] where the tag single nucleotide polymorphisms and coefficients of determination (r^2) are also reported. For celiac disease, DQ2.2 only has an effect when in combination with DQ2.5. The relative risk of DQ2.5 changes depending on the coordinate presence of other DQ types.

SLE, systemic lupus erythematosus.

viral antigens that might induce T-cell responses to self-antigens.[227–229] The very high incidence of some diseases associated with certain HLA genes assists in the diagnosis as well as the counseling of patients and their families. Several of these diseases are of particular note. Because virtually 100% of patients with narcolepsy have HLA-DQB1*06:02 (associated with HLA-DR2),[230,231] HLA typing can be used as a test of disease exclusion. Thus, a diagnosis of narcolepsy can be excluded with reasonable certainty if the patient does not have HLA-DQB1*06:02. On the other hand, the presence of HLA-DQB1*06:02 is of little predictive value in diagnosis of narcolepsy because this HLA type is relatively common in many populations and occurs frequently in the absence of disease.

Ankylosing spondylitis is so strongly associated with the *Mhc-I* allele HLA-B27 and the presence of some bacterial pathogens that it is a popular hypothesis that ankylosing spondylitis is due to the stimulation of particular T cells by HLA-B27–presented bacterial antigens that cross-react on self-tissues. These T cells are then thought to initiate an inflammatory cascade. Despite the strong association of HLA with spondyloarthropathy, critical evaluation of the literature brings a postinfectious etiology into question and certainly more studies are indicated.[232,233] Recent genome-wide studies strongly indicate that a genetic interaction between an enzyme involved in processing MHC-I–associated peptides, ERAP1, and HLA-B27 contributes to disease susceptibility.[234] The tendency of HLA-B27 to form disulfide-linked covalent dimers raises the question whether the resulting cellular pathology related to poor cell surface expression of this MHC-I molecule may be related to the inflammation of joints that is characteristic of this disease.[235,236]

Hereditary hemochromatosis is one of the most common genetic disorders in Caucasian populations (with a prevalence of 1/300 to 1/400). The gene controlling this condition (*HFE*) is *Mhc* linked, mapping approximately three Mb telomeric to the HLA-A locus.[122] The HFE protein is a class I–like molecule, the structure of which has been determined.[237,238] HFE assembles with β2m and is expressed in the intestinal mucosa and placenta, where it plays a role in regulating iron uptake and transport.[239,240] Mice that are homozygous for an induced defect of β2m as well as those with targeted inactivation of the HFE gene suffer from iron overload or hemochromatosis.[241–244] Mutations at loci other than β2m or HFE also lead to the same disease phenotype.[245] HFE regulates the affinity of the receptor for transferrin, resulting in an alteration of the efficiency of iron transport. The most common molecular defect associated with hereditary hemochromatosis involves a point mutation that results in a Cys282Tyr substitution in the α3 domain of this class I–like molecule.[122] This mutation accounts for >80% of patients with hereditary hemochromatosis.[246] The disruption of the disulfide bond in the α3 domain at this site prevents efficient folding of the molecule and impairs assembly with β2m, resulting in improper HFE protein expression. This leads to a failure to downregulate the affinity of the transferrin receptor for its ligand, transferrin, presumably causing increased iron uptake by cells and tissue damage as a result of iron overload. A second common HFE mutation, 187G, results in a His63Asp substitution and a very slight increase in susceptibility to developing hereditary hemochromatosis, depending on the genotype of the individual.[247] Incomplete penetrance of even the high-risk Cys282Tyr *HFE* genotypes can be partly explained by natural iron deficiency from limited dietary intake and menstrual losses in women.

As summarized in Tables 21.6 and 21.7 and discussed previously, there are a number of autoimmune diseases associated with particular alleles of HLA class II loci, especially with DR and DQ.[221] These diseases include type 1 (insulin-dependent) diabetes mellitus, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, thyrogastric autoimmunity, Sjögren syndrome, and many others. Rheumatoid arthritis is strongly associated with HLA-DR4 subtypes that share a common sequence motif within the DRβ chain,[248] suggesting antigen presentation of self-epitopes by these molecules. The relative risk of severe rheumatoid arthritis is increased in DR4 homozygotes and particularly in compound heterozygotes with high-risk alleles,[249,250] indicating a gene dose effect in susceptibility to autoimmune inflammation.

Although the number of different *HLA* class I and class II alleles that are associated with type 1 diabetes clearly indicates that this relatively common disease has a complex etiology, the identification of a novel *Mhc-II* haplotype in the mutant nonobese diabetic mouse,[251–255] and the recognition that particular TCRs can mediate disease,[256] suggest that a cross-reactive response to a common self- or environmental antigen may play an important role in the etiology of this disease as well. In human type 1 diabetes, the incidence of disease is significantly increased in Caucasians with HLA-

DR3-DQ2 and DR4-DQ8 haplotypes.[257–259] These haplotypes impart a synergistically increased relative risk when they occur as a heterozygous combination compared to the risk of disease conferred by either haplotype alone.[258–260] This raises the possibility of transcomplementation of HLA-DQ gene products producing new molecules involved in antigen presentation.[261] Analysis of large cohorts of patients in genome-wide marker studies offers a promising approach to identify additional genetic factors that act synergistically with HLA allelic linkages in raising the odds of disease susceptibility.[262] Online genomic maps and correlations of associations with particular loci further augments our ability to understand the relative contributions of MHC-linked and MHC-unlinked genes in autoimmune diseases such as type 1 diabetes.

Celiac disease is an underdiagnosed inflammatory disorder affecting the small intestine[263] but resulting from a Th1 hypersensitivity to wheat gluten and related proteins present in barley and rye. Normally manifest in childhood as a failure to thrive, the disease may affect adults quite later in life leading to malabsorption and wasting.[264] Celiac disease can be subclinical, presenting with anemia, osteoporosis, or neurologic symptoms.[263] This disease is strongly associated with HLA-DQ2 and/or HLA-DQ8 (DQ3)[265] as well as with autoantibodies to the enzyme tissue transglutaminase (TG2). Deamidated gluten peptides are presented to CD4+ T cells by HLA-DQ2 or HLADQ8 molecules via macrophages, dendritic cells, and B cells.[266,267] HLA-DQ2 preferentially binds peptides that contain glutamic acid residues at P4 or P6/P7. The dominant gluten peptide is thought to have a low affinity for HLA-DQ2 but this is improved if P6 glutamine is deamidated first.[266,267] HLA-DQ2 forms a network of hydrogen bonds that bind a dominant deamidated gliadin determinant.[268] This leads to enhanced Ag presentation and T-cell recognition with intestinal inflammation in susceptible patients.[266,267,269] The protease resistance of the dominant DQ2-restricted gliadin peptides contrasts with DQ8-associated disease where the dominant gliadin peptides are protease sensitive. The HLA-DQ2–associated and HLA-DQ8–associated forms of celiac disease have their own epitope patterns of deamidation dependence leading to T-cell reactivity. The DQ2-linked peptides possess single residue deamidation at P6 as compared with the preferred deamidation at positions P1 and P9 for DQ8-associated celiac disease.[270] Deamidation of both P1 and P9 to form glutamate residues engenders peptide salt-bridges with HLA-DQ8.[271] Whereas the mechanism of immunopathology of celiac disease is well defined, the exact combination of factors that trigger celiac pathology is still mysterious.[272]

HLA and Drug Reactions

Recently, a number of adverse drug reactions (ADRs) have revealed strong associations with particular HLA types[273] (Table 21.8). The most notable HLA associations are HLA-B*57:01 with abacavir hypersensitivity syndrome in the Caucasian population,[274–276] HLA-B*15:02 with carbamazepine induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Asian populations,[277–279] and HLA-B*58:01 and allopurinol hypersensitivity syndrome and Stevens-Johnson syndrome/toxic epidermal necrolysis.[280–282] Odds ratios or relative risks of these associations are greater than 500, 1000, and 800, respectively. These ADRs are all associated with HLA class I allotypes, with evidence of T-cell involvement.

TABLE 21.8 HLA–Linked Drug Hypersensitivity Reactions

	Drug	Adverse Drug Reaction	HLA Association
MHC class I associations	Abacavir	HSS	B*57:01
	Allopurinol	SJS/TEN and HSS	B*58:01
	Carbamazepine	SJS/TEN	B*15:02
	Feprazone	Fixed drug eruption	B22
	Flucloxacillin	Hepatitis	B*57:01
	Sulfamethoxazole	Fixed drug eruptions	A30, B13, Cw6
	Sulfonamides (including sulfamethoxazole)	TEN	A29, B12, DR7
	Levamisole	Agranulocytosis	B27
	Oxicam	SJS/TEN	A2, B12
	Phenytoin	SJS/TEN	B*15:02
MHC class II associations	Aspirin	Asthma	DPB1*03:01
		Urticaria	DRB1*13:02-DQB1*06:09
	Hydralazine	Systemic lupus erythematosus	DR4
	Lapatinib	Hepatotoxicity	DRB1*07:01-DQA1*02:01-DQB1*0202/0203
	NSAIDs	Anaphylactoid and cutaneous reactions	DR11
	Ximelagatran	Elevated alanine aminotransferase	DRB1*07, DQA1*02

HLA, human leukocyte antigen; HSS, allopurinol hypersensitivity syndrome; MHC, major histocompatibility complex; NSAID, nonsteroidal anti-inflammatory drug; SJS, Steven-Johnson syndrome; TEN, toxic epidermal necrolysis.
Adapted from Bharadwaj et al.[273]

The kinetics of the syndromes are also consistent with T-cell–mediated drug hypersensitivity.[276,283–286] The systemic features of many HLA-linked ADRs suggest that general mechanisms might mediate these reactions. Abacavir-pulsed APCs expressing HLA-B*57:01, but not closely related allotypes, stimulate CD8-positive T cells that are drug-specific.[287] It is now known that abacavir specifically binds the HLA-B*57:01 Ag-binding cleft straddling the central pockets and occupying the F-pocket which normally accommodates a tryptophan anchoring the carboxyl terminus of the peptide.[287a] Consequentially, the chemical environment of the cleft is changed which in turn alters the range of self-peptides that now bind HLA-B*57:01. Accordingly, abacavir induces a dramatic shift in the endogenous peptides selected for binding by HLA-B*57:01. This leads to a state of "altered self" that stimulates a broad range of CD8+ T cells reactive in a GVHD-like manner, explaining the clinical manifestations of the drug hypersensitivity.[287] The mode of abacavir binding provides a basis for understanding its exquisite specificity in binding to HLA-B*57:01 and not to closely related allotypes with polymorphisms in the C, D, E, or F pockets. It will be interesting to learn whether this represents a general mechanism for HLA-linked drug reactions applicable to other small molecule drugs that might bind specifically to HLA antigen-binding clefts. HLA testing before administration of these drugs is now recommended by many physicians and the U.S. Food and Drug Administration in order to avoid life-threatening ADRs.[273,288] The very high relative risks of abacavir, carbamazepine, and allopurinol ADRs and HLA class I allotypes suggests a very high level of specificity in HLA binding, either directly or through a drug-peptide conjugate(s) that only binds the associated HLA allotype. Interestingly, such high relative risks are not commonplace in HLA allotypes and protective immunity, perhaps because it is unusual for any single determinant or HLA allotype to control protective phenotypes.

In addition to recent progress in recognizing *Mhc* associations with adverse reactions to small molecule drugs and a variety of autoimmune and immune deficiency conditions, further analysis has identified relationships of HLA-linked markers with susceptibility and prognosis in a number of infectious diseases. Among the most striking has been the recognition that delayed progression of human immunodeficiency virus (HIV) infection to acquired immunodeficiency syndrome correlates with possession of HLA alleles that express a molecule with a "HLA-Bw4 motif" (see Table 21.2) containing isoleucine at position 80 along with a gene for the NK-cell receptor KIR3DS1. This observation suggested that KIR3DS1 offered protection early in HIV infection for those patients bearing the appropriate Bw4 allele.[289] More recent observations indicate that patients with such a KIR3DS1/B*04 ile80 genotype also exhibit resistance to late opportunistic infections.[290] Other studies now show that particular *HLA-DRB* alleles as well as *MHC-I* alleles significantly influence hepatitis B and C virus susceptibility, persistence, and response to treatment.[291–293]

Mutations in the *H2* Region

The rapid pace of improvements in deriving mouse and other models for genetic disease based on the engineering of transgenic, targeted deletion and mutation, and conditional knockout mice emphasizes the importance of understanding the valuable reservoir of *Mhc-I* and *Mhc-II* mutants originally derived by laborious methods and still available for precise studies of the role of the *Mhc* in immune responses. Mutations at the *H2* locus have been identified in animals screened by skin grafting in extensive experiments carried out over a 25-year period.[294,295] By grafting tail skin of siblings to and from each other, spontaneous or induced mutant animals that displayed either "gain," "loss," or "gain plus loss" transplantation phenotype were identified. Gain mutants are those that express a new transplantation antigen—thus their skin is rejected by their nonmutant siblings; loss mutants have lost a transplantation antigen—thus they recognize the skin of their siblings as foreign and reject that graft. Gain plus loss mutations give effects in both directions—they reject the skin of their siblings, and their skin is rejected by their siblings as well. In classic studies, Melvold, Kohn, and colleagues screened a large number of mouse progeny.[294,295] Homozygous inbred and F1 animals were examined, yielding a total of 25 *H2* mutations identified at *K*, *D*, *L*, and *Ab* loci, and an additional 80 mutations of non-*H2* histocompatibility genes. Although earlier studies suggested that all *H2* genes might be hypermutable, a more complete retrospective evaluation of the available data suggests that with the exception only of the *H2-K*^{b} gene, the spontaneous mutation rate for *H2* genes was comparable to that for non-*H2* genes. The characterization of these mutant animals, first based on peptide maps and amino acid sequences of the H2 proteins,[296–298] and later based on the nucleotide sequences of the cloned complementary DNAs or genes,[159] provided some of the basic biochemical information on which further studies of structure and function and mechanism of gene evolution were based. X-ray structure determination of the H2-K^{bm1} and H2-K^{bm8} mutant proteins suggests explanations for the differences in T-cell recognition that result from what might appear to be subtle amino acid substitutions.[165]

Control of Expression of MHC Molecules

MHC molecules, synthesized in the ER and destined for cell surface expression, are controlled at many checkpoints before their final disposition as receptors available for interaction with either T cells or NK cells. The classical MHC-I molecules are trimers, consisting of the polymorphic H2 or HLA heavy chain, the light chain β2m, and the assembled self-peptide. Thus, the cell surface expression of the assembled trimer may be reduced by a defect in the synthesis, steady state, or assembly of any of these requisite protein/peptide chains. The first level of MHC-I expression control is transcriptional; interferon-γ–dependent regulation is particularly important.[299] For the most part, MHC-I molecules are ubiquitously expressed. Their expression is dependent on a complex regulatory process that coordinately controls the transcription of both MHC-I and β2m.[300,301] The basis of the

more limited tissue specific expression of MHC-Ib molecules is of interest because of the potential importance in the role of some of the MHC-Ib molecules in tolerance to the placenta. HLA-E and HLA-G, expressed on placenta, and HLA-F, another MHC-Ib with limited tissue specific expression, have been examined in considerable detail.[116,302–305] Another critical level of control of MHC-I expression is genetic, that is, the genes for a particular chain or key components of the PLC must be present for the trimer to be assembled and then expressed at the cell surface. A decrease in heavy chain synthesis as a result of a structural mutation in a coding (such as HLA-A*30:14L [see Table 21.1]) or in a noncoding region (such as HLA-A*24:02:01:02L) would reveal the simplest level of control. In addition, the light chain is absolutely required for cell surface expression of most MHC-I molecules. Deficiency of β2m in various tumors is a relatively common occurrence.[306–308] Induced β2m defective animals (*B2m*$^{o/o}$, "β2m knockouts") lack normal levels of MHC-I expression because of this requirement for cell surface expression.[309,310] In addition, polymorphism of the MHC-I heavy chain may influence the efficiency with which the molecule can interact with the PLC or with which it binds self-peptides, and thus some alleles, because of a decreased ability to be loaded with self-peptides, may not be well expressed at the surface. The rest of the MHC-I biosynthetic pathway is dependent on proper generation of cytosolic peptides by the proteasome and delivery to the ER by TAP, appropriate core glycosylation in the ER, transport through the Golgi, and arrival at the plasma membrane.[311] A number of persistent viruses have evolved mechanisms for subverting this pathway of expression.[312] The herpes simplex virus encodes a protein, infected cell peptide 47,[313–315] that blocks the activity of the peptide transporter TAP.[316] Cowpox virus encodes two proteins, CPXV12 and CPXV203, which contribute to the downregulation of host MHC-I, CPXV12 inhibits TAP, and CPXV203 retains MHC-I in the ER.[317,318] The human disease bare lymphocyte syndrome, type I, manifested by chronic lung disease in late childhood, results from genetic lesions in either TAP1, TAP2, or tapasin, also known as TAP-binding protein.[93,319–323] Several proteins encoded by the human CMV, unique short region proteins 2 and 11 (US2 and US11), cause rapid protein degradation of MHC-I molecules.[324] Murine CMV encodes an MHC-I-like protein, m152/gp40, which inhibits MHC-I expression by diverting MHC-I molecules to rapid degradation in the endosome.[325,326]

Other molecules that assist the large DNA viruses in evading either the T-cell or NK-cell immune response include human CMV UL142.[327] Murine CMV[328] encodes a number of open reading frames considered MHC-I homologues that may function to deceive NK cells into the perception of normal MHC-I expression.[2,329–331] The adenovirus 2 protein, E3/19K, also functions to block the transfer of folded assembled MHC-I molecules from the ER to the Golgi.[332–335]

MHC-II molecules are also susceptible to regulation at multiple steps. The clear-cut tissue dependence of MHC-II expression—MHC-II molecules are generally found on cells, which have specific antigen presentation functions such as macrophages, dendritic cells, Langerhans cells, thymic epithelial cells, and B cells, but can also be detected on activated T cells of the human and rat—suggests that transcriptional regulation plays an important role. Extensive studies of the promoter activities of *Mhc-II* genes have defined a number of specific transcriptional regulatory sequences.[336] One transcription factor, MHC class II transcriptional activator (CIITA) is clearly crucial.[300,336–338] The expression of CIITA is regulated in a complex fashion, possessing at least three distinct promoters that function in a cell type–specific manner.[339] MHC-II deficiency diseases categorized as bare lymphocyte syndrome, type II, result from lesions in at least four different complementation groups, implicating defects in CIITA, RFX5, RFXAP, or RFXANK, all encoding proteins regulating MHC-II transcription.[338,340–344] Polymorphisms in MHC-II and the resulting differences in tissue and lineage-specific expression of MHC-II suggest a role in the control of autoimmune disease.[145,345–347]

A unique aspect of MHC-II regulation is the need to protect its peptide-binding site from loading with self-peptides in the ER and the requirement to traffic to an acidic endosomal compartment where antigenic peptides, the products of proteolytic digestion of exogenous proteins, can be obtained. These two functions are controlled by the type II membrane protein, invariant chain, Ii,[30,348–350] which forms a nine subunit complex consisting of three Ii and three αβ MHC-II heterodimers. The region of Ii that protects the MHC-II peptide-binding groove, the class II-associated invariant chain peptide (CLIP), is progressively trimmed from Ii and is ultimately released from the MHC-II by the catalytic action of HLA-DM in the endosome to allow exchange for peptides generated there. HLA-DO, another MHC-II–like molecule, regulates the repertoire of bound peptides, presumably by modulating the catalytic activity of the DM exchange reaction. The important role of Ii in regulating MHC-II expression has been emphasized by the behavior of induced mutant mice lacking normal Ii[351–353] that exhibit a profound defect in MHC-II function and expression.

STRUCTURE OF MHC AND MHC–LIKE MOLECULES

"There is nothing that living things do that cannot be understood from the point of view that they are made of atoms acting according to the laws of physics."

—Richard Feynman[354]

"Love hides in molecular structures, yeah,
Love is the answer."

—Jim Morrison[355]

So central are *Mhc* genes and their encoded molecules to both the regulation and the effector function of the immune system that it has been apparent almost since their discovery that an understanding of their structure and structural interactions would be fundamental to a comprehension of their physiologic effects. Structural relationships of MHC molecules came first from understanding serologic differences, then from analysis of cellular immune responses, and subsequently from biochemical studies of the MHC-I and MHC-II chains. Amino acid sequence comparisons

suggested a domain structure for the MHC-I molecules, and an exon/domain correspondence was apparent with the initial identification of genomic and complementary DNA clones.[356] Shortly thereafter, a large number of sequences were determined leading to their alignment and comparison. Databases of these sequences are maintained and updated as indicated in previous sections of this chapter, and searches of standard sequence databases may find *Mhc* gene and protein sequences of numerous species. Expression of recombinant clones encoding MHC molecules in several systems: mammalian cells, insect cells, and bacteria, then permitted the accumulation and purification of molecules for functional, binding and ultimately x-ray structural studies.

A current search of the protein data bank (www.rcsb.org/pdb) for the keyword "histocompatibility" yields three-dimensional coordinates of more than 500 MHC/peptide, MHC/peptide/TCR, MHC/peptide/coreceptor, and MHC/peptide/NK receptor complexes that allow an understanding of the function of these molecules in a detailed structural context.[357] However, these structures also pose a number of questions that may only be addressed by additional functional experiments in whole animals complemented by biophysical methods applied in vitro. The molecular biologic, functional, and structural studies have led to the development of the use of MHC multimers as extremely powerful tools for imaging specific T cells or NK cells. The goal of this section of this chapter is to summarize these developments with an eye toward explanation of function by structure and in hopes of revealing some of the current quandaries that continue to confound our understanding of the function of the *Mhc*.

Amino Acid Sequences—Primary Structure

Before the cloning of *Mhc* genes, the biochemical purification and amino acid sequence determination of the HLA-A2, HLA-B7, and H2-K^b MHC-I molecules[358,359] indicated that the MHC molecules had unique amino terminal "domains" and showed similarities to Igs in their membrane proximal regions. Early concerns were to identify the differences between allelic gene products as well as the differences between MHC proteins encoded at different loci. With the cloning of complementary DNAs and genomic clones for MHC-I molecules[156,360–362] and shortly thereafter for MHC-II molecules,[151,363–365] the encoded amino acid sequences of a large number of MHC molecules of a number of species quickly became available. The comparison of gene and complementary DNA sequences gave an indication of the exon/intron organization of the genes and explained the evolution of the MHC molecules as having been derived from primordial single domain structures of a unit size of a single Ig-domain (such as the light chain β2m) that duplicated to form the basic unit of the MHC-II chain (two extracellular domains) and the MHC-I chain (three extracellular domains).[129] The canonical MHC-I molecule has a heavy chain (also known as the α chain), which is an intrinsic type I integral membrane protein with amino terminal domains called α1, α2, and α3, is embedded in the cell membrane by a hydrophobic transmembrane domain and extends into the cytoplasm of the cell with a short carboxyl-terminal tail. The light chain of the MHC-I molecule, β2m, is a single domain soluble molecule that is encoded elsewhere in the genome.

The MHC-II molecule consists of two chains embedded in the membrane as type I proteins, an α chain and a β chain. The α and β chains both consist of two major extracellular domains, α1 and α2, and β1 and β2, respectively, each linked to a transmembrane domain and cytoplasmic sequences. An initial analysis of both MHC-I and MHC-II molecules suggests that they are noncovalently assembled heterodimers consisting of four extracellular domains, the two membrane proximal domains (α3 and β2m for MHC-I and α2 and β2 for MHC-II) of each molecule are Ig-like, while the two amino terminal domains (α1 and α2 of MHC-I and α1 and β1 of MHC-II) possess what is known as the MHC-fold. The α1 domains of both MHC-I and MHC-II lack the intradomain disulfide bond characteristic of the other extracellular domains. The cytoplasmic domain of MHC-I molecules can be regulated by splicing and differential phosphorylation or other modification, and is likely to play a role in cell surface stability and cycling between the cell surface and other intracellular compartments.[366–372] However, analysis of directed mutants of MHC-I in some systems indicates that the cytoplasmic domain is not required for cytoskeletal association or surface recycling.[373] (The cytoplasmic domain of MHC-I molecules is the target for the Nef protein of HIV, and the Nef/MHC interaction results in MHC-I downregulation in HIV-infected cells.[374,375] The MHC-II transmembrane and cytoplasmic domains have clear effects on the level of cell surface expression, the efficiency of antigen presentation, and the rate of lateral diffusion of the molecules in the cell membrane.[376,377] Single particle tracking of MHC-II molecules indicates that such lateral diffusion is governed, at least in part, by interaction with the actin-based membrane skeleton.[378] Amino acid sequence alignments of MHC-I and MHC-II proteins, particularly of the human molecules, are available in a number of databases such as www.ebi.ac.uk/imgt/hla/ and http://www.ebi.ac.uk/ipd/mhc/.

Identification of Peptides Bound by MHC Molecules

Although initial understanding of MHC molecules indicated that they are heterodimers, a complete understanding of their structure and resulting function requires the appreciation that they are, in fact, heterotrimers, in which the third component is a short peptide ranging from eight to roughly 15 amino acids in length. This characteristic of mature MHC-I and MHC-II molecules makes them a unique class of protein molecules because an integral part of their peptide composition and resulting structure derive from a heterogeneous array of peptides delivered to the assembling molecules during their maturation. Many different lines of evidence coalesced over a short period of time to demonstrate that MHC molecules function by binding peptides. From functional experiments, MHC-II–restricted T-cell responses to protein antigens were shown to be dependent on peptide fragments.[379] The first direct evidence of MHC/peptide interactions was that purified MHC-II proteins could

bind synthetic peptides in a specific, saturable, and stable manner[380,381] with measurable affinity and a remarkably slow dissociation rate.[381] For MHC-I molecules, the results were at first less clear, but the realization that some cell lines defective in MHC-I surface expression could be induced to express higher levels of surface MHC-I molecules by exposure to the appropriate peptides[382] led the way for direct measurement of MHC-I peptide binding.[383]

Several laboratories succeeded in developing methods for the partial purification and identification of the peptides that copurified with MHC molecules. A viral, antigenic peptide was identified bound to the MHC-I molecule H2-K^b by recovering MHC molecules from virus infected cells, fractionating the bound peptides chromatographically, identifying a peak of functional biologic activity in a cytotoxic T-cell assay, and determining the amino acid sequence of the recovered peptide by radiochemical techniques.[384] Another method that was applied to the identification of both virus-derived peptides as well as the "motif" of self-peptides by particular MHC-I molecules involved first the isolation of a large amount of detergent solubilized MHC-I using appropriate antibodies, the elution of the bound peptides, their partial purification as pools by reverse phase high pressure liquid chromatography, and the determination of the amino acid sequence of the bound peptides by classic amino terminal sequencing.[385–387] The unpredicted and surprising result obtained from these studies of MHC-I–derived peptides was that specific amino acid residues were favored at particular positions of the sequence, depending on the MHC-I molecule from which the peptides were obtained, and that the length of the bound peptides was well defined and short, ranging from 8 to 10 amino acids. From such experiments, a number of peptide "motifs" of peptides bound to particular MHC-I molecules and allelic products were identified. Often, specific amino acids were identified at particular Edman degradation steps, indicating a common, highly preferred residue at the same spacing from the amino terminus of the peptide. Thus, the peptide "motif" could be determined even from heterogeneous pools of peptides eluted from particular MHC-I molecules. Further refinements in methodology have included the application of mass spectrometry to the identification and sequencing of individual peptides.[388,389] Alternative approaches for identifying peptide motifs include the use of soluble analogs of MHC-I molecules to ease the purification[390,391] or the use of peptide display libraries to identify those peptides that can bind the MHC.[392,393] With steady improvements in technology, including the use of transfected soluble versions of MHC-I molecules, immunoaffinity purification of the MHC-I molecules of interest, tandem reverse phase high pressure liquid chromatography and further identification and sequencing by tandem mass spectrometry, particularly MHC-I motifs, as well as tumor antigen peptides, may now be identified routinely.[394] A summary of MHC-I peptide motifs is given in Table 21.9. Regularly maintained online databases of MHC-I peptide motifs may be found at several sites (www.imtech.res.in/raghava/mhcbn/links.html and www.syfpeithi.de).[395] Antigenic peptides that represent those observed as tumor antigens

TABLE 21.9 Peptide-Binding Motifs for Some MHC Class I Molecules[a]

Position	1	2	3	4	5	6	7	8	9 (or C-term)
MHC-I molecule									
HLA-A*01		(T)	D				(L)		Y
HLA-A*02:01		L(M)				(V)			V(L)
HLA-A*03		L(V,M)	(F,Y)			(I,M,F,V,L)	(I,L,M,F)		K,Y,F
HLA-B*07		P	(R)						L,F
HLA-B*08:01			K		K,R				L
HLA-B*27:05		R							L,F (Y,R,H,K)
HLA-B*35:01		P							Y,F,M,L,I
HLA-B*53:01		P							W,F,L
H2-K^b			Y		F (Y)			L (M,I,V)	*prefers 8-mers
H2-D^b					N				M(I,L)
H2-K^d		Y(F)							I(L,V)
H2-D^d		G	P		(R)				I(L,F)
H2-L^d		P,S							F,L,M
H2-K^k		E						I(V)	*prefers 8-mers
H2-D^k	Motif uncertain	(R)							
Qa-2		(M,L,Q)	(N,I,L)		(V,I)	(K,M,I)	H		L,I,F
H2-M3	formyl-M								
RT1.A1		(A,S,V)	F,Y						Y,F,L,M

[a] Peptide-binding motifs for the indicated MHC class I molecules are shown in the single amino acid code. Position refers to the amino acid position of the peptide from the amino terminus. Only the most common residues are shown. Assignments in parenthesis are less common than the others. These motifs are taken from the more extensive summary of www.syfpeithi.de/, www.imtech.res.in/raghava/mhcbn/links.html, and Biddison and Martin.[562] Full sequences of proteins may be queried for MHC peptides presented by particular alleles with http://bimas.dcrt.nih.gov/molbio/hla_bind/, www.immuneepitope.org/, or www.mpiib-berlin.mpg.de/MAPPP/expertquery.html.

HLA, human leukocyte antigen; MHC, major histocompatibility complex.

and available for recognition by T cells are summarized at www.cancerimmunity.org/peptidedatabase/differentiation.htm. In addition, the immune epitope database (www.immuneepitope.org)[396] offers a regularly updated and curated Web site that describes MHC motifs, allows evaluation of new proteins for MHC epitopes, and provides new computational tools for such evaluation. An algorithm that allows the prediction of candidates for MHC-I–restricted peptides based on the amino acid sequence of the protein of interest is also available (www-bimas.cit.nih.gov/molbio/hla_bind/).[397] The distinction between "motif" residues of an MHC-restricted peptide and "anchor" residues is an important one. Motif refers to those amino acid residues that are identified based on the sequences of self-peptide or antigenic peptides that have been demonstrated to bind or copurify with a particular MHC molecule. Anchor implies a biophysical function of the particular amino acid residue as specifically interacting with a particular part of the MHC molecule itself. The designation of a residue as an anchor residue may be inferred by analysis of binding to peptide variants in the context of the parental peptide. Alternatively, anchors can be defined from a knowledge of the x-ray structure of the peptide complexed to the MHC-I molecule, and predictions of likely peptide motifs for MHC molecules whose sequence is known but whose structure has not yet been determined can be made by comparison to known structures.

The identification of MHC-II–bound self-peptide-or antigenic peptides by biochemical methods similar to those employed for MHC-I molecules has been proved more difficult because the MHC-II molecules do not have the rigorous requirements for a defined amino terminus or restricted length. Whereas MHC-I molecules bind peptides with a particular motif residue at a specific position as defined by the amino terminus, resulting in the ability to identify the dominant residue at a particular step in the Edman degradation even amidst a pool of peptides, MHC-II molecules bind peptides with "ragged ends," and little information is obtained from the standard methods of sequencing of pools of peptides.[398–401] Thus, more precise fractionation of the peptides released from purified MHC-II molecules is required. Identification of MHC-II/peptide-binding motifs by bacteriophage display is also possible.[402] In accord with the view that MHC-II molecules present peptides derived from an "outside in" pathway, many of the peptides that copurify with MHC-II molecules represent molecules derived from the extracellular milieu or the medium in which the cells were grown. Analysis of MHC-II/peptide complexes with cloned T cells and monoclonal antibodies with MHC/peptide specificity reveals that, in part because of the ability of MHC-II molecules to accommodate peptides with extensions at their amino and carboxyl-termini, and in part because of the smaller role that anchor residues seem to play in peptide binding by MHC-II molecules, occasionally even a unique peptide can bind a particular MHC-II molecule in more than one frame.[403–406] As a result of structural studies (summarized in the following) and compilation of sequences of those peptides bound by particular MHC-II molecules, the general conclusion is that MHC-II molecules all have binding pockets identifiable for the particular allelic product. For some alleles, such as HLA-DR1 and IA^d, these pockets are spaced at position P1, P4, P6, and P9 (or i, i + 3, i + 5, i + 9). (Some analyses, borne out in part by structural studies, suggest that a pocket at P7 [i + 6] may also contribute to the energy of binding.) Because of the complexity and potential degeneracy of peptide motifs for MHC-II molecules, more elaborate schemes have been devised for predicting those peptides that may bind to particular MHC-II molecules.[407] A summary of identified peptide-binding motifs for MHC-II molecules is in Table 21.10, but for some alleles, the analysis of MHC-II binding peptides is not straightforward and demands sophisticated algorithms for identifying potential antigenic peptides from new proteins. The site www.imtech.res.in/raghava/mhcbn/links.html tabulates a number of links to various servers for predicting likely peptide epitopes for particular MHC-I and MHC-II molecules. Implicit in the description of MHC-I and MHC-II peptide motifs is that the bound peptides bind to the MHC molecule only in an amino to carboxyl-terminal orientation, that is, that all peptides

TABLE 21.10 Peptide-Binding Motifs for Some MHC Class II Molecules[a]

Position	i (P1)	i + 1	i + 2	i + 3 (P4)	i + 4	i + 5 (P6)	i + 6	i + 7	i + 8 (P9)
Allele									
DRB1*01:01	YVLFIAMW			LAIVMNQ		AGSTCP			LAIVNFY
DRB1*03:01	LIFMV			D		KREQN			YLF
DRB1*04:01	FYWILVM			PWILVADE		STQHR	DEHKNQR..		DEHKNQR..
DRB1*04:05	FYWVILM			VILMDE		NSTQKD			DEQ
DQA1*05:01/B1*03:01	WYAVM			A		AIVTS			QN
H2-IA^b (undefined)									
H2-IA^d	STYEVWMLI..			VLIA		AV			
H2-IA^{g7}	KHSAV			L		VA			DSE
H2-IA^k	DN			IVLN		EQ			
H2-IA^s (undefined)									
H2-IE^b	WFYILV			LIFSA		QNASTHRE			KR
H2-IE^d	WFYILV			KRIV		ILVG			KR
H2-IE^{g7}	ILVFWM			DESMV		QNASTED			RKMF

[a] Major histocompatibility complex class II peptide-binding motifs are summarized from those of Biddison and Martin[562] and www.syfpeithi.de/home.htm.

bound to MHC molecules bind with the amino terminus at the lefthand side of the peptide-binding groove, in the canonical structural representation that will be described in further detail subsequently. However, a recent analysis employing both x-ray crystallography and nuclear magnetic resonance indicates that the CLIP peptide may undergo orientational inversion, binding dynamically in the carboxyl to amino as well as the amino to carboxyl direction.[408]

High-Resolution Structures

MHC-I Molecules

A most graphic description of the relationship of form to function of the MHC molecules was made by Bjorkman et al.[409,410] who determined the three-dimensional structure of the human MHC-I molecule, HLA-A*02:01, by x-ray crystallography. For these studies, the extracellular, soluble portion of the type I membrane-associated molecule was purified by papain cleavage from the surface of tissue culture cells. At the time, there was not a clear appreciation of the role of peptide either in the assembly of the molecule or of the nature of the recognition of the MHC molecule by TCRs or, for that matter, NK receptors. Despite the fact that the first purified HLA-A2 molecules possessed a heterogeneous mixture of bound peptides, protein from these preparations crystallized readily, and electron density maps calculated from the diffraction data were interpretable, allowing modelling of the backbone molecular structure. The most important insight in the interpretation of the derived electron density map was that part of the electron density, and thus part of the structure, was due to this mixture of peptides bound tightly by the molecule, and that this density could not be modeled based on the known amino acid sequence of HLA-A2.

This first MHC-I structure clarified several important aspects of the mechanism by which the MHC-I molecule carries out its peptide binding function. The amino terminal domains (α1 and α2) form a unitary binding site for peptide. This domain unit (also called a "superdomain") consists of a floor of eight strands of antiparallel β-pleated sheet that supports two α-helices, one contributed from the α1 domain and one from the α2 domain, aligned in an antiparallel orientation. The membrane proximal α3 domain has an Ig C-type fold and pairs asymmetrically with the other Ig domain of the molecule contributed by β2m. The nature of recognition by T-cells was suggested by comparing the location of those amino acid residues that had been characterized as being strong elements in T-cell recognition, residues that distinguished closely related allelic gene products, and amino acid residues that had been identified as those that were responsible for the transplant rejection of the mutants of the *H2-K*^{b} series.[409] Amino acid residues of the MHC-I molecule responsible for T-cell recognition were clearly classified into one of two categories or an overlapping set: those residues that were "on the top of the molecule," exposed to solvent and available for direct interaction with the TCR, and those residues whose side chains pointed into the peptide binding groove and might be considered crucial in the peptide-binding specificity of the particular MHC molecule. The original publications, based on a structure determined to a resolution of 3.5 Å, focused mainly on the general structural outline of the molecule. More recently, structures of a wide variety of MHC-I molecules of different species including human, mouse, rat, and chicken, complexed with specific peptides, have been determined. Ribbon diagrams of HLA-A2 as seen from the side (Fig. 21.6A) and from the top (Fig. 21.6B), complexed with a unique synthetic peptide, indicate the design of the entire molecule: the peptide-binding site is supported by the β-sheet floor, and the floor in turn is supported by the two Ig-like domains, the α3 domain of the heavy chain and the β2m light chain. (In the canonical view of the MHC-I, HLA-A2 molecule shown in Fig. 21.6B through D, the amino terminal residue of the bound peptide lies to the left, and the carboxyl terminal residue lies to the right.)

The comparison of this structure with that of the closely related human MHC-I molecule, HLA-A*68:01 (see Table 21.2), suggested that surface depressions in the groove of the MHC-I molecule, now designated pockets A through F, would be available for interactions with some of the side chains of the bound peptide.[411,412] These six pockets are illustrated in Fig. 21.6C and with bound peptide in Fig. 21.6D. Concomitant with the determination of the x-ray structure of the human MHC-I molecule, HLA-B*27:05,[413,414] the motif of the peptides that were recovered from this molecule was determined, permitting the more precise modeling of the bound peptide in the cleft of the MHC-I. For HLA-B27, this was of particular interest because the bound peptides had a strong overrepresentation of arginine at position 2,

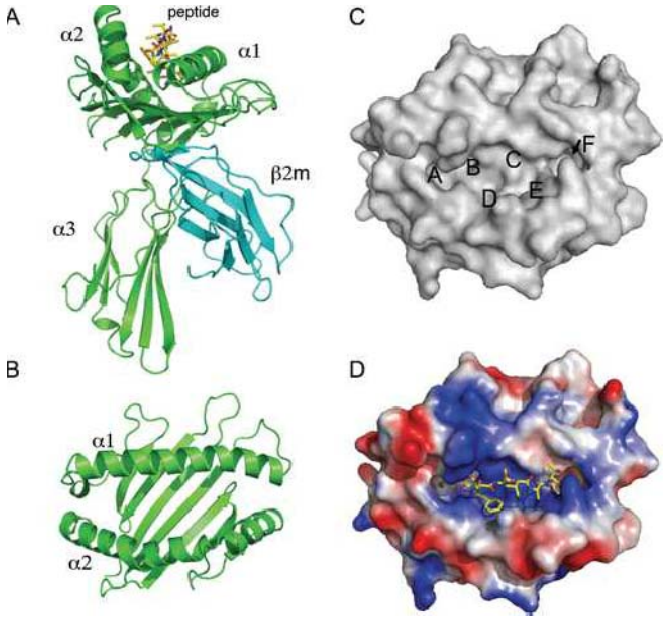

FIG. 21.6. Structure of HLA-A*02:01. **A:** Ribbon representation of HLA-A2 heavy chain *(green)*, β2m light chain *(cyan)*, and bound peptide *(yellow)*. **B:** Ribbon representation of the peptide-binding groove. **C:** Surface representation of the binding groove with pockets labeled. **D:** Surface representation of binding groove colored by electrostatic charge (*red*, acidic; *blue*, basic) with peptide shown in stick illustration. Figure generated from protein data bank[559] structure 2BSV using PyMOL.[560]

and scrutiny of the HLA-B27 structure suggested that the amino acid residues lining the B pocket, particularly glutamic acid at position 45 as well as cysteine 67, were complementary to the long, positively charged arginine side chain of the peptide amino acid at that position. Structural studies supported a view of MHC-I/peptide binding in which the side chain of the carboxyl-terminal residue of the bound peptide sits deep in the F pocket. In addition, the amino terminal amino group of the peptide forms hydrogen bonds with the hydroxyl groups of conserved amino acids tyrosine 59 and tyrosine 171 that line the A pocket. A hydrogen bond from the amino group of conserved tryptophan 147 to the backbone carbonyl oxygen of the penultimate peptide amino acid (usually position 8) also seems important, as do charge interactions and hydrogen bonds of the free carboxyl group at the carboxyl terminus of the peptide with tyrosine 84, threonine 143, and lysine 146.

Other structures of MHC-I molecules were determined involving complexes produced with homogeneous peptide, assembled either in vitro from bacterially expressed proteins with synthetic peptide[415] or exploiting MHC proteins expressed in insect cells.[416,417] The structures determined with homogeneous peptide confirmed the impression obtained from the structures obtained from molecules with heterogeneous self-peptides. Examination of the H2-K^b molecule complexed with synthetic, known antigenic peptides derived either from Sendai virus, vesicular stomatitis virus, or chicken ovalbumin revealed that the same MHC molecule can bind peptides of different sequence, length, and structure by virtue of their conserved motif residue side chains. Although small conformational changes of the MHC are detectable on binding the different peptides, the main distinction in the recognition of different peptides bound by the same MHC molecule is due to the location, context, size, and charge of amino acid side chains that are anchored in the MHC pockets. Conversely, solvent-exposed side chains contribute to the unique structure of the surface of the molecule available for interaction with TCR and other receptors.

The most consistent rule learned from the first x-ray structures and complemented by peptide recovery and early binding studies was that the carboxyl-terminal amino acid side chain of the MHC-I–bound peptide was embedded in the F pocket. A recent study revealing that the MHC-I molecule H2-D^b binds a pentapeptide well anchored in the F pocket, but lacking amino terminal interactions, is consistent with this view.[418] However, other studies indicate that MHC-I molecules may bind longer peptides that extend beyond the residue anchored in the F pocket,[419] an observation confirmed by a crystallographic structure.[420] More recently, the lack of an absolute requirement for a free C-terminal amino acid has been exploited in the engineering of single chain molecules in which peptide, β2m, and the MHC-I heavy chain are covalently linked. These molecules have unusual thermal stability and are effective MHC/peptide immunogens.[421] An additional variation on the theme of MHC–I binding peptides based on particular anchor residues includes the demonstration that glycopeptides, bound to MHC-I via amino acid side chains and termini, can expose their carbohydrate moieties to solvent and be available for TCR interaction.[422–424] An example of a 13-residue peptide bound to its MHC-I–presenting element, the rat MHC-I molecule, RT1-Aa, was crystallized and shown to produce a peptide/MHC complex with a large central bulge. Two different complexes consisting of the same MHC and peptide reveal significantly different conformations in this central bulge region.[425] "Bulged" viral peptides have also been characterized in complex with human MHC-I structures of HLA-B35 allotypes complexed with 13-mer and 11-mer peptides.[426,427] TCR recognition of such bulged peptides can involve conformational adjustments of the TCR in recognizing a fairly rigid peptide[426] or "crumplings" of the bound peptide by a largely rigid face of the TCR upon binding the MHC-I/peptide complex.[428] Another set of modified peptides that can be bound by MHC-I molecules and presented to TCR includes self-peptides modified by phosphorylation.[429,430] Although the fundamental topology of MHC-I structure and its interactions with antigenic peptide have been solved by the classic structures mentioned previously, many more MHC-I/peptide complexes have been studied crystallographically in order to define the details of particular interactions with antigenic peptides. A number of structures at a resolution of 1.40 Å or better have been deposited in the protein database. Structure determination of representative single-chain peptide-β2m-MHC-I,[431] and of MHC-I molecules containing photolabile peptides that can be exchanged with other peptides easily,[432] reinforce our basic understanding of the structure of MHC-I/peptide complexes.

MHC-II Molecules

Before any MHC-II structure had been determined experimentally, a model was constructed based on the alignment of amino acid sequences and the available MHC-I three-dimensional structure.[433] This model made several valid predictions that were borne out by the subsequent x-ray structure determination of HLA-DR1.[434] MHC-II clearly showed similarity to MHC-I and formed its binding groove by the juxtaposition of the α1 and β1 domains. The position of the electron density representing the heterogeneous peptide that copurified with the HLA-DR1 was identified. Figure 21.7A and C show a ribbon diagram of HLA-DR1 with a homogeneous bound peptide. In comparison to the MHC-I structure (see Fig. 21.6), the peptides bound to the MHC-II molecule extend through the binding groove rather than being anchored at both ends.

A comparison of the α-carbon backbone of the peptide-binding region of an MHC-I structure with that of an MHC-II structure is shown in Figure 21.8. The structures are very similar although the binding domain is built of the α1 and α2 domains from the same chain (for MHC-I) or of the α1 and β1 domains that derive from two chains (for MHC-II). The location of polymorphic residues can be determined by variability plots based on multiple sequence alignments, as originally suggested by Wu and Kabat.[435] The current bioinformatics approach to variability prefers to calculate *Shannon entropy*, a function that describes the degree of unpredictability of an event.[436,437] For protein sequence

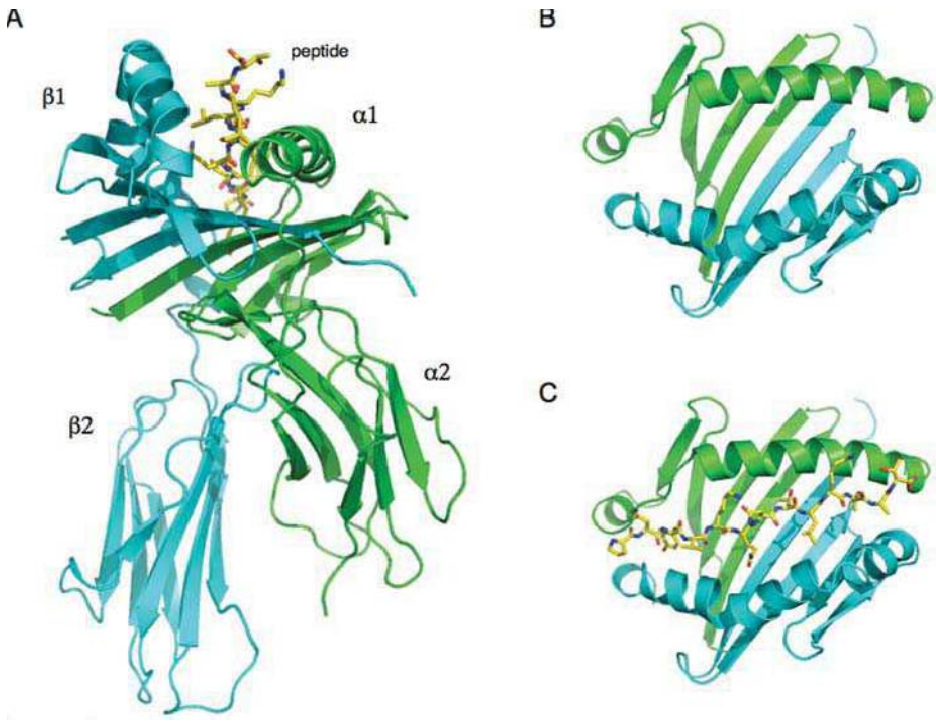

FIG. 21.7. Color Ribbon Representation of HLA-DR1. A: Side view. α chain is in *green;* β chain is in *blue;* peptide in stick representation in *yellow.* B: Top view. C: Top view with bound peptide (PKYVKQNTLKLAT) visualized. The illustration was made with PyMol[560] based on the protein data bank[559] coordinates of 1DLH.

alignments, Shannon entropy provides a sensitive measure of the diversity of particular amino acid positions.[438] Several online tools are available for implementing and displaying the results of such calculations (http://bio.dfci.harvard.edu/Tools/svs_help.html; http://imed.med.ucm.es/Tools/svs_help.html; and http://consurfdb.tau.ac.il/). Comparative ribbon diagrams (Fig. 21.9), in which the location of the amino acid residues that are polymorphic for the human MHC-I and MHC-II chains are indicated with a color map, show that the bulk of MHC polymorphism derives from amino acid variability in regions that line the peptide-binding groove. This suggests that MHC polymorphism is required to allow the MHC molecules, and as a result, the organism and its species, to respond to a changing antigenic environment.

As with MHC-I, a further understanding of the details of the interactions of peptides with the MHC-II molecule came from crystallographic studies of molecules prepared with

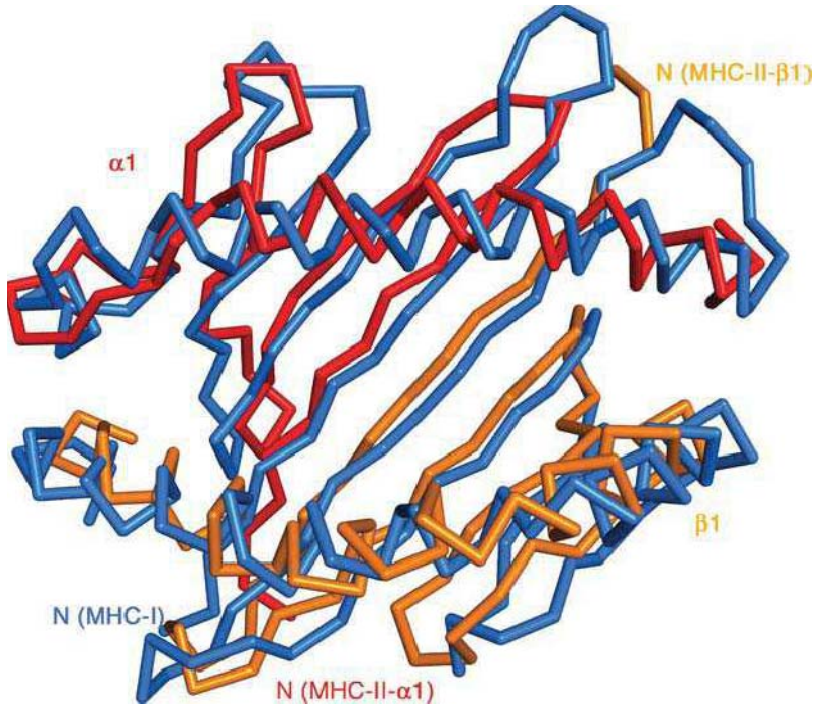

FIG. 21.8. Comparison of α-Carbon Backbone of MHC-I and MHC-II molecules. The α1α2 domains of MHC-I (human leukocyte antgien [HLA]-A2, PDB 3HLA) and the α1 and β1 domans of MHC-II (HLA-DR1, PDB 1DLH) were superposed with PyMOL[560] and displayed as a Cα carbon representation. MHC-I is *blue,* the α1 domain of MHC-II is *red,* and the β1 domain of MHC-II is *orange.*

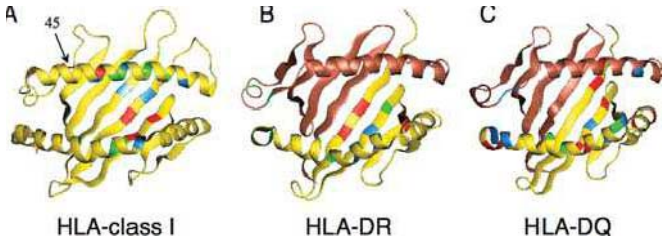

FIG. 21.9. Location of Polymorphic Amino Acid Residues in MHC-I and MHC-II molecules. Variability plots were calculated as described by Kabat and Wu[435] and level of variability illustrated on ribbon diagrams (generated in QUANTA 2000 [Accelrys] of 3HLA[412] [HLA-class I], 1DLH[439] [HLA-DR], and 1JK8 [HLA-DQ]) where greatest variability is *red*, intermediate is *green*, and least is *blue*.

homogeneous peptide—in the first case HLA-DR1 complexed with an antigenic peptide derived from the hemagglutinin of influenza virus.[439] Based on this structure, a set of pockets was initially designated, numbered for the peptide position that is bound. For the influenza peptide studied in this example, the major interactions were from peptide positions 1, 4, 6, 7, and 9 that are indicated in Figure 21.10. The deep P1 pocket accommodates the tyrosine (the third position of the peptide PKYVKQNTLKLAT) and the pockets indicated by 4, 6, 7, and 9 fit the Q, T, L, and L residues, respectively. MHC-II exploits a similar mode of binding as compared with MHC-I, but there are key differences. MHC-II lacks the requirement for free amino and carboxyl termini of the peptide, the peptide conformation is relatively extended (like that of a type II polyproline helix), and the MHC-II forms number of hydrogen bonds between conserved amino acids that line the binding cleft and the main chain (ie, amino nitrogen and carbonyl oxygen) atoms of the peptide. Although the pocket designations refer specifically to the features of the MHC-II molecule surface, occasionally the cognate peptide positions that reside in such pockets are given the same names.

Among the most provocative observations from the first MHC-II structures was that the molecule was visualized as a dimer of dimers, and this moved a number of investigators to consider the possibility that activation of the T cell via its receptor might require the dimerization or multimerization of the TCR, an event thought to be dependent on the propensity of the MHC/peptide complex to self-dimerize. The simple elegance of this dimer of dimers is illustrated in Figure 21.11. Several arguments support the dimerization hypothesis: the finding of a dimer of dimers in the crystals of HLA-DR that formed in several different space groups,[434,439] the observation that a TCR Vα domain formed tight dimers and in its crystals formed dimers of the dimers,[440] the demonstration of the ability to immunoprecipitate MHC-II dimers from B cells,[441] the apparent requirement for purified MHC-I dimers for stimulation of a T cell in an in vitro system,[442] and the finding that MHC-II/peptide/TCR complexes could form higher order multimers in solution as detected by quasi-elastic light scattering.[443] However, a number of strong counterarguments draw this hypothesis into question. Many MHC-II molecules other than HLA-DR1 that have been crystallized do not seem to form the same kind of

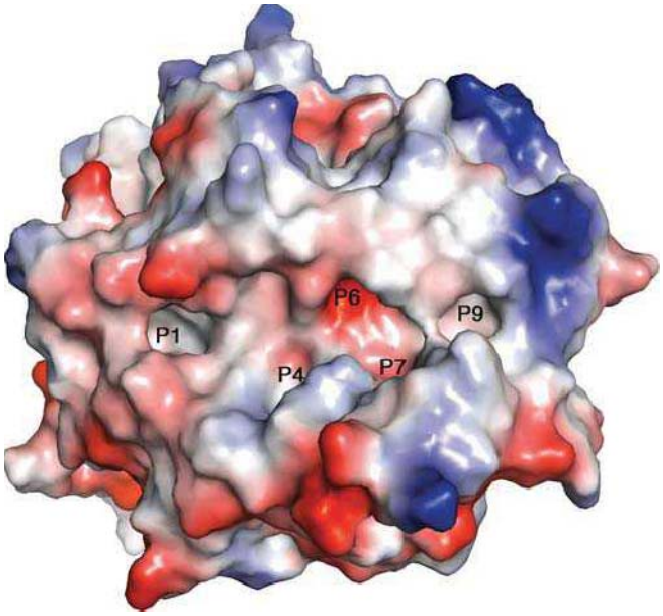

FIG. 21.10. Location of Pockets in HLA-DR1 Based on the Cocrystal of HLA-DR1 with a Peptide Derived from the Influenza Hemagglutinin. An electrostatic surface representation of HLA-DR1 (1DLH) generated in PyMOL[560] is shown with the positions of pockets P1, P4, P6, P7, and P9 indicated.

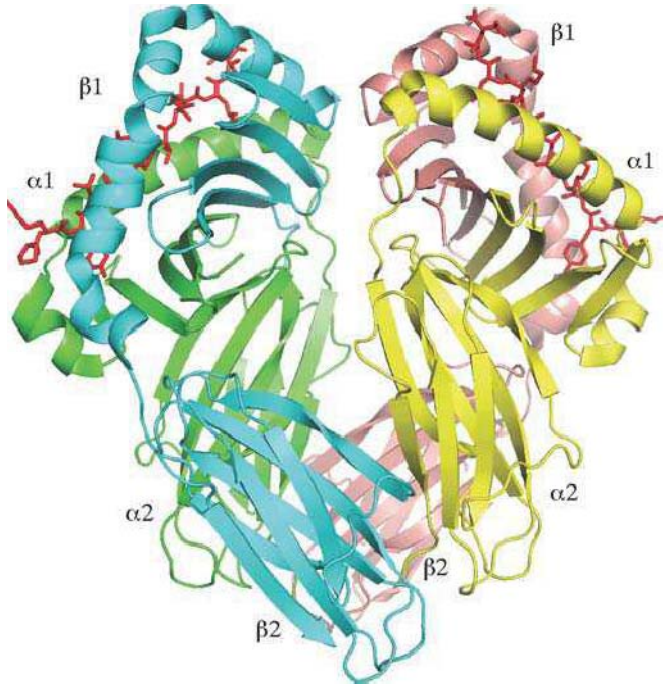

FIG. 21.11. Ribbon Diagram of the Structure of HLA-DR1 showing the dimer of dimers and the individual domains of the protein. α chains are in *green* and *yellow;* β chains are in *blue* and *salmon.* Peptide in stick representation is in *red.* The illustration was generated from PDB 1DLH with PyMOL.

dimer of dimers in their crystals.[444,445] Modes of MHC-II dimerization with distinct topology that would preclude simultaneous interaction with multiple TCRs on the same T cell have been observed. None of the MHC-I molecules that have been examined by x-ray crystallography show dimers in the same orientation as the MHC-II ones reported. A different Vα domain fails to dimerize even at high concentration.[446] Many reported x-ray structures of MHC/peptide/TCR complexes[447–450] fail to show dimerization. Despite the simple elegance of the dimer hypothesis, it is clear that additional experimentation will be required to understand the topologic requirements for T-cell activation through the αβ TCR.

Recent additions to the library of MHC-II structures include the I-A^{g7} molecule, a unique MHC-II that provides one link in the susceptibility to insulin-dependent diabetes in the mouse model.[451,452] Although the structure fails to provide direct evidence to explain the linkage to diabetes, it suggests that the novel repertoire of peptides bound by this MHC-II molecule reveals unique features of a wider peptide binding groove and resulting relatively low-affinity interaction with peptide. Until recently, all of the MHC-II/peptide structures revealed bound peptide in a canonical left to right, amino to carboxyl orientation. A detailed analysis of HLA-DR1 molecules bound to the CLIP 106 to 120 peptide revealed crystals in two different forms, which on solution and refinement indicated that this peptide could interact in either of two distinct orientations (Fig. 21.12).[408] This exception to the general rule of left to right, amino to carboxyl orientation, may reflect the unique role that the CLIP peptide must play in protecting the MHC-II–binding site while simultaneously being available for DM-mediated peptide exchange in the endosome.

MHC-Ib Molecules

H2-M3

To this point, our description of MHC-I molecules has focused on the classical MHC-Ia molecules, represented by HLA-A, HLA-B, and HLA-C in the human, and by H2-K, H2-D, and H2-L in the mouse. Several MHC-Ib molecules for which three-dimensional structures have been determined are of particular interest, the CD1 molecules,[453,454] H2-M3,[137,455] MICA,[456,457] FcRn,[458] and Rae-1.[459] Other molecules that are thought to show structural relatedness to the MHC-I fold but whose x-ray structures have not yet been reported include the stress-induced molecules H-60[173] and MULT1,[460] and the mucosally expressed MR1 molecule that selects invariant T cells in the gut.[461] H2-M3 is of particular note because of its ability to bind and present peptide antigens that contain amino terminal N-formyl groups. H2-M3 was originally identified as the MHC-Ib molecule that presents an endogenous peptide derived from the mitochondrially encoded protein ND1 known as maternally transmitted factor.[137,462] Thus, it was of interest to understand in structural terms how this molecule binds such N-formylated peptides.[455,463] The crystal structure of H2-M3 complexed with an N-formylated nonamer peptide, fMYFINILTL, revealed that the structure of the A pocket, highly conserved among MHC-Ia molecules, which have tyrosine 7, tyrosine 59, tyrosine 159, tryptophan 167, and tyrosine 171, is quite different so that it can accommodate the N-formyl group in the A pocket. In particular, H2-M3 has an A pocket reduced in size and lined by hydrophobic residues, leucine at 167 and phenylalanine at 171, and leucine 16. These structural features cause the amino terminal nitrogen of the formylated peptide to be positioned where the peptide position 2 amino nitrogen would lie in a MHC-Ia molecule. Thus, the unique peptide selectivity of H2-M3 is explained in structural terms.

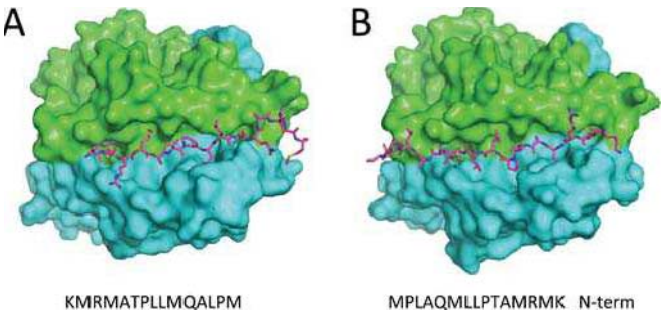

FIG. 21.12. MHC-II Binds Class II-Associated Invariant Chain Peptide in Either N to C or C to N Orientation. The structures of HLA-DR1 bound to the CLIP 106 to 120 peptide in either the N to C (A) or C to N (B) orientation is illustrated. α1 domain is in *green*; β1 domain is in *cyan*. Peptide sequences are shown at the bottom.

CD1

Another MHC-Ib molecule of great interest is CD1, representative of a class of MHC-I molecules that map outside of the MHC, that have limited tissue-specific expression, and that are capable of interaction with both αβ and γδ T cells.[464] In the human, there are two clearly distinct groups of CD1 molecules: one consisting of CD1a, CD1b, CD1c, and CD1e; and another of CD1d alone.[465] In the mouse and rat, only CD1d is expressed.[466] As a group, these are β2m–associated chains that bind hydrophobic antigens, primarily glycolipids, with the lipid moiety embedded in the CD1 heavy chain and the carbohydrate portion exposed to solvent. CD1a, CD1b, and CD1c are capable of binding and presenting various nonpeptidic mycobacterial cell wall components such as mycolic acid containing lipids and lipoarabinomannan lipoglycans.[467,468] NK T cells, a subset of αβ-TCR–bearing T cells, of independent lineage and defined by the expression of the NK1.1 marker, are restricted to CD1 recognition.[469] The crystal structures of both mouse and human CD1d1 have been determined,[453,470] revealing classic MHC-I structures with the basic protein fold and β2m association quite similar to that of the MHC-Ia molecules. Consistent with its apparent biologic function of binding hydrophobic lipid-containing molecules, its binding groove is somewhat narrower and deeper than that of either MHC-Ia molecules or MHC-II molecules. The backbone configuration of the α1α2 domain structure of CD1 is shown in Figure 21.13, where it is compared to the homologous region of H2-K^b, HLA-DR1, and another MHC-Ib molecule, a neonatal Fc receptor (FcRn). The structure of a number of CD1-glycolipid complexes

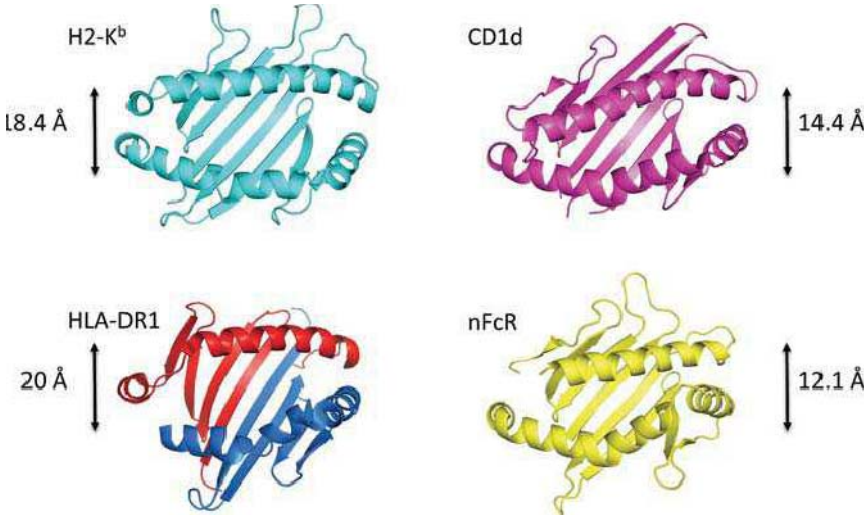

FIG. 21.13. Comparison of the Ribbon Representations and Size of the Potential Peptide-Binding Cleft. MHC-I molecule H2-K^b (2vaa), MHC-II HLA-DR1 (1DLH), and MHC-Ib molecules CD1d (1Z5L) and FcRn (1EXU) are compared. Approximate maximal distances separating the two helices are indicated.

have been determined, and human and mouse CD1 molecules bound to different glycolipids have been compared.[466] The depth of the groove of CD1 results from the merging of pockets to form what have been termed the *A' and F' pockets* in place of the MHC-Ia A through F pockets. This A' pocket is about the size of the binding site of a nonspecific lipid binding protein.

In an effort to understand more precisely how CD1 molecules bind lipid antigens, Gadola et al.[454] crystallized human CD1b complexed with either phosphatidylinositol or ganglioside GM2 and determined their x-ray structures. The structures were essentially identical for the CD1b heavy chain and β2m in the two complexes, and revealed a network of four hydrophobic channels at the core of the α1α2 domain, which accommodate four hydrocarbon chains of length from 11 to 22 carbon atoms. These channels are called A', C', and F' for the three analagous to the A, C, and F pockets of the MHC-Ia molecules, and a fourth, termed *T'* which is a distinct tunnel. Illustrations of the binding groove with the bound alkyl chains is shown in Figure 21.14, which shows views of the binding pockets of CD1a, b, and d with different ligands. These structures illustrate how the

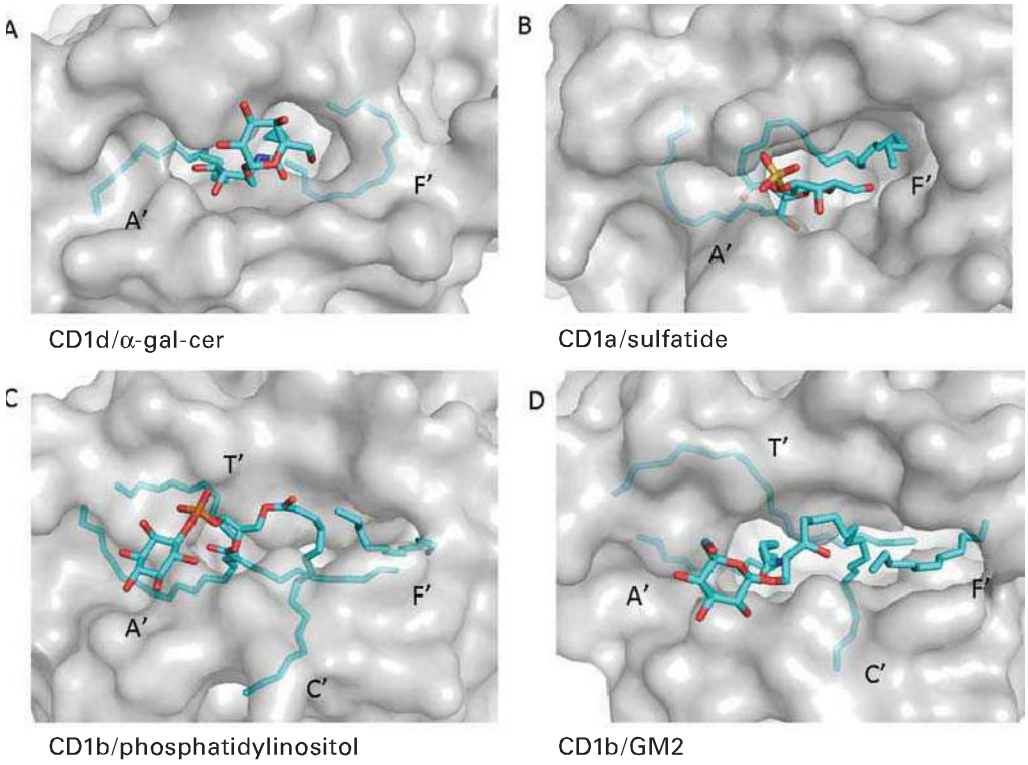

FIG. 21.14. Tunnels and Pockets of CD1 Molecules. Structures of (A) CD1d with α-galactosyl ceramide (1Z5L), (B) CD1a with sulfatide (1ONQ), (C) CD1b with phosphatidylinositol (1GZQ), and (D) CD1b with ganglioside GM2 are shown, with pockets A', C', and F' indicated and tunnel T'.

binding site of a classical MHC-I molecule may have evolved from (or to) the binding site of molecule like CD1 to provide antigen selectivity for a distinct set of molecules that would be common to a set of important mycobacterial pathogens. Complexes of CD1 with their cognate NK TCR have been reported and will be discussed subsequently.[471–474]

Neonatal Fc Receptor

Another example of an MHC-Ib molecule, noteworthy because it exemplifies a novel function of MHC molecules, is the FcRn. Originally described in the rat as a molecule of the intestinal epithelium that is involved in the transport of colostral Ig from the lumen to the bloodstream,[475,476] homologues in the mouse and human have also been described,[477–479] and the structure of the rat molecule has been determined crystallographically[458] (Fig. 21.15). As suggested by the amino acid sequence similarity of the FcRn to MHC-I proteins, the three-dimensional structure revealed considerable similarity to MHC-Ia molecules.[458] Specifically, α1 and α2 domains have similar topology to the MHC-I molecule, although, as discussed previously, what would be the peptide-binding groove in the MHC-Ia molecules is closed tightly and lacks space sufficient for a ligand. The most provocative feature of the structure of the FcRn/Fc complex is that the MHC-I–like FcRn interacts with the Fc through contacts from the α2 and β2m domains to interact with the Fc $C_\gamma2$-$C_\gamma3$ interface. As compared to the structure of the unliganded Fc, the complex reveals both conformational changes in the Fc and the presence of several titratable groups in the interface that must play a role in the pH-dependent binding and release of Ig molecules from the FcRn. The FcRn has taken the MHC-I fold and diverted its function for an interaction with the Fc of the Ig. Amino acids at what would classically be considered the "righthand side" of the peptide-binding groove make contact with the Fc interface that lies between $C_\gamma2$ and $C_\gamma3$ domains. The FcRn serves as an excellent example of similar structures in the immune system being diverted for alternate purpose. The importance of the FcRn has been underscored by observations of differences in the serum half-life of Ig in animals that, as a result of an induced deletion of β2m, lack the normal expression FcRn as well and seem to metabolize serum Ig aberrantly.[480]

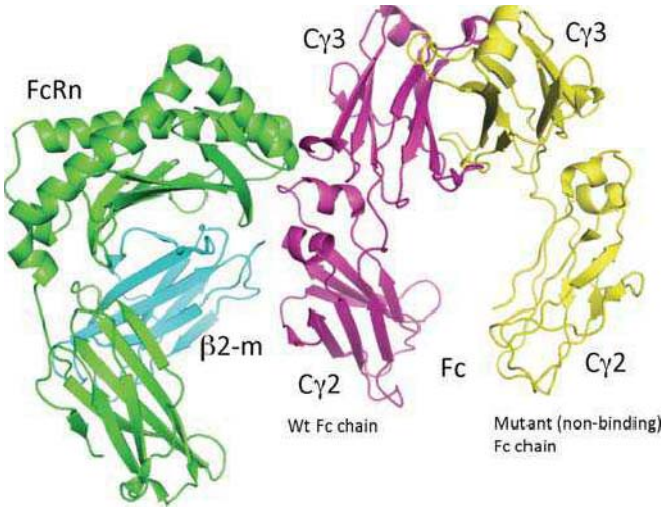

FIG. 21.15. Structure of the Rat Neonatal Fc Receptor Complexed with Fc. Ribbon diagram of rat neonatal Fc receptor complexes with the Fc heterodimer consisting of the wild-type and nonbinding chains. This is from PDB 1I1A.

MILL MHC–Like Molecules

A novel set of MHC-Ib–like genes designated *Mill* (*M*HC class *I*-*l*ike located near the *l*eukocyte receptor complex) has recently been identified in both mice and rats.[481–483] These encode MICA/B-like glycophosphatidylinsol-linked cell surface molecules that are associated with β2m and do not require TAP for cell surface expression. Their function remains unclear, although further examination indicates the presence of two family members, Mill1 and Mill2, observed on cycling thymocytes, proliferating smooth muscle cells and fibroblasts.[484]

M10 Proteins

The robust MHC fold seems to possess both the stability and flexibility requisite for the molecular function of binding ligands such as peptides, glycopeptides, phosphopeptides, and glycolipids as well as for subsequent interactions with macromolecular receptors such as NK receptors and TCRs. Likely, these qualities have allowed its adaptation for distinct function related to a class of MHC-Ib molecules, the M1 and M10 families, some members of which are associated with the V2R mouse pheromone receptors. The structure determination of M10.5 revealed a β2m-associated MHC-I–like molecule with clear three-dimensional similarity to the MHC-I molecule H2-D^d.[485]

MHC-Iv Molecules

Because viruses are constantly engaged in a struggle for survival in their interplay with the immune system of their hosts, there is little surprise to note that pathogenic eukaryotic viruses can evolve numerous approaches to evade the immune response. MHC and structurally related molecules are produced not only by vertebrates but also by CMVs, large DNA viruses of the β-herpesvirus family, which have coevolved with their vertebrate hosts over millennia and are exquisitely adapted to persist in the face of host immune responses.[2] Viral MHC-I–like molecules have been identified bioinformatically from amino acid sequences of the open reading frames predicted from genome sequence data. A number of putative MHC-Iv molecules, including UL18 and UL142 of the human CMV,[327,486] members of the "m145 family" of the mouse CMV1[487,488] the 2L molecule of tanapox,[489] and the U21 molecule of human herpesvirus 7,[490] may modulate the immune response of the host. In some cases, such as the UL18 protein of human CMV and the m144 protein of mouse CMV, the sequence homology to bonafide MHC-I molecules is sufficiently strong to unambiguously identify them as MHC-I like. However, in most cases, such as with the m145 family of mouse CMV, the sequence homology to MHC-I is very weak, and relatedness to MHC-I was originally based on structure prediction algorithms alone. We term these virally encoded MHC-I–like molecules MHC-Iv

to reflect their distinct evolutionary history, their structural deviation from typical MHC-I molecules, and their role in evading host immune responses. A survey of DNA sequences of 11 genes obtained from 26 wild murine CMV isolates and laboratory strains indicated that several of the *MHC-Iv* genes (m144, m145, and m155) revealed significant sequence variation, consistent with the view that this variation may offer some immune-evasive benefit to the virus.[491]

Structural characterization of MHC-Iv molecules begins with an evaluation of the requirement for β2m and/or peptide for stable expression. The UL18 is associated with both β2m and peptide, whereas m144 may be associated only with β2m but does not require β2m for cell surface expression. The structure of m144 has been reported,[487] revealing the preservation of all structural elements of the MHC-I fold (Fig. 21.16). The cleft of m144 does not seem capable of binding peptides as the groove is narrow and critical tyrosine residues are not conserved, results consistent with earlier biochemical studies.[492] A unique disulfide anchors the α1 helix to the β sheet. The structure of m157, a CMV MHC-I–like immunoevasin that binds the Ly49H activation receptor of C57BL/6 mice and the Ly49I inhibitory receptor of 129/J mice, has been reported.[493] This β2m-independent, peptide-free MHC-Iv molecule reveals the major features of the MHC-I fold with some unique aspects, in particular a novel amino terminal α helix. How it binds its Ly49 ligands remains unclear. The m153 protein of mouse CMV, also a member of the m145 family, reveals novel adaptations of the MHC-I fold.[1] The m153 protein does not require β2m or peptide and, in contrast to other MHC-I molecules, is a noncovalent dimer. The monomers are associated in a head-to-tail fashion. An extended N-terminus contains a unique disulfide that anchors it to the α3 domain. The m153 structure hints tantalizingly of more surprises to come as structures of other MHC-Iv molecules become available. Other MHC-Iv proteins whose structure have been determined include the 2L tanapox virus protein that binds the inflammatory cytokine TNF-α[489] and the mouse CMV-encoded m152, which sequesters both classical MHC-I and the stress-induced NKG2D ligand, RAE-1, from the cell surface.[494]

In those cases where functions have been elucidated, MHC-Iv molecules have been shown to act as immunoevasins that inhibit the NK cell to enable virus survival and persistence in the host. Both UL18 and UL142 inhibit human NK function, the former by binding to the inhibitory receptor LIR-1. The mode of action of UL142 remains to be determined.[327] The m157 binds to the inhibitory NK receptor Ly49I in mouse strains susceptible to viral infection but remarkably serves as a target for the activating NK receptor Ly49H expressed in resistant strains. The m144 protein has also been shown to inhibit NK activation,[495,496] but the ligand remains to be identified.

Complexes of MHC Molecules with Ligands

The most recent and exciting developments in MHC biology include the detailed description of the interactions of MHC-I and MHC-II molecules with their receptors and coreceptors on cells of the innate and adaptive immune system. Our structure/function survey will be completed by brief descriptions of the interactions of MHC-I and MHC-II molecules with αβ TCRs, with the T-cell coreceptors CD8 and CD4, and of MHC-I molecules with NK receptors. Details of the interactions of a number of CD1 molecules with their glycolipid antigens and specific NK T-cell receptors shed light on the biology and evolution of these molecules. A brief description of interactions of MHC-II molecules with superantigen will follow. Each of these structural studies complements a host of biologic experiments that have led to an appreciation of the importance of understanding the structural basis of these immune reactions.

MHC/T-Cell Receptor Interactions

Second to the initial visualization of the structure of the MHC-I molecule HLA-A2 by crystallographic analysis, the images of TCR/MHC/peptide complexes initially reported some 15 years ago clarified the phenomenologic details of T-cell recognition, antigen specificity, and MHC restriction. The initial structures were determined in several systems,[447–449,497,498] and there remains ongoing interest in the details of particular TCR/MHC interactions. The first examples were of MHC-I–restricted TCR, one from the mouse[448] and one from the human.[447] The mouse MHC-I molecule H2-K^b was analyzed in complex with a self-peptide, dEV8, and a TCR known as 2C,[448,499] and the human

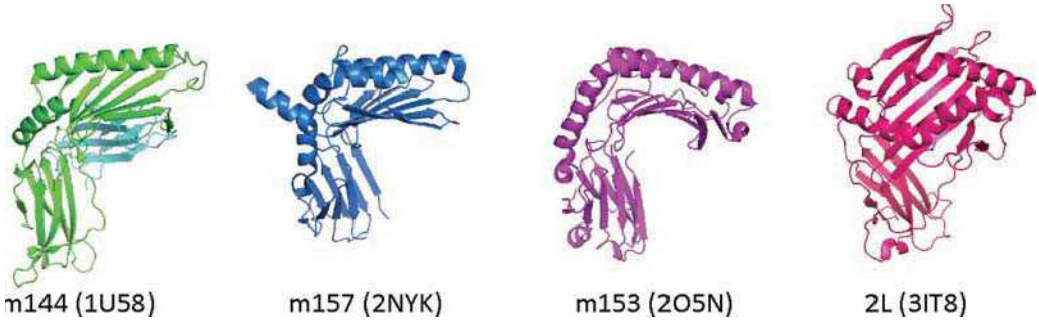

FIG. 21.16. Comparison of MHC-Iv Molecules. Structures of MHC-Iv chains of m144 (1U58), m153 (2O5N), m157 (2NYK), and the 2L protein of tanapox virus (3IT8) were superposed and are illustrated as ribbon diagrams.

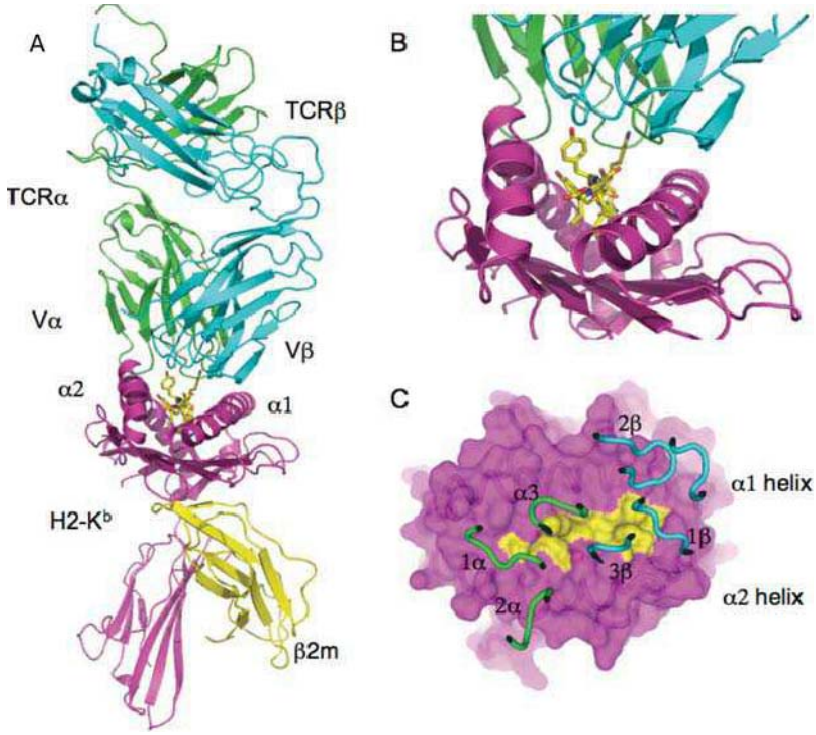

FIG. 21.17. Structure of an MHC-I/Peptide/T-Cell Receptor (TCR) Complex and the TCR Footprint on the MHC/Peptide. The structure of the H2-K^b/dEV8/2CTCR complex (2TCR) is displayed. **A:** The complex complex. **B:** A close-up of the MHC/peptide/TCR interface. **C:** The surface of the MHC *(magenta)*/peptide *(yellow)* complex with the complementarity determining region loop of the TCR Vα *(green)* and Vβ *(blue)* shown and labeled.

HLA-A2 complexed with the Tax peptide was studied with its cognate TCR derived from a cytolytic cell known as A6.[447] These structures offered a consistent first glimpse at the orientation of the TCR on the MHC/peptide complex, but additional structures, including that involving a murine MHC-II–restricted TCR suggest that more molecular variations may exist. As a canonical example of the MHC/peptide/TCR complex, we include an illustration of the H2-K^b/dEV8/2CTCR complex[499] (Fig. 21.17). This illustration shows that the complementarity determining regions (CDRs) of the TCR (labeled 1α, 2α, 3α for CDRs of Vα and 1β, 2β, and 3β for the CDRs of Vβ, respectively) sit symmetrically on the MHC/peptide complexes. For the H2-K^b/dEV8/2CTCR complex, the region contacted by the CDRs of the Vα domain of the TCR lie to the left and that contacted by the CDRs of the Vβ domain lie to the right. The regions contacted by CDR3, labeled 3α and 3β, are at the center of the bound peptide, whereas the regions contacted by CDR1 and CDR2 of both Vα and Vβ lie peripherally. Footprints of other TCR have been reviewed elsewhere.[357]

With the publication of additional MHC/peptide/TCR structures,[357] additional points have emerged: 1) There is considerable variability in the orientation of the TCR Vα and Vβ domains with respect to the MHC/peptide complex. Although the first MHC-II/peptide/TCR structure suggested that an orthogonal disposition, in which Vβ makes the great majority of contacts with the MHC-II α1 domain and the Vα predominantly interacts with the β1 domain, might be the preference for MHC-II/TCR interactions,[449] additional MHC-II/peptide/TCR structures[450,497] suggest that this disposition is not indicative of MHC-II as compared to MHC-I but rather reveals the wide variety of possibilities. 2) Considerable plasticity in the conformation of the CDR loops of the TCR, particularly long CDR3 loops, is observed in the comparison of TCR free or bound to their cognate MHC/peptide ligands.[499,500] A striking example of this is illustrated by the structure of the KB5-C20 TCR alone as compared to its complex with H2-K^b/peptide (Fig. 21.18).

Structural Insights into Alloreactivity

During T-cell development, TCRs destined to be useful to the host are selected for weak reactivity with one or more self-peptides complexed with cell surface host major histocompatibility (MHC/peptide) molecules. TCR selection is customized in each individual because of extensive polymorphism in MHC molecules that is designed to diversify peptide repertoire and optimize immune responsiveness. Thymic selection and MHC polymorphism conspire to generate antimicrobial T-cell responses that are genetically restricted to recognizing host MHC molecules while retaining Ag-specificity. MHC restriction has been a central paradigm of T-cell immunity and was the basis for the 1996 Nobel Prize awarded to Peter Doherty and Rolf Zinkernagel.

Unfortunately for transplant clinicians and their patients, the rule of MHC restriction is violated when T cells are exposed to allogeneic MHC/peptide complexes. Remarkably, up to 10% of naive T cells react strongly against allogeneic MHC/peptide in vitro (mixed lymphocyte reaction) and in vivo leading to allograft rejection and GVHD. This reaction, known as T-cell alloreactivity, is why MHC molecules were initially called transplantation or histocompatibility molecules and has puzzled immunologists for decades.

There are two main historical theories to explain the high frequency of alloreactive T cells. The first, proposed in 1977,[183] postulated that a single allogeneic MHC molecule

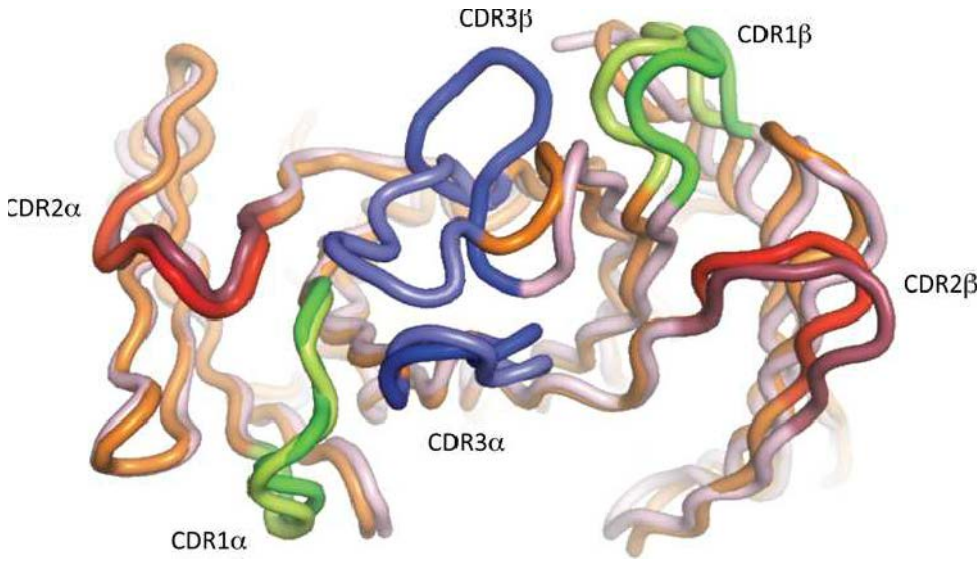

FIG. 21.18. Complementarity Determining Region (CDR)3 Plasticity. Backbone tracings of the KB5-C20 T-cell receptor CDR loops free (1KB5) or bound to the major histocompatibility/peptide complex (1KJ2) are superposed. Unliganded forms are in *darker colors*. The largest conformational difference is seen in the CDR3β loop.

could give rise to multiple binary complexes with cell surface molecules, creating "neoantigenic determinants" recognized by clonally distinct T cells. This has been reinterpreted in a "peptide-centric" hypothesis whereby as a single MHC molecule presents disparate peptides recognized by multiple different T-cell clones. The "multiple binary complex" model implies the TCR interacts with a set of amino acids shared by self- and allogeneic MHC molecules, so the cross-reaction depends crucially on the peptide antigen. However, it was later suggested that alloreactive T cells might focus on polymorphic residues exposed on the allogeneic MHC molecule itself, the so-called high determinant density model in which the TCR focus is "MHC centric," and the peptide is largely irrelevant.[501]

Colf et al.[502] solved the structure of the 2C TCR in complex with its known allogeneic ligand, H2-L^d-QL9, and then compared it to the structure of 2C in complex with its positively selecting ligand, H2-K^b-dEV8, and thus were able to provide important insight into the structural basis of alloreactivity. These two TCR footprints were typical of known MHC/peptide/TCR structures but differed from each other in a number of ways. For instance, each chain of 2C made contacts with one MHC α helix of H2-L^d-QL9, whereas both chains contacted both α helices in H2-K^b-dEV8. The geometry of 2C adopted a more perpendicular orientation on H2-L^d-QL9, and there was a relative rotation of H2-K^b-dEV8 by 20 degrees. The alloreactive complex reveals that both peptide-centric and MHC-centric interactions underpin direct T-cell allorecognition by the 2C receptor but with a heavy emphasis on MHC-centric interactions. Most surprising, however, was the small number of shared contacts between the two structures, implying a limited role for mimicry between cognate and allogeneic MHC/peptide. The H2-K^b and H2-L^d have 31 amino acid differences, and there is no sequence similarity between the H2-K^b–restricted octamer self-peptide, dEV8, and the H2-L^d–restricted nonamer, QL9. Given these differences, it was a fair bet that the cross-reactivity of 2C on H2-K^b-dEV8 and H2-L^d-QL9 would depend upon plasticity in the CDR3 regions of the TCR as documented in comparisons of bound and free TCRs, including 2C. Surprisingly, this was not the case as the TCR actually adopted very similar conformations in the two structures. In contrast to the findings with the alloreactive murine, 2C TCR, alloreactivity of the natural human LC13 TCR is based on striking molecular mimicry between the cognate and alloantigens.[503] The LC13 recognizes an Epstein-Barr viral peptide complexed with self-HLA-B*08:01 but also alloreacts with B44 allotypes (HLA-B*44:02 and 44:05) bound to an endogenous allopeptide from an adenosine triphosphate–binding cassette protein ABCD3. HLA-B*08:01, and the closely related allotypes HLA-B*44:02 and HLA-B*44:05, are distinguished by numerous polymorphisms and the LC13-reactive viral and endogenous peptides are unrelated in their sequence. Nonetheless, the LC13 TCR bound the allogeneic and cognate virus-specific HLA-peptide structures in a very similar manner.[503] Moreover, there was mimicry of the viral peptide by the allopeptide. Hence, this study demonstrates that recognition of allogeneic and cognate HLA-peptide structures can occur via molecular mimicry, even in the face of polymorphism and disparate peptide ligands.[503] Interestingly, another HLA-B*08:01–restricted TCR (CF34) with the identical Epstein-Barr virus–peptide specificity as LC13 lacks B44 reactivity because it arises when HLA-B44 is coinherited in trans with HLA-B8. Docking of the CF34 TCR focused on the N-terminus of the HLA-B*08:01-peptide while LC13 docked over the C-terminus of the viral peptide bound by HLA-B*08:01.[504] This corresponds with CF34 engagement of a polymorphic region distinguishing HLA-B8 from HLA-B44, revealing a topographical image of shifting TCR specificity to accommodate T cell self-tolerance.[504]

The role of molecular mimicry and the relative importance of peptide-centric versus MHC-centric bias in T-cell allorecognition is likely to vary in different systems. Hence, it is likely that the nature of the polymorphisms between cognate and allogeneic MHC allotypes will affect TCR focus. Thus, closely related MHC allotypes that differ by as little as one amino acid (eg, H2-K^b mutants in mice; HLA-A2, B44, and B27 families in humans) are set up for MHC mimicry to be a key component of T-cell allorecognition where *specificity* is likely to be peptide centric. For example, HLA-B*44:02 and B*44:03 allotypes differ by only a single amino acid and yet stimulate strong mutual allogeneic T-cell responses. Indeed, the potency of T-cell alloresponses between closely related MHC allotypes probably occurs because positive selection of host T cells is purposely designed to create a repertoire responsive to subtle changes in peptide display. Therefore, closely related MHC allotypes, with differences in both peptide repertoire and MHC/peptide conformation of a shared repertoire, play straight into nature's design for T-cell recognition.

Important questions concerning the nature of T-cell signaling mediated by engagement of the TCR by MHC/peptide complexes remain. What are the molecular and biophysical determinants of agonist as compared with antagonist activity of particular MHC/peptide ligands? Although it is clear that a number of parameters such as binding kinetics, half-life, and affinity (both two dimensional and three dimensional) as well as coreceptor contributions may play a role in the outcome of TCR engagement by MHC/peptide complexes, and the additional role of receptor clustering may contribute to such signaling, the precise contribution of each of these factors has not been established. A provocative approach to the question of the role of the geometry of docking of the TCR onto the MHC/peptide complex has been addressed in the analysis of the relationship of stimulatory parameters of four different peptides bound to H2-L^d with respect to the three-dimensional structure of the relevant MHC/peptide/TCR complex.[505] One of the four peptides failed to induce signaling, and remarkably, the TCR docked onto the MHC/peptide in a unique docking geometry. The authors interpreted these results to demonstrate the relationship between TCR/pMHC docking geometry, peptide cross-reactivity, and signaling as well as explaining the basis for germline bias in TCR/MHC interactions.

MHC/Coreceptor Complexes

The major coreceptors for recognition by αβ TCRs are CD8, which interacts with MHC-I molecules, and CD4, which interacts with MHC-II. Coreceptor function plays a role in signaling the T cell in addition to contributions the MHC/coreceptor interaction may provide in increasing apparent avidity between the MHC/peptide and the TCR. CD8, the coreceptor on MHC-I–restricted αβ T cells, exists as a cell surface homodimer of two α chains or a heterodimer of α and β chains and plays an important role both in the activation of mature peripheral T cells as well as in the thymic development of MHC-I–restricted lymphocytes.[506,507] Mature peripheral CD8 T cells express significant amounts of CD8αβ, whereas other lymphocytes, such as intraepithelial lymphocytes in the gut, predominantly express the CD8αα homodimer. The CD8αβ heterodimer is considered the relevant functional coreceptor on peripheral T cells. The three-dimensional structures of human and mouse MHC-I/CD8αα complexes have been described,[508,509] and more recently, a complex of mouse MHC-I/CD8αβ has been reported.[58] These structures localize the binding site of the CD8 Ig-like αα homodimer or CD8αβ heterodimer to a region on an exposed loop of the MHC-I α3 domain. This interaction is illustrated in Figure 21.19, which shows the flexible loop of residues 223 to 229 clamped into the CD8 combining site. This is one of relatively few examples of an non-Ig, Ig-like molecule exploiting an Ig-like heterodimer interface to bind another ligand. The structural identification of the CD8αα and CD8αβ on the MHC-I provides a context for understanding the contribution of CD8, which binds the kinase Lck through the cytoplasmic domain of its α chain to T-cell signaling.

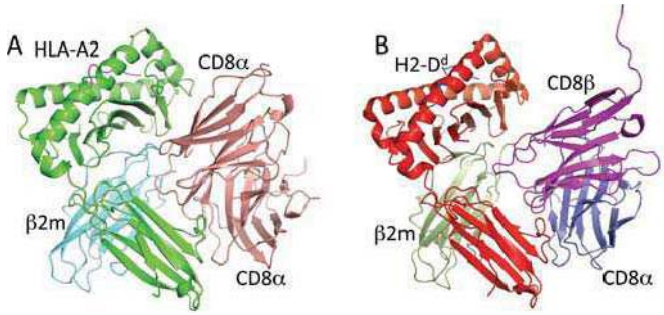

FIG. 21.19. CD8αα and CD8αβ Bind the Same Site on the MHC-I α3 Domain. The MHC-I interactions with the CD8αα (PDB 1AKJ) homodimer **(A)** and CD8αβ (PDB 3DMM) heterodimer **(B)** are shown.

The T-cell coreceptor associated with cells restricted to MHC-II antigens, CD4, has also been the subject of detailed structural studies, in part, because of its role as a receptor for attachment and entry of HIV.[510] The x-ray structure determination of the complete extracellular portion of the molecule (domains D1 through D4) indicates a degree of segmental flexibility between domains D2 and D3, and both crystallographic and biochemical data suggest that dimerization of cell surface CD4 occurs.[511] These results have been interpreted to support a role for CD4-mediated MHC-II–dependent dimerization in facilitating TCR dimerization and signaling. Although a mixed species structure of human CD4 D1-D2 domains with a mouse MHC-II molecule was reported at low resolution some years ago,[63] only recently, employing an affinity matured human CD4 molecule have diffraction quality crystals of human MHC-II/human CD4 D1-D2 molecules been obtained[64] (Fig. 21.20). This recently determined human MHC-II/hCD4 structure is remarkably similar to the low-resolution mixed species structure determined more than 10 years earlier. Both structures reveal the focus of the CD4 interaction on a stretch of amino acid residues on the Ig-like β2 domain of the MHC-II molecule. Earlier mutagenesis and functional studies of CD4-dependent MHC-II–mediated T-cell activation

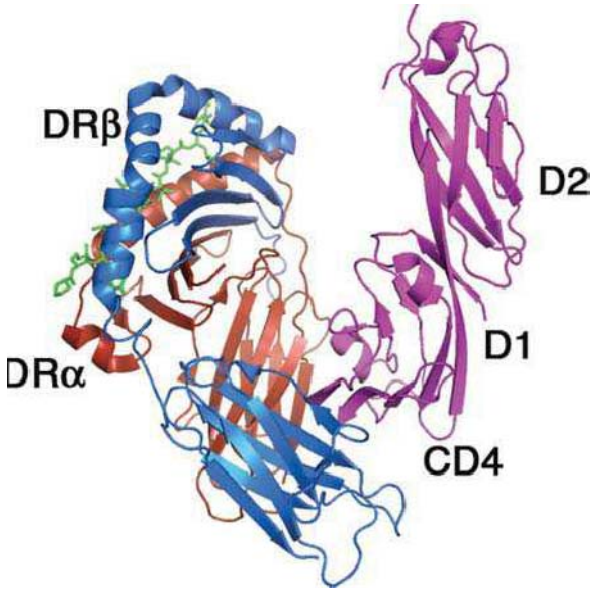

FIG. 21.20. CD4 Interacts Directly with the β2 Domain of MHC-II. A ribbon illustration of a complex of human CD4 with HLA-DR1 (PDB 3S5L) is shown. The HLA-DRα chain is in *firebrick*, HLA-DRβ chain in *blue*, peptide in *green*, and CD4 in *magenta*.

also implicated a region on the α2 domain of MHC-II.[61,512] Although the α2 domain residues do not directly interact with CD4 in these structures, it is conceivable that they play some indirect role in MHC multimerization that may contribute to signaling.

MHC/Natural Killer Receptor Complexes

NK cells provide a first line of defense against virus-infected and host cells, and their recognition of such targets is dependent on a balance of NK activating and inhibitory receptors.[513,514] In addition to their expression on NK cells, some of the NK receptors are also expressed on subsets of T cells and other hematopoietic cells. In general, each of these classes of NK receptors also falls into two different structural groups: the Ig-like receptors and the C-type lectin–like receptors. Because of their functional interactions with MHC-I and MHC-I–like molecules, and because several different systems have evolved to recognize MHC-I molecules differently, it is worthwhile to examine the recently determined structures of several MHC-I/NK receptor complexes.

In the human, the major NK receptors fall into several categories: those of the NKG2 family, the NKp molecules, and the KIRs. The NKG2 family molecules are C-type lectin–like molecules that recognize ligands grouped broadly as the NKG2D ligands, which include the MHC-like molecules, MICA and MICB, as well as the ULBP family of stress-induced molecules. NKp molecules include NKp30, NKp44, and NKp46, all members of the Ig superfamily. The KIR molecules, also Ig superfamily members, bind either HLA-C, or for the KIR3 subfamily, HLA-B molecules. Because the focus of this chapter is members of the MHC protein family, we will restrict our discussion of the human NK receptor/ligand interactions to the NKG2/MIC and ULBP complexes and to the KIR2D and KIR3D/MHC interactions.

The activating receptor, NKG2D, interacts with MICA, a stress-induced MHC-I–like molecule, allowing NK cells to eliminate virus-infected or tumor cells that are marked by the surface expression of MICA. The structure of the human NKG2D/MICA complex reveals that the homodimeric binding site of NKG2D recognizes the surface α-helical superdomain, in an orientation similar to that by which TCR see MHC-I molecules[456] (Fig. 21.21). This structure shows remarkable similarity to that of the hNKG2D bound to another MHC-Ib protein, ULBP3.[515] Similarly, the mouse NKG2D interacts, in a similar orientation, with the murine stress-induced molecule, RAE-1β.[459]

The short KIRs (eg, KIR3DS and KIR2DS molecules) are considered activating receptors because they have the potential to interact with the DAP12 signal-transducing molecule, and the long KIRs (eg, KIR3DL and KIR2DL) are inhibitory because they have cytoplasmic domains that contain immunoreceptor tyrosine-based inhibitory motifs. KIR2DL1 and KIR2DL2 have been studied extensively. They interact with the human MHC-I molecules HLA-C*04 and HLA-C*03, respectively, and show some preferences for MHC-I molecules complexed with particular peptides. Among the polymorphic amino acid residues that distinguish HLA-C*03 and HLA-C*04 are Asn80 of -C*03 and Lys80 of -C*04. Thus, it was of interest when the structures of HLA-C*03/KIR2DL2[104] and HLA-C*04/KIR2DL1 complexes were reported.[105] The KIR2DL2/HLA-C*03 interaction is illustrated in Figure 21.22. The important conclusions from this structure and those of similar KIR2DL complexes is that the recognition of MHC-I is via amino acid residues of the

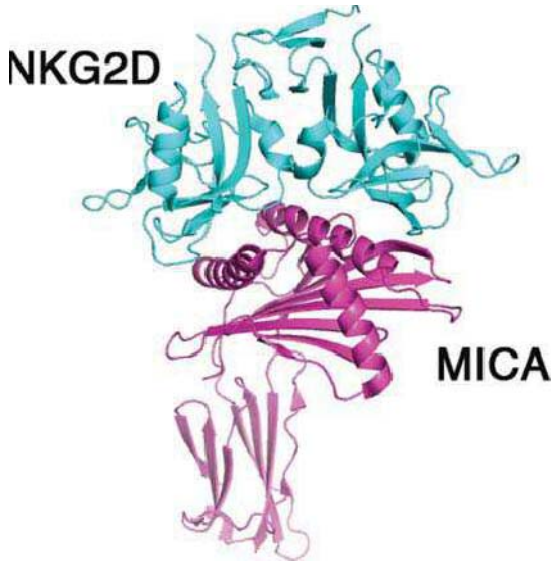

FIG. 21.21. The NKG2D sees the MHC-Ib MICA α-Helical Superdomain. The structure of the human NKG2D/MICA complex (PDB 1HYR) reveals the homodimer NKG2D sitting asymmetrically astride the MICA α-helical superdomain. The NKG2D domains are in *cyan* and MICA in *magenta*.

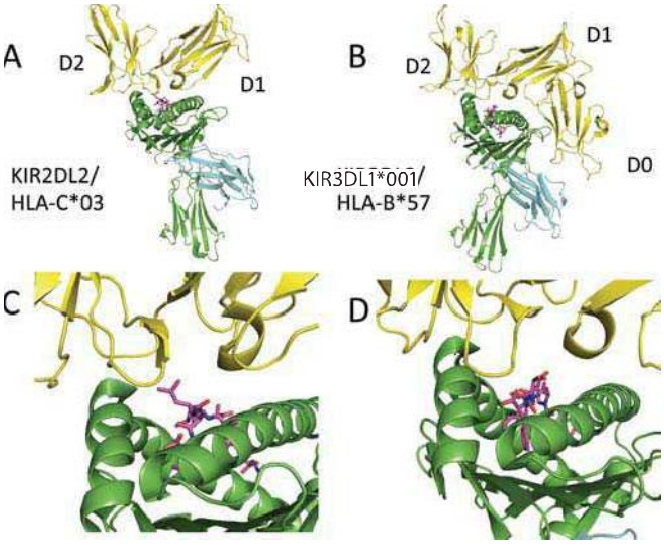

FIG. 21.22. KIR2DL2 and KIR3DL1 Interact Similarly with Their MHC-I/p Ligands. KIR2DL2 complexed with HLA-C*03 (PDB 1EFX) **(A,C)** and KIR3DL1 complexed with HLA-B*57:01 (PDB 3VH8) **(B,D)** are shown to illustrate the overall interactions **(A,B)** and close-ups of the contact with bound peptide **(C,D).**

elbow bend joining the two Ig-like domains of the KIR, and that residues that vary among different KIRs determine the molecular specificity of the interaction with the particular allelic product of HLA-C. In addition, the interaction of the KIR with the HLA-C is also modulated by the particular bound peptide, explaining the results of binding/peptide specificity studies. Structural understanding of the KIR3 molecules, which have three extracellular Ig-like domains, eluded the field for many years. Recently, a molecular complex of HLA-B*57:01 with KIR3DL1 has been reported (see Fig. 21.22). This structure reveals the contribution of the D0 amino terminal domain of KIR3DL1 in recognizing a region of the MHC-I with limited polymorphism, the D1 domain accommodates a region with sequence variation, and the D2 domain shows high complementarity.

The mouse exploits a set of NK receptors of a different structural family to provide the same function. In particular, the predominant, and best studied, mouse NK receptors are those of the Ly49 family. Functional experiments had demonstrated that Ly49A interacts with the MHC-I molecule, H2-D^d, and also that for appropriate interaction, H2-D^d needs to be complexed with a peptide. In contrast to human NK recognition, however, surveys of H2-D^d–binding peptides revealed little if any peptide preference or specificity. This would explain the function of the NK inhibitory receptor in that they are at baseline chronically stimulated by normal MHC-I on somatic cells, turning off the NK cell. When MHC-I is dysregulated by tumorigenesis or by pathogenic infection, the lower level of surface MHC-I diminishes the NKIR-mediated signal, and the NK cell is activated. The structure of the mouse Ly49A inhibitory receptor in complex with its MHC-I ligand, H2-D^d (Fig. 21.23), reveals several crucial features of the interaction: 1) the Ly49A C-type lectin–like molecule is a homodimer; 2) the Ly49A molecule makes no direct contact with residues of the MHC-bound peptide; and 3) in the x-ray structure, there are two potential sites for Ly49A interaction with the MHC molecule: site 1 at the end of the α1 and α2 helices, and site 2, an extensive region making contact with the floor of the peptide binding groove, the α3 domain of the MHC-I molecule, and the β2m domain as well. The ambiguity suggested by the x-ray structure has been resolved by extensive mutagenesis studies that are consistent with the view that site 2 is functionally significant.[516,517] Recently, several other NK receptors have been studied structurally. These include the Ly49I inhibitory receptor, which interacts functionally with H2-K^b. The Ly49I has been crystallized without a ligand, revealing a basic fold similar to that of Ly49A but with a somewhat different dimeric arrangement.[518] The Ly49C, another murine NK inhibitory receptor, has been examined in complex with its H2-K^b ligand.[519] Differences between H2-K^b/Ly49C and H2-D^d/Ly49A suggest different modes of NK receptor binding to MHC-I depending on whether the interaction is "cis" (ie, between the NK receptor and the MHC-I on the same NK cell) or "trans" (between the NK receptor on the NK cell and the MHC-I on its target).[520]

MHC-II Superantigen Complexes

Superantigens are molecules, frequently toxic products of bacteria, which bind MHC molecules on the cell surface and are then presented to a large subset of T cells, usually defined by the expression of a particular family of TCR V regions.[521] Most of the known superantigens bind MHC-II molecules, although one, the agglutinin from *Urtica dioica*, the stinging nettle, can be bound by both MHC-I and MHC-II molecules and presented to T cells of the Vβ8.3 family.[522] Its structure in complex with carbohydrate ligand has been reported.[523,524] MHC-II interactions with superantigens, such as those derived from pathogenic bacteria, are the first step in the presentation of the multivalent array of the APC-bound superantigen to T cells bearing receptors of the family or class that can bind the superantigen.[521] A number of structural studies examining the interaction

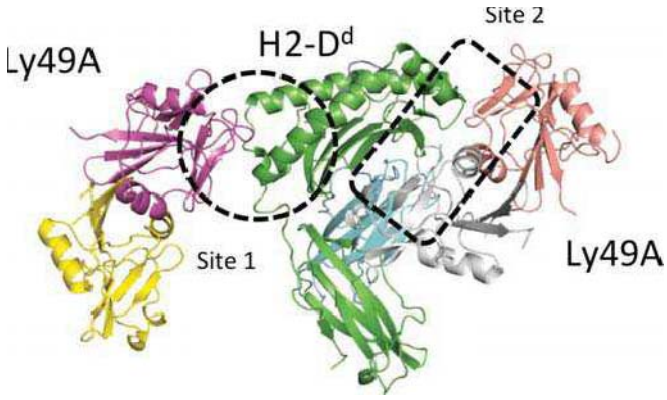

FIG. 21.23. Structure of the H2-D^d/Ly49A Complex Reveals an Alternate Site for MHC/Natural Killer Receptor Interaction. The structure of the complex (PDB 1Q03) is shown, illustrating both potential sites of interaction.

of superantigen both with their MHC-II ligands and with TCR have been reported.[525] Structural analysis of crystals derived from staphylococcal enterotoxin B complexed with HLA-DR1[526] and from toxic shock syndrome toxin-1 complexed with HLA-DR1[527] revealed that the two toxins bind to an overlapping site, primarily on the MHC-II α chain, and indicated that the staphylococcal enterotoxin B site would not be expected to be influenced by the specific peptide bound by the MHC, although the toxic shock syndrome toxin-1 site would. The view that superantigens exert their biologic effects by interaction with conserved regions of the MHC-II molecule as well as with conserved regions of the TCR has been challenged by the determination of two structures, that of *S. aureus* enterotoxin H complexed with HLA-DR1[528] and that of streptococcal pyrogenic toxin C in complex with HLA-DR2a.[529] Both of these studies indicate that these superantigens can interact with the MHC-II β chain through a zinc-dependent site that includes superantigen contacts to bound peptide. Our understanding of superantigen interactions has been augmented by the determination of ternary structures of MHC-II/superantigen/TCR complexes for two examples, that of the *Mycoplasma arthritidis* mitogen in complex with HLA-DR1 and TCR,[530] and that of *Staphylococcus aureus* enterotoxin H complexed with HLA-DR1 and TCR.[531] In both of these cases, the superantigen interacts with the MHC-II and with both TCR Vα and Vβ domains.

MOLECULAR INTERACTIONS OF MHC MOLECULES

Assays for Molecular Interactions

Whereas the crystal structures provide a vivid static illustration of the interactions of MHC molecules with their peptide, FcRn, CD8, CD4, superantigen, NK receptor, and TCR ligands, the dynamic aspects of these binding steps can be approached by a variety of biophysical methods.[532] It is important to note that affinities and kinetics of interaction of MHC/peptide complexes for TCR have been determined by several methods in a variety of systems.[533–540] In addition, MHC interactions with NK receptors[101,102,541,542] have been quantified by similar techniques. Although there are clear differences in the affinity and kinetics of binding of different TCR and NK receptors for their respective cognate MHC/peptide complexes, the generally consistent findings are that the affinities are low to moderate (ie, $K_d = 5 \times 10^{-5}$ to 10^{-7} M) and are characterized by relatively rapid dissociation rates (ie, $k_d = 10^{-1}$ to 10^{-3} sec^{-1}). Recently, attention has been drawn to the differences in measuring interactions with one component in solution (three-dimensional affinity) as compared with measurements made between multivalent displays of the interactions on two surfaces, such as might mimic the apposition of two cells. Methods exploiting microscopic techniques[543,544] or a micropipette adhesion frequency assay[545,546] suggest that the biologic readout of T-cell activation may be more readily correlated with two-dimensional rather than three-dimensional measurements of MHC/TCR affinities.

Multivalent MHC/Peptide Complexes

A major development in the past several years has been the engineering and application of multivalent MHC/peptide complexes for the identification, quantification, purification, and functional modulation of T cells with particular MHC or MHC/peptide specificity. Two general approaches have been exploited: one based on the enzymatic biotinylation of soluble MHC/peptide molecules generated in bacterial expression systems that are then multimerized by binding of the biotinylated molecules to the tetravalent streptavidin,[547] and another based on the engineering of dimeric MHC/Ig fusion proteins.[548] These reagents can be used in flow cytometric assays that permit the direct enumeration of MHC/peptide-specific T cells taken directly ex vivo. In either of these methods, multivalent MHC/peptide complexes are generated, and the relatively weak intrinsic affinity of the MHC/peptide complex for its cognate TCR is effectively magnified by the gain in avidity obtained by the increase in valency. For MHC-I molecules, the technology has been so reliable in producing multivalent (tetrameric) molecules loaded homogeneously with synthetic peptides that a wide variety of specific MHC-I/peptide multimers are available either from a resource facility sponsored by the National Institute of Allergy and Infectious Diseases (www.niaid.nih.gov/reposit/tetramer/index.html) or from commercial suppliers that offer either the tetramer, Ig multimer, or a pentamer preparation. The MHC-I/peptide multimers have also been exploited for identification of specific populations of NK cells and for assignment of various NK-receptor specificities.[549–552] For some MHC-II molecules, similar success has been achieved in the production of such multimers, using insect cell or mammalian cell expression systems for molecules produced by either the tetramer or Ig chimera strategy.[553–557] MHC-II/peptide tetramers can be effectively used for a variety of CD4 T-cell applications, but some TCRs (particularly those involved in autoimmune diseases) have an affinity for their cognate MHC-II/peptide that is beneath the threshold for detection.[545] Also, the potential for the same peptide binding to one MHC-II in multiple frames generates difficulties in the engineering and preparation of effective MHC-II tetramers.[406]

CONCLUSION

We have surveyed the *Mhc* as a genetic region and a source for molecules crucial to immune regulation and immunologic disease. These genes reflect the panoply of mechanisms involved in the evolution of complex systems and encode cell surface proteins that interact via complex orchestration with small molecules including peptides and glycolipids as well as with macromolecular receptors on T cells and NK cells. The MHC-I molecules provide the immune system with a window for viewing the biologic health of the cell in which they are expressed, and MHC-II molecules function as scavengers to taste and display the remnants of the cellular environment. Viruses and bacterial pathogens contribute enormously to the genetic dance—

they modulate and compete in the control of MHC expression, sometimes exploiting MHC mimics that they have acquired—and the host by adjusting its T-cell and NK-cell repertoire on the time scale of both the individual organism and the species, resisting the push to extinction. The immune system, dynamically, resourcefully, and creatively provides, through the concerted action of its MHC molecules, TCRs, NK receptors, and antibodies as well as a host of other regulatory molecules, an organ system vital not only to the survival of the individual but also to the success of the species. As we understand better the molecular functions of the MHC, we should better understand rational approaches to manipulating the immune system in the prevention, diagnosis, and treatment of immunologic and infectious diseases.

ACKNOWLEDGMENTS

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CHAPTER 22

Antigen Processing and Presentation

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ANTIGEN PRESENTATION PATHWAYS

As part of the adaptive immune response, T cells mount immune responses to diseased cells and abnormal cells. Remarkably, the mechanism by which T cells discriminate self from non-self is based on pathogen- or tumor-derived proteins displayed on the cell surface by self–major histocompatibility complex (MHC) molecules. More specifically, when T cells detect diseased cells displaying MHC molecules loaded with peptides derived from foreign peptides, effector mechanisms are initiated to eliminate the diseased cell. By contrast, T cells that detect cells displaying MHC molecules loaded with peptides derived from self-proteins are either eliminated during ontogeny by negative selection or suppressed by peripheral tolerance mechanisms. Thus, at the molecular level, the T-cell receptors on T cells discriminates MHC loaded with self- versus non–self-peptides displayed at the cell surface of normal and diseased cells. The mechanisms by which peptide ligands for MHC molecules are generated is referred to as *antigen processing*, whereas the mechanism by which peptide/MHC complexes are displayed at the cell surface is called *antigen presentation*.

To assure the appropriate effector mechanism is generated to detect cells infected with pathogens in the cytosol or endocytic compartments, the antigen presentation pathways for classical cluster of differentiation (CD)8+ versus CD4+ T cells are different. More specifically, antigen derived from intracellular pathogens is typically bound to MHC-I proteins that are uniquely detected by CD8 T cells, whereas antigen derived from extracellular pathogens is typically bound to MHC-II proteins that are uniquely detected by CD4 T cells. To preferentially bind peptides derived from proteins synthesized inside the cell, the processing of peptides that bind MHC-I proteins occurs in the cytosol and the loading of these peptides occurs in the endoplasmic reticulum (ER). This pathway is commonly referred to as the classical MHC-I antigen-binding pathway. By contrast, to preferentially bind peptides from proteins synthesized outside the cell, the processing and loading of antigenic peptide ligands for MHC-II proteins occurs in an endocytic pathway terminating in the lysosomes. This pathway is typically referred to the classical MHC-II antigen-binding pathway. The differences between the MHC-I and MHC-II antigen presentation pathways are determined by their differential interaction with molecular chaperones. These same molecular chaperones enforce the quality control of antigen presentation to assure appropriate T cells are activated for detection and elimination of pathogen-infected cells or tumors.

ANTIGEN PROCESSING AND PRESENTATION BY MAJOR HISTOCOMPATIBILITY CLASS I (MHC-I) PROTEINS

Origin of MHC-I binding peptides

As detailed in Chapter 21, MHC-I proteins preferentially bind peptides of 8 to 10 amino acid lengths, and which peptides bind is determined by MHC-I allele-specific polymorphic residues that form the architecture of its peptide binding groove. Each MHC-I allele typically binds peptides with a consensus binding motif requiring a relatively specific amino acid central anchor and a C-terminal hydrophobic amino acid. Given the lack of restraints in most peptide positions and the large number of proteins synthesized by each cell relative to the number of MHC molecules, the selective processes controlling which peptides get presented is of considerable importance for the detection of pathogens and malignancies.

The source of the peptides that get presented during infection has been a question of considerable investigation. It has been known for some time that MHC-I–binding peptides are derived from membrane-bound, secreted, cytosolic, and nuclear proteins that are almost exclusively generated by the proteasome in the cytosol. However, the uncertainty of peptide source is based on two apparently incongruent observations. Most proteins from which MHC-I–binding peptides are derived have slow rates of turnover in cells ranging from hours to days; viral-derived peptides are presented by infected cells to CD8 T cells within a few minutes. This disparity between the degradation versus presentation kinetics is potentially explained by cumulative observations that a fraction of newly synthesized polypeptides are rapidly degraded, providing a peptide reservoir sufficient to support a robust CD8 T-cell response. Supporting evidence for this conclusion comes from studies that block protein synthesis with cycloheximide and detected a rapid decrease in the peptide supply for MHC-I binding.[1,2] One of the more elegant ways to monitor peptide transport into the ER was by transporter associated with antigen presentation (TAP) mobility in the ER membrane by photobleaching; establishing TAP mobility was proportional to the peptides in the cytosol. This assay was used to show a 90% reduction in peptide supply after 30 m treatment with cycloheximide, thus kinetically linking presentation closely to translation.[3] Indeed, there are now several reports that within 30 minutes after synthesis, antigenic peptides are presented at

the cell surface at levels that activate T cells.[4,5] What remained unclear from these studies is the biochemical and mechanistic basis for the generation of these rapidly transcribed proteins from which MHC-I–binding peptides are derived.

In a model that has gained wide acceptance, Yewdell and colleagues proposed that a rapid supply of MHC-I ligands is derived from aberrant protein production they termed defective ribosomal products or DriPs.[6,7] In their model, it was speculated that DriPs could be derived from unfolded or misfolded proteins of the proper sequence and length, proteins with errors in sequence or posttranslational modifications, prematurely terminated proteins, and proteins translated from the wrong start codon.[8] Regarding aberrant translations, there is evidence that MHC-presented peptides can arise from alternative reading frames, generating what has been called cryptic epitopes.[9] For example, Schwab et al. showed that inhibition of the eukaryotic translational initiation factor eIF2α resulted in the synthesis of cryptic peptides and intriguingly, viruses inhibit host translation by inactivating eIF2α.[10,11] However, most MHC-I–presented peptides have native amino acid sequence and are derived from standard messenger ribonucleic acid (mRNA) translation products, suggesting cryptic epitopes represent only a minor component of MHC-presented peptides. In addition, the contribution of peptides derived from misfolded proteins in the secretory pathway is also likely modest. Although antigenic peptides from misfolded tyrosinase were found to be preferentially presented,[12] other studies show that several mutations causing misfolding do not lead to enhanced antigen presentation. Furthermore, ER quality control is typically too slow to account for the rapid supply of MHC-I–binding peptides.[13] The reason for this kinetic delay is that substrates for ER-associated degradation (ERAD) must be 1) translocated into the ER, 2) detected presumably by ER chaperones as being misfolded, 3) ubiquitinated and extracted from the ER, and 4) degraded by the proteasome in the cytosol. Thus the predominance of MHC-I–presented peptides likely does not come from cryptic epitopes or misfolded ER proteins identified by ER quality control.

