

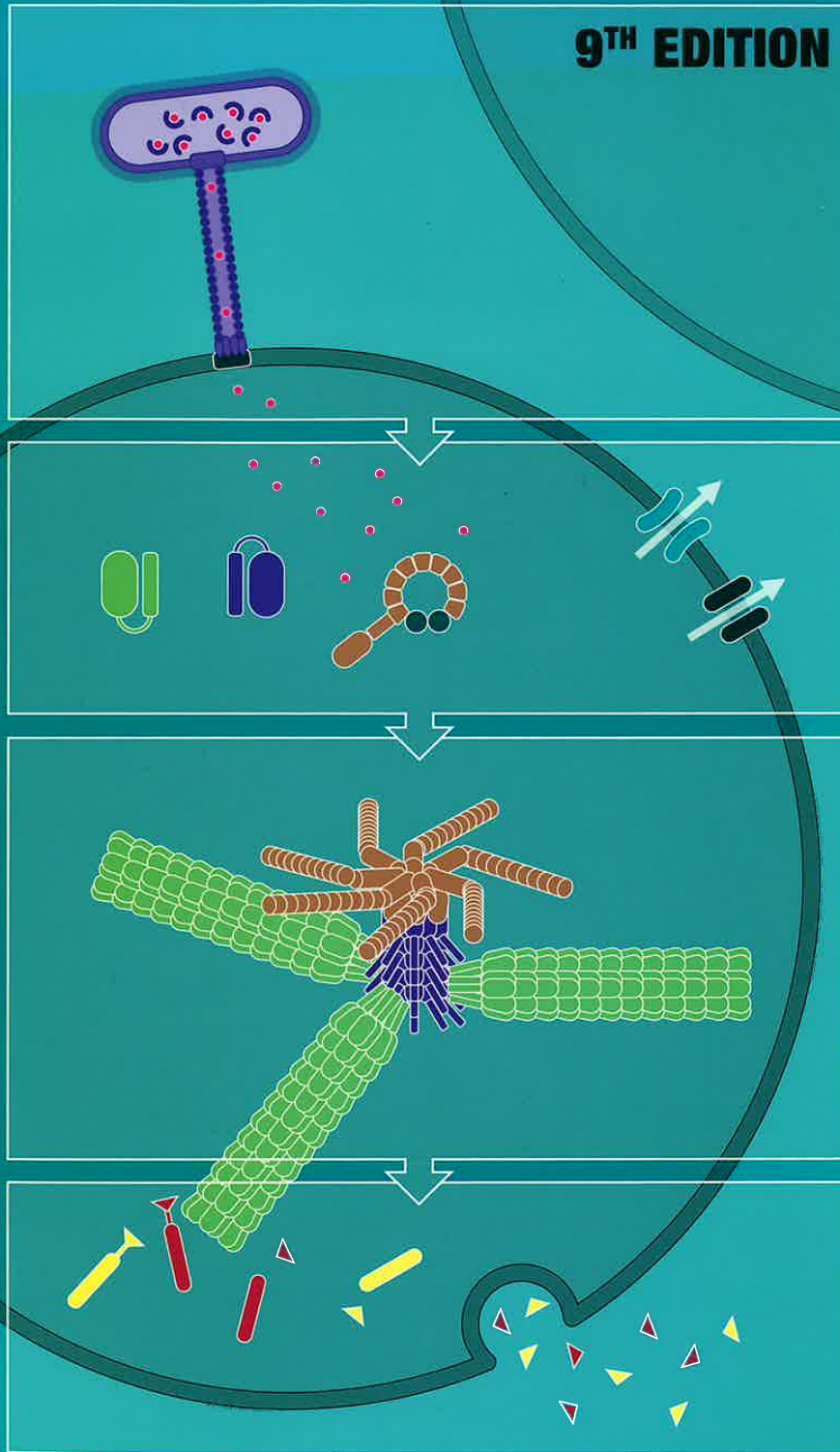
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