To obviate trafficking into the ER, misfolded secretory proteins could be degraded rapidly based on degron detection such as unshielded hydrophobicity.[14] Alternatively, early in translation a lack of signal recognition particle engagement could lead to the mistargeting of secretory proteins to the cytosol. In support of this model, Schlosser et al.[15] showed that an epitope encompassing the signal peptidase cleavage site was efficiently presented by MHC-I proteins. To rapidly generate peptides, it has been speculated that cells may have select ribosomes called "immunoribosomes."[16] Such immunoribosomes might be more adept at generating peptides with TAP access perhaps by compartmentalization and/or targeting proteins to the 20S proteasome for immediate destruction. In any case, there is considerable evidence that antigenic peptides can be rapidly presented by MHC-I within minutes after translation of the protein source. The physiologic significance of this rapid kinetics of antigen presentation is that it allows CD8 T cells to kill virus-infected cells before viral replication is completed and progeny are released. However, whether the production of peptides bound to MHC class I molecules is a deliberate process for degrading defective translation products and/or a stochastic event of normal protein translation remains to be determined.[17]

Peptide Trimming in the Cytosol

Degradation of cellular and antigenic peptides bound to MHC-I is largely mediated in the cytosol by the proteasome. The proteasome is responsible for the degradation of the majority of cytosolic and nuclear proteins, and in most cases proteasome targeted proteins are ubiquitinated. Ubiquitin (Ub) is typically coupled to internal lysine residues of proteins substrates, but coupling can also occur at the N terminus or on internal cysteine, serine, or threonine residues.[18–21] In this orchestrated process, activated Ub is transferred from one predominant Ub-activating enzyme (E1) to one of 30 to 40 mammalian Ub conjugating enzymes (E2s) and then to the substrate that is bound by one of hundreds of different ubiquitin protein ligases (E3s). The E3s are the major determinant of substrate specificity. This process can be repeated to form polyUb chains whereupon the next Ub moiety is added to one of the seven internal lysine residues of Ub. Proteins coupled with polyUb chains of four or more Ub moieties linked through Lys48 Ub residues are the prototypic signal for proteasome mediated degradation.

The cylindrical 20S catalytic particle (CP) of the proteasome is formed by four stacked rings of seven subunits each. The inner two rings of the 20S proteasome are assembled from the beta subunits, three of which have catalytic activity with chymotryptic, tryptic, or caspase activity. Thus each proteasome has six active sites with the ability to cleave after most types of peptide bonds, although to differing efficiencies based on flanking residues. The catalytic sites are exposed to the interior of the central chamber of the 20S cylinder. The two outer rings of the 20S proteasome are assembled from alpha subunits forming a pore of 13 Angstrom, mandating protein substrates be partially denatured prior to entering the interior of the chamber. The gate of the 20S CP is normally closed, and access to the interior of the 20S proteasome is controlled by the complex of proteins termed the 19S regulatory particle that binds to each end of the 20S cylinder. The full assemblage of 19S-20S-19S complex constitutes the 26S proteasome. The lid of the 19S regulator particle binds and deubiquitinates protein substrates before they gain entry in the 20S CP; the base of the 19S regulatory particle contains ATPases that promote substrate unfolding and open the gate of the 20S CP to provide access of denatured protein substrates to the catalytic activity of the inner chamber.

The evolutionarily conserved function of the proteasome is for recycling amino acids and ubiquitin moieties. However, in mammals, modifications to the proteasome have been made to promote antigen presentation. In response to interferon (IFN)γ stimulation that occurs during inflammation, three catalytic subunits of the constitutively expressed proteasome are replaced by IFNγ-induced homologs forming what is called the immunoproteasome. More specifically, subunits β1, β2, and β5 of the constitutive proteasome are replaced by subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) of the immunoproteasome. Suggesting an immunologic function, LMP2 and 5 are genetically encoded within a central

region of the mouse and human MHCs juxtaposed to genes encoding the TAP heterodimer. Consistent with their immunologic relevance, the immunoproteasomes display enhanced cleavage of protein substrates after hydrophobic and basic amino acids, thus generating peptides with C-terminal residues preferred for binding to many MHC-I alleles. IFNγ stimulation also induces the expression of PA28α and PA28β that form the 11S regulatory particle, which can also bind to either end of the 20S CP. Interestingly, the PA28 regulatory particle does not contain ATPases, but it does induce conformational changes to open the 20S CP gate. Thus, PA28 may function to prevent the overdigestion of short peptides. This is an important issue, as it has been estimated that a nonamer peptide in the cytosol has a half-life of only 7 seconds.[22] However, studies testing PA28α/β-deficient mice demonstrated that PA28α/β is not required for overall antigen presentation during virus infection, but may affect degradation of select substrates.[23] In any case, the inclusion of the IFNγ-inducible β subunits into the 20S CP of immunoproteasome enhances antigen presentation by generating peptides with preferred C-terminal anchor residues, and in some same cases inclusion of the PA28 regulatory particle may prevent overdegradation of immunologically relevant proteins.

The proteasome generates the final C-terminal cleavage of most MHC-I–binding peptides. A recently reported exception to this is the generation of the tumor antigen MAGE-A3 by insulin-degrading enzyme.[23] Whereas insulin degrading enzyme is solely responsible for the generation of the human leukocyte antigen (HLA)-A1–restricted MAGE-A3 epitope, the HLA-B40–restricted MAGE-A3 epitope was proteasome dependent. Although there are several N-terminal proteases in the cytosol, they have only a limited role in generation of the MHC-I–binding peptides. More specifically, tripeptidyl peptidase II, leucine amino peptidase, bleomycin hydrolase, and puromycin-sensitive aminopeptidase have all been found to affect generation of select MHC-I–binding epitopes, but these proteases have an overall negative effect on antigen presentation by MHC-I. Interestingly, the metalloprotease nardilysin that cleaves substrates on the N-terminus of arginine residues in dibasic pairs was implicated in three cytotoxic T lymphocyte epitopes and may be more generalizable given the preference for basic N-terminal resides for MHC-I binding.[24] However, as noted in the following, significant N terminal trimming of MHC-I–binding peptide occurs after transport from the cytosol into the ER.

Peptide Transport into the Endoplasmic Reticulum

Landmark studies of the mutagenized and immunoselected mouse cell line RMA-S resulted in the discovery of how peptides are transported from the cytosol into the ER lumen.[25–27] The low level of surface expression of MHC-I molecules by RMA-S that could be rescued by either low temperatures or culturing with known MHC-I–binding peptides was attributed to lack of peptide transport by TAP. TAP is a member of the adenosine triphosphate (ATP) binding cassette family. Mechanistically, hydrolysis of ATP is required both for peptide binding to the cytosolic face of TAP as well as peptide transport into the ER. Structurally, TAP is a heterodimeric complex of TAP1 and TAP2 subunits both encoded within the central region of the MHC of mouse and human. The translocation pore of TAP is formed by six transmembrane domains of each subunit, whereas the remaining transmembrane domains (four for TAP1 and three for TAP2) are involved in interaction with peptide loading complex (PLC).[28] Awaiting transport, both the N- and C-termini of peptides are bound to TAP[29]; peptides with higher affinity for binding TAP have a greater likelihood of transport, MHC binding, and presentation to T cells during infection.[30,31] TAP typically transports peptides of 8 to 16 residues, although longer peptides are less efficiently transported. As discussed in the next section, peptides with N-terminal extensions are trimmed in the ER.

Peptide Trimming in the Endoplasmic Reticulum

As noted previously, TAP transports many peptides with C-terminal hydrophobic residues required for MHC binding, but with N-terminal extensions, which need to be cleaved to conform to the MHC-binding motif of the expressed alleles. It is now clear that N-terminal trimming within the ER is carried out by the ER-associated aminopeptidase (ERAAP; ERAP1 in mice and ERAP1 in humans). Interestingly, unlike other aminopeptidases, ERAP1 preferentially cleaved peptides of 9 to 16 amino acids thus matching the same peptide length preference as transported by TAP.[32] More specifically, ERAP1 was found to degrade a model 13-mer to a 9-mer and then stop, an apparent adaptation to maximize optimal MHC-I binding. The length preference of ERAP1 could be explained by extended peptides binding to MHC-I before N-terminal trimming. However, this model is not supported by structural analyses of ERAP1 and the location of its active site.[33] Alternatively, recent analyses support a "molecular ruler" model. In this model, a hydrophobic pocket of ERAP1 binds the C-terminal peptide residue thereby positioning the peptide so that N-terminal cleavage occurs about nine amino acids away.[32,33] Thus, ERAAP/ERAP1 has unique structural features explaining its ability to trim extended peptide precursors while sparing ones of optimal length for MHC-I binding. As further evidence of their specialized functions in antigen presentation by MHC-I proteins, ERAP1 preferentially binds and processes peptides with hydrophobic C-terminal residues consistent with the majority of the TAP-transported MHC-I–binding peptides. Interestingly, humans have a second ER-associated peptidase with homology to ERAP1 designated ERAP2.[34] ERAP2 preferentially binds peptides with C-terminal basic residues and thus may function in human cells to provide peptides for HLA-A3, -Aw68, and -B27 alleles that bind such peptides. Additionally, in humans the combined activity of both ERAP1 and ERAP2 (most likely as heterdimers) is required for the presentation of certain epitopes.[35] By contrast, in mice ERAAP/ERAP1 is sufficient to service their MHC-I alleles, all of which bind peptides with hydrophobic C-termini. As additional evidence for their tailoring for MHC-I–antigen presentation, the expression and trimming activity of ERAP1 and ERAP2 are upregulated upon IFNγ stimulation.

The impact of ERAP on specific epitopes and the overall MHC-binding peptide repertoire has been analyzed using

cell lines[36,37] and ERAAP-knockout mice.[38,39] These studies have shown that cytotoxic T lymphocyte detection of some but not all MHC-I/peptide complexes are ERAP1-dependent. For example, in vitro ribonucleic acid interference experiments suggested that ERAP1 is involved in formation of one-third of peptide/MHC-I complexes.[36,37] ERAAP-deficient mice had a 20% lower expression of K^b, D^b, K^d, and D^d alleles at the cell surface but a 70% lower expression of L^d. Of these mouse alleles, only L^d preferentially binds peptides with the motif of Pro in the second position, although about 20% of human HLA alleles also prefer peptides with a Pro in the second position. Notably, peptides with Pro in the second position are only transported by TAP with N-terminal extensions and are thus dependent upon ERAP1 for their generation. In vivo relevance of this conclusion was demonstrated by the observation that ERAAP-deficient BALB/c mice were found to be susceptible to *Toxoplasma gondii* infection resulting from the fact that the immunodominant epitope presented to CD8 T cells is L^d restricted.[40] Interestingly the ERAAP-deficient mice were also found to present several unique peptides that elicited potent CD8 T-cell responses demonstrating ERAAP functions, as a peptide editor altering the repertoire of peptides presented.[38]

Chaperone-assisted Peptide Loading in the Endoplasmic Reticulum

Full assembly of the MHC-I heavy chains (HCs) with β2m and peptide within the ER is orchestrated by molecular chaperones that keep folding intermediates in a conformation capable of attaining full assembly (Fig. 22.1). Nascent HCs are transiently associated with the membrane-anchored

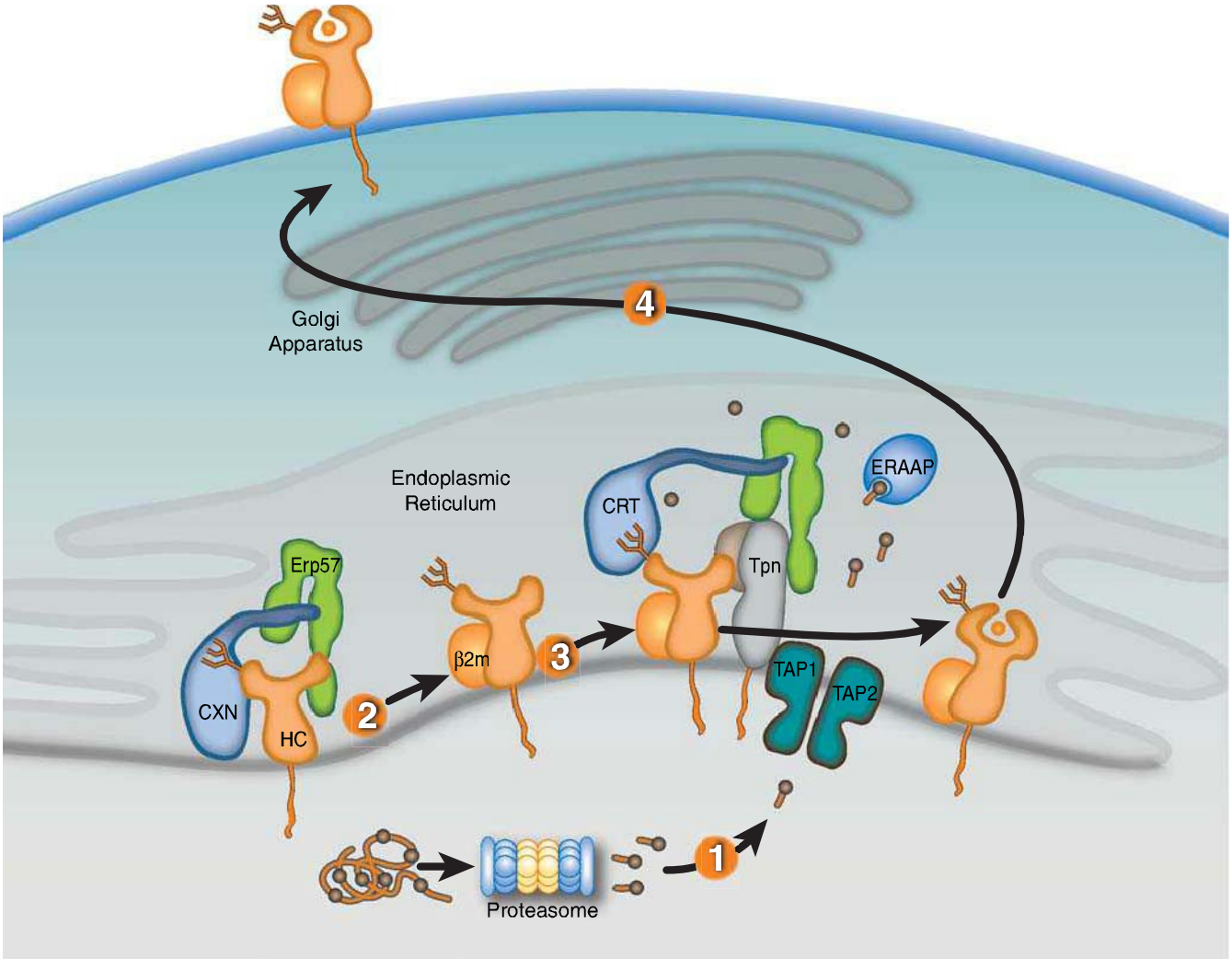


FIG. 22.1. Schematic Representation of the Sequential Events of Antigen Processing and Presentation by Major Histocompatibility Complex (MHC)-I Proteins. 1: Peptides are processed by the proteasome and translocated into the ER by TAP1/TAP2 heterodimers. 2: MHC-I heavy chains (HCs) awaiting assembly with β2m are bound to calnexin (CXN)/ERp57 complexes. 3: After assembly with β2m, HC/β2m heterodimers enter the peptide loading complex wherein tapasin (Tpn) bridges HC with transporter associated with antigen presentation (TAP)/TAP2, and calreticulin (CRT)/ERp57 complexes bridge HC with Tpn. 4: Following the binding of a suitable peptide, which may require final N-terminal trimming by ERAAP, fully assembled MHC-I proteins (HC/β2m/peptide) traffic to the plasma membrane via the secretory pathway.

lectin calnexin (CXN) that, like its soluble paralog calreticulin (CRT), functions as a general chaperone for assembly of oligomeric glycoproteins. Quality control of glycoprotein assembly by CXN and CRT is carried out by monitoring terminal glucose residues on their N-linked glycans. More specifically, the folding sensor UDP-glucose glycoprotein glucotransferase allows incompletely assembled substrates to reassociate with CXN or CRT, and cycles of glucose addition and removal continue until substrates are either correctly folded and assembled or targeted for ERAD. After the MHC-I HC assembles with β2m, the HC/β2m heterodimer exchanges CXN for CRT and enters the PLC consisting of CRT, ERp57, tapasin (Tpn), and TAP.[41–44] Optimal antigen presentation by MHC-I is dependent upon all four components of the PLC (Tpn, TAP, CRT, and ERp57) that function to improve peptide loading in MHC-I proteins and their surface display.[45,46]

Knockout cell lines, genetically deficient mouse strains, and mutagenesis studies have provided key insights in the selective roles of PLC components in MHC-I assembly. CRT association with MHC-I proteins is dependent upon the N-linked glycan at residue Asn86 of the HC. Interestingly, the location of the glycan is important for CRT but not CXN association with HCs, likely reflecting geometric constraints imposed by PLC assembly. CRT-deficient cells have suboptimal peptide loading resulting in reduced surface MHC-I expression.[47] ERp57 is a thiol oxidoreductase that mediates disulfide bond formation of different substrates and is commonly associated with CXN and CRT. Interestingly, however, the function of ERp57 as a component of the PLC appears to be independent of the MHC-I redox state. ERp57 forms a disulfide bond with Tpn that is required for PLC construction.[48,49] It was originally proposed that the ERp57-Tpn complex prevents reduction of the α2 disulfide HC bond, thereby keeping HC in a peptide-receptive state.[50] However, other studies have detected little evidence the ERp57 controls redox state of MHC-I and more recently, the crystal structure of the ERp57-Tpn complex suggests that the role of the ERp57 in the PLC is structural, facilitating recruitment of peptide-accessible MHC-I.[49,51,52] As a likely reflection of its fundamental role in PLC construction, ERp57-deficient cells were found to have impaired peptide loading, surface expression, and antigen presentation by MHC-I proteins.

Tpn, an MHC-I–dedicated molecular chaperone, is required to bridge β2m-assembled HCs with TAP.[53] Playing a redundant role with other members of the PLC, Tpn also functions in ER retention of MHC with suboptimal peptide cargo.[54,55] Perhaps most importantly, Tpn functions as a peptide editor by stabilizing peptide-accessible MHC-I proteins and optimizing peptide cargo before release from the PLC. The mechanism of peptide editing by Tpn of MHC-I proteins was revealed using recombinant Tpn tethered to HLA,[56] and using recombinant Tpn-ERp57 conjugates added to Tpn-deficient cells.[57] These and other reports support the model that Tpn promotes the peptide exchange of MHC molecules in an affinity-dependent manner. The consensus model is that Tpn stabilizes the peptide binding groove of MHC-I in an "open" peptide-accessible conformation. The association of Tpn with peptide-accessible MHC-I then promotes peptide exchange until a peptide of suitable affinity binds to complete the folding of the ligand binding groove. This peptide-induced folding then induces the release of fully assembled MHC-I from the PLC. Logistically, how peptide editing by Tpn might occur was provided by mutagenesis studies of the MHC-I/Tpn interaction site. Two sites on the HC are critical for Tpn interaction: one site is in the α3 domain (residues 227 and 229) and the other in the α2 domain (residues 128-136)[43,58,59] (Fig. 22.2A). Based on the location of the latter site and molecular dynamic modeling, it has been theorized that the α2 interaction site might function as a folding sensor for C-terminal peptide anchoring in the MHC-I F pocket.[60,61] Consistent with this model, polymorphisms within the F pocket can have a profound effect of Tpn dependencies of different MHC-I alleles.[62] Based on mutagenesis and structural analyses, a credible model was constructed by Dong et al. of the MHC-I/PLC interactions[52] (see Fig. 22.2).

Although Tpn within the PLC is thought to enforce most of the quality control of antigen presentation by MHC-I, there are also adjunct pathways. For example, the sensor UGT1 that adds a terminal glucose to unfolded ER proteins for rebinding CRT has been recently implicated in the quality control of antigen presentation by MHC-I. In support of this conclusion, using in vitro assays with recombinant components, UTG1 was reported to preferentially reglucosylate MHC-I proteins loaded with suboptimal peptides.[63] Also, as a post-ER quality control pathway, CRT was reported to use a KDEL-dependent mechanism to recycle suboptimally loaded MHC-I proteins from the early Golgi back the ER for improved peptide binding.[64]

Viral Immune Evasion of Antigen Presentation

As noted previously, several molecular components of antigen presentation were coopted from physiologic pathways of protein degradation and quality control. Using elegant mechanisms, viruses express diverse proteins that coopt these same pathways of protein degradation and quality control to evade immune detection by CD8 T cells.[65] Indeed, the specificity and potency of immune evasion proteins impairing antigen presentation makes them efficacious probes for physiologic pathways of relevance for MHC expression. Most of the well-characterized immune evasion proteins are expressed by deoxyribonucleic acid viruses with large genomes, particular viruses capable of latency or host coexistence. Strikingly, immune evasion proteins block several different steps of the antigen presentation pathway by MHC-I proteins (Fig. 22.3). For example, Epstein-Barr virus and Kaposi sarcoma–associated herpesvirus express proteins during latency that escape cytotoxic T lymphocyte detection by containing sequences that inhibit proteasome processing.[66–70] Alternatively, blocking peptide transport by inhibiting TAP function is a commonly used immune evasion strategy. For example, ICP47 of herpes simplex virus binds the cytosolic side of TAP and blocks peptide and not ATP binding,[71–76] whereas US6 of human cytomegalovirus (HCMV) binds the luminal side of TAP and induces a conformational change resulting in inhibition of ATP hydrolysis

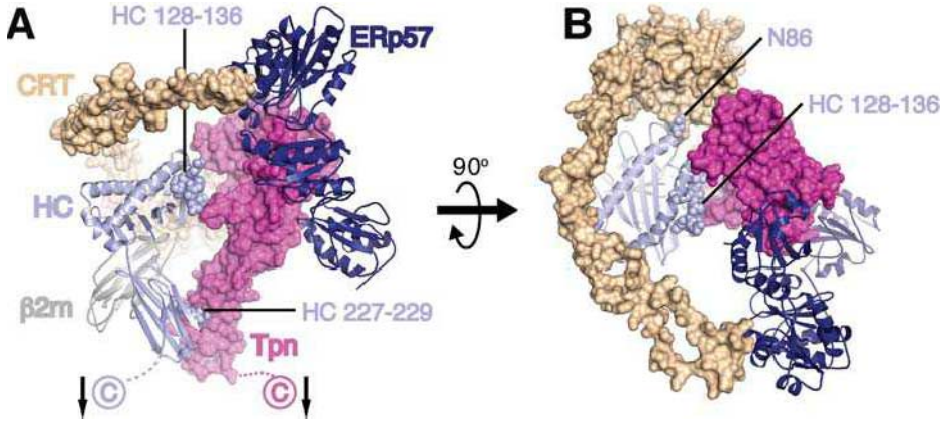


FIG. 22.2. A Model of the Peptide Loading Complex Based on Known Structures and Mutagenesis Data of Interaction Sites.[52] Heavy chains (HCs), β2m, and ERp57 are shown as ribbons, while calreticulin (CRT) and tapasin (Tpn) are shown as Connolly surfaces (1.4 Å probe). HCs and Tpn are membrane anchored as indicated with *arrows*, whereas transmembrane interactions between Tpn and TAP (not shown) attach the phospholipase C (PLC) components to TAP. HC sites predicted by mutagenesis studies to interact with Tpn (residues 227 to 229 and 128 to 136) and CRT (N86) are indicated as *spheres*. The long flexible P domain of CRT extends above the major histocompatibility complex (MHC)-I peptide binding platform before it noncovalently binds to ERp57, which is covalently attached to Tpn. **A:** PLC components are shown from the side to highlight Tpn contacts to the HC α2 and α3 domains. **B:** PLC components shown from above reveal coordinated chaperone binding of both MHCI α-helices near the antigenic peptide's C-terminus. The figure was rendered by Drs. William McCoy IV and Daved Fremont using Protein Data Bank (PDB) files kindly provided by Drs. Karin Reinisch and Peter Cresswell. The structures of the Tpn/ERp57 complex was published in Dong et al.,[52] the structure of CRT was modeled using PHYRE2[235] on its homolog calnexin,[236] and human leukocyte antigen-A2[237] is shown as a representative HC. It should be noted that the HC conformation shown is that attained after peptide occupancy, as the peptide-accessible HC conformation that binds the PLC has not been resolved.

and peptide translocation.[77–81] As a third mechanism of TAP blocking, UL49.5 of bovine herpesvirus induces TAP degradation.[82] There are also published examples of immune evasion proteins inhibiting Tpn function. For example, US3 of HCMV binds to Tpn and impairs optimization of peptide loading,[83,84] whereas E3-19K of AdV inhibits the ability of Tpn to bridge MHC-I with TAP.[85]

Immune evasion proteins also employ strategies to misdirect trafficking of MHC-I proteins. For example, the aforementioned E3-19K protein of AdV binds MHC-I proteins and blocks their transport out of the ER.[86–90] Interestingly, cowpox virus expresses two different immune evasion proteins targeting MHC-I proteins that function in tandem. Cowpox virus 12 is a TAP function blocker that curtails peptide supply, whereas cowpox virus 203 returns fully assembled MHC-I proteins from the Golgi back to the ER.[91–94] To also misdirect assembled MHC-I proteins, the Nef protein of human immunodeficiency virus and gp48 protein of murine cytomegalovirus shuttle assembled MHC-I proteins from the Golgi to the lysosome.[95–97]

Interestingly, the aforementioned immune evasion proteins for the most part do not have cellular homologs, making it difficult to track their evolution. Striking exceptions to this generality are the viral ER ubiquitin ligases called the viral MARCH (*m*embrane-*a*ssociated *R*ING-*CH*) proteins. The extensive family of MARCH proteins includes viral and cellular homologs that share a transmembrane orientation and a highly conserved atypical RING domain that confers E3 ubiquitin ligase activity.[98–100] Viral proteins mK3 of MHV68, and proteins kK3 and kK5 of Kaposi sarcoma–associated herpesvirus that function as immune evasion proteins were the founding members of the MARCH protein family.[101–103] Mechanistically, mK3 binds to TAP and awaits the entry of MHC-I into the PLC after which mK3 ubiquitinates the cytosolic tail of MHC-I HCs and thus induces their dislocation to the cytosol and degradation in the cytosol (ie, ERAD).[104–107] Of note, the extensively studied US2 and US11 proteins of HCMV also target ERAD of MHC-I proteins by recruiting cellular E3 ligases.[108–113] Indeed, studies of US2, US11 and mK3 continue to provide molecular insights into various mechanisms by which ERAD substrates are detected in the ER and dislocated to the cytosol.[114,115] In contrast to ERAD, the kK3 and kK5 MARCH ligases of Kaposi sarcoma–associated herpesvirus induce endocytosis and lysosomal degradation of MHC-I proteins. Interesting, kK3 like mK3 appears to ubiquitinate only MHC-I proteins, whereas kK5 targets other surface receptors including the T cell costimulation molecule CD86 (B7.2) as well as natural killer cell ligands, MHC class I-related chain A or B (MICA, MICB), and newly defined ligand for NKp80, activation-induced C-type lectin (AICL).[116] Inhibition of natural killer responses is of importance to the virus because downregulation of MHC-I proteins renders cells susceptible to natural killer cell lysis. Mechanistic studies of viral MARCH

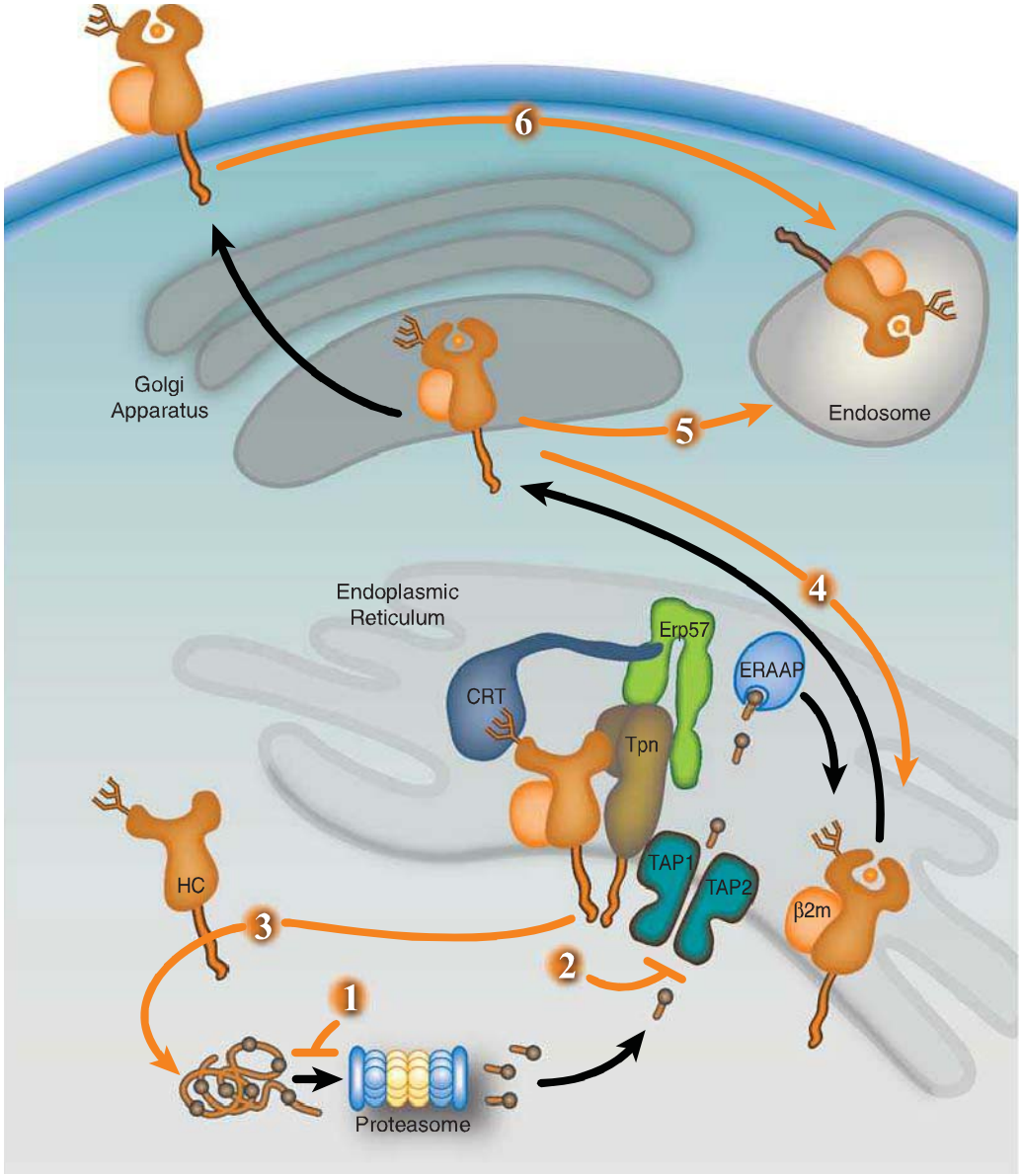


FIG. 22.3. Immune Evasion Strategies Used by Viral Proteins to Block Antigen Presentation by Major Histocompatibility Complex (MHC)-I Proteins. *Black arrows* demarcate physiologic pathways for antigen presentation, whereas the *numbered red arrows* demarcate reported immune evasion strategies by virus proteins. 1: Blocking of antigen processing by the proteasome (examples are the EDNA1 protein of Epstein-Barr virus or the LANA1 protein of Kaposi sarcoma–associated herpesvirus). 2: Blocking peptide transport by transporter associated with antigen presentation (examples include the ICP47 protein of herpes simplex virus, US6 protein of human cytomegalovirus, cpxv12 protein of cowpox virus, UL49.5 protein of bovine herpesvirus, and BNFL2a protein of Epstein-Barr virus). 3: Dislocation of MHC-I proteins from the endoplasmic reticulum (ER) to the cytosol for proteasome-mediated degradation (examples are US2 and US11 proteins of human cytomegalovirusand mK3 protein of MHV68). 4: ER retention and retrieval of MHC-I proteins (examples are E3-19K protein of AdV and the cpxv203 protein of cowpox virus). 5: Mistrafficking of MHC-I proteins from the Golgi to an endosomal compartment (examples include gp48 protein of murine cytomegalovirus and Nef protein of human immunodeficiency virus-1). 6: Rapid internalization of MHC-I proteins from the cell surface to an endosomal/lysosomal compartment (examples are kK3 and kK5 proteins of Kaposi sarcoma–associated herpesvirus).

proteins have provided several molecular insights into ubiquitin-dependent degradation pathways of particular relevance to antigen processing. In addition, there are about 10 cellular MARCH protein homologs in humans and mice, many of which detect immune receptors.[98–100] For example, as mentioned in the MHC-II section, MARCHI regulates MHC-II expression in B cells and dendritic cell (DC) subsets.[117–119] Thus, viral MARCH proteins were stolen from the host and adapted by the virus to function as immune evasion proteins using similar mechanisms.

ANTIGEN PROCESSING AND PRESENTATION BY MAJOR HISTOCOMPATIBILITY CLASS II (MHC-II) PROTEINS

Source of Antigen for MHC-II Presentation

As a general rule, the antigen-processing machinery that is utilized by MHC-I is specialized for the degradation, importation, and binding of peptides that are derived from antigens present in the cytosol of host cells. Although many viruses and bacteria thrive in the cytosol of infected cells, there are pathogens (such as *Toxoplasma gondii*) that reside in membrane-encapsulated intracellular compartments and do not access the cytosol.[120] Furthermore, almost any foreign material (including live or dead pathogens, fragments of apoptotic cells, or soluble proteins) can enter the cell by endocytosis and are therefore sequestered from the cytosol. It is for recognition of antigens such as this that MHC-II molecules exist; MHC-II binds peptides derived from antigens that gain access to intracellular compartments in cells by a variety of endocytic pathways.[121] Fluid-phase macropinocytosis is a process whereby extracellular fluid is taken up by plasma membrane protrusions that bring extracellular fluids and soluble proteins into the cell by endocytosis. Once internalized, macropinosomes fuse with early endosomes, thereby releasing their contents to the endocytic pathway. Immature (resting) DCs are particularly efficient in their capacity for macropinocytosis that enhances their significance as "sentinals" of the immune system.[122] Upon activation by encounter with foreign organisms, DCs greatly suppress macropinocytosis,[122–124] thereby limiting the ability of the activated DCs to sample their microenvironment and restricting the repertoire of antigenic peptides presented to T-lymphocytes.

As one can imagine, macropinocytosis is not a particularly efficient mechanism of antigen uptake. B cells possess antigen-specific receptors (immunoglobulins) that permit specific antigen internalization and presentation to T cells that can be 1000 times more efficient than fluid-phase endocytosis.[125] Curiously, the function and fate of B cells that generate specific MHC-II peptide complexes following immunoglobulin-mediated antigen uptake versus fluid-phase uptake of the same antigen differ significantly,[126] highlighting the importance of the receptor-mediated antigen uptake pathway in B cells. Similarly, macrophages and DCs express Fc receptors and complement receptors that allow efficient phagocytosis of large extracellular particles such as antibody-coated organisms or even apoptotic cells. Following endocytosis, the phagosome fuses with the membrane of a lysosome, forming a degradative phagolysosome that neutralizes and degrades internalized antigens into peptides.[127] Other surface receptors, such as mannose receptors[122,128,129] and DEC-205[130] on DCs, also allow for efficient antigen binding and uptake onto the endocytic pathway, and these receptors have been exploited as a vehicle to efficiently deliver bring foreign vaccine antigens into the MHC-II processing pathway in antigen-presenting cells (APCs).[131]

While antigens entering the endocytic pathway by phagocytosis, macropinocytosis, and receptor-mediated endocytosis give rise to the majority of peptides that bind to MHC-II, there is increasing evidence that cytosolic antigens are capable of entering endosomal MHC-II processing compartments and are degraded there into MHC-II–binding peptides. The most likely cellular mechanism responsible for this phenomena is process termed autophagy.[132,133] Macroautophagy is constitutively active in DCs and is a process that leads to the "engulfment" of aggregated cytosolic protein complexes to form an autophagosome. Like phagosomes from extracellular antigens, the autophagosome fuses with a lysosome and results in the degradation of autophagosome contents into antigenic peptides.[134,135] A related process, termed chaperone-mediated autophagy, results from the direct import of cytosolic proteins into the lumen of endo/lysosomes and is facilitated by the chaperone protein hsc-70 and the lysosomal integral membrane protein Lamp2a.[136] Once inside this organelle, the imported protein is degraded into antigenic peptides just like "conventionally" internalized antigens. While this pathway clearly functions in vitro, the extent to which autophagy participates in MHC-II–restricted immune responses remains to be established.

MHC-II Assembly and Transport to the Endosomal Pathway

Because MHC-II is specialized to bind peptides generated in the endocytic pathway, there must be mechanisms in APCs to prevent the premature binding of peptides to MHC-II as the molecules assemble in the ER and traffic through the Golgi apparatus before their delivery to endosomal peptide loading compartments. In addition to the inability of MHC-II to bind to TAP/tapasin and other members of the MHC-I–peptide loading complex, MHC-II is unique in that it binds to a transmembrane chaperone protein termed the invariant chain (Ii). It is the association of Ii with the MHC-II–peptide binding site that prevents MHC-II molecules in the early secretory pathway from binding peptides.[137,138] Indeed, failure of newly synthesized MHC-II to bind Ii allows for the presentation of ER-imported peptides to CD4 T cells by MHC-II.[139] A specific region of Ii, termed the class II-associated Ii peptide (CLIP), serves to block the peptide-binding groove of nascent MHC-II molecules until it is ready for removal in endo-/lysosomes. The MHC-II-CLIP complex has been crystallized, and as one might expect, the structure of this complex is nearly identical to that of a MHC-II molecule containing an "antigenic" peptide.[140] As the name implies, unlike the highly polymorphic MHC-II molecule itself, Ii is nonpolymorphic. However, the human Ii mRNA can initiate protein synthesis using one of

two translation initiation sites (Fig. 22.4A), leading to generation of a major 33 kDa form of Ii (Ii-p33) as well as a minor population of larger Ii molecules whose translation is initiated using an upstream methionine (Ii-p35[141]). The use of alternative translation initiation sites appears to be unique to humans, as it is not seen in rodents.[142] Furthermore, alternative mRNA splicing in all species examined can give rise to an additional 8 kDa domain in each form of Ii, leading to the production of Ii-p41 and (in humans) Ii-p43.[143,144] This additional exon encodes a domain (see subsequent discussion) that regulates the activity of proteases in endo-/lysosomes.

The Ii molecule itself forms a trimer even in the absence of MHC-II, and each constituent subunit of this trimer contains either Ii-p33, -p35, -p41, or -p43 isoforms (see Fig. 22.4B). Ii homotrimers and mixed heterotrimers are generated, and each trimer contains an Ii molecule whose presence in the trimer is proportional to its level of expression.[145–147] Onto this "scaffold" of an Ii trimer, pairs of MHC-II αβ heterodimers assemble, ultimately forming a mature, nine-chain MHC-II-Ii complex.[148] The partially formed MHC-II-Ii complex containing an Ii trimer and only one or two αβ heterodimers is retained in the ER by interacting with the chaperone calnexin,[149] presumably allowing the MHC-II molecule additional time to form a fully functional MHC-II-Ii nonamer. In addition, the longer p35 and p43 forms of human Ii contain an ER retention motif, and free Ii trimers containing one of these longer Ii forms are retained in the ER.[150,151] Curiously, completely folded MHC-II-Ii nonamers containing one of

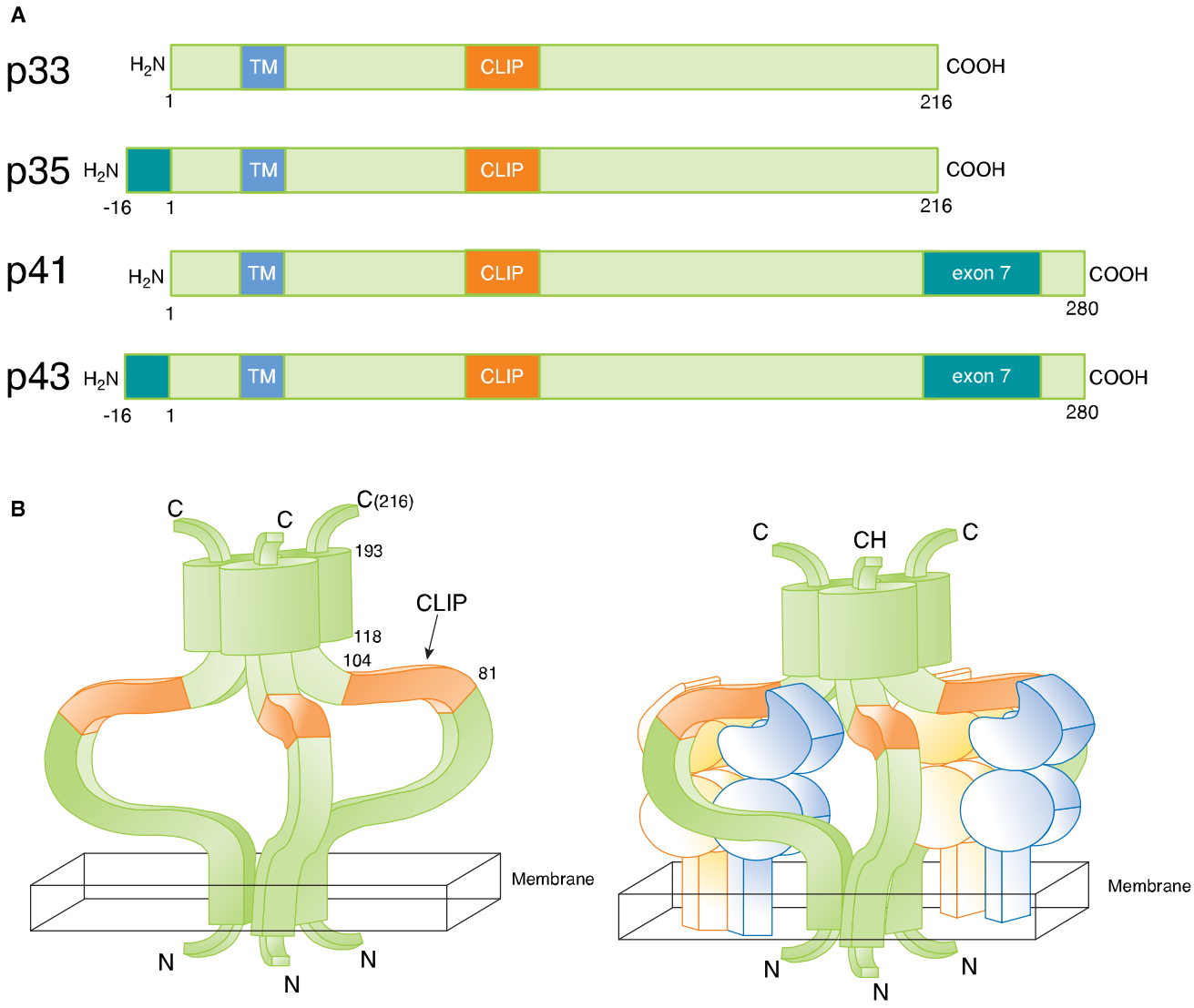


FIG. 22.4. Structure of Invariant Chain (Ii) and Major Histocompatibility Complex (MHC)-II–Ii Complexes. **A:** The major 216 amino acid form of human Ii is the 33 kDa Ii-p33 isoform. The use of an upstream initiation methionine results in the generation of Ii-p35 (which contains an endoplasmic reticulum–retention motif), and alternative splicing of each of these isoforms leads to the generation of Ii-p41 and Ii-p43 forms of Ii that possess proteinase inhibitor activity. The relative positions of the transmembrane domain (TM) and class II-associated Ii peptide region of each isoform of Ii are indicated. **B:** Models of Ii trimer (*left panel*) and the MHC-II–Ii complex (*right panel*). Only two of the three pairs of MHC-II αβ dimers are shown bound to the Ii trimer. The model shows the peptide-binding groove of MHC-II αβ dimers occupied by the CLIP region of Ii. (From Cresswell P. The biochemistry and cell biology of antigen processing. In: Paul WE. *Fundamental Immunology*. 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2003:613–629, reprinted with permission).

these longer Ii isoforms are capable of exiting the ER and moving to antigen-processing compartments,[147,152] demonstrating that assembly with MHC-II masks the ER-retention motif on the Ii trimer.

Ii association does more for MHC-II than simply prevent premature peptide binding. Assembly with Ii is critical for some alleles of MHC-II to fold property and achieve competence to leave the ER, thus revealing a role for Ii as an assembly/folding molecular chaperone.[153] After leaving the ER, the MHC-II-Ii complex moves through the Golgi apparatus and is targeted to the endosomal pathway[150,154] (Fig. 22.5A). It is likely that MHC-II-Ii complexes arrive in the endocytic pathway by two distinct mechanisms and the relative contribution of each pathway may be cell-type specific. Some MHC-II-Ii complexes target directly from the trans-Golgi network to endosomes,[155,156] while other complexes move from the trans-Golgi network directly to the plasma membrane and enter endosomes only after endocytosis.[157] The movement of MHC-II-Ii complexes to the endosomal pathway is facilitated by recognition of two dileucine sorting motifs present in the cytosolic domain of every isoform of Ii.[158,159] The machinery that recognizes these motifs in the trans-Golgi network remains to be defined; however, it is clear that these motifs are recognized at the plasma membrane by the AP-2 adaptor protein complex.[160,161] This protein complex serves as a linker between Ii and the clathrin-mediated endocytosis pathway and serves to deliver plasma membrane-localized MHC-II-Ii to the early endosomes. Recognition and internalization of MHC-II-Ii from the plasma membrane is efficient and rapid,[157] and as a consequence of this, very little MHC-II-Ii is present on the plasma membrane of APCs at any given moment.

Removal of Invariant Chain in Antigen-Processing Compartments

Regardless of the mechanism responsible for delivering MHC-II-Ii to endosomes, Ii must be removed in order to allow antigenic peptides to bind to nascent MHC-II. Ii is

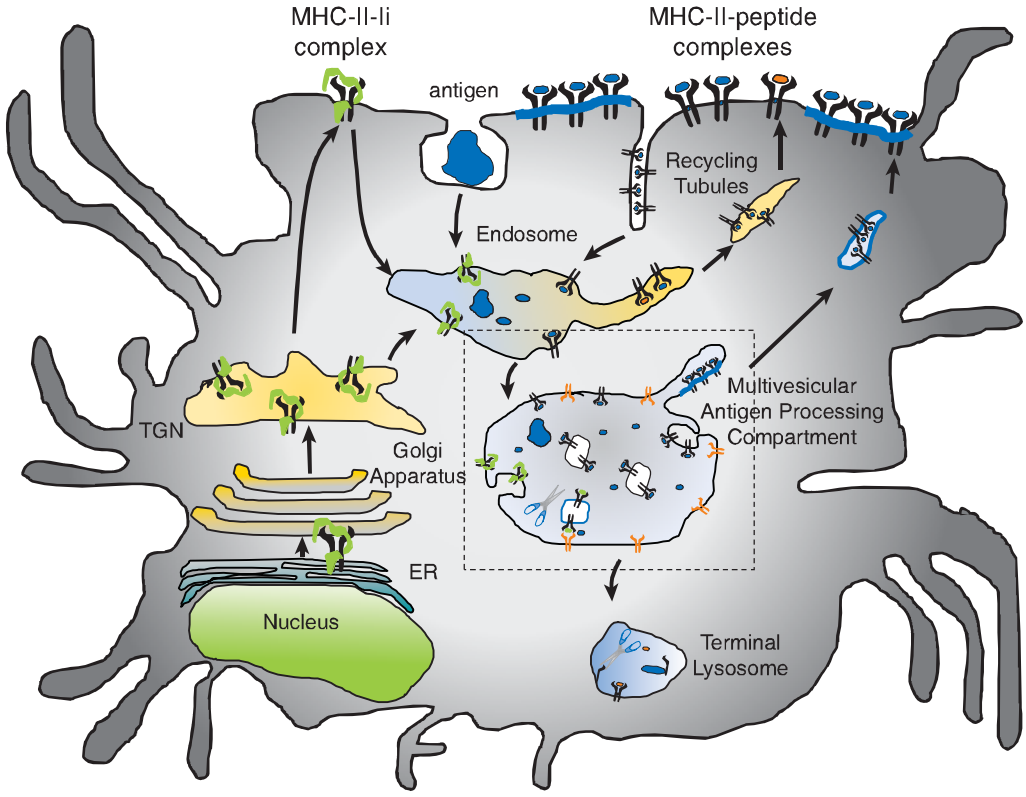


FIG. 22.5. Transport of Major Histocompatibility Complex (MHC)-II in Antigen-Presenting Cells. **A:** MHC-II associates with Ii in the endoplasmic reticulum, moves through the Golgi apparatus to the plasma membrane, and enters the endocytic pathway by clathrin-mediated endocytosis. Some fraction of MHC-II can also access these endocytic compartments directly from the Golgi apparatus/trans-Golgi network. Antigens access these same endocytic compartments by a number of different endocytosis pathways. The MHC-II–invariant chain (Ii) complex moves to late endosomal/prelysosomal antigen-processing compartments in which Ii is degraded, the MHC-II–associated class II-associated Ii peptide (CLIP) fragment of Ii is removed by DM, and peptides derived from foreign antigens then bind to MHC-II (this compartment is highlighted in *panel B*). MHC-II–peptide complexes leave these intracellular antigen-processing compartments via vesicles or tubules that fuse with the plasma membrane, thereby delivering MHC-II–peptide complexes on the antigen-presenting cell surface. Once at the surface, MHC-II–peptide complexes can recycle between the plasma membrane and the endosomal system, potentially exchanging antigenic peptides. Some fraction of internalized MHC-II is also targeted for degradation in terminal lysosomes. *(continued)*

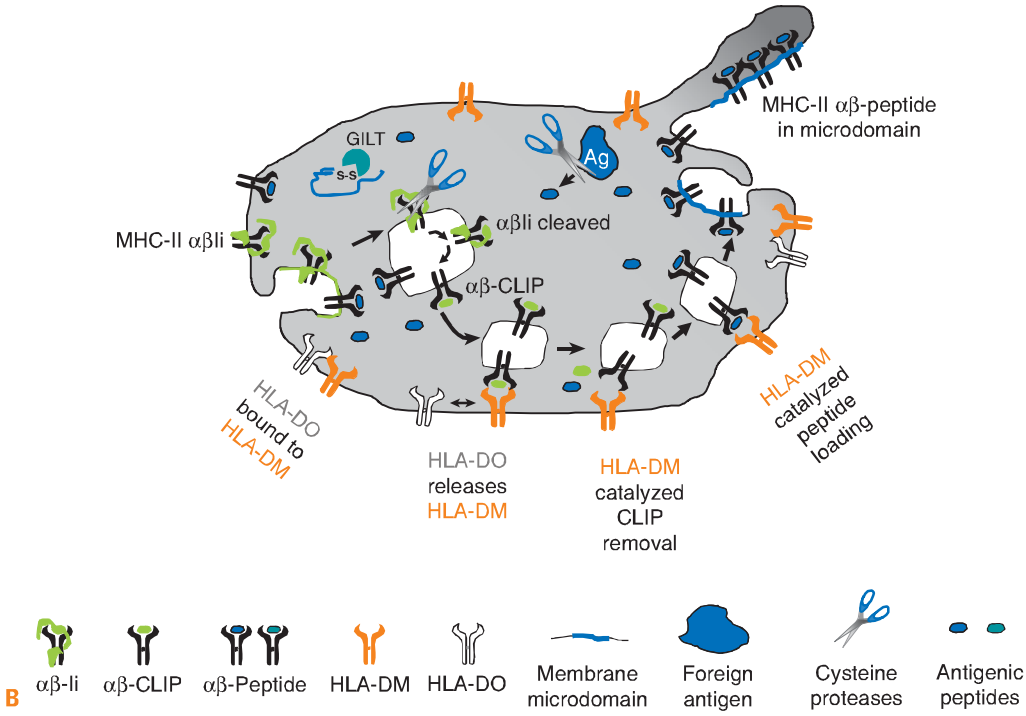


FIG. 22.5 *(continued)* **B:** Binding of peptides to MHC-II in antigen-processing compartments. The multivesicular antigen-processing compartment highlighted in *panel A* is shown here. MHC-II–Ii complexes are targeted to intraluminal vesicles of late endosomal multivesicular antigen-processing compartments where Ii proteolysis is initiated. This process results in the generation of MHC-II–CLIP complexes that are subject to editing by DM. DO binds to DM during intracellular transport and dissociates at the low pH of these compartments, thereby liberating free DM that catalyzes the removal of CLIP from the MHC-II–CLIP complex. DM also stabilizes the structure of peptide-free MHC-II until high-affinity peptide binding. Disulfide bonds present in protein antigens can be reduced by IFNγ-induced thiol reductase, thereby enhancing the formation of antigenic peptides generated by proteolysis. In this cartoon MHC-II, DM, and DO have particular membrane distributions; however, whether these distributions are maintained in all antigen-presenting cells and whether the reactions highlighted here occur on the indicated membranes remains to be determined. MHC-II molecules present in antigen-processing compartments are present in distinct membrane microdomains, and after peptide binding, microdomain-associated MHC-II is transported to the plasma membrane in vesicles or tubules that bud from the limiting membrane of the antigen processing compartments (DM, HLA-DM; DO, HLA-DO).

degraded in discrete steps by proteolysis in acidic endosomes (see Fig. 22.5B), with proteolysis initiated at the luminal C-terminus of Ii in earlier endosomes and Ii degradation completed in more acidic late endosomal/prelysosomal structures. In vitro studies have shown that many different proteinases are capable of degrading Ii; however, in vivo, the initial cleavage of Ii is initiated by a proteinase whose activity in insensitive to the protease inhibitor leupeptin while additional cleavage is strictly leupeptin-dependent.[162] Leupeptin treatment of living APCs results in the accumulation of a 21 kDa Ii degradation intermediate that retains the nonameric MHC-II-Ii stoichiometry and is unable to bind antigenic peptides.[152] After the initial cleavage of Ii, a variety of proteinases can cleave the remaining MHC-II–associated Ii molecule; however, the terminal stages of Ii degradation in B cells and DCs are dependent on the cysteine proteinase cathepsin S.[163] Inhibition of cathepsin S activity or analysis of DCs isolated from cathepsin S-deficient mice reveal the accumulation of a 10 kDa Ii degradation product, highlighting the importance of this proteinase in Ii degradation. Curiously, MHC-II–expressing epithelial cells in the thymus do not rely on cathepsin S for Ii degradation but require cathepsin L for terminal Ii degradation.[164]

Proteolysis of Ii from both the luminal C-terminus and from residues adjacent to the luminal Ii transmembrane domain ultimately leaves a small fragment of Ii bound to MHC-II. This class II-associated Ii polypeptide, termed CLIP, is bound to the peptide-binding groove of MHC-II and serves as a "placeholder" to occupy the peptide-binding site until antigenic peptides are available to bind (see Fig. 22.4). While some MHC-II alleles have a very low affinity for CLIP and CLIP spontaneously dissociates from MHC-II,[165] in most cases CLIP must be actively removed from the peptide-binding site of MHC-II prior to antigenic peptide binding. A seminal finding in antigen processing and presentation came in 1994 with the discovery of HLA-DM (DM).[166,167] HLA-DM (known as H-2M in mice) is a nonpolymorphic transmembrane heterodimer whose α- and β-subunits are encoded in the MHC gene locus.

Like MHC-II, DM is expressed exclusively in APCs. An important function of DM is to facilitate the removal of CLIP from the MHC-II-CLIP complex.[168,169] DM functions enzymatically, and while the precise mechanism by which DM promotes CLIP dissociation from MHC-II remains to be completely elucidated, it is thought that DM disrupts the hydrogen bonding network in the peptide binding groove of MHC-II and thereby enhances the kinetics of CLIP dissociation from MHC-II.[170] Not only does DM catalyze CLIP removal from MHC-II, but DM also facilitates the dissociation of weakly bound antigenic peptides from MHC-II[168,170,171]; it is for this reason that DM has been termed a "peptide editor." Unlike the MHC-II molecule itself, DM contains a tyrosine-based endocytic sorting motif in its cytosolic domain that directly interacts with clathrin-associated adaptor proteins.[172] The presence of this signal allows for the entry of DM into the endocytic pathway after arrival at the cell surface. Most DM is localized in late endocytic compartments[173] that contain MHC-II-CLIP complexes,[174] and the activity of DM is enhanced by the low pH of these later endocytic compartments.[168,169,175]

DM editing activity in APCs can be regulated by its association with another MHC-encoded transmembrane protein termed HLA-DO (DO; H-2O in mice[176]). While the precise function of DO in antigen processing and presentation remains to be defined, in vitro studies have shown that DO binds to DM and inhibits the ability of DM to catalyze CLIP removal.[177,178] Like DM, DO expression is relatively restricted to APCs. Unlike MHC-II-Ii or DM, DO does not contain any intrinsic endo-/lysosomal sorting motifs. Instead, DO associated with DM in the ER and DM serves to "escort" DO to antigen-processing compartments.[179] It is in the low pH of these compartments that DO dissociates from DM, thereby enhancing DM activity. In this respect, DO acts as an inhibitor of DM function in much the same way that the CLIP region of Ii acts as an inhibitor of MHC-II peptide binding.

Antigen Processing and Peptide Loading onto MHC-II

While MHC-II in lysosome-like antigen-processing compartments must be "prepared" for peptide binding (by degrading MHC-II–associated Ii and removing CLIP by DM), internalized foreign antigens must be similarly prepared for binding to the nascent MHC-II molecule. This task is initiated by proteolysis by any number of endocytic proteinases. Antigen-processing compartments have a pH between 4.5 and 6.5, and most proteinases in these compartments have enzymatic activity optima at this pH. There is remarkable diversity in the proteinase pool in these compartments and great redundancy in the ability of distinct proteinases to degrade particular proteins. For this reason, it has been difficult to identify specific proteinases whose activity is required to generate specific antigenic peptides. There are exceptions to this, however, as asparaginyl cysteine endoprotease is required for the generation of an immunodominant epitope to tetanus toxoid[180] and both cathepsin L and cathepsin S affect quantitative and qualitative differences in the repertoire of peptides bound to MHC-II molecules.[181] It is also a known fact that many internalized foreign (and self-) antigens contain intrachain disulfide bonds, and reduction of these bonds would alter protein conformation and render a protein more (or less) sensitive to proteolysis in antigen-processing compartments. As its name implies, the expression of IFNγ-induced thiol reductase, GILT, can be induced by exposure to IFNγ.[182] This thiol reductase is expressed in antigen-processing compartments in APCs, and the activity of GILT is important for the presentation of antigenic epitopes that are generated from proteins that contain many intrachain disulfide bonds.[183]

Just as important as generation of antigenic peptides is the ability of the antigen-processing compartment to limit excessive proteolytic degradation of antigenic peptides. While asparaginyl cysteine endoprotease positively regulates expression tetanus toxoid epitopes, this same proteinase destroys expression of the immunodominant epitope of myelin basic protein,[184] a protein thought to be involved in the demyelinating autoimmune disease multiple sclerosis. Clearly, the proteinase activity of antigen-processing compartments must be sufficient to generate antigenic peptides capable of binding to MHC-II but not so great as to completely destroy these same epitopes. Measurement of proteinase activity in various APC subtypes has revealed that macrophages have high proteinase activity and efficiently degrade proteins, but are relatively pool generators of antigenic peptides.[185] By contrast, DCs and B cells possess less proteinase activity, degrade proteins less efficiently, and are superb generators of antigen peptides.[185,186] This specialization of limiting proteinase activity helps DCs function as initiators of naïve T-cell activation and B cell capacity for immunoglobulin class switching after encounter with antigen-specific T cells.

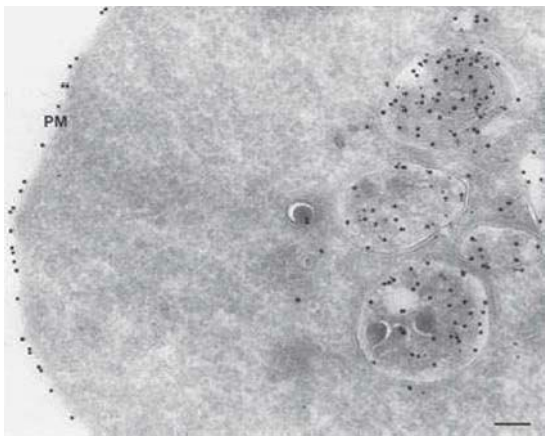
It has been proposed that binding to the MHC-II molecules can serve as a mechanism to preserve the integrity of antigenic peptides. The peptide-binding groove of MHC-II is open, and in vitro binding experiments have shown that MHC-II is capable of binding to unfolded (but otherwise intact) protein antigens.[187] The association of distinct regions within a full-length (unfolded) protein with the peptide-binding site of MHC-II could therefore protect these regions from proteolytic degradation by endo-/lysosomal proteinases. This model of "bind first trim later" has been championed as a major mechanism of peptide loading onto MHC-II[188] and has been supported by experimental studies in B cells[189]; however, the extent to which "bind first trim later" versus "trim first bind later" predominates in distinct APC subsets and for different antigens remains to be determined.

Curiously, the Ii molecule itself also can serve to limit proteinase activity of antigen-processing compartments. The additional exon that is present in Ii isoforms that are products of alternative splicing (Ii-p41 and Ii-p43) encodes a domain that functions as an inhibitor of cathepsin L.[190,191] In vitro studies showed that coexpression with Ii-p41 inhibits the kinetics of Ii-p33 degradation,[192] a finding that is consistent with the role of Ii-p41 as a proteinase inhibitor. However, no defects in function have been identified using APCs isolated from mice expression only the Ii-p41,[193] leaving the importance of this domain in APC function an open issue.

DCs are particularly interesting APCs in that they can regulate their proteinase activity upon cell activation. Immature (resting) DCs are highly phagocytic and have a remarkable capacity for macropinocytosis. Nevertheless, these cells have little proteinase activity and are relatively inefficient generators of antigenic MHC-II–peptide complexes, potentially allowing them to serve as a "depot" for antigen internalized while the cell is in the quiescent state. It has even been shown that internalized proteins can remain intact in immature DCs for days.[194,195] Activation of the immature DC by any of a number of mechanisms (either by toll-like receptor signaling or stimulation by activated T cells) leads to a number of changes in the cell, with perhaps the most important being the activation of the vacuolar proton ATPase.[196] Activation of this ATPase leads to the acidification of antigen-processing compartments, enhances the activity of endo-/lysosomal proteinases that have low pH optima, and leads to the degradation of internalized antigens to form antigenic peptides capable of binding to MHC-II. Other changes also take place following activation of immature DCs, including downregulating expression of the endogenous cysteine proteinase inhibitor cystatin C[197] and reorganizing the internal membrane structure of the antigen processing compartments[198]; however, it is likely that acidification of these compartments is the major mechanism leading to enhanced antigen processing, peptide generation, and formation of MHC-II–peptide complexes upon DC activation.

Delivery of MHC-II to the Cell Surface

The sites of antigenic protein degradation and subsequent binding of peptides onto nascent MHC-II are commonly referred to as antigen-processing compartments. At one time, it was thought that APCs possessed a "special" compartment that served as the major antigen-processing compartment[199]; however, it is now clear that "antigen-processing compartments" refer to a heterogenous collection of intracellular (generally acidic) organelles that permit peptide binding onto MHC-II.[200] These organelles generally have characteristics of typical late endosomes/prelysosomes found in all cell types (Fig. 22.6); however, in APCs these organelles contain the specialized protein machinery required for antigen processing and presentation such as MHC-II, DM, DO, GILT, and Ii-processing enzymes. A "typical" antigen-processing compartment is a late endosomal multivesicular body (MVB), an organelle that possesses intraluminal vesicles encapsulated in a limiting membrane.[201] These compartments can also have a multilamellar structure, containing numerous membrane sheets contained within an even



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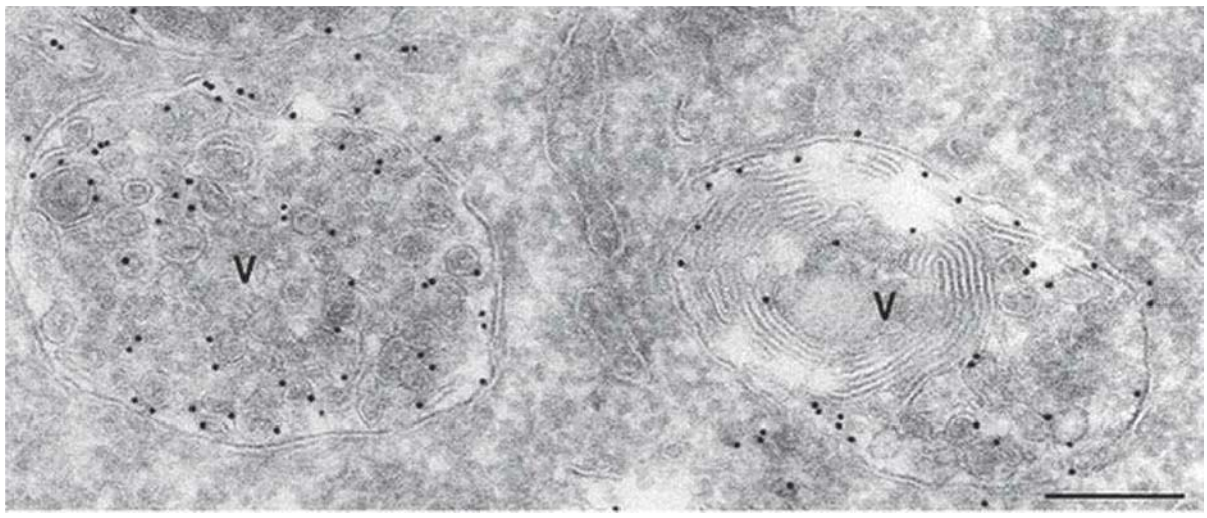

B

FIG. 22.6. Structure of Multivesicular Antigen-Processing Compartments. **A:** Ultrathin cryosections of a human B-cell line were stained with antibodies recognizing DM (10 nm gold) and total major histocompatibility complex (MHC)-II (15 nm gold). The plasma membrane stains almost exclusively for MHC-II while various forms of multivesicular antigen-processing compartments contain both MHC-II and DM (reprinted with permission Kleijmeer et al., *Methods*, 1996). **B:** Ultrathin cryosections of a mouse immature DC line were stained with antibodies recognizing MHC-II (10 nm gold). MHC-II staining is observed on the limiting membrane as well as in the intraluminal vesicles of a multivesicular body (*left*) and the numerous intraluminal membrane sheets in a multilamellar endosome (*right*) (reprinted with permission Kleijmeer et al.[198]).

more acidic lysosome-like organelles. Curiously, the in DCs majority of MHC-II is present on the intraluminal vesicles of these MVBs while the CLIP editor DM and MHC-II–peptide complexes are enriched on the MVB-limiting membrane.[198] Although most MHC-II and DM are present on distinct MVB membranes, it is unclear on which membranes the process of Ii proteolysis, CLIP release, and peptide binding to MHC-II occurs. For example, while there is in vitro evidence that DM primarily interacts with MHC-II when these proteins are on the same membrane,[202,203] whether this is the intraluminal vesicle or the MVB-limiting membrane remains to be determined.

The presence of MHC-II–peptide complexes on the intraluminal vesicles of MVB presents a topological problem for the APC, as direct fusion of the MVB with the plasma membrane would release the intraluminal vesicles from these compartments to the extracellular space. Such a process would effectively result in the secretion of MHC-II from the APC. This process does occur in DCs with some frequency, and these secreted intraluminal vesicles are termed exosomes that are capable of stimulating naïve T cells in vivo.[204] While APC-derived exosomes are being utilized as cell-free vaccines,[205] their physiologic role remains to be unambiguously determined. The majority of MHC-II in antigen-processing compartments is not released as exosomes, but is instead either degraded following fusion of the MVB with lysosomes or inserted into the plasma membrane by "vesicle"-mediated protein transport. While vesicles containing intracellular MHC-II have been observed to fuse with the plasma membrane,[206] in APCs such as DCs it is likely that these "vesicles" are actually tubules that bud from the MVB membrane and subsequently fuse with the plasma membrane. The tubulation of these compartments is stimulated in DCs by APC activation,[198,207,208] and the encounter of DCs with T cells leads to the fusion of these tubules directly to the plasma membrane region at the point of DC:T-cell contact,[208] thereby delivering MHC-II–peptides generated in DCs directly to the site of T-cell receptor concentration at the immunologic synapse.

The cellular processes that govern the movement of MHC-II to the surface of APCs are similar to those that govern the movement of any late endosomal protein the surface of any cell. In some cases, the machinery regulating discrete transport steps remains a mystery while other steps are more well characterized. For example, the mechanism leading to the delivery of intraluminal vesicle-bound MHC-II back to the MVB0limiting membrane (from which tubules are formed) is termed back-fusion,[209] and the proteins and lipids that regulate this curious process remain to be identified. By contrast, it is known that the ability of MHC-II to insert into the plasma membrane is regulated by the small GTPase ARL14/ARF7 that couples the tubulovesicle membrane with the actin cytoskeleton.[210] Like other vesicle-mediated protein transport steps, microtubules and dynein/kinesin motors[211] as well as actin and myosin motors[212] are important for the locomotion of MHC-II into and out of antigen-processing compartments.

Once at the plasma membrane, MHC-II–peptide complexes are poised to interact with antigen-specific T-cell receptors. MHC-II–peptide complexes are clustered on the plasma membrane[195,213] and associate with distinct lipid raft-[214] and tetraspanin-membrane[215] microdomains, thereby locally concentrating MHC-II on the cell surface. Biochemical studies have revealed that MHC-II associates with these membrane microdomains while still present in antigen-processing compartments even before antigenic peptide binding.[216] This finding is consistent with the observation that MVBs contain large amounts of cholesterol, a major constituent of lipid raft membrane microdomains.[217] While lipid raft- and tetraspanin-microdomain associations are important for the T-cell stimulatory function of APCs,[214,218] the molecular signals leading to membrane microdomain association of MHC-II remain to be determined.

MHC-II–peptide complexes are generated and reside in a relatively hostile (acidic, proteinase-rich) environment, and therefore it is not surprising that cell surface MHC-II has a relatively long half-life.[219,220] While this is true on activated B cells and mature DCs, MHC-II on the surface of immature DCs has a significantly reduced half-life. This is likely due to rapid endocytosis and lysosomal degradation of surface MHC-II in resting (immature) DCs.[221,222] The dichotomy between the endocytosis and survival of MHC-II in immature and mature DCs can be explained in part by the fact that internalized MHC-II is ubiquitinated in immature DCs and targeted for lysosomal degradation while internalized MHC-II is not ubiquitined in mature DCs and efficiently recycles back to the plasma membrane.[223,224] The E3 ubiquitin ligase March-I, whose expression appears to be limited to APCs, ubiquitinates the cytosolic domain of the MHC-II β-chain in APCs,[118] thereby preventing the recycling of internalized MHC-II and targeting ubiquitinated MHC-II for lysosomal degradation.[225] This mechanism ensures that the kinetics of MHC-II synthesis and degradation in immature DCs are similar and provides a means to halt the degradation of relevant MHC-II–peptide complexes upon DC activation.

Simply because MHC-II has a long half-life in B cells and DCs does not mean that these molecules do not internalize. In these APC subtypes, internalized MHC-II efficiently recycles from early (recycling) endosomes back to the plasma membrane,[221] thereby limiting their delivery to lysosomes and prolonging MHC-II half-life. The ability of internalized MHC-II to exchange one peptide for another in endosomes has been demonstrated in vitro[226,227]; however, it remains to be determined whether peptide exchange onto recycling MHC-II in vivo is a significant mechanism of peptide loading onto MHC-II. There is convincing evidence, however, that certain protein epitopes are primarily presented by recycling MHC-II.[228] Unlike presentation of epitopes from "classically processed" antigens, presentation of these epitopes does not require neosynthesis of MHC-II and is independent of Ii expression or DM activity. A particularly intriguing example of this pathway can be found in the analysis of an immunogenic epitope presentation of the influenza virus hemagglutinin,[228] in which presentation of one epitope is via the classical (late endosomal) processing and presentation pathway while another is presented via a nonclassical (early endosomal) recycling pathway.

Alternate Pathways of Antigen Presentation

In this chapter, we have reviewed what is typically called the classical MHC-I and MHC-II antigen-processing and -presentation pathways. In addition to these pathways, there are also interesting alternative antigen-presentation pathways. Although not the focus of this chapter, these nonclassical pathways provide clear evidence that antigen presentation is more complex than a simple dichotomy of the two classical pathways. The most extensively studied of these alternative pathways is cross-presentation, a process by which CD8 T cells present exogenous antigen taken up by professional APCs such as DCs. Cross-presentation is critical for initiating CD8+ immune responses to pathogens that do not infect professional APCs or tumors that do not arise in professional APCs. Although several details remain unsolved, cell biologic approaches suggest that the loading of cross-presented antigens occurs by several different pathways each with hybrid features of both of the classical pathways.[229–231]

The presentation of lipid antigens by MHC-I like CD1 molecules also occurs by a nonclassical pathway.[232,233] Human CD1a, CD1b, and CD1c isoforms present bacterial lipids to conventional αβ T cells, whereas CD1d presents endogenous and microbial lipids to invariant natural killer T cells. Interestingly, disparate targeting motifs in their cytoplasmic tails facilitate the binding of lipid antigens in the early endosomes by CD1a, in the late endosome/lysosome by CD1b and CD1d, and at both sites by CD1c. This diversity of location of lipid antigen binding by different human CD1 isoforms allows surveying of different endosomal compartments for microbes. The assembly of CD1 isoforms in the ER uses molecular chaperones CXN, CRT, and ERp57 similar to the classical MHC-I pathways. As expected, the processing and loading of lipid antigens presented by CD1 isoforms requires unique players.[234] However, the trafficking of CD1 isoforms between the plasma membrane and endosomal compartments to acquire lipid antigens has parallels with the classical MHC-II pathway. In sum, with the underlying goal of providing maximal immunity for detection and protection against diverse pathogens, mammals have two classical and other nonclassical pathways of antigen presentation.

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SECTION VI

Induction, Regulation, and Effector Functions of the Immune Response

CHAPTER 23

Immunogenicity and Antigen Structure

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THE NATURE OF ANTIGENIC DETERMINANTS RECOGNIZED BY ANTIBODIES

Haptens

In the antigen–antibody binding reaction, the antibody-binding site is often unable to accommodate the entire antigen. The part of the antigen that is the target of antibody binding is called an antigenic determinant, and there may be one or more antigenic determinants per molecule. To study antibody specificity, we need to have antibodies against single antigenic determinants. Small functional groups that correspond to a single antigenic determinant are called haptens. For example, these may be organic compounds, such as trinitrophenyl or benzene arsonate, a monosaccharide or oligosaccharide such as glucose or lactose, or an oligopeptide such as pentalysine. Although these haptens can bind to antibody, immunization with them usually will not provoke an antibody response (for exceptions, see Goodman[1]). Immunogenicity often can be achieved by covalently attaching haptens to a larger molecule, called the carrier. The carrier is immunogenic in its own right, and immunization with the hapten-carrier conjugate elicits an antibody response to both hapten and carrier. However, the antibodies specific for hapten can be studied by equilibrium dialysis using pure hapten (without carrier) or by immunoprecipitation using hapten coupled to a different (and non–cross-reacting) carrier or by inhibition of precipitation with free hapten.

This technique was pioneered by Landsteiner[2] and helped to elucidate the exquisite specificity of antibodies for antigenic determinants. For instance, the relative binding affinity of antibodies prepared against succinic acid–serum protein conjugates shows marked specificity for the maleic acid analog, which is in the cis configuration, as compared to the fumaric acid (trans) form.[3] Presumably, the immunogenic form of succinic acid corresponds to the cis form.[3] This ability of antibodies to distinguish cis from trans configurations was reemphasized in later studies measuring relative affinities of antibodies to maleic and fumaric acid conjugates[4] (Table 23.1A). Table 23.1B shows the specificity of antibodies prepared against p-azobenzenearsonate coupled to bovine gamma globulin.[5] As the hapten is coupled through the p-azo group to aromatic amino acids of the carrier, haptens containing bulky substitutions in the para position would most resemble the immunizing antigen. In fact, p-methyl–substituted benzene arsonate has a higher binding affinity than unsubstituted benzene arsonate. However, methyl substitution elsewhere in the benzene ring reduces affinity, presumably due to interference with the way hapten fits into the antibody-binding site. Thus, methyl substitutions can have positive or negative effects on binding energy, depending on where the substitution occurs. Table 23.1C shows the specificity of antilactose antibodies for lactose versus cellobiose.[6] These disaccharides differ only by the orientation of the hydroxyl attached to C4 of the first sugar either above or below the hexose ring. The three examples in this table, as well as many others,[1] show the marked specificity of antibodies for cis–trans, ortho–meta–para, and stereoisomeric forms of the antigenic determinant.

Comparative binding studies of haptens have been able to demonstrate antibody specificity despite the marked heterogeneity of antibodies. Unlike the antibodies against a multideterminant antigen, the population of antibodies specific for a single hapten determinant is a relatively restricted population due to the shared structural constraints necessary for hapten to fit within the antibody-combining site. However, the specificity of an antiserum depends on the collective specificities of the entire population of antibodies, which are determined by the structures of the various antibody-binding sites. When studying the cross-reactions of hapten analogs, some haptens bind all antibodies but with reduced K_A. Other hapten analogs reach a plateau of binding because they fit some antibody-combining sites quite

TABLE 23.1 Exquisite Specificity of Antihapten Antibodies

Hapten	Structure		K_{rel} of Antibody Specific for	
A.			Maleic (cis)	Fumaric (trans)
	Maleanilate		1.0	<0.01
	Fumaranilate		<0.01	1.0
B.			Parasubstituted benzene arsonate	
	Benzene arsonate		1.0	
	o-Methyl benzene arsonate		0.2	
	m-Methyl benzene arsonate		0.8	
	p-Methyl benzene arsonate		1.9	
C.			Lactose	
	Lactose	β Gal (1 → 4) Glu	1.00	
	Cellobiose	β Glu (1 → 4) Glu	0.0025	

Part A from Pressman and Grossberg,[4] part B from Pressman et al.,[5] and part C from Karush,[6] with permission.

well but not others (see discussion of cross-reactivity in Chapter 7). Antibodies raised in different animals may show different cross-reactivities with related haptens. Even within a single animal, antibody affinity and specificity are known to increase over time following immunization under certain conditions.[7] Thus any statements about the cross-reactivity of two haptens reflect both structural differences between the haptens that affect antigen–antibody fit and the diversity of antibody-binding sites present in a given antiserum.

Carbohydrate Antigens

The antigenic determinants of a number of biologically important substances consist of carbohydrates. These often occur as glycolipids or glycoproteins. Examples of the former include bacterial cell wall antigens and the major blood group antigens, whereas the latter group includes "minor" blood group antigens such as Rh. In addition, the capsular polysaccharides of bacteria are important for virulence and are often targeted by protective antibodies. A number of spontaneously arising myeloma proteins have been found to show carbohydrate specificity, possibly reflecting the fact that carbohydrates are common environmental antigens. In the days prior to hybridoma technology, these carbohydrate-specific myeloma proteins provided an important model for studying the reaction of antigen with a monoclonal antibody.

Empirically, the predominant antigenic determinants of polysaccharides often consist of short oligosaccharides (one to five sugars long) at the nonreducing end of the polymer chain.[8] This situation is analogous to a hapten consisting of several sugar residues linked to a large nonantigenic polysaccharide backbone. The remainder of the polysaccharide is important for immunogenicity, just as the carrier molecule was important for haptens. In addition, branch points in the polysaccharide structure allow for multiple antigenic determinants to be attached to the same macromolecule. This is important for immunoprecipitation by lattice formation, as discussed in Chapter 5. Several examples illustrating structural studies of oligosaccharide antigens are given later.

The technique used most widely to analyze the antigenic determinants of polysaccharides is called hapten inhibition.[8] In this method, the precipitation reaction between antigen and antibody is inhibited by adding short oligosaccharides. These oligosaccharides are large enough to bind with the same affinity and specificity as the polysaccharide, but because they are monomeric, no precipitate forms. As more inhibitor is added, fewer antibody-combining sites remain available for precipitation. Using antiserum specific for a single antigenic determinant, it is often possible to block precipitation completely with a short oligosaccharide corresponding to the nonreducing end of the polysaccharide chain. Besides showing the "immunodominance" of the nonreducing end of the chain, this result also shows that the structure of the antigenic determinant of polysaccharides depends on the sequence of carbohydrates and their linkage, rather than their conformation. For inhibition by hapten to be complete, the antigen–antibody system studied must be made specific for a single antigenic determinant. For optimal sensitivity, the equivalence point of antigen and antibody should be used.

We illustrate the types of carbohydrate antigens encountered by examining three classic examples in more detail: the salmonella O antigens, the blood group antigens, and dextrans that bind to myeloma proteins.

Immunochemistry of Salmonella O Antigens

The antigenic diversity among numerous salmonella species resides in the structural differences of the lipopolysaccharide (LPS) component of the outer membrane.[9] These molecules are the main target for antisalmonella antibodies. The polysaccharide moiety contains the antigenic determinant, whereas the lipid moiety is responsible for endotoxin effects. The chemical structure of LPS can be divided into three regions (Fig. 23.1). Region I contains the antigenic O-specific polysaccharide, usually made up of repeated oligosaccharide units, which vary widely among different strains. Region II contains an oligosaccharide "common core" shared among many different strains. Failure to synthesize region II oligosaccharide or to couple completed region I polysaccharide to the growing region II core results in R (rough) mutants, which have "rough" colony morphology and lack the O antigen. Region III is the lipid part, called lipid A, which is shared among all salmonellae and serves to anchor LPS on the outer membrane. Early immunologic attempts to classify the O antigens of different salmonellae revealed a large number of cross-reactions between different strains. These were detected by preparing antiserum to one strain of salmonella and using it to agglutinate bacteria of a second strain. Each cross-reacting determinant was assigned a number, and each strain was characterized by a series of O antigen determinants (in aggregate, the "serotype" of the strain) based on its pattern of cross-reactivity. Each strain was classified within a group, based on sharing a strong O determinant. For example, group A strains share determinant 2, whereas

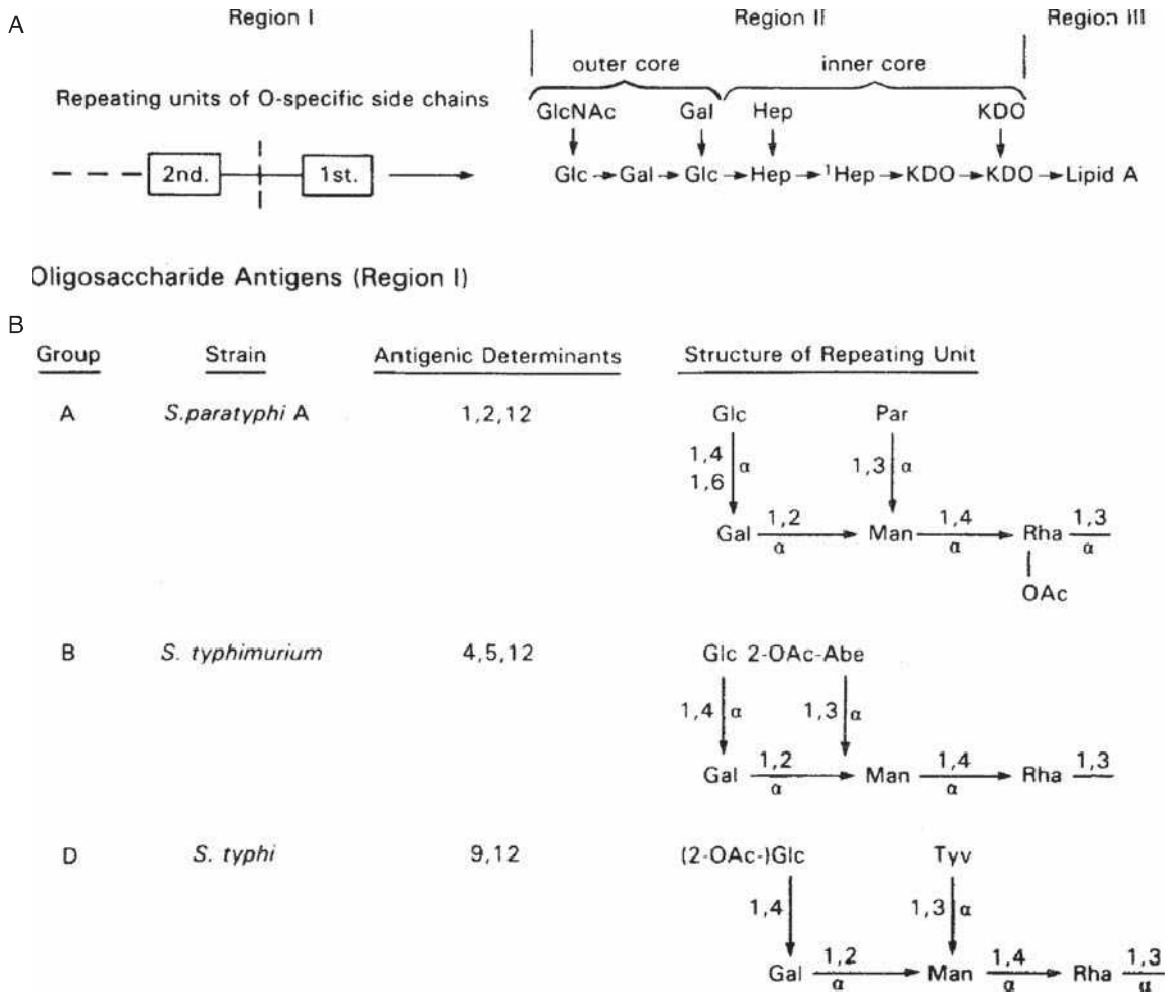

FIG. 23.1. Structure of Salmonella Lipopolysaccharide. Region I contains the unique O-antigen determinants, which consist of repeating units of oligosaccharides. These are attached to lipid moiety through the core polysaccharide. Three examples of oligosaccharide units are shown.[9] (Part A adapted from Kabat,[8] with permission; part B based on Jann and Westphal.[9])

group B strains share determinant 4 (Table 23.2). However, within a group, each strain possesses additional O determinants, which serve to differentiate it from other members of that group. Thus, determinant 2 coexists with determinants 1 and 12 on *Salmonella paratyphi* A. This problem of cross-reactivity based on sharing of a subset of antigenic determinants is commonly encountered in complex antigen–antibody systems. The problem may be simplified by making antibodies monospecific for individual antigenic determinants. To do this, antibodies are absorbed to remove irrelevant specificities, or cross-reactive strains are chosen that share only a single determinant with the immunizing strain. The reaction of each determinant with its specific antibody can be thought of as an antigen–antibody system. Thus, for the strains shown in Table 23.2, antiserum to *Salmonella typhi* (containing anti-9 and anti-12 antibodies) may be absorbed with *S. paratyphi* A to remove anti-12, leaving a reagent specific for antigen 9 (see Table 23.2). Alternatively, the unabsorbed antiserum may be used to study the system antigen 12–anti-12 by allowing it to agglutinate *S. paratyphi* B, which shares only antigen 12 with the immunogen. Because the other determinants on *S. paratyphi* B were absent from the immunizing strain, the antiserum contains no antibodies to them.

Once the antigen–antibody reaction is made specific for a single determinant, a variety of oligosaccharides can be added

TABLE 23.2 Salmonella O Antigen Serotyping

Salmonella Strain	Serogroup	O Antigenic Determinants
S. paratyphi A	A	1, 2, 12
S. paratyphi B	B	1, 4, 5, 12
S. typhi	D	9, 12

Antiserum	Absorbed	Tested on	Single Determinant Measured
Anti-*S. typhi*		*S. paratyphi* B	12
Anti-*S. typhi*	*S. paratyphi* A	*S. typhi*	9

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TABLE 23.3 Analysis of Salmonella O-Antigen Structure by Hapten Inhibition

Maximum Inhibition by Hapten (%)	Antigen System	
	1: anti-1	19: anti-19
d-Glu	—	0
Me-α-D-Glu	35	10
α-D-Glu(1 → 6)-D-Gal	80	25
Glu.Gal.Man	80	70
Gtu.Gal.Man.L-Rham	>70	>70
Deduced structure	α-D-Glu(1 → 6)-D-Gal	D-Glu-D-Gal-D-Man-L-Rham

Reprinted with permission from Kabat.[8]

to test for hapten inhibition. Because the O antigens contain repeating oligosaccharide units, it is often possible to obtain model oligosaccharides by mild chemical or enzymatic degradation of the LPS itself. Once the most inhibitory oligosaccharide is found, its chemical structure is determined. Alternatively, a variety of synthetic monosaccharide, disaccharide, trisaccharide and oligosaccharides are tested for hapten inhibition of precipitation. For example, as shown in Table 23.3, antigen 1–anti-1 antibody precipitation is inhibited by methyl-α D-glucoside. Therefore, various disaccharides incorporating this structure were tested, of which α-D-Glu-(1 → 6)-D-Gal was the most inhibitory. Then various trisaccharides incorporating this sequence were tested. The results indicate the sequence and size of the determinant recognized by anti-1 antibodies to be a disaccharide with the previously discussed structure. The test sequences can be guessed by analyzing the oligosaccharide breakdown products of the LPS, which include tetramers of D-Glu-D-Gal-D-Man-L-Rham. The results in Table 23.3 also suggest that the difference between determinants 1 and 19 is the length of oligosaccharide recognized by antibodies specific for each determinant. This hypothesis is supported by the observation that determinant 1 is found in some strains with, and in other strains without, determinant 19; whereas determinant 19 is always found with determinant 1. As shown in Table 23.3, determinant 19 requires the full tetrasaccharide for maximal hapten inhibition, including the sequence coding for determinant 1. Besides identifying the antigenic structures, these results indicate that there is variation in the size of different antigenic determinants of polysaccharides.

Blood Group Antigens

The major blood group antigens A and B were originally detected by the ability of serum from individuals lacking either determinant to agglutinate red blood cells bearing them (for reviews, see Kabat,[8] Springer,[10] Marcus,[11] and Watkins[12]). In addition, group O individuals have an H antigenic determinant that is distinct from A or B types, and individuals in all three groups may have additional determinants such as the Lewis (Le) antigens. Although the ABH and Le antigenic determinants are found on a carbohydrate moiety, the carbohydrate may occur in a variety of biochemical forms. On cell surfaces, they are either glycolipids that are synthesized within the cell (AB and H antigens) or glycoproteins taken up from serum (Le antigens). In mucinous secretions, such as saliva, they occur as glycoproteins. Milk, ovarian cyst fluid, and gastric mucosa contain soluble oligosaccharides containing blood group reactivity. In addition, these antigens occur frequently in other species, including about half of the bacteria in the normal flora of the gut.[10] This widespread occurrence may account for the ubiquitous anti-AB reactivity of human sera, even in people never previously exposed to human blood group substances through transfusion or pregnancy.

The immunochemistry of these antigens was simplified greatly by the use of oligosaccharides in hapten inhibition studies. Group A oligosaccharides, for example, would inhibit the agglutination of group A red blood cells by anti-A antibodies. They could also inhibit the immunoprecipitation of group A–bearing glycoproteins by anti-A antibodies. Because the oligosaccharides are monomeric, their reaction with antibody does not form a precipitate but does block an antibody-combining site.

The inhibitory oligosaccharides from cyst fluid were purified and found to contain D-galactose, L-fucose, N-acetylgalactosamine, and N-acetylglucosamine. The most inhibitory oligosaccharides for each antigen are indicated in Figure 23.2. As can be seen in Figure 23.2, the ABH and Le antigens all share a common oligosaccharide core sequence, and the antigens appear to differ from each other by the sequential addition of individual sugars at the end or at branch points. Besides hapten inhibition, other biochemical data support this relationship among the different determinants. Enzymatic digestion of A, B, or H antigens yields a common core oligosaccharide from each. This product cross-reacts with antiserum specific for pneumococcal polysaccharide type XIV, which contains structural elements shared with blood group determinants, as shown at the bottom of Figure 23.2. In addition, this structure, known as precursor substance, has been isolated from ovarian cyst fluid.

Starting from precursor substance, the H determinant results from the addition of L-fucose to galactose, whereas Le^a determinant results from the addition of L-fucose to N-acetylglucosamine and Le^b from the addition of L-fucose to both sugars. Addition of N-acetylgalactosamine to H substance produces the A determinant, whereas addition of galactose produces the B determinant, in each case blocking reactivity of the H determinant.

The genetics of ABH and Le antigens is explained by this sequential addition of sugars via glycosyltransferases. The allelic nature of the AB antigens is explained by the addition of N-acetylgalactosamine, galactose, or nothing to the H antigen. The rare inherited trait of inability to synthesize the H determinants from precursor substance (Bombay phenotype) also blocks the expression of A and B antigens because the A and B transferases lack an acceptor substrate. However, the appearance of the Le^a antigen on red cells is independent of H antigen synthesis. Its structure, shown in Figure 23.2, can be derived directly from precursor substance without going through an H antigen intermediate. Comparing different individuals, the appearance of Le^a antigen on red blood cells correlates with its presence in saliva, as the Le^a

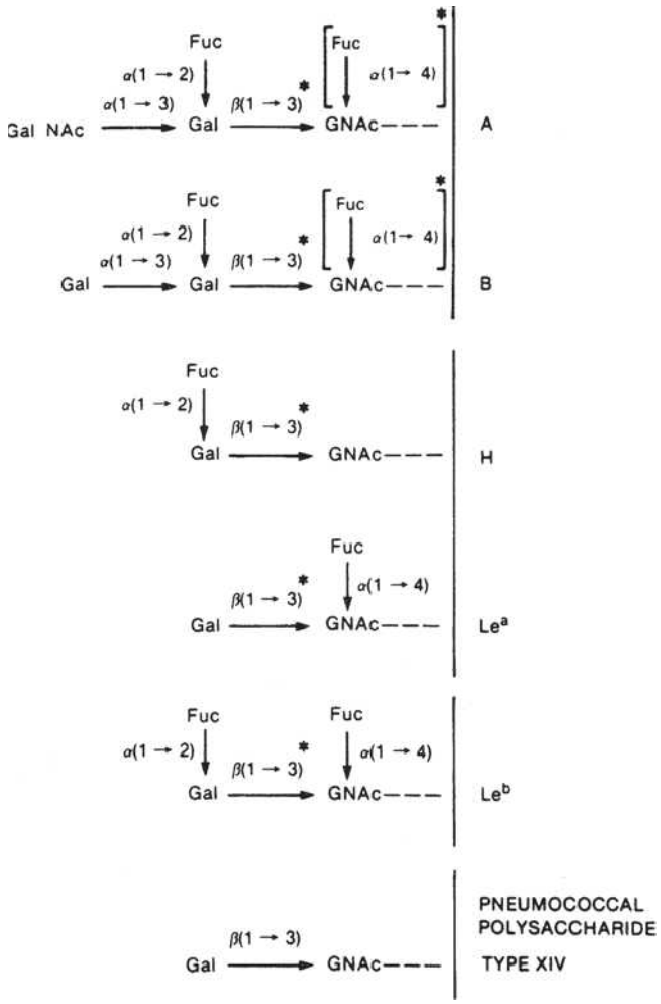


FIG. 23.2. Oligosaccharide Chain Specificity. Structure of the ABH and Le blood group antigens as determined by hapten inhibition studies.[8,11] There are two variants of each of these determinants. In type 1, the Gal-GNAc linkage is β(1 → 3), whereas in type 2, the Gal-GNAc linkage is β(1 → 4). In addition, there is heterogeneity in the A and B antigens with respect to the presence of the Le fucose attached to the GNAc. In the molecules that contain the extra fucose, when the Gal-GNAc linkage is β(1 → 3) (type 1), the fucose must be linked α(1 → 4), whereas the type 2 molecules, with the β(1 → 4) Gal-GNAc linkage, contain α(1 → 3)-linked fucose. The *asterisks* indicate the sites of this variability in linkage.

antigen is not an intrinsic membrane component but must be absorbed from serum glycoproteins, which, in turn, depend on secretion. In addition to the independent synthetic pathway, the secretion of Le^a antigen is also independent of the secretory process for ABH antigens. Therefore, salivary nonsecretors of ABH antigens (which occur in 20% of individuals) may still secrete Le^a antigen if they have the fucosyl transferase encoded by the Le gene. In contrast, salivary secretion of ABH is required for red blood cells to express Le^b.

Dextran-Binding Myeloma Proteins

Because polysaccharides are common environmental antigens, it is not surprising that randomly induced myeloma proteins were frequently found to have carbohydrate specificities. Careful studies of these monoclonal antibodies support the clonal expansion model of antibody diversity: heterogeneous antisera behave as the sum of many individual clones of antibody with respect to affinity and specificity. In the case of the Ig Aκ myeloma proteins W3129 and W3434, both antibodies were found to be specific for dextrans containing α-glu (1 → 6)glu bonds.[12] Hapten inhibition with a series of monosaccharide or oligosaccharides of increasing chain length indicated that the percentage of binding energy derived from the reaction with one glucose was 75%, two glucoses 95%, three glucoses 95% to 98%, and four glucoses 100%. This suggests that most binding energy between antidextran antibodies and dextran derives from the terminal monosaccharide, and that oligosaccharides of chain length four to six commonly fill the antibody-combining site. Human antidextran antisera behaved similarly, with tetrasaccharides contributing 95% of the binding energy. These experiments provided the first measure of the size of an antigenic determinant, four to six residues.[13,14] In addition, as was observed for antisera, binding affinity of myeloma proteins was highly sensitive to modifications of the terminal sugar and highly specific for α(1 → 6) versus α(1 → 3) glycosidic bonds. However, modification of the third or fourth sugar of an oligosaccharide had relatively less effect on hapten inhibition of either myeloma protein or of antisera reacting with dextran.

Studies with additional dextran-binding myeloma proteins[15] revealed that not all antipolysaccharide monoclonal antibodies are specific for the nonreducing end, as exemplified by QUPC 52. Competitive inhibition with monosaccharide and oligosaccharides revealed that <5% of binding energy derived from monosaccharides or disaccharides, 72% from trisaccharides, 88% from tetrasaccharides, and 100% from hexasaccharides, in marked contrast to other myeloma proteins. A second distinctive property of myeloma protein QUPC 52 was its ability to precipitate unbranched dextran of chain length 200. As the unbranched dextran has only one nonreducing end, and as the myeloma protein has only one specificity, lattice formation due to cross-linking between the nonreducing ends is impossible, and precipitation must be explained by binding some other determinant. Therefore, QUPC 52 appears to be specific for internal oligosaccharide units of three to seven chain length. The W3129 is specific for end determinants and will not precipitate unbranched dextran chains. Antibodies precipitating linear dextran were also detected in six antidextran human sera, comprising 48% to 90% of the total antibodies to branched chain dextran. Thus, antidextrans can be divided into those specific for terminal oligosaccharides and those specific for internal oligosaccharides; monoclonal examples of both types are available, and both types are present in human immune serum. Cisar et al.[15] speculated as to the different topology of the binding sites of W3129 or QUPC 52 necessary for terminal or internal oligosaccharide specificity. Both terminal and internal oligosaccharides have nearly identical chemical structures, differing at a single C–OH or glycoside bond. Perhaps the terminal oligosaccharide specificity of W3129 is due to the shape of the antibody-combining site—a cavity into which only the end can fit—whereas the

internal oligosaccharide-binding site of QUPC 52 could be a surface groove in the antibody, which would allow the rest of the polymer to protrude out at both ends. A more definitive answer depends on x-ray crystallographic studies of the combining sites of monoclonal antibodies with precisely defined specificity, performed with antigen occupying the binding site.

With the advent of hybridoma technology, it became possible to produce monoclonal antibodies of any desired specificity. Immunizing mice with nearly linear dextran (the preferred antigen of QUPC 52), followed by fusion and screening (with linear dextran) for dextran-binding antibodies, yielded 12 hybridomas,[16] all with specificity similar to QUPC 52. First, oligosaccharide inhibition of all 12 monoclonals showed considerable increments in affinity up to hexasaccharides, with little affinity for disaccharides and only 49% to 77% of binding energy derived from trisaccharides.[17] Second, all 12 monoclonals had internal $\alpha(1 \rightarrow 6)$ dextran specificity, as they could all precipitate linear dextran. Third, 9 out of 11 BALB/c monoclonals shared a cross-reactive idiotype with QUPC 52, whereas none shared idiotype with W3129.[18] These data support the hypothesis that different antibodies with similar specificity and similar groove-type sites may be derived from the same family of germline V_H genes bearing the QUPC 52 idiotype.[18]

The large number of environmental carbohydrate antigens and the high degree of specificity of antibodies elicited in response to each carbohydrate antigen suggest that a tremendous diversity of antibody molecules must be available, from which some antibodies can be selected for every possible antigenic structure. Studies of a series of 17 monoclonal anti-$\alpha(1 \rightarrow 6)$ dextran hybridomas[19,20] have investigated whether the binding sites of closely related antibodies were derived from a small number of variable region genes, for both heavy and light chains, or whether antibodies of the same specificity could derive from variable region genes with highly divergent sequences. Each monoclonal had a groove-type site that could hold six or seven sugar residues (with one exception), based on inhibition of immunoprecipitation by different length oligosaccharides. Thus, unlike monoclonals to haptenated proteins, the precise epitope could be well characterized and was generally quite similar among the entire series.

Studies of the V_κ sequences revealed that only three V_κ groups were used in these hybridomas. Use of each V_κ group correlated with the particular antigen used to immunize the animals, whether linear dextran or short oligosaccharides, so that 10 of the monoclonals from mice immunized the same way all used the same V_κ.

In contrast, the 17 V_H chains were derived from at least five different germline genes from three different V_H gene families.[21] The two most frequently used germline V_H genes were found in seven and five monoclonals, respectively, with minor variations explainable by somatic mutations. The remarkable finding is that very different V_H chains (about 50% homologous) can combine with the same V_κ to produce antibody-binding sites with nearly the same size, shape, antigen specificity, and affinity. Even when different V_H sequences combine with different V_κ sequences, they can produce antibodies with very similar properties. Dextran binding depends on the antigen fitting into the groove and interacting favorably with the residues forming the sides and bottom of the groove. The results indicate that divergent variable region sequences, both in and out of the complementarity-determining regions, can be folded to form similar binding site contours, which result in similar immunochemical characteristics. Similar results have been reported in other antigen–antibody systems, such as phenyloxazolone.[22]

Additional studies of carbohydrate binding monoclonal antibodies have revealed significant information about how the antibody variable regions can bind a carbohydrate structure with high affinity and specificity. Several examples are now available of crystal structures of carbohydrates bound to antibodies.

For example, monoclonal antibody Se155-4 is specific for the group B determinant of the salmonella O antigen, which consists of the sugars Gal-Abequose-Man.[23,24] The crystal structure of antibody bound to the polysaccharide shows that one hexose, abequose, fits into the binding pocket, while the rest of the interactions occur along the surface of the antibody, similar to the groove-type sites described previously. Binding energy depends on hydrogen bonds formed between the protein residues and the hydroxyl groups of the carbohydrate. The protein residues include aromatic amines, such as His 32, Trp 91, and Trp 96 of the light chain, as well as His 97 and His 35 of the heavy chain. In addition, one of the sugars is hydrogen bonded via a water molecule bridge to the amide bonds of the protein backbone. About three quarters of all sugar hydroxyl groups are involved in hydrogen bonds with the protein. Although each H-bond is relatively weak by itself, the combined effect of eight hydrogen bonds results in high-affinity binding. Antibody specificity derives from the fact that the carbohydrate fits into a binding pocket, where H-bond formation depends on precise interactions with amino acid residues that are oriented about the pocket. Surprisingly, most of these bonds are formed between sugar hydroxyls and aromatic amino acids that are neither charged nor very polar at neutral pH.

Similarly, monoclonal antibody BR96 and the humanized monoclonal hu3S193 are specific for the Le Y antigen, which resembles the Le B antigen described in Figure 23.2, except that the fucose-N-acetylglucosamine bond is $\beta(1 \rightarrow 3)$ instead of $\beta(1 \rightarrow 4)$. The Le Y antigen is commonly expressed on tumor cells of epithelial origin. The crystal structures have revealed the sources of the binding energy that results in affinity and specificity for this carbohydrate antigen.[25,26] These two monoclonals bind Le Y antigen in a large, deep pocket, which accommodates all four hexoses and correspond to the cavity type binding site predicted by Kabat.[15] The terminal fucose goes in first while the other three sugars are hydrogen bonded to amino acid side chains lining the pocket, including Tyr 33, Tyr 35, and Gln 52 of the heavy chain and His 27 of the light chain. Once again, hydrogen bonds between hydroxyl groups of the sugars and aromatic amines (Trp and Tyr) of the protein play a dominant role in determining affinity and specificity of binding. A smaller number of H-bonds depend on amide groups of the protein backbone.

A third example is provided by human monoclonal 2G12, which has neutralizing activity against a broad spectrum of human immunodeficiency virus (HIV) isolates. This antibody binds the mannose-rich oligosaccharide side chains that form a protective surface, called a glycoshield, on the envelope glycoprotein gp120. The crystal structure shows that the two terminal mannose sugars of each oligosaccharide bind end on into a deep pocket of the antibody, in a cavity-type site.[27,28] Twelve hydrogen bonds form between the two terminal mannose residues and the protein, depending mainly on the amide groups of the protein backbone. Additional hydrogen bonds form between the third mannose residue and the side chain of Asp 100 of the heavy chain and between the fourth mannose residue and Tyr 94 of the light chain and Tyr 56 of the heavy chain. A unique feature of this antibody is the crossover of variable regions between heavy and light chains so that each binding site is made up of the V_H from one HL pair combining with the V_L chain of the opposite pair. This arrangement allows the antibody to bind one branch of an oligosaccharide and the opposite branch of a nearby oligosaccharide and makes it ideally suited for cross-linking the densely clustered oligosaccharides of gp120.

Immunogenicity of Polysaccharide Conjugates

Capsular polysaccharides are the main target of protective antibodies against bacterial infection, and, as such, are important vaccine antigens. In adults, the chain length of the polysaccharide is an important determinant of immunogenicity, and the polysaccharides induce a T-independent response that cannot be boosted on repeat exposure. In young children, whose maternal antibodies wane by 6 months of age and who most need immunity to pathogens such as *Haemophilus influenzae* type b and *Streptococcus pneumoniae* of multiple serotypes, the T-independent response to these polysaccharides is weak, regardless of chain length. To immunize children, the polysaccharides were coupled to a protein carrier to create a new T-dependent antigen that gained immunogenicity from T-cell help and boosted antibody titers with each successive dose. This strategy has produced highly successful conjugate vaccines against *H. influenzae* type b,[29] resulting in a markedly reduced incidence of meningitis caused by this agent in immunized children[30,31] and evidence of herd immunity even among unimmunized children. The same strategy has produced an effective vaccine against invasive disease[32] and otitis media[33] caused by the most prevalent serotypes of *S. pneumoniae*.

Protein and Polypeptide Antigenic Determinants

Like the proteins themselves, the antigen determinants of proteins consist of amino acid residues in a particular three-dimensional array. The residues that make contact with complementary residues in the antibody-combining site are called contact residues. To make contact, of course, these residues must be exposed on the surface of the protein, not buried in the hydrophobic core. As the complementarity-determining residues in the hypervariable regions of antibodies have been found to span as much as 30 to 40 Å × 15 to 20 Å × 10 Å (D. R. Davies, personal communication), these contact residues comprising the antigenic determinant may cover a significant area of protein surface, as measured by x-ray crystallography of antibody–protein antigen complexes.[34–37] The size of the combining sites has also been estimated using simple synthetic oligopeptides of increasing length, such as oligolysine. In this case, a series of elegant studies[38–40] suggested that the maximum chain length a combining site could accommodate was six to eight residues, corresponding closely to that found earlier for oligosaccharides,[13,14] as discussed previously.

Several types of interactions contribute to the binding energy. Many of the amino acid residues exposed to solvent on the surface of a protein antigen will be hydrophilic. These are likely to interact with antibody contact residues via polar interactions. For instance, an anionic glutamic acid carboxyl group may bind to a complementary cationic lysine amino group on the antibody, or vice versa, or a glutamine amide side chain may form a hydrogen bond with the antibody. However, hydrophobic interactions can also play a major role. Proteins cannot exist in aqueous solution as stable monomers with too many hydrophobic residues on their surface. Those hydrophobic residues that are on the surface can contribute to binding to antibody for exactly the same reason. When a hydrophobic residue in a protein antigenic determinant or, similarly, in a carbohydrate determinant[8] interacts with a corresponding hydrophobic residue in the antibody-combining site, the water molecules previously in contact with each of them are excluded. The result is a significant stabilization of the interaction. A thorough review of these aspects of the chemistry of antigen–antibody binding is in Getzoff et al.[41]

Mapping Epitopes: Conformation versus Sequence

The other component that defines a protein antigenic determinant, besides the amino acid residues involved, is the way these residues are arrayed in three dimensions. As the residues are on the surface of a protein, we can also think of this component as the topography of the antigenic determinant. Sela[42] divided protein antigenic determinants into two categories, sequential and conformational, depending on whether the primary sequence or the three-dimensional conformation appeared to contribute the most to binding. On the other hand, as the antibody-combining site has a preferred topography in the native antibody, it would seem a priori that some conformations of a particular polypeptide sequence would produce a better fit than others and therefore would be energetically favored in binding. Thus, conformation or topography must always play some role in the structure of an antigenic determinant.

Moreover, when one looks at the surface of a protein in a space-filling model, one cannot ascertain the direction of the backbone or the positions of the helices (contrast Figs. 23.3A and 23.3B).[43–47] It is hard to recognize whether two residues that are side by side on the surface are adjacent on the polypeptide backbone or whether they come from different parts of the sequence and are brought together by the folding of the molecule. If a protein maintains its native conformation when an antibody binds, then it must similarly

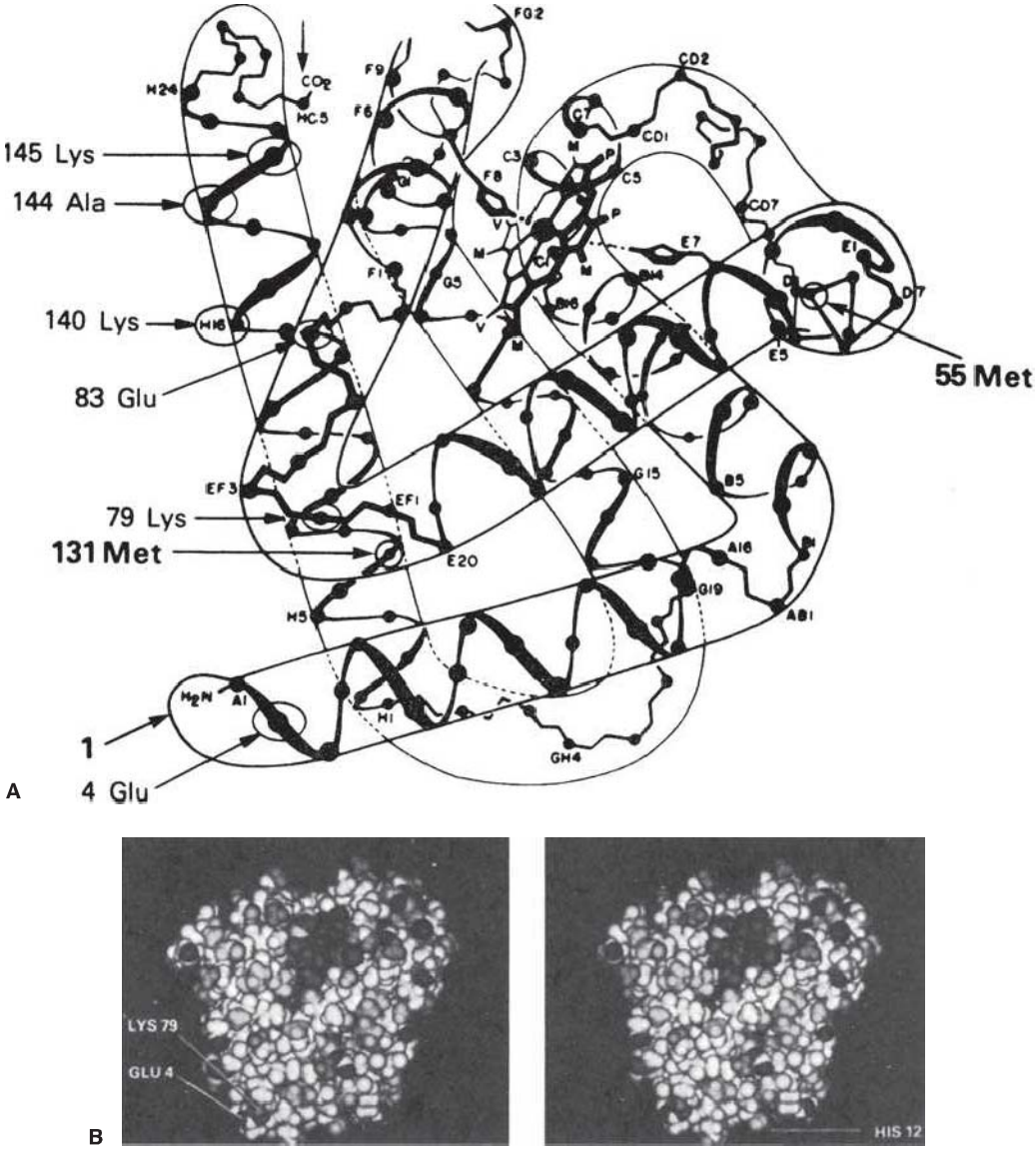


FIG. 23.3. A: Artist's representation of the polypeptide backbone of sperm whale myoglobin in its native three-dimensional conformation. The α helices are labeled A through H from the amino terminal to the carboxyl terminal. Side chains are omitted, except for the two histidine rings (F8 and E7) involved with the heme iron. Methionines at positions 55 and 131 are the sites of cleavage by cyanogen bromide (CNBr), allowing myoglobin to be cleaved into three fragments. Most of the helicity and other features of the native conformation are lost when the molecule is cleaved. A less drastic change in conformation is produced by removal of the heme to form apomyoglobin, as the heme interacts with several helices and stabilizes their positions relative to one another. The other labeled residues (Glu 4, Lys 49, Glu 83, Lys 140, Ala 144, and Lys 145) are residues that have been found to be involved in antigenic determinants recognized by monoclonal antibodies.[43] Note that cleavage by CNBr separates Lys 79 from Glu 4 and separates Glu 83 from Ala 144 and Lys 145. The "sequential" determinant of Koketsu and Atassi[44] (residues 15 to 22) is located at the elbow, lower right, from the end of the A helix to the beginning of the B helix. (Adapted from Dickerson.[45]) **B:** Stereoscopic views of a computer-generated space-filling molecular model of sperm whale myoglobin, based on the Takano[46] x-ray diffraction coordinates. This orientation, which corresponds to that in Panel A, is arbitrarily designated the "front view." The computer method was described by Feldmann et al.[47] The heme and aromatic carbons are *shaded darkest*, followed by carboxyl oxygens, then other oxygens, then primary amino groups, then other nitrogens, and finally side chains of aliphatic residues. The backbone and the side chains of nonaliphatic residues, except for the functional groups, are shown in *white*. Note that the direction of the helices is not apparent on the surface, in contrast to the backbone drawing in Panel A. The residues Glu 4, Lys 79, and His 12 are believed to be part of a topographic antigenic determinant recognized by a monoclonal antibody to myoglobin.[43] This stereo pair can be viewed in three dimensions using an inexpensive stereoviewer such as the "stereoscopes" sold by Abrams Instrument Corp. (Lansing, MI) or Hubbard Scientific Co. (Northbrook, IL). (Adapted from Berzofsky et al.[43])

be hard for the antibody to discriminate between residues that are covalently connected directly and those connected only through a great deal of intervening polypeptide. Thus, the probability that an antigenic determinant on a native globular protein consists of only a consecutive sequence of amino acids in the primary structure is likely to be rather small. Even if most of the determinant were a continuous sequence, other nearby residues would probably play a role as well. Only if the protein were cleaved into fragments before the antibodies were made would there be any reason to favor connected sequences.