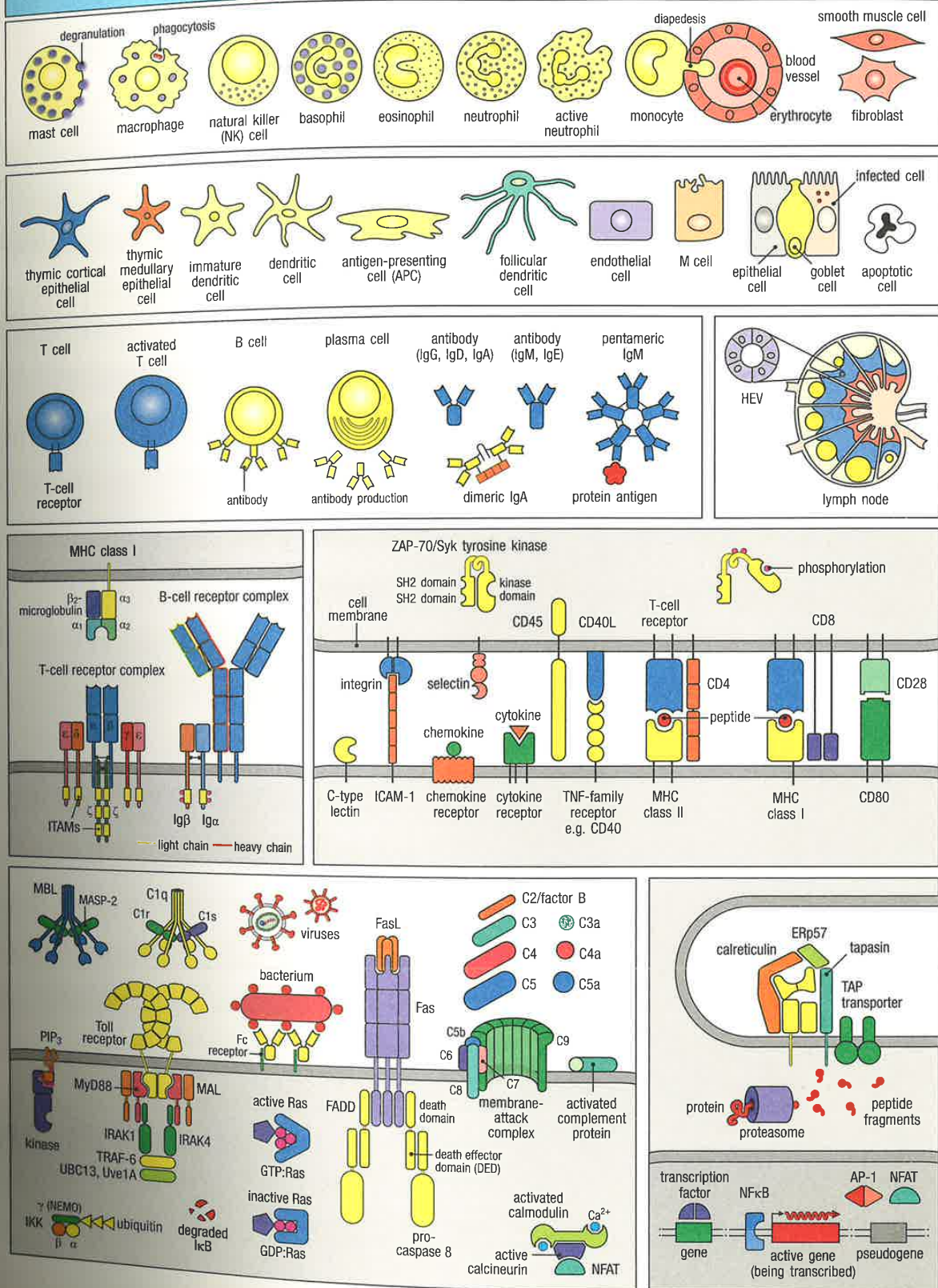
9TH EDITION