This concept was analyzed and confirmed quantitatively by Barlow et al.,[48] who examined the atoms lying within spheres of different radii from a given surface atom on a protein. As the radius increases, the probability that all the atoms within the sphere will be from the same continuous segment of protein sequence decreases rapidly. Correspondingly, the fraction of surface atoms that would be located at the center of a sphere containing only residues from the same continuous segment falls dramatically as the radius of the sphere increases. For instance, for lysozyme, with a radius of 8 Å, fewer than 10% of the surface residues would lie in such a "continuous patch" of surface. These are primarily in regions that protrude from the surface. With a radius of 10 Å, almost none of the surface residues fall in the center of a continuous patch. Thus, for a contact area of about 20 Å × 25 Å, as found for a lysozyme–antibody complex studied by x-ray crystallography, none of the antigenic sites could be completely continuous segmental sites (see following discussion and Fig. 23.4). On the other hand, other analyses did not find a correlation of epitope residues with surface accessibility, suggesting that the situation is more complex.[49]

Antigenic sites consisting of amino acid residues that are widely separated in the primary protein sequence but brought together on the surface of the protein by the way it folds in its native conformation have been called "assembled topographic" sites[50,51] because they are assembled from different parts of the sequence and exist only in the surface topography of the native molecule. By contrast, the sites that consist of only a single continuous segment of protein sequence have been called "segmental" antigenic sites.[50,51]

In contrast to T-cell recognition of "processed" fragments retaining only primary and secondary structures, the evidence is overwhelming that most antibodies are made against the native conformation when the native protein is used as immunogen. For instance, antibodies to native staphylococcal nuclease were found to have about a 5000-fold higher affinity for the native protein than for the corresponding polypeptide on which they were isolated (by binding to the peptide attached to Sepharose).[52] An even more dramatic example is that demonstrated by Crumpton[53] for antibodies to native myoglobin or to apomyoglobin. Antibodies to native ferric myoglobin produced a brown precipitate with myoglobin but did not bind well to apomyoglobin, which, without the heme, has a slightly altered conformation. On the other hand, antibodies to the apomyoglobin, when mixed with native (brown) myoglobin, produced a white precipitate. These antibodies so strongly favored the conformation of apomyoglobin, from which the heme was excluded, that they trapped those molecules that vibrated toward that conformation and pulled the equilibrium state over to the apo form. One could almost say, figuratively, that the antibodies squeezed the heme out of the myoglobin. Looked at it thermodynamically, it is clear that the conformational preference of the antibody for the apo versus native forms, in terms of free energy, had to be greater than the free energy of binding of the heme to myoglobin. Thus, in general, antibodies are made that are very specific for the conformation of the protein used as immunogen. Other more recent examples also show that antibodies can

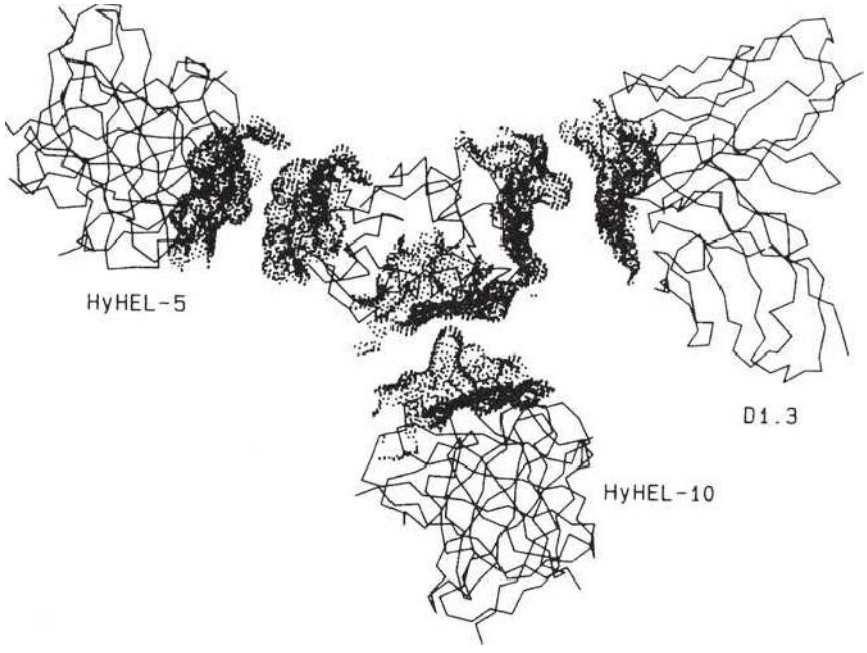


FIG. 23.4. Assembled Topographic Sites of Lysozyme Illustrated by the Footprints of Three Nonoverlapping Monoclonal Antibodies. Shown are the α-carbon backbones of lysozyme in the center and the Fv portions of three anti-lysozyme monoclonal antibodies D1.3, HyHEL-5, and HyHEL-10. The footprints of the antibodies on lysozyme and lysozyme on the antibodies (ie, their interacting surfaces) are shown by a *dotted representation*. Note that the three antibodies each contact more than one continuous loop of lysozyme and so define assembled topographic sites. Reproduced from Davies and Padlan[37] with permission.

enforce structures on disordered or denatured structures in proteins such as HIV-1 Tat[54] or influenza hemagglutinin.[55]

Synthetic peptides corresponding to segments of the protein antigen sequence can be used to identify the structures bound by antibodies specific for segmental antigenic sites. To identify assembled topographic sites, more complex approaches have been necessary. The earliest was the use of natural variants of the protein antigen with known amino acid substitutions, where such evolutionary variants exist.[50] Thus, substitution of different amino acids in proteins in the native conformation can be examined. The use of this method, which is illustrated later, is limited to studying the function of amino acids that vary among homologous proteins, that is, those that are polymorphic. It may now be extended to other residues by use of site-directed mutagenesis. A second method is to use the antibody that binds to the native protein to protect the antigenic site from modification[56] or proteolytic degradation.[57] A related but less sensitive approach makes use of competition with other antibodies.[58–60] A third approach, taking advantage of the capability of producing thousands of peptides on a solid-phase surface for direct binding assays,[61] is to study binding of a monoclonal antibody to every possible combination of six amino acids.[61] If the assembled topographic site can be mimicked by a combination of six amino acids not corresponding to any continuous segment of the protein sequence but structurally resembling a part of the surface, then one can produce a "mimotope" defining the specificity of that antibody.[61] Mimotopes have become widely used and can be combined with mutational analysis to map assembled topographic epitopes.[62] Mimetics have even been made for quaternary structural epitopes.[63] Many mimotope approaches use phase display peptide libraries to map epitopes of monoclonal antibodies.[64–66] However, other studies have been less optimistic about the ability to predict assembled topographic or discontinuous epitopes from mimotope binding[67] or random peptide libraries.[68]

Myoglobin also serves as a good model protein antigen for studying the range of variation of antigenic determinants from those that are more sequential in nature to those that do not even exist without the native conformation of the protein (see Fig. 23.3). A good example of the first more segmental type of determinant is that consisting of residues 15 to 22 in the amino terminal portion of the molecule. Crumpton and Wilkinson[69] first discovered that the chymotrypsin cleavage fragment consisting of residues 15 to 29 had antigenic activity for antibodies raised to either native or apomyoglobin. Two other groups[44,70] then found that synthetic peptides corresponding to residues 15 to 22 bind antibodies made to native sperm whale myoglobin, even though the synthetic peptides were only seven to eight residues long. Peptides of this length do not spend much time (in solution) in a conformation corresponding to that of the native protein. On the other hand, these synthetic peptides had a several hundred-fold lower affinity for the antibodies than did the native protein. Thus, even if most of the determinant was included in the consecutive sequence 15 to 22, the antibodies were still much more specific for the native conformation of this sequence than for the random conformation peptide. Moreover, there was no evidence to exclude the participation of other residues, nearby on the surface of myoglobin but not in this sequence, in the antigenic determinant.[71–74*]

A good example of the importance of secondary structure is the case of the loop peptide (residues 64 to 80) of hen egg white lysozyme.[75] This loop in the protein sequence is created by the disulfide linkage between cysteine residues 64 and 80 and has been shown to be a major antigenic determinant for antibodies to lysozyme.[75] The isolated peptide 60 to 83, containing the loop, binds antibodies with high affinity, but opening of the loop by cleavage of the disulfide bond destroys most of the antigenic activity for antilysozyme antibodies.[75]

At the other end of the range of conformational requirements are those determinants involving residues far apart in the primary sequences that are brought close together on the surface of the native molecule by its folding in three dimensions, called assembled topographic determinants.[50,51] Of six monoclonal antibodies to sperm whale myoglobin studied by Berzofsky et al.,[43,76] none bound to any of the three cyanogen bromide (CNBr) cleavage fragments of myoglobin that together span the whole sequence of the molecule. Therefore, these monoclonal antibodies (all with affinities between 2×10^8 and 2×10^9 M^{-1}) were all highly specific for the native conformation. These were studied by comparing the relative affinities for a series of native myoglobins from different species with known amino acid sequences. This approach allowed the definition of some of the residues involved in binding to three of these antibodies. Two of these three monoclonal antibodies were found to recognize topographic determinants, as defined previously. One recognized a determinant including Glu 4 and Lys 79, which come within about 2 Å of each other to form a salt bridge in the native molecule (see Fig. 23.3A, B). The other antibody recognized a determinant involving Glu 83, Ala 144, and Lys 145 (see Fig. 23.3A). Again, these are far apart in the primary sequence but are brought within 12 Å of each other by the folding of the molecule in its native conformation. Similar examples have been reported for monoclonal antibodies to human myoglobin[77] and to lysozyme[37,58] as well as the HIV-1 envelope protein (neutralizing epitopes)[78,79] and the prion protein.[80] Other examples of such conformation-dependent antigenic determinants have been suggested using conventional antisera to such proteins as insulin,[81] hemoglobin,[82] tobacco mosaic virus,[83] and cytochrome c.[84] Moreover, the crystallographic structures of lysozyme-antibody[34,36,37] and neuraminidase-antibody[35] complexes, as well as HIV-1 envelope antibody complexes,[78,79] show clearly that, in both cases, the epitope bound is an assembled topographic site.

*This is the only segmental antigenic determinant of myoglobin that has clearly been confirmed by more than one independent group of investigators. Crumpton and Wilkinson[69] did measure antigenic activity for a chymotryptic fragment 147 to 153 that overlaps one of the other reported sequential determinants.[71] However, two of the other reported sequential determinants,[71] corresponding to residues 56 to 62 and 94 to 100, have not been reproducible when tested with other antisera, even raised in the same species.[72] For related studies, see Hurrell et al.[73] and East et al.[74]

In the case of the three monoclonal antibodies binding to nonoverlapping sites of lysozyme (Fig. 23.4), it is clear that the footprints of all three antibody-combining sites cover more than one loop of polypeptide chain, and thus, each encompasses an assembled topographic site.[37] This result illustrates the concept that most antibody-combining sites must interact with more than a continuous loop of polypeptide chain and thus must define assembled topographic sites.[48] Another important example is represented by neutralizing antibodies to the HIV envelope protein that similarly bind assembled topographic sites[85,86] (see the end of this section).

How frequent are antibodies specific for topographic determinants compared to those that bind consecutive sequences when conventional antisera are examined? This question was studied by Lando et al.,[87] who passed goat, sheep, and rabbit antisera to sperm whale myoglobin over columns of myoglobin fragments, together spanning the whole sequence. After removal of all antibodies binding to the fragments, 30% to 40% of the antibodies remained that still bound to the native myoglobin molecule with high affinity but did not bind to any of the fragments in solution by radioimmunoassay. Thus, in four of four antimyoglobin sera tested, 60% to 70% of the antibodies could bind peptides, and 30% to 40% could bind only native-conformation intact protein.

On the basis of studies such as these, it has been suggested that much of the surface of a protein molecule may be antigenic,[50,88] but that the surface can be divided up into antigenic domains.[43,73,74,77] Each of these domains consists of many overlapping determinants recognized by different antibodies.

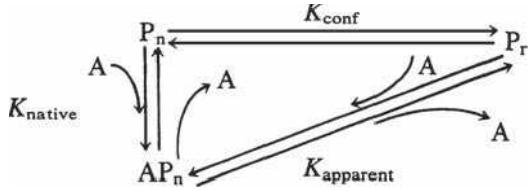
An additional interesting point is that in three published crystal structures of protein antigen–antibody complexes, the contact surfaces were broad, with local complementary pairs of concave and convex regions in both directions.[34–37] Thus, the concept of an antigen binding in the groove or pocket of an antibody may be oversimplified, and antibodies may sometimes bind by extending into pockets on an antigen.

Further information on the subjects discussed in this section is available in the reviews by Sela,[42] Crumpton,[53] Reichlin,[89] Kabat,[90] Benjamin et al.,[50] Berzofsky,[51] Getzoff et al.,[41] and Davis and Padlan.[37]

Conformational Equilibria of Protein and Peptide Antigenic Determinants

There are several possible mechanisms to explain why an antibody specific for a native protein will bind a peptide fragment in random conformation with lower affinity. Of course, the peptide may not contain all the contact residues of the antigenic determinant so that the binding energy would be lower. However, for cases in which all the residues in the determinant are present in the peptide, several mechanisms still remain. First, the affinity may be lower because the topography of the residues in the peptide may not produce as complementary a fit in the antibody-combining site as the native conformation would. Second, the apparent affinity may be reduced because only a small fraction of the peptide molecules are in a native-like conformation at any time, assuming that the antibody binds only to the native conformation. Because the concentration of peptide molecules in native conformation is lower than the total peptide concentration by a factor that corresponds to the conformational equilibrium constant of the peptide, the apparent affinity is also lower by this factor. This model is analogous to an allosteric model. A third, intermediate hypothesis would suggest that initial binding of the peptide in a nonnative conformation occurs with submaximal complementarity and is followed by an intramolecular conformational change in the peptide to achieve energy minimization by assuming a native-like conformation. This third hypothesis corresponds to an induced fit model. The loss of affinity is due to the energy required to change the conformation of the peptide, which in turn corresponds to the conformational equilibrium constant in the second hypothesis. To some extent, these models could be distinguished kinetically, as the first hypothesis predicts a faster "on" rate and a faster "off" rate than does the second hypothesis.[91] Such kinetic approaches have likewise been used to support an "encounter-docking" model related to this concept.[92]

Although not the only way to explain the data, the second hypothesis is useful because it provides a method to estimate the conformational equilibria of proteins and peptides.[52,93] The method assumes the second hypothesis, which can be expressed as follows:



(1)

where A = antibody, P_n = native peptide, and P_r = random conformation peptide so that

$$K_{apparent} = K_{conf}K_{native} \quad (2)$$

Thus, the ratio of the apparent association constant for peptide to the measured association constant for the native molecule should give the conformational equilibrium constant of the peptide. Note the implicit assumption that the total peptide concentration can be approximated by $[P_r]$. This will generally be true, as most peptide fragments of proteins demonstrate little native conformation, that is, $K_{conf} = [Pn] / [Pr]$ is much less than one. Also note that if the first hypothesis (or third) occurs to some extent, this method will overestimate K_{conf}. On the other hand, if the affinity for the peptide is lower because it lacks some of the contact residues of the determinant, this method will underestimate K_{conf} (by assuming that all the affinity difference is due to conformation). To some extent, the two errors may partially cancel out. When this method was used to determine the K_{conf} for a peptide from staphylococcal nuclease, a value of 2×10^{-4} (unitless because it is a ratio of two concentrations) was obtained.[52] Similarly, when antibodies raised to a peptide fragment were used, it was possible to estimate the fraction of time the native nuclease spends in nonnative

conformations.[93] In this case, the K_{conf} was found to be about 3000-fold in favor of the native conformation.

Antipeptide Antibodies that Bind to Native Proteins at a Specific Site

In light of the conformational differences between native proteins and peptides and the observed K_{conf} effects shown by antibodies to native proteins when tested on the corresponding peptides, it was somewhat surprising to find that antibodies to synthetic peptides show extensive cross-reactions with native proteins.[94,95] These two types of cross-reactions can be thought of as working in opposite directions: The binding of antiprotein antibodies to the peptide is inefficient, whereas the binding of antipeptide antibodies to the protein is quite efficient and commonly observed. This finding is quite useful, as automated solid-phase peptide synthesis has become readily available. This has been particularly useful in three areas: exploitation of protein sequences deduced by recombinant deoxyribonucleic acid (DNA) methods, preparation of site specific antibodies, and the attempt to focus the immune response on a single protein site that is biologically important but may not be particularly immunogenic. This section focuses on the explanation of the cross-reaction, uses of the cross-reaction, and the potential limitations regarding immunogenicity.

The basic assumption is that antibodies raised against peptides in an unfolded structure will bind the corresponding site on proteins folded into the native structure.[95] This is not immediately obvious, as antibody binding to antigen is the direct result of the antigen fitting into the binding site. Affinity is the direct consequence of "goodness of fit" between antibody and antigen, whereas antibody specificity is due to the inability of other antigens to occupy the same site. How then can the antipeptide antibodies overcome the effect of K_{conf} and still bind native proteins with good affinity and specificity? The whole process depends on the antibody-binding site forming a three-dimensional space and the antigen filling it in an energetically favorable way.

Because the peptides are randomly folded, they rarely occupy the native conformation, so they are not likely to elicit antibodies against a conformation they do not maintain. If the antibodies are specific for a denatured structure, then, like the myoglobin molecules that were denatured to apomyoglobin by antibody binding,[53] the cross-reaction may depend on the native protein's ability to assume different conformational states. If the native protein is quite rigid, then the possibility of it assuming a random conformation is quite small; if it is a flexible three-dimensional spring, then local unfolding and refolding may occur all the time. Local unfolding of protein segments may permit the immunologic cross-reaction with antipeptide antibodies, as a flexible segment could assume many of the same conformations as the randomly folded peptide.[95] On the other hand, peptides with more stable conformations may be more likely to elicit antibodies that bind both the peptide and the native protein.[96] To this end, scaffolding has been used to maintain the conformation of peptides or protein fragments to be used as immunogens/vaccines, such as for respiratory syncytial virus[97] or HIV epitopes.[98,99]

In contrast, the ability of proteins to crystallize (a feature that allows the study of their structure by x-ray crystallography) has long been taken as evidence of protein rigidity.[100] In addition, the existence of discrete functional states of allosteric enzymes[101] provides additional evidence of stable structural states of a protein. Finally, the fact that antibodies can distinguish native from denatured forms of intact proteins is well known for proteins such as myoglobin.[53]

However, protein crystals are a somewhat artificial situation, as the formation of the crystal lattice imposes order on the components, each of which occupies a local energy minimum at the expense of considerable loss of randomness (entropy). Thus, the crystal structure may have artificial rigidity that exceeds the actual rigidity of protein molecules in solution. On the contrary, we may attribute some of the considerable difficulty in crystallizing proteins to disorder within the native conformation. Second, allosterism may be explained by two distinct conformations that are discrete without being particularly rigid. Finally, the ability to generate antiprotein antibodies that are conformation specific does not rule out the existence of antipeptide antibodies that are not. All antibodies are probably specific for some conformation of the antigen, but this need not be the crystallographic native conformation in order to achieve a significant affinity for those proteins or protein segments that have a "loose" native conformation.

Antipeptide antibodies have proved to be very powerful reagents when combined with recombinant DNA methods of gene sequencing.[95,102] From the DNA sequence, the protein sequence is predicted. A synthetic peptide is constructed, coupled to a suitable carrier molecule, and used to immunize animals. The resulting polyclonal antibodies can be detected with a peptide-coated enzyme-linked immunosorbent assay plate (see Chapter 7). They are used to immunoprecipitate the native protein from a ^{35}S-labeled cell lysate and thus confirm expression of the gene product in these cells. The antipeptide antibodies can also be used to isolate the previously unidentified gene product of a new gene. The site-specific antibodies are also useful in detecting posttranslational processing, as they bind all precursors and products that contain the site. In addition, because the antibodies bind only to the site corresponding to the peptide, they are useful in probing structure–function relationships. They can be used to block the binding of a substrate to an enzyme or the binding of a virus to its cellular receptor.

Immunogenicity of Proteins and Peptides

Up to this point, we have considered the ability of antibodies to react with proteins or peptides as antigens. However, immunogenicity refers to the ability of these compounds to elicit antibodies following immunization. Several factors limit the immunogenicity of different regions of proteins, and these have been divided into those that are intrinsic to protein structure itself versus those extrinsic to the antigen that are related to the responder and vary from one animal or species to another.[51] In addition, we consider the special case of peptide immunogenicity, as it applies to vaccine development. The features of protein structure that have been suggested to explain the results include surface accessibility

of the site, hydrophilicity, flexibility, and proximity to a site recognized by helper T cells.

When the x-ray crystallographic structure and antigenic structure are known for the same protein, it is not surprising to find that a series of monoclonal antibodies binding to a molecule such as influenza neuraminidase choose an overlapping pattern of sites at the exposed head of the protein.[103] The stalk of neuraminidase was not immunogenic apparently because it was almost entirely covered by carbohydrate.

Beyond such things as carbohydrate, which may sterically interfere with antibody binding to protein, accessibility on the surface is clearly a sine qua non for an antigenic determinant to be bound by an antibody specific for the native conformation, without any requirement for unfolding of the structure.[51] Several measures of such accessibility have been suggested. All these require knowledge of the x-ray crystallographic three-dimensional structure. Some have measured accessibility to solvent by rolling a sphere with the radius of a water molecule over the surface of a protein.[104,105] Others have suggested that accessibility to water is not the best measure of accessibility to antibody and have demonstrated a better correlation by rolling a sphere with the radius of an antibody-combining domain.[106] Another approach to predicting antigenic sites on the basis of accessibility is to examine the degree of protrusion from the surface of the protein.[107] This was done by modeling the body of the protein as an ellipsoid and examining which amino acid residues remain outside ellipsoids of increasing dimensions. The most protruding residues were found to be part of antigenic sites bound by antibodies, but usually, these sites had been identified by using short synthetic peptides and so were segmental in nature. As noted previously, for an antigenic site to be contained completely within a single continuous segment of protein sequence, the site is likely to have to protrude from the surface, as otherwise residues from other parts of the sequence would fall within the area contacting the antibody.[48] However, inability of such surface or protrusion information to predict antigenic sites has also been encountered in some studies.[49]

Because the three-dimensional structure of most proteins is not known, other ways of predicting surface exposure have been proposed for the vast majority of antigens. For example, hydrophilic sites tend to be found on the water-exposed surface of proteins. Thus, hydrophilicity has been proposed as a second indication of immunogenicity.[108–110] This model has been used to analyze 12 proteins with known antigenic sites: The most hydrophilic site of each protein was indeed one of the antigenic sites. However, among the limitations are the facts that a significant fraction of surface residues can be nonpolar,[104,105] and that several important examples of hydrophobic and aromatic amino acids involved in the antigenic sites are known.[42,83,111,112] Specificity of antibody binding likely depends on the complementarity of surfaces for hydrogen bonding and polar bonding as well as van der Waals contacts, whereas hydrophobic interactions and the exclusion of water from the interacting surfaces of proteins may contribute a large but nonspecific component to the energy of binding.[113] Another study suggested that amino acid pairs were better predictors of epitopes.[114]

A third factor suggested to play a role in immunogenicity of protein epitopes is mobility. Measurement of mobility in the native protein is largely dependent on the availability of a high-resolution crystal structure, so its applicability is limited to only a small subset of proteins. Furthermore, it has been studied only for antibodies specific for segmental antigenic sites; therefore, it may not apply to the large fraction of antibodies to assembled topographic sites. Studies of mobility have taken two directions. The case of antipeptide antibodies has already been discussed, in which antibodies made to peptides corresponding to more mobile segments of the native protein were more likely to bind to the native protein.[95,115] This is not considered just a consequence of the fact that more mobile segments are likely to be those on the surface and therefore more exposed because in the case of myohemerythrin (which was used as a model), two regions of the native protein that were equally exposed but less mobile did not bind nearly as well to the corresponding antipeptide antibodies.[116] However, as is clear from the previous discussion, this result applies to antibodies made against short peptides and therefore is not directly relevant to immunogenicity of parts of the native protein. Rather, it concerns the cross-reactivity of antipeptide antibodies with the native protein and therefore is of considerable practical importance for the purposes outlined in the section on antipeptide antibodies.

Studies in the other direction—that is, of antibodies raised against native proteins—would be by definition more relevant to the question of immunogenicity of parts of the native protein. Westhof et al.[117] used a series of hexapeptides to determine the specificity of antibodies raised against native tobacco mosaic virus protein and found that six of the seven peptides that bound antibodies to native protein corresponded to peaks of high mobility in the native protein. The correlation was better than could be accounted for just by accessibility because three peptides that corresponded to exposed regions of only average mobility did not bind antibodies to the native protein. However, when longer peptides—on the order of 20 amino acid residues—were used as probes, it was found that antibodies were present in the same antisera that bound to less mobile regions of the protein.[118] They simply had not been detected with the short hexapeptides with less conformational stability. Thus, it was not that the more mobile regions were necessarily more immunogenic but rather that antibodies to these were more easily detected with short peptides as probes. A similar good correlation of antigenic sites with mobile regions of the native protein in the case of myoglobin[117] may also be attributed to the fact that seven of the nine sites were defined with short peptides of six to eight residues.[71] Again, this result becomes a statement about cross-reactivity between peptides and native protein rather than about the immunogenicity of the native protein. For reviews, see Van Regenmortel[119] and Getzoff et al.[41]

To address the role of mobility in immunogenicity, an attempt was made to quantitate the relative fraction of antibodies specific for different sites on the antigen myohemerythrin.[120] The premise was that, although the entire surface of the protein may be immunogenic, certain regions may elicit significantly more antibodies than others and therefore may

be considered immunodominant or at least more immunogenic. Because this study was done with short synthetic peptides from 6 to 14 residues long based on the protein sequence, it was limited to the subset of antibodies specific for segmental antigenic sites. Among these, it was clear that the most immunogenic sites were in regions of the surface that were most mobile, convex in shape, and often of negative electrostatic potential. Other more recent studies corroborate the greater immunogenicity of more flexible segments of protein structures.[121] The role of these parameters has been reviewed.[41]

These results have important practical and theoretical implications. First, to use peptides to fractionate antiprotein antisera by affinity chromatography, peptides corresponding to more mobile segments of the native protein should be chosen when possible. If the crystal structure is not known, it may be possible to use peptides from amino or carboxyl termini or from exon–intron boundaries, as these are more likely to be mobile.[115] Second, these results may explain how a large but finite repertoire of antibody-producing B cells can respond to any antigen in nature or even artificial antigens never encountered in nature. Protein segments that are more flexible may be able to bind by induced fit in an antibody-combining site that is not perfectly complementary to the average native structure.[41,51] Indeed, evidence from the crystal structure of antigen–antibody complexes[122–124] suggests that mobility in the antibody-combining site as well as in the antigen may allow both reactants to adopt more complementary conformations on binding to each other, that is, a two-way induced fit. A nice example comes from the study of antibodies to myohemerythrin,[123] in which the data suggested that initial binding of exposed side chains of the antigen to the antibody promoted local displacements that allowed exposure and binding of other, previously buried residues that served as contact residues. The only way this could occur would be for such residues to become exposed during the course of an induced fit conformational change in the antigen.[41,123] In a second very clear example of induced fit, the contribution of antibody mobility to peptide binding was demonstrated for a monoclonal antibody to peptide 75 to 110 of influenza hemagglutinin, which was crystallized with or without peptide in the binding site and analyzed by x-ray crystallography for evidence of an induced fit.[124] Despite flexibility of the peptide, the antibody-binding site probably could not accommodate the peptide without a conformational change in the third complementarity determining region of the heavy chain, in which an asparagine residue of the antibody was rotated out of the way to allow a tyrosine residue of the peptide to fit in the binding pocket of the antibody.[124]

Regarding host-limited factors, immunogenicity is certainly limited by self-tolerance. Thus, the repertoire of potential antigenic sites on mammalian protein antigens such as myoglobin or cytochrome c can be thought of as greatly simplified by the sharing of numerous amino acids with the endogenous host protein. For mouse, guanaco, or horse cytochrome c injected into rabbits, each of the differences between the immunogen and rabbit cytochrome c is seen as an immunogenic site on a background of immunologically silent residues.[50,84,125] In another example, rabbit and dog antibodies to beef myoglobin bound almost equally well to beef or sheep myoglobin.[126] However, sheep antibodies bound beef but not sheep myoglobin, even though these two myoglobins differ by just six amino acids. Thus, the sheep immune system was able to screen out those clones that would be autoreactive with sheep myoglobin.

Ir genes of the host also play an important role in regulating the ability of an individual to make antibodies to a specific antigen.[127] These antigen-specific immune response genes are among the major histocompatibility complex (MHC) genes that code for transplantation antigens. Structural mutations, gene transfer experiments, and biochemical studies[127] all indicate that Ir genes are actually the structural genes for MHC antigens. The mechanism of action of the MHC antigens works through their effect on helper T cells (described later in this chapter). There appear to be constraints on which B and T cells of a given specificity can help,[128,129] a process called T–B reciprocity.[130] Thus, if Ir genes control helper T-cell specificity, they will in turn limit which B cells are activated and which antibodies are made.

The immunogenicity of peptide antigens is also limited by intrinsic and extrinsic factors. With less structure to go on, each small peptide must presumably contain some non–self-structural feature in order to overcome self-tolerance. In addition, the same peptide must contain antigenic sites that can be recognized by helper T cells as well as by B cells. When no T-cell site is present, three approaches may be helpful: graft on a T-cell site, couple the peptide to a carrier protein, or overcome T-cell nonresponsiveness to the available structure with various immunologic agents, such as interleukin 2.

An example of a biologically relevant but poorly immunogenic peptide is the asparagine-alanine-asparagine-proline (NANP) repeat unit of the circumsporozoite (CS) protein of malaria sporozoites. A monoclonal antibody to the repeat unit of the CS protein can protect against murine malaria.[131] Thus, it would be desirable to make a malaria vaccine of the repeat unit of *Plasmodium falciparum* $(NANP)_n$. However, only mice of one MHC type ($H\text{-}2^b$) of all mouse strains tested were able to respond to $(NANP)_n$.[132,133] One approach to overcome this limitation is to couple $(NANP)_n$ to a site recognizable by T cells, perhaps a carrier protein such as tetanus toxoid.[134] In human trials, this conjugate was weakly immunogenic and only partially protective. Moreover, as helper T cells produced by this approach are specific for the unrelated carrier, a secondary or memory response would not be expected to be elicited by the pathogen itself.

Another choice might be to identify a T-cell site on the CS protein itself and couple the two synthetic peptides together to make one complete immunogen. The result with one such site, called Th2R, was to increase the range of responding mouse MHC types by one, to include $H\text{-}2^k$ as well as $H\text{-}2^b$.[135] This approach has the potential advantage of inducing a state of immunity that could be boosted by natural exposure to the sporozoite antigen. As CS-specific T and B cells are both elicited by the vaccine, natural exposure to the antigen could help maintain the level of immunity during the entire period of exposure.

Another strategy to improve the immunogenicity of peptide vaccines is to stimulate the T- and B-cell responses artificially by adding interleukin 2 to the vaccine. Results with myoglobin indicate that genetic nonresponsiveness can be overcome by appropriate doses of interleukin 2.[136] The same effect was found for peptides derived from malaria proteins.[137,137a]

One of the most important possible uses of peptide antigens is as synthetic vaccines. However, even though it is possible to elicit with synthetic peptides anti-influenza antibodies to nearly every part of the influenza hemagglutinin,[94] antibodies that neutralize viral infectivity have not been elicited by immunization with synthetic peptides. This may reflect the fact that antibody binding by itself often does not result in virus inactivation. Viral inactivation occurs only when antibody interferes with one of the steps in the life cycle of the virus, including binding to its cell surface receptor, internalization, and virus uncoating within the cell. Apparently, antibodies can bind to most of the exposed surface of the virus without affecting these functions. Only those antibodies that bind to certain "neutralizing" sites can inactivate the virus. In addition, as in the case of the VP1 coat protein of poliovirus, certain neutralizing sites are found only on the native protein and not on the heat-denatured protein.[138] Thus, not only the site but also the conformation that is bound by the antibodies may be important for the antibody to inactivate the virus. These sites may often be assembled topographic sites not mimicked by peptide segments of the sequence. Perhaps binding of an antibody to such an assembled site can alter the relative positions of the component subsites so as to induce an allosteric neutralizing effect. Alternatively, antibodies to such an assembled site may prevent a conformational change necessary for activity of the viral protein.

One method of mapping neutralizing sites is based on the use of neutralizing monoclonal antibodies. The virus is grown in the presence of neutralizing concentrations of the monoclonal antibody, and virus mutants are selected for the ability to overcome antibody inhibition. These are sequenced, revealing the mutation that permits "escape" by altering the antigenic site for that antibody. This method has been used to map the neutralizing sites of influenza hemagglutinin[139] as well as poliovirus capsid protein VP1.[140] The influenza escaping mutations are clustered to form an assembled topographic site, with mutations distant from each other in the primary sequence of hemagglutinin but brought together by the three-dimensional folding of the native protein. At first, it was thought that neutralization was the result of steric hindrance of the hemagglutinin-binding site for the cell surface receptor of the virus.[141] However, similar work with poliovirus reveals that neutralizing antibodies that bind to assembled topographic sites may inactivate the virus at less than stoichiometric amounts, when at least half of the sites are unbound by antibody.[142] The neutralizing antibodies all cause a conformational change in the virus, which is reflected in a change in the isoelectric point of the particles from pH 7 to pH 4.[140,143] Antibodies that bind without neutralizing do not cause this shift. Thus, an alternative explanation for the mechanism of antibody-mediated neutralization is the triggering of the virus to self-destruct. Perhaps the reason that neutralizing sites are clustered near receptor-binding sites is that occupation of such sites by antibody mimics events normally caused by binding to the cellular receptor, causing the virus to prematurely trigger its cell entry mechanisms. However, in order to transmit a physiologic signal, the antibody may need to bind viral capsid proteins in the native conformation (especially assembled topographic sites), which antipeptide antibodies may fail to do. Antibodies of this specificity are similar to the viral receptors on the cell surface, some of which have been cloned and expressed without their transmembrane sequences as soluble proteins. The soluble recombinant receptors for poliovirus[144] and HIV-1[145–147] exhibit high-affinity binding to the virus and potent neutralizing activity in vitro. The HIV-1 receptor, cluster of differentiation (CD)4, has been combined with the human Ig heavy chain in a hybrid protein CD4-Ig,[148] which spontaneously assembles into dimers and resembles a monoclonal antibody, in which the binding site is the same as the receptor-binding site for HIV-1. In these recombinant constructs, high-affinity binding depends on the native conformation of the viral envelope glycoprotein gp120. Binding of CD4 to gp120 elicits a conformational change exposing a CD4-induced epitope, and fusions of CD4 domains to gp120 can be used as vaccines to elicit such antibodies.[149]

For HIV-1, two types of neutralizing antibodies have been identified. The first type binds a continuous or segmental determinant, such as the "V3 loop" sequence between amino acids 296 and 331 of gp120.[150–152] Antipeptide antibodies against this site can neutralize the virus.[150] However, because this site is located in a highly variable region of the envelope, these antibodies tend to neutralize a narrow range of viral variants with nearly the same sequence as the immunogen. Even for this highly variable site, more broadly neutralizing antibodies can be obtained that recognize conserved conformations.[153–155] The second type of neutralizing antibody binds conserved sites on the native structure of gp120, allowing them to neutralize a broad spectrum of HIV-1 isolates. These antibodies are commonly found in the sera of infected patients,[156] and a panel of neutralizing monoclonals derived from these subjects has been analyzed.

These monoclonals can be divided into three types. One group, possibly the most common ones in human polyclonal sera, bind at or near the CD4 receptor–binding site of gp120.[79,157–161] A second type of monoclonal, called 2G12, binds a conformational site on gp120 that also depends on glycosylation, but has no direct effect on CD4 binding.[162] A third type, quite rare in human sera, is represented by monoclonal antibody 2F5[163] and binds a conserved site on the transmembrane protein gp41. Although this site is contained on a linear peptide ELDKWA, antibodies such as 2F5 cannot be elicited by immunizing with the peptide, again suggesting the conformational aspect of this site.[164,165] Indeed, the binding of antibodies to this membrane-proximal site has even been found to involve interaction of the antibody with the lipid membrane.[166] One might view this intriguing case as an example of a discontinuous or assembled topographic site created by the proximity of residues of the protein with structures in the lipid membrane, thus, spanning more than just different parts of the antigenic protein.

These monoclonals neutralize fresh isolates, as well as laboratory-adapted strains, and they neutralize viruses tropic for T cells or macrophages,[167] regardless of the use of CXCR4 or CCR5 as second receptor. These monoclonals, which target different sites, act synergistically. A cocktail combining all three types of monoclonals can protect monkeys against iv challenge or vaginal challenge with a simian immunodeficiency virus/HIV hybrid virus, indicating the potential for antibodies alone to prevent HIV infection.[168,169] Because each of the three conserved neutralizing determinants depends on the native conformation of the protein,[170] a prospective gp120 vaccine (or gp160 vaccine) would need to be in the native conformation to be able to elicit these antibodies.

ANTIGENIC DETERMINANTS RECOGNIZED BY T CELLS

Studies of T-cell specificity for antigen were motivated by the fact that the immune response to protein antigens is regulated at the T-cell level. A hapten, not immunogenic by itself, will elicit antibodies only when coupled to a protein that elicits a T-cell response in that animal. This ability of the protein component of the conjugate to confer immunogenicity on the hapten has been termed the "carrier effect." Recognition of the carrier by specific helper T cells induces the B cells to make antibodies. Thus, the factors contributing to a good T-cell response appear to control the B-cell response as well.

"Nonresponder" animals display an antigen-specific failure to respond to a protein antigen, both for T cells and antibody responses. The "high responder" phenotype for each antigen is a genetically inheritable, usually dominant trait. Using inbred strains of mice, the genes controlling the immune response were found to be tightly linked to the MHC genes.[127,171] MHC-linked immune responsiveness has been shown to depend on the T-cell recognition of antigen bound within a groove of MHC antigens of the antigen-presenting cell (APC) (discussed herein below and see Chapters 21 and 22). The recognition of antigen in association with MHC molecules of the B cell is necessary for carrier-specific T cells to expand and provide helper signals to B cells.

In contrast to the range of antigens recognized by antibodies, the repertoire recognized by helper and cytotoxic T cells appears to be limited largely to protein and peptide antigens, although exceptions such as the small molecule tyrosine-azobenzene arsonate[172] exist. Once the antigenic determinants on proteins recognized by T cells are identified, it may be possible to better understand immunogenicity and perhaps even to manipulate the antibody response to biologically relevant antigens by altering the helper T–cell response to the antigen.

Defining Antigenic Structures

Polyclonal T-Cell Response

Significant progress in understanding T-cell specificity was made possible by focusing on T-cell proliferation in vitro, mimicking the clonal expansion of antigen specific clones in vivo. The proliferative response depends on only two cells: the antigen-specific T cell and an APC, usually a macrophage, dendritic cell, or B cell. The growth of T cells in culture is measured as the incorporation of [^{3}H]thymidine into newly formed DNA. Under appropriate conditions, thymidine incorporation increases with antigen concentration. This assay permits the substitution of different APCs and is highly useful in defining the MHC and antigen-processing requirements of the APCs.

Using primarily this assay, several different approaches have been taken to mapping T-cell epitopes. First, T cells immunized to one protein have been tested for a proliferative response in vitro to the identical protein or to a series of naturally occurring variants. By comparing the sequences of stimulatory and nonstimulatory variants, it was possible to identify potential epitopes recognized by T cells. For example, the T-cell response to myoglobin was analyzed by immunizing mice with sperm whale or horse myoglobin and testing the resulting T cells for proliferation in response to a series of myoglobins from different species with known amino acid substitutions.[173] Reciprocal patterns were observed in T cells from mice immunized with sperm whale or horse myoglobin. The response to the cross-stimulatory myoglobins was as strong as to the myoglobin used to immunize the mice. This suggested that a few shared amino acid residues formed an immunodominant epitope, and that most substitutions had no effect on the dominant epitope. A comparison of the sequences revealed that substitutions at a single residue could explain the pattern observed. All myoglobins that cross-stimulated sperm whale–immune T cells had Glu at position 109, whereas all that cross-stimulated horse-immune T cells had Asp at 109. No member of one group could stimulate T cells from donors immunized with a myoglobin of the other group. This suggested that an immunodominant epitope recognized by T cells was centered on position 109, regardless of which amino acid was substituted. Usually, this approach has led to correct localization of the antigenic site in the protein,[173–175] but the possibility of long-range effects on antigen processing must be kept in mind (see the section on "Antigen Processing"). Also, this approach using natural variants is limited in that it can focus on the correct region of the molecule but cannot define the boundaries of the site. Site-directed mutagenesis may therefore expand the capabilities of this approach.

A second approach is to use short peptide segments of the protein sequence, taking advantage of the fact that T cells specific for soluble protein antigens appear to see only segmental antigenic sites not assembled topographic ones.[127,176–180] These may be produced by chemical or enzymatic cleavage of the natural protein,[178–186] solid-phase peptide synthesis,[185,187–190] or recombinant DNA expression of cloned genes or gene fragments.[191] In the case of class I MHC molecule–restricted cytotoxic T cells, viral gene deletion mutants expressing only part of the gene product have also been used.[192–194]

In the case of myoglobin-specific T cells, mapping of an epitope to residue Glu 109 was confirmed by use of a synthetic peptide 102 to 118, which stimulated the T cells.[189,195] The T cells elicited by a myoglobin with either Glu or Asp 109 could readily distinguish between synthetic peptides containing Glu or Asp at this position. Similar results were obtained with cytochrome c, where the predominant site recognized by T cells was localized with sequence variants to the region

around residue 100 at the carboxyl end of cytochrome.[174] Furthermore, the response to cytochrome c peptide 81 to 104 was as great as the response to the whole molecule. This indicated that a 24 amino acid peptide contained an entire antigenic site recognized by T cells. The T cells could distinguish between synthetic peptides with Lys or Gln at position 99, although both were immunogenic with the same MHC molecule.[196–198] This residue determined T-cell memory and specificity, and so presumably was interacting with the T-cell receptor (TCR). A similar conclusion could be drawn for residue 109 of myoglobin. However, this type of analysis must be used with caution. When multiple substitutions at position 109 were examined for T-cell recognition and MHC binding, residue 109 was found to affect both functions.[199] The ultimate use of synthetic peptides to analyze the segmental sites of a protein that are recognized by T cells was to synthesize a complete set of peptides, each staggered by just one amino acid from the previous peptide, corresponding to the entire sequence of hen egg lysozyme.[200] Around each immunodominant site, a cluster of several stimulatory peptides was found. The minimum "core" sequence consisted of just those residues shared by all antigenic peptides within a cluster, whereas the full extent of sequences spanning all stimulatory peptides within the same cluster defined the "determinant envelope." These two ways of defining an antigenic site differ, and one interpretation is that each core sequence corresponds to an MHC-binding site, whereas the determinant envelope includes the many ways for T cells to recognize the same peptide bound to the MHC.

In each case, the polyclonal T-cell response could be mapped to a single predominant antigenic site. These results are consistent with the idea that each protein antigen has a limited number of immunodominant sites (possibly one) recognized by T cells in association with MHC molecules of the high-responder type. If none of the antigenic sites could associate with MHC molecules on the APCs, then the strain would be a low responder, and the antigen would have little or no immunogenicity.

Monoclonal T Cells

Further progress in mapping T-cell sites depended on the analysis of cloned T-cell lines. These were either antigen-specific T-cell lines made by the method of Komoto and Fathman[201] or T-cell hybridomas made by the method of Kappler et al.[202] In the former method, T cells are allowed to proliferate in response to antigen and APCs, rested, and then restimulated again. After stimulation, the blasts can be cloned by limiting dilution and grown from a single cell in the presence of interleukin 2. In the second method, enriched populations of antigen-specific T cells are fused with a drug-sensitive T-cell tumor, and the fused cells are selected for their ability to grow in the presence of the drug. Then the antigen specificity of each fused cell line must be determined. The key to determining this in a tumor line is that antigen-specific stimulation of a T-cell hybridoma results in release of interleukin 2, even though proliferation is constitutive. T cells produced by either method are useful in defining epitopes, measuring their MHC associations and studying antigen-processing requirements.

Monoclonal T cells may be useful in identifying which of the many proteins from a pathogen are important for T-cell responses. For instance, Young and Lamb[203] have developed a way to screen proteins separated by SDS–polyacrylamide gel electrophoresis and blotted onto nitrocellulose for stimulation of T-cell clones and have used this to identify antigens of *Mycobacterium tuberculosis*.[204] Mustafa et al.[205] have even used T-cell clones to screen recombinant DNA expression libraries to identify relevant antigens of *Mycobacterium leprae*. Use of T cells to map epitopes has also been important in defining tumor antigens.[206–211]

Precise mapping of antigenic sites recognized by T cells was made possible by the fact that T cells would respond to peptide fragments of the antigen when they contain a complete antigenic determinant. A series of overlapping peptides can be used to walk along the protein sequence and find the antigenic site. Then, by truncating the peptide at either end, the minimum antigenic peptide can be determined. For example, in the case of myoglobin, a critical amino acid residue, such as Glu 109 or Lys 140, was found by comparing the sequences of stimulatory and nonstimulatory myoglobin variants and large CNBr cleavage fragments[212] as previously discussed, and then a series of truncated peptides containing the critical residue was synthesized with different overlapping lengths at either end.[185,189] Because solid-phase peptide synthesis starts from a fixed carboxyl end and proceeds toward the amino end, it can be stopped at various positions to produce a nested series of peptides that vary in length at the amino end. In this way, it was found that two of the Glu 109–specific T-cell clones responded to synthetic peptides 102 to 118 and 106 to 118 but not to peptide 109 to 118.[189] One clone responded to peptide 108 to 118, whereas the other did not. Thus, the amino end of the peptide recognized by one clone was Ser 108, whereas the other clone required Phe 106 and/or Ile 107. Similar fine specificity differences have been observed with T-cell clones specific for the peptides 52 to 61 and 74 to 96 of hen egg lysozyme,[182,213,214] the peptide 323 to 339 of chicken ovalbumin,[183] and the peptide 81 to 104 of pigeon cytochrome c[188]: The epitopes recognized by several T-cell clones overlap but are distinct. In addition, nine T-cell clones recognized a second T-cell determinant in myoglobin located around Lys 140, and each one responded to the CNBr cleavage fragment 132 to 153.[215] Further studies with a nested series of synthetic peptides showed that the stimulatory sequence is contained in peptide 136 to 145.[185]

These findings can be generalized to characterize a large number of epitopes recognized by T cells from a number of protein antigens (Table 23.4).[212–227] What these studies and others demonstrated about epitopes recognized by T cells is that in each case, the entire site is contained on a short peptide. MHC class I–restricted antigens also follow this rule,[228] even when the protein antigen is normally expressed on the surface of infected cells. This applies to viral glycoproteins, such as influenza hemagglutinin, which are recognized by cytolytic T cells after antigen processing[229] (see section on "Antigen Processing"). These peptides consist of no more than about 12 to 17 amino acid residues for class II MHC or 8 to 10 residues for class I. Within this size, they must contain all the information necessary to survive processing

TABLE 23.4 Examples of Immunodominant T-Cell Epitopes Recognized in Association with Class II MHC Molecules

Protein	T-Cell Antigenic Sites and Reference	Amphipathic Segments
Sperm whale myoglobin	69–78[148]	64–78
	102–118[159]	99–117
	132–145[155]	128–145
Pigeon cytochrome c	93–104[158]	92–103
Beef cytochrome c	11–25[192]	9–29
	66–80[193]	58–78
Influenza	109–119[186]	97–120
Hemagglutinin	130–140[187]	—
A/PR/8/34	302–313[187,188]	291–314
Pork insulin	B 5–16[157]	4–16
	A 4–14[189]	1–21
Chicken lysozyme	46–61[185]	—
	74–86[184]	72–86
	81–96[175]	86–102
	109–119[145]	—
Chicken ovalbumin	323–339[153]	329–346
Foot and mouth virus VP1	141–160[191]	148–165
Hepatitis B virus		
Pre-S	120–132[190]	121–135
Major surface antigen	38–52[194]	36–49
	95–109[194]	—
	140–154[194]	—
λ Repressor protein CI	12–26[195]	8–25
Rabies virus spike glycoprotein precursor	32–44[196]	29–46

Adapted with permission from Schwartz et al.[188]

within the APC, associate with the MHC antigen, and bind to the TCR, as discussed in the following sections.

Sequential Steps that Focus the T-Cell Response on Immunodominant Determinants

In contrast to antibodies that bind all over the surface of a native protein[50] (see "Protein and Polypeptide Antigenic Determinants" section), it has been observed that T cells elicited by immunization with the native protein tend to be focused on one or a few immunodominant sites.[230–232] This is true whether one deals with model mammalian or avian proteins such as cytochrome c,[179] myoglobin,[178,180] lysozyme,[182,214,233,234] insulin,[187,219] and ovalbumin,[183] or with bacterial, viral, and parasitic proteins from pathogens, such as influenza hemagglutinin[217] or nucleoprotein (NP),[228] staphylococcal nuclease,[235] or malarial CS protein.[135,236] Because the latter category of proteins shares no obvious homology to mammalian proteins, the immunodominance of a few sites cannot be attributed simply to tolerance for the rest of the protein because of homologous host proteins. Moreover, immunodominance is not simply the preemption of the response by a single clone of predominant T cells because it has been observed that immunodominant sites tend to be the focus for a polyclonal response of a number of distinct T-cell clones recognizing overlapping subsites within the antigenic site or having different sensitivities to substitutions of amino acids within the site.[182,183,188,189,200,213,214,237]

Immunodominant antigenic sites appear to be qualitatively different from other sites. For example, in the case of myoglobin, when the number of clones responding to different epitopes after immunization with native protein was quantitated by limiting dilution, it was observed that the bulk of the response to the whole protein in association with the high-responder class II MHC molecules was focused on a single site within residues 102 to 118[195] (Fig. 23.5). When T cells in the (high × low responder) F1 hybrid restricted to each MHC haplotype were compared, there was little difference in the responses to nondominant epitopes, and all the overall difference in magnitude of response restricted by the high versus low responder MHC could be attributed to the high response to the immunodominant determinant in the former and the complete absence of this response in the latter (see Fig. 23.5). Similar results were found for two different high-responder and two different low-responder MHC haplotypes.[195] Why did the response to the other sites not compensate for the lack of response to the immunodominant site in the low responders?

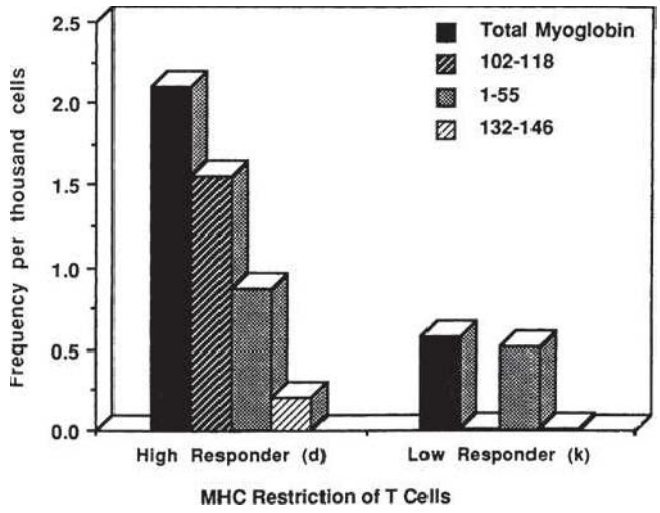


FIG. 23.5. Frequency of High- and Low-Responder Major Histocompatibility Complex (MHC)-Restricted T Cells in F1 Hybrid. High responsiveness may be accounted for by the response to a single immunodominant epitope. Lymph node T cells from (low-responder [H-2^k] × high-responder [H-2^d]) F1 hybrid mice immunized with whole myoglobin were plated at different limiting dilutions in microtiter wells with either high- or low-responder presenting cells and myoglobin as antigen. The cells growing in each well were tested for responsiveness to whole myoglobin and to various peptide epitopes of myoglobin. The frequency of T cells of each specificity and MHC restriction was calculated from Poisson statistics and is plotted on the ordinate. Most of the difference in T-cell frequency between high- and low-responder restriction types *(solid bars)* can be accounted for by the presence of T cells responding to the immunodominant site at residues 102 to 118, accounting for more than two-thirds of the high-responder myoglobin-specific T cells, in contrast to the absence of such T cells restricted to the low-responder MHC type. (Based on the data in Kojima et al.[195].)

The greater frequency of T cells specific for the immunodominant site may in part be attributed to the large number of ways this site can be recognized by different T-cell clones, as mentioned previously, but this only pushes the problem back one level. Why is an immunodominant site the focus for so many different T-cell clones? Because the answer cannot depend on any particular T cell, it must depend on other factors primarily involved in the steps in antigen processing and presentation by MHC molecules.

It has also been observed that some peptides may be immunogenic themselves, but the T-cell response they elicit is specific only for the peptide and does not cross-react with the native protein nor do T cells specific for the native protein recognize this site.[238–240] These are called cryptic determinants.[240] The reasons for these differences may involve the way the native protein is processed to produce fragments distinct from, but including or overlapping, the synthetic peptides used in experiments and also the competition among sites within the protein for binding to the same MHC molecules, as discussed further in the next section. To understand these factors that determine dominance or crypticity, one must understand the steps through which an antigen must go before it can stimulate a T-cell response.

Unlike B cells, T-cell recognition of antigen depends on the function of another cell, the APC.[241] Antigen must pass through a number of intracellular compartments and survive processing and transport steps before it can be effectively presented to T cells. Following antigen synthesis in the cell (as in a virally infected cell) or antigen uptake via phagocytosis, pinocytosis, or, in some cases, receptor-mediated endocytosis, the subsequent steps include 1) partial degradation ("processing") into discrete antigenic fragments that can be recognized by T cells, 2) transport of these fragments into a cellular compartment where MHC binding can occur, 3) MHC binding and assembly of a stable peptide-MHC complex, and 4) recognition of that peptide-MHC complex by the expressed T-cell repertoire. At each step, a potential antigenic determinant runs the risk of being lost from the process, for example, by excessive degradation or failure to meet the binding requirements needed for transport to the next step. Only those peptides that surmount the four selective hurdles will prove to be antigenic for T cells. We will now consider each step in detail, for its contribution to the strength and specificity of the T-cell response to protein antigens.

Antigen Processing

Influence of Antigen Processing on the Expressed T-Cell Repertoire. Several lines of evidence indicate that antigen processing plays a critical role in determining which potential antigenic sites are recognized and, therefore, what part of the potential T-cell repertoire is expressed upon immunization with a protein antigen. Because the T cell does not see the native antigen but only the products of antigen processing, it is not unreasonable that the nature of these products would at least partly determine which potential epitopes could be recognized by T cells.

One line of evidence that processing plays a major role in T-cell repertoire expression came from comparisons that were made of the immunogenicity of peptide versus native molecule in the cases of myoglobin[238] or lysozyme.[239] In the case of myoglobin, a site of equine myoglobin (residues 102 to 118) that did not elicit a response when H-2^k mice were immunized with native myoglobin nevertheless was found to be immunogenic when such mice were immunized with the peptide.[238] Thus, the low responsiveness to this site in mice immunized with the native myoglobin was not due to either of the classical mechanisms of Ir gene defects—namely, a hole in the T-cell repertoire or a failure of the site to interact with MHC molecules of that strain. However, the peptide-immune T cells responded only poorly to native equine myoglobin in vitro. Thus, the peptide and the native molecule did not cross-react well in either direction. The problem was not simply a failure to process the native molecule to produce this epitope because (H-2^k × H-2^s) F1-presenting cells could present this epitope to H-2^s T cells when given native myoglobin but could not present it to H-2^k T cells. Also, because the same results applied to individual T-cell clones, the failure to respond to the native molecule was apparently not due to suppressor cells induced by the native molecule. Similar observations were made for the response to the peptide 74 to 96 of hen lysozyme in B10.A mice.[239] The peptide, not the native molecule, induced T cells specific for this site, and these T cells did not cross-react with the native molecule. With these alternative mechanisms excluded, we are left with the conclusion that an appropriate peptide was produced, but it differed from the synthetic peptide in such a way that a hindering site outside the minimal antigenic site interfered with presentation by presenting cells of certain MHC types. Further evidence consistent with this mechanism came from the work of Shastri et al.,[242] who found that different epitopes within the 74 to 96 region of lysozyme were immunodominant in H-2^b mice when different forms of the immunogen were used.

Another line of evidence came from fine specificity studies of individual T-cell clones. Shastri et al.[243] observed that H-2^b T-cell clones specific for hen lysozymes were about 100-fold more sensitive to ring-necked pheasant lysozyme than to hen lysozyme. Nevertheless, they were equally sensitive to the CNBr cleavage fragments containing the antigenic sites from both lysozymes. Thus, regions outside the minimal antigenic site removable by CNBr cleavage presumably interfered with processing, presentation, or recognition of the corresponding site in hen lysozyme. Similarly, it was observed that a T-cell clone specific for sperm whale myoglobin, not equine myoglobin, responded equally well to the minimal epitope synthetic peptides from the two species.[238] Also, residues outside the actual site must be distinguishing equine from sperm whale myoglobin. Experiments using F1-presenting cells that can clearly produce this epitope for presentation to other T cells proved that the problem was not a failure to produce the appropriate fragment from hen lysozyme[239] or equine myoglobin.[238] Thus, these cases provide evidence that a structure outside the minimal site can hinder presentation in association with a particular MHC molecule.

Such a hindering structure was elegantly identified in a study by Grewal et al.[244] comparing hen egg lysozyme peptides presented by strains C57BL/6 and C3H.SW that share H-2^b but differ in non-MHC genes. After immunization with whole lysozyme, a strong T-cell response was seen to peptide 46 to 61 in C3H.SW mice but not at all in C57BL/6 mice. Because the F1 hybrids of these two strains responded, the lack of response in one strain was not due to a hole in

the T-cell repertoire produced by self-tolerance. It was found that peptide 46 to 60 bound directly to the I-A^b class II MHC molecule, whereas peptide 46 to 61 did not, indicating that the C-terminal Arg at position 61 hindered binding. Evidently, a non–MHC-linked difference in antigen processing allowed this Arg to be cleaved off the 46 to 61 peptide in C3H.SW mice, in which the peptide was dominant, but not in C57Bl/6 mice, in which the peptide was cryptic.

Even a small peptide that does not need processing may nevertheless be processed, and that processing may affect its interaction with MHC molecules. Fox et al.[245] found that substitution of a tyrosine for isoleucine at position 95 of cytochrome c peptide 93 to 103 enhanced presentation with $E\beta^b$ but diminished presentation with $E\beta^k$ when live APCs were used but not when the APCs were fixed and could not process antigen. Therefore, the tyrosine residue was not directly interacting with the different MHC molecule but was affecting the way the peptide was processed, which in turn affected MHC interaction.

Besides the mechanisms suggested previously, Gammon et al.[239] and Sercarz et al.[246] have proposed the possibility of competition between different MHC-binding structures ("agretopes") within the same processed fragment. If a partially unfolded fragment first binds to MHC by one such site already exposed, further processing may stop, and other potential binding sites for MHC may never become accessible for binding. Such competition could also occur between different MHC molecules on the same presenting cell.[239] For instance, BALB/c mice, expressing both A^d and E^d, produce a response to hen lysozyme specific for 108 to 120, not for 13 to 35,[239] and this response is restricted to E^d. However, B10.GD mice that express only A^d respond well to 13 to 35 when immunized with lysozyme. The BALB/c mice clearly express an A^d molecule, so the failure to present this 13 to 35 epitope may be due to competition from E^d, which may preempt by binding the 108 to 120 site with higher affinity and preventing the 13 to 35 site from binding to A^d. Competition between different peptides binding to the same MHC molecule could also occur.

All these results, taken together, indicate that antigen processing not only facilitates interaction of the antigenic site with the MHC molecule and/or the TCR but also influences the specificity of these interactions and, in turn, the specificity of the elicited T-cell repertoire. The molecular mechanisms behind such effects are just now being elucidated, as described in the following sections.

Processing of Antigen for T Cells Restricted to Class II Major Histocompatibility Complex Molecules. It has long been known that T-cell responses such as delayed hypersensitivity in vivo or T-cell proliferation in vitro to exogenous proteins can be stimulated not only by the native protein but also by denatured protein[176] and fragments of native protein.[219] Indeed, this feature, along with the requirement for recognition in association with class II MHC molecules, distinguishes T- from B-cell responses. In a number of cases, the site recognized by cloned T cells has been located to a discrete synthetic peptide corresponding to a segment of the primary sequence of the protein. Examples include insulin,[187,219] cytochrome c,[188] lysozyme,[182,213] and myoglobin.[178,185,189] In each case, the stimulatory peptide must contain all the information required for antigen presentation and T-cell stimulation. The lack of conformational specificity does not indicate a lack of TCR specificity. Rather, it results from antigen processing into peptide fragments that destroys conformational differences prior to binding the TCR. One way to accomplish this is via antigen processing, which involves the partial degradation of a protein antigen into peptide fragments (Fig. 23.6).

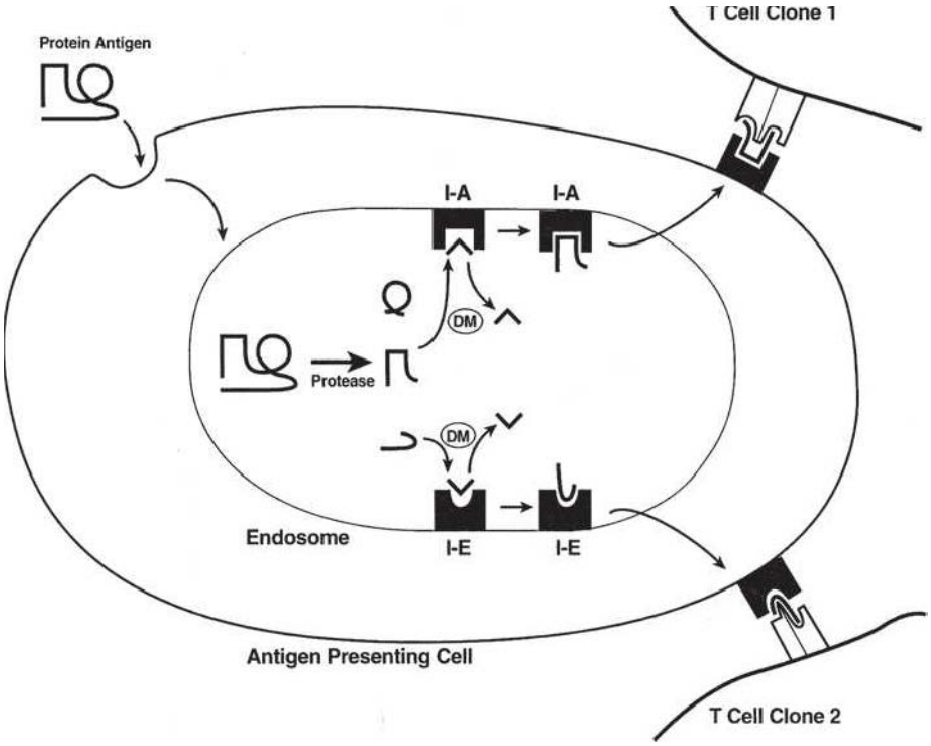


FIG. 23.6. Steps in Antigen Presentation by Class II Major Histocompatibility Complex (MHC) Molecules. Soluble antigen enters the presenting cell by phagocytosis, pinocytosis, or receptor-mediated endocytosis. It is partially degraded to peptide fragments by acid-dependent proteases in endosomes. Antigenic peptides associate with MHC class II molecules (I-A or I-E in the mouse) to form an antigenic complex that is transported to the cell surface. Before an MHC class II molecule can bind the peptide, it must release the class II–associated invariant chain peptide fragment of invariant chain from the binding groove, which is catalyzed by human leukocyte antigen (HLA)-DM. Binding of T-cell receptors to the peptide-MHC complex triggers T-cell proliferation, resulting in clonal expansion of antigen-specific T cells.

Evidence of processing came from the fact that a single protein antigen could stimulate T cells to different epitopes, each specific for a different MHC antigen. For example, when a series of myoglobin-specific T-cell clones were tested for both antigen specificity and MHC restriction, six clones were specific for a site centering on amino acid Glu 109, and all six recognized the antigen in association with I-A^d. Nine additional T-cell clones were specific for a second epitope centered on Lys 140 and were restricted to a different MHC antigen, I-E^d. Thus, the antigen behaved as if it was split up into distinct epitopes, each with its own ability to bind MHC.[215]

That T cells recognize processed antigen was demonstrated by the fact that inhibitors of processing can block antigen presentation. Early experiments by Ziegler and Unanue[247] showed that processing depends on intracellular degradative endosomes, as drugs such as chloroquine and ammonium chloride (NH4 Cl), which raise endosomal pH and inhibit acid-dependent proteases, could block the process. However, prior degradation of proteins into peptide fragments allows them to trigger T cells even in the presence of these inhibitors of processing.[248] For example, T-cell clone 14.5 recognizes the Lys 140 site of myoglobin equally well on the antigenic peptide (residues 132 to 153) as on the native protein (Fig. 23.7). The difference between these two forms of antigen is brought out by the presence of processing inhibitors. Leupeptin, for example, inhibits lysosomal proteases and blocks the T-cell responses to native myoglobin but not to peptide 132 to 153. Thus, native myoglobin cannot stimulate T cells without further processing, whereas the peptide requires little or no additional processing.[249]

Why is antigen processing necessary? For class II MHC molecules, experiments suggest that antigen processing may uncover functional sites that are buried in the native protein structure. For example, a form of intact myoglobin that has been partially unfolded through chemical modification can behave like a myoglobin peptide and can be presented by APC even in the presence of enough protease inhibitor or chloroquine to completely block the presentation of native myoglobin.[249] Denatured lysozyme could also be presented without processing to one T-cell clone.[184] This result suggests that the requirement for processing may simply be a steric requirement, that is, to uncover the two sites needed to form the trimolecular complex between antigen and MHC and between antigen and TCR. Thus, unfolding may be sufficient without proteolysis, and proteolysis may simply accomplish an unfolding analogous to Alexander's approach to the Gordian knot.

The importance of antigen unfolding for T-cell recognition and the ability of unfolding to bypass the need for antigen processing apply to a range of polypeptide sizes from small peptides to extremely large proteins. At one extreme, Lee et al.[250] found that even fibrinogen, of Mr 340,000, does not need to be processed if the epitope recognized is on the carboxy-terminal portion of the α chain, which is naturally unfolded in the native molecule. At the other extreme, even a small peptide of only 18 amino acid residues, apamin, requires processing unless the two disulfide bonds that hold it in the native conformation are cleaved artificially to allow

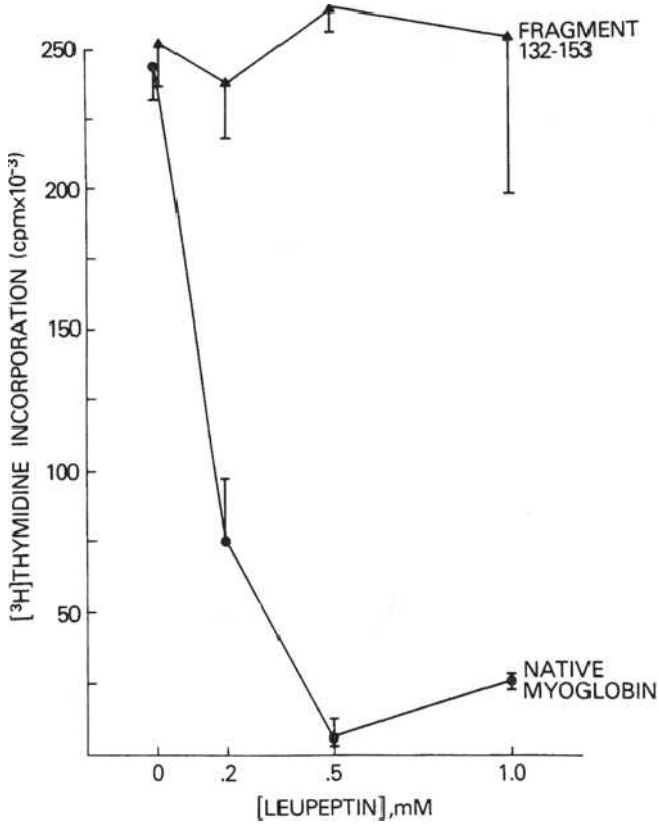


FIG. 23.7. Inhibition of Antigen Presentation by the Protease Inhibitor Leupeptin: Differential Effect on Presentation of the Same Epitope of Native Myoglobin or Peptide 132 to 153 to the Same Monoclonal T-Cell Population. Splenic cells from nonimmunized B10.D2 mice, as a source of antigen-presenting cells, were incubated with leupeptin at the concentration indicated for 15 minutes prior to and during exposure to 2 μM native myoglobin or 1 μM peptide fragment, washed, irradiated, and cultured at 400,000 cells per well with 10,000 T cells of clone 14.5; thymidine incorporation was measured after 4 days of culture.[249]

unfolding.[251] Therefore, large size does not mandate processing, and small size does not necessarily obviate the need for processing, at least for class II presentation. The common feature throughout the size range seems to be the need for unfolding. This evidence, taken together with the earlier data on unfolding of myoglobin and lysozyme, strongly supports the conclusion that unfolding, rather than size reduction, is the primary goal of antigen processing, and that either antigen presentation by MHC molecules or TCR recognition frequently requires exposure of residues not normally exposed on the surface of the native protein. This conclusion is supported by recent studies of peptides eluted from class II MHC molecules, and the crystal structures of class II MHC-peptide complexes, which show that longer peptides can bind with both ends extending beyond the two ends of the MHC groove[252–254] (see "Antigen Interaction with MHC Molecules" section).

Besides proteolysis, unfolding may require the reduction of disulfide bonds between or within protein antigens. A gamma interferon–inducible lysosomal thiol reductase (GILT) is expressed in APCs and localizes to the late endosomal and lysosomal compartments where MHC class II peptide loading

occurs.[255] Unlike thioredoxin, this enzyme works at the acid pH of endosomes and uses Cys but not glutathione as a reducing agent. APCs from GILT knockout mice were tested for the ability to present hen egg lysozyme to hen egg lysozyme–specific T-cell lines.[256] For two epitopes, the T-cell response was insensitive to the GILT defect, even though they involved a disulfide bond in the native protein. But for one epitope, located between disulfide bonds, the T-cell response was completely inhibited when the APCs lacked GILT reductase. In this case, reduction of disulfide bonds was an essential step for antigen presentation, presumably needed to generate free peptides for MHC class II binding.

Processing of Antigen for T Cells Restricted to Class I Major Histocompatibility Complex Molecules. Early studies on class I–restricted T cells, such as cytolytic T cells (CTLs) specific for virus-infected cells, assumed that they responded mainly to unprocessed viral glycoproteins expressed on the surface of infected cells. However, since the mid-1980s, it has been clear that CTLs, like other T cells, recognize processed antigens. For example, influenza NP was a major target antigen for influenza specific CTLs, even though NP remains in the nucleus of infected cells and none is detectable on the cell surface.[257] Further support came from the finding that target cells that take up synthetic NP peptide 366 to 379 were lysed by NP-specific CTLs.[228] This constitutes evidence that antigen presented in association with class I molecules requires processing into antigenic fragments. Also, the demonstration that synthetic peptides could sensitize targets for CTLs introduced a powerful tool for mapping and studying CTL epitopes.

Even for influenza hemagglutinin, which is expressed on the surface of infected cells, surface expression was not required for antigenicity, implying that it is the processed antigen that stimulates a T-cell response. Target cells expressing leader-negative hemagglutinin, which is not transported to the cell surface but remains in the cytosol, were lysed equally well as those with surface hemagglutinin.[229] Similar conclusions were drawn from anchor-negative mutants.[258] Indeed, studies of HIV-1 gp160 genes with or without a leader sequence suggest that removal of the leader sequence can increase the amount of protein that is retained in the cytosol and is available for processing and presentation through the class I MHC processing pathway.[259] The explanation may be that the signal peptide results in cotranslational translocation of the growing peptide chain into the endoplasmic reticulum (ER), whereas proteins without a signal peptide remain in the cytosol, where they are accessible to the processing machinery of the class I pathway (see subsequent discussion). This cytosolic protein processing machinery consists primarily of the 26S proteasomes.[260,261] The specificity of such proteasomes to cleave at certain positions in a protein sequence thus provides the first hurdle that a potential epitope must surmount to be presented by class I MHC molecules to be cut out correctly but not destroyed by the proteasome.

In the standard proteasome, 14 distinct subunits assemble to form a high-molecular weight complex of about 580 kD with three distinct protease activities located on different subunits. The proteasome is a barrel-shaped structure, with the protease activities arrayed on the inner surface, and unfolded proteins are believed to enter the barrel at one end, leaving as peptides at the other end. The different proteases cut preferentially after aromatic or branched chain amino acids (chymotryptic-like activity of the β5 subunit), basic amino acids (trypsin-like activity of the β2 subunit), or acidic residues (glutamate preferring of the β1 subunit).[262,263] Protease activity is increased against misfolded proteins, such as senescent proteins, which are tagged with ubiquitin and directed to the proteasome. In addition, viral proteins produced during infection and proteins synthesized with artificial amino acids are particularly susceptible to degradation by proteasomes. The products of protease digestion are peptides ranging from to 3 to 14 amino acids in length, including 9-mers, of just the right size for MHC binding. The chymotrypsin- and trypsin-like activities may be particularly important for antigenic peptides, as many peptides that naturally bind MHC end in hydrophobic or basic residues.[264]

The proteasome is the major processing machinery of the nonendosomal processing pathway. This is shown by the effect of proteasome inhibitors on MHC class I assembly and antigen presentation and by the effect of Large Multifunctional Peptidase (LMP-2) and LMP-7 mutations on antigen processing. A family of proteasome inhibitors have been described[262,263,265] that consist of short peptides, three to four amino acids in length, ending in an aldehyde, such as Ac-Leu Leu norLeu-al, carbobenzoxy-Leu Leu nor-Val-al,[263] or nonpeptides such as lactacystin.[266] Although the peptides appear to be directed primarily at the chymotrypsin-like protease activity, as false substrates, in fact, they inhibit all three types of protease activity.

By inhibiting antigen processing, these inhibitors induce a phenotype of reduced expression of MHC class I and inability to present antigen to class I–restricted CTL.[263] The MHC class I heavy chains remain in the ER, as shown by failure to become resistant to endoglycosidase H,[267] which occurs in the Golgi. They are also unable to form stable complexes with β2-microglobulin due to a lack of peptides. These effects are specific for the protease function because the inhibitors do not block presentation of synthetic peptides, which also rescue MHC class I expression, and because inhibition is reversible when inhibitor is removed. These results suggest that proteasomes are the primary supplier of antigenic peptides for class I, as other pathways are unable to compensate. However, it is also possible that the inhibitors could block other potential processing enzymes as well. An alternative processing pathway that bypasses the proteasome is provided by signal peptidase. As signal peptides are cleaved from proteins entering the ER, these hydrophobic peptides can bind MHC class I.[268] Particularly for MHC molecules such human leukocyte antigen (HLA)-A2, which prefer hydrophobic sequences, this peptidase can be an alternative source of antigenic peptides that are independent of proteasomes and transporter associated with antigen presentation (TAP)-1/2 transport (see section on "Transport")because they are formed inside the ER.

The proteins destined for proteasomal processing include some normally short-lived proteins with a half-life of about 10 minutes, which constitute about 25% of the proteins in the cell. The rest arise from long-lived proteins, with a half-life

of about 1 day, which may be synthesized incorrectly. These defective ribosomal products are ubiquitinated and marked for rapid degradation in proteasomes.[269,270] In a normal cell, these can arise from errors in ribonucleic acid transcription, protein translation, assembly, folding, or targeting. But in a virally infected cell, misfolded viral proteins provide a ready supply of antigenic peptides for antigen presentation and T-cell recognition almost as soon as the virus starts to produce new viral proteins. Similarly, incorporation of amino acid analogues, such as canavavanine in place of arginine, creates misfolded proteins that are rapidly processed and more efficiently presented via the proteasomal pathway.[271]

Interestingly, the MHC itself encodes, near the class II region, three proteins, known as LMP-2 and LMP-7 for "low-molecular-weight protein," and MECL-1, for "multicatalytic endopeptidase complex-like 1," which contribute to the proteasome structure. The LMP-2, MECL-1, and LMP-7 subunits are upregulated by interferon-γ, and substitute for the subunits β1, β2, and β5, respectively, forming what has been dubbed an "immunoproteasome," present in professional APCs. All complexes with LMPs contain proteasome proteins, but only 5% to 10% of proteasomes contain LMP-2 and LMP-7. The ones without LMPs are called constitutive proteasomes.

These MHC-encoded subunits of the immunoproteasome shift the preference of proteasomes for cleaving after certain sequences, resulting in the production of different peptide fragments.[272–274] Proteasomes lacking LMP-2 through mutation or gene knockout have the same affinity, but decreased cleavage rate, for sequences ending in hydrophobic or basic amino acids. The effect is specific for these proteolytic sites, as the activity against sequences containing acidic amino acids actually increased.[273] Despite the shift in specific peptides released, the overall level of MHC class I expression was reduced only slightly in LMP-7 knockouts[274] and not at all in the LMP-2 knockouts. However, presentation of specific epitopes of the male H-Y antigen or of influenza NP was reduced by three-fold to five-fold in these knockouts. Toes et al.[275] quantitatively compared the cleavage fragments produced by standard proteasomes and immunoproteasomes and defined the prevalence of different amino acids on each side of the cleavage site. Consistent with earlier studies, there is a strong preference for both to cleave after leucine and also to a lesser extent after other hydrophobic residues, both aliphatic and aromatic. However, the immunoproteasomes have a stronger tendency to cleave after such hydrophobic residues and a much reduced cleavage frequency after acidic residues, Asp and Glu, than standard or constitutive proteasomes. This shift in specificity is concordant with the observation that class I MHC molecules tend to bind peptides with C-terminal hydrophobic or basic residues, not acidic ones. Thus, the immunoproteasomes in professional APCs may be more effective at generating antigenic peptides that can be presented by MHC molecules.[272,275] Protein degradation by proteasomes is processive, so the peptides released after 5% digestion are the same as the fragments released after 90% digestion. This suggests that intact proteins may enter the barrel, but they are not released at the other end until processing is complete.

Immunoproteasomes were shown to be essential for production of a hepatitis B virus core antigenic epitope[276] and to increase production of epitopes from adenovirus[277] and lymphocytic choriomeningitis virus.[278] Similarly, certain epitopes of latent membrane protein 2 of Epstein-Barr virus depended on immunoproteasomes.[279] In that case, the requirement for immunoproteasomes depended on the context of the peptide within the native protein. Incomplete protein synthesis due to puromycin or expression of the epitope surrounded by protein fragments with fewer membrane spanning domains allowed epitope generation by constitutive proteasomes.

On the other hand, some epitopes are generated more effectively by the constitutive proteasome than the immunoproteasome.[275,280] When the repertoire of seven defined class I MHC-restricted epitopes was compared in an elegant quantitative study in LMP-2–deficient or wild-type C57BL/6 mice, it was found that responses to the two epitopes that are immunodominant in wild-type mice were greatly reduced in the LMP-2–deficient mice, which lack immunoproteasomes, and two normally subdominant epitopes became dominant.[281] However, from adoptive transfer experiments in both directions, it was found that the reduced response to one normally dominant epitope was due to decreased production without immunoproteasomes, but that to the other was due to an altered T-cell repertoire in the LMP-2–deficient mice, presumably due to alterations in the peptides presented in the thymus. Further, the increased response to one of the subdominant determinants was related to increased production of this peptide by the constitutive proteasomes compared to the immunoproteasomes. Thus, the immunoproteasome specificity plays a significant role in determining the repertoire of epitopes presented, and in selecting those that are immunodominant, as well as regulating the $CD8^+$ T-cell repertoire generated in the thymus.

Another protein associated with proteasomes is the proteasome activator PA28, which assembles into 11s structures.[282] Like LMP-2 and LMP-7,[272] PA28 is inducible by interferon-γ, and its induction causes a shift in proteasome function that may lead to the production of greater amounts of and different repertoires of antigenic peptides. For example, synthetic substrates were designed to test the ability of proteasomes to generate authentic MHC-binding peptides. These substrates contained the MHC-binding ligand flanked by the natural sequence as found in the original protein.

To generate the MHC-binding ligand, the proteasome would have to cleave the substrate twice.[283] By itself, the 20s proteasome was able to produce singly cleaved fragments, but with added PA28, doubly cut peptides were generated preferentially. Thus, PA28 favored the production of antigenic peptides, possibly by keeping the peptide in the proteasome until processing was complete. Alternatively, PA28 may coordinate the proteolytic activity of two adjacent sites to generate doubly cut peptides of just the right length (8- to 9-mers) to fit in the MHC groove. The distance between these nearby sites would determine the size of the peptides produced. The PA28 has been shown to increase generation of a dominant lymphocytic choriomeningitis virus epitope independently of the presence of the other interferon-γ–inducible components LMP-2, LMP-7, and MECL-1.[284]

The specificity of this proteasomal processing system determines the first step in winnowing the number of

protein segments that can become CTL epitopes, by selectively producing some peptide fragments in abundance and destroying others. Thus, it is probably not just coincidental that the C-terminal residues produced by proteasomal cleavage often serve as anchor residues for binding class I MHC molecules, or that the lengths of peptides produced are optimal for class I MHC binding.[272,285] Better understanding of the specificity of proteasomes will contribute to the new methods to predict dominant $CD8^+$ T-cell epitopes.[232]

An analysis of proteasomal target sites was based on the proteasomal degradation pattern of three proteins: beta-casein, enolase, and prions.[286] The resulting peptides were analyzed by mass spectrometry, and the cleavage sites identified, including four amino acids on the amino end of each cleavage site and two amino acids on the carboxy end. The results are summarized in Figure 23.8. The three proteolytic activities of the proteasome were represented by favorable amino acids: Arg (trypsin-like), Tyr and Phe (chymotrypsin-like), and peptidylglutamylpeptide (Asp and Glu) at position P1. At this site, Pro was unfavored, as were Asn, Lys, and Ser. Additional negative effects were observed for Asp, Pro, and Ile at position P2. On the carboxy side of the cleavage site, at position P'1, the positive effect of Tyr was noted as well as negative effects of Ile, Phe, and Val.

Additional downstream processing steps, after the proteasome, are known to be important for generation of antigenic peptides. One of these occurs in the cytoplasm, prior to TAP transport, and others occur after transport into the ER. The proteasome creates a first draft of the peptides, which are then selected and trimmed to produce the final pool of antigenic peptides of optimal size and sequence for MHC binding while protecting the nascent peptides from degradation before they can bind MHC.

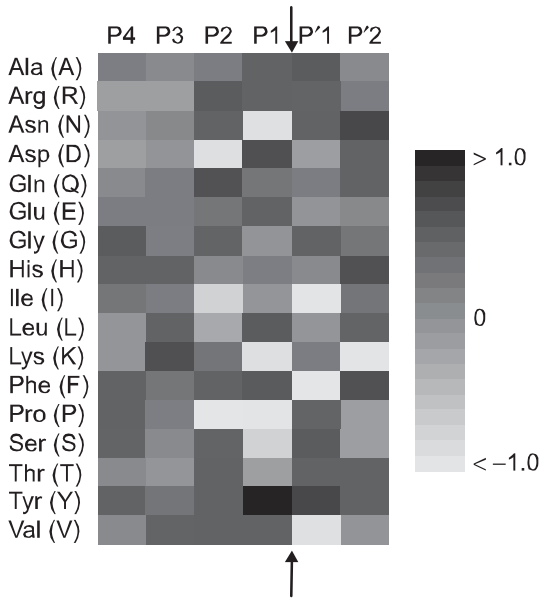


FIG. 23.8. The Effect of Specific Amino Acids on Proteasomal Cleavage between Positions P1 and P'1. Amino acids that favor cleavage are shown in *black*, whereas those that inhibit cleavage are shown in *white*. Reproduced from Donnes and Kohlbacher[286] with permission.

Recent studies have revealed that most peptides are released from the proteasome when they still need further processing to become antigenic peptides.[287,288] In the cytoplasm, the major proteolytic activity comes from an enzyme called tripeptidyl amino peptidase II (TPPII), which is located on a large particle. It has amino peptidase activity, which can remove 1 to 3 amino acids at a time and is useful for peptides of 15 amino acids or less. It also has endopeptidase activity that can cut in the middle of peptides larger than 15 amino acids, with the release of fragments of at least 9 amino acids. These are a significant source of 9-mers with new carboxyl ends, and this may be the only way to generate carboxyl ends other than the proteasome itself.

The importance of TPPII activity for antigen processing is shown by the fact that a specific inhibitor, butabindide, can prevent peptide loading of MHC, resulting in reduced surface expression. The most likely path for most protein antigens is to enter the proteasome and emerge as peptides of 15 amino acids or larger. These are then trimmed by TPPII, resulting in peptides ready for TAP transport. Longer peptides are trimmed internally by TPPII endoprotease, which may generate carboxyl ends needed for TAP binding. The resulting peptides are then transported by TAP into the ER, where they may be trimmed further to prepare them for MHC binding. An additional role of TPPII is shown by the generation of unique epitopes that are not produced by proteasomes. For example, an important T-cell epitope of the Nef protein of HIV requires TPPII processing. Proteasomal inhibitors have no effect on it, but butabindide prevents its processing and presentation to HLA-A3– or A11-restricted T cells.[289] This pathway seems particularly important for generating epitopes ending in lysine groups, which bind these two MHC types but are rarely generated by proteasomes alone. TPPII can act in parallel with proteasomes or in tandem with them to release this epitope from intact protein or its partially degraded fragments.

Transport into a Cellular Compartment Where Major Histocompatibility Complex Binding Can Occur

The second hurdle a potential epitope must surmount is to be transported into the cellular compartment for loading onto MHC molecules. These compartments are different for class I and II molecules, as noted previously.

Transport Pathways Leading to Major Histocompatibility Complex Class I Presentation. The second hurdle for peptide presentation by class I MHC molecules is to get from the cytosol, where the peptides are produced, to the ER, where the newly synthesized class I MHC molecules are assembled and loaded with peptide. The discovery of a specific active transporter suggested that specificity of transport could further restrict the repertoire of peptides available to load onto class I MHC molecules. Genetic analysis of mutant cell lines that failed to load endogenous peptides onto class I MHC molecules revealed homozygous deletions of part of the MHC class II region near the DR locus. Molecular cloning of DNA from this region revealed at least 12 new genes, of which 2, called TAP-1 and TAP-2 showed a typical sequence for ABC transporter proteins.[290–292] Their function is

to transport processed peptides from the cytosol to the ER. Once in this compartment, peptides are handed off by TAP to newly formed MHC class I molecules and stabilize a trimolecular complex with β2-microglobulin. This complex is then transported to the cell surface, where antigen presentation occurs. Without the peptide transporters, empty dimers of MHC class I with β2-microglobulin form, but these are unstable. Excess free peptide would rescue MHC class I by stabilizing the few short-lived empty complexes that reach the surface, as shown by Townsend et al.[293] and Schumacher et al.[294] Thus, MHC-linked genes coding for proteolysis, peptide transport, and presentation at the cell surface have been identified. In effect, the MHC now appears to encode a complex system of multiple elements devoted to the rapid display of foreign protein determinants on the surface of an infected cell. By continuously sampling the output of the protein synthesizing machinery, this system permits rapid identification and destruction of infected cells by CTL before infectious virus can be released.

In an infected cell, as soon as viral proteins are made, peptide fragments generated by the proteasome become available to the TAP-1 and TAP-2 transporter proteins (Fig. 23.9). These transport the peptide fragments into the ER for association with newly formed MHC class I molecules, which would carry them to the cell surface for antigen presentation, all within 30 minutes. Indeed, the finding of a physical association between TAP and the nascent class I heavy chain/β2-microglobulin complex suggests that the peptide may be directly handed off from TAP to the new MHC molecule without being free in solution.[295,296] If TAP transport is highly selective, then some cytosolic peptides may fail to enter the ER for presentation with class I, but if it is promiscuous, then some peptides may be transported that were better off not presented, such as those leading to autoimmunity.

The idea that other proteins may control accessibility of MHC class I–binding sites for peptides originally came from the observation that two rat strains with the same MHC type (RT1.A^a) were nevertheless not histocompatible, and CTL could recognize the difference between them.[297] The difference, called a cim effect, for class I modification, occurred because different peptides were binding the same MHC in the two strains.[298,299] The rat has two alleles for a peptide transporter supplying peptides to MHC. The one called TAP2A has peptide specificity matching that of RT1.A^a and delivers a broad set of peptides for MHC binding. The other transporter allele, called TAP-2B, supplies a different set of peptides that are discordant with RT1.A^a, so fewer types of peptides are bound. Although RT1.A^a would prefer to bind peptides with Arg at position 9, it has to settle for peptides with hydrophobic termini as provided by TAP-2B,[300] thereby accounting for the apparent histocompatibility difference. Thus, the specificity of TAP transport was shown to provide a selective step in narrowing the potential repertoire of CTL epitopes.

To measure TAP specificity in other species, a transportable peptide bearing an N-linked glycosylation site was added to cells permeabilized by treatment with streptolysin. If the peptide was transported by TAP, it would enter the ER and cis Golgi, where it would be glycosylated.[301–303] The extent of glycosylation served as a measure of TAP function. When competitor peptides were added as well, TAP-mediated transport of the reporter peptide decreased, indicating saturation of peptide-binding sites. In this way, a series of related peptides could be tested for the ability to compete for TAP binding and transport in order to identify the requirements for TAP binding and transport.

TAP binding and transport depended strongly on peptide length.[303,304] Mouse TAP was shown to have a strong preference for peptides of nine residues or longer.[304] For human TAP, peptides shorter than seven amino acids long were not transported, regardless of sequence.[303] Peptides 8 to 11 amino acids long were almost all transported, with some variation in binding affinities depending on sequence.

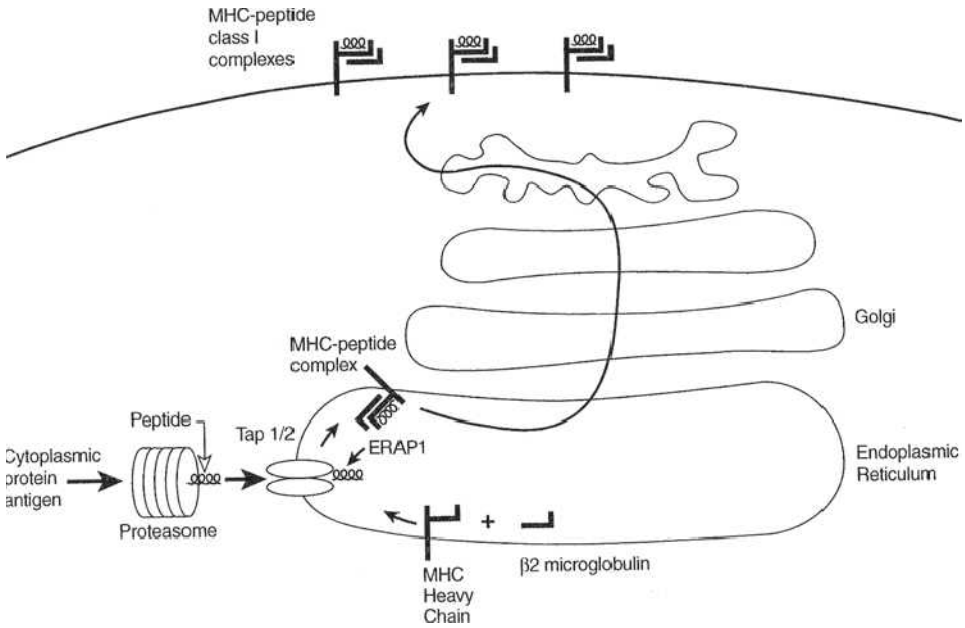


FIG. 23.9. Cytoplasmic Antigen-Processing Pathway leading to Major Histocompatibility Complex (MHC) Class I Presentation. Cytoplasmic antigen is degraded in the proteasome to fragments, which are transported into the endoplasmic reticulum by the transporter associated with antigen presentation (TAP) transporter. Peptide supplied by TAP forms a stable complex with MHC class I heavy chains and β2-microglobulin, and the complex is transported through the Golgi, where it achieves mature glycosylation, and out to the cell surface for presentation to class I–restricted T cells. Additional processing options by tripeptidyl amino peptidase II in the cytoplasm and endoplasmic reticulum aminopeptidase I in the endoplasmic reticulum, before and after TAP transport, allow greater flexibility in meeting the requirements of binding both TAP and MHC molecules.

Peptides 14 to 21 amino acids in length were transported selectively, whereas those longer than 24 amino acids were almost never transported intact. Thus, human TAP transport selected against peptides <7 or >24 amino acids in length, regardless of sequence. Unlike the rat, human and mouse TAP do not show allelic differences in peptide transport.

Although TAP can and must transport a wide variety of peptides, it may still have preferences for which peptides are transported most efficiently and which MHC types are provided with the peptides they need. For example, a self-peptide that naturally binds HLA-B27 was modified slightly to produce an N-linked glycosylation site, resulting in the sequence RRYQNSTEL.[303] Using the glycosylation of this peptide to measure transport, saturation of TAP by homologous peptides occurred with a 50% inhibitory concentration of <1 μM. Other peptides with unrelated sequences also inhibited, often with equally high affinity. Not only did natural HLA-binding peptides compete but also did peptide variants lacking the MHC-binding motif at positions 2 and 9 (see subsequent discussion). Clearly, peptides binding different MHC types were transported by the same TAP protein, and even peptides that bound mouse MHC were transported by human TAP. In another example, using rat TAP proteins, peptides with Pro at position 2, 6, or 9 were found to be poor competitors for transport of a reference peptide.[302]

In a different approach, using a baculovirus system overexpressing TAP proteins in microsomes, the affinity of TAP binding was determined for a wide variety of synthetic peptides, allowing mapping of the important residues.[305,306] Binding, rather than transport, appears to be the major step determining TAP peptide selectivity.[307] Indeed, artificial neural networks have been developed to predict peptide binding to human TAP.[308] Using this scheme, it was found that peptides eluted from three different human class I molecules had higher predicted affinities for TAP than a control set of peptides with equal binding to those class I MHC molecules, supporting the hypothesis that TAP specificity contributes to the selection of the subset of peptides able to bind a class I molecule that actually bind in vivo.[308] Unlike MHC class I, there were no anchor positions at which a specific amino acid was required. However, there were several positions where substituting the wrong amino acid caused a marked reduction in TAP binding. In a typical MHC class I binding 9-mer, the strongest substitution effects were observed at position 9 (P9), followed by substitutions at P2 and P3, followed by P1. At the carboxy-terminal P9, the preferred residues were Tyr and Phe (as well as Arg and Lys), whereas Glu was worst, causing a 3 log reduction in binding. Similarly, substituting Pro at P2 caused a 1.5 to 2 log reduction in binding, as compared to preferred residues Arg, Val, and Ile.

These binding studies were extended to 231 peptides, which were evaluated for binding to both mouse and human TAP proteins.[309] Both TAP proteins showed strong positive selection favoring certain amino acids (Tyr and Phe) at position P9 and strong negative selection against others (Glu, Asp, and Ser) at P9. However, mouse TAP showed greater negative effects from basic amino acids (Arg or Lys) at P9. Both TAPs were sensitive to Pro at P1, P2, and P3, but human TAP was also sensitive to negative effects of amino acids Asp, Glu, and Gly at these positions. In both cases, TAP binding was insensitive to amino acid substitutions at intermediate P4 through P8.

TAP preferences such as these would selectively transport some peptides more than others from cytoplasm to ER. Use of combinatorial peptide libraries independently confirmed that the critical residues influencing TAP transport were the first three N-terminal residues and the last C-terminal residue.[310]

Interestingly, these preferred residues are many of the same ones forming the MHC class I–binding motifs (P2 and P9). However, as the MHC-binding motifs differ from each other, it is not possible for TAP preferences to match them all. For example, the TAP preference for Arg at P2 and Phe, Tyr, Leu, Arg, or Lys at P9 overlaps with the binding motif of HLA-B27 and may favor the transport of peptide ligands for this MHC type. Remarkably, the variant B*2709, which does not prefer Tyr or Phe at P9, is not associated with autoimmune disease as in the more common form of HLA-B27. In contrast, HLA-B7 requires a Pro at P2, which greatly decreases TAP binding. Similarly, some peptides binding HLA-A2 have hydrophobic residues unfavorable for TAP binding, suggesting suboptimal compatibility between TAP and the most common HLA class I allele. Measurements with a series of naturally presented peptides from HLA-A2 and HLA-B27 indicated a mean 300-fold higher affinity of TAP for the HLA-B27 peptides than for those from HLA-A2, and some of the HLA-A2 peptides did not bind TAP at all.[308] How are these low-affinity peptides delivered to MHC? One suggestion is that peptide ligands for HLA-A2 and HLA-B7 may be transported as a larger precursor peptide containing the correct amino acids, which are then trimmed off to fit the MHC groove.

A series of studies support this mechanism, showing that longer peptide precursors are trimmed at the N-terminus by aminopeptidases in the ER to form HLA-binding peptides.[311] The ability to transport larger peptides, followed by trimming, could increase the range of permissible antigenic peptides, by facilitating transport of nonantigenic peptides, followed by trimming to the size and specificity needed for MHC binding. For example, following hepatitis B virus infection, certain peptide epitopes that are frequently recognized by CTL are nevertheless not bound or transported by TAP. By extending these peptides by one or two amino acids of the natural sequence at the amino end, their TAP binding was greatly enhanced, but at the expense of reduced MHC binding. When tested on permeabilized cells, the overall effect of the extended peptides was to increase MHC binding. Apparently, by improving TAP transport, these peptides were able to increase peptide delivery into the ER, where they were trimmed to produce peptides compatible with MHC binding.[312]

Similarly, it is known that peptides with a Pro at P2, needed to bind to certain MHC molecules but poorly transported, are produced from longer precursors that are transported by N-terminal trimming in the ER by aminopeptidases.[313] In fact, the inability of the aminopeptidase to cleave beyond a residue preceding a Pro naturally leads to trimming of peptides to produce ones with a Pro at P2. Alternatively, some of these peptides may derive from signal peptides and enter

the ER in a TAP-independent manner. As HLA-A2 tends to bind hydrophobic peptides, signal peptides, which are usually hydrophobic, may account for some HLA-A2-binding epitopes. The ER trimming enzyme has been identified as endoplasmic reticulum aminopeptidase I (ERAP I).[314–316] This enzyme is a 106 kD zinc metalloproteinase, and it is the major ER protease with broad specificity. It is inducible by interferon-γ and inhibited by the aminopeptidase inhibitor leucinethiol. Downregulation of ERAP I with small interfering ribonucleic acid in decreased expression of MHC class I, indicating that ERAP I contributes to the supply of peptides. ERAP I prefers peptides of 9 or 10 amino acids or longer and ignores peptides of 8 amino acids or fewer, which may allow it to generate antigenic peptides for MHC binding without degrading them beyond recognition.

The aminopeptidase provides a way to relax the requirement for proteasomes to generate ends that can simultaneously bind TAP and MHC. In contrast, the fact that a comparable carboxypeptidase has not been identified suggests that carboxyl ends generated by proteasomal processing must be suitable for TAP transport and compatible with MHC binding. This requirement is quite stringent, as the carboxyl-terminal residue is the most important for TAP binding, and it is frequently an anchor residue for MHC as well.

The significance of selective peptide transport may be to limit immunity to self-peptides. If the match between HLA-B27 and TAP specificity is too good, it may contribute to the increased incidence of autoimmune disease associated with HLA-B27.[305] An effect of human TAP specificity in loading of peptides in viral infection has confirmed the biologic significance of TAP specificity.[312] TAP-binding specificity also limited the repertoire of alloantigenic peptides presented by HLA-B27.[317] Tapasin, which is involved in the TAP-binding process, may also contribute to selective MHC loading contributing to immunodominance by favoring binding of peptides with slow dissociation rates from MHC molecules.[318]

By combining the selectivity of proteasomal processing, TAP transport and MHC binding, it has been possible to generate models that correlate with known antigenic peptides.[319,320] These can be used to analyze the sequence of any given protein and to predict epitopes that may be recognized by T cells restricted to MHC class I. These may be important for analyzing the T-cell response to viral or neoplastic antigens and for generating synthetic vaccines capable of eliciting a T-cell response to these antigens.

The importance of TAP proteins to antiviral immunity is shown by the fact that herpesviruses have targeted TAP-1 function as a way to interfere with antigen presentation to $CD8^+$ CTL. A herpes simplex virus immediate early viral protein called ICP47 binds to TAP and inhibits its function, causing reduced expression of new MHC class I molecules on the cell surface and inability to present viral or other antigens with MHC class I.[321–323] As a way to evade immune surveillance, this strategy could contribute to viral persistence in chronic infection and viral activation in recurrent disease as frequently occurs with herpes simplex virus-1 and herpes simplex virus-2. These findings also raise the possibility of making a live-attenuated ICP47-defective herpes simplex virus vaccine that would be more immunogenic than natural infection.

Transport Pathways Leading to Major Histocompatibility Complex Class II Presentation. Unlike the class I pathway, which delivers peptides to MHC, the MHC class II pathway transports MHC molecules to the endosomal compartment, where antigenic peptides are produced. During transport, the peptide-binding groove must be kept free of endogenous peptides. The cell uses one protein, called invariant chain (and its processed fragment CLIP), to block the binding site until needed, and another protein, HLA-DM, to facilitate release of CLIP peptides and their exchange for antigenic peptides as they become available.

MHC class II molecules assemble in the ER, where α and β chains form a complex with invariant chain.[324–326] Invariant chain binds MHC and blocks the peptide-binding groove, so endogenous peptides transported into the ER, for example by TAP, cannot bind.[325,327–332] The complex of α, β, and invariant chains, consisting of nine polypeptide chains in all,[333] is transported via the Golgi and directed by signals on invariant chain into endosome-/lysosome-like vesicles called MHC class II compartments. The compartments contain acid-activated proteases capable of digesting foreign proteins into antigenic peptides. In addition, they degrade invariant chain to a fragment called CLIP, corresponding to amino acids 80 to 103. As long as CLIP remains in the binding groove, antigenic peptides cannot bind, so the rate of CLIP release limits the capacity of MHC to take up antigenic peptides.

Peptide loading can be measured by its effect on MHC structure. When an MHC class II molecule binds a peptide, it changes conformation, and certain monoclonal antibodies are specific for the peptide-bound conformation.[334] Also, the α-β complex becomes more stable after peptide binding, which can be detected by running the MHC on an SDS gel without boiling. The peptide bound form runs on gels as a large α-β dimer while MHC without peptides (but still bound to CLIP) is unstable under these conditions and falls apart to give α and β chain monomers on SDS gels.[335]

Mutant cell lines have been generated with a deletion between HLA-DP and HLA-DQ genes on chromosome 6.[334,336–338] These cells express normal levels of MHC class II structural proteins, HLA-DQ and HLA-DR, but fail to present protein antigens.[338] Some of their class II MHC proteins appear on the cell surface but more are retained in the MHC class II compartments. Biochemically, they still contain CLIP peptides,[339] rather than peptide antigens, and they have not achieved the conformation[334] or SDS stability of peptide-binding MHC class II complexes.[340] The defect was discovered to be due to loss of either of the two chains of a class II molecule, HLA-DM, and the phenotype can be corrected by adding back the missing gene.[341] In the presence of normal HLA-DM, MHC releases CLIP and binds antigenic peptides for presentation to T cells.

The importance of HLA-DM function for T-cell help in vivo was studied in H2-DM knockout mice.[342] These mice have reduced numbers of T cells, their class II MHC molecules reach the cell surface bearing high levels of CLIP peptide, and their B cells are unable to present certain antigens, such as ovalbumin, to T cells. When H2-DM knockout

mice were immunized with 4-hydroxy-5-nitrophenyl acetyl ovalbumin, specific IgG antibodies were reduced 20-fold, as compared to wild type. Germinal center formation and class switching were greatly reduced, and affinity maturation was not observed. The phenotype was more pronounced for some MHC types, such as I-A^b, than for others, such as I-A^k. Due to tighter binding of CLIP peptides, these MHC types may be more dependent on H2-DM to maintain empty class II molecules in a peptide receptive state.

In vitro studies with purified MHC class II molecules and biotin-labeled peptides have shown that HLA-DM can accelerate loading of exogenous peptides into HLA-DR–binding sites.[343,344] For example, loading of myelin basic protein fragment 90 to 102 was accomplished in 9 minutes with HLA-DM versus 60 minutes without it (Table 23.5). Other peptides were also loaded at the same rate, suggesting that the rate limiting step was the same for each: removal of CLIP peptides to expose the peptide binding sites on HLA-DR. The kinetic effect was optimal between pH 4.5 and 5.8, which is typical of the endosomal/lysosomal compartment where HLA-DM operates. HLA-DM did not affect the affinity, as measured by half-maximal binding, but it had a marked effect on the kinetics of binding.

Conversely, when biotinylated peptides were allowed to saturate HLA-DR–binding sites overnight, and then free peptides were removed, the off rate could be measured over time.[343,344] As shown in Table 23.5 (adapted from Sloan et al.[343]), the off rate for different peptides could be compared in the absence or presence of HLA-DM. The half-life for CLIP peptides was reduced from 11 hours to 20 minutes by adding HLA-DM. This could explain the enhanced loading of all other peptides, as they must wait for CLIP to come off. In the case of antigenic peptides, myelin basic protein 90 to 112 was released 80-fold faster in the presence of DM than in its absence. However, another peptide, influenza hemagglutinin 307 to 319, was not affected at all. The differential effect on these antigenic peptides suggests that HLA-DM can serve a potential role in editing which peptides stay on MHC long enough to be presented and which are removed.[343] By releasing myelin basic protein preferentially and not the hemagglutinin peptide, HLA-DM would favor the stable MHC binding and presentation of hemagglutinin peptides over myelin basic protein peptides. The affinity of each peptide is determined by the fit between peptide and MHC groove, not by HLA-DM. However, DM can amplify the impact of the difference in affinity (ie, signal to noise ratio) by facilitating release of low-affinity peptides and allowing the high-affinity ones to remain. This editing function could have an important effect on which peptides get presented and elicit a T-cell response. HLA-DM could contribute to immunodominance of a peptide binding MHC with high affinity by releasing its lower affinity competitors. Alternatively, HLA-DM could contribute to self-tolerance by releasing self-peptides of low affinity before they could stimulate self-reactive T cells either at the time of positive selection (so they would fail to be positively selected) or in the periphery (so they would fail to be activated). When HLA-DM was engineered to be expressed on the cell surface, it also facilitated loading of exogenous peptides onto class II MHC molecules and affected the activation of T cells of different fine specificity for the same epitope.[345]

TABLE 23.5 Effect of Human Leukocyte Antigen-DM on Peptide on Rates and Off Rates for Binding to Human Leukocyte Antigen-DR1

Peptide	HLA-DM	Half-Time for Binding	Half-life for Release
CLIP (80–103)	–	60 min	11 hr
	+	9 min	0.3 hr
MBP (90–102)	–	62 min	86 hr
	+	9 min	1 hr
HA (307–319)	–	67 min	144 hr
	+	10 min	144 hr

CLIP, class II–associated invariant chain peptide; HA, hemagglutinin; HLA, human leukocyte antigen; MBP, myelin basic protein.
The on (association) and off (dissociation) rates of biotinylated peptide from purified soluble HLA-DR1 were measured by fluorescence assay, in the presence or absence of HLA-DM. The on rates of all three peptides are increased similarly in the presence of HLA-DM and probably reflect the rate-limiting dissociation of the bound CLIP fragment of the invariant chain. In contrast, once the peptides are bound, the off rates differ as a result of differences in affinity. Thus, HLA-DM catalyzes release of more weakly binding peptides and allows stable binding of higher affinity peptides. In effect, this is an editing function of HLA-DM.
Adapted from the data of Sloan et al.[298]

Major Histocompatibility Complex Binding and Assembly of a Stable Peptide-Major Histocompatibility Complex

Antigen Interaction with Major Histocompatibility Complex Molecules. Perhaps the most selective step a potential antigenic site must pass is to bind with sufficiently high affinity to an appropriate MHC molecule.

The response specificity of T cells to antigens on APCs or target cells provides a number of hints that antigen interacts directly with MHC molecules of the APCs to form an antigenic complex recognized by T cells. First, genes coding for immune responsiveness (Ir genes) to a specific antigen are tightly linked to genes for MHC-encoded cell surface molecules.[127,171] Second, it became apparent that T-cell recognition of antigen is the step at which MHC restriction occurs.[127,179,219,346] For example, in vitro T-cell responses to small protein and polypeptide antigens were found to parallel in vivo responses controlled by Ir genes, and T cells were exquisitely sensitive to differences in MHC antigens of the APCs in all their antigen recognition functions. This observation in vitro made it possible to separate the MHC of the T cell from that of the APC. The T-cell response to antigenic determinants on each chain of insulin depended on the MHC antigens of the APC. This was particularly apparent when T cells from an (A × B)F1 animal responded to antigen presented by APCs of either the A or B parental MHC type.[219,347] Neither parental APC stimulated an allogeneic response from (A × B)F1 T cells, and the response to antigen was now limited by the MHC of the APCs. This ability of the APCs to limit what could be presented to the T cells was termed "determinant selection."[219,347] It became obvious that even in a single (A × B)F1 animal, distinct sets of antigen-specific T cells exist that respond to each antigenic determinant only in association with MHC type A or B.[348]

Experiments on the fine specificity of antigen-specific T-cell clones suggested that the MHC of the APCs could influence the T-cell response in more subtle ways than just allowing or inhibiting it. Determinant selection implied that a given processed peptide should contain both a site for MHC interaction and a distinct functional site for TCR binding. Thus, a protein with multiple determinants could be processed into different peptides, each with a different MHC restriction, consistent with the independent Ir gene control of the response to each antigenic determinant on the same protein.[181] For example, T-cell clones specific for myoglobin responded to different antigenic determinants on different peptide fragments of myoglobin[215]: Those specific for one of the epitopes were always restricted to I-A, whereas those specific for the other were always restricted to I-E. The simplest interpretation was that each antigenic peptide contained an MHC association site for interacting with I-A or I-E. At the level of Ir genes, mouse strains lacking a functional I-E molecule could respond to one of the sites only, and those with neither I-A nor I-E molecules capable of binding to any myoglobin peptide would be low responders to myoglobin.

Evidence for a discrete MHC association site on peptide antigens came from studies with pigeon cytochrome c. The murine T-cell response to pigeon cytochrome c and its carboxy-terminal peptide (81 to 104) depends on the I-E molecules of the APCs.[179] However, distinct structural sites on the synthetic peptide antigen appear to constitute two functional sites: an epitope site for binding to the TCR and an "agretope" (for "antigen restriction tope") site for interacting with the MHC molecule of the APC.[179,196–198] Amino acid substitutions for Lys at position 99 on the peptide destroyed the ability to stimulate T-cell clones specific for the peptide, whereas the difference between Ala and a deletion at position 103 determined T-cell stimulation in association with some MHC antigens but not others, independent of the T-cell fine specificity. In addition, immunizing with the peptides substituted at position 99 elicited new T-cell clones that responded to the substituted peptide but not the original and showed the same pattern of genetic restriction, correlated with the residue at position 103, as the clones specific for the original peptide. These results implied that the substitutions at position 99 had not affected the MHC association site but independently altered the epitope site that interacts directly with the TCR. In contrast, position 103 was a likely subsite for MHC interaction, without altering the TCR-binding site.

It remained to be shown that MHC molecules without any other cell surface protein were sufficient for presentation of processed peptide antigens. This was demonstrated by Watts et al.,[349] who showed that glass slides coated with lipid containing purified I-A molecules could present an ovalbumin peptide to an ovalbumin-specific T-cell hybridoma. This result meant that no other special steps were required other than antigen processing and MHC association. Likewise, Walden et al.[350] specifically stimulated T-cell hybridomas with liposomes containing nothing but antigen and MHC molecules. Also, Norcross et al.[351] transformed mouse L cells with the genes for the I-A α and β chains and converted the fibroblasts (which do not express their own class II molecules) into I-A–expressing cells. These cells were able to present several antigens to I-A–restricted T-cell clones and hybridomas[351] and similar I-E transfectants presented to I-E–restricted T cells.[215] Thus, whatever processing enzymes are required are already present in fibroblasts, and the only additional requirement for antigen-presenting function is the expression of I-A or I-E antigens.

The planar membrane technique has been applied to determine the minimum number of MHC-antigen complexes per APC necessary to induce T-cell activation.[352] After pulsing the presenting cells with antigen, the cells were studied for antigen-presenting activity, and some of the cells were lysed to produce a purified fraction containing MHC charged with antigenic peptides. These MHC-peptide complexes were used to reconstitute planar membranes, and their potency was compared to a reference MHC preparation pulsed with a high peptide concentration in vitro and presumed to be fully loaded. In this way, the relative peptide occupancy of MHC-binding sites corresponding to any level of antigen presentation could be determined. For B cells and macrophages, the threshold of antigen loading necessary for triggering T cells was 0.2% of $I\text{-}E^d$ molecules occupied by peptide, corresponding to about 200 MHC-peptide complexes per presenting cell. For artificial presenting cells, such as L cells transfected with $I\text{-}E^d$, the threshold was 23 times greater or 4.6% of MHC occupied by peptide. Similarly, when MHC-peptide binding was measured directly, using radiolabeled peptide to determine the minimum level of MHC-peptide complexes required for T-cell triggering, B cells were capable of presenting antigen with as few as 200 to 300 MHC-peptide complexes per cell.[353] A similar number of peptide-MHC class I molecule complexes was reported to be required on a cell for recognition by $CD8^+$ cytotoxic T cells.[354] These results explain how newly generated peptide antigens can bind enough MHC molecules to stimulate a T-cell response, even in the presence of competing cellular antigens, as a low level of MHC occupancy is sufficient. In addition, this threshold of presentation may explain how multivalent protein antigens, such as viral particles, with 100 to 200 protein copies each, can be over 10^3-fold more immunogenic than the same weight of protein monomers.[355–357] Studies on the number of TCRs needed for triggering, based on titrating peptide and recombinant soluble class I MHC molecules on plastic, suggested that interaction of three to five TCRs with MHC-peptide complexes was sufficient, consistent with several T cells interacting with one APC.[358,359] Biochemical evidence for the direct association between processed peptide and MHC molecules was demonstrated by competition between peptides for antigen presentation[225,360–363] and then more directly by equilibrium dialysis,[364] molecular sieve chromatography,[365] or affinity labeling.[366] Equilibrium dialysis (see Chapter 7) was performed by incubating detergent-solubilized class II molecules with fluoresceinated or radioactive antigenic peptides, followed by dialysis against a large volume of buffer. Peptide can pass in or out of the dialysis bag, but the class II molecules are trapped inside. In the absence of binding by class II molecules, the labeled peptide would distribute itself equally between the inside and outside of the dialysis

TABLE 23.6 Correlation between Major Histocompatibility Complex Restriction and Binding to Major Histocompatibility Complex Molecules

Competitor Peptide		Ova + A^d	Myo + E^d	HEL + A^k	Cyto + E^k
Ova	323–339	++++	–	++	+
Myo	106–118	++++	–	++	+/–
Myo	132–153	–	++++	–	++
HEL	46–61	+	+	++++	+
Cytochrome c	88–104	++	+/–	++	++++

HEL, hen egg lysozyme.
Data from Früh et al.[322]

chamber. However, when the appropriate class II molecules were added to the chamber, extra peptide molecules were retained inside it due to formation of a complex with MHC class II. In this way, direct binding of antigen and MHC was shown, and an affinity constant was determined.[364,365]

A second approach was to form the antigen-MHC complex over 48 hours, followed by rapid passage over a Sephadex G50 sizing column (Sigma-Aldrich). The bound peptide came off the column early because it is the size of class II molecules (about 58 kD), while free peptide was usually included in the column and eluted later, as it is only approximately 2 kD.[365] Peptide bound to specific and saturable sites on MHC. Competitive binding showed that different peptide antigens with the same MHC restriction bind to the same site on the MHC class II molecule.[367,368] For example, Table 23.6 shows the results with peptide antigens that are known to be presented with I-A or I-E antigens of the D or K haplotype. We observe that Ova peptide 323 to 329, which is presented with I-A^d, also binds well to purified I-A^d, whereas nonradioactive peptide competes for the peptide-binding sites of the I-A^d molecule. Similarly, the other I-A^d–restricted peptide, myoglobin 106 to 118, competes with Ova 323 to 339 for the same site. However, myoglobin 132 to 153, which is not restricted to I-A^d, does not compete for it but does compete for its own restriction element, I-E^d. Similarly, pigeon cytochrome c competes best for its restriction element I-E^k rather than I-A^k or I-E^d, which do not present cytochrome. Conversely, recombinant Eβ genes have been used to map separate sites on a class II MHC molecule for binding to peptide antigen and to the TCR.[369]

Using these two biochemical methods, it has been possible to explain major losses of peptide antigenicity resulting from amino acid substitutions in terms of their adverse effect on epitope or agretope function. For example, the response of each of two ovalbumin-specific T-cell clones was mapped to peptide 325 to 335 by using a nested set of synthetic peptides. Five substitutions were made for each amino acid in the peptide, and the resulting 55 different peptides were each tested for the ability to stimulate the clone.[370] Presumably, those peptides that failed to stimulate could be defective at an epitope or an agretope functional site. In fact, only two amino acids (Val 327 and Ala 332) were essential for MHC interaction, and changes at either of these resulted in a loss of antigenicity for the clone. Seven other amino acids were critical for T-cell stimulation but did not affect MHC binding. Thus, these must be part of the functional epitope. Interestingly, certain substitutions for His 328, Ala 330, and Glu 333 had effects on MHC binding, whereas others had effects on T-cell stimulation without affecting MHC binding. These amino acids might participate in both agretope and epitope functional sites, or, alternatively, the substitutions may affect the conformation of the peptide as it binds, thus indirectly affecting T-cell recognition[371] (see following discussion). The fact that substitutions at 9 of 11 amino acids could be tolerated without affecting MHC binding is consistent with the determinant selection hypothesis in that multiple antigenic peptides are capable of interacting with the same antigen-binding site on the MHC molecule.

Similarly, by using a T-cell clone specific for peptide 52 to 61 of hen egg lysozyme, substitutions at each amino acid were analyzed for the ability to bind to I-A^k and stimulate the clone.[372] Four of eleven amino acid residues were silent, whereas substitutions at three positions resulted in reduced binding to I-A^k. Substitutions at the remaining three positions resulted in decreased T-cell stimulation without affecting MHC association. The epitope was very sensitive to substitutions, even conservative ones such as changing Leu 56 to Ile, norLeu, or Val. The results in both of these studies confirmed by competitive binding that the MHC molecule contains a single saturable site for peptide binding. This site must be capable of binding a broad range of antigenic peptides. In binding the MHC groove, antigenic peptides assume the extended conformation that exposes the epitope for recognition by the TCR.

Although a full set of general principles explaining the specificity of antigen presentation and T-cell recognition has not yet emerged, it is studies such as these, combined with complementary structural studies characterizing the antigen-interacting portions of MHC molecules[214,253,254,369,373–380] (see Chapters 21 and 22) and of TCRs[381–387] (see Chapter 11) that will ultimately lead to an understanding of these principles.

One observation that came out of this type of structure-function study was that a single peptide can bind to a class II MHC molecule in more than one way, and thus be seen by different T cells in different orientations or conformations.[371,388] The same conclusion can be reached from an entirely different type of study, in which mutations are introduced into the MHC molecule. Mutations in the floor of the peptide-binding groove, which cannot directly interact with the TCR, can differentially affect recognition of a peptide by one clone and not another.[389–391] In a particularly thoroughly studied case, it was clear that the quantitative level of peptide binding was not affected by the mutation, but rather the change in the floor of the groove imposed an altered conformation on the peptide that differentially affected recognition by different T cells.[391] If indeed the TCR cannot detect the mutation in the MHC molecule except indirectly by its effect on the peptide conformation, then

one is forced to conclude that different T cells have preferences for different conformations of the same peptide bound to (what appears to the T cell as) the same MHC molecule.

Another general observation to come from this type of study is that substitution of amino acids often affects presentation by MHC and recognition by T cells through introduction of dominant negative interactions or interfering groups, whereas only a few residues are actually essential for peptide binding.[392] Both for class II binding[392–395] and for class I MHC binding,[396] most residues can be replaced with Ala or sometimes Pro without losing MHC binding, as long as a few critical residues are retained. Of course, T-cell recognition may require retention of other residues. If many of the amino acid side chains are not necessary for binding to the MHC molecule, then one might expect side chains of noncritical amino acids to occasionally interfere with binding, either directly or through an effect on conformation. That is exactly what was observed for a helper epitope from the HIV-1 envelope protein when a heteroclitic peptide—that is, one that stimulated the T cells at much lower concentrations than did the wild-type peptide—was obtained by replacing a negatively charged Glu with Ala or with Gln, which has the same size but no charge.[392] An Asp, negatively charged but smaller, behaved like the Glu. Thus, this residue was not necessary for binding to the class II MHC molecule, but a negatively charged side chain interfered with binding to the MHC molecule as measured by competition studies. Information about residues that interfere with binding has allowed the refinement of sequence motifs for peptides binding to MHC molecules to permit more reliable prediction of binding[397] (see following discussion).

This observation also provides a novel approach to make more potent vaccines by "epitope enhancement," the process of modifying the internal sequence of epitopes to make them more potent, for example, by increasing affinity for an MHC molecule or TCR, or able to induce more broadly cross-reactive T cells specific for multiple strains of a virus.[398–401] Proof of principle that this approach can make more potent peptide vaccines has recently been obtained.[401] The modified "enhanced" helper T-cell epitope from the HIV-1 envelope protein described previously,[392] with Ala substituted for Glu, was shown to be immunogenic at 10- to 100-fold lower doses for in vivo immunization than the wild-type HIV-1 peptide to induce a T-cell proliferative response specific for the wild-type peptide. Further, when a peptide vaccine construct using this helper epitope coupled to a CTL epitope[402,403] was modified with the same Glu-to-Ala substitution, it was more potent at inducing $CD8^+$ CTL specific for the CTL epitope than was the original vaccine construct, even though the CTL epitope was unchanged (Fig. 23.10).[401] The increased potency of the vaccine construct was shown to be due to improved class II MHC–restricted help by genetic mapping using congenic strains of mice expressing the same class I MHC molecule to present the CTL epitope and the same background genes but differing in class II MHC molecules.[401] Thus, class II–restricted help makes an enormous difference in induction of class I–restricted CTL, and epitope enhancement can allow construction of more potent vaccines, providing greater protection against viral infection.[404] Further, the improved help was found to be qualitatively, not just quantitatively, different, skewed more toward Th1 cytokines.[404] The mechanism was found to involve greater induction of CD40 ligand on the helper T cells, resulting in greater interleukin 12 production by the antigen-presenting dendritic cells, which in turn polarized the helper cells toward Th1 phenotype.[404] The dendritic cells conditioned with the helper T cells and the higher affinity peptide and then purified were also more effective at activating $CD8^+$ CTL precursors in the absence of helper cells, supporting a mechanism of help mediated

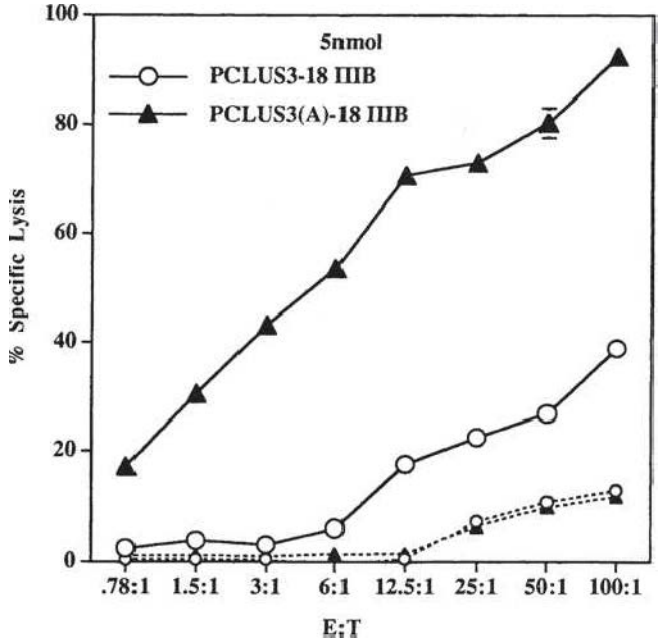


FIG. 23.10. Enhancement of Immunogenicity of a Peptide Vaccine for Induction of Class I Major Histocompatibility Complex (MHC)-Restricted Cytotoxic T-Lymphocytes by Modification of the Class II MHC-Binding Portion to Increase Cluster of Differentiation (CD)4+ T-Cell Help. Peptide vaccine PCLUS3-18 IIIB contains a class II MHC binding helper region, consisting of a cluster of overlapping determinants from the human immunodeficiency virsu-1 envelope protein gp160, and a class I MHC-binding cytolytic T cell (CTL) epitope, P18 IIIB. Modification of the helper epitope to remove an adverse negative charge by replacement of a Glu with an Ala residue was shown to increase binding to the class II MHC molecule.[392] Here, introduction of the same modification of the helper epitope, to produce PCLUS3(A)-18IIIB, is shown to greatly increase immunogenicity in vivo for induction of CTL to the class I-binding P18IIIB portion. Immunization of A.AL mice with 5 nmol of either vaccine construct subcutaneously in montanide ISA 51 adjuvant, and stimulation of resulting spleen cells with P18IIIB for 1 week in culture, resulted in 33-fold more lytic units for lysing targets coated with P18IIIB when mice were immunized with the modified second-generation vaccine than when they were immunized with the original construct with the natural sequence. Thus, class II–restricted $CD4^+$ T-cell help has a major impact on induction of class I–restricted CTL, and this process of "epitope enhancement" can be used to make vaccines more potent than the natural viral antigens. Targets: BALB/c 3T3 fibroblasts with P18IIIB (*solid lines*) or no peptide (*dashed lines*). (Modified from Ahlers et al.[401] with permission.)

through activation of dendritic cells.[405–407] This study showed also that such help was mediated primarily through upregulation of interleukin 12 production and CD80 and CD86 expression on the dendritic cell.[404] A similar or overlapping HIV envelope epitope recognized by human $CD4^+$ T cells when presented by HLA-DR13 has been mapped and has undergone epitope enhancement as well.[408] Understanding this mechanism of epitope enhancement may contribute to the design of improved vaccines.

Similar epitope enhancement has been carried out for class I MHC–binding viral or tumor peptides as well,[409–414] and in one case has been found to result in greater clinical efficacy of a melanoma vaccine used for human immunotherapy.[415] Cautions have also been raised about the use of enhanced epitopes (sometimes also called agonistic or heteroclitic epitopes), in that the amino acid sequence modifications that affect MHC binding can also alter the conformation of the MHC-bound peptide and affect the specificity of T cells elicited.[416–419] The altered or enhanced peptides that elicit the most T cells specific for the wild-type epitope are the most effective at clearing tumors.[420] Thus, it is important in using epitope-enhanced peptides that the peptides still activate a large number of T cells that recognize the wild-type sequence. These results emphasize the importance of affinity for MHC molecules vaccine efficacy[421] and suggest that rational design of vaccines with higher affinity epitopes may produce more effective second-generation vaccines.[422,423] To that end, the discovery of sequence motifs predicting MHC molecule binding and the development of bioinformatics strategies to predict peptide affinity have proven a great impetus to the field.[424]

In the case of class I MHC molecules, results defining sequence binding motifs generalize the conclusion that only a few critical "anchor" residues determine the specificity of binding to the MHC molecule (Table 23.7).[264,396,425–429] These motifs were defined by a detailed study of one peptide-MHC system[427] by sequencing the mixture of natural peptides eluted from a class I MHC molecule and finding that at

TABLE 23.7 Examples of Motifs for Peptides Binding to Class I and II Major Histocompatibility Complex Molecules

	Residue Number								
MHC Molecule	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Class I									
H-2K^d		Y							I, L, V
H-2D^b					N				M, I
H-2K^b					F, Y			L, M	
H-2L^d		P			(hydrophilic K, R)				M, L, F
H-2D^d		G	P		K, R				L
H-2K^k		E						I	
HLA-A2.1		L, M							V
HLA-A3		L	(F)						Y, K
HLA-B27	K, R G	R	I, Y, F, W						K, R
Class II									
DRB1*0101	Y, V, L, F, I, A			L, A		A, G			L, A
DRB1*0301	L, I F, M V			D		K, R E, Q			Y, L F
DRB1*0401 (DR4Dw4)	F, Y W			no R, K		N, S T, Q Aliphatic	Polar Charged		Polar Aliphatic K
DRB1*0402 (DR4Dw10)	V, I, L, M		no	D, E		N, Q, S, T K	R, K H, N Q, P		Polar Aliphatic H
DRB1*1501 (DR2b)	L, V, I			F, Y I			I, L V, M F		
DQA1*0501 DQB1*0301	F, Y, I, M L, V				V, L I, M Y		Y, F M, L, V, I		

HLA, human leukocyte antigen; MHC, major histocompatibility complex.
Data from Goodman and Sercarz,[234] Walden et al.,[350] Sette et al.,[370] Kurata and Berzofsky,[371] Allen et al.,[372] Brown et al.,[373] and Bjorkman et al.[374]

certain positions in the sequence, a single residue predominated within the pool of peptides,[425] and by separating and sequencing individual natural peptides eluted from a class I molecule and finding a conserved residue at certain positions.[426] The latter two studies also made the important observation that the natural peptides eluted from class I MHC molecules were all about the same length, eight or nine residues, and this was confirmed for a much larger collection of peptides eluted from HLA-A2 and analyzed by tandem mass spectrometry.[428] This finding was consistent with other studies demonstrating that a minimal nonapeptide was many orders of magnitude more potent than longer peptides in presentation by class I molecules to T cells.[430,431] This conservation of length was critical to the success of the approach of sequencing mixtures of peptides eluted from a class I molecule[425] because such a method requires that the conserved anchor residues all be at the same distance from the N-terminus. The fact that Falk et al.[425] could find a single amino acid at certain positions, such as a Tyr at position 2 in peptides eluted from K^d, implies not only that most or all of the peptides bound to K^d had a Tyr that could be aligned but also that the peptides were already aligned as bound to the MHC molecule, with each one having just one residue N-terminal to the Tyr. This result implies that the position of the N-terminal residue is fixed in the MHC molecule. It is this fact that has made the identification of motifs for binding to class I molecules much more straightforward than finding motifs for binding class II molecules.

This conclusion has not only been confirmed but also explained by x-ray crystallographic data on class I peptide–MHC complexes.[376–378] It appears that both the N-terminal α-amino group and the C-terminal carboxyl group are fixed in pockets at either end of the MHC groove, independent of what amino acids are occupying those positions, and that the rest of the peptide spans these fixed points in a more or less extended conformation. The minimum length that can span the distance between these pockets is 8 residues, but 9 or 10 residues can be accommodated with a slight bulge or β turn in the middle of the peptide, explaining the narrow restriction on length. Between these ends, one or two pockets in the groove can accommodate the side chain of an amino acid, usually either at P2 binding in the B pocket or at P5 binding in the C pocket, depending on the particular MHC molecule. Additionally, the side chain of the C-terminal residue serves as an anchor in the F pocket at the end of the groove. These residues that fit into pockets correspond exactly to the "anchor" residues, at P2 or P5, and P8, P9, or P10, defined by the sequence motifs, and appear to be the primary determinants of specificity for peptide binding because the rest of the interactions are largely with peptide backbone atoms, including the α-amino and carboxyl groups, and therefore do not contribute to sequence specificity. This finding can explain both the breadth of peptides that can bind to a single MHC molecule because most of the binding involves only backbone atoms common to all peptides and also the exquisite specificity of binding is determined by the anchor residues that account for the Ir gene control of responsiveness.

In contrast, when natural self-peptides were eluted from class II MHC molecules,[252,432] the lengths were much more variable, ranging from 13 to 18 residues, and several variants of the same peptide were found with different lengths of extra sequence at one end or the other ("ragged ends"). This finding suggested that both ends of the peptide-binding groove of class II MHC molecules are open, in contrast to class I, so that additional lengths of peptide can hang out either side, and trimming does not have to be precise. However, a corollary is that the peptides eluted from class II molecules would not be aligned in a motif starting from the exact amino terminus and that was indeed what was found. Although a moderately conserved motif was found in some of the peptides eluted from the murine class II molecule $I\text{-}A^d$, consistent with the motif defined based on known antigenic peptides binding to $I\text{-}A^d$,[433] the motif was neither so clearly defined nor so highly conserved as in the class I case and required aligning of sequences to identify a core motif of about nine amino acid residues.[432] Subsequently, a number of motifs for peptides binding to human class II MHC molecules have been defined.[264,434–439] Unlike peptides eluted from MHC class I grooves, these class II–binding peptides may locate the core-binding motif at various distances from the amino or carboxyl end of the peptide.

The crystal structure of a peptide bound to a human class II MHC molecule, DR1, revealed that indeed the ends of the groove are open, and the peptide can extend beyond the groove in either direction.[254,440] In addition, the more broadly defined class II motifs in Table 23.7 can be explained by less stringent requirements for amino acid side chains to interact with binding pockets in class II. In general, the MHC class II binding pockets are shallower than for class I, and a selected peptide derives less binding energy from each pocket. In fact, they form fewer H-bonds with the peptide side chains, and more H-bonds are directed at the peptide backbone, allowing a variety of different peptides to bind. Rather than requiring a specific amino acid at each position, the shallow binding pockets of MHC class II tend to exclude peptides based on unfavorable interactions, such as side chains too large to fit the binding pocket. Even one amino acid side chain that binds strongly to an MHC pocket is sufficient to anchor the peptide to MHC class II and set the frame for the interaction of the rest of the peptide with the MHC groove.

For example, binding of three peptides to the class II molecules I-A in mice or HLA-DQ in humans are shown in Figure 23.11. The first residue of the peptide motif is designated P1, the next is P2, and so on. The α-helical walls and β-sheet floor of the MHC class II groove (see Chapter 21) are peeled away to reveal the peptide backbone and side chains in relation to MHC-binding pockets. For the ovalbumin peptide $Ova_{323\text{-}329}$ binding to $I\text{-}A^d$, residues P1, P4, and P9 all point down into the binding pockets.[441] The best fit is between Val 327 and the P4 pocket, which creates mainly hydrophobic interactions with MHC and serves as the anchor residue. Residues P5 (His 328) and P8 (His 331) project upward for binding to the TCR. The shallow P4 pocket can tolerate only small hydrophobic side chains, such as Val, so it dictates which peptides can bind here. The other MHC

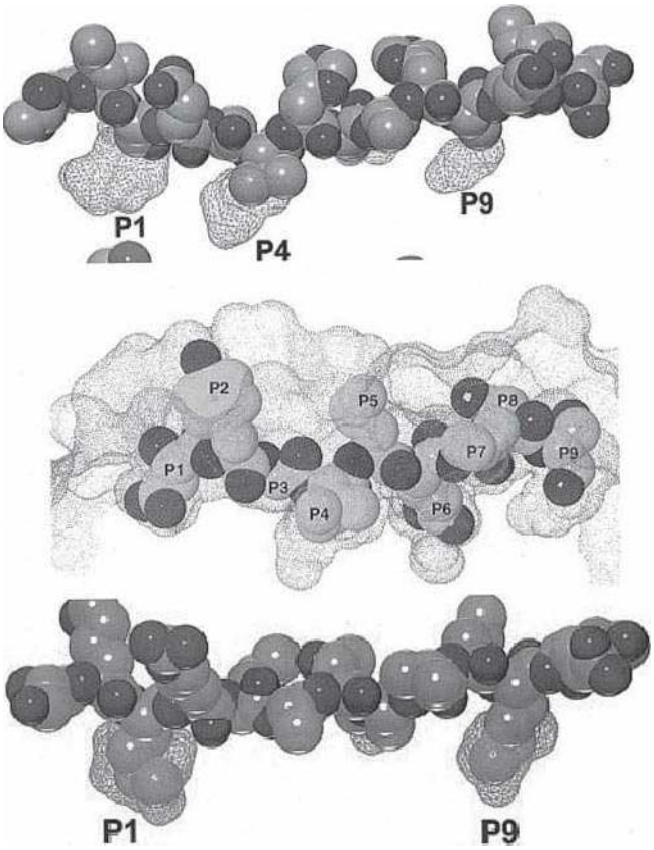


FIG. 23.11. Interaction of Peptides with the Binding Groove of Major Histocompatibility Complex Class II, as Determined by X-ray Crystallography. Anchor residue side chains fill binding pockets to a greater or lesser extent, supplemented by H-bonds to the peptide backbone. Examples include I-A^d with ovalbumin peptide 323 to 334 *(Top)*,[441] I-A^k with hen egg lysozyme 52 to 60 *(Middle)*,[442] and HLA-DR3 with invariant chain class II–associated invariant chain peptide *(Bottom)*.[443]

pockets, P1 and P9, accommodate many different residues, so they have little effect on which peptides can be presented by I-A^d.

For hen egg lysozyme $peptide_{50-62}$ binding to I-A^k, interactions with MHC are observed for P1, P4, P6, and P9.[442] The P1 interaction is very different from I-A^d, as the P1 pocket is a perfect fit for Asp and has an arginine at the end of the tunnel to neutralize charge. This structure explains why nearly all peptides presented by I-A^k must have Asp at this position. In contrast, the P4 and P9 pockets are partially filled and will tolerate a number of different side chains at these positions. The P6 pocket requires a Glu or Gln here, even though the MHC residues deep in the pocket are acidic. It is presumed that one of the Glu residues must be protonated to allow Glu binding at this position. This arrangement of the peptide leaves P2, P5, and P8 exposed to solvent in the crystal structure and to the TCR during antigen presentation.

For the CLIP peptide binding to HLA-DR3, deep pockets at P1 and P9 are more fully occupied by the peptide side chains.[443] The pH-dependent binding is important because CLIP must be stable at neutral pH and unstable at acid pH in the presence of HLA-DM in order to perform its function.

Based on affinity for MHC, these interactions explain the peptide-binding motifs for MHC class II that select which peptides can be presented to T cells. In addition, these interactions orient the peptide in the MHC groove and determine which residues are accessible for recognition by the TCR.

T-Cell Receptor Recognition

The last hurdle that a potential antigenic determinant must surmount is recognition by a TCR within the repertoire of the individual responding. This repertoire may be limited by the availability of combinations of V, D, and J genes (see Chapter 11) in the genome that can combine to form an appropriate receptor, given the lack of somatic hypermutation in TCRs in contrast to antibodies,[383,444] and then by self-tolerance, as mediated by thymic or peripheral negative selection, or by limits on the repertoire that is positively selected in the thymus on existing self-peptide–MHC complexes. The available repertoire may also be influenced by prior exposure to cross-reactive antigens. In general, however, it has been hard to find holes in the repertoire.[445] Indeed, studies examining large panels of peptides binding to particular class I molecules for correlations between peptide affinity for MHC and T-cell responses have failed to find high-affinity peptides for which no T-cell response can be raised, whereas not all lower affinity peptides elicit a response.[446,447] These results suggest that if the peptide can bind well enough to the MHC molecule, there is virtually always a T cell that can see the complex. However, the array of other MHC molecules present can influence the breadth of the T-cell repertoire.[448] Furthermore, when TCR repertoires of mice and humans were compared for peptides presented by HLA-A2.1, they seemed to be capable of seeing the same spectrum of peptides.[449] Eleven peptides from hepatitis C virus proteins, each of which had a motif for binding to HLA-A2.1, were tested for recognition by CTL from HLA-A2.1 transgenic mice and human HLA-A2.1 positive patients infected with hepatitis C virus. The same four peptides that were recognized by the T cells from the mice were the ones recognized by the human T cells, whereas the others were not recognized well by either murine or human T cells. The selection of which peptides were recognized seemed to be determined by binding to the HLA-A2.1 molecule rather than by the availability of T cells. Thus, despite the differences in TCR genes in mice and humans, the repertoires are plastic enough that if a peptide passes the other three hurdles of processing, transport, and binding to MHC molecules, T cells can be elicited to respond to it in either species.[449] Likewise, no hole in the helper T-cell repertoire could be found to explain low responders to the recombinant hepatitis B vaccine.[450]

On the other hand, evidence exists that MHC binding is not the whole story. Schaeffer et al.[451] examined 14 overlapping peptides covering the sequence of staphylococcal nuclease with different class II MHC molecules, constituting 54 different peptide-MHC combinations. Clearly, MHC binding plays a major role because 12 out of 13 immunogenic peptides were high or intermediate binders to MHC molecules, whereas only 1 of 37 poor binders were immunogenic. Of high-affinity binders, five out of five peptides

were immunogenic. However, for intermediate affinity MHC-binding peptides, only 7 out of 12 were immunogenic. Similar results were found for the class I MHC molecule HLA-B*0702, in which it was found that 7 of 7 high-affinity binders but only 9 out of 12 intermediate affinity binders, were immunogenic.[447] Thus, MHC binding alone is not sufficient to ensure immunogenicity. The T-cell repertoire was one factor suggested that might limit the spectrum of immunogenic peptides.

Indeed, examples for selection at the level of the TCR repertoire exist. A particularly elegant example described by Moudgil et al.[452] is one in which a peptide (46 to 61) of mouse lysozyme presented by I-A^k is recognized T cells from CBA/J and B10.A mice, expressing I-A^k and I-E^k, but not by T cells from B10.A(4R) mice expressing only I-A^k, even though the APC from B10.A(4R) mice can present the peptide to T cells from the other strains. T cells from the B10.A(4R) mice can respond to variant 46 to 61 peptides in which the C-terminal Arg is replaced by Ala, Leu, Phe, Asn, or Lys, indicating that the C-terminal Arg is hindering recognition, but not binding by I-A^k, and in this case, not processing as the B10.A(4R) APC can present the peptide. It appears that the hindrance interferes with recognition by TCRs available in B10.A(4R) mice, but not TCRs available in B10.A or CBA/J mice, or in (B10.A(4R) × CBA/J)F1 mice. Because the B10.A mice are congenic with the B10.A(4R) mice, the difference is not one of non–MHC-linked genes such as TCR structural genes or non-MHC self-antigens producing self-tolerance. Further, because the F1 mice respond, the difference is not due to a hole in the repertoire produced by a self-antigen of the B10.A(4R) mice. It was concluded that the CBA/J and B10.A mice contain an additional repertoire, positively selected on I-E^k or possibly an H-2D/L class I molecule, in which these strains differ, which can recognize the 46 to 61 peptide despite the hindering Arg at the C-terminus. An alternative related explanation is that strains that express I-E^k or D^k or D^d/L^d have an additional repertoire of TCRs positively selected on I-A^k–presenting self-peptides from processing of these other MHC molecules in the thymus. This example illustrates a case in point that subtle differences in TCRs, presumably caused in this case by positive selection, can lead to responsiveness or nonresponsiveness to a determinant that has already passed all of the three earlier hurdles, processing, transport, and MHC binding. Of course, there are some holes related to self-tolerance, primarily related to the loss of response to dominant determinants[453] and especially loss of high-avidity T cells.[454–457] Interestingly, not only is there no loss of response to cryptic determinants but also T cells recognizing cryptic determinants of mouse lysozyme can be positively selected on other nonmouse lysozyme self-ligands in a lysozyme knockout mouse.[453] The selective loss of high-avidity T cells to immunodominant determinants has suggested a strategy to apply epitope enhancement to modify subdominant lower affinity tumor antigen peptides to increase their affinity for MHC molecules, and thereby make them more immunogenic in order by to take advantage of a repertoire not already crippled by loss of the high-avidity clones.[458]

Another elegant example of T-cell repertoire limitations on immunodominance comes from a study of mice deficient in LMP-2, and so unable to make immunoproteasomes. Reduced response to one normally immunodominant influenza epitope was found by T-cell adoptive transfer studies to be due to an alteration in the T-cell repertoire, presumably because of altered processing of self-peptides in the thymus.[281]

A recent study found another mechanism by which the T-cell repertoire contributes to immunodominance. It was found that the relative immunodominance of a dominant determinant of the HIV envelope protein among different strains of H-2D mice all presenting the same peptide–H-$2D^d$ complex correlated with the avidity of the T cells responding in those strains.[459] The mechanism found was that high-avidity T cells proliferate faster than low-avidity ones when exposed to antigen and therefore dominate the response. This mechanism differs from those at earlier steps that progressively constrain the response, and thereby narrow the repertoire to dominant epitopes, whereas this mechanism selectively expands the dominant repertoire. This mechanism may also explain other recent findings such as the higher affinity of dominant compared to subdominant TCRs specific for human cytomegalovirus.[460] Because high-avidity T cells are better able to clear virus infection,[461,462] a recent study even suggests that the driving force for MHC polymorphism is to create a large enough repertoire to select for high-avidity T cells.[463]

Several critical parameters have been reported to be involved in more effective T-cell activation during TCR-peptide-MHC interaction. In some situations, TCR avidity for the peptide-MHC complex appears to play a more critical role than dissociation rate[464] and a threshold for maximal activity may even be defined,[465] whereas in other situations, the half-life of the TCR-peptide-MHC interaction seems most critical.[466–468] Moreover, in situ in two-dimensional surface interactions, a rapid on-rate may compensate for a faster off-rate (shorter half-life) and allow effective TCR-peptide-MHC engagement, suggesting the total cumulative duration time (including multiple repeat engagements) may be most critical.[469–472]

As more is understood about the molecular basis of TCR recognition, with crystallographic data now available,[386,387,473] it becomes possible to apply epitope enhancement in a rational way to the affinity of the peptide-MHC complex to the TCR, as was described for the peptide affinity for MHC molecules. Sequence modifications in the peptide that increase the affinity for the TCR were shown to be more effective at expanding in vivo the T cells specific for tumor antigens.[474–476] Most of these modifications were found empirically, but a systematic study of substitutions throughout a number of peptides revealed a pattern in which peptides with conservative substitutions at P3, P5, or P7 were most likely to yield increased TCR affinity, narrowing the candidate list of peptides that require empirical screening.[477] This strategy provides a second type of epitope enhancement, derived from basic immunologic principles, to produce more effective vaccines.

The advent of DNA vaccines has made it possible to link a series T-cell epitopes in tandem and express them in the form of a multiepitope vaccine. These chimeric proteins have the

potential advantages of focusing the immune response on biologically important epitopes, combining T helper epitopes with CTL epitopes, using epitopes with high affinity for MHC, and linking the epitopes in any desired order to optimize immunogenicity. Ideally, antigen processing throughout the construct would produce multiple epitopes that could be presented independently. In the case of HIV, where antigenic variation is so common, these vaccines could be designed to overcome variation by including epitopes that are conserved within each geographical region (clades).

For example, Létourneau et al.[478] expressed a string of HIV sequence fragments from the gag, pol, vif, and env genes that were highly conserved within their own clades. As they progressed from one fragment to the next, they alternated sequences from different HIV clades: Immunity to multiple epitopes would produce broad based immunity across clades. Fourteen fragments were linked in tandem. Each fragment contained multiple T-cell epitopes, for a total of 270 known epitopes recognized with human MHC. In addition, T cells from most patients infected with HIV responded to peptide pools from the vaccine, indicating that the epitopes were processed and presented to T cells. But when the multiepitope vaccine was given as a DNA vaccine followed by two viral vector boosts, it elicited relatively weak T-cell responses to two out of six peptide pools in HLA-A2 transgenic mice.

For these constructs to function as multiepitope vaccines, antigen processing between epitopes is required. In any given string, all of the epitopes are expressed equally, but different locations might be processed differently. In order to elicit T-cell immunity to multiple epitopes at the same time, there must be efficient processing and presenting of epitopes in all parts of the string. For example, Depla et al.[479] created a vaccine with 30 epitopes from hepatitis B virus that were restricted to five different MHC class I types. The immune response to the combined antigen was compared to individual peptides as an indication of the effect of antigen processing on presentation of internal epitopes. For two MHC types, the same 5 out of 12 epitopes were immunogenic, regardless of whether they were presented as a string of epitopes or as free peptides. However, for three other MHC types, whereas 14 out of 18 epitopes were immunogenic as peptides, only 3 out of 18 were immunogenic in the multiepitope vaccine. This difference suggests inefficient antigen processing at multiple sites, either because of failure to process the epitopes correctly or due to excessive degradation.

One way to control processing was suggested by Livingston et al.[480,481] These authors found that the first amino acid following each epitope is an important marker for antigen processing. They demonstrated that poor presentation of an internal epitope can be corrected by adding a spacer of as little as one amino acid between epitopes to improve the yield of processed peptides. The most favorable amino acid spacers were Lys, Arg, Asn, and Gln, followed by Cys, Gly, Ala, Thr, or Phe. The least active spacer residues were Asp, Val, Met, or Leu.[480,481] For example, an epitope from hepatitis B virus core antigen was poorly immunogenic when it was located next to a Phe residue, but its immunogenicity was enhanced by four logs when Lys was substituted for Phe at this position. These amino acid preferences may reflect the major protease activities of proteasomes: The most targeted amino acids could improve the yield of intact epitopes.

This strategy was used to construct a multiepitope vaccine from HIV antigens.[482] Twenty-one epitopes were identified, based on the T-cell response of infected humans, and these were linked together in a multiepitope DNA vaccine. Spacers of one to four amino acids were inserted between epitopes to optimize antigen processing. The DNA vaccine was used to immunize HLA transgenic mice, and the response to each epitope was compared to peptide vaccinated mice. For two HLA types, A2 and A3, the peptide and multiepitope vaccines were nearly equal, suggesting efficient processing. In addition, for HLA-A2, the potency of the T-cell response to different epitopes was largely independent of their position in the vaccine sequence, suggesting efficient antigen processing at various points throughout the chimeric protein. But for a third HLA type, B7, despite a normal peptide response, the multiepitope vaccine failed to elicit immunity to four out of seven epitopes tested, indicating that processing was limiting the response to these residues.

Design of an effective multiepitope vaccine depends on finding the optimal balance between antigen processing and epitope survival. Antigen processing between epitopes generates antigenic peptides, but processing within the epitope would destroy its antigenicity. Delamarre et al.[483] have compared pairs of proteins with the same sequence but different conformations, resulting in different susceptibilities to lysosomal processing. Ribonuclease (RNAse) A was relatively stable to lysosomal extracts of dendritic cells, whereas RNAse S was degraded quickly. Of these two forms of the same protein, RNAse A was a far more potent immunogen, as measured by T-cell proliferation or Delayed Type Hypersensitivity (DTH). Similarly, horseradish peroxidase is stable to lysosomal proteases in the native form, but is quickly degraded in the apo form. Native horse radish peroxidase (HRP) was a much more potent immunogen than the unstable apo form. These results indicate the harmful effect of excessive processing on T-cell stimulation and immunogenicity.

Multiepitope immunogens have been designed to elicit CTLs by stimulating T helper cells and CTLs simultaneously. These immunogens have combined T helper and CTL epitopes in a variety of ways. Some vaccines contain at least one T helper epitope and one CTL epitope on each polypeptide chain.[484] Others have expressed them separately: A polypeptide consisting of multiple T helper epitopes was coadministered with a DNA plasmid coding for multiple CTL epitopes.[485] A third way would be to express alternating T helper and CTL epitopes in tandem in the same multiepitope vaccine, either as DNA or polypeptide. The T-cell response to paired T helper epitopes was generally weak,[484] whereas the response to a string of multiple T helper epitopes was reportedly stronger, although it did not confer any improvement on the immune response to CTL epitopes expressed separately.[485]

As with T-cell help for B cells, a multiepitope vaccine for CTLs should combine T helper and CTL epitopes in the same construct.[402] It would deliver both epitopes into

the same APC, to facilitate helper T cell effects on CTL induction. But the epitopes should target different cellular compartments, leading to the main processing pathways: T helper epitopes should travel to endosomes for processing and presentation with MHC class II. CTL epitopes should traffic to the cytoplasm for proteasomal processing and presentation with MHC class I.

Defining the Role of Individual Amino Acids and Effects of Altered Peptide Ligands

Once an antigenic peptide is identified, the next step is to map key amino acid residues by making a series of variant peptides, each of which differs from the native sequence by a single amino acid substitution, as previously described in the section on MHC binding. One approach, called an alanine scan, substitutes Ala for the natural amino acid at each position in the peptide or uses Ser or Gly to replace naturally occurring Ala. Ala is used because the side chain is only a methyl group, so it replaces whatever functional side chain is present with the smallest one other than that of Gly, which is not used because of its effects on conformation. Thus, one can ask whether loss of the naturally occurring side chain affects function, without the introduction of a new side chain that might itself affect function. Generally, each peptide will have several amino acids where Ala substitution destroys antigenicity. Some of these correspond to contact residues for the TCR, whereas others are contact residues for MHC. In many cases, the MHC-binding residues can be determined by testing the substituted peptides in a competitive MHC-binding assay (discussed previously). The amino acid substitutions that knockout T-cell proliferation but not MHC binding are presumed to be in the epitope recognized by the TCR directly, and these can be studied with additional substitutions. For example, this technique was used to compare the residues interacting with the MHC molecule or TCR when the same HIV-1 V3 loop peptide P18 (residues 308 to 322) was presented by three different MHC molecules, a human class I molecule, a mouse class I molecule, and a mouse class II molecule (Fig. 23.12).[486,487] Interestingly, there was a striking concordance of function of several of the residues as presented by all three MHC molecules (see Fig. 23.12). For example, Pro and Phe interacted with the MHC in all three cases, and the same Val interacted with the TCR in all three cases. Also, the same Arg in the middle of the peptide interacted with both the mouse class I and II molecules, and the C-terminal Ile was an anchor residue for both human and murine class I molecules.[486,487]

In the case of autoimmune T cells, these techniques have been used to study the number and variety of epitopes recognized by self-reactive T cells. In the nonobese diabetic mouse, the β chain of insulin is a major target of T cells recovered from pancreatic islets.[488] Alanine scanning of β chain peptide 9 to 23 revealed two patterns of T-cell recognition for the same peptide. Some T cells recognize peptide 9 to 16, whereas others respond to peptide 13 to 23. Each epitope appears to have distinct sites for MHC and TCR binding, even though they come from the same peptide chain. Similarly, in systemic lupus erythematosus, human T cells specific for the Sm antigen are narrowly restricted to a few epitopes that are found on a small group of proteins.[489] On the Sm-B antigen, three epitopes were recognized. On Sm-D antigen, there were two. In each case, alanine scans showed that the same epitopes were recognized by distinct T-cell clones. These results are consistent with the hypotheses that the autoimmune response to insulin or to Sm antigen may be induced by abnormal exposure of a very few cryptic epitopes, or they may depend on selective loss of tolerance for a limited number of epitopes shared by a small subset of self-proteins.

TCRs may distinguish different chemical classes of amino acid side chains. An example of structural differences between amino acid side chains recognized by the TCR comes from an analysis of non–cross-reactive CTL that distinguishes homologous peptides from the V3 loop of different strains of HIV-1 envelope protein. The residue at P8 in the minimal determinant was identified as a key

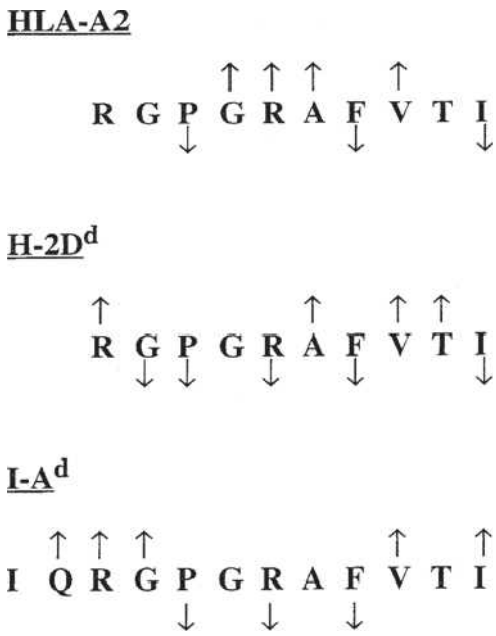


FIG. 23.12. Comparison of the Major Histocompatibility Complex (MHC)-Interacting ("Agretopic") and T-Cell Receptor (TCR)-Interacting ("Epitopic") Residues of the Same Human Immunodeficiency Virus (HIV)-1 Envelope V3 Loop Peptide as It Is Presented by Human Class I, Murine Class I, and Murine Class II MHC Molecules to Cluster of Differentiation (CD)8$^+$ Cytolytic T Cell and CD4$^+$ Helper T Cells. The sequence of the optimal binding portion of peptide P18 IIIB from the HIV-1 envelope protein V3 loop for each MHC molecule, in single letter amino acid code, is shown. *Arrows pointing up* indicate residues determined to interact with the TCR, and *arrows pointing down* indicate residues determined to interact with the MHC molecule. Mapping of residue function for binding to the human class I MHC molecule HLA-A2.1 was described in Alexander-Miller et al.,[487] and binding to the murine class I molecule H-2D^d and the murine class II molecule I-A^d was described in Takeshita et al.[486] Note the common use of the Pro and Phe for binding all three MHC molecules and the use of the key Val residue for binding all the TCRs. Also, the murine MHC molecules both use the central Arg residue as a contact residue, whereas both class I molecules use the C-terminal Ile residue as an anchor residue. Thus, there is a surprising degree of concordance.

"epitopic" TCR contact residue in both strain IIIB, which has a Val at this position, and strain MN, which has a Tyr at this position.[486,490,491] CTLs specific for strain IIIB do not recognize the MN sequence but will recognize peptides identical to MN except for the substitution of any aliphatic amino acid at that position, such as Val, Leu, or Ile.[400] In contrast, CTL specific for the MN strain do not recognize the IIIB sequence but will recognize the IIIB peptide if the Val at this position is replaced by a Tyr.[490] Moreover, they will see any MN variant in which the Tyr is replaced by another aromatic amino acid, such as Phe, Trp, or His.[400] Thus, the two non–cross-reactive TCRs see similar peptides but discriminate strongly between peptides with amino acids with aliphatic versus aromatic side chains. On the other hand, they do not distinguish strongly among different aliphatic residues or among different aromatic residues. Interestingly, however, in each category, the least active is the bulkiest member of the category, Ile and Trp, respectively, suggesting that these residues must fit into a pocket of limited size in the TCR.

The interaction of peptide ligand with TCR can be studied by introducing single substitutions of conservative amino acids at these contact residues, such as Glu for Asp, Ser for Thr, or Gln for Asn. The TCR readily distinguishes among peptides with these minor differences at a single residue, and the results have been revealing. Depending on affinity for the TCR, closely related (altered) peptides can elicit very different responses in T cells. Thus, although a substituted peptide may be very weak or nonstimulatory by itself, it may still act as a partial agonist or even a strong antagonist of an ongoing T-cell response. Antagonistic peptides can be demonstrated by pulsing APCs with native peptide antigen first so that one is not measuring competition for binding to MHC molecules, followed by pulsing with a 10-fold or greater excess of the antagonist before adding T cells. In the case of influenza hemagglutinin peptide 307 to 319 presented with HLA-DR1, peptide analogues such as Gln substituted for Asn 313 inhibited the proliferation of a human T-cell clone, even though they did not stimulate the clone. Anergy was not induced, and the antagonist peptide had to be present throughout the culture to inhibit the response.[492,493] Thus, lack of antagonist activity is another feature of the interaction between peptide (in complex with MHC molecule) and TCR that is required for the peptide to be a stimulatory antigenic determinant.

Partial agonists were first demonstrated using T-cell clones specific for an allelic form of mouse hemoglobin. These T cells were from CE/J mice, which express the Hb^s allele of mouse hemoglobin, after immunization with the Hb^d allele. The minimum antigenic peptide corresponds to amino acids 67 to 76 of the Hb^d sequence and differs from Hb^s at P72, P73, and P76.[494] Peptides substituted at each residue from amino acid 69 to 76 were tested for T-cell proliferation and cytokine release. Some substitutions, such as Gln for Asn at P72, blocked T-cell stimulation completely in both assays. Other substituted peptides, such as Asp for Glu at P73, lost T-cell proliferation but still stimulated interleukin 4 release, and these are considered partial agonists (Fig. 23.13).[495] Lack of stimulation was not due to failure to bind MHC, as both substituted peptides gave reasonable

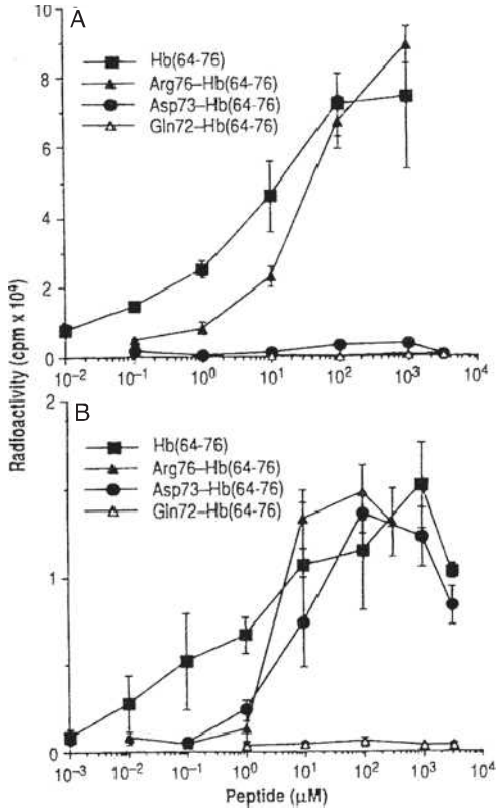


FIG. 23.13. Differential Effect of Altered Peptide Ligands on the Response to Peptide 64 to 76 from Hemoglobin. A: Proliferative response of a T-cell line incubated with antigen-presenting cells and the natural Hb (64-76) peptide or with peptides substituted at positions 72, 73, or 76. B: Interleukin 4 release by the T-cell line under the same conditions. The peptide substituted with Asp for Glu at position 73 is unable to induce T-cell proliferation, but it can still induce production of interleukin 4, so it is a partial agonist. In contrast, substitution of Gln for Asn at position 72 knocks out both responses equally. Modified from Evavold and Allen,[495] with permission.

binding in a competitive-binding assay.[496] Similar alteration of cytokine profile by altering the peptide ligand can be seen in other systems.[493,497,498]

For one of the Hb 64 to 76 specific T-cell clones, PL.17, substitutions at amino acids 70, 72, 73, or 76 reduced antigenic potency by 1000-fold or more, even though conservative amino acids were substituted. Although substitution of Ser for Ala 70 prevented T-cell stimulation in both assays, there was clearly some response to this peptide, as it induced expression of the interleukin 2 receptor.[499] In addition, once T cells were exposed to the Ser 70 peptide, they became unresponsive to subsequent exposure to the natural Hb^d peptide. This phenomenon closely resembled T-cell anergy and persisted for a week or more. The Ser 70 substitution alters a contact residue of the peptide for the TCR of clone PL.17 and affects its affinity. Other T-cell clones, however, can respond to this peptide presented on the same MHC molecule ($I\text{-}E^k$). Other Hb^d peptides substituted at this position, such as Met and Gly 70, also induced anergy but not proliferation, whereas nonconservative substitutions such as Phe, Asn, Asp, and His 70 induced neither.[500]

Another well-studied example is influenza hemagglutinin peptide 306 to 318 as presented on human HLA-DR1. Based on the known crystal structure of the peptide-MHC complex,[253] amino acid substitutions could be targeted to contact residues for the TCR at P307, P309, P310, P312, P315, and P318.[501] At each position, nonconservative substitutions often rendered the peptide inactive, whereas conservative substitutions at several sites either gave full antigenicity or gave progressively lower stimulatory activity, down to 1000-fold less than native peptide while retaining the ability to induce anergy. For example, substituting His or Gly for Lys 307 gave 1000-fold reduced stimulation of T-cell proliferation but full ability to induce tolerance. Similarly, substituting His for Lys 315 gave complete loss of stimulation but nearly full anergy inducing activity. As before, induction of the interleukin 2 receptor (CD25) was a sign of T-cell activation by these altered peptide ligands, even when they did not induce proliferation. Unlike these peptides, the antagonists do not induce interleukin receptors or secretion, and they do not cause long-lasting tolerance. Overall, a number of altered peptide ligands have now been identified that, in appropriate complexes with MHC molecules, induce anergy or act as antagonists of the TCR and block activation by agonist ligands by delivering an abortive signal.[493]

Several methods have been found to anergize T cells to a specific antigen for up to a week, and all have the common theme of delivering a partial signal via the TCR, resulting in tolerance rather than stimulation. The first method was to expose the T cells to peptide plus APCs treated with the carbodiimide cross-linker ECDI.[502] This treatment may prevent accessory molecules on the presenting cell from interacting with the TCR complex or costimulatory signals from contributing to T-cell activation. The second method was to present peptide on presenting cells with mutated I-E molecules.[503,504] The third method was to use altered peptide ligands that act as TCR antagonists as described previously.[493,500,501] The final method was to block CD4 function with a monoclonal antibody, which would delay the recruitment of CD4 to the engaged TCR.[505] Because generation of a complete stimulatory signal requires the interaction of TCR and accessory molecules, modifications that affect either component can block signaling. An altered peptide ligand, with decreased affinity for the TCR, may form an unstable complex, which cannot stay together long enough to recruit accessory molecules and generate a complete signal.[505,506] Altered peptide ligands with low affinity for the TCR can also act as partial agonists that can compete with optimal agonists and reduce T-cell stimulation based on a similar mechanism (short dwell time of peptide-MHC complex on the TCR).[199]

Abnormal TCR signaling can be demonstrated by following the activity of protein kinases. Normal signaling produces phosphorylation of TCR subunits, such as ζ chain, as well as phosphorylation and activation of receptor-associated tyrosine kinases, such as ZAP-70. These kinases generate the downstream signal needed for T-cell activation. However, in each case studied, partial antigen signaling resulted in ζ chain phosphorylation without phosphorylation or activation of ZAP-70,[500,504,505] so downstream activation did not occur. This abnormal pattern occurred regardless of the method of anergy induction.

Partial signaling may be important for T-cell survival during negative selection in the thymus or in maintaining peripheral tolerance. By responding to self-antigens as if they were altered ligands presented in the thymus, T cells could use anergy induction as a successful strategy for avoiding clonal deletion. Similarly, peripheral tolerance may be an important mechanism for preventing autoimmune disease. Immunotherapy with altered peptide ligands could be envisioned as a way to block an ongoing response or induce tolerance to a specific antigen, such as the synovium in arthritis, or foreign MHC antigens in allograft rejection. However, a potential pitfall is that different T cells recognize the same peptide differently, so a peptide that is seen as an altered peptide ligand by some T-cell clones may be seen as a complete antigen by others. In addition, the choice of peptide would vary with MHC type. To be effective, an altered peptide ligand should antagonize or anergize polyclonal T cells and should work with each patient's MHC type.

A similar mechanism may be invoked to explain the generally weak immunogenicity of tumor antigens. According to this hypothesis, the only T cells capable of responding to self-antigens on tumors may have low-affinity receptors for them. In effect, the natural sequence is the altered ligand that induces tolerance. In some cases, this anergy can be overcome with modified peptides that have greater affinity for the TCR and induce a full stimulatory signal, resulting in an effective immune response to the tumor antigens,[474] as previously described under epitope enhancement.

Prediction of T-Cell Epitopes

The fact that T cells recognize processed fragments of antigens presented by MHC molecules leads to the ironic situation that T-cell recognition of antigen, which is more complex than antibody recognition due to the ternary complex needed between TCR, antigen, and MHC molecule, may actually be focused on simpler structures of the antigen than those seen by most antibodies specific for native protein antigens. In contrast to the assembled topographic antigenic sites seen by many antibodies,[50,51] T cells specific for processed antigens are limited to seeing short segments of continuous sequence.[177,233] Therefore, the tertiary structure of the protein plays little if any role in the structure of the epitope recognized by T cells, except as it may influence processing. However, the structure of the T-cell antigenic site itself must be limited to primary (sequence) and secondary structure, the latter depending only on local rather than long-range interactions. This limitation greatly simplifies the problem of identifying structural properties important to T-cell recognition because one can deal with sequence information, which can now be obtained from DNA without having a purified protein, and with the secondary structure implicit therein without having to obtain an x-ray crystallographic three-dimensional structure of the native protein—a much more difficult task.

Because the key feature necessary for a peptide to be recognized by T cells is its ability to bind to an MHC molecule, most approaches for predicting T-cell epitopes are based on

predicting binding to MHC molecules. These approaches, which have been reviewed,[424,507–510] can be divided into those that focus on specific individual MHC molecules one at a time, such as motif-based methods, and those that look for general structural properties of peptide sequences. We shall discuss first the methods based on general properties and then those directed to individual MHC molecules.

The first structural feature of amino acid sequences found associated with T-cell epitopes that remains in use today is helical amphipathicity,[227,511–514] which is statistically significant independent of the tendency to form a helix per se.[512] Because the x-ray crystallographic structures of both MHC class I[376–378,515] and II molecules[253,443] have consistently shown peptides to be bound in extended, not α-helical, conformation, helicity per se has been abandoned as an associated structural feature of T-cell epitopes. However, as discussed in the following text, other explanations of amphipathic structures have been discovered that do not require the peptide to be bound to the MHC molecule as an α helix. Amphipathicity is the property of having hydrophobic and hydrophilic regions separated in space. It was observed that the immunodominant T-cell epitopes of myoglobin and cytochrome c corresponded to amphipathic helices.[185,189,516] DeLisi and Berzofsky[511] developed an algorithm to search for segments of protein sequence that could fold as amphipathic helices based on the idea that the hydrophobicity of the amino acids in the sequence must oscillate as one goes around an amphipathic helix. For the hydrophobic residues to line up on one side and the hydrophilic residues on the other, the periodicity of this oscillation must be approximately the same as the structural periodicity of the helix, about 100 degrees per residue (360 degrees per 3.6 residues per turn) (Fig. 23.14).

A microcomputer program implementing this analysis was published.[514] Margalit et al.[227] optimized the original approach,[511] correctly identifying 18 of the 23 immunodominant helper T cell antigenic sites from the 12 proteins in

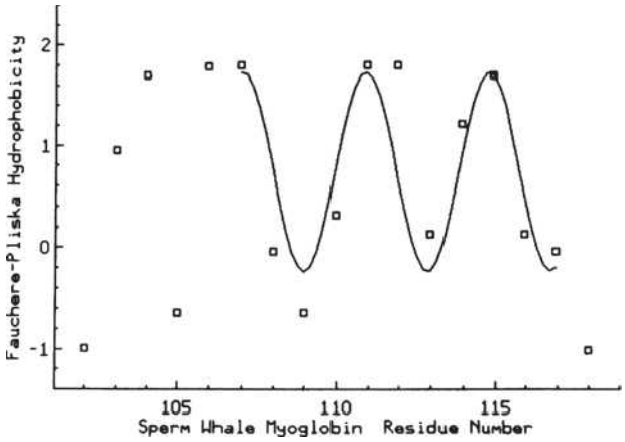


FIG 23.14. Plot of hydrophobicity of each amino acid in sperm whale myoglobin 102 to 118, according to the scale of Fauchère and Pliska,[604] as a function of amino acid sequence, showing least-squares fit of a sinusoidal function to the sequence of hydrophobicities from 107 to 117. (From Berzofsky et al.,[180] with permission.)

an expanded database ($p < .001$)[227] (see Table 23.4). Indeed, when the database was expanded to twice and then four times its original size, the correlation remained highly significant, and the fraction of sites predicted remained relatively stable (34/48 sites = 71%, $p < .003$; 61/92 sites = 66%, $p < .001$).[517,518] A similar correlation was found for 65% of peptides presented by class I MHC molecules.[518] A primary sequence pattern found in a substantial number of T-cell epitopes by Rothbard and Taylor[519] was consistent with one turn of an amphipathic helix. Also, another approach, called the "strip-of-the-helix" algorithm, which searches for helices with a hydrophobic strip down one face, found a correlation between amphipathic helices and determinants presented by both class II and I MHC molecules.[513,520]

Newer data suggest at least two explanations, not mutually exclusive, for this correlation in the absence of helical structure found in the peptides bound to MHC molecules.[521] First, crystal structures of peptides bound to class II MHC molecules have found that the peptides are bound in an extended conformation, but with a −130-degree twist like that of a type II polyproline helix,[253,443] and can be quite amphipathic because of this twist.[253] Although the −130-degree twist is distinct from that of an α helix, it gives a periodicity similar enough to be detected. Second, it was observed that spacing of the anchor residues in the motifs for peptides binding to class I and II MHC molecules was consistent with the spacing of turns of an α helix, for example, at P2 and P9 (seven residues apart like two turns of a helix) or at P5 and P9 (spaced like one turn of a helix).[521] Because the anchor residues are most often hydrophobic, this pattern resulted in an amphipathic periodicity pattern like that of an amphipathic α helix for just the anchor residues alone, seen in most motifs.[521] Thus, if the other residues have a random pattern, the anchor residue spacing alone, which is enforced by the spacing of the pockets in the MHC molecules that bind these anchor residues, will produce the amphipathic helical signal, even though the peptide is bound in an extended conformation. This amphipathic helical periodicity has held up as a correlate for peptides defined as T-cell epitopes[521] and has continued to be a useful predictive tool for identifying potential epitopes, successful in a number of studies, when one does not want to focus on individual MHC alleles or wants to find regions of high epitope density. Other structural properties such as coil content and exposure have been used as general predictors of MHC class II binding.[522]

Other approaches to predicting T-cell epitopes are generally based on sequences found to bind to specific MHC molecules.[424,507–510,523–525] The simplest approach is to apply standard sequence search algorithms to known protein sequences to locate motifs for peptides binding to particular MHC molecules, using collections of motifs identified in the literature.[264] This approach showed early success for epitopes in proteins from *Listeria monocytogenes*[526] and malaria,[527] but it also became apparent that only about 30% of sequences bearing motifs actually bound to the corresponding MHC molecules.[526,528,529] This discrepancy may relate to adverse interactions created by nonanchor residues[392,397,530] and

could be overcome to some extent by generating extended motifs taking into account the role of each residue in the sequence.[397,530]

To determine whether one could locate regions of proteins with high densities of motifs for binding multiple MHC molecules, Meister et al.[531] developed the algorithm Epimer, which determined the density of motifs per length of sequence. A surprising result was that the motifs were not uniformly distributed but clustered. This clustering may reflect the fact that many motifs are related, and that the same anchor residues are shared by several motifs, perhaps because MHC molecules are also related and their variable segments that define some of the binding pockets are sometimes exchanged by gene conversion events.[532] This hypothesis has now been confirmed and extended by studies showing that each anchor pocket can be grouped into families of MHC molecules sharing similar pockets and therefore anchor residues, but the families for the B, C, and F pockets do not coincide, so there is a reassortment between pockets.[533,534] These observations allow prediction of motifs for additional MHC molecules. In the case of HIV, the densities of motifs for class I MHC binding were anomalous at both the low and high ends of the spectrum.[535] Clustering at the high end may be due to anchor sharing and showed no correlation with conserved or variable regions of the sequence. However, at the low end, long stretches with low motif density occurred preferentially in variable regions, suggesting that the virus was mutating to escape the CTL immune system.[535] This clustering may be useful in vaccine development because identification of sequences containing overlapping motifs for multiple MHC molecules may define promiscuously presented peptides that would elicit responses in a broad segment of the population.[531]

Another type of MHC allele–specific approach is the use of matrices defining the positive or negative contribution of each amino acid possible at each position in the sequence toward binding to an MHC molecule. A positive or negative value is assigned to each of the 20 possible amino acids that can occur at each position in a peptide sequence, and these are summed to give the estimated potential of that peptide for binding. The values in the matrix are derived from either experimental binding studies using peptide panels with single positions substituted with each possible amino acid,[439,536–539] or from comparisons of peptides known to bind in a compilation of the literature, if the number known is sufficiently large.[507,540] One improvement described is an amino acid similarity matrix that disfavors substitutions with opposite charge.[541] Davenport et al.[542,543] also developed a motif method based on Edman degradation sequencing of pooled peptides eluted from MHC molecules. All of these methods have had some success in predicting peptides binding to particular MHC molecules,[507] but they all require the assumption that each position in a peptide must be acting independently of its neighbors, which is a reasonable first approximation, but exceptions are known.[544,545] The more experimental data that goes into generating the matrix, the more reliable the predictions. Therefore, the predictive success may be greater for some of the more common HLA molecules for which more data exists. This matrix approach has been used for both class I and II MHC molecules. Current predictive matrix algorithms, compared to experimental screening, have been found to detect the vast majority of $CD8^+$ T-cell epitopes detected experimentally, for example, in vaccinia virus, in which the top-ranked 300 peptides using four different algorithms predicted 40 per 49 epitopes found.[546]

A potentially very useful observation is the finding that HLA class I molecules can be grouped into families (HLA supertypes) that share similar binding motifs.[438,536,547,548] The broader motifs that encompass several MHC molecules have been called supermotifs. For example, HLA-A*0301, A*1101, A*3101, A*3401, A*6601, A*6801, and A*7401 all fall into the HLA-A3 superfamily.[547] A peptide that carried this supermotif should be active in a broader range of individuals than one which was presented by a single HLA molecule. Moreover, as several HLA supertypes have been defined, it should be possible to design a vaccine effective in a large fraction of the population with only a limited number of well-selected antigenic determinants.[549]

Another approach for predicting peptides that bind to MHC molecules is based on free energy calculations of peptides docked into the groove of a known MHC structure, for which the crystallographic coordinates are known, or on structural modeling of the MHC molecule by homologous extension from another MHC molecule, when the crystal structure is not known, followed by peptide docking calculations.[550–552] It is important to use free energy rather than energy, as the latter alone cannot find the most stable orientation of a side chain and cannot correctly rank order different side chains at the same position. This approach correctly predicts the structure of several known peptide-MHC complexes when starting with the crystal structure of a different complex, in each case to within 1.2 to 1.6 Å all-atom root-mean square deviation.[551] Using this structural modeling can allow one to extend motifs to nonanchor positions for cases where only anchor residue motifs are known and can allow one to predict new motifs for MHC molecules whose motifs have not yet been determined.

Another approach to predicting MHC-binding sequences uses a technique called threading that has been developed for predicting peptide secondary structure, based on threading a sequence through a series of known secondary structures, and calculating the energies of each structure. Altuvia et al.[553,554] showed that threading could be applied to peptides in the groove of MHC molecules, because when several peptides that bind to the same MHC molecule are compared crystallographically, the conformation of the peptides is fairly similar, as for example in several peptides cyrstallized bound to HLA-A2.1.[515] In testing the threading approach, Altuvia et al.[553,554] showed that known antigenic peptides are highly ranked among all peptides in a given protein sequence, and the rank order of peptides in competitive binding studies could be correctly predicted. The advantage of this approach is that it is independent of known binding motifs and can identify peptides that bind despite lack of the common motif for the MHC molecule in question. It can

also be used to rank a set of peptides all containing a known motif. For both class I and II MHC–binding peptides, a number of structure-based predictive approaches have been developed as well.[522,555–558]

Finally, artificial neural networks can be trained on a set of peptides that bind to a given MHC molecule to recognize patterns present in binding peptides.[559,560] When the predictions of the artificial neural networks are tested, the results can be used to further train the artificial neural networks to improve the predictive capability in an iterative fashion.

Recently, predictive algorithms for proteasomal cleavage sites[561,562] and TAP-transported peptides[319,563] have been developed. Combining these into approaches to predict epitopes based on all the steps a peptide must pass through, cleavage, transport, and MHC binding (except for TCR binding), has led to the most recent comprehensive algorithms for epitope prediction that can achieve up to 72% sensitivity.[286,320,508,564–568]

As all these methods are further developed and refined, they promise to allow accurate prediction of peptides that will bind to different MHC molecules and thus allow the design of vaccines without empirical binding studies until the end of the process. In addition, empirical high throughput methods for epitope mapping have been developed to speed the latter.[569] Further, localization of clusters of adjacent or overlapping binding sequences in a short segment of protein sequence can also be useful for selecting sequences that will be broadly recognized. Predictive algorithms for locating T-cell epitopes also have the potential use to reduce immunogenicity of therapeutic proteins (such as hormones, cytokines, and monoclonal antibodies) against which one would not want an immune response to develop.[570,571] For this purpose and others, it is useful to be able to predict all the epitopes likely to be recognized not just with a single MHC allele but with all of a person's MHC molecules or even those of a larger population; such methods have also been developed.[572]

RELATIONSHIP BETWEEN HELPER T-CELL EPITOPES AND B-CELL EPITOPES ON A COMPLEX PROTEIN ANTIGEN

As we have seen, the factors that determine the location of antigenic sites for T and B cells, with the possible exception of self-tolerance, are largely different. Indeed, if B cells (with their surface antibody) bind sites that tend to be especially exposed or protruding—sites that are also more accessible and susceptible to proteolytic enzymes—then there is reason to think that T cells may have a lower probability of being able to recognize these same sites, which may be more likely to be destroyed during processing. Certainly, assembled topographic sites will be destroyed during processing. On the other hand, there are examples in which T cells and antibodies seem to see the same, or very closely overlapping, sites on a protein,[43,185,215,573–575] although fine specificity analysis usually indicates that the antibody and T cell fine specificities are not identical. The question dealt with here is whether there are any functional or regulatory factors in T cell–B cell cooperation that would produce a relationship between helper T cell specificity and B cell specificity for the same protein antigen.

Early evidence that helper T cells might influence the specificity of the antibodies produced came from a number of studies showing that Ir genes, which appeared to act through effects of T-cell help, could influence the specificity of antibodies produced to a given antigen.[181,576–583] It was hard to imagine how MHC-encoded Ir genes could determine which epitopes of a protein elicit antibodies, when such antibodies are generally not MHC restricted. One explanation suggested was that the Ir genes first select which helper T cells are activated, and these in turn influence which B cells, specific for particular epitopes, could be activated.[130] Because, for cognate help, the B cell has to present the antigen in association with an MHC molecule to the helper T cell, the Ir gene control of antibody specificity must operate at least partly at this step by selecting which helper T cell can be activated by and help a given B cell. Conversely, if the helper T cell selects a subset of B cells to be activated on the basis of their antibody specificity, then there is a reciprocal interaction between T and B cells influencing each other's specificity. Therefore this hypothesis was called "T–B reciprocity."[130] Steric constraints on the epitopes that could be used by helper T cells to help a B cell specific for another particular epitope of the same protein were also proposed by Sercarz et al.[584]

The concept was first tested by limiting the fine specificity of helper T cells to one or a few epitopes and then determining the effect on the specificities of antibodies produced in response to the whole molecule. This was accomplished by inducing T-cell tolerance to certain epitopes[585] or using T cells from animals immune to peptide fragments of the protein.[128,586,587] In each case, the limitation on the helper T-cell specificity repertoire influenced the repertoire of antibodies produced.

One purpose of the B-cell surface Ig is to take up the specific antigen with high affinity, which is then internalized by receptor-mediated endocytosis and processed like any other antigen.[588–595] Therefore, the explanation was proposed that the surface Ig, which acts as the receptor to mediate endocytosis, sterically influences the rate at which different parts of the antigen are processed because what the B cell is processing is not free antigen but a monoclonal antibody–antigen immune complex.[130] This concept presupposes that many antibody–antigen complexes are stable near pH 6 in the endosome, and that what matters is the kinetics of production of large fragments, rather than the products of complete digestion, when both the antigen and the antibody may be degraded to single amino acids. Such protection from proteolysis of antigen epitopes by bound antibody can be demonstrated at least in vitro.[57] More recently, the effect of antigen-specific B-cell surface Ig on the fragments produced by proteolytic processing of antigen was elegantly demonstrated by Davidson and Watts.[596] They demonstrated that the pattern of fragmentation of tetanus toxoid, as measured by SDS–polyacrylamide gel electrophoresis, produced during processing by B-lymphoblastoid clones specific for tetanus toxoid varied among B-cell clones depending on their specificity for different epitopes within the antigen.

Binding to the antibody may also influence which fragments are shuttled to the surface and which are shunted into true lysosomes for total degradation. Thus, different B cells bearing different surface Ig would preferentially process the antigen differently to put more of some potential fragments than others on their surface in contrast to nonspecific presenting cells that would process the antigen indifferently. By this mechanism, it is proposed that B-cell specificity leads to selective antigen presentation to helper T cells and, therefore, to selective help from T cells specific for some epitopes more than from T cells specific for others.[130]

To test this hypothesis, Ozaki and Berzofsky[129] made populations of B cells effectively monoclonal for purposes of antigen presentation by coating polyclonal B cells with a conjugate of monoclonal antimyoglobin coupled to anti-IgM antibodies. B cells coated with one such conjugate presented myoglobin less well to one myoglobin-specific T-cell clone than to others. B cells coated with other conjugates presented myoglobin to this clone equally well as to other clones. Therefore, the limitation on myoglobin presentation by this B cell to this T-cell clone depended on the specificity of both the monoclonal antibody coating the B cell and the receptor of the T-cell clone. It happened in this case that both the monoclonal antibody and the T-cell clone were specific for the same or closely overlapping epitopes. Therefore, it appears that the site bound by the B-cell surface Ig is less well presented to T cells. This finding is also consistent with a recent study of chimeric proteins in which one or more copies of an ovalbumin helper T-cell determinant were inserted in different positions.[597] Although the position of the ovalbumin determinants did not affect the antibody response to one epitope, the position did matter for antibody production to an epitope of the chimeric protein derived from insulin-like growth factor I. An ovalbumin determinant inserted distal to this epitope was much more effective in providing help than one inserted adjacent to the same epitope, when both constructs were used as immunogens, even though both constructs elicited similar levels of ovalbumin-specific T-cell proliferation in the presence of nonspecific presenting cells in vitro as a control for nonspecific effects of flanking residues on processing and presentation of the helper T-cell determinants. However, circumstantial evidence from the Ir gene studies mentioned previously suggests that T cells may preferentially help B cells that bind with some degree of proximity to the T-cell epitope, as there was a correlation between T cell and antibody specificity for large fragments of protein antigens under Ir gene control.[127,130,181,579,580,583] Therefore, antibodies may have both positive and negative selective effects on processing. Further studies on presentation of β-galactosidase–monoclonal antibody complexes by nonspecific APCs suggest similar conclusions.[598,599] Presumably, the conjugates are taken up via Ig fragment c (Fc) receptors on the presenting cells and processed differentially according to the site bound by the antibody so that they are presented differentially to different T-cell clones. Thus, non–B-presenting cells can be made to mimic specific B-presenting cells. This also suggests that circulating antibody may have a role in the selection of which T cells are activated in a subsequent exposure to antigen.

The issue of whether bound antibody enhanced or suppressed presentation of specific determinants to T cells was explored further by Watts et al.[600] and Simitsek et al.[601] They first found that a particular tetanus toxoid–specific Epstein-Barr virus–transformed human B-cell clone 11.3 failed to present the tetanus toxoid epitope 1174 to 1189 to specific T cells, whereas it presented another epitope as well as did other B cells, and another B-cell clone presented the 1174 to 1189 epitope well. Moreover, the free 11.3 antibody also inhibited presentation of this epitope to T cells at the same time that it enhanced presentation of other epitopes by Fc receptor facilitated uptake.[600] They subsequently found that the same 11.3 B cell and antibody actually enhanced presentation of another epitope of tetanus toxoid, 1273 to 1284, by about 10-fold, even though both epitopes were within the footprint of the antibody as determined by protection from proteolytic digestion.[601] The enhancement could be mediated also by free antibody as well as Fab fragments thereof, indicating that the mechanism did not involve Fc receptor facilitated uptake. Furthermore, the 11.3 antibody had no effect on presentation of another determinant in the same tetanus toxoid C fragment, 947 to 967, which was not within the footprint of the antibody, and another antibody to the C fragment did not enhance presentation of 1273 to 1284. The authors concluded that the same antibody or surface Ig can protect two determinants from proteolysis but sterically hinder the binding of one to class II MHC molecules while facilitating the binding of the other.[601] The facilitation may involve protection from degradation. This antibody-mediated enhancement of presentation of selected epitopes to helper T cells can greatly lower the threshold for induction of a T-cell response and may thereby elicit responses to otherwise subdominant epitopes. It can also contribute to epitope spreading, for example, in autoimmune disease, in which an initial response to one dominant determinant leads to a subsequent response to other subdominant determinants, perhaps by helping for antibody production, which in turn facilitates presentation of the other determinants.

Taken together, these results support the concept of T–B reciprocity in which helper T cells and B cells each influence the specificity of the other's expressed repertoire.[130] This mechanism may also provide an explanation for some of the cases in which Ir genes have been found to control antibody idiotype.[602,603] These relationships probably play a significant role in regulating the fine specificity of immune response of both arms of the immune system. Therefore, they will also be of importance in the design of synthetic or recombinant fragment vaccines that incorporate both T- and B-cell epitopes to elicit an antibody response.

CONCLUSION

Overall, antibodies and T cells recognize different structural features in different contexts or environments, and thus complement each other to detect the spectrum of foreign (or self-) antigens encountered. Antibodies recognize three-dimensional structures on the exposed surface of molecules either in solution or on a cell surface.

Therefore, they are dependent on the conformation of the antigen and can recognize structures that are assembled on the surface of the antigen molecule by the way it folds but that are not contiguous in the primary sequence. However, they do not generally recognize structures buried within protein molecules or inside cells. In contrast, T cells are designed to recognize short segments of primary amino acid sequence of protein antigens, and thus are not dependent on the conformation of the original protein unless it affects processing. Furthermore, T cells particularly provide internal surveillance of proteins inside cells, recognizing peptide fragments of these presented by MHC molecules that carry these fragments to the cell surface. This surveillance of intracellular proteins requires a number of hurdles including proteolytic processing of the antigen into fragments, transport of these into the compartments where they are loaded onto MHC molecules, binding with appropriate specificity to the combining site of specific MHC molecules, and then finally recognition by an appropriate TCR. These hurdles limit the number of amino acid sequences that can be recognized and account in part for immunodominance. Furthermore, the two major subsets of T cells also complement each other by recognizing exogenous and endogenous proteins processed and transported through different pathways and presented by difference classes of MHC molecules. Thus, the two major classes of T cells complement the types of structures and the locales surveyed by antibodies. For example, in the case of viruses, antibodies can detect intact virions and shed viral proteins in solution as well as viral proteins expressed on the surface of infected cells; $CD4^+$ T cells can recognize viral proteins taken up by APCs and processed in an endosomal pathway and $CD8^+$ T cells can detect proteins synthesized within the infected cell, whether or not they are ever expressed intact on the cell surface or secreted. Together, they provide the immune system with a strategy to detect all forms of foreign invaders as well as to protect the host through different effector mechanisms.

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CHAPTER 24

Fc Receptors and Their Role in Immune Regulation and Inflammation

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HISTORICAL BACKGROUND

Cellular receptors for immunoglobulins (Igs) were anticipated by the description of cytophilic antibodies of the IgG class, identified by Boyden and Sorkin in 1960.[1] These antibodies conferred upon normal cells, like macrophages, the capacity to specifically absorb antigen. Using sheep red blood cells (RBCs) as the antigen resulted in rosette formation between the cytophilic antisheep RBC antibodies and macrophages and provided a convenient means of visualization of the binding of cytophilic antibodies with normal cells. Subsequent studies by Berken and Benacerraf[2] suggested that the crystallized fragment (Fc) of the cytophilic antibody interacted with a cell surface receptor on macrophages. Similar studies on B-lymphocytes extended the generality of these receptors and led to the term *Fc receptor* (FcR) to denote the surface molecules on lymphoid and myeloid cells that are capable of interacting with the Fc of immunoglobulin molecules.[3] Studies on IgE, IgM, and IgA demonstrated the existence of distinct receptors for those isotypes as well on various immune cell types. Detailed biochemical characterization of Fc receptors was inaugurated by the studies of Kulczycki et al.[4] on the high-affinity IgE FcR of mast cells, revealing a hetero-oligomeric $\alpha\beta\gamma_2$ subunit structure. A distinction between FcRs for the IgE and IgG isotypes emerged with the observation of the very high (10^{10} M^{-1}) binding affinity of IgE for its receptor in comparison with the low binding (10^{6} M^{-1}) of IgG1 to its receptor. This distinction led to the realization that the functional IgG1 ligand was exclusively in the form of an immune complex (IC), whereas IgE binding occurred through monomer interaction with its receptor. This difference in binding affinity had significant functional implications for the structures of these receptors and mechanisms by which each isotype activated its target cell. Determination of the structure of these receptors was facilitated by their molecular cloning, beginning with the IgG FcRs[5,6] and followed by the IgE FcR.[7] Two distinct types of IgG receptors, differing in their transmembrane and cytoplasmic sequences, were identified that offered a molecular explanation for the apparent contradictory activation and inhibitory activities attributed to IgG FcRs. The primary structure of the subunits of the high-affinity IgE FcR revealed homology in the ligand binding α subunit to its IgG counterparts. However, the extent of similarity between these receptors became apparent with the observation that the γ chain subunit was common to both IgG and IgE FcRs, providing both assembly and signaling functions to these activation receptors.[8,9] This common structure suggested a functional link between IC diseases and allergic reactions, a prediction that was confirmed through mouse knockout studies of IgG FcRs.[10,11] The FcRs, through their dependence on the immunoreceptor tyrosine-based activation motif (ITAM) pathway of cellular activation, belonged to the family of immunoreceptors that included the antigen receptors on B and T cells. Three-dimensional crystal structures have been solved for the low-affinity IgG FcRs[12,13] and the high-affinity IgE FcR,[14] alone and in complex with their Ig ligands,[15,16] further establishing the close structural link between these Ig receptors.

The functional roles of IgG FcRs were suggested by the distribution of these receptors on both lymphoid and myeloid cells.[17] On myeloid cells, they were presumed to mediate effector cell activation, resulting in phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and release of inflammatory mediators. However, the well-known ability of the classical pathway of complement to generate activated fragments in response to ICs capable of inducing inflammatory responses by myeloid cells complicated the interpretation of the physiological role of IgG FcRs. Thus, the contribution of IgG FcRs to the mechanism of IC-mediated inflammation, as distinct from the role of complement, remained uncertain. Insight into this distinction was gained through the generation of mouse strains specifically deficient or blocked in either FcRs[10,18–20] or components of the classical complement pathway.[21] Studies on IC-mediated inflammatory responses in these animals, such as the Arthus reaction, led to the realization that IgG FcRs and not the classical pathway of complement activation were the functional mediators of inflammatory responses triggered by ICs.[20,22,23] The situation for IgE was less confounding, and the identification and characterization of a high-affinity receptor for this isotype on mast cells offered a plausible explanation for many of the inflammatory features of allergic reactions,[24] validated later by mouse knockouts of this receptor. IgG ICs had also been observed to mediate suppression of B-cell responses; thus, the presence of an IgG FcR activity on B cells provided a possible, but uncharacterized, mechanism for this inhibitory activity. Molecular characterization of this inhibitory activity for the B-cell FcR, FcRIIB, resulted in the first detailed description of an inhibitory motif, now termed the immunoreceptor tyrosine-based inhibitory motif (ITIM),[25,26] and the signaling pathway by which it abrogates ITAM-triggered activation. The ITIM mechanism is now recognized as ubiquitous and has resulted in the recognition of a large family of inhibitory receptors on immune cells that function to maintain proper

thresholds for activation and abrogate activation responses to terminate an immune reaction.[27]

FcRs are now recognized as central mediators of antibody-triggered responses, coupling the innate and adaptive immune responses in effector cell activation.[28] In addition to these specialized roles, the IgG FcRs have served as an example of the emerging class of balanced immunoreceptors, in which activation and inhibition are tightly coupled in response to ligand binding. Perturbations in either arm of the response have been shown to lead to pathological consequences and have been taken as a paradigm of how these systems are likely to work for those paired immunoreceptors with unknown ligand-binding functions. The newly described roles for FcRs in maintaining peripheral tolerance, shaping the antibody repertoire, regulating antigen-presenting cell (APC) maturation, and promoting mast cell survival indicate the diversity of functions that these receptors possess and their central role in modulating both afferent and efferent responses in the immune response.

This chapter focuses primarily on the IgG and IgE FcRs, for which substantial data on their structure, function, regulation, and role in a variety of physiological and pathological conditions are now available. The similarity in structure and signaling between those receptors and other members of this family, such as the IgA FcR and the recently discovered Fc-receptor for mouse and human IgM (FcμR), is also discussed. These FcRs will be referred to as "canonical Fc receptors," reflecting their shared structural and functional properties. In contrast, lectins such as SIGN-R1 and DC-SIGN, which bind IgG Fc when sialylated, represent a novel class of FcRs with distinct structures and functions and will be discussed in detail in the following. Other Ig receptors with specialized functions in the transport of Igs, such as the FcRn[29] and the poly-Ig FcR,[30] will not be discussed here.

STRUCTURE AND EXPRESSION

Molecular Genetics

Two general classes of canonical FcRs are now recognized: the activation receptors, characterized by the presence of a cytoplasmic ITAM sequence associated with the receptor, and the inhibitory receptor, characterized by the presence of an ITIM sequence. These two classes of receptors function in concert and are usually found coexpressed on the cell surface. Thus coengagement of both signaling pathways is the rule, setting thresholds for and ultimately determining the physiological outcome of effector cell responses. Among the factors that determine this threshold level are the actual affinities of the individual activating and inhibitory receptors for a specific IgG ligand and the expression level of the receptor pairs on immune effector cells. Importantly, the affinity of different antibody isotypes and subclasses for their respective activating and inhibitory FcRs varies significantly, thus explaining the differential activity of antibody isotypes in vivo.[20,31,32] In addition, alleles of specific FcRs have been described that alter their affinity for individual subclasses thus accounting for the variable responses seen in a population to an antibody (see subsequent discussion). Noncanonical FcRs such as the IgM receptors FcμR, Fca/μR, the poly-Ig receptor, and the sialylated IgG Fc receptors SIGN-R1 and DC-SIGN, contain neither ITAM nor ITIM motifs.

Subunit Composition

Canonical FcRs are typically type I integral membrane glycoproteins consisting of, at the least, a ligand recognition α subunit that confers isotype specificity for the receptor. α subunits for IgG, IgE, IgM, and IgA have been described.[17,33–35] These subunits typically consist of two extracellular domains of the IgV type superfamily, a single transmembrane domain and a relatively short intracytoplasmic domain. In activation FcRs, a signaling subunit of the γ family is often found, resulting in an $\alpha\gamma_2$ complex. The inhibitory FcγRIIB molecule, in contrast, is expressed as a single-chain receptor. The α subunits have apparent molecular weights of between 40 and 75 kDa, and share significant amino acid sequence homology in their extracellular domains. Alternatively, spliced forms of FcγRIIB modify the intracytoplasmic domain of this molecule. For example, the B2 form lacks sequences that inhibit internalization and thus demonstrates enhanced internalization of ICs, in comparison with FcγRIIB1. However, all the splice variants contain the ITIM motif, a necessary and sufficient domain for mediating inhibitory signaling. The conservation of this sequence in mice and humans, its presence in all splice variants, and the hyperresponsive phenotypes generated in mice deficient in this receptor all support inhibition as the central function of FcγRIIB. The specific structures of the α subunits of the canonical FcRs are shown in Figure 24.1. The notable exceptions to the general structure just outlined are seen for the high-affinity FcγRIα subunit, which has three extracellular domains; the activation FcγRIIAα subunit, which does not require additional subunits for assembly or signaling; and the glycosylphosphatidyl-inositol (GPI)-linked FcγRIIIB, which attaches to the cell surface through a GPI linkage, rather than through a transmembrane domain.

The γ subunit is found associated with activation IgG, IgE, and IgA FcRs, as well as with non-FcR molecules, such as paired Ig-like receptor A (PIR-A) and natural killer (NK) cell cytotoxicity receptors, but not with IgM receptors, nor the lectins SIGN-R1 and DC-SIGN.[36–38] It is required for assembly of the α subunits of these receptors by protecting these subunits from degradation in the endoplasmic reticulum. The γ chain is found as a disulfide-linked homodimer, with a short extracellular domain containing the cysteine involved in dimerization, a transmembrane domain, and an intracytoplasmic domain containing the ITAM. An aspartic acid residue found in the transmembrane domain of the γ chain is often associated with a basic amino acid residue in the transmembrane domain of the α subunit. The γ subunit belongs to a gene family that includes the T-cell receptor–associated ζ chain and the NK receptor DAP-10– and DAP-12–associated molecules.[39] FcγRIIIA can associate with the ζ chain, resulting in the $\alpha\zeta_2$ complex found in human NK cells.

A third subunit is found associated with the activation FcRs FcεRI and FcγRIII, the β subunit. This 33-kDa subunit has four transmembrane-spanning domains and amino and carboxy intracytoplasmic domains, belonging to the cluster

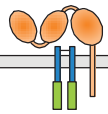
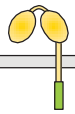
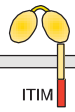
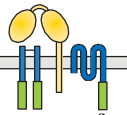
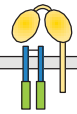
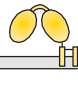
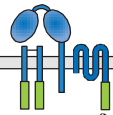
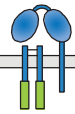
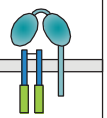
	FcγRI CD64	FcγRIIA CD32	FcγRIIB CD32	FcγRIIIA CD16		FcγRIIIB CD16	FcεRI		FcαRI CD89
Structure									
Subunit composition	γ_2 α	α	ITIM α	γ_2 α β	γ_2 α	α-GPI	γ_2 α β	γ_2 α	γ_2 α
Ka	10^8 M^{-1}	$2x10^6$ M^{-1}	$2x10^6$ M^{-1}	$5x10^5$ M^{-1}	$5x10^5$ M^{-1}	$2x10^5$ M^{-1}	10^{10} M^{-1}	10^{10} M^{-1}	$5x10^7$ M^{-1}
Binding Specificity	1. IgG1=IgG3 2. IgG4 3. IgG2	1. IgG1 2. IgG2=IgG3 3. IgG4	1. IgG1 2. IgG2=IgG3 3. IgG4	1. IgG1=IgG3	1. IgG1=IgG3	1. IgG1=IgG3	IgE	IgE	IgA_2=IgA_2
Expression	Macrophages Neutrophils Eosinophils Dendritic Cells	Macrophages Neutrophils Mast cells Eosinophils Platelets Dendritic Cells	Macrophages Neutrophils Mast cells Eosinophils Dendritic Cells FDC B cells	Mast cells Basophils	Macrophages Mast cells Basophils NK cells Dendritic Cells	Neutrophils	Mast cells Basophils	Mast cells Basophils Eosinophils Platelets Dendritic Cells	Macrophages Neutrophils Eosinophils
Class	Activation	Activation	Inhibition	Activation		Decoy	Activation	Activation	Activation
Function	-Inducible by inflammatory cytokines -Enhance effector responses at inflammatory sites -IC capturing by DC	-Effector cell activation by IC's, cytotoxic Ab	-Set threshold for effector cell activation by Fc -B cell repression -Maintain tolerance	-Dominant pathway for effector activation by IgG -In vivo ADCC -Arthus reaction -IC capture by DC -IC mediated DC maturation		-Sink for IC -Focus IC to PMN -Synergize with FcγRIIA	-Degranulation -Allergic reactions (Type I)	-Degranulation -Allergy -Antigen caption by DC	-IgA binding -IgA activation of effector cells

FIG. 24.1. Summary of Fc Receptor Structures, Expression Patterns, and in Vivo Functions. The immunoreceptor tyrosine-based activation motif signaling motif is indicated by the *green rectangle*; the immunoreceptor tyrosine-based inhibitor motif is indicated as a *red rectangle*. Alleles of FcγRIIA and FcγRIIIB and their binding properties are discussed in the text.

of differentiation (CD)20 family of tetraspan molecules.[24] An ITAM sequence is found in the intracytoplasmic carboxy domain. In mast cells and basophils, the β chain assembles into an $\alpha\beta\gamma_2$ complex with the α chain belonging to either FcγRIII or FcεRI. Its presence is required for assembly of FcεRI in rodents. In humans, however, $\alpha\gamma_2$ complexes of FcεRI are found in monocytes, Langerhans cells, and dendritic cells (DCs), in addition to the $\alpha\beta\gamma_2$ complexes found in mast cells and basophils. The ITAM motif found in the β subunit is not an autonomous activation sequence but functions as a signaling amplifier of the ITAM found in the γsubunits.[40]

Gene Organization, Linkage, and Polymorphisms

All α subunits of canonical FcRs share a common gene organization, which indicates that the evolution of this family of receptors resulted from gene duplication from a common ancestor.[41] Sequence divergence then resulted in the acquisition of distinctive specificities for these related sequences. Most of the genes belonging to the expanded FcR family, including the recently identified Fcμ-receptor, the Fcα/μR, the poly-Ig receptor, the group of FcR-homologous or -like (FCRL) proteins, the α chains of FcγRI, FcγRII, FcγRIII, and FcεRI including the common FcR-γ chain (Fig. 24.2), are found on the long arm of chromosome 1.[34,35,42–44] This region is syntenic with a comparable region on mouse chromosome 1; however, FcγRIα and several FCRLs are found on mouse chromosome 3. In humans, the α subunit of the IgA receptor is found on chromosome 19 as is the lectin DC-SIGN (CD209) and the β subunit is on chromosome 11. The FcγRII–FcγRIII locus on chromosome 1 is further linked to a variety of lupus susceptibility genes found in that region, including the *Sle1* cluster.[45] A locus linked to atopy has been identified at 11q12–13 and further delineated polymorphisms of the β chain (I181V and V183L) that are associated with a heightened risk of atopy. However, a direct functional association of these polymorphisms with the known biological activities of the β chain has not been found.[46]

Polymorphisms in the α chains of the FcγRs have been described, most notably in FcγRIIA and FcγRIIIA; these polymorphisms result in differences in binding affinity to specific IgG subclasses.[47] For example, a histidine at position 131 in FcγRIIA results in higher affinity binding to IgG2 and IgG3 than does an arginine at that position. Similarly, FcγRIIIA with valine at position 158 of the α chain has a higher binding affinity for IgG1 and 3 than does the polymorphic form with phenylalanine at that position. These polymorphisms translate into a more robust ADCC response for the FcγRIIIA val/val and the FcγRIIA his/his haplotype in vitro and has been positively correlated with a better clinical response to antitumor antibodies inclunding the CD20-specific antibody rituximab, the Her2/neu-specific antibody trastuzumab, and the epidermal growth factor receptor–specific antibody cetuximab in human lymphoma, metastatic breast cancer, and colorectal cancer patient cohorts, respectively.[48–53] This data is consistent with previous results obtained with rituximab

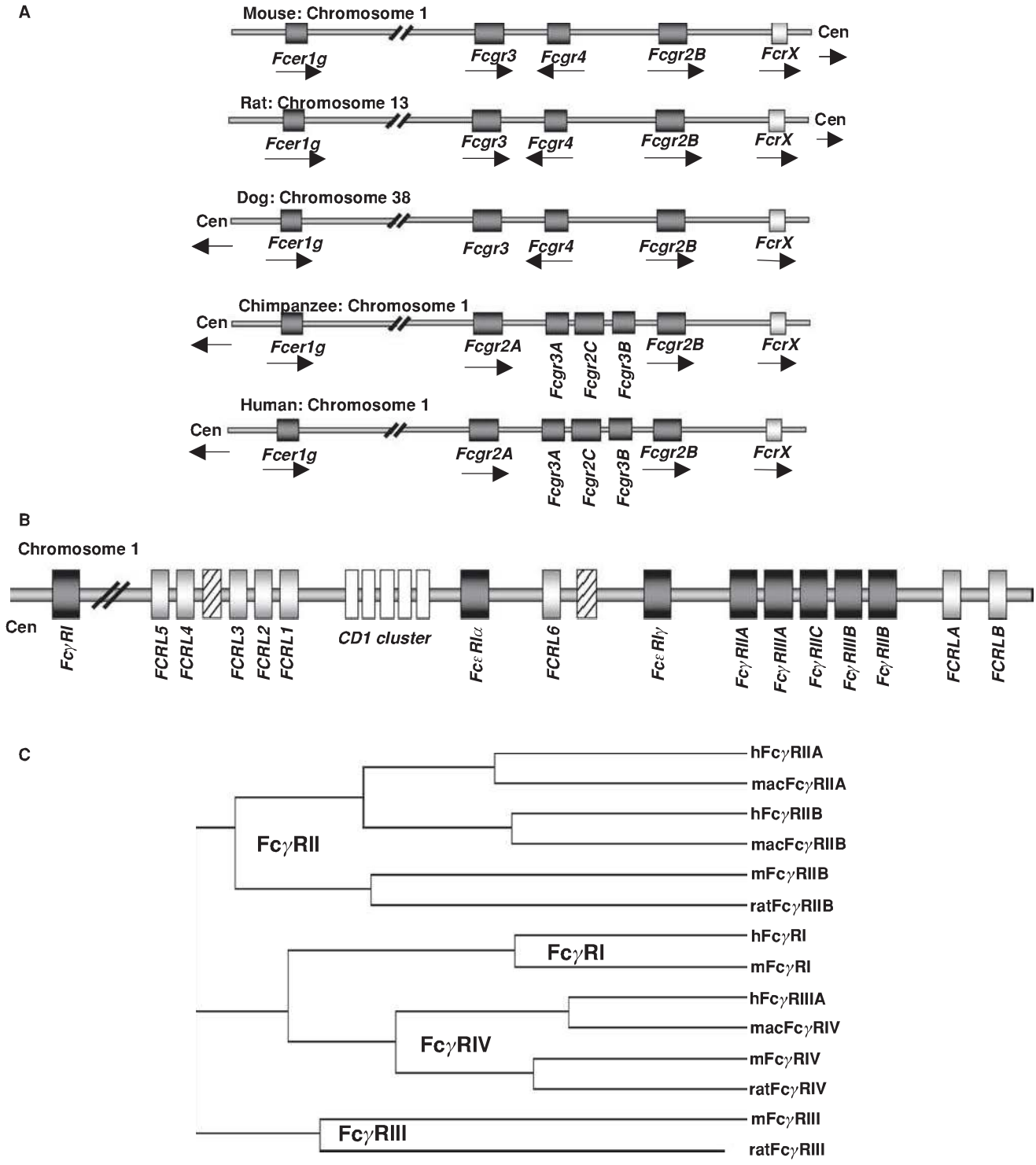


FIG. 24.2. Chromosomal Organization of Fc Receptor (FcR) and FcR-Like Genes. **A:** Localization of FcR genes in different species. **B:** The human FcR locus on chromosome 1. Classical FcR genes are shown in *dark grey*, FCRL genes as *light grey*, and the CD1 gene cluster on chromosome 1 as *white boxes*. Pseudogenes are indicated as hatched boxes. (adapted from Davis et al.[44]). **C:** The cladogramm shows the alignment of selective classical FcRs of humans (*h*), macaques (*mac*), mice (*m*), and rats.

and trastuzumab in mouse models, demonstrating an important role of cellular FcγRs for the activity of these therapeutic antibodies in vivo.[54,55] Four amino acids are polymorphic for FcγRIIIB at positions 18, 47, 64, and 88, which contribute to the neutrophil antigen polymorphisms for this receptor. Several studies have attempted to link specific FcR polymorphisms or copy number variations to autoimmune diseases, specifically to systemic lupus erythematosus (SLE).[56] Recent studies have reported associations in susceptibility to SLE in both murine and human populations with levels of FcγRIIB

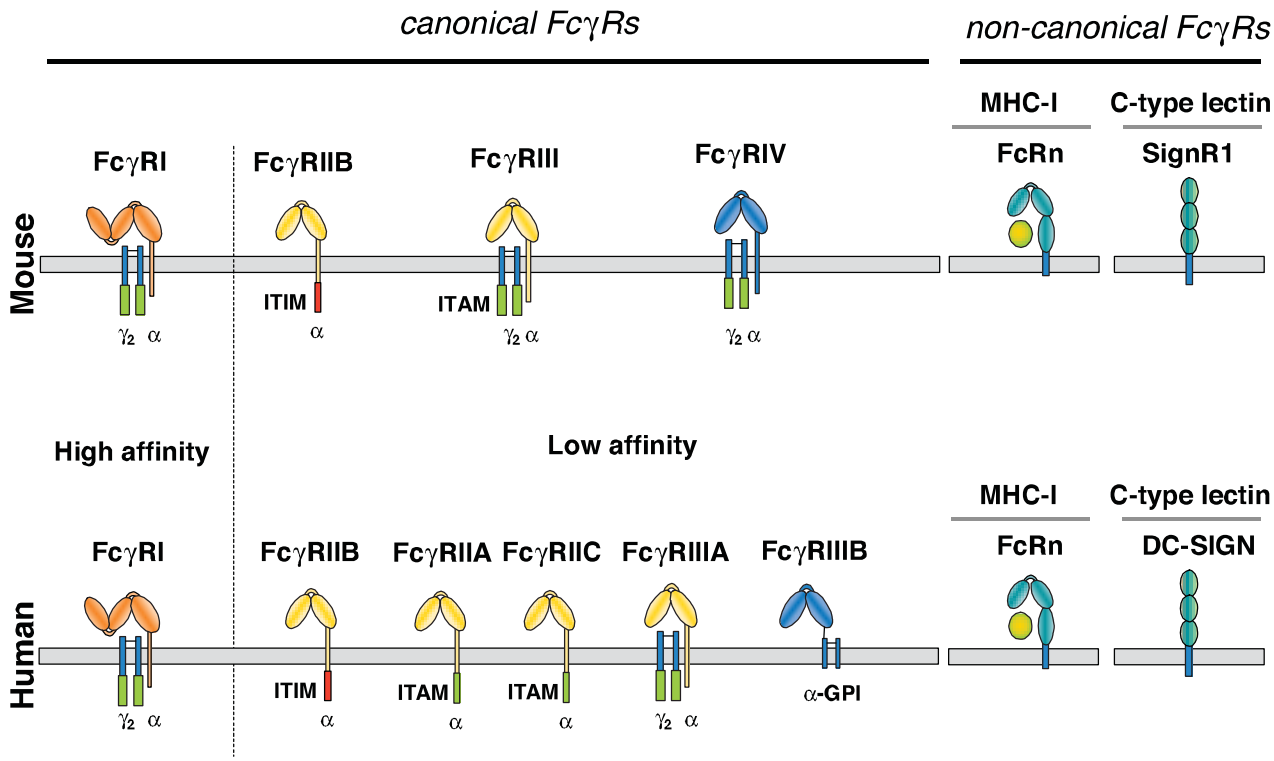


FIG. 24.3. Comparison of the Human and Mouse Canonical and Noncanonical Fcγ-Receptor Protein Family. In both species, canonical FcγRs can be distinguished by their affinity for the antibody Fc-portion (high or low affinity) and by the signaling pathways they trigger (activating versus inhibitory). The noncanonical neonatal FcR belongs to the family of major histocompatibility class I proteins and regulates immunoglobulin (Ig)G half life. Mouse SIGNR1 or human DC-SIGN are C-type lectin proteins that can only bind to IgG glycoforms rich in terminal sialic acid residues and are involved in the anti-inflammatory activity of IgG.

expression or alleles of FcγRIIB.[57] Reduced expression of FcγRIIB on activated B cells, such as memory cells, has been seen in patients with SLE and chronic inflammatory demyelinating polyneuropathy (CIDP), and is associated with a promoter polymorphism.[58,59] In mouse strains that develop a spontaneous, lupus-like disease, restoring the level of FcγRIIB expression on their B cells to a wild-type level reverses disease.[60,61] A higher incidence of an allele of FcγRIIB has been reported in several populations with autoimmune disease. This allele, found in the transmembrane domain of the receptor, is suggested to result in a hypomorphic phenotype, similar to the reduced expression observed.[57] Of note, despite increasing the risk for development of SLE, this FcγRIIB allele seems to decrease the likelihood of malaria infections and the severity of disease and can be found at increased frequencies in areas of the world where malaria is endemic.[62,63]

Species Comparisons

Detailed comparisons between the canonical FcRs in mice and humans have revealed several notable differences in both structure and expression of these molecules (Figs. 24.1 to 24.3). Whereas IgG, IgM, and IgE FcRs are conserved in these species, IgA FcRs are not. To date, a murine homolog for the IgA FcR has not been identified. In general, murine and human IgG FcRs display comparable degrees of heterogeneity and complexity.[41,64] Specific differences, however, have been noted. For example, FcγRI is encoded by a single gene in the mouse, in comparison with three genes in the human.[43] Two genes for activation FcRs, FcγRIIA and C, are found in the human and not rodents, which is notable because of their unusual single-chain activation structure.[65] Both mice and human encode a gene referred to as FcγRIIIA, although recent studies have identified a novel mouse FcR with higher homology to the human FcγRIIIA called FcγRIV.[31,64,66] As mentioned previously, FcγRIIIB is unique among FcRs in being expressed as a GPI-anchored protein (see Fig. 24.3). Its expression is limited to human neutrophils, in comparison with FcγRIIIA, which is expressed widely on cells of the myeloid lineage, such as macrophages, NK cells, mast cells, and DCs. Finally, both mice and humans have only a single gene encoding the inhibitory FcγRIIB molecule. Among the noncanonical FcRs, the lectin DC-SIGN is the functional homologue of the murine SIGN-R1 molecule with respect to its ability to bind sialylated IgG Fc and mediate an anti-inflammatory response.[67,68] While the overall structure of these proteins are similar (see Fig. 24.3), their patterns of expression are quite distinct. SIGN-R1 expression is restricted to marginal zone macrophages of the spleen and lymph node, whereas DC-SIGN expression is seen on myeloid DCs and macrophages in a variety of tissues, including spleen, bone marrow, and lymph node.[69]

The genes for the IgE FcR are conserved in mice and humans. The difference that is observed relates to the requirement for the β chain to achieve surface expression

in mice, precluding the expression of the $\alpha\gamma_2$ complex.[24] In humans, this form of the receptor is widely expressed on monocytes, Langerhans cells, and DCs, and is likely to be found on mast cells and basophils as well. This difference in FcεRI subunit composition is likely to result in functional differences as well. Although these specific interspecies differences are important, the fundamental organization of the canonical FcR system, with activation and inhibitory signaling through a shared ligand specificity coupled to opposing signaling pathways, is well conserved, as is the role of the noncanonical FcRs in mediating the immunomodulatory functions of sialylated IgG. Thus, conclusions regarding the function of this expanded FcR system in immunity by the analysis of murine models are relevant to an understanding of the role of these receptors to human immunity as well.

Expression

Canonical FcRs are expressed widely on cells of the myeloid lineage, including monocytes, macrophages, DCs, mast cells, basophils, neutrophils, eosinophils, and NK cells.[17,33] In addition, B cells and follicular DCs (FDCs) express the inhibitory FcγRIIB receptor, whereas T cells are generally negative for FcγR expression, but may express the Fc-receptor for IgM. Interestingly, the majority of FCRL proteins are expressed during varying stages of B-cell development. Despite their homology to the canonical FcRs, however, the FCRL proteins seem not to bind to Igs, rendering FcγRIIB the only IgG binding FcR on B cells. The specific expression pattern for each FcR varies, and these patterns are summarized in Figure 24.1. Because canonical FcRs represent a balanced system of activation and inhibition, the general rule of coexpression of FcRs of these classes is maintained. B cells use the B-cell antigen receptor as the activation coreceptor for FcγRIIB, whereas NK cells appear to utilize NK inhibitory receptors to modulate FcγRIIIA activation. The decoy FcγR, FcγRIIIB, is expressed exclusively on human neutrophils, on which it functions to concentrate and focus ICs without directly triggering cell activation, perhaps also playing role in neutrophil recruitment.[70] The FcγRIIA–FcγRIIB pair functions on neutrophils to modulate IC activation. FcεRI can be modulated by FcγRIIB, as demonstrated both in vitro and in vivo; mice deficient in FcγRIIB display enhanced IgE-triggered anaphylaxis[71] by virtue of the ability of IgE to bind with high affinity to FcεRI and with low affinity for FcγRIIB. Other mast cell inhibitory receptors, such as glycoprotein 49B1, modulate mast cell sensitivity to IgE: mice deficient in this molecule display enhanced anaphylactic responses to IgE stimulation.[72] Expression of the common γ chain is broad: it has been found on all myeloid and lymphoid cells examined to date. In contrast, the β chain appears to be quite restricted in its expression: it has been found only on mast cells and basophils.

Regulation of canonical FcR expression can occur at several levels. In general, cytokines involved in activation of inflammatory responses induce expression of activation FcγRs, whereas inhibitory cytokines downregulate these activation receptors. Transcriptional regulation of α chain levels has been documented for a variety of cytokines, including interferon-γ, interleukin (IL)-4, and transforming growth factor β.[73,74] In addition, complement component C5a binding to its receptor, C5aR, results in the induction of expression of activation FcγRs.[75] Induction of FcγRI, FcγRIIA, and FcγRIIIA α and γ chains in myeloid cells occurs upon interferon-γ treatment: IL-4 generally inhibits expression of these activation receptors but induces expression of the inhibitory FcγRIIB. Administration of intravenous gamma globulin, a widely used treatment for inflammatory diseases, has been shown to induce expression of the inhibitory FcγRIIB on effector macrophages and B cells in mice and humans.[59,76–78] This induction is not direct but mediated through other, noncanonical FcR pathways including molecules such as SIGNR1 and its human orthologe DC-SIGN (see Fig. 24.7). Engagement of these receptors by sialylated IgG results in an intrinsic Th2 pathway, ultimately leading to the expression of IL-4, which induces FcRIIB expression on inflammatory macrophages.[67,68,79,80] The situation in B cells is likely to be more complex, whereby regulation of FcγRIIB is critical for the maintenance of peripheral tolerance. Germinal center B cells downregulate FcγRIIB, perhaps in response to IL-4 production by T cells. Regulation of FcR expression has also been documented to occur upon binding of ligand. IgE regulates the expression of FcεRI by stabilizing the intracellular pool of receptor upon receptor engagement.[81] Thus, high IgE levels result in the induction of surface expression of FcεRI. However, this same mechanism of regulation is not seen for FcγRs: mice deficient in IgG have FcγR levels comparable with those of wild-type animals. Competition for limiting subunits also contributes to regulation of receptor expression. In mast cells, it appears that the level of γ chain is limiting. Competition between α chains for the limiting concentration of γ chain has been documented in mouse knockouts, whereby levels of one receptor increase if the α chain of the other receptor is reduced.[82] This type of reciprocal regulation has recently also been observed for FcγRs in neutrophils and is likely to be significant in the cross-regulation of FcRs by different isotypes of Ig.[83]

Three-Dimensional Structure

The crystal structures of FcγRIIA, FcγRIIB, FcγRIIIA, and FcεRI have been solved, as have the cocrystals of FcγRIIIA–IgG1 Fc and FcεRI–IgE Fc[84] (Fig. 24.4). These studies demonstrate that the receptors have a common structure in which the two extracellular Ig domains fold in a strongly bent overall structure, arranged into a heart-shaped domain structure. A 1:1 stoichiometry between the receptor and ligand is observed, with the receptor inserted into the cleft formed by the two chains of the Fc fragment (Cγ2 or Cε3). The binding region of the FcR to Fc fragments consists mainly of rather flexible loops that rearrange upon complex formation. Only domain 2 and the linker region connecting domains 1 and 2 interact in the complex with different regions of both chains of the Fc. Conserved tryptophans located on the FcRs interact with proline to form a "proline sandwich." A solvent-exposed hydrophobic residue at position 155 is conserved among all FcRs and represents a binding site for the important IgG1 residue Leu 235 (not

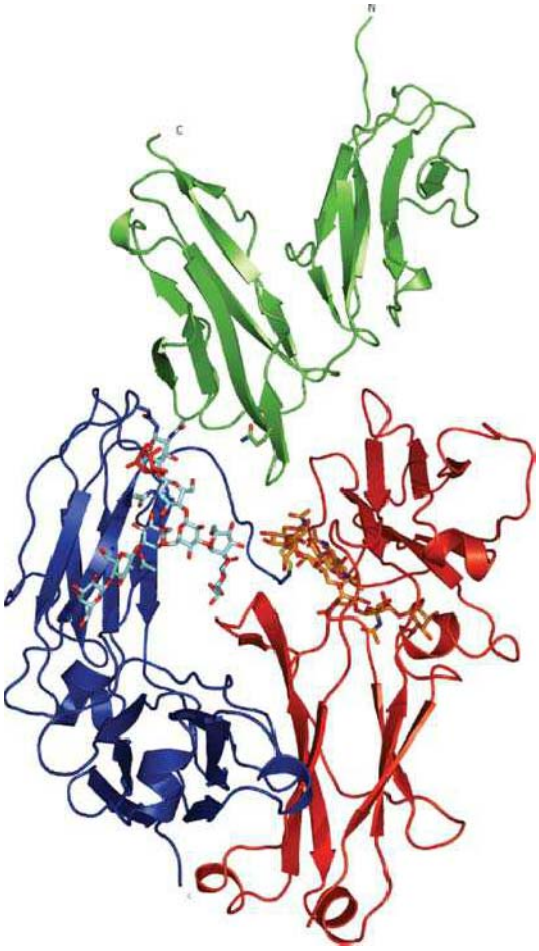


FIG. 24.4. Ribbon Diagram of Crystallizable Fragment (Fc) Receptor III–Immunoglobulin (IG)G1 Fc Structure. The carbohydrate moiety attached to both CH2 domains of the IgG Fc-fragment is shown as a stick and ball model. The extracellular domains of Fc receptor III are shown, together with the Fc fragment of IgG1. See text for details. Adapted from Sondermann et al.,[15] with permission.

found in IgE). Specificity is generated among the receptor–ligand pairs in a variable region connecting the two extracellular domains that is in contact with the lower hinge region of the Fc fragment (residues 234 to 238), a region not conserved among the IgGs and IgE. The binding region of FcRs to their Ig ligands does not overlap with other Fc binding molecules such as protein A, protein G, and FcRn.

The structure of the FcR bound to its ligand reveal that the antigen-binding fragment (Fab) arms are quite sharply bent and may adopt a perpendicular orientation toward the Fc. This arrangement would give the Fab arms maximal flexibility to bind antigen when the Fc fragment is oriented parallel to the membrane of the FcR-expressing cell. The asymmetrical interaction of the two Fc chains with a single FcR prevents a single antibody molecule from triggering dimerization of receptors and initiating signaling. Instead, dimerization is initiated by the interaction of antigen with the Fab arms, thus linking adaptive responses to effector cell triggering. Of note, the majority of the IgG-FcγR cocrystals have been generated with FcγRs produced in bacteria and therefore in an aglycosylated form. In general glycosylation at Asn 297 is critical for FcγR binding; modulation of FcγR binding by glycan modification has been observed for several classes of glycan modifications, including sialylation, fucosylation, and branching N-acetyl glucosamine.[85,86] The first IgG-FcγR cocrystal structure with differentially glycosylated IgG variants demonstrated that carbohydrate-carbohydrate interactions between IgG and FcγR are essential for high-affinity recognition of afucosylated IgG glycovariants by FcγRIIIA.[87] In contrast, low-affinity binding of FcγRIIIA to fucosylated IgG is independent of these carbohydrate interactions. These novel insights may offer new ways of optimizing IgG-FcγR interactions for enhanced therapeutic activity of IgG.

IN VITRO ACTIVITY

Binding Properties

As outlined in Figures 24.1 and 24.3, Ig binding to canonical FcRs falls into either high- or low-affinity binding classes. The high-affinity binding class is typified by FcεRI, with a binding affinity of 10^{10} M^{-1} for IgE, which ensures a monomeric interaction between IgE and its receptor. FcγRI binds with relatively high affinity for IgG1 and IgG3 (human) and IgG2a (mouse) with an affinity constant of 10^8 M^{-1}. In contrast to these high-affinity FcRs, the low-affinity receptors, such as the human FcγRIIA, FcγRIIB, FcγRIIIA, FcγRIIIB, and FcαRI, and the mouse FcγRIIB, FcγRIII, and FcγRIV bind with affinities ranging from 5×10^5 M^{-1} (FcγRIII) to 5×10^7 M^{-1}. This low-affinity binding ensures that these receptors interact with ICs and not monomeric ligands. As described subsequently, this dependence on high-avidity and low-affinity interactions ensures that these receptors are activated only by physiologically relevant ICs and not by circulating monomeric Ig, thus avoiding inappropriate activation of effector responses. In general, low-affinity FcγRs bind IgG1 and IgG3 preferentially; their binding to IgG2 and IgG4 is of even lower affinity. As mentioned previously, polymorphisms in FcγRIIA and FcγRIIIA affect binding to IgG2 and IgG1, respectively, which may have significance in vivo in predicting responses to specific cytotoxic antibodies (Table 24.1). The isolated consideration of the affinity of antibody isotypes to their activating FcγRs is not sufficient, however, to explain the differences in in vivo activity. More importantly, the ratio of the affinities of an antibody isotype to the activating FcγRs compared to the inhibitory FcγRIIB (termed A/I-ratio) predicts antibody activity more consistently.[20,64] Thus, antibody isotypes with a high A/I-ratio, such as mouse IgG2a and IgG2b, will show a greater activity than counterparts with a lower ratio (see Table 24.1). The sugar moiety of the antibody Fc-portion is essential for FcR binding and the presence or absence of certain sugar residues can significantly impact on antibody-FcR binding.[20,80,88,89] The absence of fucose, for example, will selectively increase the affinity of human IgG1 or mouse IgG2b for human FcγRIIIa or mouse FcγRIV, respectively. In contrast, the presence of terminal sialic acid on the N-linked Fc glycan reduces affinities for FcRs by an order of magnitude with a concomitant reduction in in vivo activity.[80] Subunit interactions have also

TABLE 24.1 Affinities of Mouse and Human Fc Receptors to Different Antibody Isotypes

	Mouse Soluble FcγR (K_A in M^{-1})					*Human Soluble FcγR (K_A in M^{-1})*						
	FcγRI	FcγRIIB	FcγRIII	FcγRIV	A/I	FcγRI	FcγRIIA^{131R}	FcγRIIA^{131H}	FcγRIIB	FcγRIIIA^{158F}	FcγRIIIA^{158V}	A/I[a]
mIgG1	n.b.	3.3×10^6	3.1×10^5	n.b.	0.1	n.d.	2.5×10^5	0.4×10^5	1×10^5	$< 10^{4b}$	n.d.	
mIgG2a	1.8×10^8	0.42×10^6	6.8×10^5	2.9×10^7	69	n.d.	3.2×10^5	1.7×10^5	1.6×10^5	1.0×10^5	n.d.	
mIgG2b	n.b.	2.2×10^6	6.4×10^5	1.7×10^7	7	n.d.	9.1×10^4	1.2×10^5	1.2×10^5	0.1×10^5	n.d.	
mIgG3	n.b.	n.b.	n.b.	n.b.	—	n.d.	$< 10^{4b}$	$< 10^{4b}$	$< 10^{4b}$	$< 10^{4b}$	n.d.	
hIgG1	3.8×10^6	2×10^5	3.5×10^4	2.2×10^6		9.1×10^{8d}	3.5×10^{5e}	5.2×10^{5e}	$1–3.8 \times 10^{5c,e}$	$0.4–1.1 \times 10^{6e}$	$1.9–4.8 \times 10^{6c,e}$	1/13
hIgG2	n.b.	$< 10^{4b}$	$< 10^{4b}$	n.b		n.b.	1.0×10^{5e}	4.5×10^{5e}	0.2×10^{5e}	0.3×10^{5e}	0.7×10^{5e}	1.5/3.5
hIgG3	1.2×10^6	8.3×10^4	n.b.	$< 10^{4b}$		6.1×10^{7e}	9.1×10^{5e}	8.9×10^{5e}	1.7×10^{5e}	7.7×10^{6e}	9.8×10^{6e}	45/57
hIgG4	7.2×10^4	$< 10^{4b}$	$< 10^{4b}$	$< 10^{4b}$		3.4×10^{7e}	2.1×10^{5e}	1.7×10^{5e}	2×10^{5e}	2.0×10^{5e}	2.5×10^{5e}	1

Binding constants were obtained by surface plasmon resonance analysis with immobilized antibodies (FITC-isotype switch variants) and soluble Fc receptors (FcRs) produced by transient transfection in 293T cells. Shown are the association constants (K_A in M^{-1}) of soluble FcR binding to the indicated antibody isotypes as determined by surface plasmon resonance analysis.[31,174–178] IG, immunoglobulin; n.b., no detectable binding or binding that is too low to be evaluated; n.d., that no surface plasmon resonance or other quantitative data are available.

[a]The two numbers indicate the A/I ratios for the low and high affinity allele of FcγRIII

[b]Indicates detectable but very low binding that did not allow to determine exact binding constants by surface plasmon resonance.

[c*]Maenaka et al.[174]; Okazaki et al.[175]

[d#]Paetz et al.[178]

[e§]Bruhns et al.[177]

been reported to influence affinity for ligand, as demonstrated for the common γchain associating with FcγRIIIA.[90] Its affinity for IgG1 is higher than the GPI-anchored form of this receptor, FcγRIIIB.

The crystal structures of IgG1-FcγRIIIA and IgE-FcεRI reveal similarities in the binding properties of these two complexes. Of significance is the 1:1 stoichiometry of the complexes, which ensures that a single receptor binds to a single immunoglobulin molecule.[15,16] This property in turn ensures that activation occurs upon cross-linking of receptor complexes by multivalent ligands. Two binding sites on the receptor interact asymmetrically with two sites on the Fc molecule. The FcR inserts into the cleft formed by the two chains of the Fc molecule, burying a binding surface of 895 Å for each binding site. Alterations in the Fc structure that reduce the cleft, such as deglycosylation of IgG, inhibit FcR binding. Four distinct regions have been defined in the Fc domains involved in FcR interactions. For IgE, this includes residues 334 to 336, 362 to 365, 393 to 396, and 424. The homologous regions for IgG are residues 234 to 239, 265 to 269, 297 to 299, and 327 to 332. Interactions of these residues occur with the carboxy-terminal domain 2 of the respective FcRs. In view of the similarities of these complexes and the homologies among the receptors and their ligands, an obvious question that arises concerns the molecular basis for specificity. Attempts to resolve that question have relied on mutagenesis studies of the ligands and domain exchanges between receptors. For example, exchange of the FG loop in domain 2 of Fcε to Fcγ receptors confers detectable IgE binding; similarly, variation in this loop in FcγRs may provide interactions that determine IgG specificity for these receptors. Mutagenesis of IgG1 revealed that a common set of residues is involved in binding to all FcγRs, but FcγRII and FcγRIII also utilize distinct residues.[91] Several IgG1 residues not found at the IgG–FcR interface by crystallographic determination had a profound effect on binding, which indicates the greater complexity of these interactions in solution.

The implications of these structural studies are that the Fc domain of IgG may be selectively mutated to direct its binding to specific FcγRs. Fc mutants that selectively engage activation FcγRs (IIIA and IIA) while minimally interacting with inhibitory and decoy FcγRs (IIB and IIIB) would confer optimal cytotoxic potential for tumoricidal applications. Indications that such Fc engineering is possible are suggested by IgG mutants with selective binding to FcγRIII or FcγRII.[92–94]

Effector Cell Activation

The critical step in triggering effector cell response by canonical FcRs is mediated by the cross-linking of these receptors by Ig. This can occur either by interactions of low-affinity, high-avidity IgG ICs or of IgG opsonized cells with activation FcγRs or by the cross-linking of monomeric IgG or IgE bound to FcγRI or FcεR, respectively, by multivalent antigens binding to the Fab of the antibody. Cross-linking of ITAM-bearing FcRs results in common cellular responses, determined by the cell type, rather than the FcR. Thus, for example, FcεRI or FcγRIII cross-linking of mast cells results in degranulation of these cells, whereas cross-linking of macrophage expressed FcαRI or FcγRIII by opsonized cells triggers phagocytosis. These functions underlie the functional similarity of activation FcRs in which cross-linking mediates cellular responses by ITAM-mediated tyrosine kinase cascades. In addition to degranulation and phagocytosis, activation FcR cross-linking has been demonstrated to induce ADCC, the oxidative burst, and the release of cytokines and other inflammatory cell mediators. A sustained calcium influx is associated with these functions, as is transcription of genes associated with the activated state.

Cellular activation initiated by ITAM-bearing activation FcRs can be enhanced by coengagement with integrin and complement receptors. Although the ability of these receptors to mediate phagocytosis, for example, are

modest, synergistic interactions with FcRs result in sustained activation and enhancement. Synergistic interactions between activation FcRs and toll receptors, mannose receptors, and other pattern-recognition molecules have also been reported in vitro and suggest that interplay between the innate and adaptive effector mechanisms of an immune response are involved in mediating efficient protection from microbial pathogens.

In contrast to the activation of effector cell responses triggered by cross-linking of ITAM-bearing FcRs in vitro, cross-linking of an ITIM-bearing inhibitory receptor to an ITAM-bearing receptor results in the arrest of these effector responses. Homoaggregation of FcγRIIB by its cross-linking on effector cells by ICs does not result in cellular responses; rather, it is the coengagement of ITAM- and ITIM-bearing receptors that results in the functional generation of an inhibitory signal. In vitro, it is possible to ligate any ITAM-bearing receptor to any ITIM-bearing receptor with a resulting inhibitory response. This activity is used functionally to define putative ITIMs and has proved to be a useful device in dissecting the signaling pathways induced by ITAM-ITIM coligation.

B-Lymphocyte Suppression

B-cell stimulation through the B-cell antigen receptor can be arrested by the coligation of FcγRIIB to the B-cell receptor (BCR). This occurs naturally when ICs, retained on FDCs in the germinal center, interact with both the BCR and FcγRIIB during the affinity maturation of an antibody response. In vitro suppression of B-cell activation has been demonstrated by coligation of BCR and FcγRIIB, resulting in arrest of calcium influx and proliferative responses triggered by the BCR,[25,95] the result of recruitment of the SH2-containing inositol 5′-phosphatase (SHIP)-1.[96] Calcium release from the endoplasmic reticulum is not affected, and there is thus an initial rise in intracellular calcium; however, this calcium flux is not sustained, because SHIP recruitment blocks calcium influx by uncoupling of the capacitance channel. Homoaggregation of FcγRIIB by ICs can trigger apoptosis in B cells, as demonstrated in the DT40 B-cell line and in murine splenocyte preparations.[97] This activity is retained in ITIM mutants and is dependent on the transmembrane sequence of FcγRIIB. Consistent with this, other studies showed that a SHIP-independent but BTK-, JNK1-, and cABL-dependent pathway is involved in induction of apoptosis upon FcγRIIB homoaggregation.[98] The potent inhibitory effect of FcγRIIB on B-cell activation has recently been used therapeutically to suppress autoantibody production by B cells. By engineering the Fc-fragment of CD19-specific antibodies to have increased affinity to FcγRIIB, resulting in an enhanced coengagment of the inhibitory FcγRIIB with the BCR signaling complex, it was possible to suppress humoral immunity in mice and to shut down autoantibody production by B cells.[94] In contrast to mature B cells, which coexpress the BCR and FcγRIIB, plasma cells responsible for high-level antibody production downregulate BCR expression but maintain FcγRIIB expression. It was suggested that crossliniking of FcγRIIB by ICs on bone marrow plasma cells might be involved in inducing apoptosis in a fraction of plasma cells thereby creating niches for newly generated plasma cells during exposure to a new antigenic stimulus.[99]

SIGNALING

Immunoreceptor Tyrosine-Based Activation Motif Pathways

The general features of signal transduction through ITAM receptors are conserved among all members of this family, including T-cell receptors, BCRs, and various FcRs. The 19 amino acid–conserved ITAM is necessary and sufficient to generate an activation response, as demonstrated by the analysis of chimeric receptors. With a single exception, FcRs associate with accessory subunits that contain these signaling motifs. As described previously, the common γ chain contains an ITAM and is associated with FcεRI, FcγRI, FcγRIII, FcγRIV, and FcαRI. In addition, both FcεRI and FcγRIII may associate with the β subunit in mast cells. The ITAM found in the β chain does not function as an autonomous activation cassette, as has been found for most other ITAMs. Rather, it functions to amplify the activation response generated by the γ chain ITAM by increasing the local concentration of Lyn available for activation upon aggregation of the receptor.[40] FcγRIIA contains an ITAM in the cytoplasmic domain of its ligand recognition α subunit and is thus able to activate in the absence of any associated subunit.

Upon sustained receptor aggregation, Src family kinases that may be associated with the receptor in an inactive form become activated and rapidly tyrosine-phosphorylate the ITAM sequences, creating SH2 sites for the docking and subsequent activation of Syk kinases. Ligands that rapidly dissociate from their receptors result in nonproductive signaling complexes that fail to couple to downstream events and behave as antagonists.[100] The specific Src kinase involved for each FcR depends on the receptor and cell type in which it is studied. Thus, Lyn is associated with the FcεRI pathway in mast cells, Lck is associated with FcγRIIIA in NK cells, and both of these kinases as well as Hck are associated with FcγRI and FcγRIIA in macrophages. After activation of the Src kinase, tyrosine phosphorylation of the ITAM motif rapidly ensues, leading to the recruitment and activation of Syk kinases. This two-step process is absolutely necessary to transduce the aggregation signal to a sustainable intracellular response. Once activated, Syk kinases lead to the phosphorylation or recruitment of a variety of intracellular substrates, including PI3K, Btk and other Tec family kinases, phospholipase C-γ(PLCγ), and adaptor proteins such as SLP-76 and BLNK. The Ras pathway is also activated through Sos bound to Grb2 that is recruited upon phosphorylation of Shc. Ras phosphorylates Raf, which in turn leads to MEK kinase and MAP kinase activation. A summary of these intracellular pathways is shown in Figure 24.5A. A crucial step in this sequential activation cascade occurs with the activation of PI3K by Syk. By generating phosphatidyl inositol polyphosphates, such as PIP_3, PI3K leads to the recruitment of pleckstrin homology (PH) domain–expressing proteins such as Btk and PLCγ which in turn leads to the

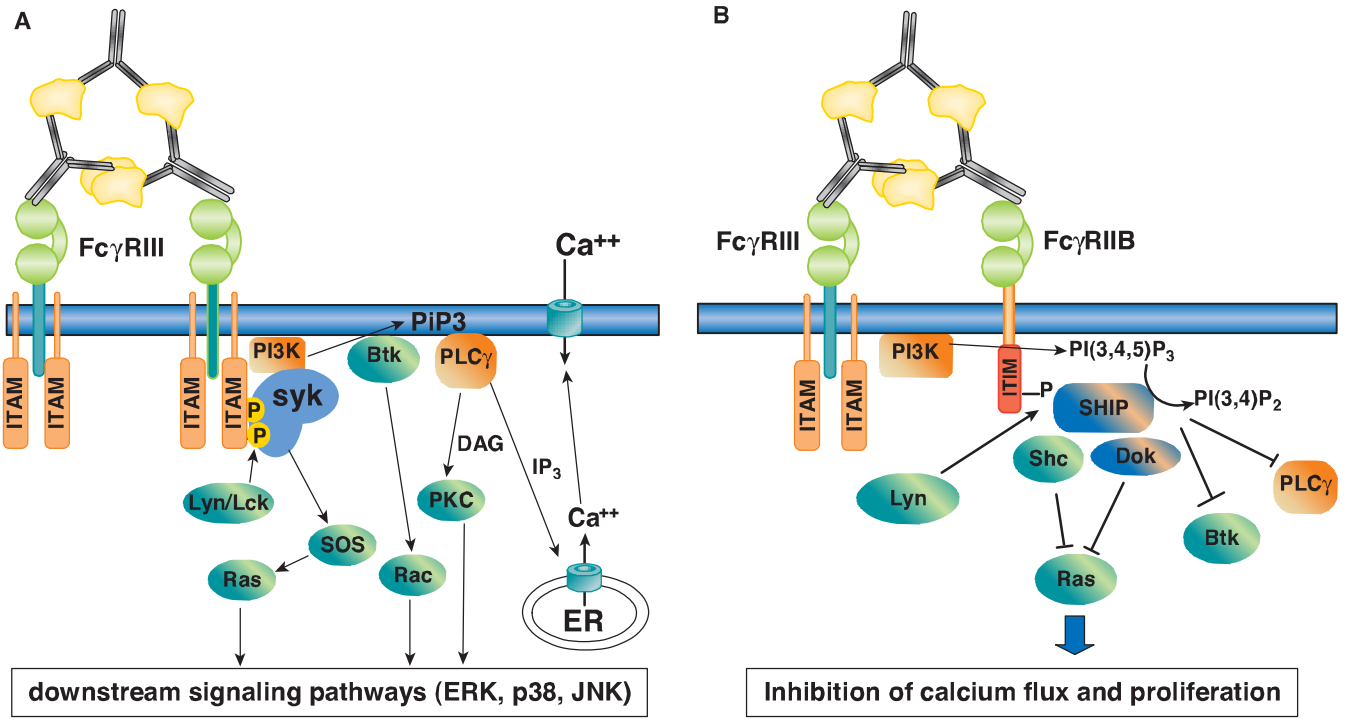


FIG. 24.5. Signaling by Activation and Inhibitory Fc Receptors (FcRs). **A:** FcγRIII signaling in natural killer cells is shown as an example of the activation class of FcRs. Cross-linking by an immune complex initiates the signaling cascade. The specific Src family kinase varies, depending on the cell type. **B:** FcR signaling in macrophages is shown as an example of simultaneous triggering of activation and inhibitory FcRs.

generation of inositol triphosphate and diacylglycerol, intermediates crucial to the mobilization of intracellular calcium and activation of protein kinase C, respectively.

Immunoreceptor Tyrosine-Based Inhibitory Motif Pathways

The inhibitory motif, embedded in the cytoplasmic domain of the single-chain FcγRIIB molecule, was defined as a 13 amino acid sequence AENTITYSLLKHP, shown to be both necessary and sufficient to mediate the inhibition of BCR-generated calcium mobilization and cellular proliferation.[25,26] Significantly, phosphorylation of the tyrosine of this motif was shown to occur upon BCR coligation and was required for its inhibitory activity. This modification generated an SH2 recognition domain that is the binding site for the inhibitory signaling molecule SHIP.[96,101] In addition to its expression on B cells, where it is the only IgG FcR, FcγRIIB is widely expressed on macrophages, neutrophils, mast cells, DCs, and FDCs, absent only from T and NK cells. Studies on FcγRIIB provided the impetus to identify similar sequences in other surface molecules that mediated cellular inhibition and resulted in the description of the ITIM, a general feature of inhibitory receptors.