IMMUNOBIOLOGY

KENNETH MURPHY & CASEY WEAVER



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The origins of vertebrate immune cells.

Principles of innate immunity.

- 1-1 Commensal organisms cause little host damage while pathogens damage host tissues by a variety of mechanisms.
- 1-2 Anatomic and chemical barriers are the first defense against pathogens.
- 1-3 The immune system is activated by inflammatory inducers that indicate the presence of pathogens or tissue damage.
- 1-4 The myeloid lineage comprises most of the cells of the innate immune system.
- 1-5 Sensor cells express pattern recognition receptors that provide an initial discrimination between self and nonself.
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- 1-12 Lymphocytes activated by antigen give rise to clones of antigen-specific effector cells that mediate adaptive immunity.
- 1-13 Lymphocytes with self-reactive receptors are normally eliminated during development or are functionally inactivated.
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PART I

AN INTRODUCTION TO IMMUNOBIOLOGY AND INNATE IMMUNITY

- 1 Basic Concepts in Immunology
- 2 Innate Immunity: The First Lines of Defense
- 3 The Induced Response of Innate Immunity

Basic Concepts in Immunology

1

Immunology is the study of the body's defense against infection. We are continually exposed to microorganisms, many of which cause disease, and yet become ill only rarely. How does the body defend itself? When infection does occur, how does the body eliminate the invader and cure itself? And why do we develop long-lasting immunity to many infectious diseases encountered once and overcome? These are the questions addressed by immunology, which we study to understand our body's defenses against infection at the cellular and molecular levels.

The beginning of immunology as a science is usually attributed to **Edward Jenner** for his work in the late 18th century (Fig. 1.1). The notion of immunity—that surviving a disease confers greater protection against it later—was known since ancient Greece. **Variolation**—the inhalation or transfer into superficial skin wounds of material from smallpox pustules—had been practiced since at least the 1400s in the Middle East and China as a form of protection against that disease and was known to Jenner. Jenner had observed that the relatively mild disease of cowpox, or vaccinia, seemed to confer protection against the often fatal disease of smallpox, and in 1796, he demonstrated that inoculation with cowpox protected the recipient against smallpox. His scientific proof relied on the deliberate exposure of the inoculated individual to infectious smallpox material two months after inoculation. This scientific test was his original contribution.

Jenner called the procedure **vaccination**. This term is still used to describe the inoculation of healthy individuals with weakened or attenuated strains of disease-causing agents in order to provide protection from disease. Although Jenner's bold experiment was successful, it took almost two centuries for smallpox vaccination to become universal. This advance enabled the World Health Organization to announce in 1979 that smallpox had been eradicated (Fig. 1.2), arguably the greatest triumph of modern medicine.

Jenner's strategy of vaccination was extended in the late 19th century by the discoveries of many great microbiologists. **Robert Koch** proved that infectious diseases are caused by specific microorganisms. In the 1880s, **Louis Pasteur**

IN THIS CHAPTER

The origins of vertebrate immune cells.

Principles of innate immunity.

Principles of adaptive immunity.

The effector mechanisms of immunity.



Fig. 1.1 Edward Jenner. Portrait by John Raphael Smith, Reproduced courtesy of Yale University, Harvey Cushing/John Hay Whitney Medical Library.

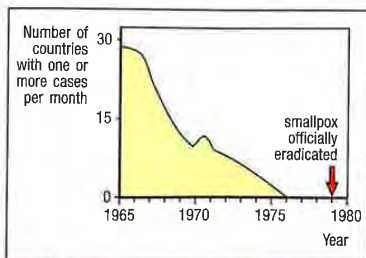


Fig. 1.2 The eradication of smallpox by vaccination. After a period of 3 years in which no cases of smallpox were recorded, the World Health Organization was able to announce in 1979 that smallpox had been eradicated, and vaccination stopped (upper panel). A few laboratory stocks have been retained, however, and some fear that these are a source from which the virus might reemerge. Ali Maow Maalin (lower panel) contracted and survived the last case of smallpox in Somalia in 1977. Photograph courtesy of Dr. Jason Weisfeld.

devised a vaccine against cholera in chickens, and developed a rabies vaccine that proved to be a spectacular success upon its first trial in a boy bitten by a rabid dog.

These practical triumphs led to a search for vaccination's mechanism of protection and to the development of the science of immunology. In the early 1890s, **Emil von Behring** and **Shibasaburo Kitasato** discovered that the serum of animals immune to diphtheria or tetanus contained a specific 'antitoxic activity' that could confer short-lived protection against the effects of diphtheria or tetanus toxins in people. This activity was later determined to be due to the proteins we now call **antibodies**, which bind specifically to the toxins and neutralize their activity. That these antibodies might have a crucial role in immunity was reinforced by **Jules Bordet's** discovery in 1899 of **complement**, a component of serum that acts in conjunction with antibodies to destroy pathogenic bacteria.

A specific response against infection by potential pathogens, such as the production of antibodies against a particular pathogen, is known as **adaptive immunity**, because it develops during the lifetime of an individual as an adaptation to infection with that pathogen. Adaptive immunity is distinguished from **innate immunity**, which was already known at the time von Behring was developing serum therapy for diphtheria chiefly through the work of the great Russian immunologist **Elie Metchnikoff**, who discovered that many microorganisms could be engulfed and digested by phagocytic cells, which thus provide defenses against infection that are nonspecific. Whereas these cells—which Metchnikoff called 'macrophages'—are always present and ready to act, adaptive immunity requires time to develop but is highly specific.

It was soon clear that specific antibodies could be induced against a vast range of substances, called **antigens** because they could stimulate *antibody generation*. **Paul Ehrlich** advanced the development of an **antiserum** as a treatment for diphtheria and developed methods to standardize therapeutic serums. Today the term antigen refers to any substance recognized by the adaptive immune system. Typically antigens are common proteins, glycoproteins, and polysaccharides of pathogens, but they can include a much wider range of chemical structures, for example, metals such as nickel, drugs such as penicillin, and organic chemicals such as the urushiol (a mix of pentadecylcatechols) in the leaves of poison ivy. Metchnikoff and Ehrlich shared the 1908 Nobel Prize for their respective work on immunity.

This chapter introduces the principles of innate and adaptive immunity, the cells of the immune system, the tissues in which they develop, and the tissues through which they circulate. We then outline the specialized functions of the different types of cells by which they eliminate infection.

The origins of vertebrate immune cells.

The body is protected from infectious agents, their toxins, and the damage they cause by a variety of effector cells and molecules that together make up the **immune system**. Both innate and adaptive immune responses depend upon the activities of white blood cells or **leukocytes**. Most cells of the immune system arise from the **bone marrow**, where many of them develop and mature. But some, particularly certain tissue-resident macrophage populations (for example, the microglia of the central nervous system), originate from the yolk sack or fetal liver during embryonic development. They seed tissues before birth and are maintained throughout life as independent, self-renewing populations. Once mature, immune cells reside within peripheral tissues, circulate in the bloodstream, or circulate in a specialized system of vessels called

the **lymphatic system**. The lymphatic system drains extracellular fluid and immune cells from tissues and transports them as **lymph** that is eventually emptied back into the blood system.

All the cellular elements of blood, including the red blood cells that transport oxygen, the platelets that trigger blood clotting in damaged tissues, and the white blood cells of the immune system, ultimately derive from the **hematopoietic stem cells (HSCs)** of the bone marrow. Because these can give rise to all the different types of blood cells, they are often known as **pluripotent** hematopoietic stem cells. The hematopoietic stem cells give rise to cells of more limited developmental potential, which are the immediate progenitors of red blood cells, platelets, and the two main categories of white blood cells, the **lymphoid** and **myeloid** lineages. The different types of blood cells and their lineage relationships are summarized in **Fig. 1.3**.

Principles of innate immunity.

In this part of the chapter we will outline the principles of innate immunity and describe the molecules and cells that provide continuous defense against invasion by pathogens. Although the white blood cells known as **lymphocytes** possess the most powerful ability to recognize and target pathogenic microorganisms, they need the participation of the innate immune system to initiate and mount their offensive. Indeed, the adaptive immune response and innate immunity use many of the same destructive mechanisms to eliminate invading microorganisms.

1-1 Commensal organisms cause little host damage while pathogens damage host tissues by a variety of mechanisms.