FcγRIIB displays multiple inhibitory activities. Coengagement of FcγRIIB to an ITAM-containing receptor leads to tyrosine phosphorylation of the ITIM by the Lyn kinase, recruitment of SHIP, and the inhibition of ITAM-triggered calcium mobilization and cellular proliferation (see Fig. 24.5B).[96,102,103] These two activities result from different signaling pathways; calcium inhibition requires the phosphatase activity of SHIP to hydrolyze PIP_3 and the ensuing dissociation of PH domain–containing proteins such as Btk and PLCγ.[104] The net effect is to block calcium influx and prevent sustained calcium signaling. Calcium-dependent processes such as degranulation, phagocytosis, ADCC, cytokine release, and proinflammatory activation are all blocked. Arrest of proliferation in B cells is also dependent on the ITIM pathway, through the activation of the adaptor protein Dok and subsequent inactivation of MAP kinases.[105,106] The role of SHIP in this process has not been fully defined, inasmuch as it can affect proliferation in several ways. SHIP, through its catalytic phosphatase domain, can prevent activation of the PH domain survival factor Akt by hydrolysis of PIP_3.[107,108] SHIP also contains phosphotyrosine-binding domains that could act to recruit Dok to the membrane and provide access to the Lyn kinase that is involved in its activation. Dok-deficient B cells are unable to mediate FcγRIIB–triggered arrest of BCR-induced proliferation, while retaining their ability to inhibit a calcium influx, which demonstrates the dissociation of these two ITIM-dependent pathways.

Another inhibitory activity displayed by FcγRIIB is independent of the ITIM sequence and is displayed upon homoaggregation of the receptor. Under these conditions of FcγRIIB clustering, a proapoptotic signal is generated through the transmembrane sequence. This proapoptotic signal is blocked by recruitment of SHIP, which occurs upon coligation of FcγRIIB to the BCR, because of the Btk requirement for this apoptotic pathway.[97] This novel activity

has been reported only in B cells and has been proposed to act as a means of maintaining peripheral tolerance for B cells that have undergone somatic hypermutation. The in vivo relevance of this pathway remains to be proven.

IN VIVO FUNCTIONS

FcγReceptors in the Afferent Response

The ability of IgG ICs to influence the afferent response has been known since the 1950s and can be either enhancing or suppressive, depending on the precise combination of antibody and antigen and the mode of administration.[109] Investigators have attempted to define the molecular mechanisms behind these activities with the availability of defined mouse strains with mutations in activation or inhibitory FcRs and through the use of specific blocking antibodies to individual receptors. Direct effects on B cells stem from the ability of the inhibitory FcγRIIB molecule to influence the state of B-cell activation and survival. Because antigen is retained in the form of ICs on FDCs, it can interact with B cells by coengaging FcγRIIB with BCR, modulating the activation state of the cell. Mice deficient in this inhibitory receptor develop anti-deoxyribonucleic acid (DNA) and antichromatin antibodies and die of a fatal, autoimmune glomerulonephritis at 8 months of age. The phenotype is strain dependent and is not seen in BALB/c or 129 strains of mice.[110] Combining FcγRIIB deficiency with defects in other inhibitory receptor pathways, such as PD-1, results in autoantibodies with different specifities and distinct pathological presentation. Thus, PD–1–deficient Balb/c mice develop cardiomyopathy resulting from anticardiac myosin antibodies. PD-1/FcγRIIB double deficient BALB/c mice develop antiuroepithelial antibodies and hydronephrosis.[111] FcγRIIB thus acts as a modifier of autoimmune disease, a conclusion further supported by studies that determined the contribution of the C57BL/6 background to the spontaneous lupus-like disease observed in those mice. The B6 background, by virtue of an incomplete light chain editing pathway, provides a source of autoreactive B cells in the periphery, which, when combined with defects in the inhibitory FcγRIIB pathway, leads to the accumulation of autoantibodies, pathogenic ICs, and disease.[112] Further support for this conclusion is provided by the observations that autoimmune disease–prone strains of mice, such as New Zealand black (NZB), BXSB, SB/Le, MRL, and nonobese diabetic, have reduced surface expression of FcγRIIB on activated B cells, attributed to DNA polymorphisms in the promoter region of the gene encoding this receptor.[113,114] This reduced expression of FcγRIIB is thus suggested to contribute to the increased susceptibility of these animals to the development of autoantibodies and autoimmune disease. Direct evidence that this is indeed the case comes from studies where FcγRIIB expression levels have been restored by retrovirus-mediated gene transfer in BXSB, NZM, and FcγRIIB knockout animals. These mice had dramatically reduced levels of autoreactive antibodies and were protected from the development of fatal autoimmune disease.[60] Consistent with these results obtained in mouse model systems, human patients with SLE and CIDP were demonstrated to have a reduced FcγRIIB expression level on B cells.[58,59]

Moreover, if FcγRIIB indeed functions in vivo to maintain peripheral tolerance, then its loss should allow for the emergence of autoantibodies when otherwise resistant animals are challenged with potentially crossreactive antigens. This hypothesis has been validated in models of collagen-induced arthritis and Goodpasture syndrome. FcγRIIB-deficient mice, with the nonpermissive H-2^b haplotype, develop arthritis when immunized with bovine type II collagen.[115] The loss of FcγRIIB thus bypasses the requirement for the specific H-2^q and H-2^r alleles previously demonstrated to be necessary in this model by allowing FcγRIIB-deficient autoreactive B-cell clones to expand and produce pathogenic autoantibodies. When the permissive DBA/1 strain (H-2^q) is made deficient in FcγRIIB, autoantibody development is augmented and disease is greatly enhanced. In a similar manner, immunization of H-2^b mice deficient in FcγRIIB with bovine type IV collagen results in crossreactive autoantibodies to murine type IV collagen, with dramatic pathogenic effects.[115,116] These mice develop hemorrhagic lung disease and glomerulonephritis with a "ribbon deposition" pattern of ICs in the glomeruli. These characteristics are indicative of Goodpasture syndrome, a human disease not previously modeled in an animal species.

Expression of the inhibitory FcγRIIB on B cells thus provides a mechanism for the suppressive effects of ICs on antibody production. Although FcγRIIB is expressed throughout peripheral B-cell development, recent data suggest that it represents a late checkpoint controlling the expansion of autoreactive IgG-positive plasma cells. In contrast, deficiency of the inhibitory receptor did not impact the generation of autoreactive IgM antibodies.[112] Taking the considerably higher pathogenic potential of IgG compared to IgM antibodies into account, this late stage of FcγRIIB-mediated regulation seems to be sufficient to prevent severe autoreactive processes.

The enhancing property of ICs on the afferent response is likely to arise from the expression of FcRs on APCs, such as DCs.[117–119] DCs express all three classes of IgG FcRs as well as FcεRI. Although in vitro studies have suggested that triggering of activation FcRs can induce DC maturation, the in vivo significance of this pathway has not been established.[120] The ability of FcRs, particularly FcγRI, to internalize ICs could provide a mechanism for enhanced presentation and augmented antibody responses, whereas the presence of the inhibitory FcγRIIB molecule appears to reduce the enhancing effect. Mice deficient in FcγRIIB display enhanced antibody responses to soluble antibody–antigen complexes, in some cases dramatically so, which is likely to result from enhanced presentation.[121,122] In addition, in vitro studies suggest that internalization through specific FcRs on APCs may influence the epitopes presented and T-cell response generated as a result. At present, a growing body of data suggests that FcRs are indeed involved in enhancement of the afferent response, by influencing antigen presentation and cognate T-cell interactions. FcγRIIB-deficient DCs pulsed ex vivo with antigen in the form of ICs induce a strong and protective cytotoxic immune response after transfer into naïve mice.[123] In contrast, wild-type DCs induce a much smaller and nonprotective response, indicating that the threshold set by co-crosslinking of the inhibitory and activating FcRs

prevents complete DC maturation. Moreover, blocking of IC binding to FcγRIIB on human DCs with an FcγRIIB-specific antibody resulted in spontaneous maturation of the cells by ICs present in low amounts in human serum.[124,125] Taken together, these data indicate that the inhibitory receptor is an important regulator of DC activation. As the DC maturation state will determine whether an activating or a tolerogenic signal will be delivered to T cells, FcRs might be important factors for the maintenance of peripheral tolerance in the cellular immune system. Transiently blocking FcγRIIB activity with monoclonal antibodies in vivo might thus be an interesting strategy to optimize immunotherapeutic and vaccination approaches. Further defining the precise role of each FcR expressed on APCs will require conditional knockouts of these molecules on specific DC populations to resolve the contribution of these systems to the generation of an appropriate antibody response.

FcγReceptors in the Efferent Response

The first canonical FcR knockout to be described was for the common activation subunit, the γchain, which resulted in the loss of surface assembly and signaling of FcγRI, FcγRIII and FcγRIV as well as FcεRI (10). Mice deficient in the common γ chain were systematically studied in diverse models of inflammation and found to be unable to mediate IgG-triggered inflammatory responses for cytotoxic or IC reaction; attributed to low-affinity activation receptors, the high-affinity FcγRI played a minimal role in the in vivo inflammatory response triggered by IgG.[22,126–128] The results were further confirmed by comparisons of mice deficient or blocked for either FcγRI, FcγRIII, or FcγRIV.[19,20,32,129] The loss of FcεRI ablated IgE-mediated anaphylaxis; this was demonstrated independently by gene disruption in the α subunit of that receptor.[11] Subsequent studies on mice deficient in the inhibitory FcγRIIB molecule established the opposing action of this receptor, in which mice deficient in that receptor displayed enhanced B-cell responses, autoimmunity, and augmented IgG-mediated inflammation in a subclass and effector cell–dependent manner.[18,20,32,55,128] The general finding, which is discussed in detail subsequently, illustrates that IgGs initiate their effector responses in vivo through coengagement of activating and inhibitory FcRs. The physiological response is thus the net of the opposing activation and inhibitory signaling pathways that each receptor triggers and is determined by the level of expression of each receptor and the selective avidity of the IgG ligand (see Table 24.1). This also explains the longstanding observation that different IgG isotypes have a differential activity in vivo (Fig. 24.6A). The absence of a murine homolog for FcαRI has precluded similar studies for that receptor. Studies on mice bearing a human transgene of FcαRI suggest that this receptor is involved in IgA nephropathy (Berger disease).[130]

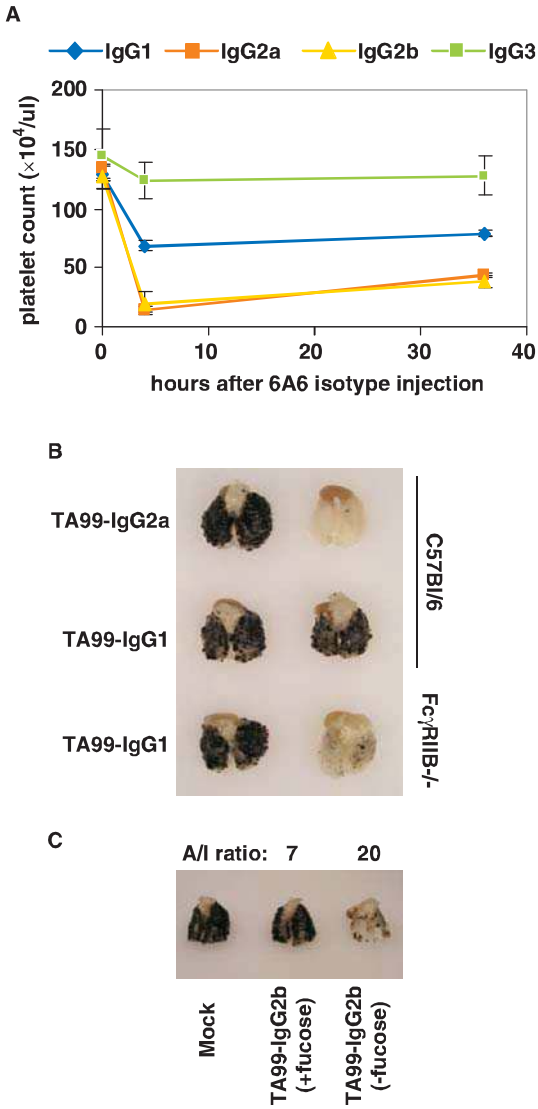


FIG. 24.6. Antibody Activity is Determined by Activating and Inhibitory Fc Receptors (FcRs). **A:** Mice were injected with the same amount of different 6A6 antibody isotypes, which recognize an integrin on mouse platelets and lead to FcR-dependent clearance of platelets from the blood as observed by the drop in platelet count after antibody injection. **B:** Wild-type or FcγRIIB knockout mice were injected intravenously with B16 melanoma cells on day 0 and with IgG1 and IgG2a isotype switch variants of the monoclonal antibody TA99 on alternate days. **C:** Mice were injected with antitumor antibodies containing or lacking branching fucose residues, thereby enhancing binding to activating FcRs and changing the A/I ratio (see text for details). Lungs were harvested on day 14. Adapted from Nimmerjahn and Ravetch,[20] with permission.

Type I: Immediate Hypersensitivity

Both cutaneous and systemic models of passive anaphylaxis, induced by IgE, were studied in FcRγchain–deficient mice and were found to be absent, a finding fully consistent with the observations obtained in FcεRI-deficient mice and confirming the role of the high-affinity IgE receptor in mediating IgE-induced anaphylactic responses.[10,11,40,131] FcγRIIB-deficient mice, challenged in this model, displayed an unexpected enhancement of IgE-mediated anaphylaxis, which suggests a physiological interaction between this inhibitory receptor and FcεRI.[71] The molecular basis for this modulation of FcεRI signaling by FcγRIIB has not been

determined, although previous studies have indicated that IgE can bind with low affinity to FcγRII/FcγIII, which suggests that there exists a mechanism for coengagement of these receptors. Deletion of the mast cell inhibitory receptor glycoprotein 49B1 also results in enhanced IgE-induced anaphylaxis.[72] In addition to FcεRI, mast cells also express the IgG FcγRIIB and FcγRIII, but not FcγRIV. Passive systemic anaphylaxis induced by IgG was attenuated in FcRγ chain–deficient and FcγRIII-deficient mice, which indicates the capacity of IgG and FcγRIII to mediate mast cell activation in vivo. FcγRIIB-deficient mice displayed enhanced IgG-induced anaphylaxis. Active anaphylaxis, induced by immunization with antigen in alum, was enhanced in FcεRI-, FcγRIIB-, and glycoprotein 49B1–deficient mice and attenuated in FcRγ- and FcγRIII-deficient mice. All these animals displayed antigen-specific antibodies for IgE and IgGs, which indicates that the active anaphylaxis seen was attributed primarily to IgG antibodies. The reason for the enhancement of anaphylactic responses in FcεRI-deficient animals resulted from the increased expression of FcγRIII on mast cells in these mice, normally limited by competition of α chains for the available pool of the common γ chain.[82] In the absence of FcεRI α chain, FcRγ chain is available to associate with FcγRIII α chain and assemble on the cell surface as a functional signaling receptor. A similar type of regulation was also described for expression of FcγRIII and FcγRIV on mouse neutrophils, where deletion of one receptor resulted in enhanced expression of the other γ-chain–dependent molecule.[83] These studies indicated the importance of the γ chain in regulating the level of surface expression of FcεRI and FcγRIII. Because γ chain is also associated with other members of the activation/inhibition paired receptors expressed on mast cells, such as PIR-A/PIR-B, the intracellular competition between these diverse α subunits and the common γ chain determines the level of surface expression of individual receptors and thus their ability to respond to specific biological stimuli. The absolute level of surface expression of FcRs on mast cells is clearly of therapeutic significance in both IgE- and IgG-mediated inflammatory responses; modulation of γ chain expression could thus represent a new therapeutic avenue for intervention in diseases such as anaphylaxis and asthma.

Type II Inflammation: Cytotoxic Immunoglobulin G

Cytotoxic IgGs are found in a variety of autoimmune disorders and have been developed for therapeutic indications in the treatment of infectious and neoplastic diseases. The mechanisms by which these antibodies trigger cytotoxicity in vivo have been investigated in FcR knockout mice. Anti-RBC antibodies trigger erythrophagocytosis of IgG-opsonized RBCs in an FcR-dependent manner; γ chain–deficient mice were protected from the pathogenic effect of these antibodies, whereas complement C3–deficient mice were indistinguishable from wild-type animals in their ability to clear the targeted RBCs.[132,133] FcγRIII plays the exclusive role in this process for the mouse IgG1 isotype. Murine IgG2a anti-RBC antibodies utilize primarily the FcγRIV receptor pathway despite the singular ability of murine IgG2a antibodies to bind as monomers to FcγRI. These and other studies suggest that the role of the high-affinity FcγRI in IgG-mediated inflammation is likely to be restricted to augmenting the effector response (determined by FcγRIII and IV) in situations that involve high concentrations of murine IgG2a antibodies which are found at localized inflammatory sites where FcγRI expression is induced on recruited macrophages.

Experimental models of immune thrombocytopenic purpura (ITP) in which murine IgG1 antiplatelet antibodies trigger thrombocytopenia and yielded results similar to those of the anti-RBC studies cited previously. The specific FcγR involved depended on the subclass of antibody used. IgG1 antibodies mediated their activity exclusively through FcγRIII, while IgG2a and 2b were FcγRIV dependent. In contrast, FcγRI- or C3-deficient mice were fully susceptible to antibody-induced thrombocytopenia.[20,31] FcγRIIB-deficient mice showed an isotype-specific enhancement of antibody-mediated platelet depletion, with the strongest impact on IgG1 and much smaller increases for IgG2a and IgG2b isotypes. This is consistent with the affinities of these isotypes for their specific activating and the inhibitory receptor, which will determine antibody activity in vivo (see Table 24.1). In a passive protection model of *Cryptococcus neoformans*–induced disease, passive immunization with mouse IgG1, IgG2a, and IgG2b antibodies resulted in protection in wild-type animals but not in FcRγchain–deficient animals.[134]

IgG antibodies raised to murine glomerular basement membrane preparations induce acute glomerulonephritis in a model of Goodpasture disease in wild-type but not FcRγ- or FcγRIV-deficient animals.[77,135,136] FcγRIIB-deficient animals displayed enhanced disease in this model, which indicates that the effector cells involved were constitutively expressing significant levels of FcγRIIB. Similar results were obtained when DBA/1 animals were immunized with bovine type II collagen to induce arthritis. Deficiency of FcRγ chain protected these mice from the pathogenic effects of the anticollagen antibodies that were generated.[137] As mentioned previously, deficiency of FcγRIIB in the DBA/1 collagen-induced arthritis model resulted in enhanced disease, through increased autoantibody production and elevated effector responses.

A dramatic example of the importance of these pathways in determining the in vivo activity of cytotoxic antibodies was obtained in models of antitumor antibody response. In a syngenic murine model of metastatic melanoma, a murine IgG2a antimelanocyte antibody was able to reduce tumor metastasis in wild-type animals but was ineffective in FcRγ- or FcγRIV-deficient mice.[20,83,138] In the absence of FcγRIIB, the activity of an IgG1 antibody, matched in its antigen binding domain, was enhanced, which indicates that the in vivo cytotoxic activity of the antibody was the net of activation and inhibitory receptor engagement[20,55] (see Fig. 24.6B). These studies, together with similar studies performed with antiplatelet antibodies or defucosylated antibodies, demonstrated that the in vivo activity of a cytotoxic antibody could be predicted by a simple equilibrium binding model in which the ratio of the monomeric affinity constants for

the activation and inhibitory Fc receptors (A/I ratio) are the dominant parameters, as demonstrated for T-cell receptor–major histocompability complex interactions.[139] An example for the enhanced cytotoxic activity of an antitumor antibody due to increased affinity for activating FcRs is shown in Figure 24.6C. The generation of an afucosylated antitumor antibody resulted in an increase of the A/I ratio of 7 to 20 for this IgG subclass, resulting in enhanced antitumor activity in vivo. Xenograft models of human tumors transplated into nude mice demonstrated, for a variety of tumors and cytotoxic antibodies, the requirement for FcR effector activity. For example, human breast carcinoma or lymphoma lines transplanted into nude mice and treated with either the humanized IgG1 or chimerized IgG1 antibodies (trastuzumab and rituximab), respectively, revealed that the ability of these antibodies to modulate tumor growth was abrogated in FcRγ chain–deficient mice. A point mutation that eliminated FcR binding of the anti-Her2/neu murine IgG1 antibody 4D5 abolished the in vivo cytotoxic activity of the antibody against a human xenograft but did not affect the in vitro growth inhibitory activity; this again illustrates the difference between in vivo and in vitro mechanisms. Similar results were obtained for T-cell lymphoma xenograft models and anti-CD2 antibodies, among others.[140,141] The general conclusions that can be drawn from these studies support a dominant role for the low-affinity activating FcγRs in mediating cytotoxicity by IgG antibodies. FcγRIIB restricts the effector response for those antibodies with low A/I ratios and in situations in which the effector cell expresses this inhibitory molecule.

The relevance of these murine in vivo studies to the treatment of human populations with antitumor cytotoxic antibodies has been demonstrated in two studies that investigated the differential responses of patients treated with anti-CD20 (rituximab) for lymphoma.[48,52] Both studies demonstrated a highly significant correlation between patient response, as measured by the time to relapse, and alleles of FcγRIIIA. In patients with an allele of this low-affinity activation receptor (158V) that confers higher binding affinity for human IgG1 Fc, improved outcome was observed, as compared to those with a lower binding allele of this receptor (158F). In addition, lymphoma patients with the high affinity allele showed a significantly better clinical response after receiving anti-idiotype vaccination.[51]

Type III Responses: Immune Complex–Mediated Inflammation

The classic example of this reaction, the Arthus reaction, has been studied in a variety of FcR- and complement-deficient animals. The initial studies were performed by using the cutaneous reverse passive Arthus reaction, in which antibody was injected intradermally and antigen was given intravenously. An inflammatory response, characterized by edema, hemorrhage, and neutrophil infiltration, developed within 2 hours. This reaction was elicited in a variety of complement- and FcR-deficient animals. The results from several independent studies confirmed the initial observations: that IgG ICs triggered cutaneous inflammatory reactions even in the absence of complement but displayed an absolute requirement for FcγR activation.[22] FcγRIIB modulated the magnitude of the response, with enhanced Arthus reactions observed in FcγRIIB-deficient strains.[18] The effector cell in the cutaneous reaction was determined to be the mast cell, as demonstrated by the use of mast cell–deficient strains and by mast cell reconstitution studies.[131] The generality of this result was demonstrated in similar reactions performed in the lung, illustrating the FcR dependence and relative complement independence of this response.[116] Thus, all studies have demonstrated an absolute dependence on FcR expression in the Arthus reaction. One model for IC-induced arthritis, the KRN/nonobese diabetic model, in which IgG1 anti-GPI antibodies are responsible for IC deposition in the synovium,[142] has been shown to depend on both FcγRIII and C3 but not on components of the classical complement pathway, such as C1q and C4; transfer of serum to animals deleted for FcγRIII or C3 prevented the development of disease.[143,144] Deficiency in the late components of complement, such as C5a or its receptor, have also been reported to result in a partial reduction in the magnitude of the response in IC-induced lung inflammation[145] and to result in a complete block in the KRN/nonobese diabetic arthritis model. The likely mechanism by which C5a exerts its effects is through upregulation of activating FcγRs, resulting in an amplification loop.[75] C5a is generated in this system as a result of FcR activation of effector cells and is independent of the classical, alternative, and mannan-binding protein pathways. Binding of C5a to the C5aR results in upregulation of activation receptors on these effector macrophages, thus augmenting the inflammatory response triggered by FcRs. These studies have led to a revision of the hypotheses about the mechanism of IC-mediated inflammation, typified by the Arthus reaction, in which there is an absolute requirement for activating FcγRs in initiating mast cell activation by ICs. FcγR activation is, in turn, modulated by the inhibitory receptor FcγRIIB. The A/I value of a specific IgG antibody and the densities of these opposing signaling receptors determines the concentration threshold for IC activation and the magnitude of the effector response that can be obtained. The classical pathway of complement activation is not required; however, C5a activation, through the FcγR pathway, may enhance the response under some circumstances through an amplifying loop. The release of inflammatory mediators such as vasoactive amines, chemokines, and cytokines leads to the hallmarks of this reaction: edema, hemorrhage, and neutrophil infiltration at the site of IC deposition.

The significance of the FcR pathway in initiating IC inflammation in autoimmune disease was further established by investigating a spontaneous murine model of lupus, the B/W F1 mouse. The Arthus reaction results predicted the absolute requirement of activation FcγR in initiating inflammation and tissue damage in IC diseases such as lupus. The FcRγ chain deletion was backcrossed onto the NZB and New Zealand white strains for eight generations, and the intercrossed progeny were segregated into B/W FcRγ−/− and FcRγ+/−. Anti-DNA antibodies and circulating ICs developed in all animals; IC and complement C3 deposition was similarly observed in all animals. However, mice deficient in the common γ chain showed no evidence of glomerulonephritis

and had normal life expectancy, despite comparable levels of circulating ICs and glomerular deposition of these complexes along with complement C3. Mice heterozygous for the γchain mutation were indistinguishable from B/W F1 animals with wild-type γchains in developing glomerulonephritis and displaying reduced viability.[127] This spontaneous model supports the conclusions stated previously about the absolute requirement for FcγRIII in the activation of inflammatory disease by ICs: in the absence of this receptor, deposited ICs and C3 are not sufficient to trigger effector cell activation, which indicates that it is possible to uncouple pathogenic ICs from inflammatory disease by removing activating FcR engagement. Similar conclusions were reached in a murine model of Goodpasture disease where IC deposition, composed of mouse IgG2b antibodies, resulted in a fulminant glomerulonephritis.[77] Blocking the relevant activation FcR, FcγRIV, with a monoclonal antibody, protected the animals from fatal disease. These results further indicate that intervention in the effector stage of IC diseases, such as lupus and rheumatoid arthritis, would be accomplished by blocking activation Fcγ Rs to prevent initiation of effector cell responses.

Immunoglobulin G–Dependent Neutralization and Induction of Agonistic Signaling

In contrast to these FcR-dependent activities, IgG molecules, by virtue of their exquisite specifity for discrete antigens, are expected to mediate FcγR-independent effects, such as neutralization of bacterial toxins or viruses. Here, IgG molecules would prevent the toxin or microorganism from binding to its cellular receptor, thereby protecting the host from the pathogenic effects of the infecting microbe. This picture changed, however, as it was demonstrated that both IgG-mediated toxin and virus neutralization can be dependent on cellular FcRs in vivo. Thus, neutralization of the anthrax toxin (called protective antigen component) by anti–protective antigen antibodies required FcR expression in vivo and anti–protective antigen antibodies that differed only by their Fc subclasses showed a hierarchy of protection with IgG2a and IgG2b antibodies affording a better protection than IgG1 antibodies, consistent with previous observation for cytotoxic IgG.[146,147] Similar results were obtained for the mechanism of activity of neutralizing antibodies protecting from influenza or human immunodeficiency virus infection.[148,149] These results suggest that current in vitro assays using only the pathogen-specific antibody, a target cell line might miss an important component required for antibody activity in vivo, and that novel assays including the FcR-dependent component might provide a better predictability of IgG activity.[150]

Beyond the central function of IgG in host defense, agonistic antibodies triggering signaling pathways in target cells are being evaluated for their therapeutic potential. For example, agonistic antibodies for tumor necrosis factor receptor family members such as anti-CD40 or anti-DR5 (drozitumab) are under evaluation for their ability to enhance tumor cell clearance by both direct and indirect mechanisms. Interestingly, the apoptosis inducing activity of anti-DR5 agonistic antibodies was found to be dependent on FcRs in vivo.[151] A similar dependence on FcRs for in vivo activity was demonstrated for agonistic anti-Fas antibodies.[152] A variety of CD40-specific agonistic antibodies have been used to deliver costimulatory signals to augment and sustain T-cell responses to result in tumor clearance.[151] Recent studies showed that the inhibitory FcγRIIB was essential for this agonistic activity and could not be replaced by activation FcγRs.[153,154] Although the exact mechanism of these FcR-dependent pathways remain to be established, these findings open the path toward generating improved agonistic anti-tumor necrosis factor receptor antibodies by Fc engineering. Consistent with this notion, a recent study showed that generating anti-CD40 antibodies with enhanced FcγRIIB binding considerably augmented their in vivo adjuvant activity.[154]

DISEASE ASSOCIATIONS

Autoimmunity and Tolerance

In view of their functional capacity to link autoantibodies to effector cells, FcRs have naturally been considered to have a pathogenic role in the development of autoimmune diseases. Several studies have attempted to correlate specific polymorphisms in FcγRIIA, FcγRIIIA, or FcγRIIIB with incidence or severity of lupus or rheumatoid arthritis.[155] In view of the heterogeneity of these diseases, it is perhaps not surprising that inconsistent results have been obtained. Alleles that increase the ability of FcγRIIA to bind IgG2 or FcγRIIIA to bind IgG1 might be expected to correlate with disease severity in some populations. Indeed, these types of associations have been reported in some studies but not in others.[156] These variable results have often been explained as an indication that other genes may be in linkage disequilibrium with the FcR alleles under investigation. This is a plausible explanation when viewed in light of the autoimmunity susceptibility genes mapping in or near the region of the FcR genes, chromosome 1q21–24.[157] This region of chromosome 1 has been implicated in a variety of human and murine linkage studies. For example, the Sle1 alleles derived from NZB flank the FcγRIIB gene and form a linkage group with the ability to break tolerance to nuclear antigens, resulting in production of antichromatin antibodies. Epistatic interactions between FcγRIIB and lupus susceptibility genes have been demonstrated in the murine lupus model of B6.RIIB. Crossing the *yaa* gene to this strain accelerates the development of disease; 50% survival is decreased from 8 months to 4 months, with 100% fatality by 8 months. This increase in severity correlates with a change in the specificity of the autoantibodies, from diffuse antinuclear antibodies to antibodies that stain with a punctate, nucleolar pattern on antinuclear antibody staining. Recently, it has been shown that the yaa susceptibility locus contains a duplication of the toll-like receptor 7 gene in the pseudoautosomal region of the y-chromosome (158). Moreover, toll-like receptor 7 and toll-like receptor 9 with their ability to recognize potential self-antigens such as ribonucleic acid or DNA, respectively, were shown to be important components in the generation of pathogenic autoreactive antibodies.[159,160] Compared to other Yaa-independent SLE susceptibility loci, FcγRIIB may be a dominant factor for the development of autoantibodies

and the systemic activation of the immune system as demonstrated by a study using NZB/BXSB F1 animals congenic for the wild-type *fcgr2b* gene. In these mice, a dramatic reduction in the production of autoantibodies and immune cell activation was noted compared to mice with impaired FcγRIIB expression.[161] These data are consistent with studies that overexpressed FcγRIIB in autoimmune-prone mouse strains resulting in suppression of autoantibody production.[60,61]

Two types of polymorphisms in the FcγRIIB gene have been associated with SLE. Promoter polymorphisms have been described resulting in reduced expression of FcγRIIB on activated B cells in both mouse and human SLE and CIDP populations.[59,113,162] In addition, a polymorphism in the transmembrane domain of FcγRIIB has been identified that is associated with susceptibility to SLE in Japanese populations. This polymorphism results in an FcγRIIB protein with reduced ability to enter lipid rafts and thus behaves as a hypomorphic allele for inhibitory function.[163]

Together, these studies point to FcγRIIB as a susceptibility factor in the development of autoimmunity with the ability to interact with other susceptibility factors to modify both the afferent and efferent limbs of the autoimmune response.

Inflammation

Antibody-mediated inflammatory diseases have been clearly demonstrated to involve the coupling of pathogenic autoantibodies or ICs to cellular FcRs. Therapeutics targeted to disrupt these interactions are in development, beginning with a monoclonal antibody to human IgE that functions to reduce IgE binding to its high-affinity receptor and thereby prevent allergic and anaphylactic reactions.[164] Because IgE is required for the survival of mast cells as well as in the regulation of FcεRI expression, reduction in IgE has synergistic effects on the ligand, receptor, and effector cell. The success of this approach will undoubtedly lead to other approaches that target the receptor or its signaling pathway. Blocking FcγRIIIA or IIA is expected to mimic the phenotype of FcRgamma-deficient animals in models of IgG-induced disease. Early attempts to use this approach in ITP were promising but limited by the crossreactivity to receptors on neutrophils, which led to neutropenia and the development of immune response to the murine antibody.[165] Development of second-generation anti-FcγRIIIA antibodies with greater specificity and reduced toxicity now appears to be a viable approach for the treatment of autoimmune diseases. Moreover, using small molecules to inhibit signaling molecules such as Syk seems to have promising effects in preventing autoantibody dependent platelet depletion in mice and humans.[166]

An alternative approach to limiting the activation of FcRs is to utilize the endogenous inhibitory pathway to abrogate IgE or IgG activation of their cognate receptors through coligation to FcγRIIB. This mechanism has been proposed to explain the ability to induce desensitization for the treatment of allergic diseases.[33,167] Inducing production of IgG antibodies to an allergen may facilitate cross-linking of FcγRIIB to FcεRI. A similar approach involving anti-CD19 antibodies containing an engineered Fc-fragment with enhanced binding to FcγRIIB has been successfully used to shut down autoantibody production by human B cells in a xenograft mouse model system.[168] The ability to exploit the inhibitory pathway to reduce the activity of activation FcγRs has been demonstrated to account for some of the anti-inflammatory activity associated with high-dose intravenous gamma globulin (IVIG), which consists of the pooled IgG fraction of serum from thousands of donors.[76] The use of IVIG for the treatment of ITP and other autoimmune diseases is well established, although the mechanism of action has been elusive. In murine models of ITP, arthritis, and nephritis, it has been demonstrated that protection by IVIG is dependent on the presence of FcγRIIB; deletion of FcγRIIB or blocking FcγRIIB by a monoclonal antibody eliminates the ability of IVIG to protect the animal against an inflammatory response.[76,78,80] IVIG was demonstrated to lead to the in vivo induction of FcγRIIB on splenic effector macrophages, which would raise the threshold required for platelet clearance by activating FcγRs on these cells.[20,31,78,83] A similar upregulation of the human inhibitory FcγRIIB was demonstrated in patients with CIDP receiving IVIG treatment as a first-line therapy.[59] Recently, it has become clear that the sialic acid–rich fraction of IgG antibodies in the IVIG preparation is responsible for the anti-inflammatory activity and FcγRIIB upregulation, enabling for the first time the generation of a recombinant IVIG replacement consisting of a sialic acid–rich IgG Fc fragment.[79,80] Human and mouse sialic acid–rich IgG has a reduced affinity for classical FcRs and acquires the capacity to bind to SIGNR1 strongly expressed on mouse splenic marginal zone macrophages.[68,80] By using SIGNR1-deficient mice expressing human DC-SIGN, it was demonstrated that human DC-SIGN expressed on macrophages or DCs can substitute for mouse SIGNR1 affording a candidate molecule essential for the anti-inflammatory activity of IVIG in humans.[67] Both molecules belong to the C-type lectin family and have been implicated in recognition of pathogenic microorganisms including human immunodeficiency virus and *Mycobacterium tuberculosis*.[169] Therefore, SIGNR1 and human DC-SIGN can be considered as noncanonical FcRs recognizing specific IgG glycovariants and, together with the family of toll-like receptors, provide a prime example for the dual usage of one molecule in the recognition of self- and non–self-ligands. Moreover, infusion of sialic acid–rich IgG resulted in an IL33-dependent expansion of basophils and IL4 production. IL4 is one of the interleukins known to upregulate FcγRIIB on innate immune effector cells, consistent with the upregulation of FcγRIIB noted in mouse model systems and humans. Together with previous data on the dependence of FcγRIIB upregulation on colony-stimulating factor-1–dependent macrophages, so called sensor macrophages, this suggests the following model for the anti-inflammatory activity of IVIG (Fig. 24.7). In this model, sialic acid–rich IVIG binds to mouse SIGNR1 or human DC-SIGN on sensor macrophages or DCs, which results in the production of IL33 that in turn leads to IL4 production by basophils, inducing the upregulation of FcγRIIB on effector macrophages, thereby raising the threshold for activation.[67] There is evidence that this

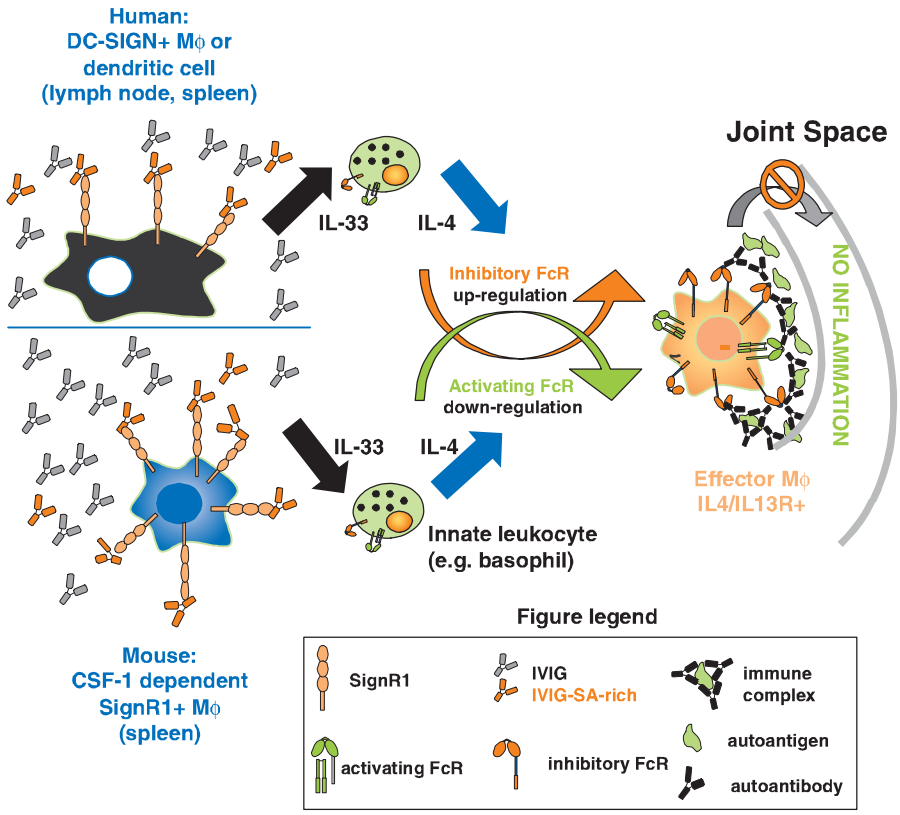


FIG. 24.7. Model for the Anti-inflammatory Activity of Intravenous Gamma Globulin (IVIG) in Mice and Humans. Sialic acid–rich antibodies in the IVIG preparation lose affinity for classical FcγRs and acquire the capacity to bind to molecules such as mouse SIGNR1 or human DC-SIGN on the surface of colony-stimulating factor-1–dependent sensor macrophages or dendritic cells. This results in the production of IL33 and the expansion of innate leukocytes such as basophils followed by IL4 release resulting in the upregulation of the inhibitory FcγRIIB and downmodulation of activating FcγRs on effector macrophages inhibiting the release of inflammatory mediators and tissue destruction.

anti-inflammatory pathway triggered by IgG glycovariants rich in terminal sialic acid residues is of general importance for maintaining immune homeostasis. Thus, patients with rheumatoid arthritis and autoimmune-prone mouse strains have reduced levels of serum IgG–rich containing terminal sialic acid and galactose residues during active phases of the disease.[170,171] A similar reduction of this anti-inflammatory IgG variant is observed during aging, consistent with an increased probability to develop autoimmune disease.[172] This downmodulation of sialic acid–rich IgG was also observed during normal immunization conditions in mice, suggesting the general importance of this phenomenon during proinflammatory conditions.[80] The opposite type of regulation is seen in women with rheumatoid arthritis during pregnancy.[173] Parallelling the decreased frequency of arthritic flares an increased level of IgG glycoforms rich in terminal galactose and sialic acid residues was observed. In addition, inducing expression of FcγRIIB might be a clinically feasible approach and could be effective at modulating pathogenic autoantibodies from activation effector cell responses through activating FcRs.

Studies on the FcαRI receptor have demonstrated a role for this molecule in the pathogenesis of IgA nephropathy, in which circulating macromolecular complexes are deposited in the mesangium, resulting in hematuria and eventually leading to renal failure. Soluble FcαRI is found in the circulating IgA complexes, which suggests a role for the receptor in the formation of these pathogenic complexes. A transgenic mouse expressing FcαRI spontaneously develops IgA nephropathy resulting from the interaction of polymeric mouse IgA and the human FcαRI receptor to release soluble receptor–IgA complexes, which leads to deposition in the mesangium and the sequelae of IgA neuropathy.

CONCLUSION

Receptors for the Fc of immunoglobulins provide an essential link between the humoral and adaptive response, translating the specificity of antibody diversity into cellular responses. The canonical FcRs mediate their biological responses through the coupling of Fc recognition to ITAM-/ITIM-based signaling motifs. A diverse array of biological responses depends on the FcR system, influencing both the afferent and efferent limbs of the immune response. Detailed biochemical, structural, and molecular biological data have provided a detailed understanding of how these receptors are regulated, are assembled, bind their ligand, and transduce specific cellular signals. FcRs play a significant role in vivo in maintaining peripheral tolerance by limiting the accumulation of autoreactive B cells that escape central tolerance checkpoints, such as light chain editing or potentially arise during somatic hypermutation in germinal centers, in modulating T-cell responses by regulating both antigen presentation and maturation by DCs, and in mediating the coupling of antigen recognition to effector-cell activation. They are the primary pathways by which pathogenic IgG and IgE antibodies trigger inflammatory responses in vivo. Allergic reactions, cytotoxic IgG responses, and IC-mediated inflammation are all critically dependent on FcR cross-linking and have resulted in a fundamental revision of the mechanisms

underlying such classic immunological responses as the Arthus reaction. Blocking of these receptors uncouples the pathogenic potential of autoantibodies and represents an important new therapeutic target for the development of anti-inflammatory therapeutic agents. Central to the correct functioning of these responses is the balance that is maintained through the pairing of activation and inhibitory receptors that coengage the IgG ligand; perturbations in either component can result in pathological responses. The study of FcRs defined the ubiquitous inhibitory motif, the ITIM, and has provided a paradigm for how these pathways modulate ITAM-based activation responses. Studies in mice deficient in individual FcRs have provided the necessary insights for defining comparable activities in human autoimmune diseases and suggest ways in which manipulation of the IgG–FcR interaction may lead to new classes of therapeutics for the treatment of these diseases. Modulation of the inhibitory response, a novel activity associated with IVIG to account for some of its anti-inflammatory activity in vivo, represents a novel approach to the regulation of Ig-mediated inflammation and suggests that therapeutic agents based on those pathways are likely to be effective. It seems clear that this anti-inflammatory pathway is of general importance for maintaining immune homeostasis, suggesting that IgG glycoforms rich in terminal sialic acid residues might be considered as a molecular switch either keeping the immune system in a resting state or, during infection or autoimmune responses, loosening the brakes thereby allowing full blown inflammation. In this pathway, noncanonical FcRs including SignR1 and human DC-SIGN recognizing specific IgG glycoforms are of central importance. Conversely, engineering of therapeutic antibodies targeted to eliminate infectious or neoplastic disease will probably benefit from optimization of their Fc domains for interaction with specific canonical FcRs.

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CHAPTER 25

Type I Cytokines and Interferons, and Their Receptors

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OVERVIEW AND ISSUES OF NOMENCLATURE

Cytokines are proteins that are secreted by cells and transduce signals via specific cell surface receptors on either the cytokine-producing cell (autocrine actions) or on other target cells (paracrine actions). From this operational type of description, it is clear that the distinction between cytokines, growth factors, and hormones may be imprecise. In general, cytokines and growth factors are similar, except that the term cytokine most often refers to molecules involved in host defense that have actions on leukocytes, whereas the term growth factor more often refers to molecules acting on other somatic cell types. Cytokines most often act locally. For example, in the interaction between a T cell and an antigen-presenting cell, cytokines are produced and usually exert potent actions locally, with rather limited biologic half-lives in the circulation. In contrast, hormones are released and then disseminated via the bloodstream throughout the body, with actions on distal target organs. Nevertheless, this distinction between cytokines and hormones is not absolute, with certain cytokines acting at longer distances as well.

In the immune system, terms such as *monokines* and *lymphokines* were originally devised to identify the cellular source for cytokines.[1] Specifically, monokines included molecules such as interleukin (IL)-1, which was first recognized to be produced by monocytes, and lymphokines included molecules such as IL-2, which was first described as a T-cell growth factor produced by T lymphocytes. The monokine/lymphokine nomenclature can be problematic; however, when a cytokine is synthesized by more than one type of cell. This resulted in the adoption of the term cytokine, as proposed by Stanley Cohen in 1974.[2,3] The term cytokine refers to a protein made by a cell ("cyto") that acts ("kine") on target cells. Cytokines can have very broad ranges of actions, including on cell development, differentiation, growth, cytolytic activity of effector cells, survival, apoptosis, and chemotaxis.

Many cytokines are referred to as *interleukins* to indicate molecules that are produced by one leukocyte and act on another.[4] However, this term is also problematic as some interleukins (eg, IL-1 and IL-6) are additionally produced by cells other than leukocytes and/or exert actions on cell types beyond the immune system. For example, IL-7 is produced by stromal and epithelial cells rather than by typical leukocytes. Furthermore, nomenclature can be inconsistent. For example, IL-7 is highly related to another cytokine that is denoted as thymic stromal lymphopoietin (TSLP) rather than as an interleukin (see section on IL-7 and Thymic Stromal Lymphopoietin), even though both can be produced by stromal and epithelial cells and share a receptor component as well as select actions on lymphocytes. The more descriptive name of TSLP correctly describes its production by thymic stroma but obscures its production by skin epithelial cells, and that major target cells include dendritic cells (DCs) and cluster of differentiation (CD)4+ T cells.

Among the many classes of cytokines, the "type I" cytokines are distinctive in their sharing a similar four α-helical bundle structure, as detailed in the section on Type I Cytokines—Structural Considerations, and correspondingly, their receptors share characteristic features that have led to their description as the cytokine receptor superfamily, hematopoietin receptors, or type I cytokine receptors.[5–8] Although many interleukins are type I cytokines, some are not. For example, two of the major "proinflammatory cytokines," IL-1 and IL-6, are interleukins, but IL-6 is a type I cytokine, whereas IL-1 is not. One interleukin, IL-8, is a CXC family chemokine, an entirely different type of molecule involved in chemotaxis. Moreover, IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29 are more similar to interferons (IFNs) and are denoted as type II cytokines.

In summary, the term interleukin indicates a relationship to leukocytes, whereas the identification of a cytokine as a type I or type II cytokine indicates general properties of its three-dimensional structure. Knowing that a molecule is a type I cytokine is instructive as it indicates a likely general structure for the cytokine receptor as well as the mechanism of signal transduction. In contrast, the identification of a molecule as an interleukin provides little information other than that it often, but not always, is a type I or type II cytokine of immunologic interest.

In addition to molecules of primarily immunologic interest, other important proteins, such as growth hormone, prolactin, erythropoietin (Epo), thrombopoietin, and leptin, are type I cytokines and their receptors are type I cytokine receptors. Despite having their major actions outside the immune system, these cytokines nevertheless share important signal transduction pathways with type I cytokines of immunologic interest. By focusing on type I cytokines and IFNs and their receptors, this chapter necessarily focuses on cytokines that are evolutionarily related and share common signaling pathways, instead of focusing on common functions per se. For example, although IL-6 has overlapping actions with IL-1 and tumor necrosis factor (TNF)-α, these latter proinflammatory cytokines are not discussed in this chapter

because they are not type I cytokines, and the signaling pathways they use are distinct from those used by IL-6. This illustrates the important concept that similar end functions can be mediated via more than one type of signaling pathway. This is not to minimize the observation that many type I cytokines in fact do have similar/overlapping functions, as detailed in the section on "cytokine redundancy."

The field of IFN research is older than the cytokine field, but both fields more recently have developed in parallel. In fact, one can consider the IFNs to be the first cytokines that were identified. IFN was discovered as an antiviral activity in 1957. This turned out to be type I IFN (IFNα/β). Type II IFN (IFNγ) was discovered in 1965. Over time, it was recognized that type I cytokines and IFNs/type II cytokines share a number of common features, including signaling pathways.

An unfortunate nomenclature issue exists that should be noted. There are specialized popuplations of T cells that include T helper 1 (Th1) and T helper 2 (Th2) cells (see following discussion), which produced specialized sets of cytokines, such as IFNγ by Th1 and IL-4 by Th2 cells. These are sometimes called type 1 and type 2 cytokines (for Th1 and Th2 cytokines); unfortunately, this jargon can result in confusion as IL-4 is a type I (ie, four α-helical bundle) cytokine that is functionally a type 2 cytokine (in that it is produced by Th2 cells), where IFNγ is a type II structure cytokine produced by Th1 cells.

TYPE I CYTOKINES AND THEIR RECEPTORS

Type I Cytokines—Structural Considerations

Type I cytokines typically share only limited amino acid sequence identity, but strikingly, all type I cytokines whose structures have been solved by nuclear magnetic resonance and/or x-ray crystallographic methods achieve similar three-dimensional structures,[5–9] and those whose structures have not yet been solved are believed to share similar three-dimensional structures. Type I cytokines contain four α helices and thus are designated as four α-helical bundle cytokines (Fig. 25.1). Within their structures, the first two and last two α helices are each connected by long overhand loops, resulting in an "up-up-down-down" topologic structure, as the first two helices (A and B) can be oriented in an "up" orientation and the last two helices (C and D) can be oriented in a "down" orientation, as viewed from the N- to C-terminal direction. As shown in Figure 25.1, the N- and C-termini of the cytokines are positioned on the same part of the molecule.

Type I cytokines are either "short chain" or "long chain" four α-helical bundle cytokines based on the lengths of the α helices.[8] Some of the short-chain cytokines include IL-2,

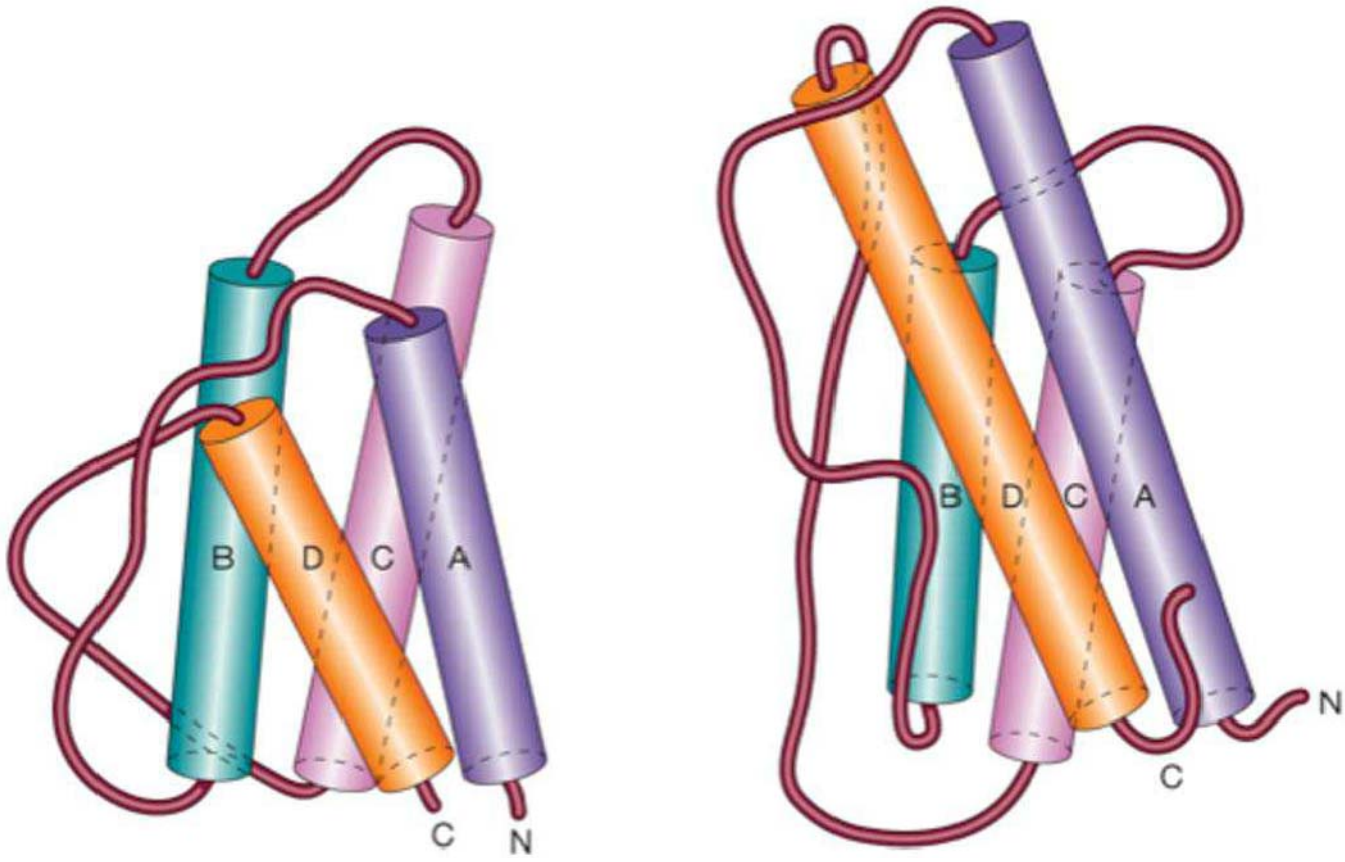

FIG. 25.1. Schematic of Four α-Helical Bundle Cytokines. Schematic drawing showing typical short-chain and long-chain four helical bundle cytokines. Although these both exhibit an "up-up-down-down" topology to their four α helices, note that in the short-chain cytokines, the AB loop is in behind the CD loop, whereas in the long-chain cytokines the situation is reversed. See text. The figure was provided by Dr. Alex Wlodawer, National Cancer Institute.

TABLE 25.1 Four Helical Bundle Cytokines

Short-Chain Cytokines	Long-Chain Cytokines
IL-2	IL-6
IL-4	IL-11
IL-7	Oncostatin M
IL-9	Leukemia inhibitory factor
IL-13	CNTF
IL-15	Cardiotropin-1
IL-21	NNT-1/BSF-3
TSLP	
IL-3	Growth hormone
IL-5[a]	Prolactin
GM-CSF	Erythropoietin
	Thrombopoietin
M-CSF[a,b]	Leptin
SCF[b]	G-CSF

BSF-3, B cell-stimulating factor-3; CNTF, ciliary neurotrophic factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; M-CSF, macrophage-colony stimulating factor; NNT-1, novel neurotrophin-1; SCF, stem cell factor; TSLP, thymic stromal lymphopoietin.

[a] Dimers.

[b] Different from the other four helical bundle cytokines in that the M-CSF and SCF receptors (CSF-1R and c-kit, respectively) have intrinsic tyrosine kinase activity and are not type I cytokine receptors.

IL-3, IL-4, IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-7, IL-9, IL-13, IL-15, IL-21, macrophage-colony stimulating factor (M-CSF), stem cell factor (SCF), and TSLP, whereas long-chain cytokines include growth hormone, prolactin, Epo, thrombopoietin, leptin, IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), novel neurotrophin-1 (NNT-1)/B cell-stimulating factor-3 (BSF-3)/cardiotrophin-like factor (CLC), and granulocyte-colony stimulating factor (G-CSF) (Table 25.1).[8,10,11] The α helices are approximately 15 amino acids long in short-chain helical cytokines and 25 amino acids long in long-chain cytokines. Additional differences include differences in the angles between the pairs of helices, and the AB loop is "under" the CD loop in the short cytokines but "over" the CD loop in the long cytokines (see Fig. 25.1).[7,8,12] Moreover, short-chain cytokines have β structures in the AB and CD loops, whereas long-chain cytokines do not.

The division of type I cytokines into short-chain and long-chain cytokines has evolutionary considerations and correlates with grouping of their receptor chains for these two subfamilies of type I cytokines. An analysis of short-chain cytokines has revealed that 61 residues comprise the family framework, including most of the 31 residues that contribute to the buried inner core. The similarities and differences in the structures of IL-2, IL-4, and GM-CSF have been analyzed.[5] Among these cytokines, there is considerable variation in the intrachain disulfide bonds that stabilize the structures. For example, IL-4 has three intrachain disulfide bonds, GM-CSF has two, and IL-2 has only one. In IL-4, the first disulfide bond (between Cys 24 and Cys 65) connects loop AB to BC, the second disulfide bond (between Cys 46 and Cys 99) connects helix B and loop CD, and the third disulfide bond (between Cys 3 and Cys 127) connects the residue preceding helix B with helix D. In GM-CSF, the N-terminus of helix B and the N-terminus of β strand CD are connected by one disulfide bond, whereas the other disulfide bond connects the C-terminus of helix C and a strand following helix D. In IL-2, a single essential disulfide bond between Cys 58 and Cys 105 connects helix B to strand CD. Thus, each cytokine has evolved distinctive disulfide bonds to stabilize its structure, although it is typical that helix B is connected to the loop between helices C and D. The structures formed by helices A and D are more conserved than those formed by helices B and C, primarily due to the interhelical angles; helix D and the connecting region are the most highly conserved elements among the three cytokines.[5] This is of particular interest, as the regions of type I cytokines that are most important for cytokine receptor interactions (based on analogy to the growth hormone receptor structure, see subsequent discussion) include helices A and D and residues in the AB and CD loops, whereas helices B and C do not form direct contacts.[5]

Variations on these typical four α-helical bundle structures can occur. For example, IL-5 is unusual in that it is a dimer, wherein the ends containing the N- and C-termini are juxtaposed, and helix D is "exchanged" between the two covalently attached monomers so that helix D of each molecule actually forms part of the four helix bundle of the other.[13] M-CSF is also a dimer, but no exchange of helix D occurs.[12]

The IFNs form related albeit distinctive structures from type I cytokines and are designated as type II cytokines.[8] IFNβ has an extra helix that is positioned in place of the CD strand.[14] IFNγ is a dimer, each of which consists of six α helices[15] (Fig. 25.2). Two of these helices are interchanged, including one from each four α-helical bundle.[12,15] IL-10, which is closely related to IFNγ, has a similar structure,[16] as

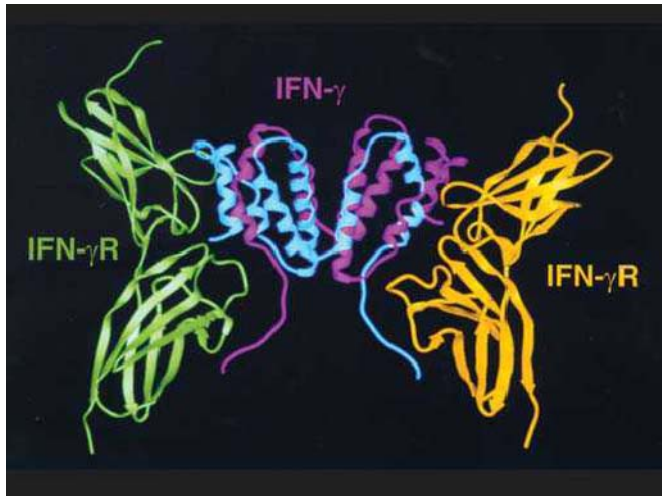

FIG. 25.2. Structure of the Interferon γ Receptor (IFNγR). Shown are ribbon diagrams of the structures of the IFNγ/IFNγR complex as an example of a type II cytokine/cytokine receptor. In the IFNγR, only IFNGR-1 complexed to the IFNγ dimer is shown, as the full structure with IFNGR-2 is not available. See text for discussion of the structure. The IFNγ-IFNGR-1 structure is from Walter et al.[401] The figure was provided by Dr. Alex Wlodawer, National Cancer Institute.

presumably do the more recently identified IL-10–like molecules, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29. Interestingly, although not universally the case, most four α-helical bundle cytokines have four exons, with helix A in exon 1, helices B and C in exon 3, and helix D in exon 4.[7] A related organization is found for IFNγ, as well as for the long-chain helical cytokines, growth hormone, and G-CSF. However, there are exceptions, for example, IL-15 is divided into nine exons, whereas the IFNαs and IFNβ are encoded by single exons.

Receptors for Type I Cytokines

The first report suggesting that type I cytokines interacted with receptors with similar features identified similarities in the sequences of the erythropoietin receptor and the IL-2 receptor β chain,[17] and subsequent analysis of a large number of type I cytokine receptors established these receptors as a superfamily.[18] Type I cytokine receptors are generally type I membrane–spanning glycoproteins (N-terminus extracellular, C-terminus intracellular). The only exceptions are proteins such as the CNTF receptor α chain (see the following discussion), which lacks a cytoplasmic domain and instead has a glycosylphosphatidylinositol (GPI) anchor; however, the orientation of this protein is otherwise similar to that of a type I membrane protein. In their extracellular domains, a number of conserved features have been noted (Table 25.2). These include four conserved cysteine residues that are involved in intrachain disulfide bonding, and a tryptophan residue, located two amino acids C-terminal to the second conserved cysteine. In addition, a membrane proximal WSXWS (Trp-Ser-X-Trp-Ser) motif is generally conserved, although again exceptions exist, for example, in the growth hormone receptor, the motif is a substantially different YGEFS (Tyr-Gly-Glu-Phe-Ser) sequence, and in the IL-23R, it is WQPWS (Trp-Gln-Pro-Trp-Ser). In some cases, such as the common cytokine receptor β chain, β_c, shared by the IL-3, IL-5, and GM-CSF receptors (see subsequent discussion), the extracellular domain is extended, with a duplication of the domains containing the four conserved cysteines and the WSXWS motif. Another shared feature of type I cytokine receptors is the presence of fibronectin type III domains.

The two pairs of conserved cysteine residues are typically encoded in two adjacent exons, and the exon containing the WSXWS motif is typically just 5′ to the exon encoding the transmembrane domain. Although serines can be encoded by six different codons (ie, sixfold degeneracy in codon usage), only two of these (AGC and AGT) dominate as the codons used for the serines in WSXWS. All of these features indicate a common ancestral type I cytokine receptor.

Overall, analogous to limited sequence identity between type I cytokines, there is only limited sequence identity among type I receptor molecules. Nevertheless, they appear to form similar structures, based on the known structures for the receptors for growth hormone, prolactin, erythropoietin, IL-4, IL-13, IL-6, and IL-2[9,19–25] as well as the modeling of other cytokine receptor molecules based on the known structures. The cytokines and their receptors have presumably coevolved, with the differences in amino acid sequences between different cytokines allowing for their distinctive interactions with their cognate receptor chains. Despite amino acid differences, there are also several sets of cytokines that coevolved to interact with shared receptor chains, which form type I cytokine and cytokine receptor subfamilies.[8,11]

In addition to these noted similarities in the extracellular domains, there are sequence similarities that are conserved in the cytoplasmic domain of cytokine receptors. In particular, membrane proximal "Box 1/Box 2" regions are conserved (see Table 25.2), with the proline-rich Box 1 region being the most conserved.[26] This will be discussed in greater detail related to its role in the binding of JAK kinases.

Type I Cytokine Receptors Are Homodimers, Heterodimers, or Higher Order Receptor Oligomers

The first cytokine receptor structure solved was that for growth hormone (Fig. 25.3).[19] Prior to x-ray crystallographic analysis, it was believed that growth hormone bound to its

TABLE 25.2 Features Common to Type I Cytokine Receptors

Extracellular domain

1. Four conserved cysteine residues, involved in intrachain disulfide bonds
2. WSXWS motif
3. Fibronectin type III modules

Cytoplasmic domain

1. Box 1/Box 2 regions—The Box 1 region is a proline-rich region that is involved in the interaction of Janus family tyrosine kinases.

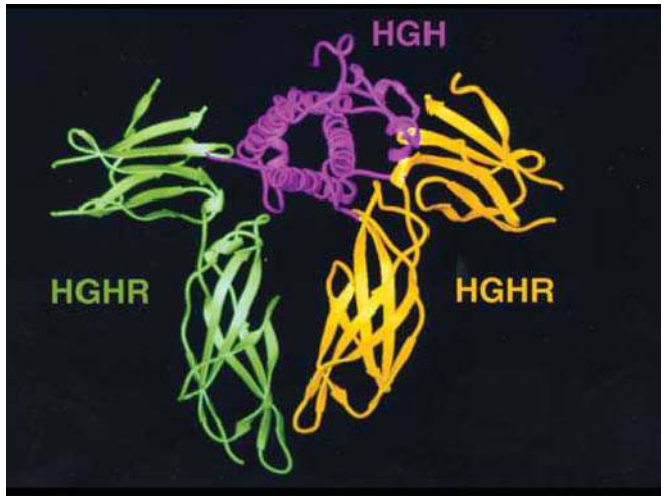

FIG. 25.3. Structure of the Growth Hormone Receptor. Shown are ribbon diagrams of the structure of the growth hormone receptor as an example of a type I cytokine receptor. For growth hormone, both growth hormone receptor monomers are shown. See text for discussion of the structures. The growth hormone/growth hormone receptor structure is from de Vos et al.[19] The figure was provided by Dr. Alex Wlodawer, National Cancer Institute.

receptor with a stoichiometry of 1:1, but x-ray crystal solution structure revealed that a single growth hormone molecule interacts with a dimer of the growth hormone receptor, in which each receptor monomer contributes a total of seven β strands. Perhaps the most striking finding is that totally different parts of growth hormone interact with the same general region of each receptor monomer. The three-dimensional structure for the growth hormone/growth hormone receptor complex is shown in Figure 25.3. Solving the structure also clarified the basis for the assembly of the growth hormone receptor complex.[19] Growth hormone first interacts with one receptor monomer via a relatively large and high-affinity interaction surface (site I), spanning ~1230 Å^2. A second receptor monomer then interacts with the growth hormone/growth hormone receptor complex via two contact points: one on growth hormone (spanning ~900 Å^2) (site II) and the other on the first receptor monomer (spanning ~500 Å^2) (site III), located much more proximal to the cell membrane. Thus, three extracellular interactions are responsible for the formation and stabilization of the growth hormone/growth hormone receptor complex. Mutations in critical residues in site I should prevent growth hormone binding to its receptor, whereas inactivating mutations in site II can be predicted to prevent dimerization and signal transduction, suggesting a basis by which different classes of antagonists might be identified.

The growth hormone/growth hormone receptor structure revealed that the growth hormone receptor extracellular domain is composed of two fibronectin type III modules, each of which is approximately 100 amino acids long and contains seven β strands, resulting in the formation of an immunoglobulin (Ig)-like structure. The contact surface between ligand and receptor occurs in the hinge region that separates these two fibronectin type III modules. Analysis of a growth hormone–prolactin receptor complex revealed a similar structure for the prolactin receptor.[20]

The growth hormone/growth hormone receptor structure served as a paradigm for the structures of other type I cytokine receptors. As a receptor homodimer, it immediately served as a model for other homodimers, such as the Epo receptor, whose structure was solved[21] using a small protein mimetic (20 amino acid long peptide) of Epo.[27] The Epo receptor structure is similar to that of the growth hormone receptor, although the "site III" stem region interaction surface in the Epo receptor is much smaller than that in the growth hormone receptor, comprising only 75 Å^2.[21]

In addition to the structural similarities for the growth hormone and Epo receptors and perhaps other homodimeric type I cytokine receptors, a similar structure was achieved by the heterodimeric growth hormone/growth hormone receptor/prolactin receptor structure,[20] in which one of the growth hormone receptor monomers is replaced by a prolactin receptor molecule. Thus, the two surfaces of growth hormone interact either with two identical monomers of growth hormone receptor or with two nonidentical monomers in the case of the growth hormone receptor/prolactin receptor heterodimer.

It seems reasonable that cytokine-receptor systems with a homodimeric receptor are evolutionarily older than those with heterodimeric receptors, and that the coordination of two different receptor chains in heterodimeric receptors would have evolved in order to allow higher levels of specialization. In this regard, it is interesting that growth hormone and Epo, whose actions are vital for growth and erythropoiesis, bind to receptor homodimers, whereas the heterodimeric structures that typify the immune system are perhaps more "specialized" functions that arose later in evolution.

Interestingly, all type I cytokines known to interact with homodimers (growth hormone, prolactin, Epo, and G-CSF) are long-chain helical cytokines, although other long-chain helical cytokines (eg, cytokines whose receptors contain gp130; see following discussion) interact with heteromeric receptors. The short-chain cytokines that signal through homodimers are SCF and M-CSF, but in these cases, the receptors (c-kit and CSF-1R, respectively) are different from type I cytokine receptors in that they contain intrinsic tyrosine kinase domains. Thus, SCF and M-CSF are not typical type I cytokines and all other short-chain cytokines signal through heterodimers or more complex receptor structures (eg, IL-2 and IL-15 receptors have three components).

Heterodimeric receptors are involved when site II on a cytokine has evolved to a point where it interacts with a different receptor molecule than site I does. This latter situation is the case for many cytokines, including all short-chain type I cytokines except for SCF and M-CSF. Overall, several sets of type I cytokines fall into distinct groups, wherein each group shares at least one common receptor component. This phenomenon is observed for certain sets of both short-chain and long-chain four α-helical bundle cytokines, and depending on the set of cytokines, the shared chain interacts either with site I or site II.

The structures of the low- and high-affinity forms of the IL-2 receptor have now been solved[9,23,28]; these are the first complete structures for a short-chain cytokine/receptor complex.[29] Moreover, they are of added interest in that the low-affinity receptor involves the interaction of IL-2 with IL-2Rα, which is not a type I cytokine receptor protein but instead is a distinctive sushi-domain containing protein, whereas the high-affinity receptor includes not only IL-2Rα but also IL-2Rβ and γ_c, which are both type I cytokine receptor proteins that are shared either with IL-15 (IL-2Rβ) or with IL-4, IL-7, IL-9, IL-15, and IL-21 (γ_c),[30] as discussed in the following text. In the structure of IL-2 bound to its high-affinity receptor (Fig. 25.4), there is a long peptide connecting the IL-2Rα globular head and transmembrane segment, allowing the binding site on this protein to extend relatively far from the cell surface in order to bind the dorsal surface of IL-2. Both the IL-2/IL-2Rα and IL-2/IL-2Rβ contacts are independent, and IL-2Rα does not appear to contact either IL-2Rβ or γ_c; however, IL-2/IL-2Rβ forms a composite surface with γ_c, somewhat analogous to the composite surface formed by growth hormone and one growth hormone receptor monomer for binding to a second monomer. As anticipated, the surface interaction between IL-2 and γ_c is smaller than that between IL-2 and either of the other chains. In addition to the heterodimeric IL-2 receptor structure,[23] the IL-6[31,32] and LIF[33] receptor complexes,

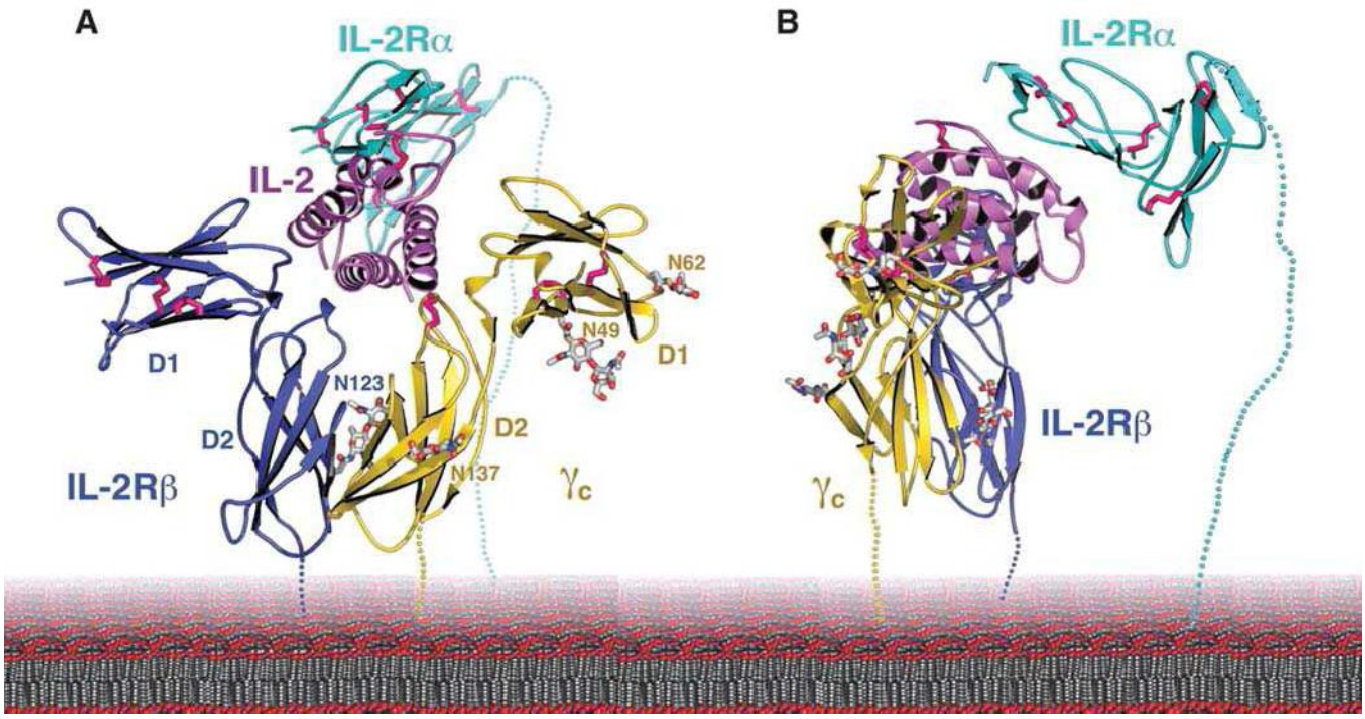

FIG. 25.4. Structure of the High-Affinity Interleukin (IL)-2 Receptor. This is the first structure for a short-chain type I cytokine complexed to its complete receptor. It is particularly interesting in that it includes the sushi-domain containing IL-2Rα chain as well. Reprinted from Wang et al.,[23] with permission of Dr. Garcia and *Science* magazine.

the IL-4/IL-13 receptor[34] complexes, and GM-CSF receptor structure[35] have been solved.

TYPE I CYTOKINE RECEPTOR FAMILIES AND THEIR RELATIONS

Cytokines that Share the Common Cytokine Receptor γ Chain (Interleukin-2, Interleukin-4, Interleukin-7, Interleukin-9, Interleukin-15, and Interleukin-21)

The receptors for six different immunologically important cytokines, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, share the common cytokine receptor γ chain, γ_c (CD132).[11,30,36–45] These cytokines are all short-chain four α-helical bundle cytokines; basic features of these cytokines are summarized in Table 25.3. The properties of these cytokines and their distinctive receptor chains are summarized in the following text, followed by a discussion of the discovery that they share a common receptor component and the implications thereof.

IL-2 is important not only for its function but also historically, as it was the first type I cytokine that was cloned,[46] the first type I cytokine for which a receptor component was cloned,[47,48] and the first short-chain type I cytokine whose receptor structure was solved.[23] Many general principles have derived from studies of this cytokine, including its being the first cytokine that was demonstrated to act in a growth factor–like fashion through specific high-affinity receptors, analogous to the growth factors being studied by endocrinologists and biochemists.[49,50]

Mature IL-2 is a 133 amino acid long peptide that can act as a major T-cell growth factor, in keeping with its original discovery as a T-cell growth factor.[51] Although IL-2 is not produced by resting T cells, it is rapidly and potently induced following antigen encounter with resting CD4+ T cells, and transcription and synthesis of IL-2 are often used as indicators of T-cell receptor (TCR)-mediated cellular activation. Although the antigen determines the specificity of the T-cell immune response, the interaction of IL-2 with high-affinity IL-2 receptors regulates the magnitude and duration of the subsequent response, based on the amount of IL-2 produced, the levels of high-affinity receptors expressed, and the duration of IL-2 production and receptor expression. IL-2 can act in either an autocrine or paracrine fashion, depending on whether the producing cell is also the responding cell or whether the responding cell is a nonproducing cell. The gene encoding IL-2 is located on chromosome 4,[52] and like many other helical cytokines, its gene consists of four exons.[7] IL-2 binds to three different classes of receptors. These are formed by different combinations of three different chains, IL-2Rα,[47,48,53] IL-2Rβ,[54–57] and a protein initially called IL-2Rγ[36] but now known as the common cytokine receptor γ chain, γ_c.[11,30,37] These different classes of IL-2 receptors are discussed in the following text.

In addition to its being a T-cell growth factor, IL-2 has other important actions as well (see Table 25.3).[29] For example, it can increase Ig synthesis and J chain transcription in B cells,[50,58,59] potently augment the cytolytic activity of

TABLE 25.3 Features of Cytokines Whose Receptors Share γ_c

Cytokine	Major Source	Size[a]	Actions	Chromosome Location (h/m)	Genomic Org
IL-2	Activated T cells (Th1 cells)	h153 aa/20aa m169aa/20aa 15.5 kDa	T-cell growth factor B-cell growth, Ig production, J chain expression Induces LAK activity Induces tumor infiltrating lymphocyte activity Augments NK activity Critical roles in antigen-induced cell death Stimulates macrophage/monocyte Antitumor effects Promotes T_{reg} development Promotes Th1 and Th2 differentiation Inhibits Th17 differentiation	4q26–27/3	Four exons
IL-4	Activated T cells (Th2 cells) CD4+NK1.1+ natural T cells	h153/24 aa m140 aa/20 aa 18 kDa	B-cell proliferation Ig class switch: IgG1, IgE production Augment MHC II, Fcε receptors, IL-4Rα, and IL-2Rβ expression Th2 cell differentiation Antitumor effects	5q31.1/11	Four exons
IL-7	Stromal cells	h177 aa/25aa m154aa/25aa 17–25 kDa	Thymocyte growth T-cell growth Pre–B-cell growth in mice but not human Survival and growth of peripheral T cells CD4+ and CD8+ T-cell homeostasis	8q12–13/3	Six exons
IL-9	Activated Th cells	h144aa/18aa m144aa/18aa 14 kDa	Th helper clones Erythroid progenitors B cells Mast cells/allergic responses Fetal thymocytes	5q31–35/13	Five exons
IL-15	Monocytes and many cells outside the immune system[b]	h162aa/48aa m162aa/48aa 14–15 kDa	Mast cell growth NK cell development and activity T-cell proliferation CD8+ T-cell homeostasis	4q31/8	Nine exons
IL-21	Activated CD4+ T cells	h162aa/31 aa m146aa/24 aa	Comitogen for T-cell proliferation Inhibits B-cell proliferation to anti-IgM + IL-4 Augments B-cell proliferation to anti-CD40 Conflicting reports related to NK cells Cooperates with IL-7 and IL-15 to expand CD8 cells Antitumor effects Drives terminal B-cell differentiation to plasma cells Proapoptotic for B and NK cells Promotes Tfh differentiation Promotes Th17 differentiation	4q67–27/3	Five exons

CD, cluster of differentiation; Ig, immunoglobulin; IL, interleukin; LAK, lymphokine activated killer; MHC, major histocompatibility complex; NK, natural killer; Tfh, T follicular helper; Th, T helper; T_{reg}, regulatory T.

[a] h and m refer to human and mouse, respectively. The number of amino acids refers to the length of the open reading frame/length of signal peptide. The number of amino acids in the mature protein is therefore the difference between these numbers. Note that for IL-15, residues 1 to 29 have been identified as a signal peptide and 30 to 48 as a propeptide.

[b] More IL-15 messenger ribonucleic acid is produced in skeletal muscle, kidney, placenta, and lung than in thymus or spleen. It is important to note, however, that IL-15 messenger ribonucleic acid is widely expressed without concomitant production of IL-15 protein so that the source of biologically meaningful IL-15 may be more limited.

natural killer (NK) cells,[60–62] induce the cytolytic activity of lymphokine activated killer cells, promote the elimination of autoreactive cells in a process known as antigen-induced (or activation-induced) cell death,[29,50,63] and promote the differentiation of regulatory T (T_{reg}) cells,[64,65] cells that suppress inappropriate responses and are important for immunologic tolerance. Low-dose IL-2 is sufficient to promote T_{reg}-cell survival, and thereby, for example, can protect mice from developing autoimmune diabetes.[66] Interestingly, IL-2 can also prime CD8+ T cells during a primary response to undergo enhanced proliferation in vivo during a secondary response,[67,68] and autocrine IL-2 is believed to be required for secondary expansion of CD8+ memory T cells.[69] There appears to be a complex interplay between IL-2 and inflammatory signals to regulate effector and memory cytolytic T-lymphocytes generation in lymphocytic choriomeningitis virus infection, with persistent IL-2 promoting effector rather than memory cytotoxic T-lymphocyte development.[70,71] In *Listeria monocytogenesis* infection, Th1 effector memory cells highly express the transcription factor T-Bet and IL-2Rα, and IL-2Rα appears to be critical for the development of these cells.[72] Presumably because of its critical role in T_{reg} development, the absence of IL-2, IL-2Rα, or IL-2Rβ leads to autoimmunity. Interestingly, the production of IL-2 by mast cells has been reported to contribute to the suppression of chronic allergic dermatitis by its increasing the relative number of T_{reg} cells at the site of inflammation.[73]

IL-2 is also important in inducing or inhibiting Th differentiation. It is required for efficient Th1[74] and Th2 differentiation,[75–78] which are populations of T helper cells first described based on patterns of cytokine production by mouse T cells.[79] This theme was then extended to human cells as well,[80–84] although there are some variations in humans and mice in terms of the degree of how tightly restricted cytokine expression is. IFNγ is the cytokine most reliably produced by Th1 cells; IL-2 is also produced by these cells, although without as rigorous an association. In contrast, IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are produced by Th2 cells. In both species, certain type I cytokines (eg, IL-3 and GM-CSF) are produced by both Th1 and Th2 cells. IL-12 and the transcription factors signal transducer and activator of transcription (STAT)4 and T-Bet are the major transcription factors promoting Th1 differentiation, whereas IL-4 and the transcription factor STAT6 drive Th2 differentiation. For Th1 cells, IL-2 acts by STAT5-dependent induction and expression of IL-12Rβ1/IL-12Rβ2 and T-Bet,[74] whereas for Th2 differentiation, IL-2 via STAT5 promotes IL-4[75,76] and IL-4Rα[77] expression. In addition, IL-2 also affects chromatin remodeling at the *Ifng* locus during Th1 differentiation.[85] IL-2 also inhibits Th17 differentiation,[86] in part via downregulation of IL-6Rα/gp130 expression[74] and by inducing T-bet,[74,87] which prevents Runx1-mediated activation of RORγt, a transcription factor that drives Th17 differentiation.[87]

Like IL-2, IL-4 is produced primarily by activated CD4+ T cells.[88,89] IL-4 is also produced by CD4+NK1.1+ "natural" T cells, denoted as natural killer T (NKT) cells,[90] and by mast cells and basophils.[88] IL-4 is the major B-cell growth factor, and it promotes Ig class switch, enhancing the production and secretion of mouse IgG1 (human IgG4) and being essential for the production of IgE.[88] IL-4 is involved in the physiologic response to parasites, including helminths, and for allergen sensitization. IL-4 induces expression of class II major histocompatibility complex molecules and increases cell surface expression of the CD23 (the low-affinity IgE receptor) on B cells. In addition to its actions on B cells, IL-4 can also act as a T-cell growth factor, inducing proliferation in both human and mouse T cells, and is critical for normal differentiation of Th2 cells.[89] Moreover, when IL-4 and transforming growth factor (TGF)-β are combined, cells that produce IL-9 (Th9) cells are induced (see following discussion). When combined with phorbol 2-myristate 3-acetate, IL-4 is also a potent comitogen for thymocytes. Importantly, IL-4 can inhibit certain responses of cells to IL-2.[91] Moreover, IL-4 can exert actions on macrophages, hematopoietic precursor cells, stromal cells, and fibroblasts.[92] The gene encoding IL-4 is located on human chromosome 5 (5q23.3–31.2) and mouse chromosome 11,[92,93] in the same region as IL-3, IL-5, IL-13, and GM-CSF. The type I IL-4 receptor is expressed on T cells and other hematopoietic cells consists of the 140 kDa IL-4Rα protein[88,94–96] and γ_c.[38,39] Expression of IL-4Rα tends to be quite low, and cells that potently respond to IL-4 often express only a few hundred receptors per cell. In addition to the type I IL-4 receptor, an alternate form of the receptor (the type II IL-4 receptor), containing IL-4Rα and IL-13Rα1, although not expressed on mature T cells, is expressed on many other cell types and can transduce IL-4 signals into these cells. For example, IL-13Rα1 is expressed on neonatal Th1 cells and the type II IL-4 receptor has been implicated as mediating apoptosis of these cells.[97]

IL-7 is not produced by lymphocytes but instead is a 152 amino acid long cytokine that is produced by stromal cells and certain other cells.[98–100] Based on the analysis of patients with X-linked severe combined immunodeficiency (SCID), JAK3-deficient SCID, and IL-7Rα-deficient SCID, IL-7 receptor-dependent signaling is essential for T-cell development in humans (discussed subsequently). The major role of IL-7 is to enhance thymocyte survival, growth, and differentiation[101–105] as well as low-affinity peptide-induced proliferation, and thus it promotes homeostatic proliferation of naive and memory CD8+ T cells.[100,105–107] Additionally, IL-7 can regulate the homeostasis of CD4+ memory T cells[108,109] and also can stimulate the growth of mature T cells.[30,105,110,111] Although IL-7, as noted previously, is believed to primarily be a stromal factor, IL-7 has also been noted to be produced by the liver and kidney but not spleen or lymph nodes after toll-like receptor (TLR) signaling. Although basal liver production is low, after TLR signaling, hepatic IL-7 can promote CD4 and CD8 T-cell survival and promotes antigen-specific T-cell responses.[112] In addition, in the mouse, IL-7 is vital for the growth of mouse pre-B cells,[96,98,103,104,111] and transient IL-7 signaling can inhibit Ig heavy chain gene rearrangements.[113] IL-7 via STAT5 has also been shown to inhibit Ig-κ recombination in pro-B cells.[114] In contrast to its requirement for B-cell development in the mouse, there is normal B-cell development in patients with defective IL-7 signaling, as is found in patients with X-linked SCID, JAK3-deficient

SCID, and IL-7Rα-deficient SCID (see following discussion). Thus, human B cells can develop normally in the absence of IL-7 responsiveness, demonstrating that in humans, IL-7 is not vital for the growth of human pre-B cells,[30,115] and it remains unknown whether IL-7 plays important roles in human B-cell biology. Interestingly, IL-7 also acts on DCs, and this appears to limit the homeostatic proliferation of T cells under lymphopenic conditions.[100,116] Clinically, IL-7 has been of interest in terms of its ability to induce T-cell proliferation and to thereby increase T-cell numbers, with a greater effect on CD8+ than on CD4+ T cells, as well as to augment the diversity of the TCR repertoire[117]; IL-7 also can increase antigen-specific responses following vaccination[100] and can promote antiviral immunity, apparently in part due to its induction of IL-22 and repression of SOCS3.[118] The gene encoding IL-7 is located on human chromosome 8q12 to 8q13[119] and mouse chromosome 3. The functional IL-7 receptor contains the 75 kDa IL-7Rα[120] and γ_c.[37,40] Interestingly, signaling via the TCR, IL-2 or IL-7 can downregulate IL-7Rα expression,[121,122] with PI 3-kinase/AKT and GFI1B being implicated in its downregulation in T cells.[122–124] The downregulation of IL-7Rα not only can both decrease responsiveness of cells but can also increase the availability of the cytokine for other cells that are poised to respond.[11] Induction of IL-7Rα requires PU.1 in B cells[125] and another Ets family protein, GABP, in T cells.[123]

IL-9 was originally described as a mouse T-cell growth factor[126] that is produced by activated T cells and can support the growth of T-helper clones but not of cytolytic clones.[127] In contrast to IL-2, its production is delayed, suggesting its involvement in later, perhaps secondary signals. In the mouse, IL-9 can exert proliferative effects on erythroid progenitors, B cells, B-1 cells, mast cells, and fetal thymocytes. IL-9 is identical to mast cell growth–enhancing activity, a factor present in conditioned medium from splenocytes,[128] and synergizes with IL-3 for maximal mast cell proliferation. IL-9 is highly expressed in the lung of patients with asthma,[129] and overexpression leads to airway inflammation and Th2 cytokine production.[130] Interestingly, IL-9 is substantially made in the lung by innate lymphoid cells, and in these cells neutralizing antibodies to IL-9 lowered expression of IL-13 and IL-5, supporting a link between IL-9 and the regulation of the Th2 response.[131] Consistent with the action of IL-9 on thymocytes in vitro, IL-9 transgenic mice develop thymic lymphomas, and IL-9 is a major antiapoptotic factor for such tumors.[132] Nevertheless, IL-9 knockout mice have normal T-cell development[133]; instead, they exhibit a defect in pulmonary goblet cell hyperplasia and mastocytosis following challenge with *Schistosoma mansoni* eggs, a synchronous pulmonary granuloma formation model. However, there was no defect in eosinophilia or granuloma formation.[133] Mice expressing an IL-9 transgene in the lung exhibit airway inflammation and bronchial hyperresponsiveness; nevertheless, IL-9−/− mice exhibit normal eosinophilia and airway hyperreactivity in an ovalbumin-induced inflammatory model.[134–136] Thus, although IL-9 can contribute to allergic/pulmonary responses, there are compensatory cytokines that substitute for IL-9 in at least certain settings. IL-9 can be produced by the Th9 populations of cells, which can be induced by IL-4 plus TGF-β in a fashion that is dependent on PU.1 and interferon regulatory factor (IRF)-4.[137] Interestingly, the addition of IL-25 further enhances the production of IL-9.[138] IL-9–producing cells are typically IL-9+IL-10+Foxp3− effector T cells and can be derived from Th2 cells.[139,140] Overall, IL-9 is believed to play a key role in allergic responses; additionally, given the ability of both Th17 and T_{reg} cells to produce IL-9 during autoimmunity and transplantation, it will be interesting to further clarify the role of IL-9 in autoimmunity.[141] Currently, there is some confusion, as one study showed that antibody blockade of IL-9 or IL-9Rα blocks experimental autoimmune encephalomyelitis (EAE) disease progression,[142] whereas another study showed that IL-9Rα knockout mice develop more severe EAE.[138] While mouse IL-9 is active on human cells, human IL-9 is not biologically active on mouse cells (the opposite situation from that for IL-2). Human IL-9 is located on chromosome 5q31 to 5q35,[143] which is also the location for the genes encoding IL-3, IL-4, IL-5, IL-13, and GM-CSF. In contrast, mouse IL-9 is "isolated" on chromosome 13, while IL-3, IL-4, IL-5, IL-13, and GM-CSF are clustered on chromosome 11. IL-9 binds to the 64 kDa IL-9Rα binding protein, which is similar in size to γ_c,[144] and the functional IL-9 receptor consists of IL-9Rα plus γ_c.[30,41,42]

IL-15 was identified as a T-cell growth factor that also unexpectedly was expressed in the supernatant of a human T-lymphotropic virus type I (HTLV-I)–transformed T-cell line.[145,146] Although IL-15 messenger ribonucleic acid (RNA) is produced by a range of nonlymphocytic cell types, it is difficult to detect physiologic levels of IL-15 protein.[147] Its main site of synthesis appears to be DCs and monocytes, and unlike IL-2, IL-15 is not produced by activated T cells.[148] IL-15 receptors are widely expressed; although IL-15 is perhaps most important for the development of NK cells[149–151] and CD8+ memory T cells,[150,151] it also has both paracrine and autocrine actions on DCs, including promoting the survival of these cells.[11] Interestingly, it also regulates the TCR repertoire of γδ intraepithelial lymphocytes[152] and cross talk between different types of DCs.[153] The receptor for IL-15 on T cells contains IL-2Rβ,[43,147,154] γ_c,[43] and an IL-15–specific protein, IL-15Rα. IL-15Rα shares a number of structural similarities with IL-2Rα, including that it is a sushi domain-containing protein (IL-2Rα has two sushi domains, whereas IL-15Rα has one),[155] and the *IL2RA* and *IL15RA* genes are closely positioned on human chromosome 10p14.[156] A distinctive feature of IL-15 signaling is that it signals substantially by a process called *transpresentation,* wherein IL-15Rα on the surface of dendritic cells or monocytes will transpresent IL-15 to responding cells such as CD8+ T cells or NK cells that express IL-2Rβ + γ_c.[148,157] IL-15Rα has a longer cytoplasmic domain than IL-2Rα and appears to be critical for IL-15Rα function, although potentially not for transpresentation.[158] Interestingly, transpresentation can be utilized by IL-2 as well.[159] The very high affinity of IL-15Rα for IL-15 is explained by a large number of ionic interactions mediated by the sushi domain.[160] Note that these responding cells can also express IL-15Rα. In contrast to IL-2, which is a growth factor as well as a mediator of antigen-induced (or activation-induced) cell death and promoter of T_{reg} differentiation, the

role of IL-15 appears to be more focused on growth of CD8+ T cells,[161] maintaining long-lasting, high-avidity T-cell responses to foreign pathogens (ie, CD8+ T-cell memory).[148,162] This ability has suggested it could have potential as a vaccine adjuvant.[163] Overall, the fact that IL-15 promotes the proliferation and differentiation of B, T, and NK cells; the cytolytic activity of CD8 T cells; and the maturation of dendritic cells, yet, unlike IL-2, fails to stimulate immunosuppressive T_{reg} cells indicates that it has an array of properties that make it potentially promising as an anticancer therapeutic agent; as a result, a number of ongoing clinical trials are now in progress.[164]

IL-21 is the most recently identified member of the IL-2 family of cytokines. IL-21 can bind to specific receptors and exert actions on T, B, NK cells, DCs, and macrophages.[165] It augments T-cell proliferation as a comitogen,[166] can cooperate with IL-7 or IL-15 to drive the expansion of freshly isolated mouse CD8+ T cells, and it can augment the antitumor activity of CD8+ T cells.[167] IL-21 promotes the differentiation of Th17 cells[168,169] as well as the generation of T follicular helper cells.[170] In the case of Th17 differentiation, IL-21 is part of an IL-6 to IL-21 to IL-23 signaling cascade[169,171,172] to drive the differentiation of these cells in an RORγ-dependent manner.[173] Polarization of Th17 cells is dependent on TCR stimulation, TGF-β, and IL-6 but is independent of IL-23, which instead may be required for maintaining/expanding these cells.[174–177]

The actions of IL-21 on B cells are particularly complex. It augments B-cell proliferation when combined with anti-CD40 or lipopolysaccharide (LPS) but inhibits proliferation in response to anti-IgM + IL-4[166]; this inhibition is reversed if anti-CD40 is additionally provided. It induces apoptosis of incompletely activated B cells, perhaps serving a role analogous to that of IL-2 in activation-induced cell death of T cells to eliminate incompletely activated cells.[165] In contrast, IL-21 drives terminal differentiation to plasma cells of more fully activated cells. Strikingly, IL-21 can drive plasma cell differentiation of both peripheral memory B cells and cord blood B cells, at least in part explained by its ability to induce expression of BLIMP1.[178,179] Strikingly, IL-21 regulates not only BLIMP-1 but also a broad range of genes via a functional cooperation between STAT3 and IRF4.[180] In IL-21R knockout mice, following immunization, IgG1 is diminished related to a role for IL-21 in class switching to IgG1 and IgG3, whereas IgE is elevated, related to the ability of IL-21 to inhibit Cε transcription[181] and/or to its ability to augment the apoptosis of IgE-producing B cells. Analysis of IL-21R/IL-4 double knockout mice has revealed that IL-21 cooperates with IL-4 to globally regulate Ig production in that these mice exhibit a panhypogammaglobulinemia, mimicking the T-cell phenotype in humans with X-linked SCID.[182] IL-21 can also cooperate with IL-15 and Flt-3 ligand to increase development of NK cells[166] and augment antitumor activity[183]; however, it was also reported to oppose the actions of IL-15[184] and can also direct NK cell apoptosis. Interestingly, IL-21 exerts potent antitumor effects, including against large established solid tumors.[11,165] In the pMEL-1 CD8 TCR transgenic system, the effector cells recognize a melanoma antigen. When cells are adoptively transferred into tumor-bearing mice and animals are vaccinated with the cognate antigen, the addition of IL-21 or IL-21 plus IL-15 has potent antitumor effects.[167] Moreover, treatment of cells with IL-21 in vitro prior to adoptive transfer results in markedly enhanced antitumor effects in vivo, conferring a distinctive differentiation program from that conferred by IL-2.[185] IL-21 is now in phase II clinical trials for cancer. In addition to its anticancer activity, IL-21 can also promote autoimmunity. Elevated IL-21 levels have been reported in the BXSB-*Yaa* mouse model of systemic lupus erythematosus,[178] and elevated IL-21 levels have been found in a subset of humans with systemic lupus erythematosus (P. Lipsky, personal communication). Elevated IL-21 has also been associated with other autoimmune processes, including in the non-obese diabetic (NOD) mouse.[181] More importantly, in mouse models of type 1 diabetes, lupus, and experimental allergic uveitis, no autoimmune disease develops on an IL-21R knockout background.[186–188] IL-21 unexpectedly can also be immunosuppressive via its induction of IL-10.[189,190] Consistent with this, an IL-21/IL-10/STAT3 pathway is required for normal development of memory CD8+ T cells after lymphocytic choriomeningitis virus (LCMV) infection[191]; similarly, STAT3, presumably by an overlapping mechanism is important for human T-cell memory development, as evidenced by studies in patients with autosomal dominant hyper-IgE syndrome, which is caused by dominant negative mutations in STAT3.[192] IL-21 has complex roles in viral responses. For LCMV, IL-21 is required to control chronic infection,[193] whereas IL-21 mediates the pathogenic response after infection with pneumonia virus of mice.[194] Interestingly, for hepatitis B virus, IL-21 appears to be critical in determining the age-dependent effectiveness of immune responses, wherein decreased IL-21 production in younger individuals hinders the generation of critical lymphoid responses to the virus, whereas more robust IL-21 production in adults is associated with viral clearance that occurs in 95% of patients.[195]

The receptor for IL-21 consists of IL-21R plus γ_c.[30,44] IL-21R is most related to IL-2Rβ, and like IL-2Rβ, its expression is induced following cellular stimulation with anti-CD3 or phytohemagglutinin, and in addition, its expression is augmented in T cells following transformation with HTLV-I.[196] Both human and mouse IL-21 can act on cells of the other species. IL-21 is on human chromosome 4q26 to 4q27, while its receptor is on chromosome 16p11, immediately downstream of the *IL4R* gene.

Thus, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 collectively exhibit partially overlapping roles related to T cells, NK cells, B cells, and mast cells, and together would be expected to play vital roles for normal development and/or function of these cellular lineages. As discussed in the following, IL-2, IL-7, IL-9, and IL-15 utilize primarily STAT5A and STAT5B; IL-4 activates primarily STAT6; and IL-21 activates STAT1, STAT3, STAT5A, and STAT5B, with STAT3 being the dominant STAT for this cytokine.[11] The fact that these six cytokines share γ_c is of particular interest, especially as the gene encoding γ_c is mutated in patients with the most common form of severe combined immunodeficiency in humans.

X-Linked Severe Combined Immunodeficiency Disease Results from Mutations in the Gene Encoding γ_c

The γ chain was originally identified as a third component of the IL-2 receptor[36] after it became clear that IL-2 receptor α and β chains alone were not sufficient to transduce an IL-2 signal. The hypothesis that the γ chain was a shared component of receptors for cytokines in addition to IL-2 was motivated from a comparison of the clinical phenotypes in humans that result from defective expression of IL-2 versus. the γ chain. In 1993, it was discovered that mutations in the gene encoding the γ chain resulted in X-linked SCID (the disease is also designated as SCIDX1).[30,197] X-linked SCID is characterized by profoundly diminished numbers of T cells and NK cells[30,45,197–201] (Table 25.4). Although the B cells are normal in number, they are nonfunctional, apparently due to a lack of T-cell help as well as an intrinsic B-cell defect.[45,200,202] In contrast to the profoundly decreased number of T cells in patients with X-linked SCID, IL-2–deficient patients[203,204] and mice[205] have normal numbers of T cells (the phenotypes of mice deficient in type I and type II cytokines and their receptor, JAK kinases, and STAT proteins are summarized in Table 25.15). This observation indicated that defective IL-2 signaling was unlikely to be responsible for X-linked SCID, making the finding that the gene encoding the γ chain was mutated in X-linked SCID all the more unexpected. Thus, the conundrum was why a defect in a component of a receptor would cause a more severe than a defect in the corresponding cytokine. This led to the hypothesis that the γ chain was critical for other cytokine receptors as well.[197] In this model, defective IL-2 signaling either did not contribute to the defects in X-linked SCID or these defects were explained by the simultaneous inactivation of multiple signaling pathways.[45,197] Indeed, it was found that the γ chain was also an essential component of both the IL-4 and IL-7 receptors on T cells,[37–40] leading to it being renamed as the common cytokine receptor γ chain, γ_c.[37,38] IL-9, IL-15, and IL-21 were subsequently also shown to share γ_c[30] (Fig. 25.5).

The sharing of γ_c by six different cytokine receptors revealed that X-linked SCID is a disease of defective cytokine signaling. The major deficiencies in X-linked SCID can be attributed to defects related to different cytokines. Based on the dramatically diminished T-cell development not only in IL-7–deficient[104] or IL-7Rα–deficient[103] mice but also in *IL-7R*-deficient humans with T–B+NK+ SCID,[206,207] yet normal T-cell development in mice deficient in IL-2,[205] IL-4,[208,209] both IL-2 and IL-4,[210] IL-9,[133] IL-15,[151] IL-15Rα,[150] IL-21R,[182,184] and most if not all of the defect in T-cell development in patients with X-linked SCID can be attributed to defective IL-7 signaling.[30] In addition to profoundly diminished numbers of T cells, humans with X-linked SCID lack NK cells. As discussed previously, NK-cell development is defective in IL-15– and IL-15Rα–deficient mice, indicating that it is defective IL-15 signaling that is responsible for the defective NK-cell development in X-linked SCID.[30]

In contrast to the greatly diminished number of T cells and absent NK cells in patients with X-linked SCID, B-cell numbers are normal. This is in contrast to the greatly diminished numbers of B cells in γ_c-deficient mice[211,212] as well as mice deficient in either IL-7 or IL-7Rα, indicating that IL-7 is not required for pre-B-cell development in humans and underscoring a major difference for IL-7 in human versus mouse biology. Indeed, patients with *IL-7R*-deficient SCID have a T–B+NK+ form of SCID.[206,207] Although B cells develop in patients with X-linked SCID, they are nonfunctional. This is due in part to a lack of T-cell help (given the near absence of T cells in X-linked SCID), but a variety of data indicate an intrinsic B-cell defect as well.[30] As discussed

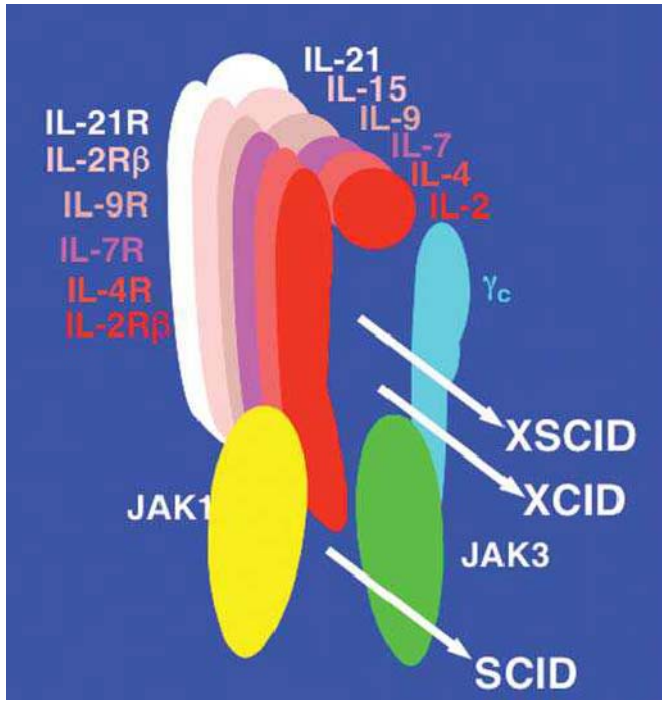

FIG. 25.5. Schematic of the Receptors for Interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21, Showing Interactions with JAK1 and JAK3. The figure shows that IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 all share γ_c. IL-2Rα and IL-15Rα are not shown. Whereas the distinctive chains associate with JAK1, γ_c associates with JAK3. Mutations in γ_c cause X-linked severe combined immunodeficiency or more moderate forms of X-linked immunodeficiency. Mutations in JAK3 cause an autosomal recessive form of severe combined immunodeficiency (see text).

TABLE 25.4 Features of X-Linked Severe Combined Immunodeficiency

1. Absent or profoundly diminished numbers of T cells and mitogen responses
2. Absence of NK cells
3. Normal numbers of B cells, but defective B-cell responses
4. IgM can be normal but greatly diminished Igs of other classes
5. XSCID carrier females exhibit nonrandom X-inactivation patterns in their T cells and NK cells; the X-inactivation pattern is random in surface IgM-positive B cells but nonrandom in more terminally differentiated B cells.

Ig, immunoglobulin; NK, natural killer; XSCID, X-linked severe combined immunodeficiency.

previously, analysis of IL-4/IL-21R double knockout mice indicate that defective signaling by IL-4 and IL-21 appear to explain the intrinsic B-cell defect in X-linked SCID.[182]

Rationale for the Sharing of γ_c

Why should there have been evolutionary pressure to maintain the sharing of γ_c, given the obvious increased risk associated with sharing a receptor component when it is mutated? There are at least two different types of models.[45,213] First, the sharing of γ_c could be a basis for shared actions. For example, given that IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 can each act as T-cell growth factors, at least in vitro, it is possible that γ_c might couple to signal transducing molecule(s) that promote T-cell growth. Second, the sharing of γ_c might represent a means by which one cytokine using γ_c can modulate the signals of the others. To understand this model, it is important to recognize that, in contrast to antigen receptor complexes, cytokine receptor components individually are targeted to the cell surface, and the formation/stability of receptor complexes is promoted or stabilized by ligand binding, as noted in the discussion of growth hormone, wherein the second receptor monomer recognizes the combined surface of growth hormone and the first growth hormone receptor monomer[19] or for the IL-2 receptor where γ_c "sees" a combined IL-2/IL-2Rβ surface.[23] This latter finding is consistent with γ_c originally having been coprecipitated with IL-2Rβ in the presence but not in the absence of IL-2,[214] and dimerization of IL-2Rβ and γ_c is known to be required for efficient signaling.[215,216] Thus, receptor heterodimerization at a minimum is stabilized by the cytokine and physiologically may be absolutely dependent on the presence of the cytokine. In the absence of stable preformed cytokine receptor complexes between γ_c and the other receptor chains, one can envision that γ_c might be differentially recruited to different receptors based on the relative amount of a cytokine or its binding efficiency. In a situation where γ_c is limiting, a cytokine might then not only induce its own action but could also simultaneously inhibit the action of another cytokine that was less efficient at recruiting γ_c to its cognate receptor complex.

An analysis of mice deficient in IL-2,[205] IL-2Rα,[217] IL-2Rβ,[218] and γ_c[211,212] provides the interesting observation that although the mice lacking γ_c have defective signaling in six different cytokine pathways, mice deficient in IL-2, IL-2Rα, and IL-2Rβ appear to be less healthy than the γ_c-knockout mice with more markedly activated T cells and autoimmunity that is not evident in γ_c-deficient mice. This is at least in part explained by the fact that IL-2–deficient mice lack T_{reg} cells and thus develop autoimmune disease,[65] whereas γ_c-deficient mice lack T cells due to defective IL-7 signaling and thus lack effector T cells. These and other data indicate that γ_c plays a major role in regulating lymphoid homeostasis, as originally indicated.[219]

Cytokines whose Receptors Share the Common β Chain, β_c (Interleukin-3, Interleukin-5, and Granulocyte Macrophage-Colony Stimulating Factor)

The hematopoietic cytokines, IL-3, IL-5, and GM-CSF (Table 25.5) are all synthesized by T cells and exert effects on cells of hematopoietic lineage.[220–222] These cytokines are vital for proliferation as well as differentiation of myeloid precursor cells. Of these three cytokines, IL-3 is the most pluripotent[221] and historically was also called multi-CSF, reflecting the large number of lineages on which it can act. It can act to promote proliferation, survival, and development of multipotent hematopoietic progenitor cells and of cells that have become dedicated to a range of different lineages, including granulocyte, macrophage, eosinophil, mast cell, basophil, megakaryocyte, and erythroid lineages. It induces a range of effects, including, for example, inducing the production off IL-4 by basophils.[223] IL-3 also can exert end-function effects, such as enhancing phagocytosis and cytotoxicity. GM-CSF is mainly restricted to the granulocyte and monocyte/macrophage lineages, but its actions are nevertheless still quite broad.[220] It is both a growth and survival factor. In addition, it can expand the number of antigen-presenting cells, such as DCs, and thereby may greatly expand the ability of the host to respond to antigen. IL-23 and RORγt can augment GM-CSF production in Th cells, whereas IL-12, IFNγ, and IL-27 negatively regulate its expression, and GM-CSF is now known to be required for the initiation of autoimmune neuroinflammation in experimental autoimmune encephalitis. Collectively, these results suggest that GM-CSF is important for encephalitogenic actions of both Th1 and Th17 cells.[224,225] GM-CSF mediates autoimmune effects at least in part by enhancing IL-6–

TABLE 25.5 Features of Cytokines whose Receptors Share the Common β Chain, β_c

Cytokine	Major Source	Size	Cellular Targets	Chromosome Location (h/m)	Exons
IL-3	T cells	h152/19aa m166/26 aa 22–34 kDa	Multiple lineages	5q31.1/11	5
IL-5	T cells	h139/22aa m133/21 45 kDa dimer	Eosinophils B cells (?)	5 q31.1/11	4
GM-CSF	T cells	h144/17aa m141/17aa 23 kDa	Granulocytes Macrophages	5q31.1/11	4

GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin.

dependent Th17 cell development and survival.[226] Whereas IL-3 and GM-CSF can act on eosinophils, they act at much earlier stages than IL-5, presumably expanding the number of eosinophil-committed precursors cells. IL-5 stimulates the eosinophilic lineage and eosinophil release from the bone marrow and is essential for expanding eosinophils after helminth infections,[227,228] and can mediate the killing of *S. mansoni*. IL-5 can also induce Ig production in B cells activated by contact with activated Th cells in mouse systems, and IL-5 and IL-5Rα knockout mice have diminished CD5+ B1 cells and decreased thymocytes until approximately 6 weeks of age.[227,228] Interestingly, the production of IL-5 by memory Th2 cells is driven by GATA3 but downregulated by expression of eomesodermin, which interacts with GATA3. Most memory Th2 cells produce the transcription factor Eomes, but the $CD62^{lo}CXCR3^{lo}$ population has decreased Eomes and is the population of cells that produces IL-5.[229]

On cells that express receptors for more than one of these cytokines, such as eosinophilic progenitors that express receptors for IL-3, IL-5, and GM-CSF, or on mouse pre-B cells, which express receptors for IL-3 and IL-5, the signals induced are indistinguishable.[221,222] Thus, the differential lineage specificities of these cytokines are determined by the cellular distribution of their receptors rather than by fundamental differences in the signals that are induced by each cytokine. These observations are explained by studies demonstrating that each of these three cytokines has its own unique 60 to 80 kDa α chain (ie, IL-3Rα, IL-5Rα, and GM-CSFRα),[230–236] but that they share a common 120 to 130 kDa β chain, β_c.[222,237–239] The α chains are the principal binding proteins for the cytokines, whereas the shared β_c subunit augments binding affinity but does not exhibit binding activity in the absence of the proper α chain. The α chains have relatively short cytoplasmic domains (approximately 55 amino acids long for IL-3Rα, IL-5Rα, and GM-CSFRα) and are not believed to play major roles in signaling function, whereas β_c, with its cytoplasmic domain of 432 amino acids, is the primary determinant of the signal. As a result, there is a relative compartmentalization of binding and signaling function for these cytokines, although the cytoplasmic domains of the GM-CSFRα and IL-5Rα chains (and by analogy, perhaps the IL-3Rα chain), as well as β_c, appear to be capable of at least modulating the growth signals in transfected cells.[222,240–242] In any case, the sharing of β_c helps to explain why the signals induced by IL-3, IL-5, and GM-CSF are similar on cells that can respond to more than one of these cytokines. The situation for the β_c family of cytokines is therefore quite different from the receptors for γ_c family cytokines, wherein the chains with the largest cytoplasmic domains (IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, and IL-21R) not only contribute most to signaling specificity but also are the proteins principally involved in ligand binding (note that for IL-2 and IL-15, IL-2Rα and IL-15Rα, respectively, serve important roles as well). The shared chain, γ_c, serves a vital accessory function (hence the development of X-linked SCID when the *IL2RG* gene is mutated), but it contributes less to cytokine binding and does not provide an obvious basis for signaling specificity (see subsequent discussion).

An interesting feature of the β_c family of hematopoietic cytokines is that there appears to be considerable redundancy of function so that knockout mice that lack the ability to respond to all three cytokines (GM-CSF, IL-3, and IL-5) due to deletion of β_c as well as the mouse IL-3–specific β_c-like protein (discussed in the following text) nevertheless exhibit relatively normal hematopoiesis. These observations do not minimize the potency of these particular cytokines but instead underscore a substantial redundancy for a particularly important set of functions.[221,222,243] It is also noteworthy that β_c-deficient mice exhibit defective host responses to infectious challenge, suggesting that these hematopoietic cytokines play a vital role in promoting immune function.

Cytokines whose Receptors Share gp130 (Interleukin-6, Interleukin-11, Oncostatin M, Ciliary Neurotropic Factor, Leukemia Inhibitory Factor, Cardiotrophin-1, Novel Neurotrophin-1/B Cell-Stimulating Factor-3/ Cardiotrophin-Like Factor, and Interleukin-27)

There are now eight cytokines that are known to utilize gp130 as a signal transducing molecule.[244–255] Some of the properties of these cytokines are summarized in Table 25.6. This family is often referred to as the IL-6 family of cytokines and includes IL-6, IL-11, OSM, LIF, CNTF, CT-1, NNT/BSF-3/CLC, and IL-27. This group of cytokines comprises molecules with a diverse range of actions, ranging beyond the hematopoietic and

TABLE 25.6 Cytokines whose Receptors Share gp130[a]

Cytokine	Chromosome Location (h/m)
IL-6	7p21/5
IL-11	19q13.3–13.4/7
LIF	22q12.1–12.2/11
OSM	22q12.1–12.2/11
CNTF	11q12.2/19
CT-1	16p11.1–11.2/7
NNT-1/BSF-3/CLC	11q13/19
IL-27	

Overlapping actions of several gp130 cytokines

	IL-6	IL-11	LIF	OSM	CNTF	CT-1
Growth of myeloma cells	+	–	+	+	+	?
Maintenance of embryonic stem cell pluripotency	–	–	+	+	+	+
Induction of hepatic acute phase proteins	+	+	+	+	+	+
Induction of cardiac hypertrophy	–	+	+	+	+/–	+
Induction of osteoclast formation	–	+	+	+	?	?
Enhanced neuronal survival/differentiaion	+	+	+	+	+	+
Inhibit adipogenesis	?	+	+	?	?	?

BSF-3, B cell-stimulating factor-3; CLC, cardiotrophin-like factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; IL, interleukin; LIF, leukemia inhibitory factor; NNT-1, novel neurotrophin-1; OSM, oncostatin M.

[a]Most of the data in this table are derived from Yin et al.[248]

Note that NNT-1 can support survival of chicken embryonic and sympathetic neurons, can induce amyloid A, and analogous to IL-6 can induce B-cell hyperplasia.

immune systems to the central nervous and cardiovascular systems, making them even more "multifunctional" than the γ_c and β_c families of cytokines, whose actions are more restricted to the lymphoid and hematopoietic systems.

IL-6 was originally identified and then cloned as a B-cell differentiation factor that stimulated terminal differentiation/maturation of B cells into antibody-producing plasma cells.[256–258] However, IL-6 also can exert effects for T-cell growth and differentiation (and thus is a thymocyte "comitogen"), induce myeloid differentiation into macrophages, induce acute-phase protein synthesis of hepatocytes, and exert actions on keratinocytes, mesangial cells, hematopoietic stem cells, the development of osteoclasts, and neural differentiation of PC12 cells.[250] IL-6 is also a mediator of amplified lymphocyte trafficking during febrile inflammatory responses, which is dependent on IL-6–mediated activation of L-selectin.[259] IL-6 binds to an 80 kDa IL-6 binding protein, denoted IL-6Rα, which has a comparatively short 82 amino acid long cytoplasmic domain.[257,260] This IL-6-IL-6Rα complex then interacts with and recruits the 130 kDa signal transducing molecule, gp130, which together with IL-6Rα can form a functional IL-6 receptor.[250] From a structural perspective, gp130 contains a total of six fibronectin type III modules, with the four conserved cysteine residues and the WSXWS motif being located in the second and third of these modules, starting from the N-terminus. As such, these regions are topologically positioned at greater distance external to the cell membrane than is the case for the other type I cytokine receptors discussed previously.

The IL-6 system illustrates a novel twist related to the properties of their principal binding proteins: The cytoplasmic domain of IL-6Rα is superfluous for signaling; a soluble form of the IL-6Rα extracellular domain is in fact sufficient for ligand binding and coordination with gp130, a process termed *IL-6 transsignaling*.[261] Thus, in the presence of soluble IL-6Rα and IL-6, many cell types that express gp130 but not IL-6Rα are capable of signaling in response to IL-6. It was observed that IL-6 signaling requires the dimerization of gp130 and that the overall complex containing two molecules each of IL-6, IL-6Rα, and gp130 (a dimer of a trimer or a hexamer),[32,262–265] providing a possible paradigm for the stoichiometry of subunits for other members of the IL-6 family of cytokines.[24]

IL-11 was identified as a factor produced by a stromal cell line in response to stimulation with IL-1.[266,267] It has a number of effects on hematopoiesis, particularly in combination with IL-3 and SCF. Because IL-11 exhibited "IL-6–like activities," a complementary deoxyribonucleic acid (DNA) was isolated based on the presence of IL-6–like activity in the presence of antibodies to IL-6.[268] Other actions of IL-11 include the ability to stimulate the proliferation of lymphoid and hematopoietic progenitor cells, stimulate megakaryocytic progenitors and megakaryocyte maturation, and stimulate erythroid progenitors (an action not shared by IL-6).[266,267] Overall, it acts on T cells, B cells, hematopoietic cells, epithelium, endothelial cells, and osteoclasts.[269] Like IL-6, IL-11 induces acute-phase proteins and augments antigen-specific B-cell responses, but it does not stimulate human myeloma cells.[266–268,270] Subsequently, adipogenesis inhibitory factor was cloned and found to be identical to IL-11,[271] revealing another action of IL-11. IL-11 is also produced by lung eosinophils and various structural cells in the lung and is expressed in patients with modest-to-severe asthma.[272] IL-11 signals via a receptor complex containing both IL-11Rα[273] and gp130.[244] Interestingly, IL-11Rα messenger RNA can be alternatively spliced to yield a form lacking the cytoplasmic domain, and like IL-6Rα, a soluble form of IL-11Rα can coordinate with IL-11 to transsignal in cells expressing gp130.[274] Studies on the stoichiometry of the IL-11 receptor complex failed to reveal dimerization of gp130 to itself or LIFRβ. Thus, assuming that it forms a hexameric receptor complex, only five of the members are known: two molecules of IL-11 and IL-11Rα, respectively, and one of gp130, suggesting that another component may still be found.[275] IL-11 and STAT3 are expressed in gastrointestinal cancers associated with inflammation, suggesting a possible link to cancer.[269]

LIF is another multifunctional cytokine originally cloned based on the activity associated with its name.[276] LIF can suppress the differentiation of pluripotent embryonic stem cells, inhibit adipogenesis (like IL-11), and induce monocyte differentiation of the M1 murine leukemia cell line, thus mimicking a number of the actions of IL-6.[277] In addition, it exerts a number of actions in the central nervous system, and LIF was shown to be identical to cholinergic neural differentiation factor.[278] and can induce acetylcholine synthesis while simultaneously suppressing catecholamine production, thereby inducing cholinergic function while suppressing noradrenergic function.[278] LIF has been shown to be essential for embryo implantation.[279] LIF binds to a receptor (LIFRβ) that is structurally related to gp130,[280] but the functional LIF receptor (LIFR) receptor requires the heterodimerization of LIFRβ and gp130 as well.[246] Interestingly, whereas IL-6 promotes Th17 differentiation, this is inhibited by LIF, apparently based on LIF's ability to activate ERKs and to augment the expression of SOCS-3, which can inhibit STAT3 activation.[281] Interestingly, LIF can also promote the differentiation of T_{reg} cells and may exhibit therapeutic potential in multiple sclerosis.[282] LIF maintains mouse embryonic stem cells, but LIF is not capable of maintaining human embryonic stem cells or mouse epiblast stem cells in the pluripotent state.[283]

CNTF was discovered based on its ability to promote neuronal survival.[284,285] CNTF signals through a receptor comprising LIFRβ and gp130 but requires a specific binding protein,[286,287] now denoted CNTF receptor (CNTFR)α. Interestingly, CNTFRα lacks transmembrane and cytoplasmic domains and instead is a GPI-linked receptor molecule. CNTFRα appears to provide a receptor-cytokine surface with which gp130 and LIFRβ can interact. Thus, CNTF is like IL-6 in that each requires initial binding to a receptor component (CNTFRα or IL-6Rα) that does not require its own cytoplasmic domain for signaling. Whereas IL-6 signaling involves homodimerization of gp130, CNTF signaling involves the heterodimerization of LIFRβ and gp130. In fact, the functional CNTFR appears to be a hexameric structure containing two molecules of CNTF, two of CNTFRα, and one each of gp130 and LIFRβ.[288] The receptor is expressed largely within the nervous system and in skeletal muscle, accounting for largely restricted actions of CNTF.[286]

OSM is a growth regulator that was originally identified based on its ability to inhibit the growth of A375 human melanoma cells.[289,290] OSM is a potent growth factor for Kaposi sarcoma in patients with acquired immunodeficiency syndrome.[291,292] Not only OSM can bind directly to gp130 and signals through a receptor combination of gp130 and LIFRβ[246] but also has an alternative receptor comprising a specific OSM receptor subunit (OSMRβ) and gp130.[293] These are now known as the type I and type II OSM receptors (OSMRs), respectively. OSM can enhance the development of both endothelial cells and hematopoietic cells, possibly by increasing hamangioblasts, a common precursor for endothelial and hematopoietic cells.[251]

CT-1 was initially isolated based on its actions on cardiac muscle cells.[294] However, it is now clear that it is a multifunctional cytokine with hematopoietic, neuronal, and developmental effects, in addition to its effects on cardiac development and hypertrophy.[295,296] Like OSM and LIF, CT-1 can also signal through a heterodimer of LIFRβ and gp130.[249] Interestingly, the CT-1 receptor on motor neurons may involve a third receptor component, possibly GPI linked.[297,298]

NNT-1/BSF-3, like CNTF, can also support the survival of chicken embryonic sympathetic and motor neurons.[253] Interestingly, in mice, NNT-1/BSF-3 can augment the effects of IL-1 and IL-6 and is a B-cell–stimulating factor (hence the term BSF-3). The NNT-1 receptor contains LIFRβ and gp130.[253] NNT-1 is also known as CLC, and this forms a complex with a soluble receptor protein known as cytokine-like factor-1. Together, this complex is a second ligand for CNTFR.[299] It appears that CLC can also interact directly with soluble CNTFR to form a related cytokine.[254]

IL-27 is an IL-6–related cytokine[300] that represents a dimer of the p28 protein (also known as IL-30) and EB13 (Epstein-Barr virus–induced gene 3) (also known as IL-27B), which can induce proliferation of naive CD4+ T cells. p28 can exist by itself independently of EB13 and is capable of antagonizing gp130-mediated signaling by IL-6, and indeed, overexpression of p28 in transgenic mice prevents normal development of germinal centers and antibody production.[301] Together with IL-12, IL-23, CLC/cytokine-like factor-1, and CLC/soluble CNTFR, this is one of five cytokines that represent dimers including a type I cytokine and a soluble receptor-like protein. IL-27 signals via gp130 and the WSX-1/TCCR receptor,[300] which is discussed in the section on diseases of cytokine receptors and related molecules. IL-27 has proinflammatory and anti-inflammatory effects. Although it was suggested to promote Th1 responses, it is clear that it can also antagonize such responses, and IL-27 can promote effector responses of both CD4+ and CD8+ T cells, of NK cells, and it additionally stimulates mast cells. The anti-inflammatory effects of IL-27 are indicated by the absence of WSX-1 resulting in increased mast cell and macrophage responses.[302,303]

Thus, eight cytokines (IL-6, IL-11, LIF, CNTF, OSM, CT-1, NNT-1/BSF-3, and IL-27) all have receptors that are dependent on gp130.[304] These can be divided into two sets of cytokines: those known to not require LIFRβ (IL-6, IL-11, and IL-27) and those that use both gp130 and LIFRβ (LIF, CT-1, OSM, CNTF, and NNT-1/BSF-3) (Table 25.7), with OSM having two forms of receptors, each of which contains gp130 but only one of which contains LIFRβ. When cytokines share essentially the same receptor, one can hypothesize that two cytokines might exert identical actions on cells that can respond to both cytokines. It is clear that the presence of IL-6Rα, IL-11Rα, and CNTFRα (either on the cell surface or as a soluble receptor form, discussed subsequently) determines whether a cell can respond to IL-6, IL-11, and CNTF. This raises the interesting question as to whether functional homologues of these proteins will also exist for LIF, OSM, CT-1, NNT-1/BSF-3, and IL-27.

TABLE 25.7 Composition of Receptors for the Interleukin-6 Family of Cytokines

Cytokines whose receptors do not contain LIFRβ	
IL-6	IL-6Rα + gp130
IL-11	IL-11Rα + gp130
OSM	OSMRβ + gp130
Cytokines whose receptors contain LIFRβ	
LIF	LIFRβ + gp130
OSM	LIFRβ + gp130
CNTF	CNTFRα + LIFRβ + gp130
CT-1	LIFRβ + gp130 + ?CT1Rα
NNT-1/BSF-3 /CLC + CLF-1	CNTFRα + LIFRβ + gp130
CLC + soluble CNTFRα	LIFRβ + gp130
Cytokines whose receptors contain OSMRβ	
OSM	OSMRβ + gp130
IL-31	IL-31R + OSMRβ

BSF-3, B cell-stimulating factor-3; CLC, cardiotrophin-like factor; CLF-1, cytokine-like factor-1; CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; CT-1, cardiotrophin-1; IL, interleukin; LIF, leukemia inhibitory factor; NNT-1, novel neurotrophin-1; OSM, oncostatin M.

Note that the sharing of CNTFRα by CNTF and the dimeric NNT-1/BSF-3/CLC – CLF-1 ligand helps to explain why the phenotype in CNTF–/– mice is less severe than that found in CNTFRα–/– mice.

Significance of the Sharing of Receptor Chains

Interestingly, γ_c, β_c, and gp130 all contribute to signaling, but none of these shared cytokine receptor proteins has primary binding activity for any known cytokine. Instead, they each increase binding affinity in the context of the primary binding protein for each cytokine. Consequently, the capacity of a cell to respond to a given cytokine is determined by the unique binding chain(s), but signaling pathways can be shared.

Other Receptors with Similarities to gp130 (Granulocyte-Colony Stimulating Factor Receptor, OB-R, Interleukin-12Rβ1, Interleukin-12Rβ2, and Interleukin-31R)

As noted previously, LIFRβ and OSMRβ bear some similarities to gp130.[293] In addition, the G-CSF receptor, the leptin receptor (also denoted OB-R, for obesity receptor), IL-12/IL-23 receptor components, and IL-31R all resemble gp130. The amino acid identity among these different receptors, compared pairwise, ranges from 18% to 32%, with LIFRβ and OSMRβ being the most similar. Although G-CSF is granulocyte-colony stimulating factor, it may have

a broader role. For example, G-CSF has been shown to stimulate myoblast proliferation and thereby to affect skeletal muscle formation,[305] indicating broad pleiotropic actions by this cytokine.

Leptin

Leptin is the product of the obesity (ob) gene, an adipose tissue–derived cytokine that plays a role in body weight homeostasis.[306–308] Additionally, leptin affects thymic homeostasis, increasing thymocyte number and having antiapoptotic effects in the thymus and periphery. It is proinflammatory, promoting Th1 differentiation, and augmenting the production of TNF-α, IL-1, and IL-6 by monocytes/macrophages.[308] The leptin receptor, OB-R, was cloned and found to be most closely related to the gp130 signal transducer, G-CSF receptor, and LIFRβ.[309] Interestingly, this receptor is encoded by the "diabetes gene," which is mutated in db/db mice.[310]

Interleukin-12, IL-23, and IL-35

As noted previously, IL-12 is the major inducer of Th1 cells. IL-12 is primarily produced by phagocytic cells in response to bacterial and intracellular parasites, such as *Toxoplasma gondii*, but it is also produced by other antigen-presenting cells, such as B cells.[311] IL-12 potently induces the production of IFNγ by NK cells and T cells and is also a growth factor for preactivated but not resting NK and T cells. IL-12 was originally discovered as NK cell stimulatory factor.[312] IL-12 can also induce the production of IL-2, IL-3, GM-CSF, IL-9, TNF-α, and M-CSF, although inducing IFNγ is perhaps its most important known action.[311,313,314] As is discussed in the section on immunodeficiency diseases, IL-12 is essential for the proper clearing of mycobacterial infections. It is interesting that Th2 cells do not respond to IL-12; the lack of responsiveness of these cells to IL-12 results from their loss of expression of the IL-12Rβ2 subunit of the IL-12 receptor.[315] Apparently, IL-4 inhibits IL-12Rβ2 expression, whereas IL-2[74] and IFNγ[315] induce its expression. The abilities of mice to survive infections is critically linked to the Th patterns of cytokines. For example, the ability to survive *T. gondii* infection is dependent on IFNγ/IL-12 production (a Th1 pattern).[83]

IL-12 can be thought of as having vital roles in both innate immunity and acquired immune responses. It is rapidly produced by NK cells and then T cells in response to antigens or foreign pathogens. This rapid response facilitates the activation of first-line defense against infections. In addition, however, IL-12 is also required for the subsequent differentiation of specialized T-cell populations, including its STAT4-dependent priming of Th1 cells for optimal production of IFNγ and IL-2. IL-12 can act synergistically with hematopoietic growth factors, such as IL-3 and SCF, to support the proliferation and survival of hematpoietic stem cells.[311] Structurally, IL-12 is a covalently linked dimer of 35 (IL-12A) and 40 (IL-12B) kDa peptides[311]; thus, successful production of IL-12 requires that a cell transcribe both the p35 and p40 genes.[316] Interestingly, whereas p35 bears sequence similarity to IL-6 and G-CSF, p40 is homologous to the extracellular domains of IL-6Rα, CNTFRα, and G-CSF receptor and bears some of the features typical of type I receptors, including four conserved cysteines, a conserved tryptophan, and a WSEWAS motif, which has similarity to the typical WSXWS motif.[311] Moreover, as both IL-12 receptor (IL-12Rβ1 and IL-12Rβ2) chains bear some similarity to gp130,[312,316,317] one can think of p40 as a functional homologue of the soluble p80 IL-6Rα chain. Thus, for this cytokine, part of the "receptor" has become part of the cytokine. Interestingly, all cells that produce IL-12 synthesize much more p40 than p35, suggesting that the careful control of signaling is at the level of the "primordial" p35 cytokine part of IL-12. p40 is on human chromosome 5q31 to 5q33 while p35 is on 3p12 tp 3p13.2.[311] As noted previously, it is interesting that IL-2 induces expression of IL-12Rβ1 and IL-12Rβ2.

IL-23 is similar to IL-12 in that it also contains p40.[318] However, rather than also containing p35, IL-23 is a dimer of a p40 and p19 (IL-23A). IL-23 signals via a receptor that contains IL-12Rβ1 but not IL-12Rβ2.[319] Instead, another receptor chain, denoted IL-23R, is the second component of the IL-23R[320]; both IL-12Rβ2 and IL-23R are located on chromosome 1 within 150 kb of each other.[320] Both IL-12 and IL-23 activate JAK2 and TYK2. IL-12 and IL-23 can activate STAT1, STAT3, STAT4, and STAT5. STAT4 is the dominant STAT protein activated by IL-12.[320] Interestingly, as compared to human IL-23R, mouse IL-23R contains a 20 amino acid duplicated region that spans the WQPWS motif. IL-23 appears to play a vital role in the maintenance, rather than in the initial differentiation of Th17 cells (IL-17–producing cells), and IL-23 is essential for resistance to certain diseases, such as EAE, which were originally believed to be Th1-mediated diseases, as elimination of IL-23 by eliminating either the p40 subunit shared by IL-12 and IL-23 or by eliminating p19 confers resistance to EAE.[175,321] Whereas IL-23 promotes Th17 cell numbers, IL-27 can negatively regulate their development.[321] In the CD40-mediated model of inflammatory bowel disease, IL-12 p35 secretion controlled wasting disease and serum cytokine production but did not affect mucosal pathology, which instead was associated with IL-23/p19.[322] Interestingly, IL-23R has been identified as an inflammatory bowel disease gene,[323] and IL-23 can drive intestinal inflammation via direct actions on T cells.[324] Separate from Th17 cells, a population of innate lymphoid cells that produce IL-17, IL-22, and IFNγ in response to IL-23 have been identified as the mediators of inflammatory bowel disease.[325] IL-23 and its receptor are nevertheless critical for the terminal differentiation of Th17 cells.[169,326] IL-27 is a related cytokine (discussed previously), but it can act as an inhibitor of Th1 responses associated with intracellular infections such as *T. gondii*. Whereas host defense to *T. gondii* is dependent on IL-12 and IFNγ, IL-27–deficient mice control the parasite replication but develop a lethal inflammatory disease that is dependent on CD4+ T cells. Additionally, IL-27 can inhibit Th2 actions.[303]

IL-35 is produced by T_{reg} cells and plays a role in immunosuppression. It is a heterodimer of IL-12A (p35) and IL-27B (EBI3).[327] IL-35 treatment of naive mouse or human T cells induces regulatory cells that have been called iTR35 cells[328] and promote tolerance and tumor progression.

Interleukin-31

IL-31 is a four α-helical bundle cytokine that fits into the greater IL-6 extended family in that its receptor consists of IL-31RA and the OSMRβ.[329] IL-31R is a gp130-like type 1 receptor, with four splice variants. IL-31 is produced primarily by activated T cells and when overexpressed results in pruritis, alopecia, and skin lesions, indicating a potential role in dermatitis.[329,330]

Other Examples of Shared Receptor Molecules

Interleukin-7 and Thymic Stromal Lymphopoietin Share Interleukin-7 Receptor α Chain

In addition to IL-7, a second stromal factor, TSLP has been identified that shares at least some actions with IL-7.[11,331–333] The TSLP receptor is a heterodimer of TSLPR and IL-7Rα.[334,335] Interestingly, TSLPR is 24% identical to γ_c, making it the cytokine receptor most like γ_c.[334] Human and mouse TSLP share only 43% amino acid identity, and human and mouse TSLP receptors share only 39% identity.[336] This is very low for human and mouse orthologues of cytokine receptors, for example, human and mouse γ_c are 70% identical.[337] In addition to their wide sequence divergence, mouse and human TSLP were originally suggested to substantially differ in their actions. Mouse TSLP was first reported to be a B-cell differentiation factor that is important for the development of IgM+ immature B cells from pre-B cells and to also be a weak thymic comitogen. In contrast, human TSLP appeared not to exert effects on these lineages but was found to be an epithelial-derived cytokine that potently activated DCs related to Th2 allergic responses, an action not then known to be shared by mouse TSLP.[338–340] However, it is now clear that mouse TSLP can also activate DCs and that it plays a critical role in both atopic disease and asthma in mouse models, and that these effects of TSLP are mediated via actions on both DCs and CD4+ T cells.[341–347] TSLP can also promote immunity to helminth parasites,[333] and TSLP is also important for mucosal healing in the dextran sulfate sodium–induced colitis model.[348] Interestingly, human TSLP is expressed by Hassall corpuscles[349] and was suggested to promote selection of T_{reg} cells in human thymus, thus contributing to central tolerance.[350] It is striking, however, that mice lacking TSLP receptor have normal numbers of T_{reg} cells, indicating that TSLP is not absolutely essential for this function, and that other molecules can at least substitute for TSLP. Indeed, IL-7 is such a molecule.[350a] TSLP is now known to be produced by a range of cell types, including epithelial cells, fibroblasts, keratinocytes, stromal cells, basophils, mast cells, and DCs,[11] and to in turn act on multiple lineages, including DCs, CD4+ and CD8+ T cells, mast cells, NKT cells, eosinophils, and B cells.[11,333,351] Interestingly, TSLP promotes Th2 differentiation and has been shown to promote metastasis in breast cancer and pancreatic cancer.[352–354] From a signaling perspective, whereas IL-7 activates JAK1 and JAK3, TSLP activates JAK1 and JAK2, making it the only type 1 cytokine to use this combination of JAK kinases to activate STAT5.[347] Although IFNγ also uses JAK1 and JAK2, it instead activates STAT1.[355]

Two Types of Interleukin-4 Receptors, One of which also Responds to Interleukin-13

As detailed previously, on T cells, IL-4 acts through a receptor comprising IL-4Rα and γ_c (the type I IL-4 receptor)[38,39]; however, IL-4 can also signal through type II IL-4 receptors comprising IL-4Rα and IL-13Rα1 but not γ_c.[356] IL-13 is another cytokine that shares some actions with IL-4 and was originally described as a T-cell–derived cytokine capable of inhibiting inflammation,[357] although it is now clear that other cells such as NK cells and mast cells can also produce IL-13. IL-13 can induce identical signals to IL-4 on non-T cells that respond to IL-4 but lacks effects on mature T cells, as these cells do not bind IL-13.[358,359] The shared actions of IL-4 and IL-13 include the ability to 1) decrease expression of inflammatory cytokines, 2) induce major histocompatibility complex class II expression, 3) induce CD23 expression and IgE production by B cells in humans, 4) inhibit IL-2–induced proliferation of chronic lymphocytic leukemia cells of B-cell origin, and 5) costimulate with anti-CD40 antibodies. This is explained by the observation that the type II IL-4 receptor consists of IL-4Rα plus IL-13Rα1,[360,361] with both IL-4 and IL-13 inducing indistinguishable signals on cells expressing this receptor. Interestingly, IL-4 binds primarily to IL-4Rα and IL-13 binds primarily to IL-13Rα1. This situation may be analogous to the situation for LIF, CT-1, and OSM, which can all act through receptors containing LIFRβ and gp130 but differ in their abilities to directly interact with each of these receptor proteins. An additional IL-13 binding protein, IL-13Rα2, has much higher binding affinity for IL-13 than does IL-13Rα1.[362] IL-13Rα2 is nonfunctional in terms of signaling and instead acts as a "decoy" receptor.

Although IL-13 was originally believed to be substantially redundant with IL-4, it has important distinctive actions as well. IL-13 may be vital in asthma, as blocking IL-13 can inhibit pathophysiologic changes of asthma.[363,364] It also is clear that IL-13 regulates eosinophilic infiltration, airway hyperresponsiveness, and mucus secretion, and that it is a potent mediator of fibrosis.[357] Moreover, IL-13 that is produced by innate natural helper cells in the lung contributes to asthma.[365] The phenotype of IL-13 knockout mice suggested a role for IL-13 the ability to expel helminths,[366] and it is clear that IL-13 specifically can modulate resistance to intracellular organisms, including *Leishmania major* and *Listeria monocytogenes*.[357] IL-13 has also been identified as a factor that is secreted by and can stimulate the growth of Hodgkin and Reed-Sternberg cells.[367]

Although IL-4 and IL-13 are both critical for host defense to helminths, the use of reporter mice revealed that Th2 cells produce both cytokines. Tfh cells in lymph nodes as well as basophils make IL-4, whereas innate helper type 2 cells secrete IL-13. Thus, although both are produced during helminth infection, there are distinctive sites of production, correlating with distinctive roles.[368]

An Example of Multiple Affinities of Binding for a Single Cytokine: Three Classes of Interleukin-2 Receptors

Although cytokines typically signal via a single class of high-affinity cell surface receptor, more complex situations exist.

TABLE 25.8 Classes of Interleukin-2 Receptors

Affinity	K_d	Where Expressed	Composition	Functional
Low	10^{-8} M	Activated cells	IL-2Rα	No
Intermediate	10^{-9} M	Resting cells	IL-2Rβ and γ_c	Yes
High	10^{-11}M	Activated cells	IL-2Rα, IL-2Rβ, and γ_c	Yes

IL, interleukin

The IL-2 system provides the very interesting illustration of a system with three classes of affinities of receptors (Table 25.8). In addition to the high-affinity receptor (IL-2Rα + IL-2Rβ + γ_c, $K_d \approx 10^{-11}$ M), there are both low-affinity (IL-2Rα alone, $K_d \approx 10^{-8}$ M) and intermediate-affinity (IL-2Rβ + γ_c, $K_d \approx 10^{-9}$ M) receptors.[29,50] Low- and high-affinity receptors are expressed on activated lymphocytes, whereas intermediate-affinity receptors are found on resting lymphocytes, particularly on NK cells. Both intermediate- and high-affinity receptors can signal, suggesting that IL-2Rβ and γ_c are necessary and sufficient for signaling, in keeping with the theme of dimerization indicated previously. Given that the intermediate-affinity form is functional, what then is the rationale for having a high-affinity IL-2 receptor that also contains IL-2Rα? This is a particularly important question in view of the fact IL-2Rα has an extremely short cytoplasmic domain that does not appear to play a role in signaling. The importance of IL-2Rα is clearly demonstrated by the severely abnormal phenotype of IL-2Rα–deficient mice, which exhibit autoimmunity, inflammatory bowel disease, and premature death,[217] and by the observation that IL-2Rα mutations can cause an autoimmune syndrome in humans as well.[369] A clue to the importance of IL-2Rα comes from the kinetics of association of IL-2 with each chain. Although the IL-2Rα appears to lack a direct signaling function, it has a very fast on-rate for IL-2 binding.[370] Thus, the combination of this rapid on-rate with the slow off-rate from IL-2Rβ/γ_c dimers results in high-affinity binding that is vital for responding to the very low concentrations of IL-2 that are physiologically present in vivo. Moreover, as activated T cells express approximately 10 times as many low-affinity than high-affinity receptors, IL-2Rα may serve as an efficient means of recruitment and concentration of IL-2 on the cell surface, allowing more efficient formation of IL-2/IL-2Rβ/γ_c signaling complexes.

As noted previously, IL-2Rα is not a type I cytokine receptor and in fact has homology to the recognition domain of complement factor B.[371] Subsequently, the IL-15 receptor α chain was shown to also have a similar structure to IL-2Rα,[155] with both IL-2Rα and IL-15Rα having what are called "sushi" domains, which contribute to ligand binding. The fact that IL-2 and IL-15 both have related α chains is consistent with the close relationship between IL-2 and IL-15, and that the receptors for both IL-2 and IL-15 contain both IL-2Rβ and γ_c.

As IL-2Rα cannot transduce a signal by itself, the detection of IL-2Rα on the cell surface does not necessarily reflect IL-2 responsiveness. Because IL-2Rα was discovered before IL-2Rβ and γ_c, many papers in the literature have evaluated IL-2 receptor expression based on IL-2Rα expression alone, and this may not reflect IL-2 responsiveness. IL-2Rα is also expressed on a subpopulation of double negative thymocytes, but it remains unclear if IL-2 physiologically acts on these cells. Each of the components of the IL-2 receptor is located on a different chromosome: Human IL-2Rα is located on chromosome 10p14 to 10p15,[372] IL-2Rβ is at 22q,[373,374] and γ_c is at Xq13.1,[197] while the mouse homologues are located at chromosomes 2, 15, and X.

Erythropoietin, Thrombopoietin, and Stem Cell Factor

Epo was the first cytokine that was biochemically purified and is vital for erythropoiesis, whereas thrombopoietin is critical for thrombopoiesis. These cytokines each bind to receptors that are homodimers.[21,375] Interestingly, Epo signaling may depend in part on the functional cooperation of the Epo receptor and c-kit, the receptor for SCF.[376] This latter receptor has intrinsic tyrosine kinase activity and is not a type I cytokine receptor. Epo was the first cytokine with demonstrated clinical efficacy, for example, in renal insufficiency. Although it is dominantly associated with erythrocyte production, Epo receptors have been found more broadly on immune cells. Recently, Epo has been shown to have effects on a number of cell types, blocking NF-κB p65 and resulting in inhibition of the induction of TNF-α and inducible nitric oxide synthase in activated macrophages, with diminished control of *Salmonella* infection, whereas in a model of chemical-induced colitis, such inhibition of NF-κB–dependent immune modulators limits tissue damage and disease severity.[377] It will therefore be important to determine the full scope of immunologic actions that are mediated by Epo.

CYTOKINE AND CYTOKINE RECEPTOR PLEIOTROPY AND REDUNDANCY

It is well recognized that many cytokines exhibit the phenomena of cytokine "pleiotropy" and "redundancy."[304] Cytokine pleiotropy refers to the ability of a cytokine to exert many different types of responses, often on different cell types, whereas cytokine redundancy refers to the fact that many different cytokines can induce similar actions. One set of cytokines that exhibit cytokine pleiotropy is the γ_c family of cytokines. For example, IL-2 can induce T-cell growth, augment B-cell Ig synthesis, increase the cytolytic activity of lymphokine-activated killer and NK cells, and play an essential role in mediating activation-induced cell death; IL-4 can induce B-cell growth and Ig class switch; and IL-7 not only plays a major role in thymocyte development but also can stimulate mature T cells, and at least in the mouse, can act as a pre–B-cell growth factor. The gp130 family cytokines also exhibit broad actions. For example, IL-6 exerts effects ranging from that of

a comitogen for thymocyte activation to that of a mediator of the acute-phase response in liver. Regarding cytokine redundancy, it has already been highlighted that IL-2, IL-4, IL-7, and IL-15 receptors contain γ_c can act as T-cell growth factors, and that IL-3 has actions that overlap with IL-5 and GM-CSF.

The recognition that cytokines not only have overlapping actions but also share receptor components, led to the concepts of "cytokine receptor pleiotropy" and "cytokine receptor redundancy."[304] The first of these terms can be defined by the ability of a single cytokine receptor subunit to function in more than one receptor. Thus, examples include the sharing of γ_c, β_c, gp130, IL-4Rα, LIFRβ, and OSMRβ, as summarized previously, as well as the sharing of IL-2Rβ by IL-2 and IL-15 receptors, the sharing of IL-4Rα and IL-13Rα in type II IL-4 receptors and IL-13 receptors, and the sharing of IL-7Rα by the IL-7 and TSLP receptors. Another way of viewing receptor pleiotropy is that certain receptor chains are useful "modules" that function in more than one context.

The final term, cytokine receptor subunit redundancy, is the one with fewest examples. There is one well-documented example in mice but not in humans. IL-3 signals through IL-3Rα plus either β_c or an alternative unique IL-3Rβ that shares 91% amino acid identity with β_c and appears to be a completely functionally redundant protein for IL-3 signaling, but IL-3Rβ cannot substitute for β_c in the context of IL-5 or GM-CSF signaling.[239] Other potential examples exist. For example, in type I and type II IL-4 receptors, IL-4Rα coordinates with either γ_c or IL-13Rα1, respectively, and there are two types of OSM receptors, both of which contain gp130, but one of which contains a specific OSM receptor while the other contains LIFRβ. What remains unknown, however, is whether the signals mediated by these different types of receptors are truly identical so that there is redundancy, or whether there are distinctive features to the signals that IL-4 and OSM induce via the different receptors. Indeed, mice lacking IL-13Rα1 have exacerbated Th2 responses and is essential for allergen-induced airway hyperreactivity, suggesting that type 2 IL-4 receptor signaling (either via IL-4 or IL-13) is not identical to that mediated by the type 1 IL-4 receptor (via IL-4).[378]

In addition to these examples related to type I cytokines, the IL-10 subfamily of type II cytokines is interesting in that IL-10Rβ (IL-10R1), IL-20Rα (IL-10R1), IL-20Rβ (IL-20R2), and IL-22Rα (IL-22R) are each shared receptor components, collectively affecting signaling in response to IL-10, IL-19, IL-20, IL-22, and IL-24.[304,379] Specifically, IL-10 signals via a receptor containing IL-10R1 and IL-10R2, IL-19 signals via a receptor containing IL-20R1 and IL-20R2, IL-20 signals via receptors containing IL-20R2 and either IL-20R1 or IL-22R, IL-22 signals via receptors containing IL-22R and IL-10R2, and IL-24 signals through receptors containing IL-20R2 plus either IL-20R1 or IL-22R. Two other IL-10 family members, IL-28 and IL-29, both signal via IL-28R + IL-10R2.[380] Interestingly, IL-10 is believed to be a dimer; IL-26 may also be a dimer, but the other family members are monomers.[379]

SOLUBLE RECEPTORS

Soluble forms of many cytokine receptors have been identified, including those for IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-13Rα2, GM-CSF, type I and type II IFNs, IL-22, and TNF.[381–383] As is clear from this list of cytokines, soluble receptors are not restricted to receptors for type I cytokines, and in the case of IL-2, the principal soluble receptor protein is IL-2Rα, which is not a type I cytokine receptor. Soluble receptors can be created by alternative splicing that truncates the protein N-terminal to the transmembrane domain, resulting in a secreted protein rather than a membrane-anchored membrane in the case of IL-4Rα, IL-5Rα, IL-6Rα, IL-7Rα, IFNAR-2, and GM-CSFRα. Alternatively, they can be created by proteolytic cleavage of the membrane receptor as is found for the receptors for IL-2Rα and TNFRI and TNFRII[382] (Table 25.9). Although it is conceivable that a distinct gene might encode the soluble forms of a receptor, no examples have been reported. In the cases where proteolytic cleavage occurs, the identity of the proteases has not been identified. The major questions related to these soluble receptors are as follows: 1) Do they have physiologic or pathophysiologic functions? 2) How do their affinities compare to the corresponding cell surface receptor? 3) Do they have diagnostic, prognostic, and therapeutic applications?

Unfortunately, there is little information available on the in vivo role of soluble receptors. In general, when analyzed using in vitro studies, soluble receptors can compete with their corresponding cell surface receptors, thereby serving negative regulatory roles. For example, this appears to be the case for the IL-22 soluble receptor. However, soluble IL-6Rα exerts an agonistic role because, as summarized previously, IL-6 signaling occurs equally well via gp130 when the soluble rather than transmembrane form of IL-6Rα interacts with IL-6. Nevertheless, a mutated form of IL-6Rα that cannot interact with gp130 but still binds IL-6 can effectively inhibit the actions of IL-6.[384] In the case of IL-2Rα, there is no reported physiologic function for soluble IL-2Rα, as the affinity of the released receptor is, as expected, similar to that of the low-affinity receptor ($K_d \approx 10^{-8}$ M), making it unlikely to effectively compete with the high-affinity cell surface receptor ($K_d \approx 10^{-11}$ M). However, this and other soluble receptors could serve as cytokine carrier proteins and potentially could increase stability of a cytokine by protecting it from proteolysis.[382] This theoretically could be similar to the role of IL-2/anti–IL-2 conjugates in exhibiting higher activity.[385] Moreover, there are potential diagnostic and prognostic uses for measuring the level of shed receptors (Table 25.10).

TABLE 25.9 Soluble Cytokine Receptors

Generated by Alternative Splicing	Generated by Proteolytic Cleavage of Mature Receptor
sIL-4Rα	sIL-2Rα
sIL-5Rα	sTNFR
sIL-6Rα	
sIL-7Rα	
sGM-CSFRα	
sIFNAR-2	

GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; TNFR, tumor necrosis factor receptor.

TABLE 25.10 Soluble Interleukin-2 Receptors in Human Disease

Malignancies

Hematologic
- Adult T-cell leukemia
- Hairy cell leukemia
- Acute lymphocytic leukemia
- Chronic lymphocytic leukemia (B cell)
- Acute myelogenous leukemia
- Chronic myelogenous leukemia (especially in blast crisis)
- Malignant lymphomas
- Hodgkin disease
- Non-Hodgkin lymphomas

Nonhematologic
- Adenocarcinoma of lung, breast, pancreas
- Small cell bronchogenic carcinoma
- Ovarian, cervical, and endometrial cancers
- Nasopharyngeal carcinoma
- Melanoma

Infections
- Human immunodeficiency virus
- Tuberculosis
- Rubeola
- Infectious mononucleosis

Other diseases
- End-stage renal disease
- Rheumatoid arthritis
- Systemic lupus erythematosus
- Scleroderma
- Sarcoidosis

After transplantation

After interleukin-2 administration

In adults, the mean sIL-2Rα levels are 280 ± 161 units/mL (levels tend to be higher in pediatric populations). The situations where the levels exceed 5000 units/mL are adult T-cell leukemia, hairy cell leukemia, chronic myelogenous leukemia, and after interleukin-2 administration. The situations where levels are between 1000 and 5000 units/mL include acute myelogenous leukemia, chronic lymphocytic leukemia, non-Hodgkin lymphomas, acquired immunodeficiency syndrome associated with Kaposi sarcoma, tuberculosis, rubeola, and end-stage renal disease. Data are from Kurman et al.[381] and Fernandex-Botran et al.[382]

TABLE 25.11 Type II Cytokines

		Chromosomal Location (h/m)
Type I IFNs		
IFNα	Many genes	9p22/4
IFNβ	Single gene	9p21/4
IFNω	Single gene	9p21/4
IFNτ	Many genes	
Type II IFN		
IFNγ	Single gene	12q14/10
IL-10 family cytokines		
IL-10	Single gene	1q31–32/1
IL-19	Single gene	1q32/1
IL-20	Single gene	1q32/1
IL-22	Single gene	12q14–15/10
IL-24	Single gene	1q32/1
IL-26	Single gene	12q14–15/
IL-28	Single gene	19q13/7
IL-29	Single gene	19q13

IFN, interferon; IL, interleukin.

INTERFERONS (TYPE II CYTOKINES) AND THEIR RECEPTORS

IFNs represent an evolutionarily conserved family (Table 25.11) of cytokines that are related to the IL-10 family of type II cytokines. IFNs were discovered in 1957 on the basis of their antiviral activity and were the first cytokines that were discovered.[386] IFNs are known as either type I or type II IFNs,[387–396] where type I interferons include IFNα (originally known as leukocyte IFN) and IFNβ (originally known as fibroblast IFN), and IFNε, IFNκ, IFNω, IFNδ, and IFNτ. IFNδ and IFNτ are absent in humans. IFNω is closely related to the IFNαs and was formerly designated as an IFNα. There are multiple (at least 12) IFNαs in mice and humans. In contrast, the other type I IFNs, IFNβ and IFNω, are each encoded by single human and mouse genes near the IFNα cluster, and in addition, there are multiple pseudogenes most closely related to IFNα and IFNω. The type I IFNs are clustered on human chromosome 9 and mouse chromosome 4. Type II interferon is IFNγ,[314] which is encoded by a single gene on human chromosome 12 and mouse chromosome 10. IFNα/β is produced after viral infection in many cell types, although plasmacytoid DCs appear to be the largest producers of IFNα/β. These type I IFNs inhibit viral replication and induce the apoptosis of virally infected cells. In addition, IFNα/β mediate the activation of macrophages and NK cells, and they additionally affect the proliferation and survival of CD8+ T cells.

The grouping of the IFNαs and IFNβ together as type I IFNs is logical not only because of the similar amino acid sequences and structures of these IFNs but also based on the fact that they share the same receptor and induce essentially the same signals.[397,398] Although DNA array analysis does show some differences in the genes induced by IFNα and IFNβ, the basis for these differences are unclear.[399] These signals include not only antiproliferative and antiviral activities but also the ability to stimulate cytolytic activity in lymphoyctes, NK cells, and macrophages. In contrast, IFNγ has a distinct receptor. Type I and type II IFN receptors share a sufficient degree of similarity to each other so as to form a family.[400] The structure of the IFNγ receptor[401] is shown in Figure 25.2. IFN receptors are referred to as type II cytokine receptors, and there are substantial differences between these receptors and the type I cytokine receptors.[8] Because both type I and type II IFNs bind to type II cytokine receptors, IFNs are occasionally referred to as type II cytokines but more generally are referred to as IFNs. Based on the similarity of the

IL-10 receptor to the IFNγ receptors,[8] IL-10 was designated as a type II cytokine, and indeed when its x-ray crystal was determined, IL-10 was found to be topologically related to IFNγ.[16] As noted previously, more recently a series of IL-10–related cytokines has been identified, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29, and these are also designated as type II cytokines, with IL-28A, IL28B, and IL-29 also known as IFNλs. Among type II cytokines, IFNγ has helices similar to those of the type I short-chain helical cytokines, but its short helices that occupy the AB and CD loops positions exhibit the long-chain cytokine-like AB over CD topology. IL-10 and IFNαβ have long-chain structures.[8] Thus, the theme of short-chain and long-chain type I cytokines also extends to the IFNs and the IL-10 family of type II cytokines.

Type I IFNs signal through a receptor known as the type I IFN receptor.[388,392,394,396,397] The receptor consists of at least two chains.[402–405] In contrast to the α and β chain nomenclature typical for type I cytokine receptors, IFN receptor chains are officially denoted as IFNAR-1 (previously also denoted IFN-αR1, IFNAR1, and IFN-Rα) and IFNAR-2 (previously also known as IFNα/β receptor, IFNαR2, IFNAR2, and IFNRβ).[406] IFNAR-2 has both short and long forms as well as a soluble form.[407] The long form has a much larger cytoplasmic domain and serves a more important role in signal transduction. Whereas IFNAR-1 cannot bind IFNα, IFNAR-2 binds with low affinity, and the combination of both chains results in high-affinity binding[407] and function. As detailed in the following, IFNAR-1 binds the Janus family tyrosine kinase TYK2, whereas IFNAR-2 binds JAK1. In addition to these cellular receptors, it is interesting that vaccinia virus and other orthopoxviruses encode a soluble form of type I IFN receptor that is related to the IL-1 receptors and that is capable of binding IFNα, IFNβ, and IFNω.[408,409] This form of IFN receptor is therefore not a member of the type II cytokine family but instead is an Ig superfamily protein. IFNαs are typically species specific, although IFNα8 is a human type I IFN that can bind to the mouse receptor. IFNAR-1 confers species specificity of binding. As noted previously, type I IFNs are induced by viral infection. Infection with viruses results in the generation of nonself RNAs in the cell, which are recognized by retinoic acid inducible gene-I (RIG-I)–like receptors, which include RIG-I, MDA5, and LGP2, each of which contains a helicase domain. Retinoic acid inducible gene-I–like receptors are essential for the production of tpe I interferons as well as IL-6, and cells lacking both RIG-I and MDA5 are completely defective in the production of type I IFNs.[410]

IFNγ was first recognized in 1965 as immune IFN, named as IFNγ in 1980, cloned in 1982, and then confirmed to be the same as primary macrophage activating factor.[388,397,411–413] In addition to its antiviral activity, IFNγ also has a range of actions on proinflammatory and anti-inflammatory host responses.[393] For example, IFNγ can prime macrophages to manifest antimicrobial and antitumor effects. Moreover, following IL-12–mediated differentiation of Th1 cells, IFNγ is a major secreted product. This production is critical for host response to a range of infectious pathogens, including, for example, *T. gondii*. IFNγ inhibits the generation of Th2 cells as well as Th17 cells. IFNγ is produced by a range of cells, including T_{reg} cells and in this context appears to result in apoptosis of naive cells and Th2 effector T cells. It is produced by NK cells and can mediate NK-dependent lysis of tumor cells, augment IgG2a isotype switching of B cells, and activate DCs. Moreover, IFNγ can either contribute to disease protection or can be protective, depending on the context.[413] Finally, IFNγ- or anti-IFNγ–based therapy are being tried in a range of conditions.[314]

IFNγ is encoded by four exons on chromosome 12. IFNγ forms a functional homodimer with an apparent molecular weight of 34 kDa, whereas little of the monomeric form can be detected and it is not biologically active. Each IFNγ monomer has six α helices, four of which resemble the short-chain helical cytokines, and there is no β sheet structure. The subunits interact in an antiparallel fashion. In contrast to the ability of many different cells to produce IFNα, IFNγ is more restricted, with it being produced primarily by NK cells, CD8+ T cells, and the Th1 subclass of CD4+ T cells.[388] Many signals, including antigen stimulation, IL-12, and IL-18, can induce the production of IFNγ. IFNγ exerts its effects through specific receptors that are expressed on all types of cells except erythrocytes. Interestingly, even platelets express IFNγ receptors, raising the possibility that they can serve a function in transporting IFNγ in the circulation.[388] The functional human receptor consists of two chains[414]: IFNGR-1, formerly also denoted IFNγR1 or IFNγRα[406,415] a 90 kDa protein whose gene is located on human chromosome 6q16 to 6q22 and mouse chromosome 10,[412] and IFNGR-2, also denoted as IFNγRβ,[416,417] located on human chromosome 21q22.1 and mouse chromosome 16.[412] IFNGR-1 is required for ligand binding, whereas IFNGR-2 plays a role in signaling. JAK1 associates with the Leu-Pro-Lys-Ser sequence in the membrane proximal region of the cytoplasmic domain of IFNGR-1,[418] whereas JAK2 binds to IFNGR-2.[419] The fact that IFNγ is a homodimer explains how its binding induces the homodimerization of IFNGR-1, which then allows the recruitment of IFNGR-2. Thus, the functional IFNγ receptor is believed to contain two molecules each of IFNGR-1 and IFNGR-2. Normal IFNγ production is dependent on IL-12, and defective IFNγ signaling is associated with failure to appropriately clear mycobacterial and other infections (discussed subsequently).

Both type I and type II IFNs are important for cancer immunoediting, a process in which neoplastic growth is suppressed by the immune system and tumor immunogenicity is shaped. IFNγ can suppress tumor development, but type I IFNs are required to initiate the antitumor response, at least in part via actions on DCs.[420]

Interleukin-10, a Type II Cytokine, and the Related Cytokines Interleukin-19, Interleukin-20, Interleukin-22, Interleukin-24, Interleukin-26, Interleukin-28A, Interleukin-28B, and Interleukin-29

IL-10 is a type II cytokine that originally was identified as cytokine synthesis inhibitory factor,[380,421,422] produced by Th2 cells. However, it then was observed to be produced by T_{reg} cells as well, and it is now clear that IL-10 is much

more broadly expressed, including by Th1, Th17 cells, B cells, macrophages, NKT, NK, T_{reg}, monocytes, and myeloid DCs but not plasmacytoid DCs, keratinocytes, as well as mast cells and eosinophils.[423,424] IL-10 production by T_{reg} cells helps to explain their suppressive effects, and IL-10 production by T_{reg} cells is required for their suppression of Th17-mediated inflammation.[425] The IL-10 gene contains five exons and is located on chromosome 1 in both mice and humans.[422] IL-10 has an open reading frame of 178 amino acids, including the signal peptide, and the mature protein is 18 kDa. Human IL-10 receptor maps to 11q23.3. IL-10 can inhibit the production of a number of cytokines, including IL-2, IL-3, IFNγ, GM-CSF, and TNF-α. Production of IL-10 is substantially dependent on the transcription factor E4BP4 (also called NFIL-3).[426] IL-10 production is induced by pathogen-derived products, with TLR2 signaling being critical for its induction by antigen-presenting cells and macrophages, although TLR-independent induction of IL-10 has also been observed.[423] Interestingly, IL-21 has been shown to be a potent inducer of IL-10 and to mediate IL-27 and part of the IL-6–mediated induction of IL-10 as well.[189,190] IL-10 inhibits monocyte/macrophage/DC-dependent T-cell proliferation, in part, by markedly decreasing synthesis of a variety of cytokines, expression of costimulatory molecules, and chemokines, to name just some of its inhibitory effects.[380] In addition to these indirect effects on T cells, IL-10 can exert direct stimulatory effects on thymocytes and T cells in vitro and promote the development of B1 cells and activity of NK cells. IL-10–deficient mice develop a form of inflammatory bowel disease that is similar to Crohn disease.[422,427] Interestingly, the BCRF1 protein that is encoded by Epstein-Barr virus is very similar to IL-10 and shares many of its biologic properties as a macrophage "deactivating" factor and as a costimulator of proliferation of B cells.[428] The Epstein-Barr virus IL-10 homologue is a selective agonist, although its binding to the IL-10 receptor is somewhat impaired.[428] IL-10 is a major inhibitor of Th1 functions.[422] Although it was originally suggested that IL-10 might also favor Th2 development, IL-4 is the major mediator of Th2 cell development, and IL-10 instead plays a major role in limiting and terminating inflammatory responses.[422]

The IL-10 receptor is most closely related to IFN receptors, making it a type II cytokine receptor[429,430] and corresponding to the close structural relationship of IL-10 to IFNγ. The receptor for IL-10 consists of an IL-10R1 chain and IL-10R2[422]; this latter protein is the same as CRF2-4, which was first identified as an "orphan" IFN receptor family member that is located on chromosome 21 within 35 kb of IFNGR-2.[431,432] The major STAT protein used by IL-10 is STAT3.[423]

A series of IL-10–related cytokines have been been identified, including IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29.[379,424,433–437] IL-19 was discovered as a gene that was induced in LPS-stimulated monocytes. IL-19 has been suggested to potentially be involved in the pathogenesis of chronic inflammatory diseases, such as psoriasis. Like IL-19, IL-20 is produced by myeloid cells; it was also found to be produced by epidermal cells, with overexpression resulting in aberrant epidermal differentiation. IL-20 is believed to have a role in psoriasis, and in psoriatic skin, expression of IL-20 is primarily detected in DCs.[379,438] IL-20 also appears to contribute to rheumatoid arthritis, atherosclerosis, and stroke; recently, IL-20 has been implicated in bone loss, with elevated IL-20 in patients with osteopenia and osteoporosis in in ovariectomized mice. Moreover, IL-20R1–deficient mice have increased bone density as did treatment with anti–IL-20.[439]

IL-22 was found as an IL-9–induced cytokine that can in turn induce acute-phase reactant production by hepatocytes and accordingly that it might be involved in inflammatory responses. It was first observed to be produced by activated Th1 cells but was subsequently noted to be more broadly produced by CD4+ and CD8+ T cells, NK cells, lymphoid tissue-inducer cells and mucosal RORγt+NKp46+ cells in humans and mice,[440–442] and to be produced by Th17 cells. IL-22 is often coexpressed with IL-10, and STAT3, the Notch pathway, and the aryl hydrocarbon receptor promoter production of these cytokines; in contrast, TGF-β, ICOS, and IL-27 augment IL-10 production yet inhibit IL-22.[424] IL-22 mediates cross talk between leukocytes and tissue epithelia, with target cells primarily in the digestive and respiratory systems, but it also can act on keratinocytes, can promote innate immunity,[443] and can cooperate with IL-17A to effect epithelial and healing responses. In particular, IL-22 can contribute to epithelial resistance to injury after microbial infection of the gut or lungs, and thereby may play a role in host defense and tissue repair.[442] Interestingly, the transcription factor c-Maf mediates suppression of IL-22 production by TGF-β.[444]

IL-24 was originally discovered as melanoma differentiation-associated antigen 7. IL-24 is induced in peripheral blood mononuclear cells by mitogen stimulation and is produced not only by Th2 cells but also by myeloid cells. Overexpression of IL-24 can induce apoptosis in a wide range of tumor cells, including, for example, melanoma and malignant gliomas, and infection with adenoviral-drive melanoma differentiation-associated antigen 7 can sensitize tumors to ionizing radiation.[380] Like IL-19, IL-20, and IL-22, IL-24 is expressed in psoriatic but not normal skin and like these other cytokines, functional receptors are highly expressed on keratinocytes. IL-10, IL-19, IL-20, IL-22, and IL-24 are all increased in synovial fluid cells in rheumatoid arthritis and thus are potential mediators of this autoimmune disease. IL-20 and IL-22 in particular may be associated with lupus.[379]

IL-26 was originally discovered as AK155. Relatively little is known regarding the function of IL-26.

IL-28A and IL-28B (IFNλ1 and IFNλ2) and IL-29 (IFNλ3) are also called type III IFNs and are induced in plasmacytoid DCs by viral infection and exhibit antiviral activity, analogous to type I IFNs; nevertheless, they bind to a receptor consisting of IL-10R2 and IL-28R[445] and differ in terms of potency and kinetics from IFNα.[379] IL-28A, IL-28B, and IL-29 induce relatively weak antiviral responses, and the IL-28R chain is relatively restricted in its expression, as compared to the IFNα receptor. IL-28A appears to enhance antiviral activity induced by TLR3 and TLR9 and to be important for host defense to influenza A.[379] Interestingly, the genes encoding IL-10, IL-19, IL-20, and IL-24 colocalize at human chromosome 1q32, whereas those encoding IL-22

and IL-26 are at 12q14 to 12q15 near the *IFNG* gene, and IL-28 and IL-29 are at 19q13.[380,446] Thus, these type II cytokines can be subdivided into three different groups based at least in part on chromosome localization.

Overall, IL-10 family cytokines are diverse and can be subdivided into the IL-19, IL-20, IL-22, IL-24, and IL-26 subfamily that acts on tissue epithelial cells and in the case of IL-20 in wound healing, whereas IL-28A, IL-28A, and IL-29 exhibit antiviral activity and are more IFN-like in their biology.[379]

SPECIES SPECIFICITY OF CYTOKINES

There are no general rules for the species specificity of human and mouse cytokines, and how the cytokines and their receptor chains have coevolved. To provide illustrative examples of each situation, human IL-2 can stimulate both human and mouse cells, whereas mouse IL-2 exhibits little action on human cells.[59] Conversely, human IL-12 does not work on mouse cells, whereas mouse IL-12 is biologically active on both mouse and human cells.[311] This selective property of IL-12 is dependent on the species origin of p35. Finally, IL-4 exhibits rather strict specificity so that human and mouse IL-4 only induce responses on human and mouse cells, respectively.[92,93] As noted previously, IFNαs are generally specifies specific, although IFNα8 is not. In addition to these examples, varying degrees of relative species specificity have been demonstrated, depending on the cytokine. Thus, a cytokine may or may not be restricted in its specificity to its own species, and if it does occur, cross-species activity may have attenuated potency.

SIGNALING THROUGH INTERFERON AND CYTOKINE RECEPTORS

Our understanding of signaling through IFN and cytokine receptors has tremendously increased in the past few years. Multiple signaling pathways/molecules have been observed for various cytokines. Collectively, these include the JAK-STAT pathway, IRF family proteins, Ras/mitogen-activated protein (MAP) kinase pathway, Src and Zap70 and related proteins, PI 3-kinase, IRS-1 and IRS-2, and phosphatases. Each of these pathways will be discussed in turn. The JAK-STAT pathways are important for both type I and IFNs/type II cytokines, while the IRF proteins play central functions more so for the IFNs than type I cytokines.

OVERVIEW OF JAKS AND STATS

The JAK-STAT pathway[30,355,397,447–449] is particularly exciting in that it serves as a rapid mechanism by which signals can be transduced from the membrane to the nucleus. JAK kinases are known as Janus family tyrosine kinases, and the acronym "STAT" denotes signal transducer and activator of transcription. STAT proteins are substrates for JAK kinases. A tremendous amount of information is now available on JAKs and STATs that demonstrate their importance related to development, differentiation, proliferation, cellular transformation, and tumorigenesis.

JAKs

The JAK kinases are 116 to 140 kDa, and comprise approximately 1150 amino acids.[355,397] The seven regions of conserved sequences in JAK kinases, denoted JH1 to JH7, are depicted in Figure 25.6. One of the hallmark features of these kinases is that in addition to the presence of a catalytic tyrosine kinase domain (JH1), there is also a pseudokinase region (JH2). The name Janus kinase reflects the two faces of the mythological Roman god, with one face representing the true kinase and the other the pseudokinase. Although the JH nomenclature was used historically, it has obvious limitations in that except for the JH1 catalytic and JH2 pseudokinase domains, and it remains unclear whether or not the other JH regions correspond to discrete domains. Modeling has indicated five important regions, including an N-terminal domain, a FERM (4.1, ezrin, radixin, moesin) domain, and SH2, kinase-like (which spans two of the JH domains), and kinase domains.[450]

There are four mammalian JAK kinases, JAK1,[451] JAK2,[452] JAK3,[453,454] and TYK2,[455] each of which was identified as part

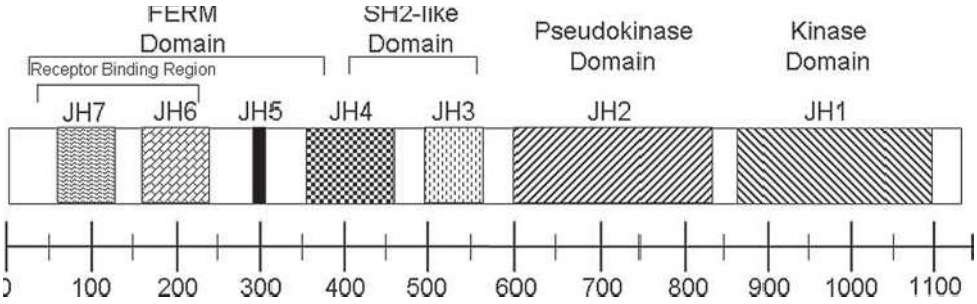

FIG. 25.6. Schematic of JAK Kinases. Shown are the locations of the seven JH domains. JH1 is the catalytic domain. JH2 is the pseudokinase domain, the presence of which prompted the naming of this family as Janus family tyrosine kinases. As noted in the text, the JH nomenclature has limitations and in fact masks the presence of an SH2 domain that spans parts of the JH4 and JH3 domains. Also shown is the conserved tyrosine (Y1007 in JAK2) whose phosphorylation is required for maximal catalytic activity.

TABLE 25.12 Features of Janus Family Kinases

Kinase	Inducible versus Constitutive	Size	Chromosomal Location (H/M)
JAK1	Constitutive	135 kDa	1p31.3/4
JAK2	Constitutive	130 kDa	9p23–24/19
JAK3	Inducible	116 kDa	19p13/8
TYK2	Constitutive	140 kDa	19p13.2/9

of a study intended to identify new kinases.[355] At least one JAK kinase is activated by every IFN and cytokine, and some cytokines activate two or three JAK kinases.[355,397,447,448] Table 25.12 lists a number of features of each JAK kinase, whereas Table 25.13 summarizes the JAK kinases that are activated by a variety of cytokines.

Because JAK1, JAK2, and TYK2 are ubiquitously expressed, each cell type expresses either three or in some cases all four JAK kinases (eg, lymphoid cells that also express JAK3). The JAK kinases that are activated by a specific cytokine are those that can bind to its receptor's cytoplasmic domain. JAK kinases physically bind to the membrane proximal Box 1/Box 2 region of the cytoplasmic domains,[454–459] and the N-terminal region of the JAK is required for this function.[460–462] In cytokine receptors, the Box 1 region is proline rich,[26] suggesting that JAK kinases may have SH3-like domains in their N-terminal regions to mediate these interactions. As each cytokine or IFN receptor is a homodimer, heterodimer, or higher order oligomer, it is reasonable to assume that at least two JAK molecules (either two molecules of one JAK or one molecule each of two different JAKs) will be activated, as one JAK will be associated with each receptor chain. In accord with their ubiquitous expression, JAK1, JAK2, and TYK2 are activated by a variety of different sets of cytokines (see Tables 25.12 and 25.13). For example, JAK1 is activated not only by type I and II IFNs but also by the γ_c family of cytokines (eg, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), whereas JAK2 is activated not only by IFNγ but also by growth hormone, Epo, prolactin, and the hematopoietic cytokines, IL-3, IL-5, and GM-CSF.[355,447] TYK2 is somewhat more restricted in that it is activated by IFNα/β and IL-12/IL-23; the significance of its activation by gp130 family cytokines is less clear. Interestingly, JAK1, JAK2, and TYK2 are recruited by each of the cytokine receptors that share gp130 as a signal transducing molecule, raising the question as to whether these cytokines require all three JAK kinases for optimal function or whether any one or two is/are sufficient. At least for IL-6, JAK1 is vital,[463,464] whereas the importance of JAK2 and TYK2 is less clear.

JAK3 is different from the other JAK kinases in that it is much more inducible. Moreover, JAK3 is only activated by cytokines whose receptors contain γ_c.[30,45] Interestingly, each cytokine whose receptor contains γ_c activates not only JAK3 but also JAK1. The basis for the activation of both JAK1 and JAK3 by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 is that JAK1 associates with each of the distinctive signaling chains (IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, and IL-21R), whereas JAK3 associates with γ_c.[30,45] Although this could be a coincidental result of coevolution of these cytokine systems, these observations raise the possibility that JAK1 is the JAK kinase that most efficiently functionally cooperates with JAK3.

Given the wide range of cytokines that activate any particular JAK kinase and that in some cases multiple cytokines can activate the same set of JAKs, it is clear that the JAK kinases by themselves do not determine signaling specificity. For example, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 all activate JAK1 and JAK3 but induce a range of actions. Moreover, JAK2 is the only JAK kinase that is activated by growth hormone and erythropoietin, cytokines with different target cells and biologic functions. Interestingly, JAK2 is the JAK that interacts with all cytokine receptors that form homodimers. Given that homodimeric receptors are likely the oldest cytokine receptors in evolution, this suggests that JAK2 might be the "primordial" JAK kinase from which others evolved.

TABLE 25.13 Cytokines and the JAKs They Activate

Cytokine	JAK Kinase(s) Activated
IFNα/β	JAK1, TYK2
IFNγ	JAK1, JAK2
Growth hormone	JAK2
Prolactin	JAK2
Erythopoietin	JAK2
Thrombopoietin	JAK2
IL-10	JAK1, TYK2
IL-12, IL-23	JAK2, TYK2
G-CSF	JAK1, JAK2
γ_c family	
IL-2, IL-4,[a] IL-7, IL-9, IL-15, IL-21	JAK1, JAK3
β_c family	
IL-3, IL-5, GM-CSF	JAK2, ?JAK1
gp130 family	
IL-6, IL-11, CNTF, LIF, OSM, CT-1	JAK1, JAK2, TYK2
TSLP	JAK1, JAK2

CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin M; TSLP, thymic stromal lymphopoietin.

[a]Note that IL-4 activates JAK1 and JAK3 when it acts through the type I IL-4 receptor (IL-4Rα + γ_c, found for example on T cells). However, JAK3 is not activated when IL-4 signals through the type II IL-4 receptors (IL-4Rα + IL-13Rα1, a form of receptor that is expressed on a number of non-T cells, including fibroblasts).

Importance of JAK Kinases in Signaling

In addition to the activation of JAK kinases by multiples cytokines and IFNs, a variety of other data indicate their importance for signaling. One of the vital series of experiments that led to the establishment of the critical role of JAK kinases in IFN signaling involved a group of mutant cell lines that were defective for IFN signaling, but wherein signaling could be rescued by genetic complementation.[397] Defective signaling in response to IFNα and IFNβ was found in a mutant fibroblast cell line (U1 cells) lacking TYK2; defective signaling in response to IFNα,

IFNβ, and IFNγ was found in a mutant cell line lacking JAK1 (U4 cells), and defective IFNγ signaling was found in cells lacking JAK2.[397] A variety of other data have indicated the importance of JAK kinases in cytokine signaling pathways. First, dominant negative JAK2 inhibits signaling by erythropoietin and growth hormone,[465,466] while a dominant negative JAK3 inhibits signaling in response to IL-2[467]; as noted previoulsy, JAK1 is vital for IL-6 signaling. Second, humans[468,469] and mice[470–472] deficient in JAK3 exhibit developmental and signaling defects. Third, humans with an activating mutation in JAK2 develop polycythemia vera,[473] and a JAK2 inhibitor inhibited the growth of acute lymphoblastic leukemia cells in vitro.[474] Fourth, a patient with mutation in TYK2 developed a form of immunodeficiency.[475] Fifth, in Drosophila, the *hopscotch* gene encodes a JAK kinase, wherein loss-of-function alleles result in lethality and decreased proliferation, whereas a gain-of-function allele, *hopscotch*$^{Tumorous\text{-}lethal}$ results in melanotic tumors and hypertrophy of the hematopoietic organs.[476,477] Sixth, in zebrafish, JAK1 is vital for normal cell migration and anterior specification.[478] Seventh, as discussed in the following, JAK kinases are constitutively activated in many cell lines infected with a number of viruses, including HTLV-I, v-Abl, spleen focus-forming virus, and with v-Src.[479–482] These data together underscore the vital roles of JAKs in cytokine signaling. Depending on the function of the particular cytokine (eg, development, differentiation, or proliferation), the particular JAK kinases potentially may be involved in a variety of processes, and when dysregulated, in at least certain settings, they appear to contribute to cellular transformation.

JAK3 Mutations Result in an Autosomal Recessive Form of Severe Combined Immunodeficiency that is Indistinguishable from X-Linked Severe Combined Immunodeficiency

A very large number of different mutations in γ_c have been observed in X-linked SCID. As might be expected, in cases where it has been examined, amino acid substitutions in the extracellular domain result in defective cytokine binding. In contrast, mutations or truncations in the γ_c cytoplasmic domain result in defective signaling. Analysis of a family in which a number of males exhibit a moderate form of X-linked combined immunodeficiency revealed that this disease also resulted from a mutation in γ_c[41] (Leu 271 → Gln) that resulted in a decrease, but not total loss of JAK3 association, in contrast to the loss of JAK3 interaction seen with mutations in the γ_c cytoplasmic domain that cause X-linked SCID. Thus, the severity of the immunodeficiency inversely correlated with the degree of JAK3 activation.[30,45] Moreover, it was predicted that JAK3 was required for T-cell and NK-cell development, and that mutations in JAK3 might result in a clinical phenotype indistinguishable from that in X-linked SCID.[41] Indeed, this is the case in humans.[468,469] As one would hypothesize, many mutations in JAK3 have been identified.[355] Presumably, any mutation that interferes with its ability to interact with γ_c, with its catalytic activity, or with recruitment of substrates could result in clinical disease, ranging from moderate-to-severe immunodeficiency.

Analogous to the similarity of X-linked SCID and JAK3-deficient SCID, mice deficient in either γ_c or JAK3 also have indistinguishable phenotypes.[211,212,470–472] These in vivo data underscore the vital role of JAK3 in mediating γ_c-dependent functions and suggested that JAK3 is essential for most, if not all, γ_c functions. Some in vitro data indicate that γ_c may do more than to recruit JAK3,[467,483] and interestingly, γ_c can interact with calpain,[484] but the recruitment of JAK3 is clearly essential, and the defects in T-cell and NK-cell development associated with γ_c or JAK3 deficiency are indistinguishable. Because JAK3 deficiency is not clinically or phenotypically more severe than γ_c deficiency in humans and mice, it seems likely that γ_c is the major, if not only, protein with which JAK3 associates. Moreover, as had been predicted based on the association of γ_c with JAK3,[41] JAK3 inhibitors are immunosuppressive, and one JAK inhibitor has now been approved by the FDA.[485] Although JAK3 was suggested to associate with CD40,[486] a functional role for JAK3 in CD40 signaling has been questioned.[487] The phenotypes of mice lacking each of the four JAK kinases are summarized in Table 25.14.

JAK2 Mutations and Translocations

The JAK2V617F mutations has now been identified in most of patients with polycythemia vera,[473,488] some of whom have homozygous and some have heterozygous mutations. The homozygous patients had duplications of the mutated allele. This mutation results in a constitutively activated kinase and presumably confers factor-independent growth, something that has been demonstrated in transfected cell lines. Moreover, patients with polycythemia exhibit erythropoietin-independent BFU-E. Furthermore, the *JAK2* locus is involved in a chromosomal translocation to create the TEL-JAK2 protein that is causally related to a human leukemia,[489] further underscoring the relationship of JAK2 to the growth of hematopoietic cells.

TYK2 Mutations and Human Immunodeficiency

A mutation in TYK2 has now been identified in a patient with primary immunodeficiency.[475] The patient had clinical defects more severe than anticipated from TYK2-deficient mice, with increased susceptibility to viral infections and atypical mycobacterial infections and defective signaling in response to IL-23, IL-6, and IL-10. Additionally, the patient had hyper-IgE production, which perhaps corresponds to enhanced Th2 cell–mediated allergic inflammation in TYK2−/− mice.[490] Because this was a single patient, it remains to be determined if other individuals with TYK2 deficiency will have this full range of clinical problems.

ACTIVATION OF JAKS AND THE JAK-STAT PARADIGM

The paradigm of JAK-STAT activation is shown in Figure 25.7, which also shows activation of other pathways, such as the Ras-MAP kinase and PI 3-kinase/AKT/p70 S6

TABLE 25.14 Phenotypes of Mice Deficient in Type I and Type II Cytokines, Their Receptors, JAKs and STATs

Type I Cytokines and Their Receptors	
γ_c family	
IL-2[205,692,693]	Normal thymic and peripheral T-cell development. Decreased polyclonal T-cell responses in vitro, but more normal in vivo responses to pathogenic challenges. Autoimmunity with marked changes in levels of serum immunoglobulin isotypes. Ulcerative colitis–like inflammatory bowel disease.
IL-4[208,209]	Defective Th2 cytokine responses and class switch; defective IgG1 and IgE production.
IL-2/IL-4[210]	Some features of both IL-2 and IL-4 knockout mice. No gross abnormalities of T-cell development.
IL-7[104]	Greatly diminished thymic and peripheral T-cell development and B lymphopoiesis, resulting in profound lymphopenia.
IL-9[133,135]	No T-cell defect. Defect in pulmonary goblet cell hyperplasia and mastocytosis following challenge with *Schistoma mansoni* eggs. No defect in eosinophilia or granuloma formation.
IL-15[151]	Defective NK-cell development. Defect in CD8 memory T-cell homeostasis.
IL-2Rα[217]	Normal initial lymphoid development, but massive enlargement of peripheral lymphoid organs, polyclonal T- and B-cell expansions, and activated T cells, with impaired activation-induced cell death. Autoimmunity with increasing age, including hemolytic anemia and inflammatory bowel disease.
IL-2Rβ[218]	Severe autoimmunity including autoimmune hemolytic anemia. Death within approximately 3 months. Deregulated T-cell activation. Dysregulated B-cell differentiation and altered Ig profile.
γ_c[211,212]	Greatly diminished thymic development but double negative, double positives, and single positives all represented. Age-dependent accumulation of peripheral CD4+ T cells with an activated memory phenotype. Greatly diminished numbers of conventional B cells, although B1 cells are present. No NK cells or $\gamma\delta$ cells. Absent gut-associated lymphoid tissue, including Peyer patches.
IL-4Rα[694]	Like IL-4–/– mice, they have defective Th2 cytokine responses and class switch; defective IgG1 and IgE production. In addition, they cannot expel *Nippostrongylus brasiliensis*, presumably because of defective IL-13 signaling.
IL-7Rα[103]	Greatly diminished thymic and peripheral T-cell development and B lymphopoiesis, resulting in profound lymphopenia.
IL-15Rα[150]	Defective NK cell development. Defect in CD8 memory T-cell homeostasis.
IL-13[366,695]	Defective Th2 cell development and the ability to expel helminths. Impaired diarrhea to OVA.
IL-4/IL-13 DKO[695]	Produce almost no IgE, highly resistant to OVA-induced diarrhea.
IL-13Rα1[378,695]	Exacerbated Th2 responses, with diminished mortality after infection with *S. mansoni* and greater susceptibility to *N. brasiliensis*. However, partially impaired allergic diarrhea to OVA.
IL-21R[182,184]	Decreased IgG1, elevated IgE.
IL-21R/IL-4 DKO[182]	Panhypogammaglobulinemia (mimicks the B-cell phenotype in humans with XSCID).
β_c family	
IL-5[227]	Decreased basal level of eosinophils and defective induction of eosinophils following infectious challenge. Developmental defect in CD5+ B1 cells. Normal antibody and cytotoxic T-cell responses.
GM-CSF[696,697]	Normal basal hematopoiesis. Unexpected abnormalities of the lung; abnormal pulmonary homeostasis.
IL-3Rβ[698]	No defects (due to redundant function of β_c).
β_c[243,698]	Defective responses to IL-5 and GM-CSF but normal responses to IL-3 (due to redundant function of IL-3Rβ). Diminished eosinophils: both basal levels and in responses to infectious challenge. Unexpected abnormalities of the lung characterized by pulmonary proteinosis and reduced phagocytosis by alveolar macrophages. In other words, the defects are a combination of those found in the IL-5– and GM-CSF–deficient mice.
IL-3Rβ + β_c DKO[698]	Same phenotype as β_c-deficient mice, except that they cannot respond to IL-3.
gp130 family	
IL-6[699]	Impaired acute-phase responses following infection or tissue damage. Decreased numbers of hematopoietic progenitor cells.
gp130[700,701]	Embryonic lethal. Extreme hypoplastic development of the myocardium; although the ventricular wall was very thin, trabeculation within the ventricle chamber was normal. Hematologic abnormalities characterized by greatly reduced CFU-S and somewhat reduced CFU-Gm and BFU-E. Markedly diminished size of thymus and numbers of thymocytes. Reduced primordial germ cells in embryonic gonads. Diminished size of placenta.
LIF[702–704]	Decreased hematopoietic progenitor cells. Normal sympathetic neurons but deficient neurotransmitter switch in vitro. Defective blastocyst implantation.
LIFRβ[705,706]	Postnatal lethality. Normal hematopoietic and germ cell compartments but multiple neurologic, skeletal, placental, and metabolic defects. The greater severity than found in LIF-deficient mice reflects that LIFRβ is shared by several cytokines, including CNTF, LIF, OSM, and CT-1.
CNTF[707]	Progressive atrophy and loss of motor neurons.

TABLE 25.14 Phenotypes of Mice Deficient in Type I and Type II Cytokines, Their Receptors, JAKs and STATs *(Cont.)*

CNTFRα[708]	Severe motor neuron deficient resulting in perinatal mortality. The more severe phenotype than in CNTF-deficient mice was unexpected and suggests another cytokine may utilize CNTFRα.
IL-11Rα[709]	Blastocysts can implant but decidualization cannot occur, associated with failure of pregnancy. Fetal lethal phenotype.
IL-12 p40[710]	Impaired but not completely lacking in their ability to produce IFNγ and to mount a Th1 response in vivo. Elevated secretion of IL-4; normal production of IL-2 and IL-10. Substantially decreased CTL responses. Resistant to infection with intracellular pathogens.
IL-12Rβ1[711]	Defective IL-12 signaling. IL-2 but not IL-12 could augment NK activity. Defective IFNγ production in response to ConA or anti-CD3. Severe defect in Th1 differentiation.
IL-12 p35[175,712]	Selective loss of IL-12 function without loss of IL-23. Less resistant to infection than p40 and IL-12Rβ1 KO.
IL-12Rβ2[713]	Selective loss of IL-12 function without loss of IL-23. Less resistant to infection than p40 and IL-12Rβ1 KO.
IL-23 p19[175,714]	Complete resistance to EAE, defective DTH response.
EBI3[715]	Increased pathologic features of colitis and shorter survival than in p28 KO mice, indicating that IL-35 rather IL-27 is protective.
IL-27 p28[715]	Decreased pathologic features of colitis and longer survival than in EBI3 KO mice, indicating that IL-35 rather than IL-27 is protective.
Epo, Tpo, G-CSF, and M-CSF	
Epo[716]	Embryonic lethal. Complete block of fetal liver erythropoiesis, resulting in severe anemia, yet normal development of BFU-E and CFU-E progenitor cells.
EpoR[717]	Same as Epo-deficient mice.
TpoR[718]	Decreased megakaryocytes and platelets, but other hematopoietic cells are present in normal numbers.
G-CSF[719]	Neutropenia and impaired neutrophil mobility. Diminished granulocytes and macrophage precursors.
M-CSF[720,721]	Osteopetrosis, absence of teeth. Females are infertile, suggesting an unexpected role for M-CSF.
M-CSF + GM-CSF[721]	A combination of defects of both M-CSF and GM-CSF, with osteopetrosis and alveolar proteinosis. Early death of pneumonia.
Type II cytokines and their receptors	
IFNAR1[722–724]	Normal development. Defective immune defense against most viral infections tested, including lymphocytic choriomeningitis virus, Semliki Forest virus, Theiler virus, vesicular stomatitis virus. Normal resistance to *Listeria monocytogenes*, *Leishmania major*, *Mycobacterium bovis*, and *Mycobacterium avium*.
IFNγ[725–727]	Normal lymphoid development. Impaired resistance to *Listeria monocytogenes*, *Leishmania major*, *M. bovis*, and *M. avium*. Can mount curative responses to a number of viruses. CD4+ effector cells default to the Th2 pathway after infection with *Leishmania*. Succumb to infection with *Toxoplasmosis gondii*.
IFNGR1[723,724,728]	Normal lymphoid development. Impaired resistance to *Listeria monocytogenes, Leishmania major, M. bovis,* and *M. avium* Can mount curative responses to a number of viruses.
IL-10[422,427]	Normal lymphocyte development and antibody responses. Chronic enterocolitis, anemia, and growth retardation. Augmented inflammatory responses.
IL-19[729]	Increased susceptibility to dextran sulfate sodium–induced colitis, increased accumulation of macrophages, and production of proinflammatory cytokines and IFNγ.
IL-20R1[39]	Increased bone density with defective osteoclast differentiation.
IL-20R2[730]	Anti-TCR stimulation results in elevated IL-2 and IFNγ production, with increased antigen-specific CD4 and CD8 cells that produce IFNγ, but diminished IL-10.
IL-22[731]	Increased susceptibility to infection with *Citrobacter rodentium*, with augmented bacterial burden, intestinal epithelial damage, and mortality.
IL-28A[732]	Normal clearance of a range of viruses but diminished antiviral activity in response to TLR3 and TLR9 agonists. Expression of IL-28α on nonhematopoietic cells appears to be the most critical.
STATs and JAKs	
STAT1[557,558]	Defective responses to both type I and type II IFNs. Defective response to certain viruses and bacterial antigens.
STAT2[559]	Defective signaling in response to type I IFNs; interestingly, the defect in not as severe in STAT2-deficient macrophages as it is in STAT2-deficient fibroblasts.
STAT3[560–564]	Embryonic lethal. Embryos implant but cannot grow. The fact that this phenotype is even more severe than that seen with gp130 suggests a role for STAT3 via a gp130-independent cytokine. By Cre-lox methodology, *STAT3* was also selectively targeted in T cells, which exhibit defective IL-2–induced proliferation that correlates with a defect in IL-2–induced IL-2Rα expression; as expected, T cells also exhibit defective signaling in response to IL-6. *STAT3*-deficient neutrophils and macrophages show defective IL-10 signaling. STAT3 is also essential for normal involution of the mammary epithelium, for wound healing, and for normal hair cycle processes. *STAT3*-deficient DCs exhibit defective Flt3L-dependent expansion of DCs.

(continued)

TABLE 25.14 Phenotypes of Mice Deficient in Type I and Type II Cytokines, Their Receptors, JAKs and STATs *(Cont.)*

STAT4[566,567]	Defective Th1 development. Essentially, the same phenotype as in IL-12–deficient mice, including defective IL-12–mediated boosting of NK cell cytolytic activity.
STAT5A[511,571]	Defective lobuloalveolar development in the mammary gland, a syndrome resulting from defective prolactin signaling. Defective IL-2–induced IL-2Rα expression and associated defects in IL-2–induced T-cell proliferation. Defective superantigen-induced expansion of Vβ8 T cells. Defective antigen-induced recruitment of eosinophils into the lung as well as antigen-induced IgG1 production.
STAT5B[512,572]	Defective growth analogous to Laron dwarfism, a disease of defective growth hormone signaling. Defective IL-2–induced IL-2Rα expression. More severe defects in IL-2–induced T-cell proliferation and NK-cell proliferation. Defective antigen-induced recruitment of eosinophils into the lung as well as antigen-induced IgG1 production. Defective NK cytolytic activity.
Partial STAT5A/STAT5B[513,514]	Defective signaling in response to prolactin and growth hormone. Absent NK-cell development. Major defect in T-cell proliferation and TCR signaling. Anemia.
Complete STAT5A/STAT5B[573]	Fetal lethality; absent T-cell development.
STAT6[568–570]	Defective Th2 development. Essentially, the same phenotype as IL-4–deficient mice. Defective B-cell proliferation.
JAK1[463]	Perinatal lethality, with defective signaling by gp130-dependent cytokines (IL-6, IL-11, CNTF, OSM, LIF, CT-1, and NNT-1/BSF-3). Defective signaling by γ_c-dependent cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21; of these, IL-2 and IL-7 were formally evaluated).
JAK2[733,734]	Fetal lethality with profound anemia due to defective signaling in response to Epo.
JAK3[470–472]	Very similar and possibly identical to γ_c-deficient mice. Defective signaling in response to γ_c-dependent cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21). Greatly diminished T cells in thymus and spleen, but then age-dependent peripheral expansion of CD4+ T cells. Unlike humans with JAK3 mutations, Jak3-deficient mice have greatly diminished B-cell numbers as well.
TYK2[735–737]	Mice lacking TYK2 exhibit diminished signaling in response to IFNα/β and IL-12, but it is not abrogated. Primarily STAT3 activation is diminished, even though STAT1/STAT2 and STAT4, respectively, are the STAT proteins that are primarily activated by IFNα/β and IL-12. Additionally, there are diminished responses to IFNγ and IL-18.

BFU, burst forming unit; BSF-3, B cell-stimulating factor-3; CD, cluster of differentiation; CFU, colony forming unit; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DKO, double knockout; DTH, delayed type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; Epo, erythropoietin; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; Ig, immunoglobulin; IL, interleukin; KO, knockout; LIF, leukemia inhibitory factor; M-CSF, macrophage-colony stimulating factor; NK, natural killer; NNT-1, novel neurotrophin-1; OSM, oncostatin M; STAT, signal transducer and activator of transcription; TCR, T-cell receptor; Th, T helper; Tpo, thrombopoietin; XSCID, X-linked severe combined immunodeficiency.

kinase pathways. Following IFN or cytokine engagement, dimerization or higher order oligomerization of receptor complexes is induced. This in turn allows the juxtapositioning of JAK kinases, facilitating transphosphorylation and activation. In receptors with only two chains, the direct transphosphorylation of one JAK by the other seems likely to occur. In more complex receptors, such as the IFNγ system, because the receptor is a heterotetramer with two IFNGR-1 chains that each bind JAK1 and two IFNGR-2 chains that each bind JAK2, it is not clear if JAK1 and JAK2 transactivate each other or if one of the JAK kinases plays a dominant role. Indeed, one study suggests that JAK2 may phosphorylate both itself and JAK1, thereby increasing the catalytic activities of both kinases.[491] JAK1 in turn then phosphorylates IFNGR-1, allowing the recruitment of STAT1. In this model, it is additionally suggested that JAK2 phosphorylates STAT1.[491] Interestingly, a kinase-dead mutant of JAK1 was able to mediate the induction of certain IFNγ-induced genes, indicating a potential "structural" role for JAK1, but catalytically active JAK1 was essential for the establishment of the antiviral state,[491] emphasizing the essential role of both JAK1 and JAK2 for normal IFNγ function.

The previous discussion assumes that transphosphorylation of JAK kinases is a mechanism for the amplification of catalytic activity. Unless other kinases are involved, however, implicit to this idea is that the JAK kinases themselves must exhibit some basal activity that is amplified to a higher level by autophosphorylation or transphosphorylation. It is reasonable to speculate that both models may be operative, depending on the system. Consistent with JAK kinases being activated by phosphorylation, mutagenesis of a critical tyrosine in TYK2[492] or JAK2[493] (eg, tyrosine 1007 in the case of JAK2) in the activation loop of the kinase domain inhibits activity. It is also conceivable that phosphorylation of other tyrosines on the JAKs may create appropriate motifs for the recruitment of additional signaling molecules.

The function of the pseudokinase domain remains unclear. No other metazoan protein tyrosine kinases contain such a domain. The JH2 lacks the third glycine in the critical Gly-X-Gly-X-X-Gly motif, is missing an aspartic acid that serves as the proton acceptor that is typically conserved in the catalytic loop of both tyrosine and serine kinases, and is missing the conserved phenylalanine in the Asp-Phe-Glu motif that binds adenosine triphosphate[355] together, pre-

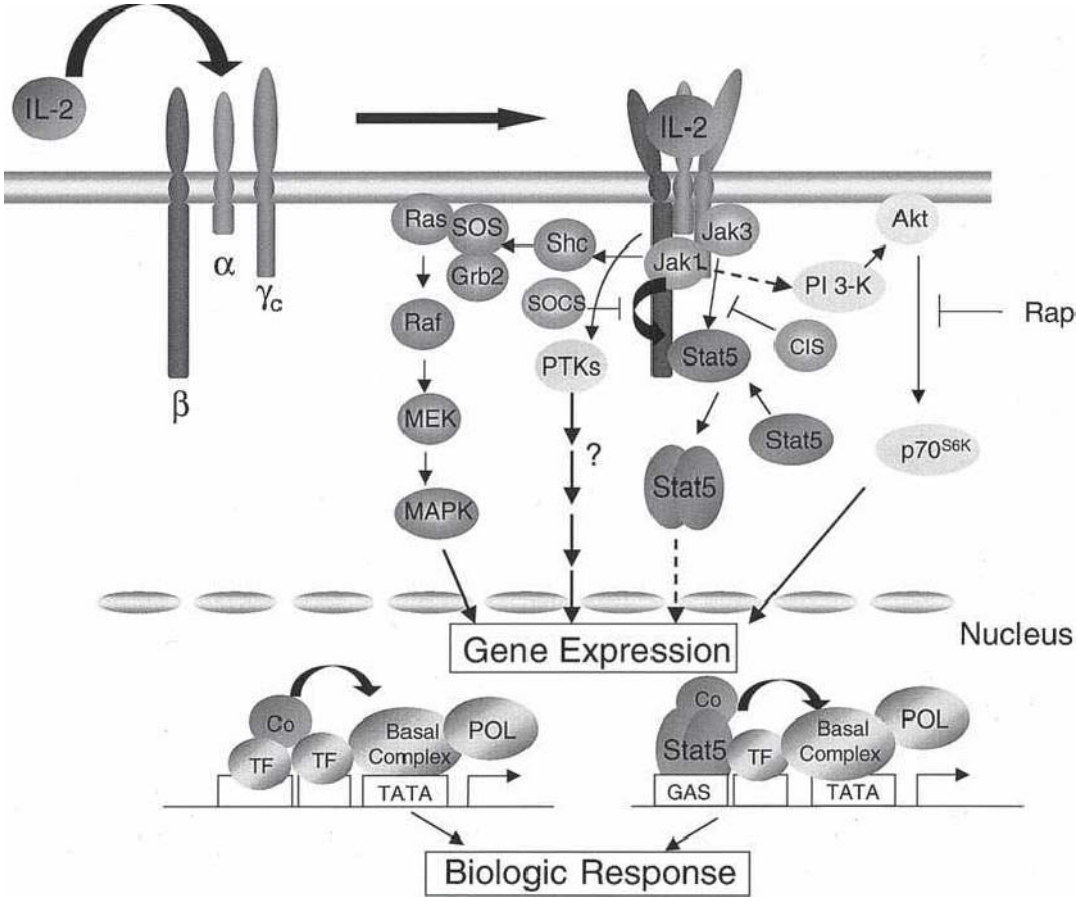

FIG. 25.7. Schematic of Cytokine Signaling Showing Multiple Signaling Pathways Activated by Interleukin (IL)-2. Shown is the association of JAK1 and JAK3 with different chains of the receptor. Activation of JAK kinases results in tyrosine phosphorylation of IL-2Rβ. This allows the docking of STAT5 proteins via their SH2 domain. The STATs themselves are tyrosine phosphorylated, dimerize, and translocate to the nucleus where they modulate expression of target genes. The schematic also indicates that another phosphotyrosine mediates recruitment of SHC, which then can couple to the Ras/Raf/MEK/mitogen-activated protein kinase pathway. Also shown is the important phosphatidyl inositol 3-kinase pathway. These and other pathways are activated by many type I cytokines.

sumably explaining the lack of catalytic function of the JH2 pseudokinase domain.[452] Despite the lack of catalytic activity, there are data in support of vital functions for this region. While the kinase domain alone can act as an active kinase, it is interesting that a mutation in the JAK kinase JH2 domain can hyperactivate the Drosophila (*hop*$^{Tum\text{-}l}$/DSTAT) JAK-STAT pathway, and that the corresponding Glu695 to Lys mutation in mouse JAK2 also resulted in increased autophosphorylation of JAK2 and phosphorylation of STAT5 in transfected cells.[494] Moreover, the JH2 domain may play an important role in mediating the interaction of JAKs with STAT proteins.[495]

Given that JAK kinases are associated with a wide range of IFN and cytokine receptors, it was predicted that JAKs would mediate signals involved in multiple processes. These include important roles in development (as demonstrated by the lack of T-cell and NK-cell development associated with JAK3 deficiency, a defect at least partially due to defective signaling in response to IL-7 and IL-15), signaling in response to cytokines that are mitogenic growth factors (eg, IL-2, IL-3, etc.), and in the antiviral response (IFNs). Studies in zebrafish revealed not only a role for JAK1 in early vertebrate development but also revealed that during early development JAK1 kinase was exclusively of maternal origin.[478] These developmental roles for JAK1 in zebrafish are consistent with the importance of a JAK kinase in early Drosophila development as well.[496]

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION PROTEINS ARE SUBSTRATES FOR JAKS THAT AT LEAST IN PART HELP DETERMINE SPECIFICITY

Given that there are only four JAK kinases but scores of cytokines, it is clear that JAKs by themselves cannot fully determine specificity. Indeed, different cytokines with different actions activate the same JAKs. One level of specificity comes from the same JAKs having different substrates, de-

pending on the receptor. The best characterized substrates for JAKs are the STAT proteins,[355,447,448] and these appear to provide some, but not all of the specificity, particularly given that there are only seven STAT proteins. Among the mutant cell lines with defects in IFN signaling, in addition to the ones with defects in JAKs noted previously, others were defective in STAT proteins, providing perhaps the earliest data proving a vital role for STAT proteins in signaling in response to IFNs.

STAT proteins are classically considered to be latent transcription factors that initially exist in the cytosol but then must be activated by phosphorylation, with subsequent translocation to the nucleus. STATs were first discovered as factors that bound to the promoters of interferon-inducible genes. The seven mammalian STAT proteins are STAT1,[497] STAT2,[498] STAT3,[499,500] STAT4,[501,502] STAT5A,[503–507] STAT5B,[505,507,508] and STAT6.[509] Table 25.15 summarizes the cytokines that activate each of the STATs. Additionally, some STATs (eg, STAT3) are known to have more than one isoform with distinct functions.[510] Although the STATs conserve a reasonable level of homology, STAT5A and STAT5B are unusually closely related, being 91% identical at the amino acid level.[505–508] It is interesting that mouse and human STAT5A are more related than STAT5A and STAT5B within a single species. The same is true for mouse and human STAT5B, suggesting that there has been evolutionary pressure to maintain the difference between STAT5A and STAT5B and that these two proteins might have certain distinctive functions. In this regard, STAT5A and STAT5B knockout mice exhibit both similarities as well as some differences in their phenotypes[511–514]; however, given different levels of each STAT5 protein in certain tissues, it remains to be determined if the different phenotypes result from different intrinsic actions of STAT5A and STAT5B versus differences in the total level of STAT5.

As active STAT proteins exist as dimers, the ability of at least some STATs to form heterodimers (eg, STAT1 with STAT2 or STAT3[448]) increases the number of different complexes that can form. In addition, further complexity can be generated by the ability of at least some of the STATs to exist in alternatively spliced forms,[508,515] some of which are inactive.

A schematic of STAT proteins is shown in Figure 25.8. The STATs can be divided into two basic groups: those that are longer (STAT2 and STAT6, approximately 850 amino acids) and those that are shorter (STAT1, STAT3, STAT4, STAT5A, and STAT5B, between 750 and 800 amino acids). The STAT genes cluster in three locations. Mouse STAT2 and STAT6 are both located on chromosome 10; STAT1 and STAT4 are located on chromosome 1; and STAT3, STAT5A, and STAT5B are located on chromosome 11.[516] Correspondingly, human STAT5A and STAT5B are closely positioned on chromosome 17q.[508]

The classic model is that in order for STATs to be "activated" and to be able to function as transcriptional activators, a number of cellular events must occur. They first bind to phosphorylated tyrosines on cytokine receptors, are tyrosine phosphorylated, dissociate, dimerize, translocate from the cytosol to the nucleus, bind to target DNA sequences, and activate gene expression. A number of conserved structural features common to all STATs help to explain these functions. These include an SH2 domain, a conserved tyrosine residue, a DNA-binding domain, and a C-terminal transactivation domain, and an N-terminal STAT tetramerization region (Fig. 25.8). In addition, there is now evidence for the biologic function of nuclear STATs that are not phosphorylated, as discussed subsequently.

TABLE 25.15 Cytokines and the Signal Transducer and Activator of Transcriptions They Activate

Cytokine	STATs Activated
IFNα/β	STAT1, STAT2, STAT4
IFNγ	STAT1
Growth hormone	STAT5A, STAT5B
Prolactin	STAT5A, STAT5B
Erythopoietin	STAT5A, STAT5B
Thrombopoietin	STAT5A, STAT5B
IL-10	STAT3
IL-12	STAT4, STAT3
G-CSF	STAT3
γ_c family	
IL-2, IL-7, IL-9, IL-15, IL-21	STAT5A, STAT5B, STAT3, STAT1
IL-4	STAT6, STAT5A, STAT5B
IL-13	STAT6
TSLP	STAT5A, STAT5B
β_c family	
IL-3, IL-5, GM-CSF	STAT5A, STAT5B
gp130 family	
IL-6, IL-11, CNTF, LIF, OSM, CT-1	STAT3
Leptin	STAT3

CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; IL, interleukin; LIF, leukemia inhibitory factor; STAT, signal transducer and activator of transcription; TSLP, thymic stromal lymphopoietin.

Note that for IL-2, IL-7, IL-9, and IL-15, STAT5A and STAT5B appear to be the major STATs that are activated, although STAT3 in particular and STAT1 to a lesser degree can also be activated. For IL-21, STAT3 is the dominant STAT protein activated, followed by STAT1 and STAT5A/STAT5B.[44,585]

Docking of Signal Transducer and Activator of Transcription on Receptors or Other Molecules, their Tyrosine Phosphorylation, and Dimerization

Each STAT protein has an SH2 domain that plays two important roles: 1) for receptor docking, as for example, has been shown for STAT1 docking on IFNGR-1,[517] STAT2 docking on IFNAR-1,[518] STAT3 docking on gp130,[519] STAT5A and STAT5B docking on IL-2Rβ and IL-7Rα,[356,520] and STAT6 docking on IL-4Rα[521]; and 2) for STAT dimerization that is mediated by the SH2 of one STAT interacting with the conserved phosphorylated tyrosine of another STAT protein. In the case of the IFNα receptor, no STAT1 docking site on IFNAR-1 or IFNAR-2 has been identified, and it is believed that STAT1 may interact with STAT2 after STAT2 is itself tyrosine phosphorylated.[355] It is also possible that STATs can dock on JAKs, given the ability to directly coprecipitate JAKs and STATs.[480,495] After

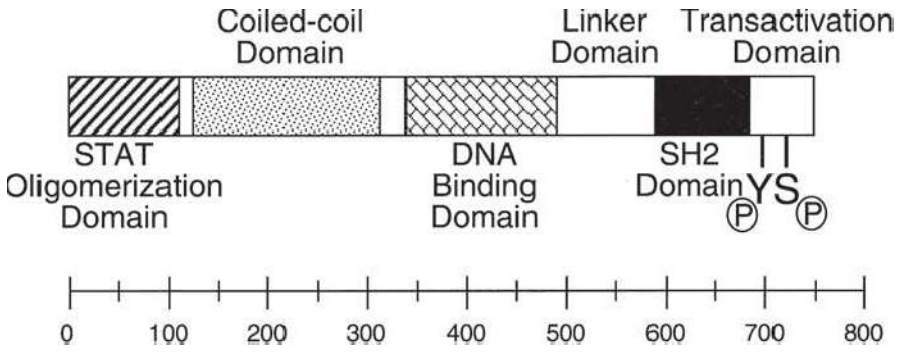

FIG. 25.8. Architecture of a Typical Signal Transducer and Activator of Transcription (STAT) Protein. Shown are the locations of the following important regions: 1) The N-terminal region has been shown to mediate the interaction of STAT dimers bound to adjacent gamma activated sites (known to be important for STAT1 but presumably true for all STATs), 2) the DNA–binding domain, 3) the SH2 domain that mediates STAT docking on receptors and STAT homodimerization/heterodimerization following tyrosine phosphorylation, and 4) the location of the conserved tyrosine whose phosphorylation allows the SH2-mediated dimerization. At least in STAT1 and STAT3, Serine 727, which is C-terminal to the conserved tyrosine, is an important site for phosphorylation. In the case of STAT1, p48 interacts downstream of the STAT dimerization domain. CBP/p300 interacts with two sites at both the N-terminus and C-terminus. Although it has been suggested that the region between the DNA–binding domains and the SH2 domain is an SH3 domain, this remains unproven, and no interactions with proline-rich regions have been reported; as a result, the labeling of this region as an SH3 domain has been omitted. Note that this structure is typical of that for STAT1, STAT3, STAT4, STAT5A, and STAT5B. The main features are conserved in STAT2 and STAT6, but these are approximately 50 to 100 amino acids longer.

docking has occurred, a conserved tyrosine (tyrosine 701 in STAT1, tyrosine 694 in STAT5A, etc.) can be phosphorylated. This phosphorylation is required for SH2 domain–mediated STAT dimerization, and the phosphorylation likely occurs while the STAT is docked on the receptor in physical proximity to receptor-associated JAK kinases. Following STAT phosphorylation, the STAT protein dissociates from the receptor, and its dimerization with itself or another STAT is presumably then favored over its reassociation with the cytokine receptor chain given that STAT dimerization involves two reciprocal phosphotyrosine-SH2 interactions, whereas docking on a receptor involves only one interaction. Thus, efficient activation of STATs requires the presence in STATs of a conserved SH2 domain and a critical tyrosine.

Whereas some receptor proteins such as IFNGR-1[517] and IL-7Rα[356] have a single STAT docking site (for STAT1 and STAT5, respectively), a number of receptor molecules, including IL-2Rβ,[356,520] IL-4Rα,[521] gp130,[519] Epo receptor (EpoR),[522] and IL-10R1,[523] have more than one docking site for their respective STATs. The presence of more than one site not only provides functional redundancy but also potentially could allow the simultaneous activation of two STAT molecules, providing a high local concentration of phosphorylated STATs to facilitate their dimerization.

Signal Transducer and Activator of Transcription Nuclear Translocation and Deoxyribonucleic Acid Binding

Following dimerization, the STATs translocate into the nucleus where they can bind to DNA as transcription factors. The mechanism for nuclear translocation was originally mysterious given the absence of an obvious nuclear localization signal.[524] However, it was shown that tyrosine-phosphorylated STAT1 dimers can directly interact with importin-α5, allowing internalization. This suggested that there indeed was a nuclear localization signal that is normally masked, and mutation of Leu407 does not interfere with tyrosine phosphorylation, dimerization, or DNA binding but prevents nuclear localization.[524] Following its dephosphorylation, nuclear STAT1 is exported to the cytosol by a process that is dependent on the chromosome region maintenance 1 (CRM1) export reporter.[525] Thus, both import and export of STAT1 appear to be regulated processes.[526]

Whereas most STAT dimers directly bind to DNA, in the case of IFN-α/β, STAT1-STAT2 heterodimers are formed, and these bind DNA in conjunction with a 48 kDa DNA-binding protein known as IRF9; the STAT1-STAT2-IRF9 complex is known as IFN-stimulated gene factor 3 (ISGF3).[448] In the case of other STAT dimers, accessory proteins are not required for DNA binding. The motif recognized by ISGF3 complexes is AGTTTNCNTTTCC (known as an ISRE, for IFN-stimulated response element), whereas the other STAT complexes bind more semipalindromic TTCNmGAA motifs that are generally denoted as gamma-activated site (GAS) motifs for IFNγ-activated sequences, reflecting their original discovery in the context of IFNγ.[355,397,447]

A series of chimeric STAT proteins were used to delineate a DNA-binding domain of approximately 180 amino acids, with two conserved subdomains. Although multiple STATs bind to the same motifs, their relative efficiencies vary, indicating fine specificities conferred by the different

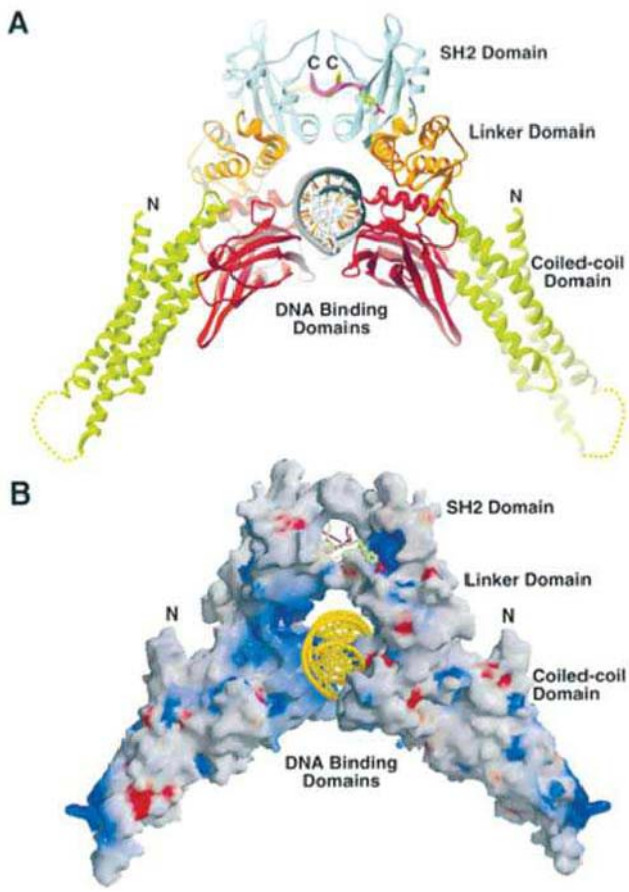

FIG. 25.9. Three-Dimensional Structure of a Signal Transducer and Activator of Transcription 1 Dimer Bound to Deoxyribonucleic Acid. Reproduced from Chen et al.[527] and Becker et al.,[528] with permission of Dr. Kuryian and Cell Press.

DNA-binding domains. For example, whereas STAT1 homodimers favor a TTCN3GAA motif, STAT6 prefers a TTCN4GAA motif.[521] These differences partially explain why different STATs modulate the expression of nonidentical sets of target genes. The structures of STAT1 and STAT3β bound to DNA[527,528] (Fig. 25.9) almost resembles that of a vertebral column, wherein the DNA represents the spinal cord. The N-terminal and coiled-coil domains are spatially the furthest from the DNA, whereas the DNA-binding domain, linker, and SH2 domain surround the DNA, with the stability apparently being provided by the SH2-phosphotyrosine interaction between the STAT monomers and each STAT monomer–DNA interaction with the DNA, presumably via a "half GAS site,"[529] as discussed under STAT tetramerization.

N-terminal regions mediate cooperative DNA binding of STAT proteins when multiple STAT-binding sites occur in close proximity,[530,531] for example, in the IFNγ gene.[530] In the IL-2Rα gene, IL-2 response elements have been described in both the 5′ regulatory region and the first intron, each of which has tandem GAS motifs that functionally cooperate to mediate IL-2–induced IL-2Rα transcription.[532–535]

STAT proteins were the first transcription factors that were identified as targets for tyrosine phosphorylation. Previously, tyrosine phosphorylation was primarily associated with membrane proximal events, but the tyrosine phosphorylation of STATs is associated with a membrane proximal event, as the STATs dock on cell surface receptors. This phosphorylation then allows the rapid dimerization that facilitates nuclear localization and DNA binding. STATs can interact directly with JAK kinases (first shown for STAT5 and JAK3),[480] suggesting that STATs may at times dock on JAKs rather than on receptors.

Nonphosphorylated Signal Transducer and Activator of Transcription Proteins Can Be Nuclear

In addition to the classical tyrosine phosphorylation–mediated dimerization and nuclear translocation, it is now clear that STAT proteins can exist in the nucleus even without being tyrosine phosphorylated and in that context can modulate gene expression.[536–538] Moreover, nonphosphorylaed STATs (eg, STAT4) can form N-domain–mediated dimers.[539]

Optimal Binding Sites for Signal Transducer and Activator of Transcription Proteins

The optimal binding motifs for several STAT proteins have been determined. For STAT1, STAT3, and STAT4, a TTCCSGGAA motif was defined,[448,530] while STAT5A and STAT5B optimally bind a TTCYNRGAA motif[529] and STAT6 binds a TTCNTNGGAA motif (where Y is C or T and R is G or A).[521,529] Whereas dimeric STAT protein binding strongly preferred canonical motifs, the range of sequences recognized by STAT5 tetramers is broader, often occurring to two imperfect motifs or with one canonical motif with an associated "TTC" or "GAA" half GAS motif. The optimal inter-GAS motif spacing is 11–12 bp, with relative secondary peaks of 6–7 bp and 16 bp as well.[529a] The presence of suboptimal GAS motifs spaced at appropriate distances to allow tetrameric binding may allow greater specificity via cooperative binding of STAT oligomers. A subset of genes bind STAT5 tetramers and STAT5 tetramers are critical for cytokine responses and normal immune function.

Transcriptional Activation by Signal Transducer and Activator of Transcription

In addition to tyrosine phosphorylation, some STATs can be phosphorylated on serine, and STAT1 and STAT3 phosphorylation at Serine 727 in the C-terminal transactivation domain is required for full activity.[540–542] The Serine 727 region resembles a MAP kinase recognition site, and one study indicated that MAP kinase activity is required for IFNα/β-induced gene expression.[543] Serine 727 mediates the interaction of STAT1 with MCM5, a member of the minichromosome maintenance family of proteins; this interaction presumably is important for maximal transcriptional activation.[544] In contrast, STAT2 is not serine phosphorylated.[448] STAT5A and STAT5B are serine phosphorylated,[545] and this phosphorylation may be important in cellular transformation.[546,547]

In addition to this regulated modification of the STAT proteins, STATs can also interact with other factors. For

example, as noted previously, STAT1-STAT2 heterodimers bind DNA only after interacting with IRF9 to form ISGF3.[448] STAT1 interacts with and synergizes with Sp1 for transcriptional activation in the ICAM-1 gene[548] and with TRADD to influence IFNγ signaling.[549] An alternatively spliced shorter form of STAT3, denoted STAT3β, associates with c-Jun to enhance transcriptional activity,[550] and full-length STAT3 can also associate with c-Jun.[551] Moreover, certain STATs can interact with the potent transcriptional coactivators CBP/p300.[552–554] In the case of STAT1, this is mediated by interactions involving both the N- and C-terminal regions of STAT1 and the CREB and E1A binding regions of CBP, respectively.[553] STAT5A has been shown to associate with the glucocorticoid receptor.[555] Additionally, the IL-2–response element in the IL-2Rα gene requires not only STAT5 binding but also the binding of Elf-1, an Ets family transcription factor, to a nearby site.[532] Thus, active STAT complexes appear to involve the coordination of STAT proteins with other factors. The corepressor silencing mediator for retinoic acid receptor and thyroid hormone receptor was identified as a potential STAT5-binding partner that binds to both STAT5A and STAT5B and potentially plays a negative regulatory role.[556]

Specificity of Signal Transducer and Activator of Transcription Proteins

Analogous to the JAKs, the same STATs are activated by multiple cytokines. The phenotypes of mice lacking expression of each of the seven STAT proteins are known, and these knockout models have helped to clarify which cytokines critically depend on a given STAT protein. STAT1 knockout mice exhibit defects that are very selective for the actions of type I and type II IFNs,[557,558] suggesting that STAT1 is only vital for the actions of IFNs, even though a variety of other cytokines have been reported to activate STAT1. Although it is possible that STAT1 plays an important but redundant role for at least some of these other cytokines, the phenotype of STAT1-deficient mice indicates a need for caution in the interpretation of in vitro experiments that use high concentrations of cytokines or cell lines. STAT2-deficient mice exhibit defects consistent with selective inactivation of IFNα/β signaling.[559] STAT3-deficient mice exhibit fetal lethality; the embryos implant, but they exhibit defective development and growth.[560] Deletion of STAT3 within specific lineages has revealed that mice lacking STAT3 in T cells[561] have normal lymphoid development but exhibit a defect in IL-2–induced IL-2Rα expression, somewhat analogous to what is seen in STAT5A- and STAT5B-deficient mice (see the following discussion). They also exhibit defective Th17 differentiation due to defective signaling by IL-6 and IL-21.[169] Neutrophils and macrophages lacking STAT3 exhibit defective signaling to IL-10, and it is known that STAT3 is important for the normal involution of the mammary epithelium, for wound healing, and for normal hair cycle.[562,563] DCs lacking STAT3 exhibit a defect in Flt3L-dependent differentiation,[564] and antigen-presenting cells that are deficient in STAT3 exhibit disruption of priming of antigen-specific CD4+ T cells.[565] STAT4-deficient mice exhibit a phenotype similar to that of IL-12–deficient mice (ie, defective Th1 development), consistent with the observation that STAT4 is activated by IL-12.[566,567] Analogously, STAT6-deficient mice exhibit a phenotype similar to that of IL-4–deficient mice (ie, defective Th2 development),[568–570] in keeping with the observation that STAT6 is only activated by IL-4 and the closely related cytokine, IL-13. Interestingly, mice lacking STAT5A exhibit a defect in prolactin-mediated effects, including defective lobuloalveloar proliferation,[571] whereas mice lacking STAT5B have defective growth similar to that found in Laron dwarfism.[572] Thus, although STAT5A and STAT5B appear to always be coordinately induced, each of these STATs is important in vivo. In addition to these defects, both STAT5A- and STAT5B-deficient mice have defects in T-cell development and signaling.[511,512] STAT5A-deficient mice have diminished numbers of splenocytes and exhibit a defect in IL-2–induced IL-2Rα expression.[511] STAT5B-deficient mice have similar defects but also have diminished thymocytes.[512] Most dramatically, these mice have a major defect in the proliferation of freshly isolated splenocytes[512] and defective NK-cell development.[512] Mice lacking both STAT5A and STAT5B in lymphoid cells exhibit a dramatic defect in T-cell development,[573] and even hypomorphic expression of both STAT5A and STAT5B is sufficient to result in a lack of NK-cell development.[513] Presumably, the lack of T-cell development relates to defective IL-7 signaling, whereas the lack of NK-cell development relates to defective IL-15–dependent STAT5 activation. STAT5A/STAT5B double knockout mice exhibit lethality that is associated with severe anemia that develops in these mice.[574]

Signal Transducer and Activator of Transcription Proteins are Evolutionarily Old

Just as Drosophila has a JAK kinase, there is a Drosophila STAT, denoted as either DSTAT or STAT92E.[575,576] The existence of JAK kinases and STAT proteins in lower organisms suggest that the system is evolutionarily old. A STAT has been identified in *Dictyostelium* that recognizes the sequence TTGA,[577] has highest sequence similarity to STAT5B, and can bind mammalian IFN-stimulated response elements.[577] Interestingly, *Saccharomyces cerevisiae* do not appear to have STATs as no SH2 domains have been identified in the entire *S. cerevisiae* genome.

What Types of Functions Are Mediated by Signal Transducer and Activator of Transcriptions?

First and foremost, STAT proteins can translocate to the nucleus and bind to regulatory regions of target genes and influence transcription. Although STATs are generally activators of transcription, they can repress. For example, STAT1 can function as a transcriptional repressor of the c-myc gene.[578]

STAT proteins were discovered based on the study of IFN-inducible genes as part of studies intended to understand the cellular differentiation events that lead to development of the antiviral state. In addition to roles in differentiation, STAT proteins can contribute, directly or indirectly, to survival and/or mitogenic/proliferative responses that typify he-

matopoietic and immunologic cytokines, such as IL-3, IL-5, GM-CSF, IL-2, and IL-4.[355] First, a number of in vitro systems have demonstrated that viruses or viral oncogenes are associated with activated JAK-STAT pathways, suggesting a role for STATs in cellular transformation. Indeed, STAT3 and STAT5 have been implicated as oncogenes, with compelling data for transformation capability of STAT3 in cell lines, persistently tyrosine-phosphorylated STATs in several types of leukemia and lymphoma, and the development of T-lymphoblastic lymphomas in STAT5 transgenic mice.[579] The role of STAT3 in the tumor microenvironment makes it an attractive target for cancer immunotherapy.[580] Second, there is diminished proliferation in a number of the STAT knockout mice that have been analyzed. For example, STAT4-deficient cells exhibit diminished cellular proliferation to IL-12[566,567]; STAT6-deficient cells exhibit diminished proliferation to IL-4[568,569]; and STAT5-deficient mice have diminished T-cell proliferation to IL-2.[511,512] Some of these effects are indirect, based on modulation of receptor expression. For example, STAT6-deficient mice exhibit decreased IL-4Rα expression. Similarly, the absence of STAT5 results in decreased IL-2–induced IL-2Rα expression as well as decreased proliferation.[511,512] Thus, in at least some cases, decreased proliferation results at least in part from decreased expression of receptor components. STAT5A and STAT5B regulate BCL-XL induction, indicating their ability to affect cell survival.[574] Finally, STAT1 has been linked to cell growth arrest and induction of the cdk inhibitor $p21^{WAF1/CIP1}$,[581] and activation of STAT1 occurs in thanatophoric dysplasia type II dwarfism as the result of a mutant fibroblast growth factor receptor.[582] In this chondrodysplasia, the mutant fibroblast growth factor receptor induces nuclear translocation of STAT1, expression of $p21^{WAF1/CIP1}$, and growth arrest, suggesting a possible relationship to the disease. Thus, different STATs may potentially mediate either growth expansion or growth arrest. Moreover, STATs may potentially play other types of roles, as well. For example, STAT3 has been reported to serve as an adapter to couple PI 3-kinase to the IFNAR-1 component of type I IFN receptors.[583]

A more complete understanding of the actions of the different STAT proteins may be facilitated with a compilation of the genes that are regulated by each STAT. Progress in this area has been made for a number of cytokines by microarray or RNA-Seq analysis (eg, for the IFNs[399]). It is also important to recognize that not all cytokine signals are dependent on STATs, as illustrated, for example, by STAT1-independent IFN signals,[584] STAT5-independent IL-2 signals,[520] and STAT1/STAT3-independent IL-21 signals.[585]

Do Other Proteins Bind to GAS Motifs?

At least one non-STAT protein can bind to GAS motifs. The *BCL6* gene is often mutated or has undergone translocations in diffuse large cell B-cell lymphomas. Interestingly, BCL6 binds to GAS motifs capable of binding STAT6 and specifically can inhibit IL-4 action.[586] Mice lacking BCL6 expression exhibit defective germinal center formation, suggesting that formation of germinal centers may be at least partially dependent on BCL6 regulated (presumably negative) control of certain STAT-responsive genes.[586]

OTHER LATENT TRANSCRIPTION FACTORS AS EXAMPLES OF CYTOPLASMIC TO NUCLEAR SIGNALING (NF-κB, NFAT, AND SMADS)

An exciting feature of STAT proteins is that they exist in an inactive form in the cytosol and then are rapidly translocated to the nucleus. The rapid activation within minutes of signals from cell membrane to nuclear DNA binding makes the STAT acronym apt, analogous to the urgency associated with "STAT" emergency physician orders in clinical medicine. The rapid activation of STAT proteins is somewhat analogous to several other transcription factors.[587] NF-κB also undergoes rapid nuclear translocation but by a completely different mechanism from STATs. In contrast to STAT proteins where the tyrosine phosphorylation of the STATs is an initiator of nuclear translocation, for NF-κB, it is the serine phosphorylation and/or uquitination of IκB that results in its dissociation and/or destruction, allowing the release and translocation of NF-κB. There is the classical NF-κB pathway used by many cytokines, which involves the IKKα/IKKβ/IKKγ-dependent phosphorylation of IκB, resulting in the activation of NFκB1 (p50)/Rel-A (p65) heterodimers as well as the alternative pathway in which a homodimer of IKKα mediates the activation of NFκB2 (p52)/Rel-B heterodimers.[588–594] A third example of cytosolic to nuclear translocation occurs with nuclear factor of activated T-cell (NFAT) family proteins,[595–597] which are vital for regulating transcription of a number of cytokines, including for example IL-2, IL-4, and GM-CSF. NFAT is translocated to the nucleus where it associates with activator protein 1 (AP-1) family proteins to form a functional complex. It is the activation of calcineurin and dephosphorylation of NFAT that allows its nuclear translocation. A fourth example of cytosolic to nuclear translocation occurs with the SMAD proteins that mediate TGF-β signaling, which contributes to Th17 differentiation (discussed subsequently). For SMADs, the phosphorylation is on serine and the kinase is intrinsic to the receptor, but like STATs, activation of these latent transcription factors is rapid and initiated by the binding of a growth factor. Thus, several mechanisms, each involving phosphorylation or dephosphorylation, have evolved to allow cytoplasmic to nuclear translocation of latent transcription factors.

OTHER SUBSTRATES FOR JAKS

As JAKs are potent cytosolic tyrosine kinases, it is evident that the JAKs may do more than just phosphorylate tyrosine residues on receptors where STAT proteins dock as well as phosphorylating the STATs. In vitro data indicate that JAK kinases also can phosphorylate receptor tyrosines other than the docking sites for STATs. For example, in the case of IL-2Rβ, JAK1 can phosphorylate not only tyrosines 392 and 510, which are STAT docking sites, but also tyrosine 338, which is a docking site for SHC,[520] all of which are required for maximal proliferation. JAK kinases are known to autophosphorylate themselves or transphosphorylate other JAKs. Other molecules are potentially phosphorylated by JAK kinases, including the STAM adapter molecule[598] and the p85 subunit of PI 3-kinase.[599]

Interferon Regulatory Factor Family Proteins

IRFs form a family of nine proteins that are regulated by type I IFNs.[395,600,601] They each have a well-conserved N-terminal DNA-binding domain that forms a helix-loop-helix structure as well as a C-terminal interaction domain. IRFs are critical for a number of actions, including type I IFN–dependent gene transcription.[395] Select IRFs play critical roles in TLR-mediated IFN induction. IRFs can have negative as well as positive effects. For example, IFNγ-mediated repression of IL-4 is mediated by IRF protein(s).[602]

OTHER SIGNALING MOLECULES IMPORTANT FOR CYTOKINES

Other Tyrosine Kinases besides JAKS

In addition to their activation of JAK kinases, a number of cytokines can activate Src family kinases. For example, IL-2 can activate p56lck[603,604] in T cells and p59fyn and p53/p56lyn in B-cell lines.[605,606] The activation of some of these kinases has been reported to be mediated by associating with the "A" region of IL-2Rβ. Another tyrosine kinase, Syk, has been reported to associate with the S region of IL-2Rβ.[607] However, the significance of these interactions is less clear than that for JAK kinases. First, cells lacking Lck can vigorously proliferate in response to IL-2.[608,609] Second, when the A region is deleted, proliferation still occurs, albeit at a lower level than seen with wild-type IL-2Rβ.[610] However, Y338, which is required for the recruitment of SHC, is in the A region and is required for normal proliferation.[520] Thus, it is possible that the decrease in proliferation associated with deletion of the A region relates more to the loss of Y338 than it does to the loss of association of Lck. Moreover, in *Il2rb*−/− mice reconstituted with an IL-2Rβ A-region mutant, proliferation is increased rather than decreased.[611] Additional investigation is required to clarify the role of activation of Src family kinases by IL-2 and other cytokines as well as the significance of the Syk interaction with IL-2Rβ. As Syk and JAK1 both associate with the S region of IL-2Rβ, mutations that delete the S region simultaneous prevent both associations, making it hard to determine the specific role of Syk. Syk-deficient mice exhibit normal IL-2 proliferation,[612] further suggesting that Syk may not play an important role in IL-2–induced proliferation. The G-CSF receptor also can form a complex with Lyn and Syk,[613] but again Syk-deficient mice do not exhibit a defect in G-CSF signaling.[612] β_c has also been reported to interact with Src family kinases[614]; gp130 has been reported to associate with a number of other kinases, including Btk, Tec, and Fes[615–617]; and IL-4Rα has been shown to interact with Fes.[618] However, relatively little is known about the significance of tyrosine kinases besides JAKs in cytokine signaling. Additionally, PI 3-kinase, p38, ERK, and JNK can each become activated, leading to activation of many downstream transcription factors, including NF-κB, AP1, PU.1, IRF1, IRF4, and IRF8, to mediate a program of gene expression.

IRS Proteins

IRS-1 was discovered as a tyrosine phosphorylated substrate of the insulin receptor.[619] IRS proteins have a large number of phosphotyrosine docking sites, particularly for the p85 subunit of PI 3-kinase, and they presumably serve to recruit important accessory molecules. Interestingly, both insulin and IL-4 could induce tyrosine phosphorylation of an IRS-1–like molecule in hematopoietic cells. The 32D myeloid progenitor cells lack IRS-1 and could only signal in response to insulin or IL-4 when they were transfected with IRS-1.[620] Both the insulin receptor and IL-4Rα proteins contain NPXY sequences that are important for IRS-1 or IRS-2 binding; in IL-4Rα, this is contained within a sequence denoted as the I4R motif.[621]

Other cytokines have subsequently been shown to activate IRS-1 and/or IRS-2. For example, growth hormone can induce phosphorylation of IRS-1[622] and IRS-2[623]; IFNγ and LIF induce phosphorylation of IRS-2[623]; and the γ_c-dependent cytokines IL-2, IL-7, and IL-15 can induce tyrosine phosphorylation of IRS-1 and IRS-2 in T cells.[624] The significance of these findings remain unclear, as 32D cells reconstituted with a complete IL-2 receptor can proliferative vigorously in response to IL-2,[608] whereas as noted previously, in these same cells, IL-4 responsiveness requires coexpression of both IL-4Rα and IRS-1, indicative of differing roles for IRS proteins for different cytokines.

Phosphatidylinositol 3-Kinase

PI 3-kinase is a lipid kinase that consists of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit.[625] PI 3-kinase phosphorylation and activation can be induced by a number of cytokines,[626–629] and the use of inhibitors, such a wortmannin or LY294002, have demonstrated its importance in signaling for at least certain cytokines. IRS-1 has multiple docking sites for PI 3-kinase (YXXM motifs) and thus for some cytokines, such as IL-4, the association of IRS-1 might be the mechanism by which PI 3-kinase can be recruited.

The Ras/Mitogen-Activated Protein Kinase Pathway

Another major signaling pathway for a number of cytokines is the Ras/MAP kinase pathway.[630] This pathway presumably is used by cytokines whose receptors recruit the SHC adaptor molecule, which in turn mediates the recruitment of Grb2 and Sos, eventually leading to the activation of Ras. In turn, Ras couples to the MAP kinase pathway through a well-defined signaling cascade. Certain cytokines, such as IL-2 and IL-3, appear to use this pathway, whereas others, such as IL-4, do not, indicating that this pathway is differentially important depending on the cytokine that is being used.

DOWNMODULATION OF CYTOKINE SIGNALS

Much of the previous discussion has centered on the mechanisms by which cytokines induce signals. However, the mechanisms by which cytokine signals can be terminated are also extremely important. There are multiple levels at which negative regulation can occur. These include 1) regulating a balance between the production (transcriptional and translational control) of the cytokine, its receptor, and/or downstream signaling molecules and the degradation

of these same molecules; and 2) regulation of the activation state of the receptor and downstream signaling molecules. For example, these can be mediated by transcriptional repressors or molecules that either inhibit cytokine signaling (eg, SOCS proteins) or reverse activated states (eg, phosphatases).

Transcriptional control of cytokine production is a widely used mechanism. Many T-cell–derived cytokines such as IL-2, IL-3, and IL-4 are only produced by activated T cells, and their production is lost with the loss of activation. IL-15 provides an example where translation of the protein is carefully regulated, in part by the existence of multiple upstream antithymocyte globulins.[147] Most cytokine receptor chains are constitutively expressed, but some like IL-21R and IL-2Rβ are regulated in part by signals that act through the TCR. The most regulated receptor chain may be IL-2Rα, whose expression is absent on resting lymphocytes but strongly induced following stimulation with antigens, mitogens, and certain cytokines, but the transcriptional/translational control of most cytokine receptors is poorly studied.

Because phosphorylation events are vital for the creation of phosphotyrosine docking sites, dephosphorylation is an obvious mechanism of control. Indeed, two tyrosine phosphatases, Shp-1 (formerly also known as SHP, HCP, SH-PTP1, and PTP1C) and Shp-2 (formerly also known as Syp and PTP1D) have been shown to play roles related to cytokine signaling.[631,632] Shp-1 mutations cause the motheaten (*me*) and viable motheaten (*mev*) phenotypes in mice.[633,634] The viable motheaten mouse had a less severe phenotype that is associated with increased numbers of erythroid progenitor cells and hyperresponsiveness to Epo,[635] suggesting that Shp-1 might normally diminish responsiveness to Epo. Indeed, it was demonstrated that Shp-1 binds directly to the EpoR when Y429 is phosphorylated.[636] This tyrosine is located in a "negative" regulatory region of the EpoR, and when mutated, Epo-responsive cells can grow in lower concentrations of Epo. Following Shp-1 binding to Y429, dephosphorylation and inactivation of JAK2 is facilitated.[636] Thus, the negative regulation of Epo signaling appears to be at the level of a receptor-dependent inactivation of a JAK kinase. Shp-1 has also been shown to interact with β_c and to mediate diminished IL-3–induced signaling[637] and to be able to associate with both TYK2[638] and JAK2.[639]

Shp-2 has generally been considered primarily an "activating" phosphatase; it is therefore interesting that it can also interact with JAK1, JAK2, and TYK2.[640] In addition to the presumed dephosphorylation of JAK kinases by phosphatases, STAT proteins also appear to be regulated at the level of tyrosine dephosphorylation,[641] and interestingly, it has been shown that dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the two monomers within the dimer, something that is facilitated by the N-terminal domain that is involved in tetramerization.[642] Finally, another type of phosphatase, namely the lipid phosphatases, known as SHIP and SHIP2, can act as negative regulators of cytokine signals.[643]

In addition to Shp-1 and Shp-2, CD45, PTP1B, and T-cell PTP (TCPTP) have been reported to regulate JAK kinases. CD45 appears to play roles related to Epo and IFN signaling. PTP1B has been reported to dephosphorylate JAK2 and TYK2, and TCPTP has been reported to dephosphorylate JAK1 and JAK3.[449] In the cytosol, Shp-2 and PTP1B have also been reported to dephosphorylate cytosolic STAT5 and nuclear STAT1 and STAT3, whereas TCPTP has been reported to dephosphorylate STAT1 and STAT3 in both cytosol and nucleus, but additional work is needed to determine whether these events are occurring physiologically in vivo.[449]

In addition to dephosphorylation, another mode of negative regulation is by degradation. In addition to the degradation of receptor molecules, STAT1 itself is a target of the ubiquitin-proteasome pathway.[644] Finally, it is possible that regulation also can occur at the level of alternative splicing. In this regard, alternatively spliced versions of some of the STATs[448,508,515] have been reported.