We recognize four broad categories of disease-causing microorganisms, or **pathogens: viruses, bacteria** and archaea, **fungi**, and the unicellular and multicellular eukaryotic organisms collectively termed **parasites** (**Fig. 1.4**). These microorganisms vary tremendously in size and in how they damage host tissues. The smallest are viruses, which range from five to a few hundred nanometers in size and are obligate intracellular pathogens. Viruses can directly kill cells by inducing lysis during their replication. Somewhat larger are intracellular bacteria and mycobacteria. These can kill cells directly or damage cells by producing toxins. Many single-celled intracellular parasites, such as members of the *Plasmodium* genus that cause malaria, also directly kill infected cells. Pathogenic bacteria and fungi growing in extracellular spaces can induce shock and sepsis by releasing toxins into the blood or tissues. The largest pathogens—parasitic worms, or helminths—are too large to infect host cells but can injure tissues by forming cysts that induce damaging cellular responses in the tissues into which the worms migrate.

Not all microbes are pathogens. Many tissues, especially the skin, oral mucosa, conjunctiva, and gastrointestinal tract, are constantly colonized by microbial communities—called the **microbiome**—that consist of archaea, bacteria, and fungi but cause no damage to the host. These are also called **commensal microorganisms**, since they can have a symbiotic relationship with the host. Indeed, some commensal organisms perform important functions, as in the case of the bacteria that aid in cellulose digestion in the stomachs of ruminants. The difference between commensal organisms and pathogens lies in whether they induce damage. Even enormous numbers of microbes in the intestinal microbiome normally cause no damage and are confined within the intestinal lumen by a protective layer of mucus, whereas pathogenic bacteria can penetrate this barrier, injure intestinal epithelial cells, and spread into the underlying tissues.