THE CIS/SOCS/JAB/SSI FAMILY OF INHIBITORY ADAPTER PROTEINS

In 1995, the protoype molecule for an interesting class of proteins was discovered. The prototype molecule was named CIS, for cytokine inducible, SH2-containing protein, and it was shown to negatively regulate the actions of a set of cytokines.[645,646] CIS is rapidly induced by a variety of cytokines, including IL-2, IL-3, GM-CSF, and Epo, to physically associate with both the β_c and Epo receptors.[645,646] Subsequently, a related protein, variably denoted SOCS-1, JAB (JAK-binding protein), and SSI-1 (STAT-induced STAT inhibitor-1) was identified that could negatively regulate the activity of other cytokines, including IL-6[647–649] and IL-2, among others. Interestingly, this protein could associate with JAK family kinases, whereas this function has not been reported for CIS. A total of eight CIS/SOCS/JAB/SSI family members have been identified that collectively regulate signals in response to multiple cytokines.[650–654] These proteins are now generally known as SOCS proteins and share a central SH2 domain and a region known as a C-terminal region known as a SOCS box. Additional SOCS box–containing proteins lack SH2 domains and include proteins known as ASBs (ankyrin repeat-containing proteins with a SOCS box), SSBs (SPRY domain-containing proteins with a SOCS box), and WSBs (WD40 repeat–containing proteins with a SOCS box). SOCS proteins tend to be expressed at very low levels in nonactivated cells and to be induced by cytokines and pathogens. Following their induction, they negatively influence cytokine signaling, serving as negative feedback regulators. Their actions can extend beyond type I cytokines, for example, SOCS-1 can negatively regulate LPS responses.[655]

Knockout mice for a number of the SOCS proteins have been prepared, alone and in combination. CIS knockout mice are relatively normal, but CIS transgenic mice exhibit low body weight, failure of lactation, and diminished numbers of NK and NKT cells as well as altered TCR-mediated responses, presumably related to its ability to inhibit STAT5-dependent responses including those related to growth hormone, prolactin, IL-15, and IL-2, as well as others. SOCS-1 knockout exhibit multiorgan inflammation and neonatal lethality as the result of augmented re-

sponsiveness to IFNγ, and this lethality can be prevented if the mice are crossed to IFNγ-deficient mice. Interestingly, SOCS-1−/− mice also indicate an essential role for SOCS-1 in thymocyte differentiation, and there are diminished numbers of maturing B cells. Deletion of SOCS-1 within thymocytes/T cells/NKT cells results in multiple lymphoid abnormalites with increased CD8+ T cells due to increased sensitivity to γ_c-dependent cytokines, including IL-7.[656] SOCS-2 knockout mice exhibit gigantism, ostensible due to dysregulated growth hormone signaling. SOCS-2 knockout T cells exhibit enhanced Th2 differentiation, and knockout mice exhibit elevated Th2 responses after helminth infection.[657] SOCS-3 knockout mice exhibit embryonic lethality due to placental insufficiency and dysregulated responses to LIF and IL-6 as well as hematopoietic defects. Conditional SOCS-3 knockout mice have revealed a physiologic role for SOCS-3 in regulating G-CSF signaling in myeloid cells and for "emergency" granulopoiesis[658] as well as for IL-6/gp130 signaling.[659,660] SOCS-6 knockout have mild growth retardation.[449,652,661] Additional SOCS knockout mice and various combinations continue to be generated, adding additional information to this important area of negative regulation of STAT-dependent signaling.

PROTEIN INHIBITORS OF ACTIVATED SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION PROTEINS

Protein inhibitors of activated STAT (PIAS) proteins are other negative regulators of cytokine actions,[449,662–666] with PIAS-1 being an inhibitor of STAT1-binding activity and PIAS-3 being a similar inhibitor of STAT3-binding activity. These PIAS proteins block the DNA-binding activity of STAT dimers. Two other members, PIASx (which has both α and β splice variants) and PIASy, have also been described, with PIASx inhibiting STAT4 and PIASY inhibiting STAT1. They appear to act at least in part as transcriptional corepressors by recruiting other proteins such as histone deacetylase. In addition, PIASx and PIASy have added actions not restricted to the context of STAT inhibition. For example, PIASy is a nuclear matrix-associated SUMO E3 ligase (a ubiquitin-related protein) that can repress the activitity of the Wnt-responsive transcription factor, LEF1.[665] PIASy coexpression results in the covalent modification of LEF1 by SUMO. Thus, as a class, PIAS proteins have more than one type of action.[449,667]

DISEASES OF CYTOKINE RECEPTORS AND RELATED MOLECULES

A Range of Cytokine-Related Causes of Severe Combined Immunodeficiency

As detailed previously, mutations in the γ_c cause X-linked SCID, and mutations in JAK3 cause a similar T−B+NK− SCID. Given that γ_c-dependent cytokines activate primarily STAT5A and STAT5B as signaling molecules downstream of the JAKs, it remains an open question as to whether mutations in these STAT proteins also cause human disease. Although one could hypothesize that mutations in either STAT5A or STAT5B alone might not cause a phenotype due to potential redundancy, mice lacking STAT5A or STAT5B exhibit defects in T- and NK-cell numbers as well as T-cell proliferation and NK cytolytic activity,[511,512] and mice lacking both STAT5A and STAT5B have an even more profound defect, with absent T-cell[573] and NK-cell development.[513] Moreover, STATB mutations have been found and cause growth hormone insensitivity, IGF-1 deficiency, and immune dysfunction.[573a,573b]

It can be predicted that human immunodeficiencies might also result from mutations in some of the cytokines whose receptors contain γ_c or from mutations in other components of the receptors for these cytokines. Indeed, as noted previously, patients with IL-2 deficiency exhibit a SCID-like syndrome, due to inadequate function of their T cells, and recently, an unusual immunodeficiency has been found to result from a mutation in IL-2Rα.[369] One patient with defective IL-2Rβ expression also had an immunodeficiency syndrome characterized by autoimmunity,[668] somewhat analogous to IL-2Rβ–deficient mice. Given that mutations in IL-7Rα in humans cause T−B+NK+ SCID,[206] mutations in IL-7 might be predicted to cause a similar syndrome. The one major difference might be that IL-7–deficient humans might not be capable of receiving a successful bone marrow transplant if stromal IL-7 is required for the graft. Such patients have not yet been identified. Given the defective NK-cell development and CD8+ memory T-cell development in IL-15– and IL-15Rα–deficient mice, it is likely that these types of defects would also occur in humans lacking either of these proteins. However, again, such patients have not yet been identified. Although IL-9 transgenic mice develop lymphomas,[669] IL-9–deficient mice exhibit defects related to mast cells and mucous production rather than lymphoid defects. Thus, defects related to the IL-9 system seem unlikely as causes of SCID. At present, defective expression of IL-2, IL-2Rα, IL-2Rβ, IL-7Rα, γ_c, and JAK3 are the only cytokine-related mutations that have been found to cause SCID, with TYK2 being implicated in another form of immunodeficiency. More time will be required to determine whether mutations in other cytokines, cytokine receptors, JAKs, or STATs can also cause SCID.

Defects in the Ability to Clear Mycobacterial Infections and Chronic Mucocutaneous Candidiasis

A number of immunodeficiencies have been characterized where affected individuals cannot properly clear mycobacterial infections. These have also turned out to be diseases of defective cytokine signaling. Mutations have been found in the components of either IL-12 itself or in the IFN or IL-12/IL-23 receptors, with mutations having been found in either the gene encoding the p40 subunit of IL-12 (which as noted previously is also a component of IL-23),[670] in IL-12Rβ1 (a component of both the IL-12 and IL-23 receptors),[671] or in either the IFNGR-1 or IFNGR-2 components of IFNγ receptors.[672,673] The critical role of IL-12 for Th1 cell–mediated differentiation and production of IFNγ provides the explanation for finding similar clinical syndromes in humans lacking the p40, IL-12Rβ1, IFNGR1, or IFNGR-2. Moreover,

one patient with a mutation in the STAT1 gene was also identified with a similar clinical syndrome,[674] indicating that as anticipated, STAT1 is a critical mediator of IFNγ signaling. Interestingly, this patient had a mutation on only one STAT1 allele, but the mutation was a dominant negative mutations that selectively inhibits the formation of STAT1 dimers (hence abrogating IFNγ signaling) but yet had at most only a modest effect on the ability to form ISGF3, hence leaving signaling in response to IFNα/β relatively intact. Chronic mucocutaneous candidiasis can be caused by autosomal dominant IL-17F or autosomal recessive IL-17RA deficiency or by gain-of-function heterozygous STAT1 mutant alleles that impair IL-17 immunity.[675]

Somatic mutations that activate STAT3 occur in benign liver adenomas, suggesting a role for IL-6–STAT3 pathway in human hepatocellular tumorigenesis.[676]

Mutations in the WSX-1/TCCR Type I Receptor

Interestingly, there is a type I cytokine receptor denoted as TCCR or WSX-1 that is related to IL-12β2 and its mutations results in defective Th1-related responses and diminished IFNγ production, resulting in susceptible to *Leishmania major* and *Listeria monocytogenes*.[254,677,678] TCCR/WSX-1 is an essential component of the receptor for IL-27[300] and also has been found to be required for resistance to *Trypanosoma cruzi*.[679,680]

Other Diseases Associated with Cytokine Receptors

A number of other diseases have been reported that related to cytokine receptors. First, mutations in the growth hormone receptor have been found in a form of dwarfism (Laron dwarfism)[681] in which target cells cannot respond to growth hormone. Interestingly, some aspects of STAT5B deficiency are related to this syndrome. Second, a single patient with a form of congenital neutropenia (Kostmann syndrome) has been found to have a mutation in one of his G-CSF receptor alleles.[682] Third, a kindred of patients with familial erythrocytosis has truncation in the erythropoietin receptor, resulting in hypersensitivity to Epo.[683] Fourth, an altered virally encoded form (v-mpl) of the thrombopoietin receptor (c-mpl) was originally identified as the oncogene of the myeloproliferative leukemia virus.[684] Finally, gain-of-function mutations in *IL7R*[685] and genomic aberrations of *TSLPR* (also known as *CRLF2*)[686–688] have been found in childhood acute lymphoblastic leukemias, sometimes associated with mutations in JAK2 or JAK1 or TLSPR itself.

Modulation of Cytokines and the Clinic

Certain diseases are associated with increased levels of cytokines or other situations where treatment with an anticytokine receptor antibody is a rationale therapy. One major example is Castleman disease, which is associated with overproduction of IL-6 by lymph node cells, leading to the successful treatment of this disease with IL-6 receptor blockade.[257,689] Anti–IL-6R–based therapy also has utility for rheumatoid arthritis and possibly for Crohn disase.[257,690] Blocking the IL-2 receptor has been used in the treatment of patients with adult T-cell leukemia and other neoplasias, and the use of humanized and conjugated antibodies have produced responses in a number of individuals. Humanized anti-Tac monoclonal antibody, marketed under the name daclizumab, has shown very strong efficacy in the treatment of allograft rejection as well as in T-cell–mediated autoimmune disorders, including multiple sclerosis, uveitis, and tropical spastic paraparesis. Conjugated antibodies to both IL-2Rα and IL-2Rβ are also being tested for use in a variety of malignant disorders, wherein the malignant cells express these proteins. Thus, there is the possibility of therapy, either based on blocking the cytokine or based on eliminating the responding cells.[691]

CONCLUSION

Type I cytokines and IFNs are involved in the regulation of an enormous number of immunologic and nonimmunologic processes. There has been a progressive transition from viewing these as discrete molecules with special actions to sets of molecules that can be grouped according to shared receptor components and common signaling pathways. Signaling is one area where our understanding has greatly expanded; the pathways that are activated are similar for many cytokines, even when the biologic functions they induce are dramatically different. Although some of the differences can be explained by "compartmentalization" according to which cells produce the cytokine and which cells express receptors that allow them to respond to the cytokine, a tremendous amount still needs to be learned about how distinctive signals are triggered as well as more regarding the sets of genes that are induced by each cytokine. These will provide vital information important to the quest to completely understand the mechanisms by which type I cytokines and IFNs can effect their actions. At the same time, the generation of knockout mice for most cytokines and their receptors, as well as many signaling molecules, has provided in vivo clues as to vital functions served by these cytokines. Caution is clearly needed, however, in generalizing from these findings to human biology, given some apparently major differences in roles served, such as the essential role played by IL-7 in both humans and mice for T-cell development, whereas IL-7 is also essential for B-cell development in mice but not in humans. The identification of so many humans disorders associated with cytokines and cytokine receptors has tremendously helped to teach us more about normal human biology as well.

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CHAPTER 26

The Interleukin-1 Family

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INTRODUCTION

More than any other cytokine family, the interleukin (IL)-1 family of ligands and receptors is primarily associated with acute and chronic inflammation. Also, more than any other cytokine family, the IL-1 family plays a fundamental role in the nonspecific innate response to infection that facilitates specific immunologic responses such as antibodies and cytotoxic T-lymphocytes. This nonspecific response to infection is now termed the "innate immune response." The cytosolic segment of each member of the IL-1 receptor family contains the toll-IL-1-receptor (TIR) domain. This domain is also present in each toll-like receptor (TLR), receptors that respond to microbial products, viruses, and nucleic acids. TIR is the functional domain for both the TLR and IL-1 receptor families, as mutations in this domain result in loss of response to IL-1 and TLR agonists. The biologic properties of both IL-1 family ligands and TLR agonists characteristically are proinflammatory and act as adjuvants for specific immune responses to antigen. Thus, the IL-1 family of ligands and receptors is fundamental to innate immunity. Of the 11 members of the IL-1 family, IL-1β has emerged as a therapeutic target for an expanding number of systemic and local inflammatory conditions termed "autoinflammatory" diseases. These diseases are distinct from autoimmune diseases and include rare hereditary conditions. But autoinflammatory diseases are also common diseases such as heart failure, gouty arthritis, and type 2 diabetes. For these, neutralization of IL-1β results in a rapid and sustained reduction in disease severity. Another member of the IL-1 family, IL-1α, is also a mediator of inflammation but is classified as an "alarmin" because the cytokine is present in most cells and readily released upon cell death. Whereas treatment for autoimmune diseases often includes immunosuppressive drugs, neutralization of IL-1β or blocking the IL-1 receptor is mostly anti-inflammatory.

With one exception, all members of the IL-1 family are initially translated as precursors lacking a signal peptide for secretion via the Golgi. The precursors are found in the cytosol and exit the cell following death by necrosis, not apoptosis. For example, once released, IL-1α, IL-33, and IL-36 can be processed extracellularly by neutrophil proteases into active cytokines. Although IL-1β is primarily processed intracellularly by the cysteine protease caspase-1, the IL-1β precursor can also be cleaved extracellularly into an active cytokine by similar serine proteases of neutrophils. The one member of the IL-1 family that is readily secreted is the IL-1 receptor antagonist (IL-1Ra). IL-1Ra is translated with a signal peptide (Fig. 26.1), although an intracellular form also exists.[1] IL-1Ra is produced in health and is found circulating in mice and humans where the antagonist serves as a brake on inflammation driven by endogenous IL-1α or IL-1β. IL-1Ra binds to the IL-1RI and blocks the receptor from binding to either IL-1α or IL-1β (see Fig. 26.1C). Mice as well as humans born with a deficiency in functional IL-1Ra exhibit increased systemic and local inflammation; in humans, a deficiency in IL-1Ra is lethal. The IL-36 receptor antagonist (IL-36Ra), another member of the IL-1 family, inhibits the activity of endogenous IL-36α, β, and γ. Although IL-36Ra is not readily secreted, individuals with a mutation in IL-36Ra develop a severe form of psoriasis. One may conclude that most members of the IL-1 family primarily promote inflammation and enhance specific acquired immune responses. But there are also members that provide a brake on inflammation. The primary characteristics of the each member of the IL-1 family are depicted in Table 26.1.

INTERLEUKIN-1 FAMILY AND INNATE RESPONSES

Independent of the type of organism or its products, the innate response is one of inflammation in which the host musters its defenses to increase the production and infiltration of phagocytic cells to the area of the invading microbe in an attempt limit infection and kill-off the invader. Systemically, the liver increases the synthesis of acute phase proteins, include antiproteases. Even in humans, in most cases this process protects the subject without the use of antibiotics. For example, a break in the skin allows bacteria to gain access to the dermis and subsequent inflammation provides activation of complement, the release of preformed cytokines from keratinocytes, an increase in vascular wall adhesions molecules, and the extravasation of neutrophils. This response has functioned to battle against invaders for millions of years and can be traced back to fruit flies.

The skin, lung, and intestinal tract each provide a first line of defense against microbial invasion and the lining cells, whether keratinocytes of the skin, the alveolar epithelial cells of the pulmonary tree, or the epithelial cells of the entire gastrointestinal tract, each contain preformed IL-1α, IL-18, and IL-33 as well as the members of the IL-36 subfamily. Because these members of the IL-1 family are each preformed in these cells, their release is a consequence of injury and is immediate. Therefore, they are termed "alarmins" as they alert the host to initiate the response. There are other "alarmins" from the lining cells that participate in defense, for example, defensins, which are directly antimicrobial.

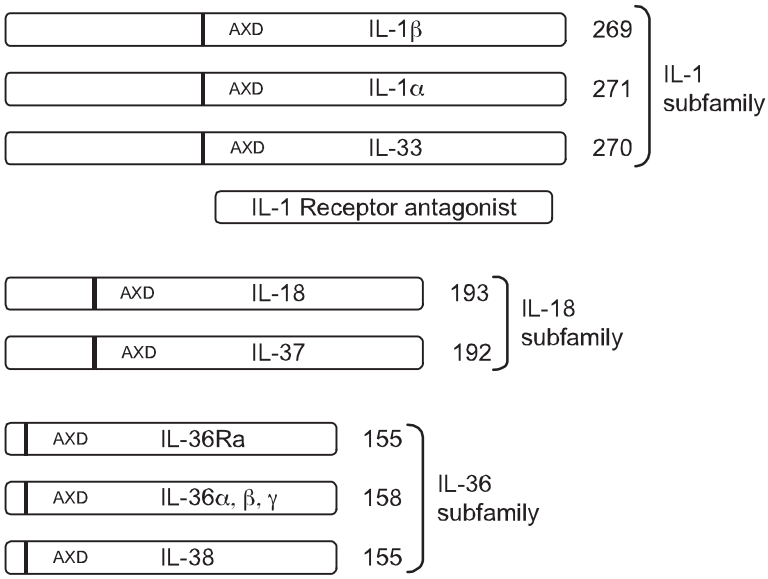

FIG. 26.1. Organization of the Interleukin (IL)-1 Family into Three Subfamilies. The number of amino acids of the full-length of each member is shown at the C-terminal end. The consensus sequence (A-X-D) is common to all IL-1 family members and serves to locate the N-terminus nine amino acids forward from this site, as shown by the *dark vertical bar*. The N-terminus results in propieces of various lengths. The IL-1Ra has a bonafide signal peptide and is shown by comparison.

Each of the constitutively present IL-1 family members in lining cells is present as a precursor. In the case of IL-1α, the precursor is fully active; in the case of the other members, the precursors are weakly active at first but are converted to more active cytokines upon the infiltration of neutrophils and processing by extracellular neutrophil proteases. In the end, the infection is contained, the invading microorganism is eliminated, and skin begins its process of repair.

Following the cloning of the mouse IL-1 receptor,[2] the cytosolic domain of the IL-1 receptor was found to be homologous to toll of the fruit fly.[3] Moreover, at the same time, the TIR domain for IL-1 signaling (Fig. 26.2A) was shown by Heguy to be required for IL-1 signaling.[4] Toll had been initially studied since its discovery in 1985 because of its central role in establishing dorsal ventral polarity in *Drosophila*. Only since 1996 was toll linked to survival in fruit flies infected with fungi.[5] However, it had already been reported, back in 1988, that a member of the IL-1/TLR family, human IL-1β, protected mice from lethal *Pseudomonas* infection.[6] As noted previously, the TIR domain is essential for both IL-1 receptor family and TLR family signaling; a mutation in the TIR domain severely impairs responses to IL-1 family ligands as well to a large number of microbial products.[7]

The TIR domain binds MyD88 (see Fig. 26.2A and E), itself a TIR domain–containing protein, through TIR/TIR interactions triggering a cascade of kinases that propagate the IL-1 signal and result in transcription of a large number of genes, the majority of which code for other cytokines, chemokines, and a host of inflammatory mediators. Of these is IL-1 and other members of the IL-1 family such as IL-36 and IL-18.

The "innate immune response" regulates to the "acquired immune response." The late Charles Janeway proposed that the innate response assists the host in mounting an acquired immune response. This relationship between a nonspecific cytokine providing help for a specific response to a microbial antigen is simply the adjuvant property of some cytokines. The adjuvant property of some cytokines functions by upregulating lymphocyte growth factors such as IL-2, IL-4, and IL-6, or lymphocyte receptors resulting in expansion of lymphocyte clones, which will either rid the host of the invading microorganism with neutralizing antibodies or in generation of cytotoxic T cells to eliminate viral infections. In 1979, purified human IL-1β, a nonspecific macrophage product, was shown to augment the T-cell response to specific antigen.[8] It was nearly 20 years later that TLR were identified as inducing IL-1β from monocytes.

TABLE 26.1 Interleukin-1 Family Members

Family Name	Name	Property
IL-1F1	IL-1α	Agonist
IL-1F2	IL-1β	Agonist
IL-1F3	IL-1Ra	Receptor antagonist
IL-1F4	IL-18	Agonist
IL-1F5	IL-36Ra	Receptor antagonist
IL-1F6	IL-36α	Agonist
IL-1F7	IL-37	Anti-inflammatory
IL-1F8	IL-36β	Agonist
IL-1F9	IL-36γ	Agonist
IL-1F10	IL-38	Receptor antagonist
IL-1F11	IL-33	Agonist

IL, interleukin.

ORGANIZATION OF THE INTERLEUKIN-1 FAMILY OF LIGANDS AND THE CONSENSUS SEQUENCE

As depicted in Figure 26.1, the IL-1 family can be divided into subfamilies according to the length of the precursor and the length of the propiece for each precursor. The IL-1 subfamily is comprised of IL-1α, IL-1β, and IL-33. This subfamily has the longest proteins with the longest propieces. In the case of IL-1β, the propiece is cleaved intracellularly by caspase-1 and then the mature cytokine is secreted. In the case of IL-1α, cleavage appears to be by the membrane protease calpain, but

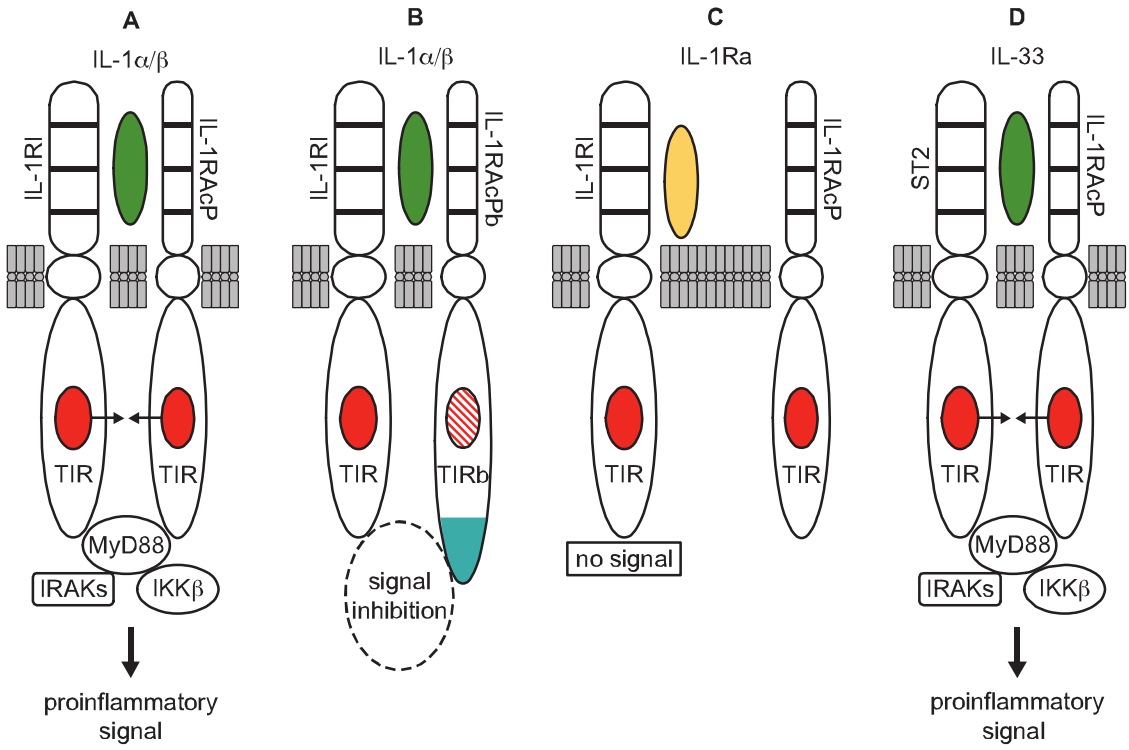

FIG. 26.2. Interleukin (IL)-1 Subfamily. **A:** IL-1α or IL-1β binds to the IL-1RI and recruits the coreceptor IL-1RAcP. The heterodimeric IL-1 receptor complex results in a close approximation of the toll-IL-1-receptor (TIR) domains on each receptor chain (*arrows*), resulting in the binding of intracellular MyD88 to the complex followed by phosphorylation of MyD88. Subsequent phosphorylations of IRAKs and IKKβ, increased NF-κB, and IL-1R AcP-1 translocation to the nucleus take place followed by expression of proinflammatory genes. **B:** In the central nervous system, IL-1α or IL-1β bind IL-1RI recruiting IL-1RAcP but can also recruit the coreceptor IL-1RAcPb. IL-1RAcPb contains an altered TIR domain, which results in a reduced signal. **C:** IL-1Ra binds to IL-1RI. There is no recruitment of the co-receptor IL-1RAcP, no approximation of the TIR domains, and no signal. **D:** IL-33 binds to its specific receptor, ST2, and recruits the coreceptor IL-1RAcP, the TIR domains approximate, and signal transduction is initiated resulting in the induction of proinflammatory gene profile.

extracellular neutrophil proteases can also cleave the IL-1α precursor. Extracellular neutrophil proteases account for the cleavage of the propiece of IL-33. The exception in the IL-1 subfamily is IL-1Ra, which contains a signal peptide.

The IL-18 subfamily is comprised of IL-18 and IL-37. By comparison, this subfamily has a smaller propiece. IL-18 requires the cleavage of its propiece by caspase-1 in order to be active. IL-37 is part of the IL-18 subfamily because the cytokine binds to the IL-18Rα chain. It is unclear how the propiece of IL-37 is removed. The IL-36 subfamily comprised of IL-36α, β, and γ, as well as IL-36Ra. In addition, IL-38 likely belongs to this family due to its binding to the IL-36R. The IL-36 subfamily has the shortest propiece.

A consensus sequence in all members of the IL-1 family is A-X-D, where A is an aliphatic amino acid such as isoleucine, methionine, or leucine; X is any amino acid; and D is aspartic acid. The aspartic acid of the consensus sequence is not the aspartic acid of the caspase-1 cleavage recognition site. The A-X-D motif is conserved in the IL-1 family where it plays a role in three-dimensional structure of the active cytokine. The actual N-terminus is often located nine amino acids before the A-X-D site. By eliminating the amino acids before the N-terminus, the first beta-sheet structure common to all members of the IL-1 family can form. For example, with the tenth amino acid before A-X-D consensus site as the N-terminus, the specific activity of the IL-36 subfamily (IL-36α, IL-36β, IL-36γ and the IL-36Ra) is low. However, with the ninth amino acid as the N-terminus, there was a marked increased in the activity.[9] In the case of IL-1β, the ninth amino acid before the A-X-D site coincides exactly with the N-terminal alanine generated by the caspase-1 site.

THE INFLUENCE OF INTERLEUKIN-1 FAMILY ON TH17 RESPONSES

The IL-1 family plays a significant role in interferon (IFN)γ production, which is essential for the defense against intracellular pathogens. On the other hand, Th2 cells are characterized by the production of IL-4 and are important in the host defense against parasitic infections. For more than one decade, the dichotomy between Th1 and Th2 has been the focus of studies on differentiation of cluster of differentiation (CD)4+ T-lymphocytes. More recently, Th17 helper cells have been described and are characterized by their production of IL-17. IL-17 plays a major role in neutrophil recruitment and host defense against extracellular bacteria and fungi. Th17 cells produce a distinct cytokine profile, namely IL-17A, IL-17F, IL-21, and IL-22. The cytokines produced

by Th17 cells, in addition to activating neutrophils, are also crucial for nonimmune cells, for example, induction of defensins by IL-22 in epithelial cells and keratinocytes, which are part of mucosal and skin defenses. It has become apparent that Th17 responses are associated with chronic inflammation and autoimmune diseases such as multiple sclerosis, type 1 diabetes, Crohn disease, and psoriasis. Furthermore, Th17 responses are fundamental for host defense against many microorganisms, although they also contribute to the inflammation during infection.

Whereas IL-4 and IL-12 were the first cytokines described as influencing Th-cell differentiation, cytokines of the IL-1 family also influence cytokine differentiation. IL-18 was initially described as IFNγ-inducing factor due to its strong stimulatory effect on Th1/IFNγ responses.[10] It is now known that IL-18 is, in fact, a crucial cytokine directing the development of Th1 cells, and one role of IL-12 is the induction of the expression of IL-18 receptors. In contrast, binding of IL-33, another member of the IL-1 family, to its ST2 receptor plays a role in inducing Th2 responses,[11] and it thus appeared as if distinct members of the IL-1 family of cytokines directed Th1 versus Th2 differentiation. Considering these effects of IL-18 and IL-33, it came as no surprise that IL-1, the most well-known member of the family, participates in the function of Th cells.

Known for over 30 years that IL-1 enhances T-cell activation and recognition of antigen, one of the early names of IL-1 was lymphocyte activation factor. The specificity of this response was, however, not known. Although initially only IL-23, IL-6, IL-21, and transforming growth factor-β were suggested to play a role in the development of Th17 responses in mice, there is no dearth of data that a more complex picture exists. Thus, IL-1β, IL-6, and transforming growth factor-β have been reported to induce the development of Th17 cells, whereas IL-23 has been reported to be important for the maintenance of Th17 cells. The combination of IL-23 and IL-1β induce the development of human Th17 cells expressing IL-17A, IL-17F, IL-22, IL-26, the chemokine CCL20, and transcription factor RORγt.[12] Interestingly, these cells also released IFNγ, displaying a phenotype common to both Th17 and Th1 cells.[12] The strong capacity of IL-1 to induce Th17 differentiation has been also linked to its well-known capacity to induce the release of prostaglandins, as reviewed in Dinarello.[13] PGE2 induced by COX-2 is a stimulator of Th17 induction, and inhibitors of cyclooxygenase decrease IL-17 production.[14] On the other hand, engagement of the aryl hydrocarbon receptor, a pathway demonstrated to be crucial for the generation of Th17 cells, has been shown to strongly induce IL-1β.[15] In addition to inducing IL-17 production from the Th17 subset of lymphocytes, IL-1β is required for the production of IL-17 by natural killer (NK) T cells[16] and of IL-22 from NK cells.[17]

Thus, cytokines of the IL-1 family have an important role in the differentiation of the Th subsets, with IL-1β strongly inducing Th17 responses, IL-18 being crucial for the generation of Th1 cells, and IL-33 being important in Th2 responses. Interestingly, reciprocal regulation has been demonstrated between the various Th subsets, with cytokines released by Th2 cells inhibiting Th1 responses, whereas IFNγ release from Th1 cells impairing both Th2 and Th17 responses.

INTERLEUKIN-1α

From an evolutionary viewpoint, IL-1α is the oldest member of the IL-1 family and its primary amino acid sequence is closely related to that of the fibroblast growth factor family. Like fibroblast growth factor, IL-1α does not have signal peptide, binds to nuclear deoxyribonucleic acid (DNA), exits the cell upon death, and binds to its receptor as an unprocessed precursor. As shown in Figure 26.2A, IL-1α binds to the IL-1RI and recruits the IL-1R accessory protein (IL-1RIAcP) to form a heterodimeric complex, which signals to induce inflammation. In health, primary cells contain constitutive levels of the IL-1α precursor but not IL-1β.[18] The IL-1α precursor is present in keratinocytes, thymic epithelium, hepatocytes, endothelial cells, the epithelial cells of mucus membranes, including the entire gastrointestinal tract, and fibroblasts, regardless of their location. The propiece of IL-1α precursor can be cleaved extracellularly by neutrophil proteases, a step that increases its biologic activity. However, IL-1α can also be active as a membrane-associated cytokine. Most cell lines, including tumor cell lines, contain constitutive levels of IL-1α.[19–21] Using an epithelial cell line, what was considered to be intrinsic IFNγ activities depended largely on constitutively expressed IL-1α. IFNγ activities were inhibited by antibodies to IL-1α, but not to IL-1β.[20] The concept that IL-1α acts as an autocrine growth factor assumes that the intracellular IL-1α precursor regulates normal cellular differentiation, particularly in epithelial and ectodermal cells. In support of the concept, an antisense oligonucleotide to IL-1α reduces senescence in endothelial cells.[22] In fibroblasts, the constitutive IL-1α precursor binds to HAX-1, a nonreceptor substrate for tyrosine kinases in hematopoietic cells. In fibroblasts, the IL-1α HAX-1 complex translocates to the nucleus.[23] Although the concept is that IL-1α acts as an autocrine growth factor in fibroblasts or endothelial cells in vitro, the data should be interpreted carefully as mice deficient in IL-1α show no demonstrable defects in growth and development, including skin, fur, epithelium, and gastrointestinal function.[24] However, mice deficient in IL-1α still retain the N-terminal propiece, which functions as nuclear factor.21 In fact, in another study, the N-terminal propiece of IL-1α was shown to bind HAX-1.[25]

Is there is a role for intracellular precursor IL-1α in normal cell function? The IL-1α precursor is present in cells that also contain large amounts of the intracellular form of the IL-1Ra (icIL-1Ra), as reviewed in Arend.[1] This form of the IL-1Ra also binds to the IL-1 receptor and prevents signal transduction. In fact, icIL-1Ra is thought to compete with the intracellular pool of precursor IL-1α for nuclear binding sites.

Membrane-Associated Interleukin-1α

Precursor IL-1α can be found on the surface of several cells, particularly on monocytes and B-lymphocytes, where it is referred to as membrane IL-1α.[26] Membrane IL-1α is biologically active[27]; its biologic activities are neutralized by antibodies to IL-1α but to IL-1β. Endothelial cells undergoing stress-induced apoptosis release membrane apoptotic body-like particles containing nuclear fragments and histones as well as full-length IL-1α precursor and the processed ma-

ture form.[28] When injected into mice, apoptotic body-like particles containing the IL-1α precursor induce neutrophilic infiltration that was prevented by neutralization of IL-1α but not IL-1β.[28]

Processing and Secretion of Interleukin-1α

Although the IL-1α precursor is biologically active, the processed form is more active. Furthermore, the binding of IL-1α to the IL-1RI has been modeled using recombinant IL-1α with an N-terminus at 113. The processing of the IL-1α precursor is accomplished by calpain II, a membrane-associated, calcium-dependent cysteine protease.[29] In macrophages treated with hydroquinone, calpain II levels fall and are associated with inhibition of IL-1α precursor processing.[29] Not surprisingly, calcium influx induced IL-1α secretion of the processed form.[30] The secretion of IL-1α requires the presence of IL-1β, as IL-1β–deficient mice do not secrete IL-1α.[31] IL-1α binding to IL-1β has been reported in which IL-1β acts as a chaperone for the secretion mechanism via caspase-1.[31] In another study, IL-1β was shown to bind to, and enhance the activity of, high-mobility group protein B1 (HMGB1).[32] It is thus possible that both IL-1α exits the cell bound to IL-1β and HMGB1.

Biologic Functions of Constitutive Interleukin-1α: Interleukin-1α and Sterile Inflammation

Large numbers of reports use bacterial and fungal products to induce cytokines as models of inflammatory disease; however, most inflammatory diseases are sterile. For example, the inflammation associated with atherosclerosis, myocardial infarction, stroke, cancer, and renal and liver failure is sterile. The hypoxic insult that takes place in ischemia results in local necrosis and release of cellular contents, including nucleic acids. Members of the IL-1 family contribute to sterile inflammation, and IL-1α plays a significant role in this regard. Upon cell death by necrosis, the IL-1α precursor is released[33,34] and binds to the IL-1 receptor on nearby tissue macrophages and epithelial cells, triggering a response.[35,36] For example, infiltration of neutrophils occurs first and followed by influx of monocytes.[35] Extracts of tumor cells induce neutrophilic inflammation, which does not occur in mice deficient in IL-1RI and is prevented by neutralization of IL-1α, not neutralization of IL-1β.[37] Sterile inflammation is independent on TLR2 and TLR4.[37]

Thus, IL-1α, either the unprocessed precursor or the calpain cleavage form, is classified as an "alarmin" because the cytokine is preformed and triggers an inflammatory response rapidly. Endothelial cells subjected to nutritional stress release inflammatory apoptotic bodies, which contain both the precursor and processed forms of IL-1α.[28] Inflammatory apoptotic bodies induce chemokine and neutrophilic infiltration into the peritoneal cavity, both of which are IL-1α dependent.[28] Platelets also contain IL-1α as well as IL-1β.[38] Platelet-derived IL-1 induces chemokines such as IL-8 from endothelial cells[39] and monocyte chemotactic protein (MCP-1) from monocytes.[40] Platelet-derived IL-1α is important in brain injury in stroke models[41] and in atherosclerosis.[42]

Studies in Interleukin-1α–Deficient Mice

Mice deficient in IL-1α are born healthy and develop normally. In some models of local inflammatory responses, wild-type and IL-1α–deficient mice develop fever and acute-phase proteins, whereas IL-1B–deficient mice do not.[24] In addition, although the inflammation-associated induction of glucocorticoids was suppressed in IL-1β–deficient mice, this suppression was not observed in IL-1α–deficient mice. However, expression of IL-1β messenger ribonucleic acid (mRNA) in the brain decreased 1.5-fold in IL-1α–deficient mice, whereas expression of IL-1α mRNA decreased more than 30-fold in IL-1β–deficient mice. These data suggest that IL-1β exerts greater control over production of IL-1α than does IL-1α over the production of IL-1β. In caspase-1–deficient mice, IL-1α production is also reduced,[43] further suggesting that production of IL-1α is under the control of IL-1β. It is important that caspase-1–deficient mice are also deficient in caspase-11.[44]

In mice fed a high-fat diet, serum amyloid A protein, a marker of inflammation in atherogenesis, was markedly lower in IL-1α–deficient mice compared to wild-type or IL-1β–deficient mice.[45] IL-1α–deficient mice had significantly higher levels of non–high-density lipoprotein cholesterol. The beneficial effect of IL-1α deficiency was due to hematopoietic cells transferred from the bone marrow of IL-1α–deficient mice, resulting in a reduction in aortic lesion size twice that observed in mice transplanted with IL-1β–deficient bone marrow cells. Therefore, IL-1α appears to play a greater role in the pathogenesis of lipid-mediated atherogenesis than IL-1β, and this may be due to an effect of membrane IL-1α.

INTERLEUKIN-1β

Interleukin-1β: The Master Cytokine in the Interleukin-1 Family

More than any other member of the IL-1 family, IL-1β has been the focus of most studies. IL-1β is a highly inflammatory cytokine, particularly in humans, as reviewed in Dinarello.[46] As shown in Figure 26.2A, IL-1β and IL-1α bind to the same IL-1RI and trigger a proinflammatory signal. The interest in IL-1β is also due, in part, to it being a secreted cytokine from macrophages and to the importance of the macrophage in antigen presentation before the era of dendritic cells. The inactive IL-1β precursor is converted into an active cytokine by the intracellular cysteine protease caspase-1. In particular, persons with activating mutations in one of the key genes that control the activation of caspase-1 can develop life-threatening systemic inflammation, which is reversed by either blocking the IL-1 receptor or through the use of a neutralizing antibody to IL-1β. Other chronic inflammatory diseases are mediated by IL-1β, as neutralizing antibodies have been used to treat a broad spectrum of diseases.

The IL-1β–mediated illnesses fall into the category of "autoinflammatory" diseases, which are to be distinguished from the classic "autoimmune" diseases. Although inflammation is common to both autoinflammatory and autoimmune diseases, in the case of IL-1–mediated disease, there is no evidence for role of adaptive immunity in its induction.

Interleukin-1β is an Inducible Cytokine

Unlike IL-1α, the IL-1β precursor is not present in health. Also unlike IL-1α, IL-1β is primarily a product of monocytes, macrophages, and dendritic cells, as well as B-lymphocytes and NK cells. In health, circulating human blood monocytes or bone marrow cells do not constitutively express mRNA for IL-1β. Endothelial cells, skin keratinocytes, fibroblasts, and epithelial cells contain constitutive IL-1α and constitutive IL-33 as precursors as well as mRNA, but these cells do not express IL-1β mRNA even upon stimulation with TLR ligands. Melanoma cells do express IL-1β as a precursor, and the more aggressive and metastatic the melanoma, the greater the likelihood of active caspase-1 and IL-1β secretion.[47] In the bone marrow neutrophil precursors, IL-1β gene expression is inducible but mature neutrophils in the circulation no longer produce IL-1β. Neutrophil IL-1β plays a pathologic role in the severe inflammation of mice with a mutant form of the phosphatase SHP1.[48] Several malignant tumors do express IL-1β as part of their neoplastic nature, particularly acute myelogenous leukemia, melanoma, multiple myeloma, and juvenile myelogenous leukemia, each of which exhibit constitutive expression of IL-1β. Unlike most cytokine promoters, IL-1β regulatory regions are distributed over several thousand base pairs upstream from the transcriptional start site. In addition to a cAMP response element, there are NF-κB–like and activating protein-1 sites. IL-1β gene regulation has been reviewed in detail.[49] Although steady-state mRNA levels for IL-1β may be present, there is distinct dissociation between transcription and translation of the IL-1β precursor. Non-TLR ligands such as the complement component C5a, hypoxia, adherence to surfaces, or clotting of blood induce the synthesis of large amounts of IL-1β mRNA in monocytic cells without significant translation into the IL-1β protein. In these cells, the IL-1β mRNA assembles into large polyribosomes, but there is no significant elongation of the peptide.[50] This failure to complete the translation into IL-1β protein may be due to the instability element present in the coding region. This instability region is also found in IL-18 and IL-37, and appears to limit the mRNA of these cytokines.[51] However, completion of translation of the mRNA into the respective cytokines can be accomplished by adding low concentrations of TLR ligands or IL-1 itself to the "primed" monocytes.[52]

Processing and Secretion of Interleukin-1β via the Caspase-1

Nearly all microbial products induce IL-1β via TLR activation; in addition, IL-1 (either IL-1α or IL-1β) induces itself both in vivo and in monocytes in vitro.[53] Other studies supporting this concept of IL-1–induced IL-1 have been reported.[54–57] Regardless of the stimulus, processing and secretion of IL-1β requires conversion of procaspase-1 to active caspase-1, although in some studies processing of the IL-1β precursor is caspase-1 independent.[58] The activation to active caspase-1 is dependent on a complex of intracellular proteins termed the "inflammasome" by the late Juerg Tschopp.[59,60] The critical component of the inflammasome is NACHT, LRR, and PYD domains containing protein 3 (NLRP3). NLRP3 is also termed cryopyrin as the gene was initially discovered in patients with "familial cold autoinflammatory syndrome," a genetic disease characterized by constitutional symptoms, fevers, and elevated acute-phase proteins following exposure to cold.[61]

As monocytes exit the bone marrow, they circulate in the bloodstream for approximately 3 days. In the absence of disease, it is likely that these cells do not enter tissues but are destroyed in the spleen or undergo apoptosis. There is no dearth of reports that circulating human blood monocytes release processed IL-1β upon stimulation starting 4 hours after stimulation with TLR agonists and continue to release the cytokine during the following 20 to 40 hours. Following lipopolysaccharide (LPS), IL-1β mRNA levels rise rapidly within 15 minutes but begin to decline after 4 hours due to the short half-life of their mRNA or the action of micro RNA. In contrast, using IL-1 itself as a stimulant, IL-1β mRNA levels are sustained for over 24 hours.[52] Raising intracellular cAMP levels with histamine enhances IL-1–induced IL-1 gene expression and protein synthesis. Monocytes of patients with autoinflammatory diseases such as cryopyrin-associated periodic syndrome (CAPS) and hyper IgΔ syndrome (HIDS) release IL-1β even without TLR stimulation during a 24-hour incubation.[62,63]

When obtained from the venous blood of healthy subjects, human blood monocytes contain active caspase-1. Active caspase-1, as determined by its cleavage into the active dimer, is present even in the absence of stimulation.[64] Active caspase-1 present in freshly obtained monocytes is nevertheless dependent on the presence of the key components of the inflammasome, namely ASC and NLRP3.[64] However, during subsequent incubation, extracellular levels of adenosine triphosphate (ATP) increase in the supernatant as IL-1β also increases and inhibition of ATP by oxidized ATP reduces the secretion of IL-1β.[64] The inhibition of IL-1β secretion by oxidized ATP is consistent with the role of the P2X7 receptor, which binds ATP and opens the potassium channel for release of intracellular potassium. The presence of active caspase-1 in circulating blood monocytes suggests that the rate limiting step in the processing and release of IL-1β is at the level of gene expression.

However, upon differentiation of the same blood monocytes into macrophages in vitro, TLR-induced IL-1β release requires activation of caspase-1 by exogenous ATP.[64] The assembly of the inflammasome components with inactive pro–caspase-1 takes place following a fall in intracellular potassium triggered by ATP binding to the P2X7 receptor. ATP activation of the P2X7 receptor opens the potassium channel, and simultaneously, as potassium levels fall, caspase-1 is activated by the inflammasome.[65–69] Without exogenous ATP, there is little or no processing of the IL-1β precursor in differentiated monocyte-derived macrophages. Alveolar macrophages obtained from the lungs of healthy human also do not release IL-1β with LPS stimulation unless exogenous ATP is added.[64] In addition to ATP activation of P2X7, activation of IL-1β processing can also take place with a cathelicidin-derived peptide termed LL37, which is released from neutrophils.[69]

The cleavage of the IL-1β precursor by active caspase-1 can take place in the specialized secretory lysosomes or in the cytoplasm. However, more than one pathway seems available for processed IL-1β to exit the cell. These include by exocytosis of the secretory lysosomes,[65,66] shedding of plasma membrane microvesicles, and direct release via transporters or multivesicular bodies containing exosomes.[70] In general, the release of processed IL-1β takes place before there is significant release of lactate dehydrogenase,[71] although in vitro cell death eventually takes place. Pyroptosis is a caspase-1–dependent form of cell death and is induced by certain bacteria using Ipaf, a member of the Nod-like receptor (NLR) family of intracellular receptors.[72] An increase in intracellular calcium is also required for the mature IL-1β to exit the cell and is phospholipase C dependent.[66]

Gain of Function Mutation in Cryopyrin

Diseases associated with single amino acid activating mutations in cryopyrin are termed CAPS. In monocytes from patients with CAPS, activation of caspase-1 occurs without a requirement for a rapid fall in the level of intracellular potassium.[57] Therefore, mutated cryopyrin allows for the assembly of the complex of interacting proteins in the presence of normal intracellular levels of potassium. Although often studied using LPS-induced synthesis of the IL-1β precursor,[73] it is unlikely that LPS plays a role in autoinflammatory diseases. On the other hand, spontaneous secretion of IL-1β from monocytes of patients is due to endogenous IL-1β stimulation. In patients with CAPS, there is a decrease in steady state levels of pro–caspase-1 mRNA with IL-1Ra treatment,[54] suggesting that IL-1β stimulates its own production and processing. Thus, in any disease process that includes an increase in the steady state levels of pro–caspase-1 mRNA, components of the inflammasome or the IL-1β precursor explain the "autoinflammatory" nature of the disease. Type 2 diabetes appears to be an example of an autoinflammatory disease where glucose induces IL-1β production from the insulin-producing beta cell and IL-1β induces the beta cell to produce its own IL-1β.[74]

Polymorphisms in P2X7 and the Activation of the Inflammasome

Patients with classic autoinflammatory diseases such as Familial Mediterranean Fever (FMF) or CAPS have nearly identical clinical parameters, secrete more IL-1β, and respond dramatically to IL-1 receptor blockade yet have no mutation in NALP3. It is therefore possible that mutations in P2X7 itself or regulation of the other genes controlling potassium channels[75] may account for dysfunctional secretion of IL-1β. For example, monocytes from patients with rheumatoid arthritis are more sensitive to release of IL-1β following ATP activation of the P2X7 receptor compared to monocytes from healthy controls.[76] However, monocytes from subjects with a P2X7 Glu496Ala loss-of-function polymorphism secrete significantly less IL-1β.[77] Monocytes from subjects homozygous for this polymorphism also released significantly less IL-18.[78] Another P2X7 receptor polymorphism is associated with increased mortality in patients undergoing allogenic stem cell transplantation.[79] Bacteremia was documented in 68% of patients with this polymorphism compared to 18% in wild-type control patients.[79]

In mice deficient in P2X7 receptors, inflammation, pain, and IL-1β–mediated IL-6 production are markedly reduced.[80] In addition to a fall in intracellular potassium, ATP triggers formation of peroxynitrite, which is required for caspase-1 activation because peroxynitrite scavengers prevent IL-1β secretion.[81] Pannexin-1, a mammalian protein that functions as a hemichannel for the uptake of dyes, is required for caspase-1 processing and release of IL-1β via the P2X7 receptor.[82] Pannexin-1 can also function for LPS-induced IL-1β synthesis in the absence of TLR4.[83] P2X7 receptor activity is also regulated by "regeneration and tolerance factor."[84]

Non–Caspase-1 Processing of Interleukin-1β

Non–caspase-1 mechanisms also exist to generate active forms of IL-1β. For example, sterile inflammation induces fever, elevated IL-6, and increased production of hepatic acute–phase proteins. These responses are absent in mice deficient in IL-1β but present in mice deficient in caspase-1.[85,86] Sterile inflammation is often associated with neutrophilic infiltration and neutrophils produce IL-1β. Because neutrophils are short-lived cells dying within hours upon emigration, release of the IL-1β precursor from intracellular stores is not unexpected. Processing of the IL-1β precursor extracellularly into an active cytokine has been reported for the common neutrophil protease, proteinase-3.[86,87] Proteinase-3 also contributes to the processing of IL-18.[88] Other proteases such as elastase, matrix metalloprotease 9, and granzyme A process the IL-1β precursor extracellularly. In addition, a mast cell chymase generates active IL-1β.

Mice with a targeted IKKβ deletion in myeloid cells are more susceptible to LPS-induced shock than control mice,[55] and markedly elevated levels of IL-1β are found in the circulation associated with a prominent neutrophilia.[55] The elevated levels of IL-1β are lethal as blockade with IL-1Ra protects these mice from death. The source of the IL-1β in these mice is the neutrophil. When incubated with proteinase-3, cleavage of the IL-1β precursor is observed yielding molecular weights of 25,000 and 15,000 Daltons.[55] Because the cleavage of the IL-1β precursor by proteinase-3, elastase, and cathepsin G are within three amino acids of the caspase-1 cleavage site, the products of the non–caspase-1 cleavage are biologically active.[86,87] Therefore, in inflammatory conditions such as urate crystal arthritis, which is characterized by a prominent neutrophilic infiltration, proteinase-3 cleavage of extracellular IL-1β precursor likely takes place.[89] Mice deficient in caspase-1 are not protected against urate-induced inflammation. Although IL-1Ra is effective in treating gout, IL-1Ra would be equally effective in any disease with extracellular processing of the precursor.[90–92] The importance of extracellular processing of the IL-1β precursor by serine proteases may explain, in part, the anti-inflammatory properties of alpha-1-antitrypsin.[93]

Reactive Oxygen Species and Interleukin-1β Processing

Is there a role for reactive oxygen species (ROS) in the activation of the IL-1β inflammasome? It was reported that uric acid crystals added to human monocytes result in the generation of ROS, which bind to and activate NLRP3 with subsequent secretion of IL-1β.[94] However, mice deficient in ROS production exhibit a proinflammatory phenotype.[95] Humans with chronic granulomatous disease (CGD) due to mutations in p47-phox cannot generate ROS and are severely affected by inflammatory granuloma. Uric acid crystal activation of primary monocytes from persons with CGD produced fourfold higher levels of IL-1β compared to monocytes from unaffected persons.[96] In contrast to previous studies,[94] the small molecule ROS inhibitor diphenyleneiodonium, which reduces the production of IL-1β, does so due to inhibition of IL-1β gene expression rather than decreased caspase-1 activation.[96] Another study identified phagocyte oxidase–defective monocytes from CGD patients as a source of elevated IL-1β.[97] These findings support the concept that ROS likely dampens inflammasome activation and may explain the presence of an inflammatory phenotype characterized by granulomas and inflammatory bowel disease occurring in patients with CGD. In fact, patients with CGD-related inflammatory bowel disease improve upon IL-1 receptor blocking therapy.[98]

Effects in Mice Deficient in Interleukin-1β

After 10 years of continuous breeding, mice deficient in IL-1β exhibit no spontaneous disease. However, upon challenge, IL-1β–deficient mice exhibit specific differences from their wild-type controls. The most dramatic is the response to local inflammation induced by a subcutaneous injection of an irritant. Within the first 24 hours, IL-1β–deficient mice do not manifest an acute-phase response, do not develop anorexia, have no circulating IL-6, and have no fever.[85,99] These findings are consistent with those reported in the same model using anti–IL-1R type I antibodies in wild-type mice.[85,99] IL-1β–deficient mice also have reduced inflammation due to zymosan-induced peritonitis.[85,100] In contrast, IL-1β–deficient mice have elevated febrile responses to LPS, IL-1β, or IL-1α compared to wild-type mice.[101] Nevertheless, IL-1β–deficient mice injected with LPS have little or no expression of leptin mRNA or protein.[102]

Mice deficient in IL-1β were compared to mice deficient in IL-1α after exposure to chemical carcinogens.[103] In IL-1β–deficient mice, tumors developed slower or did not develop in some mice. A deficiency in IL-1α, on the other hand, did not impair tumor development compared to wild-type mice injected with the same carcinogen. In IL-1Ra–deficient mice, tumor development was the most rapid. A leukocyte infiltrate was found at the site of carcinogen injection. The neutrophilic infiltrate was almost absent in IL-1β–deficient mice, whereas in IL-1Ra–deficient mice, a dense neutrophilic infiltrate was observed. In wild-type mice, the leukocytic infiltrate was sparse and the infiltrate that was observed in IL-1α–deficient mice was similar to that of control mice. These findings may reflect the fact that IL-1β is secreted into the microenvironment resulting in the emigration of monocytes and neutrophils, whereas IL-1α remaining cell-associated is less likely to affect the microenvironment.

Interleukin-1α and Autophagy

Autophagy is an ancient process of recycling cellular components, such as cytosolic organelles and protein aggregates, through degradation mediated by lysosomes. Autophagy is activated in conditions of cell stress, hypoxia, starvation, or growth factor deprivation; it promotes cell survival by generating free metabolites and energy through degradation of the endogenous cellular components.[104] However, in addition to its role in the pathophysiology of cancer, neurodegenerative diseases, or aging, autophagy is also a modulator of inflammation.[105] A role for autophagy in production of proinflammatory cytokines, particularly of IL-1β, has emerged with deletion of ATG16-L1. For example, macrophages from *ATG16L1*-deficient mice produce higher levels of IL-1β and IL-18 after stimulation with TLR4 ligands.[106] The data suggest that higher activation of caspase-1 in the *ATG16L1*-deficient mice accounts for the higher production level.[106] This observation was related to the specific degradation of the IL-1β precursor in autophagosomes in mouse macrophages.[107] Additional studies in the *ATG16L1*-deficient mice point toward a regulatory effect of autophagy on caspase-1 activation through modulation of the NLRP3 inflammasome.[94,108,109]

This role of autophagy in the secretion of IL-1β was also observed in human primary monocytes, in which specific inhibition of autophagy leads to increased production of IL-1β.[110] However, in the same cells, tumor necrosis factor (TNF)α production was decreased by autophagy inhibition. These data suggest divergent effects of autophagy on the production of these two important proinflammatory cytokines. In mice, the increase in IL-1β production is ascribed to increased activation of the inflammasome, but in human cells, it is IL-1β mRNA transcription that is elevated when autophagy was inhibited, whereas no effects were observed on caspase-1 activation.[106,107,110] Despite these differences between mouse and human cells, the inhibition of autophagy increases the production of IL-1β but not TNFα.

The modulation of inflammation by autophagy in humans has been studied in Crohn disease. Genome-wide association studies in large cohorts of patients with Crohn disease have revealed that genetic variants in two autophagy genes, *ATG16L1* and *IRGM*, result in increased susceptibility to the disease. A nonsynonymous polymorphism in *ATG16L1* on chromosome 2q37.1 and two polymorphisms in *IRGM* on chromosome 5q33.1 were significantly associated with Crohn disease risk.[111,112] Another study revealed a significant association of Crohn disease susceptibility with an intronic polymorphism in the autophagy gene *ULK1*.[113] Moreover, autophagy defects have been reported in individuals bearing nucleotide oligomerization domain *(NOD)2* mutations and are consistent with the concept that impaired bacterial clearance and increased bacterial persistence are part of the pathogenesis of Crohn disease.[114]

The mechanism through which polymorphisms in autophagy genes influence susceptibility to Crohn disease appears to involve IL-1β production. The *ATG16L1* 300Ala risk allele was associated with elevated production of IL-1β and IL-6; however, this finding was only observed in cells stimulated with the NOD2 ligand muramyl dipeptide. In contrast, the expected levels of IL-1β and IL-6 were produced upon stimulation with TLR2 and TLR4 ligands.[115] The increased production of IL-1β was associated with an increase in the steady state levels of IL-1β mRNA rather than increased activation of the inflammasome.[115] Studying the same polymorphism (*ATG16L1* Thr300Ala) in human dendritic cells, Cooney et al. reported defective NOD2-induced, but not TLR-induced, autophagy and antigen presentation.[116] Furthermore, effects of this polymorphism on antibacterial autophagy in epithelial cells have been observed.[117] The specific effect of the *ATG16L1* polymorphism on the NOD2 pathway, and not on TLR-induced stimulation, is likely related to the fact NOD2 and ATG16L1 form a protein complex that is essential for NOD2-induced autophagosome formation.[118] Because the *ATG16L1* Thr300Ala polymorphism affects protein stability,[119] defective induction of autophagy and therefore enhanced IL-1β mRNA transcription upon triggering of NOD2 may be due to the presence of defective complex.

INTERLEUKIN-33

Interleukin-33 as a Member of the Interleukin-1 Subfamily

Formerly termed IL-1F11, IL-33 belongs to the IL-1 subfamily and has been studied for its role in the Th2 paradigm of immune responses. IL-1β is also linked to the Th2 response. The existence of IL-33 was predicted in 1994 following the discovery of a novel member of the IL-1 receptor family termed ST2.[120] ST2 is the ligand binding chain for IL-33 (Table 26.2) and is structurally similar to the ligand binding chain of IL-1α and IL-1β. In addition, the coreceptor for IL-33 is the IL-1RAcP, which is also the coreceptor for IL-1α and IL-1β. It was not until 2005 that IL-33 was reported as the ligand for ST2.[11] ST2 is regulated by the estrogen inducible transcription factor Fos,[120] and this property of estrogens may be related to the large number of studies on the effect of estrogens to regulate IL-1 and inflammation.

Similar to most members of the IL-1 receptor family, ST2 is comprised of three extracellular immunoglobulin domains and an intracellular TIR domain. Although the name ST2 is still used, the correct term is the IL-33 receptor α chain (IL-33Rα). As shown in Figure 26.2D, the IL-33Rα chain similar to the IL-1R1 in that it is the ligand binding chain for IL-33 but requires the IL-1RAcP to signal.[121,122]

Before the discovery of IL-33, several studies suggested that the putative ligand (IL-33) for the ST2 orphan receptor was playing a role in allergic type diseases. It became clear that activation of ST2 was uniquely driving Th2 responses. Structurally, IL-33 is closer to IL-18 than IL-1β. Biologically, IL-33 is closest to IL-1α, as the precursors for IL-1α and IL-33 are constitutively present in all endothelial cells. As discussed in the following, like IL-1α, IL-33 functions as a DNA-binding molecule. The dominant property of IL-33 is the induction of IL-4, IL-5, and IL-13, as well as other properties anticipated for a Th2 type cytokine. Diseases thought to be due to increased immunoglobulin production may also be related to IL-33. IL-33 induces the production of IL-6, IL-1β, and PGE2 from mast cells.

Interleukin-33 and Th2 Responses

The properties of recombinant IL-33 recapitulate much of the existing data that ST2 promotes Th2-type responses. For example, before its discovery, a role for IL-33 in the Th2 response was observed using soluble extracellular forms of ST2.[11] However, IL-33 has properties that go beyond its role in the Th2 paradigm because, similar to IL-1α, IL-1β and IL-36, IL-33 forms a heterodimeric complex with IL-1RAcP for signal transduction.[121,122] Although the IL-1RAcP is expressed on most nucleated cells, ST2 is somewhat restricted to low expression on most cells with the notable exception of mast cells.

There are several mechanisms by which IL-33 favors the Th2 response. Similar to IL-1β, IL-33 induces IL-6, an adjuvant for antibody production. IL-33 induction of IL-6 is prevented by a blocking antibody to IL-1RAcP.[122] IL-33 initiates signal transduction via activation of NF-κB, which

TABLE 26.2 Interleukin-1 Receptor Family

Name	Designation	Ligands	Coreceptor
IL-1RI	IL-1R1	IL-1α, IL-1β, IL-1Ra	IL-1RAcP (IL-1R3)
IL-1RII	IL-1R2	IL-1β, IL-1β precursor	IL-1RAcP (IL-1R3)
IL-1RAcP	IL-1R3	IL-1α, IL-1β, IL-33, IL-36	Not applicable
ST2/IL-33Rα	IL-1 R4	IL-33	IL-1RAcP (IL-1R3)
IL-18Rα	IL-1R5	IL-18, IL-37	IL-18Rβ (IL-1R7)
IL-1Rrp-2	IL-1R6	IL-36α, β, γ	IL-1RAcP (IL-1R3)
IL-18Rβ	IL-1R7	IL-18	Not applicable
TIGIRR-2/IL-1RAPL	IL-1R8	Unknown	Unknown
TIGIRR-1	IL-1R9	Unknown	Unknown
SIGIRR	TIR8	Unknown	Unknown

IL, interleukin; SIGIRR, single immunoglobulin IL-1–related receptor; TIGIRR, three immunoglobulin IL-1–related receptor.

is typical of IL-1α, IL-1β and IL-18,[11] but other studies have shown that antibody cross-linking of ST2 does not result in activation of NF-κB but rather activating protein-1. IL-33 treatment also increased serum immunoglobulin A and immunoglobulin E, an expected response for a switch from Th1 to Th2.

Processing of the Interleukin-33 Precursor

Initially, IL-33 was considered closely related to IL-1β and IL-18 because the IL-33 precursor contains a caspase-1 site, which upon activation would cleave the IL-33 precursor and release the active cytokine,[11] similar to that for IL-1β and IL-18. Indeed, the first recombinant forms of IL-33 were produced with an N-terminus at the caspase-1 site.[11] Although recombinant IL-33 was active, the concentrations required for activity were considerably higher than those of other members of the IL-1 family. Indeed, subsequent studies revealed that caspase-1 actually results in loss of IL-33 activity and that the full-length IL-33 precursor binds to ST2 and is active,[123] similar to the ability of the IL-1α precursor to bind to IL-1RI. In addition, it was reported that the caspase-1 cleavage site at 178 is similar to the consensus sequence for caspase-3 and that intracellular IL-33 precursor is a substrate for caspase-3.[123]

Using immobilized IL-33 precursor, neutrophil proteinase 3 (PR3) was isolated from human urinary proteins.[124] Neutrophil PR3 is known to process the IL-1β precursor into an active cytokine.[86] PR3 converted human and mouse precursor IL-33 proteins to biologic active forms; however, increasing the incubation time of PR3 abrogated IL-33 activities.[124] Using the consensus amino acid sequence sites for PR3, six human and mouse recombinant IL-33 proteins were produced and assessed for biologic activities; varying levels of activity were reported.[124] Another study also demonstrated cleavage of the IL-33 precursor by neutrophil proteases such as PR3, neutrophil elastase, and cathepsin G,[125] resulting in the generation of IL-33 with different N-termini and varying levels of activity. These studies support the concept that extracellular IL-33 is released as a precursor, is rapidly processed by neutrophil enzymes, and generates active forms with varying levels of activity. The implications for generation of active IL-33 by neutrophil enzymes for Th2 polarization remain unclear. It may be more relevant to study the effect of proteases from eosinophils in the processing of the IL-33 precursor. Nevertheless, the IL-33 precursor binds to ST2 and recruits the accessory chain for signal transduction, but compared to IL-33 generated by neutrophil proteases, the activity of IL-33 precursor is weak.[124,125]

There was no dearth of studies on ST2 tissue–specific localization, regulation of its expression, effects in transgenic mice overexpressing ST2, as well as deletion, neutralization, and antibody cross-linking of ST2. Elevated levels of the soluble form of ST2 were present in the circulation of patients with a various inflammatory diseases and that exogenous administration of pharmacologic doses of soluble ST2 neutralized endogenous levels of the then putative ligand IL-33 and reduced inflammation.[126] IL-33 activates Th2 lymphocytes, mast cells, basophils, and eosinophils, as well as NK T cells and blood monocytes. One of the most studied properties of IL-33 is the induction of IL-5 and IL-13 and their respective roles in lung inflammation such as allergic type asthma. For example, instillation of IL-33 into the airways triggers an immediate allergic response in the lung of naïve mice and worsens the response in mice sensitized to antigen peripherally but challenged by exposure of antigen in the lung.[127]

Mice deficient in ST2 do not develop a Th2 response to *Schistosoma* egg antigen. Indeed, several studies have focused on the role of IL-33 in the pathogenesis of helminth worm infections. The Th2 response by the host contributes to the elimination of these worm infestations, which are worldwide and afflict hundreds of millions. The role of IL-33 in the induction of IL-4, IL-5, and IL-13 is of paramount importance in terms of pulmonary and intestinal complications that reduce lifespan. Using mice deficient in IL-33, a crucial role was demonstrated in mice to rid an infection with *Strongyloides venezuelensis*.[128] The infection induces a unique class of cells called natural helper cells or nuocytes, which upon activation by IL-33 produce IL-5 and IL-13, resulting in eosinophilic infiltration into the lungs. In this model, pulmonary inflammation causes damage via eosinophilic infiltration, which is IL-33 and IL-5 dependent.[128]

Mice injected with human IL-33 exhibit impressive pathologic changes in the arterial walls, lungs, and intestinal tissues.[11] Of particular relevance to the concept that IL-33 drives a Th2 response, eosinophilic infiltration was a prominent finding in the lung and in allergic rhinitis as well as allergic conjunctivitis.[129] These initial observations have been confirmed by other reports.[130] Although the interpretation of in vivo effects following the administration of an exogenous cytokine should be conservative, the findings are clearly consistent with IL-33 being a proinflammatory ligand of the IL-1 receptor family. Even before the ability to test IL-33–mediated activation, others had reported that neutralization of the putative ST2 ligand using soluble ST2 markedly reduced joint inflammation, synovial hyperplasia, and joint erosion when given in the therapeutic phase of collagen-induced arthritis in mice.[126]

Interleukin-33 as an Anti-inflammatory Cytokine

Members of the IL-1 family of ligands bind to their specific cell surface receptors and recruit an accessory chain. The IL-1RIAcP is used by IL-1α and IL-1β but also by IL-36 and IL-33. The accessory chain for IL-18 is related to the IL-1RIAcP but is encoded by a distinct gene. We now recognize that other members of the IL-1 receptor family will bind more than one cytokine. The best example is IL-1α and IL-1β. Both bind with similar affinities to IL-1RI, but the three-dimensional structure of IL-1α and IL-1β are hardy identical.[131] The IL-1β precursor binds to IL-1RII as well as a processed form with the first 112 amino acids cleaved from the precursor. IL-37 binds to the IL-18 receptor alpha chain,[132] and both IL-36 and IL-38 bind to the IL-36 receptor.[133]

IL-33 forms a complex with ST2 IL-1RIAcP but also with single immunoglobulin IL-1–related receptor (SIGIRR).[134]

This complex plays a role in the Th2 response by reducing IL-33 signaling,[134] and consistent with these observations, Th2 responses are increased in mice deficient in SIGIRR. Furthermore, there is high expression of SIGIRR in Th2 polarized cells and in models of Th2 antigen sensitization; SIGIRR-deficient mice exhibit a greater Th2 response.[134] The complex with SIGIRR and IL-33 may explain the anti-inflammatory properties of IL-33. ST2 can sequester TLR adaptor molecules such as MyD88 and Mal.[135]

In mice deficient in ST2, there is myocardial hypertrophy, ventricle dilation, and fibrosis upon pressure overload, suggesting that IL-33 plays a protective role in the heart.[136] Furthermore, elevated levels of the extracellular domain of ST2 predict outcomes in patients with systolic heart failure or following a myocardial infarction.[136] In a model of cardiomyocyte hypertrophy induced by chronic administration of phenylephrine, administration of recombinant IL-33 inhibited the phosphorylation of IκB and reduced the hypertrophy and fibrosis.[136] One of the more challenging aspects of the properties of IL-33 to act as a Th2 cytokine is its role as an antagonist in the ApoE-deficient mouse model of atherosclerosis. In this model, arterial wall plaques of mice on a high-fat diet contain IL-33 and ST2. In mice treated with IL-33, the atherosclerotic plaques were markedly reduced.[137] In mice treated with soluble ST2 to neutralize IL-33, the disease worsened.[137]

Interleukin-33 as a Transcription Factor

Similar to IL-1α, there is another side to IL-33. Although IL-33 binds to its specific surface receptor, IL-33 is identical to a nuclear factor dominantly expressed in high endothelial venules.[138] This nuclear factor is termed NF-HEV. In addition to endothelial cells, constitutive nuclear localization of IL-33 has been reported in several cell types such as type II lung epithelial cells,[128] epithelial cells,[139] and pancreatic stellate cells.[140] In fact, IL-33 binding to DNA and acting as a nuclear factor is similar to IL-1α binding to chromatin and functioning as a nuclear factor.[21,33,141] A short IL-33 peptide similar to a sequence in Kaposi sarcoma virus binds chromatin.[142] The full-length IL-33 precursor, but not mature IL-33, binds to the N-terminal Rel homology domain of NF-κB p65.[143] In cells overexpressing the IL-33 precursor, there was a reduction in IL-1β–induced TNFα.[143] These data are consistent with other data that IL-33 possesses anti-inflammatory properties (see previous discussion) and the mechanism for this property of IL-33 appears to be nuclear sequestration similar to that of IL-1α.[33]

INTERLEUKIN-18 AND INTERLEUKIN-37 SUBFAMILY

Interleukin-18

Background

IL-18 was first described in 1989 as "IFNγ-inducing factor" isolated in the serum of mice following an injection of endotoxin. The mice had been pretreated with *Proprionibacterium acnes*, which stimulates the reticuloendothelial system, particularly the Kupffer cells of the liver. Many investigators concluded that the serum factor was IL-12. With molecular cloning of "IFNγ-inducing factor" in 1995,[144] the name was changed to IL-18. Surprisingly, the new cytokine was related to IL-1 and particularly to IL-1β. Similar to IL-1β, IL-18 lacks a signal peptide, is first synthesized as an inactive precursor, and remains as an intracellular cytokine. The tertiary structure of the mature form of IL-18 closely resembles that of IL-1β,[144] although the IL-18 precursor is closely related to the IL-37 precursor. Since 1995, many studies have used neutralization of endogenous IL-18 or IL-18–deficient mice to demonstrate the role for this cytokine in promoting inflammation and immune responses.[145] However, the biology of IL-18 is hardly the recapitulation of IL-1β. There are several unique and specific differences between IL-18 and IL-1β. For example, in healthy human subjects and also in healthy mice, gene expression for IL-1β in blood mononuclear cells and hematopoietic cells is absent, and there is no evidence that the IL-1β precursor is constitutively present in epithelial cells.[146] In contrast, in the same blood cells, large amounts of the IL-18 precursor are present. Peritoneal macrophages and mouse spleen contain the IL-18 precursor in the absence of disease.[146] The IL-18 precursor is also present in keratinocytes and nearly all epithelial cells. In this regard, IL-18 is similar to IL-1α and IL-33.

Processing of the Interleukin-18 Precursor

The IL-18 precursor has a molecular weight of 24,000 and is processed by caspase 1 cleavage into a mature molecule of 18,000. Compared to wild-type mice, following an injection of endotoxin into caspase-1–deficient mice, circulating IFNγ is absent. IL-12–induced IFNγ is also absent in caspase-1–deficient mice.[147] Importantly, any phenotypic characteristic of capsase-1–deficient mice must be studied as to whether the deficiency is due to reduced IL-1β or IL-18 activity. For example, the caspase-1–deficient mouse is resistant to colitis,[148] but the IL-1β–deficient mouse is susceptible in the same disease. Because neutralizing antibodies to IL-18 are protective in the colitis model, caspase-1 deficiency appears to prevent processing of IL-18.[148,149] On the other hand, there are examples where caspase-1 processing of IL-18 is not required. For example, Fas ligand stimulation results in release of biologically active IL-18 in caspase-1–deficient murine macrophages.[150] Similar to IL-1β processing, proteinase-3 appears to activate processing to mature IL-18.[88]

Similar to IL-1α and IL-33, the IL-18 precursor is constitutively expressed in endothelial cells, keratinocytes, and intestinal epithelial cells throughout the gastrointestinal tract. Macrophages and dendritic cells are the primary sources for the release of active IL-18, whereas the inactive precursor remains in the intracellular compartment of mesenchymal cells. Also, similar to IL-1α and IL-33, the IL-18 precursor is released from dying cells and processed extracellularly, most likely by neutrophil proteases such as proteinase-3.

The IL-1 family consensus sequence (A-X-D) in IL-18 is I-N-D at amino acid 50, but the N-terminus generated by caspase-1 is 14 amino acids before the consensus sequence rather than 9 amino acids (see Fig. 26.1). Although the IL-18 as well as the IL-1β precursor can be cleaved extracellularly by proteianse-3, there is an additional enzyme that cleaves the IL-18 precursor into an active cytokine. Merprin β is a

member of the merpin family of zinc metalloproteinases, which are expressed on the membrane surface of epithelial cells of the intestine and kidney but also on myeloid cells.[151,152] Merprin α will cut the IL-1β precursor into an active cytokine, and inhibitors of merprin α markedly reduce serum levels of IL-1β in mice subjected to cecal ligation and puncture.[151] Merprin β will cleave the IL-18 precursor and generate an active cytokine on cells bearing the IL-18R.[153] In a model of dextran sulfate sodium (DSS) colitis, serum IL-18 is elevated on days 3 and 5, during which time the mice develop inflammatory colitis. However, in merprin β–deficient mice, there is a statistically significant reduction in serum IL-18.[153] The role of IL-18 and caspase-1 in DSS colitis is discussed in the following.

Signal Transduction by Interleukin-18

As shown in Figure 26.3A, IL-18 forms a signaling complex by binding to the IL-18 alpha chain (IL-18Rα), which is the ligand binding chain for mature IL-18; however, this binding is of low affinity. In cells that express the coreceptor, termed IL-18 receptor beta chain (IL-18Rβ), a high-affinity complex is formed, which then signals. The complex of IL-18 with the IL-18Rα and IL-18Rβ chains is similar to that formed by other members of the IL-1 family with the coreceptor, the IL-1R accessory chain IL-1RAcP. Following the formation of the heterodimer, the TIR domains approximate, and it appears that the cascade of sequential recruitment of MyD88, the four IRAKs, and TRAF-6 followed by the degradation of IκB and release of NFκB are nearly identical as that for IL-1.[154] There are differences between IL-1 and IL-18 signaling that remains unexplained. With few exceptions, IL-1α or IL-1β are active on cells in the low nanogram/mL range and often in the picogram/mL range. In contrast, IL-18 activation of cells expressing the two IL-18 receptor chains requires 10 to 20 ng/mL and sometimes higher levels.[155,156]

Although nearly all cells express the IL-1RI, not all cells express IL-1RAcP. Similarly, most cells express the IL-18Rα but not all cell express the IL-18Rβ. IL-18Rβ is expressed on T cells and dendritic cells but not commonly expressed in mesenchymal cells. The best example is the A549 cell. This cell line, derived from a lung carcinoma epithelial cell, does not express IL-18Rβ,[157] and there is no signal unless IL-12 is added to induce IL-18Rβ.[10] In the absence of IL-18Rβ, IL-18 binds to IL-18Rα without a proinflammatory signal. In A549 cells transfected with IL-18Rβ, IL-18 induces IL-8 and a large number of genes. One of these genes is the former IL-2-induced gene termed NK4,[158] now termed IL-32.[157] IL-32 is not a member of the IL-1 family but plays an important role in the regulation of cytokines such as IL-1β and TNFα.

Interleukin-18 as an Immunoregulatory Cytokine

Together with IL-12, IL-18 participates in the Th1 paradigm. This property of IL-18 is due to its ability to induce IFNγ either with IL-12 or IL-15. Without IL-12 or IL-15, IL-18 does not induce IFNγ. IL-12 or IL-15 increases the IL-18Rβ, which is essential for IL-18 signal transduction. Without IL-12 or IL-15, IL-18 plays a role in Th2 diseases.[159] The importance of IL-18 as an immunoregulatory cytokine

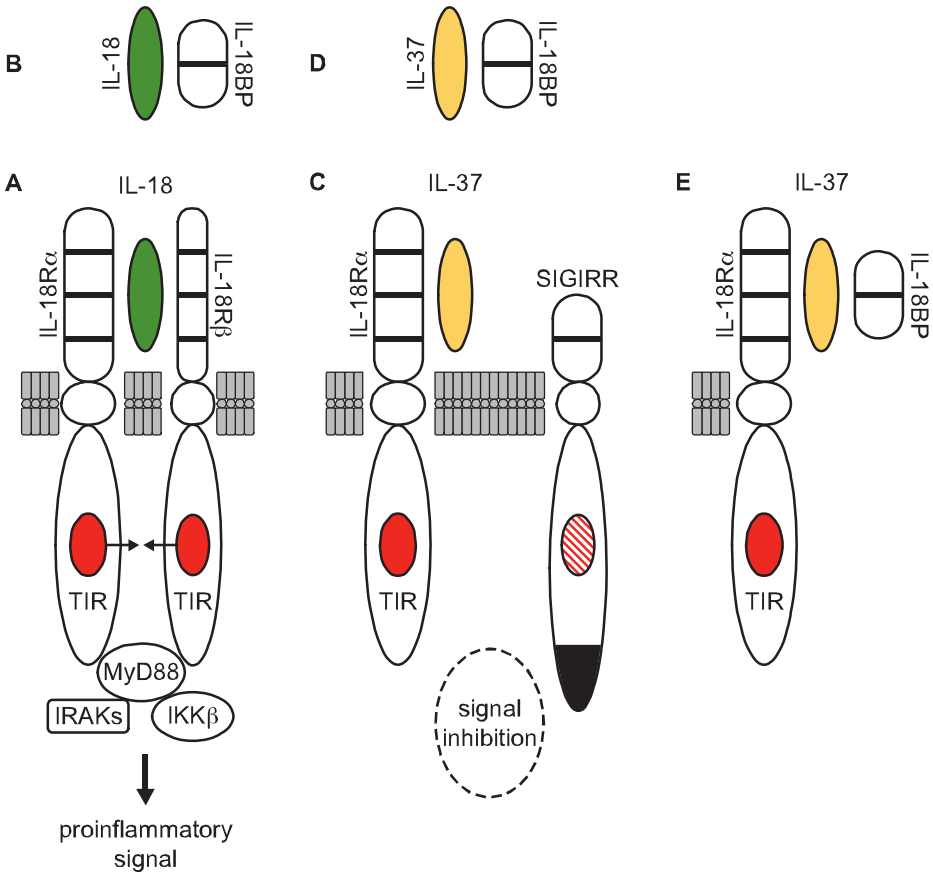

FIG. 26.3. Interleukin (IL)-18 Subfamily. **A:** IL-18 binds to the IL-18Rα chain and recruits the coreceptor IL-18Rβ. The signaling cascade of the IL-18 receptor complex is nearly the same as that of IL-1α and IL-1β, resulting in the expression of proinflammatory genes. **B:** The natural occurring IL-18BP binds IL-18, thus neutralizing the activity of the cytokine. **C:** IL-37 also binds to the IL-18Rα but with an affinity lower than that of IL-18 binding to the same receptor. Furthermore, the binding of IL-37 to IL-18Rα does not recruit the coreceptor IL-18Rβ and therefore there is no proinflammatory signal. The anti-inflammatory properties of IL-37 require single immunoglobulin IL-1–related receptor, which may act as a "decoy" for MyD88. **D:** IL-18BP also binds to IL-37, thus preventing binding of IL-37 to the IL-18Rα. **E:** IL-37 binds to IL-18BP forming a complex, which then binds to the IL-18Rα, enhancing the anti-inflammatory property of IL-18BP.

is derived from its prominent biologic property of inducing IFNγ from NK cells. Macrophage-colony stimulating factor induces human blood monocytes to develop into a subset of macrophages; these cells express a membrane-bound form of IL-18.[160] Membrane IL-18 is expressed in 30% to 40% of macrophage-colony stimulating factor–primed macrophages. In contrast, monocytes, dendritic cells, and monocytes differentiated into M1 macrophages did not express membrane IL-18. Although the expression of membrane IL-18 is caspase-1 dependent,[160] LPS treatment was necessary for the release of membrane IL-18.[160] A major immunoregulating role for IL-18 is on the NK cell. Upon shedding of membrane IL-18 into a soluble form, NK cells expressed CCR7 and produced high levels of IFNγ. As expected, IFNγ production was prevented by neutralization of IL-18. This mechanism may account for the role of IL-18 as major IFNγ-inducing factor from NK cells and the role of NK cells in the pathogenesis of autoimmune diseases.

The induction of IFNγ by IL-18 has been studied with coinducer IL-12. For example, mice injected with the combination of IL-18 plus IL-12 develop high levels of IFNγ and die with hypoglycemia, intestinal inflammation, and inanition.[161] In leptin-deficient mice, IL-18 plus IL-12 induce acute pancreatitis.[162] Several human autoimmune diseases are associated with elevated production of IFNγ and IL-18. Diseases such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, Crohn disease, psoriasis, and graft-versus-host disease are thought to be mediated, in part, by IL-18.

Proinflammatory Properties of Interleukin-18

IL-18 exhibits characteristics of other proinflammatory cytokines, such as increases in cell adhesion molecules, nitric oxide synthesis, and chemokine production. Blocking IL-18 activity reduces metastasis in a mouse model of melanoma; this is due to a reduction in IL-18–induced expression of vascular call adhesion molecule-1.[163] A unique property of IL-18 is the induction of Fas ligand (FasL), which may account for the hepatic damage that takes place in macrophage activation syndrome.[150,164] The induction of fever, a well-studied property of IL-1α and IL-1β as well as TNFα and IL-6, is not a property of IL-18. Injection of IL-18 into mice, rabbits, or humans does not produce fever.[165,166] Unlike IL-1 and TNFα, IL-18 does not induce cyclooxygenase-2 and hence there is no production of prostaglandin E2.[156,167] IL-18 has been administered to humans for the treatment of cancer in order to increase the activity and expansion of cytotoxic T cells. Not unexpectedly and similar to several cytokines, the therapeutic focus on IL-18 has shifted from its use as an immune stimulant to inhibition of its activity.[145,168]

Because IL-18 can increase IFNγ production, blocking IL-18 activity in autoimmune diseases is an attractive therapeutic target as anti–IL-12/23 reduces the severity of Crohn disease as well as psoriasis. As discussed subsequently, there appears to be a role for blocking IL-18 in Crohn disease. However, there are several activities of IL-18 that are independent of IFNγ. For example, IL-18 inhibits proteoglycan synthesis in chondrocytes,[169] and proteoglycan synthesis is essential for maintaining healthy cartilage. IL-18 also increases vascular call adhesion molecule-1 expression in endothelial cells independently of IFNγ. Vascular call adhesion molecule-1 plays a major role in multiple sclerosis, other autoimmune diseases, as well as in the metastatic process.[170]

Role of Interleukin-18 in Models of Inflammatory Bowel Disease

Inflammatory bowel disease such as Crohn disease is a complex autoimmune disease. Treatment is initially based on immunosuppressive drugs. Not surprisingly, anticytokines such as neutralizing monoclonal antibodies to TNFα[171] or to IL-12/23 provide effective treatment for many patients.[172,173] The reduction of IFNγ in Crohn disease is linked to the clinical response to these agents.[173] IL-18 is found in affected intestinal lesions from patients with Crohn disease as a mature protein, but the IL-18 precursor form is present in uninvolved intestinal tissues.[174] This observation was confirmed in a similar assessment of mucosal biopsies from patients with Crohn disease.[175] Antisense RNA to IL-18 decreased IFNγ production in lamina propria mononuclear cells.[175]

A commonly used mouse model for colitis is DSS, which is added to the drinking water and which damages the intestinal wall. Thus in DSS-induced colitis, the epithelial barrier defenses against luminal bacterial products are breeched. In this model, reducing IL-18 with a neutralizing antibody is protective and linked to a reduction in IFNγ.[149] Blocking IL-18 with the IL-18 binding protein (IL-18BP) also reduces colitis induced by antigen sensitization.[176] Because generation of active IL-18 requires caspase-1, studies have also been performed in mice deficient in caspase-1 and subjected to DSS colitis. Nevertheless, despite many studies, the role of caspase-1 in DSS colitis remains unclear. The first study showed that mice deficient in caspase-1 were protected.[148,177] In addition, treatment of mice with a specific caspase-1 inhibitor was also effective in protecting against the colitis.[178–180] In both studies, the effect of caspase-1 deficiency was linked to reduced IL-18 activity, whereas reducing IL-1 activity with the IL-1Ra was ineffective.[148] In support of the role of IL-18 in DSS colitis, inhibition of endogenous merprin β to reduce the generation of active IL-18 was protective in DSS colitis.[153]

However, a conundrum has developed whether caspase-1 deficiency is protective or detrimental in DSS colitis. DSS colitis is not the optimal model for Crohn disease, as the model is one of rapid loss of the protective barrier of the intestinal epithelium exposing the lamina propria mononuclear cells to a large amount and variety of bacterial products. Using the same DSS model, mice deficient in the adapter protein inflammasome component ASC experienced increased disease, morbidity, and precancerous lesions compared to wild-type mice exposed to DSS.[181] Similarly, mice deficient in caspase-1 died rapidly from DSS compared to wild-type mice,[182] whereas mice deficient in caspase-12, in which caspase-1 is enhanced, were protected.[182] Administration of exogenous IL-18 restored mucosal healing in caspase-1–deficient mice.[182] Also, mice deficient in NLRP3 were more susceptible to either DSS or 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and exhibited decreased IL-1β as well as decreased beta-defensins.[183] Macrophages from NLRP3-deficient mice failed to respond

to muramyl dipeptide.[183] Mice deficient in NLRP6 are also more vulnerable to DSS,[37,184] and the susceptibility appears to be due to lack of sufficient IL-18.

How to reconcile these data in mouse models of colitis was addressed by Siegmund[185]? It is likely that IL-18 being constitutive in the intestinal epithelium has a protective role in that the cytokine contributes to maintaining the intestinal barrier. With loss of the barrier, the microbial products stimulate macrophages in the lamina propria and caspase-1–dependent processing of IL-18 results in inflammation. In this model, inhibition of IL-18 production in caspase-1–deficient mice or treatment of wild-type mice with anti–IL-18 antibodies or caspase-1 inhibitors is protective. Worsening of disease in mice deficient in caspase-1 or NLRP3 or NLRP6 may lower the levels of active endogenous IL-18 needed to protect the epithelial barrier. Similarly, active endogenous IL-1β may be needed to protect to maintain the epithelial barrier by inducing growth factors.

Although it remains unclear why caspase-1 deficiency worsens DSS colitis, in humans with Crohn disease, natalizumab, the antibody that blocks the very late antigen-4, is highly effective in treating the disease. Very late antigen-4 is the α4 subunit of the β-1 integrin. Anti–very late antigen-4 binds to the surface of macrophages and other myeloid cells, and prevents the binding of these cells to the very late antigen-4 receptor on endothelial cells known as vascular cell adhesion molecule-1. Thus, the antibody disables the function of vascular cell adhesion molecule-1 and prevents the passage of macrophages and other myeloid cells into tissues such as the intestine in Crohn disease and the brain in multiple sclerosis. Because IL-18 induces vascular cell adhesion molecule-1, blocking IL-18 would also reduce the passage of cells through the endothelium into to intestine.

Interleukin-18, Hyperphagia, and the Metabolic Syndrome

Whereas there is no constitutive gene expression for IL-1β in freshly obtained human peripheral blood mononuclear cells (PBMC), the same cells express constitutive mRNA for IL-18.[146] In western blot analysis from the same cells, the IL-18 precursor was present but not the IL-1β precursor. Similar observations were also made in mice.[146] These findings suggest that IL-18 may act as regulator of homeostasis. Starting at age 16 weeks of age, IL-18–deficient mice start to overeat, become obese, and exhibit lipid abnormalities; there is increased atherosclerosis, insulin resistance, and diabetes mellitus reminiscent of the metabolic syndrome.[186] IL-18Rα–deficient mice also develop a similar phenotype. The higher body weight is attributed to enhanced food intake, in which the IL-18–deficient mice begin to diverge from wild-type animals at a relatively early age, and to reach values 30% to 40% higher than that of wild-type mice. Others have observed similar findings.[187] A striking finding was an increase of more than 100% in the percent of adipose tissue in the IL-18–deficient animals that was accompanied by fat deposition in the arterial walls. The insulin resistance in these mice is corrected by exogenous recombinant IL-18. Mice deficient in IL-18 respond normally to a challenge with exogenous leptin, suggesting that expression of the leptin receptor is unaffected. The unexpected and unique mechanism is responsible for the higher food intake in the IL-18–deficient animals appears to be due to a central nervous system loss of appetite control. IL-18–deficient mice eat throughout the day, whereas wild-type mice eat once, nocturnally.

Interleukin-18 as a Protected Cytokine

As stated previously, mice deficient in caspase-1 experience increased disease severity when subjected to DSS colitis and that administration of exogenous IL-18 restored mucosal healing in these mice.[182] In addition, mice deficient in NLRP3 were more susceptible to DSS colitis, which is thought to be due to decreased IL-18.[183] Mice deficient in NLRP6 are also more vulnerable to DSS,[37,184] and the susceptibility appears to be due to lack of sufficient IL-18. Thus, there are a growing number of studies that support a protective role for IL-18. The fact that mice deficient in IL-18 develop a metabolic syndrome–like phenotype is consistent with a role for IL-18 in homeostasis. A study in age-related macular degeneration is also consistent with a protective role for IL-18. In that study, drusen, which is mixture of complement-derived and apolipoproteins and lipids, was shown to activate NLRP3 and induce the production of mature IL-1β and IL-18.[188] In a mouse model of "wet" age-related macular degeneration, the disease was worse in mice deficient in NLRP3 but not in IL-1RI–deficient mice.[188] Therefore, IL-18 rather than IL-1α or IL-1β were protective; upon administration of IL-IL-18, the disease severity improved. Taken together, there is a case for IL-18 being a protective rather than inflammatory cytokine.

Interleukin-18 Binding Protein

The discovery of the IL-18BP took place during the search for the soluble receptors for IL-18.[189] IL-18BP is a constitutively secreted protein with an exceptionally high affinity for IL-18 (400 pM) (see Fig. 26.3B). Present in the serum of healthy humans at a 20-fold molar excess compared to IL-18,[190] IL-18BP may contribute to a default mechanism by which a Th1 response to foreign organisms is blunted in order to reduce triggering an autoimmune responses to a routine infection. Although IL-18BP is readily secreted, it falls into the functional category of being a shed soluble receptor. As shown in Figure 26.3B, IL-18BP contains only one immunoglobulin G domain, whereas the type II IL-1 receptor contains three domains. In this regard, the single immunoglobulin G domain of IL-18BP is similar to SIGIRR, which also has a single immunoglobulin G domain and also functions as a decoy receptor. The salient property of IL-18BP in immune responses is in downregulating Th1 responses by binding to IL-18 and thus reducing the induction of IFNγ.[159] Because IL-18 also affects Th2 responses, IL-18BP also has properties controlling a Th2 cytokine response.[159] IL-18BP has a classic signal peptide and therefore is readily secreted. Serum levels in healthy subjects are in the range of 2,000 to 3,000 pg/mL compared to the levels of IL-18 in the same sera of 80 to 120 pg/mL/mL. Moreover, IL-18BP binds IL-18 with an affinity of 3 to 5 nM,[189] an affinity significantly higher than that of IL-18Rα. Because a single IL-18BP molecule binds a single IL-18 molecule, one can calculate bound versus free IL-18 in a mixture of both molecules.[190]

If one examines immunologically mediated diseases where IFNγ plays a pathologic role such as Wegener granulomatosis

and systemic lupus erythematosus, one must consider the level of free IL-18 compared to IL-18 bound to IL-18BP. In fact, in these diseases both IL-18BP and IL-18 are high,[191,192] but the level of IL-18BP is not sufficiently high enough to neutralize IL-18 and therefore, the level of free IL-18 is higher than in healthy subjects. In macrophage activation syndrome where IFNγ plays a pathologic role, both IL-18BP and IL-18 are also high but the clinical and hematologic abnormalities correlate with elevated free IL-18.[164]

A unique property of IL-18BP is that the molecule also binds IL-37[193] and in doing so, enhances the ability of IL-18BP to inhibit the induction of IFNγ by IL-18. IL-37 binds to the IL-18Rα with a very low affinity but in mice expressing human IL-37, a profound anti-inflammatory effect is observed,[194] particularly of LPS-induced cytokines and dendritic cell maturation.[194] Human IL-37–expressing mice are also resistant to colitis.[195] Thus, the anti-inflammatory property of IL-37 can be affected by the concentration of IL-18BP. As the concentration of IL-18BP increases and binds IL-37, there is the possibility that IL-37 becomes less available as an anti-inflammatory cytokine. Indeed, this has been observed in mice injected with IL-18BP. At low dosing of IL-18BP, there is reduced inflammation in a model of rheumatoid arthritis but as the doing of IL-18BP increases, the anti-inflammatory properties of IL-18BP are lost.[196]

IL-18BP is highly regulated at the level of gene expression and unexpectedly, IFNγ increases gene expression and synthesis of IL-18BP.[197,198] Therefore, IFNγ driving an increase in the natural and potent inhibitor of IL-18 falls into the category of a negative feedback loop. The concept is supported by clinical data showing that patients being treated with IFNα for hepatitis have elevated levels of IL-18BP.[199,200] IL-27, like IFNγ, functions as both a pro- as well as an anti-inflammatory cytokine and both may accomplish their roles as anti-inflammatory cytokines at the level of increased production of IL-18BP. In the skin, IL-27 also acts through a negative feedback loop for inflammation. IL-27 is acting, as is IFNγ, by induction of IL-18BP gene expression and synthesis.[201]

Viral Interleukin-18 Binding Protein

Natural neutralization of human IL-18 by IL-18BP takes place during a common viral infection. In *Molluscum contagiosum* infection, characterized by raised but bland eruptions, there are large numbers of viral particles in the epithelial cells of the skin, but histologically there are few inflammatory or immunologically active cells in or near the lesions. Clearly, the virus fails to elicit an inflammatory or immunologic response. Amino acid similarity exists between human IL-18BP and a gene found in various members of the poxviruses; the greatest degree of homology is found to be expressed by *Molluscum contagiosum* gene.[202] The ability of viral IL-18BP to reduce the activity of mammalian IL-18 likely explains the lack of inflammatory and immune cells in the infected tissues and provides further evidence for the natural ability of IL-18BP to interfere with IL-18 activity.

Interleukin-37

IL-37 was formerly termed IL-1F7. IL-37 lacks a signal peptide and has a caspase-1 site, but the secretion of IL-37 has not been documented with any certainty. It is likely, however, that similar to IL-1α and IL-33, with loss of membrane integrity upon cell death, the IL-37 precursor exits from the cell. The recombinant form of the IL-37 precursor suppresses LPS-induced IL-1β, IL-6, and TNFα. However, this is observed primarily in macrophages that have been differentiated into the M1 phenotype by 5 days in the presence of granulocyte macrophage-colony stimulating factor. There are two consensus sequences (A-X-D) in N-terminal domain of IL-37: IHD and LED. A recombinant form of IL-37 with an N-terminus nine amino acids from the IHD site is active in suppressing LPS-induced TNFα and IL-6. Whether this short form of recombinant IL-37 exists in nature is unclear.

Interleukin-37 Reduces Interleukin-1β and Lipopolysaccharide-Induced Inflammation in Vivo

A mouse homologue for human IL-37 has not been identified. Therefore, to define the in vivo functional role of IL-37, a strain of transgenic mice was generated.[194] The full-length IL-37 complementary DNA was inserted into a vector using the standard cytomegalovirus (CMV) promoter for constitutive expression of the transgene in all cells. Both heterozygous and homozygous IL-37 transgenic mice (IL-37 tg) mice breed normally and exhibit no obvious phenotype. Despite the presence of the CMV promoter, the IL-37 transcript is not constitutively expressed in the tissues of the IL-37 transgenic mice. The failure to express IL-37 is likely due to a functional instability sequence found in IL-37, which limits the half-life of IL-37 mRNA.[51] Nevertheless, upon stimulation with LPS or IL-1β, levels of IL-37 increase after 4 to 24 hours. Once the transcript is present, the IL-37 precursor can be found in peripheral blood cells taken from the transgenic mice.[203]

IL-37 transgenic mice are protected against LPS challenge compared to similarly challenged wild-type mice. IL-37 transgenic mice exhibit significantly less hypothermia, acidosis, hyperkalemia, hepatitis, and dehydration.[194] In addition, circulating cytokines are significantly reduced as well as cytokines induced in whole blood cultures and in lung and spleen cell homogenates. In addition to LPS-induced cytokines, whole blood cultures from IL-37 transgenic mice produce significantly less IL-6 and TNFα when stimulated by IL-1β or the combination of IL-12 plus IL-18. The anti-inflammatory activity of IL-37 was not limited to a reduction of the cytokines and chemokines of innate immunity. Dendritic cells isolated from the spleen of IL-37 transgenic mice upon LPS stimulation revealed a marked reduction (75% and 89%) in expression of CD86 and MHC II, respectively.[194] The total numbers of dendritic cells, macrophages, NK cells, and CD4+ T cells were similar in all strains and experimental conditions.

A Role for Interleukin-37 During Experimental Colitis

IL-37 transgenic mice have been subjected to DSS-induced colitis. Despite the presence of a CMV promoter to drive expression of IL-37, mRNA transcripts were not present in colons in the resting state.[195] Expression was observed only upon disruption of the epithelial barrier, with a six- to sevenfold increase on days 3 and 5 after continuous exposure to DSS. During the development of colitis, clinical disease

scores were reduced by 50% and histologic indices of colitis were one-third less in IL-37 transgenic mice compared with wild-type counterparts. Reduced inflammation was associated with decreased leukocyte recruitment into the colonic lamina propria. In addition, release of IL-1β and TNFα from ex vivo colonic explant tissue was decreased 5- and 13-fold, respectively, compared with wild-type mice, whereas IL-10 was increased 6-fold. However, IL-10 was not required for the anti-inflammatory effects of IL-37 because IL-10 receptor antibody blockade did not reverse IL-37–mediated protection. Mechanistically, IL-37 originating from hematopoietic cells was sufficient to exert anti-inflammatory effects because wild-type mice reconstituted with bone marrow from IL-37 transgenic mice were protected from colitis.

A Nuclear Role for Interleukin-37

In stable transfectants of human IL-37 in RAW macrophages stimulated with LPS, levels of TNFα, IL-1α, IL-6, as well as the chemokine MIP-2 were substantially reduced (72% to 98%) compared with LPS-stimulated cells transfected with the empty plasmid.[204] Similar to IL-1α and IL-33, IL-37 translocates to the nucleus following stimulation.[204] In mouse RAW macrophages stably expressing IL-37, the mature carboxyl-terminal was detected in the nucleus. Furthermore, a specific caspase-1 inhibitor markedly reduced nuclear entry of IL-37.[204] The data demonstrate that IL-37 translocates to the nucleus after caspase-1 processing and may act as a transcriptional modulator reducing the production of LPS-stimulated proinflammatory cytokines, consistent with IL-37 being an anti-inflammatory member of the IL-1 family.

IL-37 was identified in a proteomics-based search for proteins that interacted with Smad3.[205] To test for a functional interaction of Smad3 with IL-37, IL-37 was transfected into A549 cells. IL-37 colocalized with phospho-Smad3 and was found in perinuclear and cytosolic regions, and a IL-37-Smad3 complex was observed.[194] A specific inhibitor of Smad3 reversed the inhibition of IL-6 and IL-1β expression in RAW cells stably transfected with IL-37. In stable human macrophage lines expressing IL-37, depletion of Smad3 by lentiviral introduction of short hairpin RNA that inhibits Smad3 expression prevented the ability of IL-37 to reduce IL-1β- or LPS-induced production of IL-8, IL-6, and TNF. These in vitro findings were confirmed in vivo. IL-37 transgenic mice were pretreated intranasally with a Smad3 specific small interfering RNA and then challenged with intranasal LPS. The reduction of lung cytokines in IL-37 transgenic mice was reversed in transgenic mice with a lung knockdown of Smad3.[194]

Role of Interleukin-18Rα for Interleukin-37

From the first reports on IL-37, it was observed that the recombinant forms bound to the IL-18Rα.[132,206] This is shown in Figure 26.3C and E. The binding of IL-37 to IL-18Rα has also been observed in cells from IL-37 transgenic mice using immunofluoresence, immunoprecipitation, and fluorescence resonance energy transfer analysis.[207] IL-37 specifically binds to the third domain of the IL-18Rα.[193] Despite these studies showing binding of IL-37 to the IL-18Rα chain, IL-37 does not act as a classical receptor antagonist for IL-18 in that the ability of recombinant IL-18 to induce IFNγ is not inhibited by high concentrations of IL-37. However, in the presence of low concentrations of IL-18BP, recombinant IL-37 modestly reduces IL-18–induced IFNγ.[193] The concept that IL-37 binds to the IL-18Rα and reduces cytokine production is supported, in part, with the finding embryonic fibroblasts from mice deficient in IL-18Rα produce 10-fold more IL-6 in response to IL1β than do wild-type embryonic fibroblasts.[208] In addition, silencing of IL-18Rα in primary human blood monocytes results in a fourfold increase in the secretion of LPS-induced IL-1β, IL-6, IFNγ, and CD40 ligand.[208] Thus, the seemingly paradoxical hyperresponsive state in cells deficient in IL-18Rα supports the concept that IL-18Rα participates in both pro- and anti-inflammatory responses and that the endogenous ligand IL-37 engages the IL-18Rα to deliver an inhibitory signal.

Role of Single Immunoglobulin Interleukin-1–Related Receptor in the Anti-inflammatory Property of Interleukin-37

The mechanism by which an IL-1β or an LPS signal is suppressed by IL-37 requires an understanding of SIGIRR (Fig. 26.4G). The IL-1RAcP serves as the coreceptor for IL-1α, IL-1β, IL-36α, IL-36β, IL-36γ, and IL-33, each a proinflammatory cytokine. However, in the IL-1 family of receptors, three coreceptors contain unusually long intracellular domains. These are SIGIRR (Fig. 26.4G), a variant of the IL-1RAcP termed IL-1RAcPb (Fig. 26.4F) and receptors termed "three immunoglobulin IL-1–related receptors" (TIGIRRs). As shown in Figure 26.4H, there are two TIGIRRs: TIGIRR-1 and TIGIRR-2. IL-1RacPb is expressed only in the brain, and TIGIRR has limited expression. However, SIGIRR is expressed in most cells. The TIR domain of the three coreceptors is also different from that of other members of the IL-1 coreceptor family in that the TIR domain contains an amino acid sequence different from that of wild-type TIR.[209] As shown in Figure 26.2B, IL-1RAcPb forms the expected complex with IL-1 and IL-1R1 but does not recruit MyD88 or phosphorylate IRAK4.[209] Therefore, most IL-1 signaling is arrested. But as some genes are increased in response to the formation of the IL-1RI/IL-1RAcPb complex, partial IL-1 signaling must take place. Nevertheless, IL-1RAcPb functions as an inhibitory receptor chain but only in the brain. Mice deficient in IL-1RAcPb exhibit a normal inflammatory response in the periphery but greater neurodegeneration in the brain. As such, IL-1RAcPb could play a role in chronic inflammatory responses in the brain by "buffering" IL-1–mediated neurodegeneration.

Like IL-1RAcPb, SIGIRR contains the same amino acids differences in its TIR domain, termed TIRb. Compared to wild-type TIR, TIRb likely has reduced binding of MyD88.[209] In addition to an altered TIR domain, SIGIRR has a carboxyl extension of 140 amino acids. Carboxyl extensions are also present in IL-1RAcPb as well as the two TIGIRRs. TIGIRR-2, which is associated with an X-linked cognitive deficiency, is apparently independent of IL-1 function. Little is known whether these C-terminal segments contribute to the inhibitory properties of these receptors. Nevertheless,

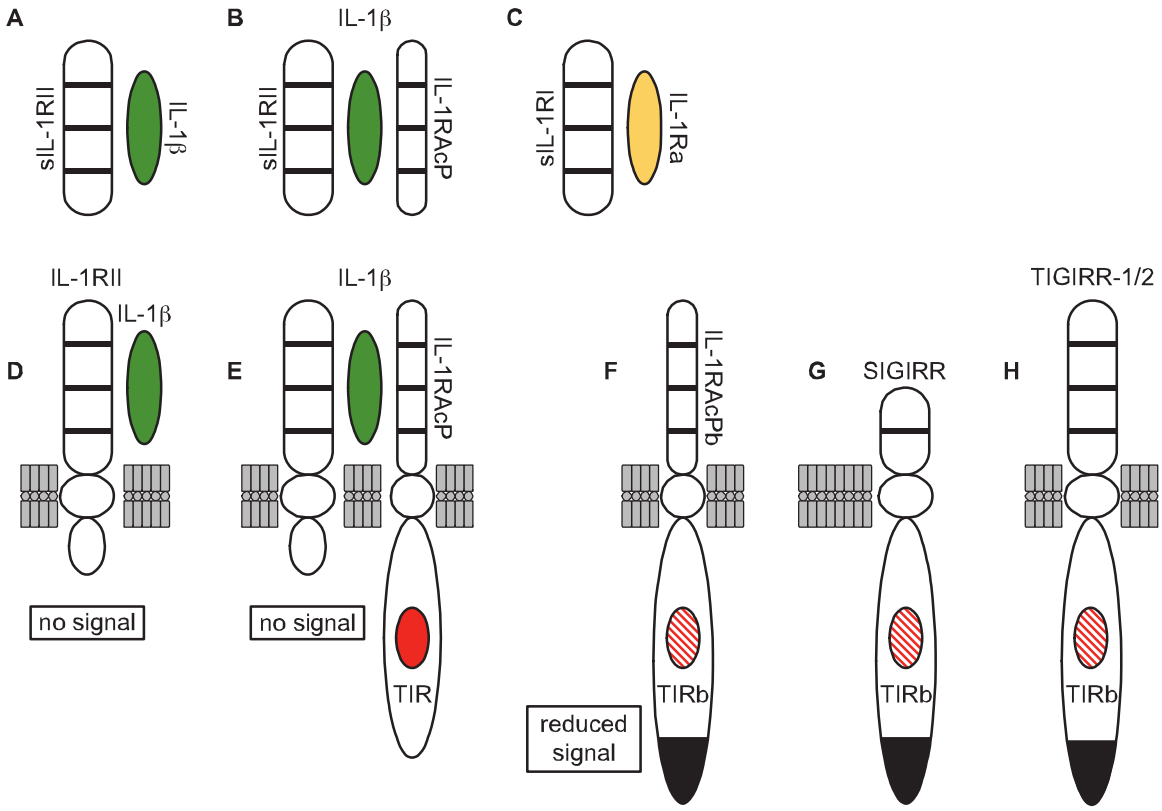

FIG. 26.4. Anti-Inflammatory Receptors of the Interleukin (IL)-1 Receptor Family. **A:** Soluble (extracellular) IL-1RII (sIL-1RII) binds and neutralizes either the precursor or mature form of IL-1β. **B:** A complex of IL-1β, sIL-1RII, and sIL-1RAcP neutralizes IL-1β activity. **C:** Soluble (extracellular) IL-1RI (sIL-1RI) binds IL-1Ra and prevents IL-1Ra from its function as a receptor antagonist. Similarly, IL-1α can bind to the sIL-1RI (not shown). **D:** IL-1RII expressed on the membrane binds mature IL-1β acting as a decoy receptor, preventing IL-1β from binding to its signaling receptor. **E:** A complex of membrane IL-1RII, IL-1β, and membrane IL-1RAcP also prevents IL-1β from signaling. **F:** The IL-1RAcPb is expressed in brain tissue (see Fig. 26.2B). **G:** Single immunoglobulin IL-1–related receptor is expressed in myeloid cells and epithelial cells and function in damped inflammation due to the TIRb domain. **H:** Three immunoglobulin IL-1–related receptor is also an anti-inflammatory member of the IL-1 due to the toll-IL-1-receptor b domain.

it seems likely that the alternative sequence in the TIRb domain of SIGIRR may act as a partial decoy for MyD88. MyD88 is phosphorylated upon TLR4 as well as IL-1β and IL-18 signaling, and results in downstream phosphorylation of IRAK-4. In cells expressing SIGIRR and activated by IL-37 binding to the IL-18Rα, the signal from either IL-1 or LPS initiates phosphorylation of MyD88. However, the decoy effect by the mutated TIRb of SIGIRR reduces the degree of phosphorylation of MyD88 and thus the phosphorylation of IRAK-4. The reduction, however, is partial. Indeed, the suppression by IL-37 added to blood macrophages is in the range of 20% to 50% and unlike the total loss of activation by a deficiency in MyD88.

Upon binding to the IL-18Rα, the IL-37 precursor may activate SIGIRR and provide a negative signal (see Fig. 26.3C). An inhibitory signal from IL-37 is enhanced by a low concentration of IL-18BP.[193] As shown in Figure 26.3D, IL-18BP binds IL-37[193] and likely presents the complex of the cytokine with the binding protein to the IL-18Rα (see Fig. 26.3E). Because A549 cells express SIGIRR, it is likely that the inhibitory signal from IL-37 activates SIGIRR or alternatively IL-37 recruits SIGIRR as the accessory chain. The inhibitory signal of SIGIRR is established in several mouse models of inflammation in which SIGIRR-deficient mice exhibit more inflammation compared to wild-type control mice.[210] In differentiated human blood M1 macrophages, recombinant IL-37 suppresses LPS-induced TNFα and IL-6 production 50% to 70%. A source of IL-18BP in this culture may be fetal calf serum. During the differentiation of macrophages into the M1 subset by granulocyte macrophage-colony stimulating factor, it is possible that SIGIRR expression increases whereas the level of IL-18Rβ decreases (see Fig. 26.3A). In the absence of IL-18Rβ, a proinflammatory complex is not formed with IL-18Rα and thus IL-37 binding to IL-18Rα may recruit or activate SIGIRR. Thus, expression of SIGIRR and the absence of IL-18Rβ would best explain the inhibitory properties of recombinant IL-37 reducing the response to LPS induction of IL-6 and TNFα.

INTERLEUKIN-36 SUBFAMILY

The IL-1 family members IL-1F5, IL-1F6, IL-1F8, and IL-1F9 are now termed IL-36Ra, IL-36α, IL-36β, and IL-36γ, respectively.[211] As shown in Figure 26.5A, each member of the IL-36

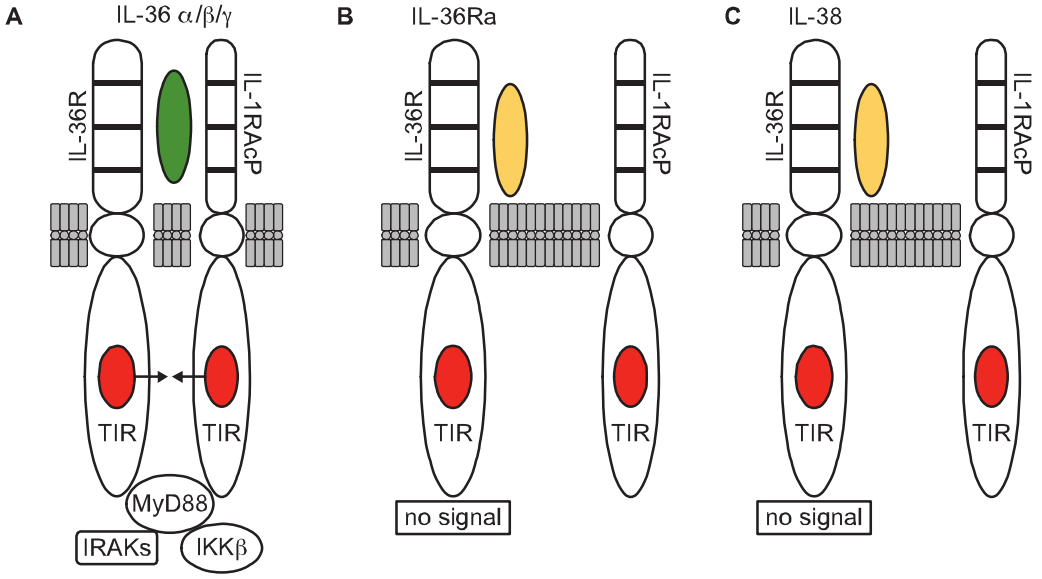

FIG. 26.5. Interleukin (IL)-36 Subfamily. **A:** IL-36α, IL-36β, or IL-36γ bind to the IL-36R and recruit the coreceptor IL-1RAcP. The heterodimeric IL-36 receptor complex results in a close approximation of the toll-IL-1-receptor (TIR) domains on each receptor chain (*arrows*), resulting in the binding of intracellular MyD88 to the complex with phosphorylation of MyD88. Subsequent phosphorylations of IRAKs and IKKβ, increased NF-κB, and activating protein-1 translocation to the nucleus take place, followed by expression of proinflammatory genes. **B:** IL-36Ra binds to IL-36R. There is no recruitment of the coreceptor IL-1RAcP, no approximation of the TIR domains, and no signal. **C:** IL-38 binds to IL-36R but there is no signal and acts similar to IL-36Ra.

subfamily binds to the IL-1Rpr2, now termed IL-36R.[212] Because IL-38 also binds to the IL-36R (Fig. 26.5C),[133] IL-38 is included in the IL-36R subfamily. The IL-36 subfamily is closely related to the IL-1 subfamily because, similar to the IL-1α and IL-1β and IL-33, the IL-36R forms a signaling complex with the IL-1RAcP (Fig. 26.5A).[122,212] Thus of the 11 members of the entire IL-1 family, 6 members use IL-1RAcP as the coreceptor for signal transduction.

Interleukin-36

The IL-36R is the ligand binding chain and therefore is comparable to the ligand binding IL-1R1 and the IL-18Rα. However, two members of the IL-36 subfamily bind to the IL-36R but do not signal, the IL-36 receptor antagonist (IL-36Ra) and IL-38 (see Fig. 26.5B and C). As such, these function as receptor antagonists.[9,133] An unusual property of IL-38 is that low concentrations (1 to 10 ng/mL) are able to reduce the activity of endogenous IL-36,[133] whereas in the case of IL-1Ra, higher concentrations are required to prevent the activating of endogenous IL-1α or IL-1β.

None of the members of the IL-36 subfamily have a signal peptide, indicating the generation of the N-terminus and secretion via the Golgi. In addition, each member of the IL-36 subfamily has an unusually short propiece compared to those of the IL-1α, IL-1β and IL-33 (see Fig. 26.1). Like IL-1β and IL-18, there is no true caspase-1 cleavage site for generation of an N-terminus in the IL-36 subfamily. It remains unknown which specific proteases generate the various N-termini of the IL-36 subfamily; nevertheless, each member has a unique N-terminus with a different levels of biologic activity.[9] What determines the N-terminus with optimal biologic activity in the IL-36 subfamily? Each member of the IL-36 subfamily contains the IL-1 family consensus sequence of A-X-D. The aspartic acid is not the recognition amino acid for caspase-1 or caspase-3, but rather participates in the stabilization of the first beta sheet of the three-dimensional structure that characterizes the entire IL-1 family.

The "A" of the consensus sequence is for any aliphatic amino acid, for example, leucine or isoleucine. Nine amino acids before the "A" of the consensus sequence is the N-terminal site, which results in the cytokine with the greatest activity.[9] For example, the biologic activity of IL-36γ increases by a factor of 1,000 when the N-terminus is at the site nine amino acids before the consensus sequence and in the case of the IL-36β, there is a 10,000-fold increase.[9] The nine amino acid forward site from the consensus sequence is not only the N-terminus for the agonist members of the IL-36 family but also the IL-36 receptor antagonist (IL-36Ra),[9] which increases from a low level of blocking of IL-36 family ligands to a high degree of blockade. It is unclear what specific protease cleaves at this site as the amino acid is different for each member of the IL-36 subfamily. Moreover, the site for the N-terminus of the IL-36Ra (valine) is but one amino acid from the N-terminal precursor methionine and yet IL-36Ra with an N-terminus at the valine site is 10,000-fold more potent that the IL-36 precursor. It is also an unusual situation that proteases that usually

are inflammatory in processing members of the IL-1 family in that case of IL-36Ra generate an anti-inflammatory molecule.

Interleukin-36α, β, and γ, Proinflammatory Members of the Interleukin-36 Subfamily

IL-36α was highly expressed in the murine model of glomerulonephritis[213] where the cytokine was localized to the kidney epithelium and also in CD3 T cells surrounding the tubules. IL-36α is also found in the kidneys of the MRL/lupus, nephritic syndrome, and streptozotocin-induced diabetic models.[213] IL-36γ increases IL-8, CXCL3, and the Th17 chemokine CCL20 in human lung fibroblasts,[214] and thus may account for acute neutrophilic lung inflammation. In addition to CD4+ T cells, human articular chondrocytes and synovial fibroblasts express the IL-36R.[215] In chondrocytes, there is also constitutive gene expression of IL-36β. Following stimulation with IL-1β or TNFα, levels of the IL-36β precursor rise intracellularly but the cytokine is not secreted. Although IL-36β levels were detected in the joint fluids of patients with rheumatoid arthritis as well as in serum samples, there was no correlation with disease severity.[215] It is likely that IL-36 ligands are functional only when released from dying cells and can be processed extracellularly by enzymes present in inflammatory conditions such as the joints of patients with rheumatoid arthritis. It unclear to what extent IL-36β plays a role in joint disease, although constitutive expression in primary chondrocytes may indicate a role for the cytokine in osteoarthritis.[215]

High levels of this cytokine are found in mouse embryonic tissues rich in epithelial cells.[216] In humans, IL-36α is observed in keratinocytes, not fibroblasts, and is thought to contribute to the inflammation of psoriasis. Upon forming the heterodimer with IL-36R and IL-1RAcP, IL-36α activates NF-κB similar to that of IL-1β.[212] In addition to NF-κB activation, IL-36α also activates MAPK, JNK, and ERK1/2.[212] In the mouse, bone marrow–derived dendritic cells and CD4+ T cells express IL-36 receptors in health. In a comparison with IL-1 as a stimulant, the three IL-36 ligands are more active in inducing IL-1β, IL-6, IL-12, TNFα, and IL-23.[217] In addition, IL-36 ligands induced the production of IFNγ, IL-4, and IL-17 from CD4+ T cells. Not unexpectedly, cytokines induced by IL-36 lands were prevented by 100- to 1,000-fold excess IL-36Ra.[217]

Interleukin-36 in Psoriasis

Several studies implicate IL-36 ligands in the pathogenesis of psoriasis.[218–220] IL-36γ is highly expressed in keratinocytes from healthy human skin and increases upon stimulation with TLR polyI:C.[221] Furthermore, polyI:C induced caspase-3, which resulted in cell death and the release of IL-36γ. Unexpectedly, stimulation of IL-36γ gene expression was dependent on caspase-1.[221] The caspase-1 dependency may be due to IL-18 as this cytokine is constitutively present in keratinocytes as is IL-1α. With the release of IL-36γ by polyI:C and the subsequent death of the cell, IL-36γ falls into the category of being an alarmin in the skin, particularly due to infection.[221] There is also a role for IL-36 in the production of IL-17: studies suggest that each of the IL-36 ligands is expressed in the skin and dependent on IL-22.[222] Furthermore, the expression of IL-36 ligands in the psoriatic skin correlated with IL-17.[222] Similar to other models in the IL-1 family, auto- and coinduction accounts for a role in a pathologic process.