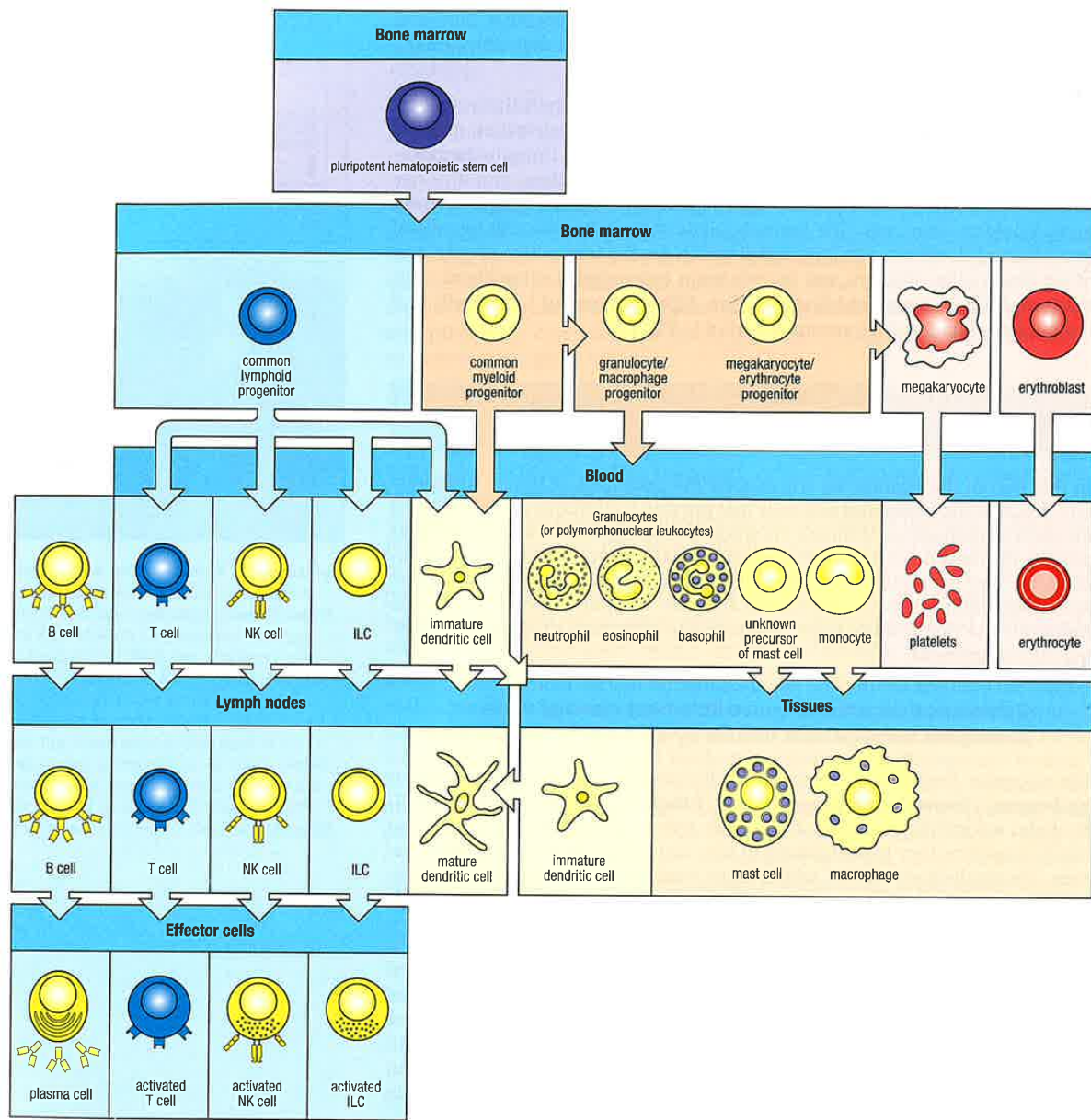


Fig. 1.3 All the cellular elements of the blood, including the cells of the immune system, arise from pluripotent hematopoietic stem cells in the bone marrow. These pluripotent cells divide to produce two types of stem cells. A common lymphoid progenitor gives rise to the lymphoid lineage (blue background) of white blood cells or leukocytes—the innate lymphoid cells (ILCs) and natural killer (NK) cells and the T and B lymphocytes. A common myeloid progenitor gives rise to the myeloid lineage (pink and yellow backgrounds), which comprises the rest of the leukocytes, the erythrocytes (red blood cells), and the megakaryocytes that produce platelets important in blood clotting. T and B lymphocytes are distinguished from the other leukocytes by having antigen receptors and from each other by their sites of differentiation—the thymus and bone marrow, respectively. After encounter with antigen, B cells differentiate into antibody-secreting plasma cells, while

T cells differentiate into effector T cells with a variety of functions. Unlike T and B cells, ILCs and NK cells lack antigen specificity. The remaining leukocytes are the monocytes, the dendritic cells, and the neutrophils, eosinophils, and basophils. The last three of these circulate in the blood and are termed granulocytes, because of the cytoplasmic granules whose staining gives these cells a distinctive appearance in blood smears, or polymorphonuclear leukocytes, because of their irregularly shaped nuclei. Immature dendritic cells (yellow background) are phagocytic cells that enter the tissues; they mature after they have encountered a potential pathogen. The majority of dendritic cells are derived from the common myeloid progenitor cells, but some may also arise from the common lymphoid progenitor. Monocytes enter tissues, where they differentiate into phagocytic macrophages or dendritic cells. Mast cells also enter tissues and complete their maturation there.

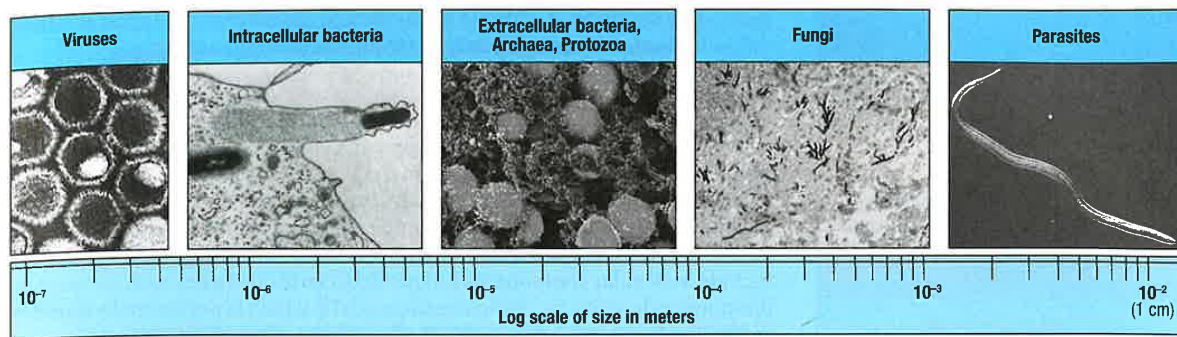


Fig. 1.4 Pathogens vary greatly in size and lifestyle.