Overexpression of IL-36 in mice results in inflammatory skin lesions that resemble psoriasis in humans, as reviewed in Towne and Sims.[223] Likewise, mice deficient in endogenous IL-36Ra results in a severe lesion similar to that of humans with pustular psoriasis. The role of IL-36 in pustular psoriasis may include IL-1α, as humans with pustular psoriasis responds to an antibody that neutralizes IL-1α. Both IL-36 and IL-1α are found in the keratinocytes in healthy skin.

A Role for Interleukin-36R in Metabolic Regulation

Obesity is characterized by chronic low-grade inflammation originating from expanding adipose tissue. Human adipogenic tissue levels of IL-36α is primarily present in adipose tissue resident macrophages and induced by inflammation; however, IL-36β is absent.[224] IL-36α, but not IL-36β, reduces adipocyte differentiation, as shown by a significant decrease in PPARγ gene expression. Both IL-36α and IL-36β induce inflammatory gene expression in mature adipocytes.[224] Therefore, IL-36α and IL-36β are present in adipose tissue and are involved in the regulation of adipose tissue gene expression. Importantly, IL-36α inhibits PPARγ expression, which may lead to reduced adipocyte differentiation suggesting metabolic effects of this cytokine.

Although IL-36Ra is known to occupy the IL-36R and act as a receptor antagonist, earlier studies revealed that IL-36Ra inhibited the induction of IL-1β by LPS. The role of IL-36Ra was also examined in the brain. IL-36Ra injected into the rat brain induced IL-4 and also in glial cells in vitro.[225] Moreover, the reduction in LPS-induced IL-1β was not observed in cells deficient in IL-4 and also not observed in cell deficient in SIGIRR.[225] However, these unique properties of IL-36Ra were not observed in peripheral monocytes or dendritic cells but only in the brain.

Role of Interleukin-36 in Human Disease

The importance of any cytokine in human biology can be found in mutations that result in a profound clinical picture. In the case IL-1, a mutation in the naturally IL-1Ra (see Fig. 26.5B) results in severe systemic inflammation with erosive bone lesions, sterile meningitis, and death; the syndrome is called deficiency of IL-1Ra.[226,227] In case of the IL-36 family, persons with a mutation in the naturally occurring IL-36Ra suffer with a severe form of pustular psoriasis.[228,229] These human studies are consistent with the data from transgenic mice overexpressing IL-36α in the skin and with the ability of IL-36Ra to suppress the

severity of the inflammation.[218] In mice overexpressing IL-36a in the skin, crossing the mice to generate a strain of mice heterozygous for natural IL-36Ra knockout results in worsening of the skin lesions.[218]

Interleukin-38

IL-38 is the name for the IL-1 family member 10. During the nomenclature revision of the IL-1 family in 2010,[211] the term IL-38 was assigned to IL-1F10 without any known biologic function. Since then, IL-38 has been shown to bind to the IL-36 receptor (formerly IL-1Rrp2) (see Fig. 26.5C).[133] In order to find the receptor for IL-38, each member of the IL-1 receptor family was immobilized and recombinant IL-38 precursor containing 152 amino acids was added and binding assessed using an antibody to the ligand. IL-38 bound only to the IL-36 receptor, as did IL-36Ra.[133] To assess the biologic function of IL-38, heat-killed *Candida albicans* was used to stimulate memory T-lymphocyte cytokine production in freshly obtained human peripheral blood mononuclear cells from healthy subjects. The addition of recombinant IL-38 inhibited the production of T-cell cytokines IL-22 and IL-17. The dose-response suppression of IL-38 as well as that of IL-36Ra of *Candida*-induced IL-22 and IL-17 was not that of the classic IL-1 receptor antagonist, because low concentrations were optimal for inhibiting IL-22 production.[133] These data provide evidence that IL-38 binds to the IL-36R, as does IL-36Ra, and that IL-38 and IL-36Ra have similar biologic effects on immune cells by engaging the IL-36 receptor.

CONCLUSION

In conclusion, IL-1 family of cytokines is one of the most important families of proinflammatory mediators, that have profound local and systemic effects. The importance of these molecules has been demonstrated in homeostasis, as well as in autoinflammatory diseases, and treatment strategies based on modulation of IL-1 cytokines have begun to be employed successfully. Furthermore, important and novel biologic roles are currently being described for the members of IL-1 family of cytokines, and it is to be expected that this will represent one of the most dynamic areas of research in immunology in the coming years.

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CHAPTER 27

Tumor Necrosis Factor–Related Cytokines in Immunity

Carl F. Ware

INTRODUCTION

Lymphocytes are highly mobile, transiting the vascular and lymphatic circulatory systems with limited stopovers in organized lymphoid tissues (lymph nodes, spleen, and Peyer's patches) in which they commune to initiate immune responses. Cytokines, both secreted and membrane-anchored proteins, serve as the communication media of the immune system. Cytokines in the tumor necrosis factor (TNF) superfamily (TNFSF) help orchestrate the development, homeostasis, and effector actions of cells in the immune system. The diversification of the TNFSF is evidenced by its roles in regulating cells of neuronal, skeletal, and ectodermal origin. This chapter's focus is on members in the TNFSF that are primarily involved in regulating immunity.

The ligands belonging to the TNFSF initiate intercellular communication by binding specific receptors on the surface of the receiving cell. The TNF-related ligands are membrane-bound and require cell-cell contact to initiate signaling, although some ligands may be secreted in soluble form influencing cellular responses at locations distant from the source of production. The TNF receptors (TNFRs) form a corresponding superfamily of cognate membrane proteins that initiate intracellular signaling pathways that influence cellular growth, differentiation, and survival. Each ligand-receptor pair forms a "system" with over 40 distinct ligand-receptor systems. Many of the ligands and receptors engage more than one cognate, thereby forming "circuits" of signaling systems, which often function together as coordinated or integrated networks with other cytokines to regulate a specific cellular process. The signals delivered to the cytosol activate transcription factors such as NF-κB and AP1, which in turn initiate the expression of hundreds of genes that alter cellular differentiation. Additionally, some TNFRs activate cell death pathways, both apoptotic and necrotic, terminating cellular life. Genetic mutations have revealed the importance of individual TNFSF members in human immune responses and development. Moreover, the beneficial effect of TNF antagonists in patients with certain autoimmune diseases brings the need to understand the complexities of the TNFSF into sharp focus.

The nomenclature of the TNFSF is a bit of a morass for students new to the field, although introduction of a numerical system standardizing gene names (www.genenames.org/) helps in accessing genomic databases. However, the use of more common, often colorful, acronyms continues. The name, rank, and genomic identification of each ligand (Table 27.1) and receptor (Table 27.2) is tabulated along with additional pertinent information for human and mouse genes.

TUMOR NECROSIS FACTOR LIGAND FAMILY

The TNF-related cytokines are type II transmembrane proteins (intracellular N-terminus) with a short cytoplasmic tail (15 to 25 residues in length) and a larger extracellular region (~150 amino acids) containing the signature TNF homology domain where the receptor binding sites are located (Fig. 27.1). The TNF homology domain assembles into trimers, the functional unit of the ligand. Atomic analysis of several members of the family[1–5] revealed the ligands have a highly conserved tertiary structure folding into a β sheet sandwich, yet amino acid sequence conservation is limited to < 35% among the family members. The conserved residues defining this superfamily are primarily located within the internal β strands that form the molecular scaffold, which promote assembly into trimers. The residues in the loops between the external β-strands are variable and in specific loops make contact with the receptor. Although most of the TNF ligands self-assemble into homotrimers, heterotrimers can also form between LTα and LTβ[6] and APRIL and BAFF.[7] The stoichiometry of the LT heterotrimer is 1:2 (LTαβ2), which imparts its distinct receptor specificity from the LTα homotrimer. Interestingly, complement component C1q and several related proteins are structurally related to the TNF family, containing a TNF homology domain, a rather surprising finding given the apparent functional divergence between the complement and TNF systems.[8] Alternate splicing can also generate distinct ligands, such as the splice form joining TWEAK and APRIL (TWE-PRIL),[9] the alternate ligands for ectodysplasin receptor,[10] and a cytosolic form of LIGHT.[11]

The genetic organization of TNF ligands is highly conserved and typically encoded in three or four exons, with the fourth exon encoding most of the extracellular TNF homology domain. The genes encoding TNF, LTβ, and LTα reside adjacent to each other in a compact loci in the class III region of the major histocompatibility complex (MHC) (in humans at chromosome 6p21) sandwiched between the antigenic-peptide presenting MHC proteins encoded by MHC class I and II genes.[6,12] Three other genetic clusters of TNF-related cytokines are found within the corresponding MHC paralogous regions, located on chromosomes 1, 9, and 19. These genetic clusters share a remarkably conserved gene structure and transcriptional orientation, and a similar function linked to regulating cellular immune responses.[11] The evolutionary pressures retaining this genetic configuration of the MHC in general is encompassed in the paralogy theory of genome

TABLE 27.1 Tumor Necrosis Factor Superfamily Chromosomal Locations

Gene Name/ Alias	Chromosomal Location: Human	Chromosomal Location: Mouse	Ligand Symbol
TNF	6p21.3	ch17 (19.06 cm)	TNFSF1A
LTα	6p21.3	ch17 (19.06 cm)	TNFSF1B
LTβ	6p21.3	ch17 (19.06 cm)	TNFSF3
OX40-L	1q25	ch1 (84.90 cM)	TNFSF4
CD40-L, CD154	Xq26	chX (18.0 cM)	TNFSF5
Fas-L	1q23	ch1 (85.0 cM)	TNFSF6
CD27-L, CD70	19p13	ch17 (20.0 cM)	TNFSF7
CD30-L, CD153	9q33	ch4 (32.20 cM)	TNFSF8
4-1BB-L	19p13	ch17 (20.0 cM)	TNFSF9
TRAIL	3q26	ch3	TNFSF10
RANK-L, TRANCE	13q14	ch14 (45.0 cM)	TNFSF11
TWEAK	17p13	ch11	TNFSF12
APRIL/TALL2	17P13.1	ch13	TNFSF13
BAFF, BLYS, TALL1	13q32-q34	ch8 (3 cM)	TNFSF13B
LIGHT	19p13.3	ch17 (D-E1)	TNFSF14
TL1A	9q33	ch4 (31.80 cM)	TNFSF15
G1TRL, A1TRL	1q23	Unknown	TNFSF18
EDA1	Xq12-q13.1	chX (37.0 cM)	EDA1
EDA2	Xq12-q13.1	cX (37.0 cM)	EDA2

CD, cluster of differentiation; LT, lymphotoxin; TNF, tumor necrosis factor; TNFSF, tumor necrosis factor superfamily; TRAIL, TMF-related apoptosis inducing ligand.
From Ware CF. The TNF superfamily-2008. *Cytokine Growth Factor Rev.* 2008;19:183–186.

TABLE 27.2 Tumor Necrosis Factor Receptor Superfamily

Gene Name/ Aliases	Chromosomal Location: Human	Chromosomal Location: Mouse	Gene Symbol
TNFR-1, p55-60	12p13.2	ch6 (60.55 cM)	TNFRSF1A
TNFR2, p75-80	1p36.3-36.2	ch4 (75.5 cM)	TNFRSF1B
LTβR	12p13	ch6 (60.4 cM)	LTβR
OX40	1p36	ch4 (79.4 cM)	TNFRSF4
CD40	20q12-q13.2	ch2 (97.0 cM)	CD40
FAS, CD95	10q24.1	ch19 (23.0 cM)	TNFRSF6
DcR3	20q13	unknown	TNFRSF6B
CD27	12p13	ch6 (60.35)	TNFRSF7
CD30	1p36	ch4 (75.5 cM)	TNFRSF8
4-1BB	1p36	ch4 (75.5 cM)	TNFRSF9
TRAILR-1, DR4	8p21	Unknown	TNRSF10A
TRAIL-R2, DR5	8p22-p21	ch14 (D1)	TNFRSF10B
TRAILR3, DcR1	8p22-p21	ch7 (69.6 cM)	TNFRSF10C
TRAILR4, DcR2	8p21	ch7 (69.6 cM)	TNFRSF10D
RANK, TRANCE-R	18q22.1	ch1	TNFRSF11A
OPG, TR1	8q24	ch15	TNFRSF11B
FN14	16p13.3	ch17	TNFRSF12A
TRAMP, DR3, LARD	1p36.3	ch4 (E1)	TNFRSF25
TACI	17p11.2	ch11	TNFRSF13B
BAFFR	22q13.1-q13.31	ch15	TNFRSF13C
HVEM, HveA, ATAR	1p36.3-p36.3	ch4	TNFRSF14
P75NTR, NGFR	17q12-q22	ch11 (55.6 cM)	TNFRSF16
BCMA	16p13.1	ch16 (B3)	TNFRSF17
AITR, GITR	1p36.3	ch4 (E)	TNFRSF18
RELT	11q13.2	unknown	TNFRSF19L
TROY, TAJ	13q12.11-q12.3	ch14	TNFRSF19
EDAR	2q11-q13	ch10	EDAR1
EDA2R	Xq11.1	ch X	EDA2R
DR6	6P12.2-21.1	ch17	TNFRSF21
IGFLR1, Tmem149	19q13.12	ch7	IGFLR1

CD, cluster of differentiation; HVEM, herpesvirus entry mediator; LT, lymphotoxin; OPG, osteoprotegerin; TNFR, tumor necrosis factor receptor; TNFRSF, tumor necrosis factor receptor superfamily; TRAIL, TMF-related apoptosis inducing ligand.
From Ware CF. The TNF superfamily-2008. *Cytokine Growth Factor Rev.* 2008;19:183–186.

evolution.[13] Conservation of gene structure of the TNF ligands outside these MHC paralogs is limited. An evolutionarily conserved pathway in *Drosophila melanogaster*, Eiger-Wengen system, is structurally and functionally related to the TNFSF-related signaling pathway, although this system is predominantly expressed in the nervous system in invertebrates.[14–16]

TUMOR NECROSIS FACTOR RECEPTOR FAMILY

Members of the TNF receptor superfamily (TNFRSF) include proteins of vertebrate and viral origin. Most of the signaling receptors in the TNFRSF are type I transmembrane glycoproteins (N-terminus exterior to the cell). However, several TNFRSF members lack a membrane-anchor domain, are proteolytically cleaved from the surface, or are anchored via glycolipid linkage (eg, TNF-related apoptosis inducing ligand [TRAIL]R3). These soluble receptors, termed "decoy receptors," retain their ligand-binding properties and compete with cellular receptors for the specific ligands, thus earning the title of decoy. The structural motifs in the cytoplasmic domains further categorize the TNFR into two groups based on their signaling properties: those contain a death domain (DD) and others that engage TNFR-associated factors (TRAFs).

The cysteine-rich domain (CRD) in the extracellular, ligand-binding region defines membership in the TNFRSF (see Fig. 27.1). Each CRD typically contains six cysteine residues forming three disulfide bonds. The CRD is pseudorepeated in different members ranging from one to six. Based upon the crystal structures solved for several TNFRSF members, the CRD confers an elongated shape and sidedness to the ectodomain.[17,18] The crystal structure of the complex between TNFR1 and one of its ligands, the LTα homotrimer, revealed

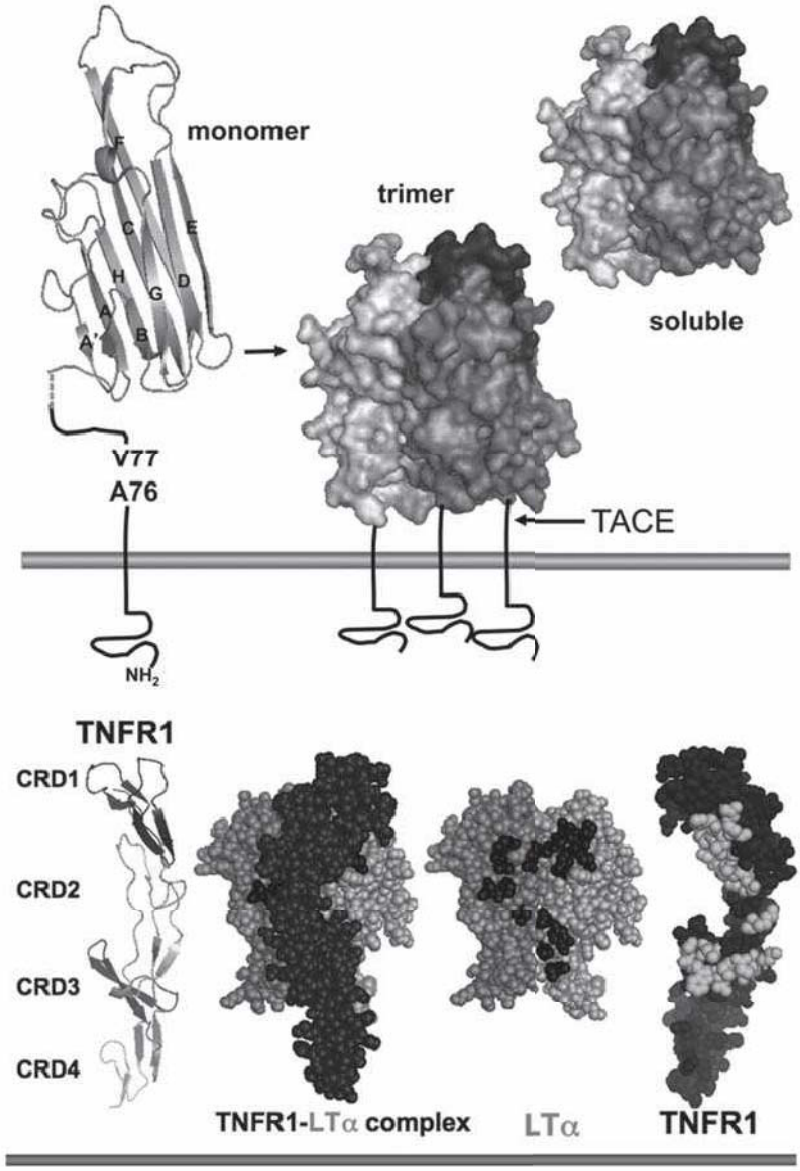

FIG. 27.1. Structure of Tumor Necrosis Factor (TNF) and TNF Receptors (TNFRs). **Top:** TNF. The β-sandwich of the TNF monomer (1A8M.pdf) (shown as a *ribbon diagram*) contains two stacked β-pleated sheets each formed by five antiparallel β strands (*wide ribbons given letter designations, A–G*) that fold into a Greek key or "jelly-roll" topology.[219] The inner β-sheet (strands A, A′, H, C, and F) is involved in contacts between adjacent subunits, which promotes assembly into a trimer. The trimer is formed such that one edge of each subunit is packed against the inner sheet of its neighbor. The outer β sheet (strands B, B′, D, E, and G) is surface exposed. The trimeric structure is characteristic of all TNF-related ligands. The type II configuration of TNF (N-terminus inside the cell) anchors TNF to the membrane. The TNF trimer is ~60 Å in height with a relatively flat base residing close to the membrane, resembling a bell-shape (shown as surface representation with *different shades of gray* used for each subunit of the trimer). The surface exposed loops between A-A′ and D-E strands are involved in receptor binding. TNF is cleaved by TNFα-converting enzyme (TACE), a member of the ADAM family of metalloproteinases (ADAM17) involved in processing of many cell surface proteins. TACE is a type 1 transmembrane protein that cleaves TNF between residues Val77 and Ala76, when all three sites in the trimer are cleaved TNF is released from the membrane. **Bottom:** TNFR and ligand complex. The ectodomain of TNFR1 forms an elongated molecule with CRD1 proximal to the N-terminus (*ribbon diagram*). The face of TNFR1 on the left engages LTα. In the ligand-receptor complex, the elongated receptor (*dark*) lies along the cleft formed between adjacent subunits. Shown (space-filling) is a single TNFR1 in complex with two subunits of LTα (*lighter shades*); the receptor N-terminus points upward, closest to the base of the ligand (transorientation). In the exploded view, TNFR1 is removed from in front of the ligand revealing the contact residues in the ligand, which are primarily located in the D–E and the A-A″ β-strands (*dark shade*). TNFR1 is rotated 180 degrees, exposing the contact residues in the receptor (*light shade*). Structures from 1TRN.pdf[17] as visualized with MacPyMOL (www.pymol.org).

that residues in CRD2 and 3 of TNFR1 contact the ligand. Variation in binding interactions has been identified; for example, the receptors for BAFF have one functional CRD.[2,19]

RECEPTOR-LIGAND COMPLEX

The binding specificity of the various members of the TNF ligand and receptor superfamilies show monotypic interaction, yet several members interact with multiple partners[20] (Figs. 27.2 and 27.3). The binding interactions between TNF-related ligands and TNFR are typically high affinity, with equilibrium binding constants measured in the high pM to low nM range. The membrane position of the ligands further enhances the binding interaction with their receptors. In the membrane anchored position, the ligands and receptors must be in trans to form a complex. In the LTα-TNFR1 complex, the surface loops between A-A″ and D-E β strands contain many of the amino acid residues that make contact with the receptor, with the receptor binding site formed as a composite of adjoining subunits in the trimeric ligand[17] (see Fig. 27.1).

The trimeric architecture of the TNF ligands, containing three equivalent receptor-binding sites, provides the basis for initiating signaling through aggregation or clustering of receptors. This concept is supported by the finding that receptor-specific bivalent antibodies can act as agonists mimicking the signaling activity of the natural ligand.[21] Indeed, antibodies or peptide mimetic to TNFR can function as antagonists, blocking the ability of the natural ligand to bind the receptor while simultaneously activating the receptor as an agonist.[22] Some ligands such as FasL, TRAIL, and BAFF form higher ordered oligomers of the basic trimer. These higher ordered oligomers promote supraaggregation of receptors, enhancing or sustaining signaling pathways in the receptor-bearing cell.[23,24] Overexpression of TNFR in cells can also lead to ligand-independent signaling, a

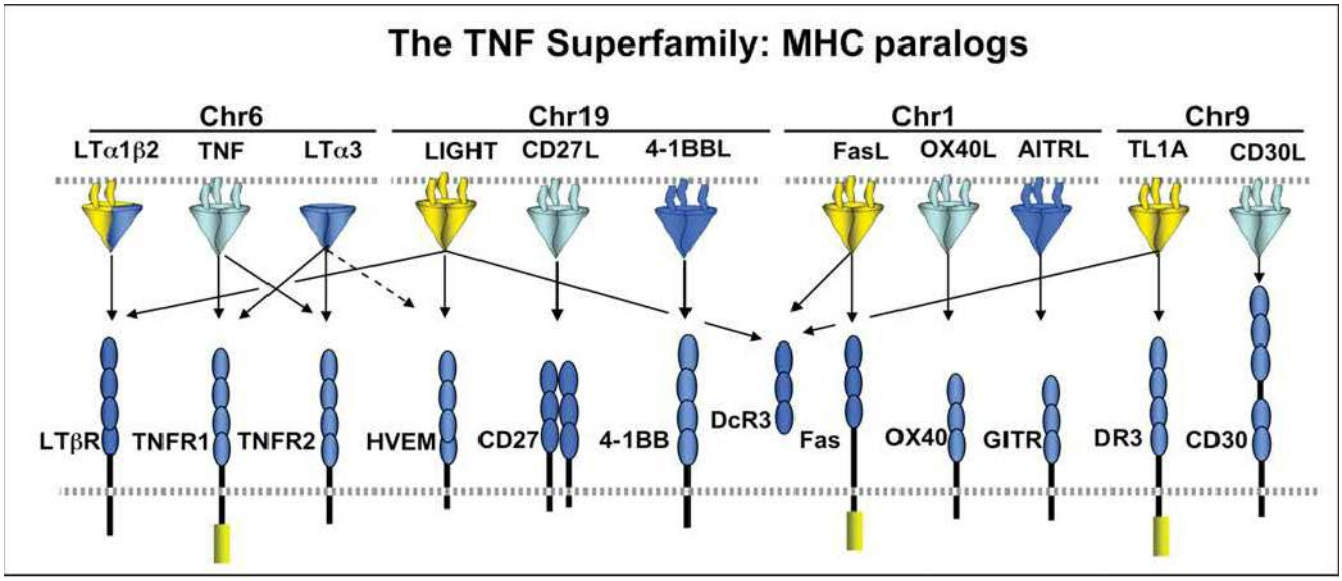

FIG. 27.2. The Tumor Necrosis Factor (TNF) Superfamily–Major Histocompatibility Complex (MHC) Paralogs. Members of the TNF ligand superfamily (*above*) and their corresponding receptors (*below*) are identified by connecting *arrows*. The ligands are grouped according to their chromosomal locations in the MHC paralogous regions. The number of cysteine-rich domains are depicted for each TNF receptor, and TNF receptors containing a death domain are identified by cylinder in the cytoplasmic tail. (Modified from Ware[336]).

feature bestowed in part by the propensity of the cytosolic tails to self-associate.[25] In the physiologic setting, the expression level and compartmentalization of these receptors are tightly controlled. A subregion in the first CRD of TNFR1 known as the preligand assembly domain may restrict the orientation of the unligated receptor to prevent spontaneous activation.[26] It is not known if this mechanism applies to all TNFRSF. Interestingly, some patients with periodic fever syndrome have mutant TNFR1 that form abnormal disulfide-linked oligomers that are retained intracellullarly and provoke misfolded protein response.[27]

Alternate Ligands

There is significant divergence in the ligands and the mechanisms of ligand binding by the TNFR family. A major branch point is exemplified by the ligands for p75 neurotrophin receptor (nerve growth factor and the other neurotrophins), which are structurally unrelated to TNF ligand family. Molecular contacts between NGF and p75NTR occur through two spatially separated binding regions located at the first and second CRD and the junction between the CRD3 and CRD4.[28] The p75NTR functions in complex with two other proteins, Nogo66 and LINGO, to engage myelin-associated inhibitory factors. Taj/TROY can supplant p75NTR in this complex.[29] Like p75 NTR, DR6, ILGF1R, and TROY/Taj do not bind any of the known TNF ligands but do engage other ligands. The pathways activated by p75NTR and TROY/Taj systems show both positive and inhibitory regulation of axonal regeneration.[29,30]

The herpesvirus entry mediator (HVEM; TNFRSF14) provides an example of a TNFR system that binds alternate ligands. Although HVEM engages two TNF-related ligands, LIGHT and LTα, it also engages two members of immunoglobulin (Ig) superfamily, B- and T-lymphocyte attenuator (BTLA)[31] and cluster of differentiation (CD)160.[32] BTLA binding to HVEM occurs in CRD1, on the opposite face of where LIGHT/LTα bind in CRD2 and 3. The BTLA binding site in CRD1 of HVEM is a region also targeted by herpesviruses.[33–35] Recent evidence indicates the neurotrophin and chondrocyte growth factor–like protein progranulin interacts with TNFR1 and TNFR2 and competes with TNF for binding.[36]

An insulin growth factor–like protein was recently identified as a ligand for a TNFR-like type 1 transmembrane protein (insulin growth factor–like R, formerly TMEM149).[37] Insulin growth factor–like R has conserved positioning of cysteines delineating a CRD1, but atypical CRD2/3. Insulin growth factor–like messenger ribonucleic acid (mRNA) is expressed in psoriatic skin lesions and the receptor is detected in T cells suggesting a possible role in skin inflammation.

Death receptor-6 (DR6) engages the growth factor–like domain in β-amyloid precursor protein[38,39] and thus may function as a negative regulator more like p75 neurotrophin receptor.[40] Emerging evidence suggests DR6 has a role in neuroimmune function. In a mouse experimental autoimmune encephalomyelitis (EAE) model, DR6-deficiency was shown to protect against central nervous system demyelination and leukocyte infiltration, but also enhanced overall CD4+ T-cell proliferation and T_H2 differentiation, underscoring a role for DR6 in mediating T_H1-specific immune responses in EAE progression.[41] Taj/TROY and RELT also do not bind any of the known TNFSF ligands; however, recent evidence indicates that Taj/TROY functions more like p75NTR, capable of binding myelin inhibitory factors.[29,30] A role for Taj/TROY and RELT in immune function is presently unclear.

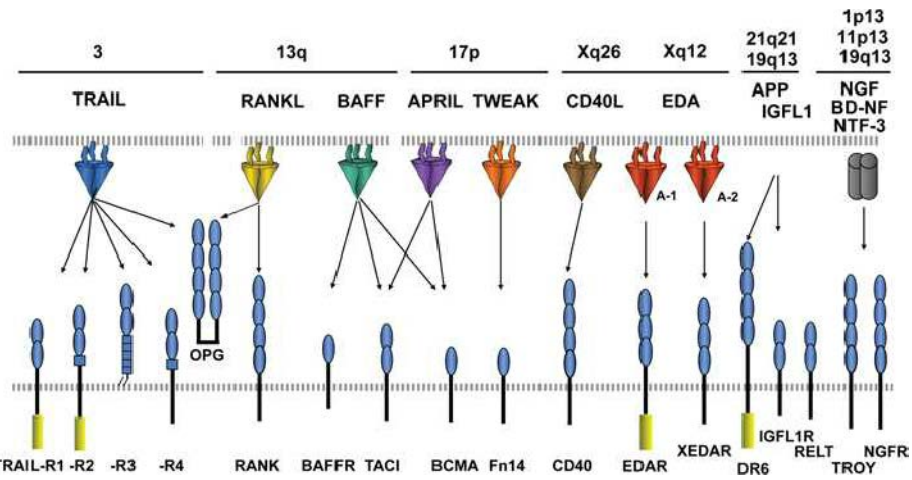

FIG. 27.3. The Tumor Necrosis Factor (TNF) Superfamily—Part 2. Members of the TNF ligand superfamily (*above*) and their corresponding receptors (*below*) are identified by connecting *arrows*. The ligands are grouped according to their chromosomal locations. The number of cysteine-rich domains are depicted for each TNF receptor, and TNF receptors containing a death domain are identified by cylinder in the cytoplasmic tail. (Modified from Ware[336]).

The engagement of ligands distinct from TNFSF members implicates a higher level of integration with other signaling pathways.

Viral Orthologs

TNFR-like proteins are found in the genomes of several viral pathogens representing captured cellular genes that have evolved as part of that pathogen's immune evasion strategy (Table 27.3). Poxviruses were the first pathogens identified harboring a version of a cellular TNFR.[43] Poxvirus TNFR displays significant sequence homology to TNFR2 and binds TNF and LTα. The rabbit poxvirus protein T2 is secreted by virally infected cells and contributes to the virulence of infection.[44,45] Smallpox virus, the former scourge of mankind, also harbored viral versions of TNFR2.[46]

SIGNALING PATHWAYS AND CELLULAR RESPONSES

Regulation

The cellular response activated by a TNF-related cytokine depends on several factors including the temporal patterns of expression of the ligands and receptors on the interacting cells, and the cellular context (the state of differentiation of the responding cell). Regulation is achieved at the level of transcriptional and translational controls, and by modulating the availability of the ligand or receptors (Fig. 27.4). For some ligands, transcriptional activation is a critical feature controlling the duration of mRNA expression. Some ligands exhibit inducible, transient expression of mRNA following signals from antigen receptors or innate receptor recognition systems. The half-life of mRNA

TABLE 27.3 Viral Orthologs and Modulators of the Tumor Necrosis Factor Superfamily

Virus	Name/ORF	Ortholog[a]	Mechanism
Poxvirus			
Myxoma	T2	Soluble TNFR2	TNF and LTα decoy
Variola	crm B (G2R)	Soluble TNFR2	TNF and LTα decoy
Cowpox	vCD30	Soluble CD30	CD30 ligand inhibitor
Herpesvirus			
HSV1&2	Glycoprotein D	BTLA	Entry; HVEM blockade
HCMV	UL144	HVEM	BTLA activation
EBV	LMP1	CD40 intracellular	TRAF activation
γHV	vFLIP	FLIP	Caspase 8 blockade
Adenovirus	E3-10.4, 14.5, 6.7	?	Fas and TRAILR downmodulation
Retrovirus			
EIAV	Envelope gp90	?	HVEM entry factor
FIV	Envelope gp95	?	Ox40 entry factor
ASLV	Envelope gp85	?	TRAILR entry factor
Rabies virus	Envelope RVG	?	NTRp75 entry factor

ASLV, avian sarcosis and leukosis virus; BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HSV, herpes simplex virus; γHV, equine gamma herpesvirus; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor; TRAIL, TMF-related apoptosis inducing ligand; ?, no homology recognized.
[a]Relationship determined by sequence or structural homology to the indicated TNF superfamily member.

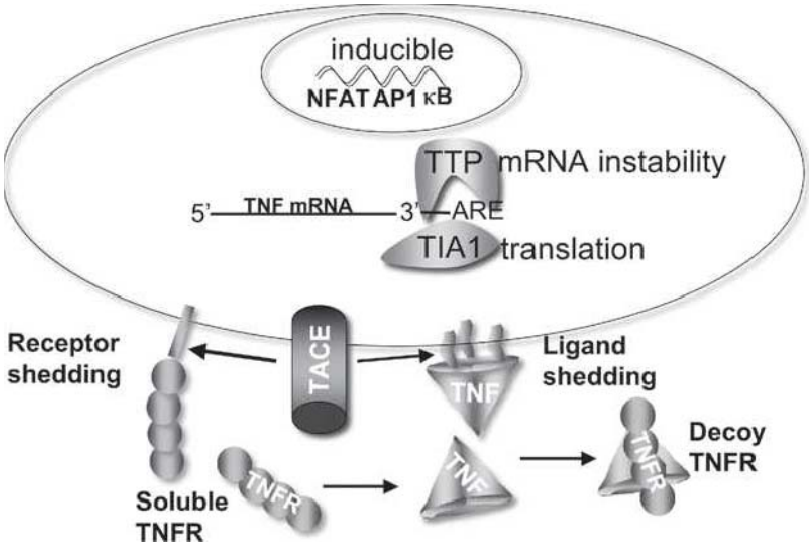

FIG. 27.4. Regulation of Tumor Necrosis Factor (TNF) Bioavailability. The expression of TNF is regulated at the transcriptional and translational levels, and bioavailability by altering its physical location and cellular receptors. TNF transcription is regulated by the action of multiple transcription factors including nuclear factors of activated T cells (NFAT), activated protein-1 (AP1), and NF-κB. NFAT is a predominant acting transcription factor regulating TNF transcription in T- and B-lymphocytes, and NF-κB and AP1 are important in myeloid lineage cells following activation via innate pattern recognition receptors, such as the toll-like receptors. TNF messenger ribonucleic acid (mRNA) stability is controlled by an adenylate-uridylate–rich element (ARE) in the 3′ untranslated region present in many transiently expressed inflammatory genes.[337] Stability of TNF mRNA is decreased by the action of tristetraprolin, and T-cell intracellular antigen silences translation of TNF mRNA through the ARE.[338] TACE proteolytically controls TNF at the membrane, generating the soluble form of TNF. TACE also cleaves TNFR1 and 2,[339] downregulating cell surface receptors and releasing soluble receptors that retain TNF binding activity. Soluble TNFR can stabilize the TNF trimer at sub saturating concentrations, and at higher, saturating concentrations act as decoys competing for TNF binding to cellular receptors.[340]

for TNF is short, controlled by an adenylate-uridylate–rich element in the 3′ untranslated region.[47] Deletion of the adenylate-uridylate–rich element in TNF mRNA in mice leads to a profound inflammatory disease.[47,48] TNF mRNA is inducible in macrophages by multiple pathways, particularly innate activation pathways such as toll-like receptor signaling, whereas other ligands like 41BBL and OX40L are constitutively expressed in differentiated antigen-presenting dendritic cells (DCs). T- and B-lymphocytes require activation prior to expression of TNF. In these cells, signals via the antigen receptor utilizing nuclear factor of activated T cells (NFAT) transcription factors are needed for the induction of TNF transcription. The inducible or constitutive patterns of expression are also observed with some receptors as well.

Posttranslational regulation of signaling is achieved by proteolytic cleavage of the ligand or receptor from the cell's surface, which places the protein into the soluble phase, where its half-life may be dramatically shortened. TNF and Fas ligand, for example, are shed from the surface by membrane proteases. ADAM17 (also known as TNFα-converting enzyme [TACE]), the enzyme that processes TNF into a soluble form, is involved in cleaving multiple cell surface proteins including transforming growth factor α, L-selectin, and TNFR1 and 2. Production of soluble TNFR1 and 2 may be important in regulating TNF bioavailability (see Fig. 27.4).

Most of the TNF-related ligands are expressed by DCs, activated lymphocytes, and myeloid cells, particularly macrophages, but can also be produced by nonlymphoid cells. TNF is an example of a ligand expressed by many cell types depending on the stimulus. Expression of TNFR is widespread. TNFR1 is expressed on most cells, while TNFR2 is limited to cells of hematopoietic origin and is expressed following activation of B or T cells. Macrophages are a primary source of TNF in response to toll-like receptor signaling, and T cells produce TNF when activated by antigenic stimuli. Fibroblasts also produce TNF in response to virus infection, and nonlymphoid tumor cells may ectopically express TNF. FasL is another example of a ligand with a varied cellular expression pattern. FasL is expressed by effector T cells and natural killer (NK) cells as a component of their cell lytic activity, yet FasL mRNA is also detected in reproductive organs and epithelium of the eye, which may use this TNFSF member to kill organ infiltrating inflammatory cells as a mechanism to dampen inflammation.

Signal Transduction Pathways

TNF receptors initiate signaling pathways that alter gene expression patterns, changing the differentiation status of a cell, as well as apoptotic pathways that terminate cellular life. The propagation of signals from receptor to enzymatically active proteins is mediated by two distinct types of signaling motifs in the cytosolic domain of the TNFR: DDs and TRAF-binding motifs. TNFR that contain a DD include Fas, TNFR1, DR3, and TRAILR1 and 2. Other TNFRs have TRAF recruitment motifs. Three basic schemes are used by TNF receptors to connect to enzymatically-driven signaling pathways (Fig. 27.5A). Adaptor molecules are required to establish the signaling connections between the receptor and signaling enzymes. The DD connects TNFR to cytosolic proteins containing a Death Effector Domain (DED), which in turn link to caspases (cysteine based, aspartic acid specific proteinases). Alternately, the TRAF proteins connect to the cytosolic domain of the TNFR, altering ubiquitin-dependent pathways that regulate key serine kinases that activate NFκB and AP1 transcription factors. The third scheme involves an indirect link between the death domain and TRAFs via the adaptor TNFR-associated death domain (TRADD).

The apoptotic and NF-κB pathways activated by the TNFR family help regulate cellular homeostasis by controlling cell death and survival. In the immune system, apoptosis is essential for homeostasis and for eliminating antigen-bearing cells from the host. Many TNFR can induce activation of survival or death pathways depending in part on the differentiated state of that cell. In all nucleated cells, apoptosis is the default pathway; that is to say, all of the constituents of the pathway are expressed and ready to be activated (Fig. 27.6), whereas cellular survival requires transcriptional control of genes that encode regulatory proteins that suppress the progression of the apoptotic pathway.

Apoptosis

Ligation of TNFR promotes assemble of the death-inducing signaling complex (DISC) that promotes dimerization of procaspase 8 forming an active enzyme complex.[49] Activated caspase 8 acts directly to cleave procaspase 3 and 7, which are known as the executioner caspases as they directly cleave critical cellular substrates leading to apoptotic death. Caspase 8 also cleaves BID, a crucial connector to the mitochondria-associated death mechanism, which greatly amplifies the apoptotic process.[50] A cell must be capable of actively transcribing and translating genes to resist apoptosis signaling. A variety of genes can inhibit apoptosis and cell death pathways. For example, the cellular inhibitor of apoptosis (XIAP) is a direct caspase inhibitor that is regulated at the level of gene expression by transcription factor NF-κB. Another regulator, FLIP (FLICE inhibitory protein), is also an NF-κB–regulated gene that contains a DED and a pseudocaspase domain that attenuates the apoptotic pathway by blocking conversion of procaspase 8 to the active form.[51] Many viral pathogens parasitize the transcriptional capabilities of the cell and prevent the cell from making new survival proteins, allowing apoptosis to proceed. As expected, viruses have evolved many different strategies to alter pro-apoptotic pathways (eg, viral orthologs of XIAP and FLIP prevent premature death of the cell) (see Fig. 27.6).

Cell Survival Signaling

Activation of transcription factors by TNFR utilizes the TRAF adaptors. There are seven members of the TRAF family (given numerical designations) that play key roles in regulating TNFR signaling and activation of host defenses. Each TRAF appears to play different roles in modulating signaling. Each TRAF protein contains a TRAF homology domain that binds TNFR and a RING and/or zinc finger domain characteristic of E3 ubiquitin ligases.[52–54] Ubiquitination plays an essential role in regulating signal transduction by TNFR[55,56] and in the pathogen recognition receptors as part of innate host defenses.[57]

Clustering of the TNFR promotes the recruitment of TRAFs into a complex with the receptor through two different mechanisms. TRAF2, 3, and 5 bind directly to the receptor's cytosolic domain recognizing a consensus PXQS/T motif,[58] whereas TNFR1 uses TRADD to couple to TRAF2. TRAF3 and TRAF6 are preassociated with different proteins, including protein kinases involved in multiple signaling pathways including the pathogen recognition and interferon (IFN) pathways.[53,59] TRAF adaptors link TNFR directly to protein kinase cascades, which in turn lead to activation of transcription factors including NF-κB and AP1.

The NF-κB family of transcription factors control expression of genes critical for cell survival, inflammatory, and immune responses. In mammalian cells, the NF-κB family consists of five members: RelA, RelB, c-Rel, p50/NFκB B1, and p52/NFκB B2 (proteolytic processing of p105 and p100 yields the active forms p50 and p52, respectively).[60,61] Activation of NF-κB releases inhibitors that restrict nuclear translocation.[62] Homo- and heterodimers of NF-κB family members are held inactive in the cytosol by inhibitors of κB (IκB), such as IκBα, that mask nuclear localization motifs on the NF-κB dimers. A complex consisting of the kinase catalytic subunits IKKα and IKKβ and the regulatory/scaffold subunit IKKγ form the IKK complex that mediates phosphorylation and ubiquitination of IκB, leading to its degradation and release of the active transcription factor. IκB is the common target of a variety of signals that control the activation of the RelA NF-κB transcription factor, which in turn regulates expression of many proinflammatory genes within a signal responsive cell.[63]

A distinct pathway regulates the activation of RelB NF-κB through the NF-κB–inducing kinase (NIK) and IKKα kinases. In unstimulated cells, NIK levels are maintained at vanishing low levels by ubiquitination and proteosome degradation through an E3 ligase complex comprised of TRAF3, TRAF2, and CIAP1/2.[52] Receptor ligation releases the active form of NIK by competitively displacing TRAF3, preventing further ubiquitination and allowing NIK to accumulate.[64] NIK phosphorylates IKKα, which induces the proteosome dependent processing of p100 to p52, degrading the inhibitory domain of p100 and allowing the RelB/p52 complex to activate gene transcription.

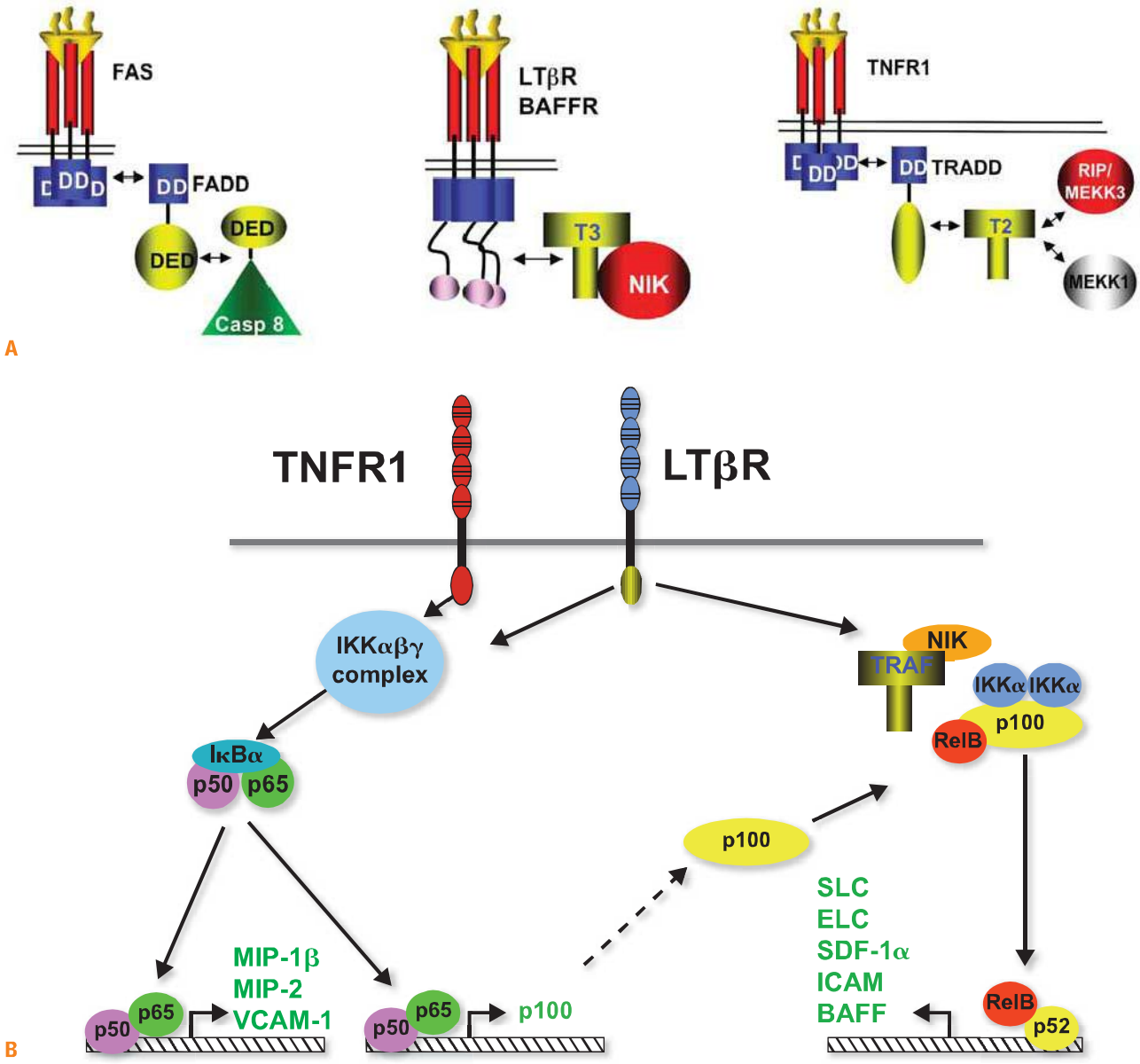

FIG. 27.5. Signaling Pathways and NF-κB. **A:** Adaptors link tumor necrosis factor receptors (TNFRs) to proteinases and kinases (*upper panel*). Three basic schemes link activated TNFRs to signaling pathways: the death inducing signaling complex formed with Fas is initiated by ligand clustering of Fas, promoting death domain (DD) interactions, which recruits Fas-associated death domain (FADD) (heterotypic interaction). The death effector domain of FADD links to the death effector domain of procaspase 8. The proximity of multiple procaspase 8 domains forms an active enzyme that can process other caspase 8 molecules. By contrast, TNFRs bind TNF-associated factor (TRAF) adaptors via short peptide motifs that release TRAF from associated kinases, such as NF-κB–inducing kinase (NIK). The third scheme is a combination of DD and TRAF recruitment. TNFR1 uses the DD protein TRADD to recruit RIP and TRAF2, promoting the activation of NF-κB. There are currently seven TRAF members with distinct interaction patterns with the TNFR family. TRAF proteins may function as regulators of key kinases. TRAF2, 3, and 6 function as modulators of several different kinases involved in toll-like receptor signaling, induction of type 1 interferon responses, and signaling by some TNFR family members. TRAF proteins contain an N-terminal RING finger motif, a coiled coil domain (isoleucine zipper), and the receptor association domain (TRAF) domain. The TRAF are trimers formed through their TRAF and coiled domains. The TRAF domain can bind to several different TNFR through a relatively short proline-anchored sequence that is responsible for binding directly to the mushroom head of various TRAF molecules.[341] The zinc RING of TRAF6 functions together with Ubc13 and Uev1A as ubiquitin E3 ligase targeting proteins for proteosome degradation. TRAF2, 3 and cIAP form the ubiquitin E3 ligase that targets NIK. Modified with permission from Ware CF. Tumor necrosis factors. In: Bertino JR, ed. *Encyclopedia of Cancer*. San Diego, CA: Academic Press, Inc.: 2002;475–489. **B:** NF-κB activation. TNFR1 and LTβR induce distinct forms of the NF-κB family of transcription factors. TNFR1 signaling is a potent activator of RelA/p50 but does not activate the RelB pathway, whereas lymphotoxin βR can activate both. The RelA and RelB forms of the κB family control transcription of distinct sets of genes; however, the two pathways are interrelated through the control of p100 expression by the RelA/p50 complex. Modified from Dejardin et al.[66]

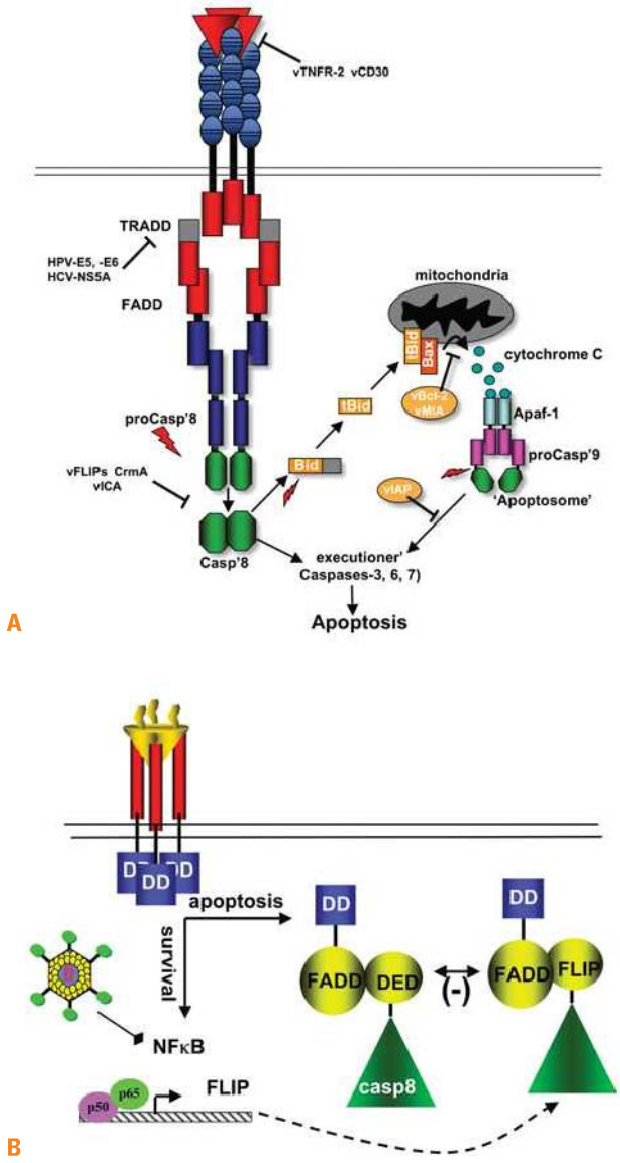

FIG. 27.6. Apoptosis and Survival Signaling. **A:** Ligation of tumor necrosis factor (TNF) receptor 1, Fas, or TNF-related apoptosis inducing ligand 1 and 2 activates apoptosis pathways. The nascent death-inducing signaling complex can directly cleave the executioner caspases 3, 6, and 7. Caspase 8 also cleaves BID to tBID, activating the mitochondrial regulated apoptotic pathway, dramatically accelerating cellular death. A number of viral proteins interfere with apoptosis induced through TNF pathway including human papilloma virus, hepatitis C virus, herpesvirus vFLIP, and virus orthologs of BCL2 and vMIA act on the mitochrondria. Adapted with permission.[111] **B:** The components of the apoptotic pathway are preformed in the cytosol enabling the cell to respond rapidly to death signaling. In contrast, the key regulators of the apoptotic pathway, including Bcl2 and inhibitor of apoptosis, require new gene expression controlled by NF-κB. An important NF-κB–induced gene is cellular FLIP, which contains a pseudocaspase domain. The cFLIP protein interferes with caspase 8 activation, blocking the prodeath pathway. If the cell is transcriptionally inactive or unable to activate NF-κB, which often occurs in a pathogen-infected cell, then the proapoptotic pathway dominates the signals emanating from the TNF receptor 1 signaling complex leading to apoptosis and curtailing parasitism of the cell.

As examples, TNFR1 and LTβR activate separate yet related pathways that lead to distinct forms of NF-κB, the RelA/p50, and RelB/p52 complexes.[65] Each form of NF-κB activates a large number of genes with distinct roles in physiology (see Fig. 27.5B). TNFR1 signaling rapidly mobilizes (within minutes) the RelA/p50 complex, which controls expression of many proinflammatory and survival genes. By contrast, the processing of p100 and accumulation of nuclear RelB/p52 takes several hours after the initial stimulus. RelB-dependent genes are often involved in lymphoid tissue organogenesis and homeostasis. NIK is also required for NF-κB RelB and RelA activation by CD27, CD40, and BAFF-R, but not by TNFRI, which is restricted to activating RelA/p50 complex.[66,67] Components of the NF-κB pathway, including TRAF3, cIAP, NIK, and A20, are frequently mutated in cancer, leading to constitutive expression of survival factors such as the BCL2 family.[68]

THE IMMEDIATE LYMPHOTOXIN AND TUMOR NECROSIS FACTOR FAMILY

TNF (formerly TNFα or TNFSF2) and lymphotoxin (LT)α (formerly TNFβ or TNFSF1) were originally pursued and characterized as inducers of tumor cell death, holding promise as antitumor therapeutics. However, the potent inflammatory action of TNF, particularly in the cardiovasculature, was quickly realized by the response of cancer patients injected with recombinant protein. We now recognize that TNF and LTα are two components of an interconnected network of "signaling circuits" that include LT-β, LIGHT (TNFSF14), and their specific receptors and regulatory proteins (Fig. 27.7). Each individual pathway has unique and cooperative signaling activities with other members of this immediate family. The immunologic processes controlled by this cytokine network are extensive, ranging from the development and homeostasis of lymphoid organs to the mobilization of innate defense systems to cosignaling activity promoting adaptive immune responses.[69–71]

TNF, LTα, LTβ, and LIGHT define the immediate group of TNF-related ligands that bind four cognate cell surface receptors with distinct but shared specificities. TNF and LTα both bind two distinct receptors, TNFR1 (p55-60, *TNFR1A*) and TNFR2 (p75-80, *TNFR1B*). The heteromeric LTαβ2 complex binds the LTβR, which also binds LIGHT (TNFSF14). LIGHT also engages the HVEM (TNFRSF14), which acts as a ligand for BTLA and CD160,[31] an Ig superfamily member. Two distinct human herpesviruses, herpes simplex virus and cytomegalovirus, target the HVEM-BTLA pathway using different mechanisms[35,72] (see Fig. 27.7).

TNF mediates a diverse range of cellular and physiologic responses linked to acute and chronic inflammatory processes. The diversity of responses is due in part to the broad expression of TNFR1 and the release of TNF as a soluble mediator where it can act in a systemic fashion. TNF production is triggered by many of the innate recognition systems, such as the toll-like receptors and by T and B cells. In chronic inflammation, TNF production can lead to tissue damage and organ failure. Genes expressed in response to TNF signaling

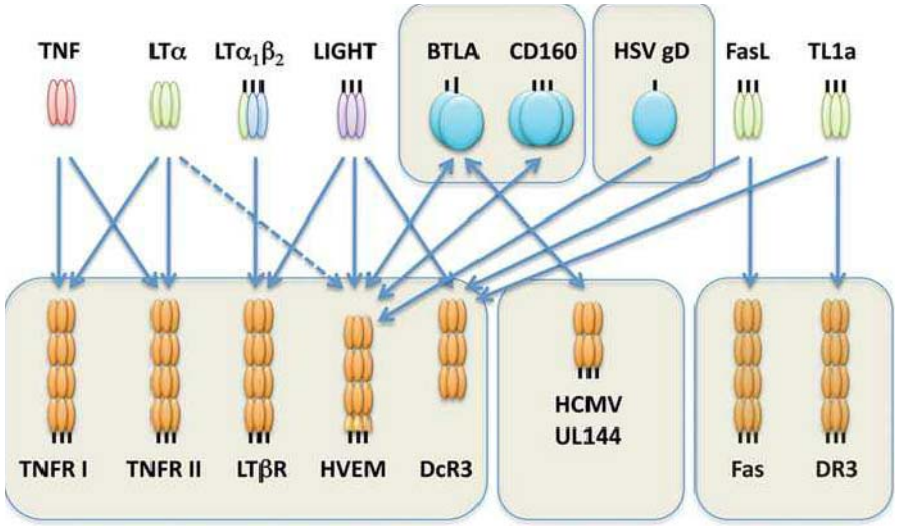

FIG. 27.7. The Immediate Tumor Necrosis Factor (TNF) Lymphotoxin (LT) Network. The cartoon depicts the signaling network formed between TNF, LTα, LTβ, and LIGHT, and their receptors. Each *arrow* indicates a ligand-receptor interaction. The network is defined by the extensive cross-utilization of ligand and receptors. Herpesvirus entry mediator (HVEM) forms a switch between positive cosignaling through LIGHT-HVEM interaction and inhibitory signaling through B- and T-lymphocyte attenuator (BTLA). LIGHT bound to HVEM activates TNF receptor–associated factor–dependent activation of NF-κB, whereas HVEM-BTLA acts through an immunoreceptor tyrosine-based inhibitory motif of BTLA to recruit the phosphatase SHP2, attenuating kinases activated by T-cell receptor signaling. The herpes simplex viron envelope protein gD attaches to HVEM acting as an entry step for infection. UL144 gene of human cytomegalovirus binds BTLA, but not LIGHT, selectively mimicking the inhibitory pathway of HVEM-BTLA. Fas Ligand and TL1A are included in this network through their interaction with decoy receptor-3.

coordinate the physiologic responses during inflammation (Table 27.4). The responses will reflect the characteristic of the inflamed organ, the local or systemic source of TNF, and the duration of TNF signaling. Acute inflammatory responses involve rapid changes in hemodynamics (plasma leakage and edema) and leukocyte adherence, extravasation, and organ infiltration induced by TNF. TNF production during chronic inflammation may contribute to systemic metabolic derangements and wasting (cachexia) or loss of organ structure and function (bone erosion in joints of patients with rheumatoid arthritis [RA]). In some models, TNF can promote an inflammatory environment that promotes tumor formation.[73–75]

Elucidation of the functions associated with this TNF/LT signaling network has been aided by studies with genetically modified mice engineered with null or transgene expression of the cytokine or receptor (Table 27.5). Deficiency in TNF or TNFR1, but not TNFR2, have similar phenotypes with alterations in host defense to intracellular bacterial pathogens, like *Listeria monocytogenes* and *Mycobacterium tuberculosis*, but surprisingly modest susceptibility to some viral pathogens. These results demonstrated a role for TNF in acute-phase response of the host defense system. In contrast, LTα-deficient mice showed a failure in formation of peripheral lymphoid organs, a phenotype not observed in mice deficient in either TNFR or TNF, which implicated the LTαβ complex signaling through the LTβR as a key developmental pathway for lymphoid organogenesis.

Lymphotoxinαβ-LymphotoxinβR, A Mammalian Organ Development Pathway

Gene-deficient mice sharing a common phenotype of no lymph nodes revealed the framework of a signaling pathway involved in mammalian organ development. LTα-, LTβ-, or LTβR-deficient mice fail to develop secondary lymphoid tissues.[76,77] Several other knockout mice, including the transcriptional regulators Ikaros, ID2, and RORγt, also lack lymph nodes,[78–82] as do mice deficient in components of the NF-κB

TABLE 27.4 Physiologic Correlates of Tumor Necrosis Factor–Mediated Gene Induction

Induced Gene	Response
iNOS	Vasodilation, edema
VCAM-1	Leukocyte margination and extravasation
IL-8	Leukocyte chemotaxis
MHC-1	Antigen presentation
Caspase 8 activation	Apoptosis
LPL	Cachexia

Caspase 8, cysteine-dependent aspartic acid specific proteinase-8; IL-8, interleukin-8 (CXC chemokine); iNOS, inducible nitric oxide synthetase; LPL, lipoprotein lipase; MHC-1, major histocompatibility complex-1; VCAM-1, vascular cell adhesion molecule-1.

TABLE 27.5 Phenotypes in Mice Deficient in Lymphotoxin and Tumor Necrosis Factor Immediate Family

	Phenotypes					
Gene Deletion	**LN[a]**	**PP[b]**	**Architecture[c]**	**NK[d]**	**NKT[e]**	**DC[f]**
LTα	–	–	Disrupted	Impaired	Impaired	CD8-DC
LTβ	–	–	Disrupted	Impaired	Impaired	CD8-DC
LIGHT	+	+	+	+	+	+
LTβ-B[g]	+	+	Disrupted	nr	nr	nr
LTβ-T[g]	+	+	+	nr	nr	nr
TNF[h]	+	+	+	+	+	Maturation
LTβR	–	–	Disrupted	–	–	CD8-DC
TNFR1	+	–	Disrupted MZ	+	+	Maturation
TNFR2	+	+	+	+	+	+

–, absent; +, normal; CD, cluster of differentiation; DC, dendritic cell; LN, lymph node; LT, lymphotoxin; MZ, marginal zone; NK, natural killer; NKT, natural killer T; nr, not reported; PP, Peyer's patches; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.
[a]LTβ–/– mice have ~75% of mesenteric LN; LIGHT/LTβ–/– mice have fewer mesenteric nodes than LTβ–/– mice.[76,342,343]
[b]See Rutschmann et al.[344] and Neumann et al.[345]
[c]Architecture of the splenic white pulp includes T- and B-zone segregation, MZ, germinal center and follicular DC network.
[d]NK-cell deficiency includes reduced cell numbers and enhanced tumor susceptibility.[348]
[e]NKT cells Vα14 subset.[347]
[f]CD8– DC subsets in spleen are diminished from failure to proliferate.[95]
[g]LTβ conditionally deleted in B cells or T cells.[348] LTβ-B cells showed partial disruption in architecture; normal for LTβ-T, but combined knockout in both B and T cells was worse than LTβ-B.
[h]Normal architecture observed in TNF point mutant[344]; abnormal architecture in TNFR1–/– mice.[345]
Modified with permission from Ware.[70]

activation pathway, including TRAF6, NIK, IKKα, and Rel B, and their gene targets. Target genes including CXCR5, the receptor for CXCL13, show defective lymphoid organ structure, as do CCR7–/– mice (receptor for CCL19/CCL21). The developmental program of secondary lymphoid organ formation initiates at 9 days post coitus progressing in organized fashion from dorsal to lateral movement, with intestinal Peyer's patches forming during the first postnatal week.[83] The defect is irreversible in that transferring LTαβ-sufficient bone marrow into an adult LT-mutant mouse failed to induce lymph node formation. Accumulating studies indicate that the formation of lymphoid organs involves two distinct cell types, an embryonic LTβR-expressing mesenchymal stromal cell that responds to LTαβ expressed in a cell of hematopoietically derived lineage, termed the lymphoid tissue inducer cell. The lymphoid tissue inducer cell develops separately from lymphocytes and myeloid cells in a pathway dependent on ID2 and RORγt and different cytokines including interleukin (IL)7, RANK ligand (TRANCE, TNFSF11), and TNF. These cytokines induce surface LTαβ in lymphoid tissue inducer cells that differentially engenders formation of lymph nodes and Peyer's patches.[84] Cells of the lymphoid tissue inducer lineage are maintained in the adult tissues at low levels (0.5%), presumably aiding in the homeostasis of lymphoid organs.[85,86]

The microarchitecture of the white pulp in the spleen[87] is disrupted in LT- and TNF-deficient mice.[88] Multiple abnormal features of the architecture are observed in LT- and TNF-deficient mice, including missing macrophages in the marginal sinus and the loss of positional segregation of T and B cells into discrete zones. The segregation of T and B cells into discrete compartments depends on expression of the tissue organizing chemokines CCL19 and CCL21, which attract T cells, and CXCL13, which attracts B cells. CCL19 and CCL21 act through the chemokine receptor CCR7 expressed on T cells, and CXCL13 binds CXCR5 on B cells to promote localization in the follicles. An LT-chemokine circuit is formed by migration of B cells to LTβR+ stromal cells expressing CXCL13, which in turn induces expression of LTαβ on B cells.[89–91] Circulating B cells lack surface LTαβ, but expression is regained upon reentry into the CXCL13 rich microenvironment.[91] The formation of the splenic microarchitecture depends on B-cell expression of LTαβ, which induces differentiation of specialized stromal cells (eg, secretion CCL20 and CXCL13 chemokines) in the spleen during postnatal maturation. Remodeling of the microarchitecture of the secondary lymphoid organs occurs during immune responses, which requires both TNF and LT pathways signaling on fibroblastic reticular cells.[92] A viral pathogen, cytomegalovirus, can induce specific changes in the splenic microenvironment through modulation of CCL21 expression.[93]

The LTβR and TNFR pathways facilitate lymphocyte entry into lymphoid tissues in part by modulating expression of adhesion molecules, such as peripheral node and mucosal addressins on high endothelial venules.[94] LTβR signaling is necessary to maintain networks of follicular DCs involved in capturing antigen and immune complexes that aid in activating B cells. These cellular interactions are further enhanced by LTβR signals that provide growth signals for some conventional myeloid DC (CD8– subsets) within the lymphoid organ, whereas TNF plays a role in differentiation of DC progenitors in bone marrow.[95]

T and B cells can communicate with stromal and myeloid cells via the LTαβ-LTβR pathway and thus modify their immediate microenvironment during the course of an immune response. Nonlymphoid tissues suffering from chronic inflammation associated with autoimmune disease, graft

rejection, or microbial infection often contain organized accumulations of lymphocytes reminiscent of secondary lymphoid organs, called tertiary lymphoid organs. Activated T and B cells provide the source of LTαβ that helps drive the process of forming these structures, but as antigen is cleared, immune responsiveness subsides and these structures resolve. A gradation of features may be found in the tertiary lymphoid organs including presence of DCs, expression of chemokines, high endothelial venules, segregated regions of T and B cells, and germinal centers, but these structures typically lack the permanence of a lymph node. TNF also contributes to formation of granuloma that assists in walling off bacteria.[96] Thus, the LTαβ and TNF pathways operative in embryonic life also play critical roles in the adult in the formation of tertiary lymphoid structures.

Influence of the Lymphotoxinαβ Pathway on Lymphocyte Development

Mounting evidence indicates the LTαβ-LTβR pathway contributes to the ontogeny of unconventional T cells, including γδ T cells and invariant NK T cells, whereas conventional T cell subsets are normal in mice deficient in the TNF and LTβR pathways. The LTβR pathway seems to operate at distinct levels during thymic development.[97] Double positive thymocytes regulate the differentiation of early thymocyte progenitors and γδ T cells via the LTβR pathway,[98] yet the LTβR is not expressed in thymocytes, suggesting an indirect mechanism. In this regard, LTβR signaling is required for the proper formation and function of the thymic stroma, which influences T-cell development.[99] The thymic medulla appears to control the export of invariant NK T cells from the thymus.[100,101] In addition, LTβR signaling in thymic stroma affects central tolerance to peripherally restricted antigens, which may be either dependent or independent upon the autoimmune regulator *Aire*.[102] Thymic differentiation depends on the LTβR pathway to mediate the cellular communication between lymphoid and stromal compartments.

Autoimmunity

Dysregulated expression of several members of the TNFSF leads to autoimmune-like diseases in humans and animal models. For example, enforced expression of TNF or LIGHT, which overrides the normal transient expression, causes severe autoimmune and inflammatory processes in mice.[103–105] LTα or LTαβ transgenic expression in the pancreas leads to insulitis and formation of tertiary lymphoid structures.[106] These types of results have implicated members of the TNF superfamily as immune regulators and support the notion that LTαβ and LIGHT pathways contribute to inflammation and tissue destructive processes.

Infectious Diseases

TNF is a major inflammatory cytokine required for the acute-phase response to bacterial infection. For instance, lipopolysaccharide in gram-negative bacteria is a potent inducer of TNF secretion through the TLR4 innate recognition system. In mice, lipopolysaccharide induces a shock syndrome that is rapidly lethal owing to profound changes in blood circulation. However, mice survive lipopolysaccharide in the genetic absence of TNFR1 or if treated with an TNF-neutralizing antibody, indicating that host-derived TNF mediates pathogenesis.[107–109] On the other hand, TNFR1 is essential for resistance to infection with a live organism, such as *Listeria monocytogenes*, through multiple processes including enhancement of phagocytosis and bacteriocidal destruction by macrophages. By contrast, T-cell immunity is not overtly impaired in TNFR1−/− mice. Humans treated with TNF inhibitors show some increase in susceptibility to selective pathogens, particularly *Mycobacterium tuberculosis*, reinforcing the role of TNF as a critical host defense system.[110]

In contrast, LT-deficient mice showed significant variability in susceptibility to individual pathogens (Table 27.6). Increased susceptibility resulted from either developmentally controlled aspects of lymphoid organ structure (eg, lymphocytic choriomeningitis virus, *Leishmania*) or a requirement for LTαβ-LTβR pathway as an effector system in innate and adaptive immune systems (eg, murine cytomegalovirus). Viewed from an evolutionary perspective, this variation in the requirement for LT signaling may reflect specific contributions from the pathogen used to evade the broader TNF- and LT-dependent pathways.[35,111]

TUMOR NECROSIS FACTOR SUPERFAMILY AND T-CELL COSIGNALING

Antigen recognition together with cooperating signaling "cosignaling" systems determine the quality of the adaptive immune responses. Lymphocyte responses to antigen are dynamic processes that start with the activation of naïve cells and transition through effector and memory phases. Cosignaling systems assist these phases by promoting more efficient engagement of antigen-binding TCR molecules to enhance initial cell activation and cell division (clonal expansion), augment cell survival (clonal contraction or memory cell differentiation), or induce effector functions such as cytokine secretion or killer function. Negative signals (inhibitory cosignaling) may also be delivered to T cells depending upon the particular system, which may prevent initial cellular activation or eliminate excess activated cells to dampen inflammation. Cosignaling can be quantitative, modifying thresholds of common signaling intermediates, or qualitative, involving signals distinct from other cosignaling systems or the TCR. Cosignaling receptors and ligands can be up- or downregulated at the transcriptional and protein levels depending on the stage of the T-cell response and the inflammatory milieu. In the absence of cosignaling, T cells may become unresponsive (anergic) or die.

The TNFRSR is one of two major families of cosignaling regulators that modulate T cells. The other cosignaling systems belong to the Ig superfamily, such as CD28,[112] cytotoxic T-lymphocyte (CTL)A-4,[113] ICOS,[114] PD1,[115] and BTLA.[116,117] TNFRSF members involved in T-cell cosignaling include OX40, 41BB, DR3, CD27, CD30, and HVEM,[118–122] whereas CD40 and BAFFR are more involved in cosignaling in B lymphocytes.[123,124] However, considerable crossover of activities in both lymphocyte populations can be demonstrated. Death

TABLE 27.6 Lymphotoxins in Host Defense: Mouse Models

Pathogen[a]	Mouse Model[b]	Susceptibility	Mechanism	Reference
Herpesvirus				
MHV68	LTα–/–	Minimal	nd	349
HSV-1	LTα–/–	Increased	Decreased effector CD8+ T cells	350
MCMV	LTα–/–;B-LTβ	Increased	IFN response; adaptive immunity lost	351–353
MCMV	LTβR–Fc Tg	Increased	Poor innate defenses	354
LCMV	LTβ–/–; LTα–/–	Increased	Defective architecture	354–356
LCMV	LTβR-Fc	Decreased	Decreased CD8+/IFNγ	357
Theiler virus	LTα–/– LTβR–Fc	Increased	Defective architecture	358
Influenza	LTα–/–	Minimal	nd	359
Vesicular stomatitis virus	LTβ–/–	Increased	Defective architecture	356
Bacteria and Parasites				
Mycobacterium tuberculosis	LTβR–/–	Increased	NO_2 synthase decreased	360
Mycobacterium tuberculosis	LTα–/–	Increased	No T cells in granuloma	361
Mycobacterium bovis	LTβR–Fc	Increased	Poor granuloma formation	362
Listeria monocytogenes	LTβR–/–	Increased	nd	360
Leishmania major	LTβ–/–	Increased	Defective architecture	363
Leishmania donovani	LIGHT–/–	Increased	Defective T cell	364
Toxoplasma gondii	LTα–/–	Increased	NO_2 synthase decreased	365
Plasmodium berghei	LTα–/–	Decreased	Decreased LTα dependent inflammation	366

CD, cluster of differentiation; HSV, herpes simplex virus; IFN, interferon; LCMV, lymphocytic choriomeningitis virus; LT, lymphotoxin; MCMV, murine cytomegalovirus; MHV murine herpesvirus; nd, not done.
[a]Virus: MHV68, HSV1 (α-herpesvirus), MCMV, LCMV. Bacteria: *Mycobacterium, Listeria monocytogenes.* Parasite: *Leishmania major, Leishmania donovani, Toxoplasma gondii, Plasmodium berghei.*
[b]Studies conducted in gene deficient mice (–/–); LTβR-Fc Tg, mice expressing LTβR-Fc as a transgene; LTβR-Fc, mice injected protein.
Modified with permission from Ware.[70]

receptors such as Fas and TNFR1 are thought to be involved in clonal contraction through apoptosis of activated effector cells, although TNF via TNFR2 also shows costimulatory action in naïve T cells. In tissue culture models, several of the TNF-related signaling pathways show costimulatory activities for T cells, which probably reflects the common induction of NF-κB–dependent survival genes, a common trait of TNFRSF members. However, analyses of physiologic models using genetically deficient mice reveal distinct roles for these molecules in the life cycle of T cells. Cosignaling systems are emerging as important targets to enhance immune responses to tumors or attenuate autoimmune diseases.[114,125–129]

Expression and Function of Tumor Necrosis Factor Receptor Superfamily Cosignaling Molecules During Primary T-Cell Responses

The TNFRs encoded on Chr 1p36 share a common function as cosignaling systems for T cells.[120,122] This region (human 1p36.33-1p36.21; syntenic with mouse 4E2-D3) contains the genes encoding GITR, OX40, HVEM, DR3, 41BB, CD30, and TNFR2, and is paralogous to the region on Chr 12p13 where genes for TNFR1, LTβR, and CD27 reside. The TNF-related ligands for these receptors are linked in the MHC paralogs on Chr 1, 6, 9, and 19, reflecting a common functional link as costimulatory molecules modulating T-cell differentiation (see Fig. 27.2).

Expression of TNFRSF members by T cells follows several distinct patterns during T-cell activation: expression on naïve T cells in peripheral lymphoid tissues (eg, CD27 and HVEM) that are upregulated or downregulated, respectively, following activation. OX40, 4-1BB, TNFR2, and CD30 are only expressed by T cells after initial activation[120]; TNFR2 is rapidly downmodulated following cleavage by TACE-generating soluble receptors that modulate TNF availability. The differing time course of expression of these molecules determines the phase of when they act in T-cell differentiation.

Experimental activation of TNFRSF members can be achieved with agonist monoclonal antibodies. Antibodies specific to OX40, 4-1BB, CD30, or CD27 all augment T-cell clonal expansion and TCR-induced cytokine expression in tissue culture and mouse models. For example, CD27, which is expressed at very early stages of T-cell activation, mediates initial cytokine production, cell division, and/or survival,[130,131] whereas OX40 and 4-1BB, which are expressed after cellular activation, promote T-cell survival and proliferation during the later stages of the primary response.[132–134] CD30-CD30L interactions promote primary CD8+ T-cell expansion and with OX40 act to promote CD4+ T-cell memory,[135,136] suggesting functional parallels with other TNFRSF molecules. Cowpox virus encodes a specific CD30 mimic to block the action of CD30L as part of its immune evasion strategy (see Table 27.6).

Death receptor-3 (DR3) engages TL1A, promoting IFNγ expression by proinflammatory TH1 cells in mouse[137] and humans,[138] especially in intestinal tissues.[139] Polymorphisms in TL1A are associated with inflammatory bowel disease.[140,141] The LIGHT-HVEM-LTβR pathway is also associated with T-cell inflammatory processes in mucosal tissues.[104,142] Similar cosignaling activities are reported for GITR,[143] with the potential of these systems to affect different subsets of T cells, such as GITR expression on T-regulatory subsets. The role of TNFRSF in regulating different T-cell subsets is an active area of research.

HVEM appears to play a role as an inhibitor of naïve T-cell activation through engagement of the Ig superfamily members BTLA and CD160.[144,145] In this situation, HVEM acts as a ligand for BTLA, which limits signaling by antigen receptor–associated tyrosine kinases through a phosphatase recruitment domain (immunoreceptor tyrosine-based inhibitory motif) in its cytosolic tail.[146,147] Naïve T cells coexpress HVEM and BTLA, forming an intrinsic complex that limits HVEM activation by its ligands expressed in the surrounding microenvironment. The BTLA binding site on HVEM is distinct from the position occupied by LIGHT, yet membrane LIGHT can disrupt the HVEM-BTLA complex, suggesting that HVEM acts as a regulatory switch between inhibitory and stimulatory cosignaling. Interestingly, like LIGHT, BTLA and CD160 can activate HVEM in trans, promoting cell survival gene expression in cells expressing HVEM.[148] This pathway appears particularly effective in controlling CD4 T-cell activation,[144,149] but it is also important in regulating differentiating memory CD8 T cells.[150]

T-Cell Memory

Cosignaling through several TNFRSF members promotes the differentiation of memory T cells. Mice deficient in OX40-OX40L, CD27-CD70, CD30-CD30L, or HVEM pathways all display defective memory responses to antigen.[131,135,151–154] The OX40 system is illustrative of the costimulatory signals provided to T cells by these TNFR. Pharmacologic stimulation of OX40 with an agonist antibody or ligand during a primary antigen-specific response in mice enhances the generation of memory CD4 T cells.[155] The enhanced response of T cells includes increased accumulation of antigen-specific effector T cells producing effector cytokines such as IFNγ at the site of inflammation.[156] Moreover, blockade of OX40-OX40L interactions during recall responses with monoclonal antibody specific to Ox40L ameliorates inflammation in models of collagen-induced arthritis[157] and experimental allergic lung inflammation.[158] In OX40-deficient mice, CD8 T cells expand normally, but their accumulation and survival at later times in the primary response was significantly impaired. T cells from OX40-deficient mice also failed to fully differentiate as measured by loss of expression of effector cell surface markers and decreases in synthesis of cytokines and cytotoxic activity. By contrast, the formation of extrafollicular plasma cells, germinal centers, and antibody responses was independent of OX40. OX40 signaling induces transcription of survival genes such as BCL2 and survivin in T cells, which may serve as the key targets that allow memory cell differentiation.[159,160]

The discovery that HVEM can elicit inhibitory signaling in T cells through engagement with the Ig family member BTLA highlights the possibility that, in certain situations, TNFRSF molecules can counter regulate the stimulatory actions during T-cell responses. Indeed, 4-1BB–deficient CD4+ T cells in some models are hyperresponsive,[161] and stimulation of 4-1BB can suppress immune responses in several models of autoimmune disease.[162–165] Although activation of 4-1BB enhances the expansion of human antiviral memory CD8+ T cells in vitro,[166] several studies demonstrate that the absence of 41BB in mice leads to enhanced T-cell responses,[161,167] reminiscent of the phenotype associated with HVEM-deficient mice.[168,169] Moreover, CD30 signals can, in some circumstances, suppress cytotoxic T-lymphocyte activity.[170] The expression of TNFRSF molecules such as GITR or OX40 on T-regulatory cells suggests that costimulatory pathways may enhance the suppressive function of these cells during immune responses.

Mouse models of autoimmune diseases such as EAE and diabetes uncover critical roles for OX40-Ox40L.[171,172] Blockade of the CD27-CD70 pathway also ameliorates EAE through the suppression of TNF induction, but not T-cell priming.[173] Furthermore, blockade of OX40, 4-1BB, CD27, or CD30 costimulation can all reduce transplant rejection and/or graft-versus-host disease.[174–177]

Stimulation of these pathways may also be applicable for the treatment of diseases such as cancer and infectious diseases where antigen-specific T-cell responses are ineffective. Stimulation of OX40, 4-1BB, CD30, and CD27 with agonist antibodies augments T-cell–mediated killing of a variety of tumors. The critical roles of the cosignaling TNF-TNFRSF members are discerned in immune responses to infectious pathogens. Similar to the immediate TNF family, the role of a specific cosignaling pathway in mediating protective T-cell responses during infection depends on the pathogen (Table 27.7).

CD40L AND BAFF SYSTEMS

The CD40 and BAFF systems play a major role in coordinating a range of costimulatory signals important to B-cell function, such as affinity maturation, isotype switching, Ig production, and clonal expansion.[123,178–181] In common with other TNFSF members, the CD40 system (see Fig. 27.3) is a key communication mechanism in host defense, autoimmunity, and cancer. Often, these pathogenic processes are centered on the function of B cells. CD40 ligand maps to the X chromosome and is regulated at the transcriptional level by NFAT, resulting in inducible CD40 ligand expression by T helper cells that promotes humoral immunity. CD40 ligand is also expressed in a variety of other cells including activated B cells, endothelial cells, basophils, mast cells, and platelets. CD40 is expressed on many cells, prominently on B cells, and utilizes both TRAF3 and 6 to activate NF-κB and AP1 transcription factors, among other signaling pathways.[182,183] Epstein-Barr virus, a B-cell–transforming herpesvirus, mimics the CD40 pathway though its LMP1 protein, driving naïve B cells into a memory state.[184–186]

TABLE 27.7 Tumor Necrosis Factor Superfamily Cosignaling Pathways in Infectious Diseases

	Cosignaling Pathway			
Pathogen	**CD27-CD27L**	**Ox40-Ox40L**	**4-1BB-41BBL**	**CD30-CD30L**
Influenza	CD4+ and CD8+[131,367]	CD4+[153,156,368]	CD8+[151]	nd
LCMV	nd	CD4+[156]	Normal CD8+[151]	nd
MCMV		CD4+[369,370]	CD8+[371]	CD8+[372]
Leishmania major	No role	CD4+ Th2[373]	No role	No role
Helminths	nd	CD4+ Th2[374]	nd	nd
Cryptococcus neoformans	nd	Enhanced CD4 Th2[375]	nd	nd
Mycobacterium avium	No role[376]	No role[376]	No role[376]	CD4+ and CD8+[376]
Listeria monocytogenes	CD4+ and CD8+[377]	nd	CD8+[378]	CD8+[379]

CD, cluster of differentiation; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; nd, not done.
Unless indicated, the function of the T cells was impaired.
Adapted from Croft.[120]

CD40 signaling is necessary for induction of activation-induced cytidine deaminase, a key enzyme in class switching and somatic mutation of Ig genes. This process occurs in B cells in contact with T helper cells, which express CD40 ligand. The importance of CD40 system in the activity of B cells was revealed in patients with hyper-IgM syndrome. Several mutated genes underlie this syndrome including CD40 ligand and CD40,[187,188] the RelA NF-κB activating kinase subunit, IKKγ (NEMO), and activation-induced cytidine deaminase, among others.[189] Patients with hyper-IgM syndrome with mutated CD40L have elevated levels of IgM, often with no serum IgG, IgE, or IgA, and display increased susceptibility to bacterial and opportunistic infections. Treatment of these patients with passive transfer of human immune IgG corrects infection by bacteria, but not opportunistic pathogens, such as *Pneumocystis carinii*. This latter result reflects the importance of CD40 system in T-cell–mediated macrophage activation, which controls these opportunistic pathogens. In some patients with hyper-IgM syndrome, significant amounts of IgA and IgE are present in serum, even in the complete absence of CD40L, a result indicating that another mechanism(s) can induce Ig class switching. Evidence has emerged that the BAFF/APRIL system can induce Ig class switch independent of CD40 system.[190]

Since the discovery of B-cell–activating factor of the TNFSF (BAFF, TNFSF13B; BLyS) and a proliferation-inducing ligand (APRIL, TNFSF13), much has been elucidated with regard to CD40L-independent isotype switching and B-cell development and survival in the periphery, as well as the implications of BAFF-APRIL dysregulation in autoimmune disease and lymphomas.[124,191] Although human *BAFF* maps to Chr 13q34 and APRIL to 17p13.1, the proteins are strikingly more conserved (~50% homology) than other TNF ligands. TWEAK (TNF-related ligand with weak apoptosis activity) maps adjacent to APRIL, part of a clade of genetically similar members that also includes ectodermal dysplasin (EDA1/EDA2). BAFF and APRIL form homo- and heterotrimers that interact with multiple receptors to form a complex signaling circuit (see Fig. 27.3). Membrane BAFF is shed via a furin protease at the cell surface, while APRIL is processed in the Golgi and secreted only in soluble form,[192] although the receptor binding domain of APRIL can be membrane-anchored via an unusual splice variant with the TWEAK cytosolic and transmembrane domain (TWE-PRIL).[193] The significance of membrane-restricted local BAFF expression to systemic availability of soluble BAFF and APRIL remains to be established, although BAFF-APRIL heterotrimers are detected in serum from patients with systemic rheumatic diseases.[7] BAFF and APRIL are inducibly expressed in monocytes, macrophages, DCs, and T cells in the spleen and lymph nodes in response to certain cytokines in different cell types, such as type I and II IFN, IL-10, and other TNF family members like CD40 and LTβR signaling. BAFF can be expressed in some B-cell–derived chronic lymphocytic leukemia cells, and some epithelial-derived cancer cell lines and primary tumor tissues express APRIL. Stromal cell expression of BAFF maintains the major mature B-cell pool, whereas inducible BAFF expression in myeloid cells aids in local B-cell survival at sites of inflammation.

Three cognate receptors mediate the biological actions of BAFF. BAFFR (TNFRSF13C), transmembrane activator and calcium signal modulating cyclophilin ligand interactor (TACI; TNFRSF13B), and B-cell maturation antigen (BCMA; TNFRSF17) are expressed on B cells and on other cell types. APRIL also engages matrix proteoglycans with weak binding, which may serve to enhance access to TACI and BCMA or function as a distinct receptor-ligand interaction.[194] Signaling through BAFFR and BCMA are required for B-cell survival and differentiation at different stages of B-cell–mediated humoral response. BAFFR−/− mice display an almost complete loss of mature and marginal zone B cells beginning from the late transitional phase of B-cell maturation, whereas BCMA deficiency impairs the survival of long-lived bone marrow plasma cells.[195,196]

By contrast, APRIL−/− mice develop normally but have altered class switching to IgA.[197,198] TACI signaling may antagonize BAFFR and BCMA, as TACI−/− mice have elevated numbers of mature B cells.[199] Both classical and alternative NF-κB pathways have been implicated BAFFR and BCMA survival signals, with BCL2 expression rescuing the B-cell defect in BAFFR−/− mice. BAFFR and TACI engagement

has also been shown to provide the necessary signals for CD40L-independent class switch recombination, to IgG and IgE for BAFFR, and IgG, IgE, and IgA for TACI, though IgA production seems specifically dependent upon APRIL-TACI interaction.[200] BAFFR is also connected to promoting the B-cell coreceptor complex CD19/CD21/CD81, which binds to C3b-opsonized antigens to enhance rapid T-independent antibody responses. Overall impairment of T-independent antibody responses appears to be greatest in TACI−/− mice, and is dependent upon APRIL-TACI interactions in B1 B cells. BAFFR, and not TACI, has also been proven to be important in T cell costimulation in in vitro and in vivo mouse models of allograft rejection response, where BAFFR−/− and BAFF−/−, but not TACI−/− nor BCMA−/−, mouse recipients prolong graft survival due to weakened alloproliferative response.[201]

Elevated levels of BAFF and APRIL have been detected in the serum, and especially the synovial fluid, of patients suffering from inflammatory autoimmune disorders such as systemic lupus erythematosus, RA, Sjörgen syndrome, and multiple sclerosis.[202] These levels tend to correlate with increasing amounts of autoreactive antibodies in serum, and in mouse models of lupus and collagen-induced arthritis, soluble TACI-Ig treatment can lessen the severity of disease symptoms and progression.[203] It is hypothesized that these excess quantities of BAFF and APRIL may contribute to the formation of autoreactive B-lymphocytes and loss of tolerance during development and perpetuating their survival as plasmablasts. Emerging evidence connects the BAFF and APRIL system to the pathology of B-cell–derived cancers like B-cell chronic lymphocytic leukemia, non-Hodgkin lymphoma, and multiple myeloma. Malignant myeloma cells express high levels of BAFF and APRIL and receive ligand-induced survival signals, with upregulation of antiapoptotic Bcl-2 and Mcl-1 molecules.[204] Therefore, BAFF and APRIL may bolster tumor survival via autocrine and paracrine signaling mechanisms.

TWEAK-FN14 SYSTEM

The TWEAK-Fn14 system is emerging as an important signaling system between the immune system and epithelial and stromal tissues.[205] TWEAK is limited to a monogamous interaction with Fn14, its cognate receptor, which is a fibroblast growth factor-2–inducible gene (see Fig. 27.3). Fn14 has one CRD and binds TWEAK homotrimers in a manner similar to BAFF, APRIL, and their receptors.[206,207] TWEAK, like BAFF and APRIL, is cleaved by furin protease to produce an active soluble form.[206] Human and mouse TWEAK share ≥ 90% sequence identity in the receptor binding domain and manifest high affinity cross-species interaction to respective Fn14 homologues.[192] The biologic functions of TWEAK-Fn14 system are broad and perhaps paradoxical, promoting both cell survival and death. TWEAK/Fn14 signaling induces apoptosis in some cancer lines (HT29, HSC3) and primary neurons.[208–210] TWEAK is produced primarily by leukocytes, whereas Fn14 expression is induced during inflammatory and tissue destructive processes. TWEAK-Fn14 system may provide important homeostatic functions to promote regenerative processes that conclude inflammatory responses. These homeostasis-promoting functions include induction of survival and growth in endothelial cells, migration and wound closure in endothelial cells, and angiogenesis.[206,207] Fn14 contributes to the activation of progenitor cell types including liver, skeletal muscle, and mesenchymal lineage progenitors such as osteoblast, chondrocyte and adipocyte, and neuronal progenitors.

TWEAK regulates the shift from innate to adaptive immune responsiveness by repressing innate inflammatory cytokines (IL-12, IFNγ) important in promoting TH1 immunity—a potential counterbalance to the proinflammatory actions of TNF.[208] TWEAK may also have a potentiating influence in chronic tissue inflammation when dysregulated, such as its role in excessive demyelination and ultimate progression to excessive autoimmune encephalomyelitis.[211] In a mouse collagen–induced arthritis model, elevated levels of TWEAK in serum correspond to severe disease progression and enhanced arthritogenic mediator molecules.[212] In obese patients with type 2 diabetes, TWEAK-Fn14 signaling augments proinflammatory cytokine release by adipocytes.[213] In rats, TWEAK may prevent excessive luteinization after gonadotrophin-induced ovulation by controlling progesterone production.[214] The seemingly contradictory functions of TWEAK/Fn14 are less puzzling when TWEAK/Fn14 is viewed as an important signaling system that aids in achieving homeostasis following inflammation, as cell death and survival are essential for homeostasis.

DEATH RECEPTORS AND LIGANDS

Fas Ligand and Fas System

The Fas (CD95, TNFRSF6)-Fas ligand (TNFSF6) system is an example of a direct signaling pathway to apoptotic cell death. The importance of the Fas-FasL system in immune regulation was revealed by the recognition that mice with the autoimmune-like disorders, *lymphoproliferative* (lpr) and *generalized lymphproliferative disorder* (gld) harbored mutations in Fas and Fas ligand and humans with the autoimmune lymphoproliferative syndrome have mutations in Fas.[215–217] Mice with *lpr* or *gld* mutations display autoimmune phenotypes with high accumulation of activated T cells in the periphery and CD4−CD8− T cells in the lymph nodes (lymphoadenopathy) and autoantibody production. The Fas-FasL system is involved in proinflammatory responses, tumor survival, and nonimmune tissue homeostasis in osteoclastogenesis and angiogenesis.[218] Activated lymphocytes are particularly sensitive to Fas-induced apoptosis, suggesting a role for Fas in the clonal contraction phase of the immune response. The epithelial cells in the eye express FasL, which may limit inflammation in organs sensitive to immune damage. FasL is thought to be one of several proapoptotic systems used as a killing mechanism by cytotoxic T cells and NK cells. In primates, Fas ligand binds Fas and decoy receptor-3, which also binds LIGHT and TL1A.[219]

FasL can undergo cleavage by matrix metalloproteases to produce soluble FasL, which may induce markedly different events when it engages Fas compared to the membrane-bound form, such as loss in cytotoxicity.[220]

Other findings suggest that soluble FasL may counteract the proinflammatory properties of membrane-associated FasL as an immunosuppressive, particularly in the eye and tumor environments.[221,222] Conversely, Fas has also been linked to lymphoproliferation and lymphocyte activation via NF-κB–signaling pathways.

Essential biologic roles FasL-Fas include regulating lymphocyte homeostasis (via contraction of clonally expanded effector T and NK cells), directing CTL-mediated apoptosis and lysis (immune surveillance), and establishing immune privileged organs and sites. Fas is expressed in a wide variety of tissues such as the thymus, spleen, heart, and liver, whereas FasL can be found on activated T cells, NK cells, tumor cells, immune privileged sites, lung, and other tissues.[218,223,224] In mature activated T and NK cells, Fas-FasL signaling plays a critical role in inducing apoptosis during the latter stages of inflammation, or activation-induced cell death, and shifts lymphocyte population numbers back to baseline.[225] In certain organs like the liver, lung, and small intestine, FasL on stromal cells can be upregulated to control the overabundance of activated T cells after an inflammatory response and ultimately reduce tissue damage. Alternately, dysregulation during hepatitis C infections can lead to death of hepatocytes and acute liver damage.[226] The Fas system has also been implicated in curtailing autoantibody production by damping T cell helper–induced B-cell activation and direct elimination of activated B cells, where CD40 stimulation also upregulates Fas expression.[218]

The Fas-FasL system, together with perforin and granzymes, is a mechanism vital to CTL-mediated destruction and clearance of tumor and virally infected cells. Viruses have evolved multiple mechanisms of suppressing Fas signaling,[111] including downmodulation of Fas[227] or blockade of caspase 8 activation.[228] Tumor genome instability may lead to mutations that aid in escaping or resisting death pathways, conducive to developing the metastatic phenotype.[229] Uterine and breast cancer patients may have elevated levels of soluble FasL and Fas, which may aid in neutralizing CTL- and NK cell–induced lysis or even killing Fas-expressing effector cells. Herpes simplex virus-1 infection in vitro of human neonatal neutrophils upregulates Fas and FasL surface expression, leading to apoptosis, exemplifying a theme of pathogenic modulation of apoptotic signaling events to evade or attenuate immune response.[230] FasL is constitutively expressed in the eye, central nervous system, testis, fetal trophoblast, and placenta, where any lymphocyte infiltration could lead to unrestricted, irreversible bystander damage to these tissues.[231] The implications of immune privilege have been applied to tissue transplantation rejection therapies; however, conflicting results as to whether FasL expression reduces likelihood of allograft rejection[232,233] or potentates effector responses[234,235] emphasize that induction of immune privilege by the Fas L system is complex. Fas-FasL interactions are at the core of graft-versus-host disease, where contaminating donor effector T cells in an allograft expand to damage the recipient's tissues. In a mouse model for acute graft-versus-host disease, p53-dependent upregulation of Fas on host stem cells leads to subsequent bone marrow depletion through FasL-mediated apoptosis.[236] With regard to tissue homeostasis, FasL has been reported to enhance RANKL-mediated osteoclastogenesis and differentiation in mouse bone marrow–derived macrophages.[237] Fas is also implicated in pulmonary inflammatory diseases resulting in fibrosis after acute lung injury.[223]

TRAIL RECEPTOR SYSTEM

TRAIL (TNFSF10) is closely related to FasL and TNF based on sequence homology. Interest in TRAIL was spurred by its ability to selectively induce apoptosis in tumor cells.[238] TRAIL binds five different receptors: TRAILR1 (DR4, TNFRSF10A), TRAILR2 (DR5, TNFRSF10B), TRAILR3 (DcR1, TNFRSF10C), TRAILR4 (DcR2, TNFRSF10D), and osteoprotegerin (OPG, OCIF, TNFRSF11B) (see Fig. 27.3). TRAILR1, 2, and 4 are type I transmembrane proteins and are membrane associated, whereas the more distantly related OPG is a dimeric, soluble decoy receptor. TRAILR3 is a type III transmembrane protein.[239] TRAILR1 and 2 contain highly homologous DD in their cytoplasmic tails that form the death-inducing signaling complex[240,241] responsible for apoptosis. TRAILR3 is anchored to the membrane via a glycophosphatidyl inositol tail, whereas TRAILR4 has a truncated, nonfunctional DD.[219,242,243] The lack of a functional DD indicates TRAILR3 and 4 antagonize TRAIL-induced apoptosis. Alternative splicing is a common feature amongst TRAIL and its receptors, and is believed to regulate programmed cell death.[244–246] The genetic organization of the TRAIL receptor loci in the mouse differs from that of the human. The mouse genome has TRAILR2, 3, and 4 homologues, but the TRAILR4 homologue does not bind TRAIL.

TRAIL-TRAILR signaling system induces apoptosis and to a lesser degree NF-κB activation. For example, the apoptotic function of TRAIL system is necessary for regulating the response of memory CD8+ T cells to rechallenge with antigen. In the absence of CD4 T-cell help, CD8 T cells undergo apoptosis mediated by TRAIL.[247,248] In much the same way as Fas, TRAILR death domains are thought to recruit FADD and initiator caspases 8 and/or 10 into the DISC to activate downstream effector caspases in apoptosis. Caspase 8 contribution to this process is well defined, but caspase 10 involvement was less so until recent studies in patients with autoimmune lymphoproliferative syndrome II and some carcinoma cell lines found that mutant caspase 10 ablates TRAIL-induced apoptosis.[242] In some cell types, the extrinsic mitochondrial pathway is important in amplifying DISC signals in TRAIL-induced apoptosis, which is analogous to mechanisms for Fas-FasL signaling.[249] TRAIL is being intensely pursued as an anticancer therapeutic and has proved particularly potent in combination with chemotherapy, because TRAILR2 expression can be increased in response to deoxyribonucleic acid damage.[250]

TRAIL seems most involved in regulating immune homeostasis and immune surveillance, or clearing virally infected and cancerous cells. IFNγ treatment of tumor cells increases sensitization to TRAIL-induced death. This has been observed in melanoma and ovarian carcinoma cells,[242] and is analogous to Fas-FasL apoptotic signaling in the human colorectal carcinoma line, HT-29. IFNα possesses

powerful antiviral and antitumor properties as well, and have been shown to upregulate TRAIL via JAK-STAT pathways in stimulated human multiple myeloma in vitro. TRAIL is also upregulated on NK cells in response to IFNγ, enhancing antitumor effector function.[251]

TL1A-DR3 SYSTEM

The TL1A-DR3 system provides essential signals that promote the expansion of effector T cells at the sites of inflammation.[139] However, the broad tissue expression profile of DR3 indicates its involvement is a range of cellular responses beyond T cells. TL1A (TNFSF15) is a paralog of FasL, LIGHT, and LTβ, and its receptor, DR3, is a homolog of TNFR1. TL1A also binds decoy receptor-3, potentially limiting any TL1A signaling mediated by DR3. Gene deletion experiments of TL1A or DR3 in mice revealed poor T-cell responses, but no overt developmental abnormalities.[137,252–254] Specific mutations in the ligand-binding domain of DR3 are linked to RA and interestingly appear to parallel other TNFRSF members and associated autoimmune pathologies.[255]

RANK LIGAND, RANK, AND OSTEOPROTEGERIN SYSTEM

OPG (TNFRSF11B) was initially identified as key mechanism regulating bone density[256] (see Fig. 27.3). As a soluble receptor, OPG is a regulator of the RANK (receptor activator of NF-κB, TNFRSF11A) and RANK ligand (also known as TRANCE) signaling pathway involved in the differentiation of bone-resorptive osteoclasts from hematopoietic progenitors (osteoclastogenesis).[257–259] However, this system is also crucial for development of mammary glands,[260] lymph nodes, and DC–T-cell interactions.[261,262] Bone remodeling needed to sustain skeletal integrity and calcium homeostasis is regulated by a dynamic equilibrium between osteoblasts (bone forming) and osteoclasts (bone resorbing), which is regulated by RANKL-RANK-OPG system. Dysregulation of the RANKL-RANK-OPG system has consequences in inflammatory, osteologic, and cancer pathophysiology, such as bone loss in inflammatory autoimmune diseases, preferential breast and prostate tumor metastasis to the bone, and gender bias in osteoporosis.

OPG functions as a decoy receptor that binds RANKL with high affinity, and also binds TRAIL, albeit at low affinity in comparison to RANKL.[263,264] Despite such a weak binding, in vitro studies show that OPG can block TRAIL-mediated apoptosis, which suggests the OPG-TRAIL system contributes to osteoclastogenesis. RANK interacts with TRAF2, 5, and 6. The membrane-proximal TRAF site is highly specific for TRAF6, and several studies have shown TRAF6 to be essential in RANKL-RANK signaling in osteoclastogenesis and lymph node genesis.[265,266] Several "osteotropic" factors have been reported to regulate RANKL and OPG expression in osteoblast and osteoclast lineages, such as transforming growth factor β, IL-1, TNF, estrogen, prostaglandin E2, glucocorticoids, and vitamin D3.[265,267] In a few cases, OPG and RANKL are differentially modulated by the same factor; for example, parathyroid-related protein increases RANKL while decreasing OPG mRNA expression in osteoclast-like odontoclasts.[268] The components in the RANK signaling triad are widely expressed. RANK is upregulated on CD40L-stimulated maturing DCs, while RANKL is upregulated during T-cell activation and is constitutively expressed in some tumors and mammary gland epithelial cells.

RANK−/− and TRAF6−/− mice lack NF-κB activation in osteoclasts and exhibit abnormally high bone density (osteopetrosis).[269,270] Osteoblasts and stromal cells stimulate differentiation via direct cell contact with osteoclast precursors. Macrophage colony stimulating factor cooperates with RANKL-expressing osteoblasts to transmit positive signals through c-Fms and RANK expressed on precursors promoting their differentiation,[259] whereas OPG negatively regulates by competitively binding RANKL, thus ablating all signals through RANK. RANK−/− and RANKL−/− mice phenocopy each other, displaying severe osteopetrosis due to the lack of osteoclasts.[271] Complementing the observations in RANKL and RANK deficiencies, OPG blocks osteoclast differentiation in a dose-dependent manner. OPG overexpression in transgenic mice causes acute osteopetrosis, while OPG-deficient mice suffer from osteoporosis due to excessive osteoclastogenesis. In humans, OPG and RANK mutations are linked to juvenile Paget disease, familial expansile osteolysis, and other osteolytic disorders.[258,265]

The RANKL-RANK system is proving to be an important enhancer of cell-mediated immune responses by promoting DC survival and naïve T-cell proliferation. RANKL stimulates DCs to produce proinflammatory cytokines like IL-12.[272,273] The RANK pathway may serve to bolster CD40-CD40L interaction between DC and T cells after T-cell receptor stimulation, or prolong survival of activated DCs to ensure the establishment of T-cell memory. For instance, in CD40L−/− mice, RANKL-RANK costimulation is protective against *Leishmania* infection by inducing IL-12 secretion and consequent T_H1 immune response.[274]

Bone loss in autoimmune and infectious models of inflammatory disease is mediated through slightly different mechanisms. In recent studies, activated, CD4+RANKL+ T cells have been shown to support osteoclastogenesis and mediate bone loss in humanized mouse models of *Actinobacillus*-specific periodontal disease, suggesting a role for T_H1 immunity in inflammatory bone destruction.[275,276] In patients with human immunodeficiency virus, the viron envelop gp120 induces disease-related osteoporosis via induction of RANKL on CD4+ T cells and augmentation of osteoclastogenesis.[277]

In mouse models of tumor metastasis, high OPG and RANK levels are risk factors for metastasis to the bone and subsequent osteolysis, especially in breast tumors.[278] OPG from human bone marrow stroma can protect prostate tumors from TRAIL-mediated apoptosis,[279] and treatment with anti-TRAIL neutralizing antibodies reduces osteoclastogenesis in mixed lymphocyte-bone marrow cultures from patients with multiple myeloma, presumably by interfering with OPG-TRAIL complexes formed by activated T cells expressing TRAIL, OPG, and RANKL.[280] The

TABLE 27.8 Human Genetic Diseases Associated with the Tumor Necrosis Factor Superfamily

System	Disease	Mutation
TNFR1	Familial periodic fever	Mutation in first cysteine-rich domain
CD40 Ligand	Hyper-IgM syndrome	Multiple mutations affecting receptor binding and processing
EDA	X-linked hypohidrotic ectodermal dysplasia	Multiple mutations affecting receptor binding, trimerization, and secretion
RANK	Familial expansile osteolysis	Mutation in the signal peptide
Fas	Autoimmune lymphoproliferative syndrome	Multiple mutations/ deletion in Fas

CD, cluster of differentiation; EDA, ectodermal dysplasin A; Ig, immunoglobulin; TNFR, tumor necrosis factor receptor.

RANKL-RANK-OPG system has also been implicated in diabetes-associated osteopenia, as serum levels of OPG are higher in patients with type I and II diabetes.[281] Interestingly, these patients are at greater risk for developing atherosclerotic plaques, which is perhaps reflected in the phenotype of OPG-deficient mice. These mice suffer from arterial calcification,[282] presumably from unrestricted bone resorption and calcium release. Although elevated endogenous OPG levels are considered a risk factor in certain disease manifestations, the complexity of the cytokine networks that regulate RANKL-RANK-OPG interactions and vice versa render the clinical data difficult to interpret.

TUMOR NECROSIS FACTOR SUPERFAMILY IN THE THERAPY OF HUMAN DISEASE

Tumor Necrosis Factor Inhibitors

The TNFSF is associated with a wide range of human diseases, evidenced by genetic deficiencies or mutations in individual components of the TNFSF and in the therapeutic efficacy of TNF inhibitors in autoimmune diseases (Table 27.8). Although cancer therapy was the original motivation in developing TNF, the success of TNF inhibitors in limiting symptoms in several autoimmune diseases provides significant motivation to explore therapeutics targeting other members of the TNFSF, currently an active area of research. Research into the function of TNFSF in human diseases is providing strong rationale for testing both inhibitors and agonists of these cytokines (Table 27.9).

TNF inhibitors are specific antibodies or soluble receptors and are classified as biologics (protein-based drugs). These biologic-based drugs provide specificity and desirable pharmacodynamic properties (Fig. 27.8). Both antibody- and receptor-based drugs function as competitive antagonists, sterically hindering the binding of ligand to receptor. The approved antibody-based drugs include a partially humanized mouse monoclonal antibody to human TNF (infliximab), two fully human antibodies (adalimumab and golimumab), and a monovalent Fab fragment of a TNF antibody (certolizumab pegol). Certolizumab is derivatized with polyethylene glycol to enhance half-life. To create a soluble "decoy" receptor, the extracellular domain of TNFR-2 was genetically linked to the Fc region of human IgG1, forming a bivalent molecule (etanercept) that has higher avidity for TNF than naturally occurring soluble receptors. Etanercept binds both TNF and LTα, whereas anti-TNF antibodies are specific for TNF and do not cross-react with LTα.

TABLE 27.9 Tumor Necrosis Factor Superfamily Therapeutics

TNFRSF Member	Drug	Therapeutic Designation	Indication	Status
TNF	Chimeric Ab	Remicade (infliximab)	RA, Ps, IBD	Approved
TNF LTα	TNFR-Fc	Enbrel (etanercept)	RA, Ps	Approved
TNF	Hu Fab-PEG	Cimzia (certolizumab)	RA, Ps, IBD	Approved
TNF	Hu mAb	Humira (adalimumab)	RA, Ps, IBD	Approved
TNF	Hu mAb	Simponi (golimumab)	RA, Ps, IBD	Approved
BAFF	Hu mAb	Benlysta (belimumab)	Lupus	Approved
CD40 L	chimeric mAb	ruplizumab	Lupus	Stopped
"	Hu Fab-PEG	CDP7657	Lupus	In trials
RANK L	Hu mAb	Prolia (denosumab)	Osteoporosis	Approved
"	Hu mAb	Xgeva (denosumab)	Bone metastasis	Approved
TRAIL	Recom cytokine		Cancer	In trials
TRAILR1	Hu mAb	mapatumumab	Cancer	In trials
TRAILR2	Hu mAb	conatumumab	Cancer	In trials
LTβR	LTβR-Fc	baminercept	Autoimmune diseases	In trials
LTα	Hu mAb	MLTA3698A	Autoimmune disease	In trials
OX40	chimeric mAb	9B12	Cancer	In trials
TWEAK	chimeric mAb	BIIB 023	Lupus nephritis	In trials

Fab-PEG, fragment of antibody polyethyleneglycoylated; IBD, inflammatory bowel diseases; LT, lymphotoxin; Ps, psoriasis; RA, rheumatoid arthritis; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TNFRSF, tumor necrosis factor receptor superfamily.

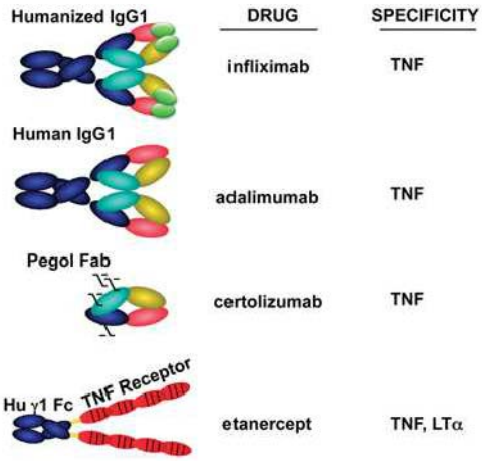

FIG. 27.8. Tumor Necrosis Factor (TNF) Inhibitors. The diagram depicts the structural features of the antibody- and receptor-based drugs targeted at TNF that are approved for use in patients with autoimmune diseases. Monoclonal antibody specific for human TNF derived from mice and partially humanized (infliximab), a fully human antibody (adalimumab) containing both heavy and light immunoglobulin chains, and an antibody-binding fragment (Fab) of a human anti-TNF antibody (certolizumab). Certolizumab is monovalent and derivatized with polyethyleneglycol to extend half-life in vivo. TNF decoy receptor (etanercept) created as a genetic fusion protein of the ectodomain of TNF receptor 2 with the Fc region of human immunoglobulin G1 forms a disulfide-linked dimer. Both immunoglobulin and receptor-based inhibitors are approved for use in treating autoimmune diseases: rheumatoid arthritis, psoriasis, and inflammatory bowel diseases.

Bivalent antibodies directed to TNFRs can function as agonists, mimicking the ligand but specific for a single receptor, a feature that may distinguish an antibody from ligand such as TRAIL, which has multiple receptors. Moreover, antibodies are inherently more stable than the native ligand, providing better pharmacodynamics (serum half-life and bioavailability). Agonist receptor antibodies (eg, anti-TRAILR, anti-Ox40) are in preclinical studies and clinical trials.

Impact of Tumor Necrosis Factor Blockade in Inflammatory Diseases

TNF inhibitors are approved for use in the treatment of RA, psoriatic arthritis, ankylosing spondylitis, juvenile rheumatoid arthritis, Crohn disease, and ulcerative colitis. RA is a chronic autoimmune joint disease that occurs in genetically predisposed individuals. Treatment of RA is usually initiated with disease-modifying antirheumatic drugs such as methotrexate or corticosteroids to improve symptoms and reduce joint damage. The American College of Rheumatology uses clinical and laboratory measures to assess improvement in response to therapy in RA. A patient with a 70% or greater improvement according to these parameters is designated as having an ACR-70 response. TNF inhibitors in combination with methotrexate (the most widely used disease-modifying antirheumatic drugs) produce ACR-70 responses in 33% to 40% of patients with early RA and in 10% to 27% in established RA. In comparison to patients receiving methotrexate alone, the ACR-70 responses occurred in 19% to 21% of early RA patients and fewer than 5% of patients with established disease.[283] In RA, joint damage begins early in the disease course as articular erosions when visualized by radiographic techniques in 40% of patients in the first year of disease and 90% by the second year.[284] TNF inhibitors slow radiographic progression in RA.[285] Interestingly, in a randomized controlled trial of 428 patients with active RA, the combination of infliximab and methotrexate reduced the radiographic progression.[286]

The inflammatory bowel diseases, ulcerative colitis (limited to colon and bowel), and Crohn disease (entire intestine) are chronic inflammatory conditions where TNF is disproportionately expressed when the disease is active. Monocytes from patients with inflammatory bowel disease produce significantly higher levels of TNF after stimulation by lipopolysaccharide.[287] The formation of fistulating inflammatory granulomas is a pathologic feature of Crohn disease. Clinical trials demonstrated an initial response in 59% to 69% of patients, and a dose-dependent clinical remission that ranged between 28% to 38% in nonfistulating Crohn disease and 64% to 97% of patients with complete fistula disease, when categorized by location.[288,289] In ulcerative colitis, controlled trials (800 patients with active ulcerative colitis) showed an initial response in 8 weeks with 65% response in patients treated with anti-TNF (infliximab) versus 35% in the placebo group, with remission achieved in 35% and 15% in patients treated with infliximab and placebo, respectively.[290] Anti-TNF (adalimumab) was efficacious in controlled trials in patients with moderate to severe Crohn disease.[288] By contrast, soluble TNFR2-Fc (etanercept) at doses known to be effective in RA failed to produce benefit in a controlled trial for Crohn disease.[291]

Erythrosquamous lesions containing infiltrating leukocytes and epidermal hypertrophy characterize psoriasis, a chronic inflammatory skin disease. Skin lesions of psoriatic patients contain elevated levels of TNF, and following treatment with TNF inhibitors, serum and skin levels of TNF decrease with remarkable clinical response.[292,293] A significant subset of patients with psoriasis develops inflammatory, erosive arthritis.[294] In clinical trials, both etanercept and infliximab significantly inhibited disease activity and improved the quality of life in patients with psoriatic arthritis.[295–299]

Serious Adverse Effects

TNF inhibitors are inherently safe because of their specificity. However, side effects occur with the use of TNF inhibitors and include lupus-like syndrome, aplastic anemia, hepatotoxicity, interstitial lung disease, optic neuritis, and exacerbations of quiescent multiple sclerosis. TNF inhibition is associated with diminished host defense, perhaps expected based on the role of TNF in innate and adaptive immune responses. The rate of serious skin and soft-tissue infections is higher in treated patients, and there is an increase risk of intracellular bacterial infections, primary tuberculosis and reactivation of latent

Mycobacterium tuberculosis among patients treated with TNF inhibitors.[300,301] Risk due to infection can be minimized and controlled (eg, a tuberculosis skin test prior to treatment in patients that have not been previously vaccinated for *Mycobacterium*). The contribution of anti-TNF therapy to the increase the risk of cancer is still unclear. A systematic review of randomized clinical trials using infliximab and adalimumab found a dose-related increase of malignancies in patients with RA patients.[302] Some of the increase risk of malignancy (ie, lymphoma) is difficult to dissect due to the increased association of lymphoma with RA.[301] TNF has been implicated in the pathogenesis of cardiac dysfunction, in part due to the negative ionotropic activities in vitro and in vivo; however, TNF blockade significantly increased the incidence of cardiac death. This observation led to the recommendation that TNF inhibitors be discontinued in patients with cardiac dysfunction.[303–305] Limiting adverse effects have been observed with biologics targeting other TNFSF members. Although interference with CD40L ameliorated autoimmune disease and allograft rejection in animal models,[306–310] anti-CD40L had an unacceptable impact on platelet function in transplantation and autoimmune diseases. The use of monovalent Fab of anti-CD40L may avoid these problems.[311–313] Another TNFRSF member, OPG, was dropped out of clinical investigation because the recombinant protein induced antibody responses that limited its therapeutic effects. Thus, dose- or mechanism-limiting toxicities can impact physiology in unanticipated ways, limiting successful clinical outcomes.

Anti-RANK Ligand

The central function of RANK-RANKL system in osteoclastogenesis has led to the development of anti-RANKL antibodies to treat bone-resorbing conditions. Human anti-RANKL monoclonal antibody (denosumab) to disrupt RANKL-RANK signaling has been approved for the treatment of osteoporosis in postmenopausal women and for treatment of skeletal changes from metastasis of solid tumors. Anti-RANKL therapy reduces osteoclastic bone resorption, thereby increasing bone mineral density at both the lumbar spine and the hip. Treatment with anti-RANKL for 36 months decreased the risk of vertebral, nonvertebral, and hip fractures.[314] There is no apparent increased risk of cancer or serious infection associated with anti-RANKL therapy in subjects with osteoporosis, although longer study periods are needed.

Treatment of patients with prostate cancer with androgen deprivation therapy and patients with breast cancer with aromatase inhibitors leads to increased bone loss and increased risk of fracture. Anti-RANKL is prescribed to increase bone mass both of these patient populations.[315,316] In a double-blind phase III clinical trial, women with nonmetastatic breast cancer who received anti-RANKL therapy had an increased lumbar spine bone mineral density of 7.6% after 24 months of treatment compared to placebo.[316] Similarly, patients with prostate cancer on androgen deprivation therapy that were treated with anti-RANKL in a phase III clinical trial had increased bone mineral density at all measured sites (hip, femoral neck, radius, and whole body) compared to placebo.[315] Anti-RANKL reduced the overall risk of bone fracture in the prostate cancer trial, while the phase III trial of patients with breast cancer treated with aromatase inhibitors was not designed to evaluate risk of fracture.[315,316]

In addition to patients with prostate and patients with breast cancer undergoing hormone deprivation therapy, anti-RANKL aids in prevention of skeletal-related events in patients with bone metastasis from solid tumors.[317–319] Three independent phase III clinical trials compared anti-RANKL with zoledronic acid, a drug prescribed to alleviate bone pain and reduce fracture risk in cancer patients with bone metastasis.[317–319] Anti-RANKL therapy delayed the time to the first skeletal changes in patients with metastatic breast cancer and metastatic, castration-resistant prostate cancer.[317,319] In a third phase III trial of patients with solid tumors (excluding breast and prostate) or multiple myeloma, anti-RANKL was equivalent to zoledronic acid in reducing fracture risk.[318]

Anti-BAFF

Systemic lupus erythematous is characterized by hyperreactive B cells, production of antideoxyribonucleic acid antibodies, and overproduction of BAFF.[320,321] The anti-BAFF antibody (belimumab) was approved in 2011 for the treatment of systemic lupus erythematosus, becoming the first new drug to gain approval for the condition in 56 years. In a phase III clinical trial, anti-BAFF decreased the rate of disease flares and increased the time between disease flares compared with placebo.[322] Anti-BAFF treatment increased systemic lupus erythematosus responder index (a reduction of SELENA-SLEDAI score of four or more points) compared to placebo.[322]

Tumor Necrosis Factor Superfamily in Cancer Therapy

Tumor angiogenesis, the formation of vessels to support cancer growth, is a process essential for tumor growth. The ability of TNF to induce apoptosis of tumor-associated endothelial cells can result in the impairment of the tumor vasculature leading to necrosis of the tumor. This mechanism involves perturbation of cell-cell adhesive junctions and inhibition of $\alpha v\beta 3$-integrin signaling in tumor-associated vessels.[323] However, clinical phase I and II studies in cancer patients receiving recombinant TNF reported "septic shock–like syndrome," a dose-limiting toxicity in quantities 10 times lower than the calculated antitumor dose extrapolated from animal studies.[324,325] Animal studies also revealed the strong hemodynamic effects of TNF in the mediation of septic shock.[326] Although there are anecdotic reports of tumor regression following TNF administration,[327] due to the induction of vasoplegia, TNF use as an antitumor agent has been limited to treatment of locally advanced tumors by isolated limb perfusion, which limits systemic toxicity of TNF.[328]

A number of studies are in progress to determine whether TRAIL and its death receptors can be used in treatment of

cancer.[329] In phase 1/2 studies, TRAIL and agonist mAb to TRAILR1 or R2 showed no dose-limiting toxicities.[330–333]

Early clinical trials are in progress for other members of the TNFSF. For example, antibodies to LTα and LTβR-Fc decoy are in clinical trials for suppressing inflammation in autoimmune diseases. Agonist antibodies to OX40 appear promising for induction of tumor immunity.[334] Redirecting T cells using a retroviral-based vector incorporating 41BB can eliminate leukemic cells.[335] It is not surprising that several other TNFSF systems are being targeted to modify inflammatory, infectious, and malignant diseases.

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