Intracellular pathogens include viruses, such as herpes simplex (first panel), and various bacteria, such as *Listeria monocytogenes* (second panel). Many bacteria, such as *Staphylococcus aureus* (third panel), or fungi, such as *Aspergillus fumigatus* (fourth panel), can grow in the extracellular spaces and directly invade through

tissues, as do some archaea and protozoa (third panel). Many parasites, such as the nematode *Strongyloides stercoralis* (fifth panel), are large multicellular organisms that can move throughout the body in a complex life cycle. Second panel courtesy of Dan Portnoy. Fifth panel courtesy of James Lok.

1-2 Anatomic and chemical barriers are the first defense against pathogens.

The host can adopt three strategies to deal with the threat posed by microbes: **avoidance**, **resistance**, and **tolerance**. Avoidance mechanisms prevent exposure to microbes, and include both anatomic barriers and behavior modifications. If an infection is established, resistance is aimed at reducing or eliminating pathogens. To defend against the great variety of microbes, the immune system has numerous molecular and cellular functions, collectively called mediators, or **effector mechanisms**, suited to resist different categories of pathogens. Their description is a major aspect of this book. Finally, tolerance involves responses that enhance a tissue's capacity to resist damage induced by microbes. This meaning of the term 'tolerance' has been used extensively in the context of disease susceptibility in plants rather than animal immunity. For example, increasing growth by activating dormant meristems, the undifferentiated cells that generate new parts of the plant, is a common tolerance mechanism in response to damage. This should be distinguished from the term **immunological tolerance**, which refers to mechanisms that prevent an immune response from being mounted against the host's own tissues.

Anatomic and chemical barriers are the initial defenses against infection (Fig. 1.5). The skin and mucosal surfaces represent a kind of avoidance strategy that prevents exposure of internal tissues to microbes. At most anatomic barriers, additional resistance mechanisms further strengthen host defenses. For example, mucosal surfaces produce a variety of **antimicrobial proteins** that act as natural antibiotics to prevent microbes from entering the body.

If these barriers are breached or evaded, other components of the innate immune system can immediately come into play. We mentioned earlier the discovery by Jules Bordet of **complement**, which acts with antibodies to lyse bacteria. Complement is a group of around 30 different plasma proteins that act together and are one of the most important effector mechanisms in serum and interstitial tissues. Complement not only acts in conjunction with antibodies, but can also target foreign organisms in the absence of a specific antibody; thus it contributes to both innate and adaptive responses. We will examine anatomic barriers, the antimicrobial proteins, and complement in greater detail in Chapter 2.

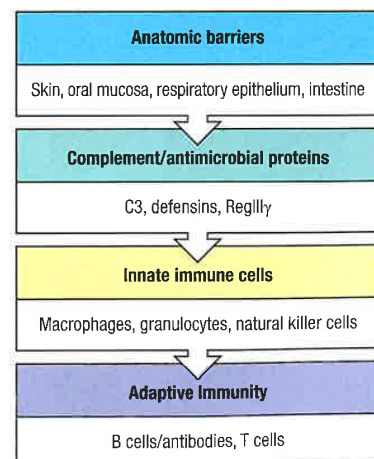


Fig. 1.5 Protection against pathogens relies on several levels of defense.

The first is the anatomic barrier provided by the body's epithelial surfaces. Second, various chemical and enzymatic systems, including complement, act as an immediate antimicrobial barrier near these epithelia. If epithelia are breached, nearby various innate lymphoid cells can coordinate a rapid cell-mediated defense. If the pathogen overcomes these barriers, the slower-acting defenses of the adaptive immune system are brought to bear.

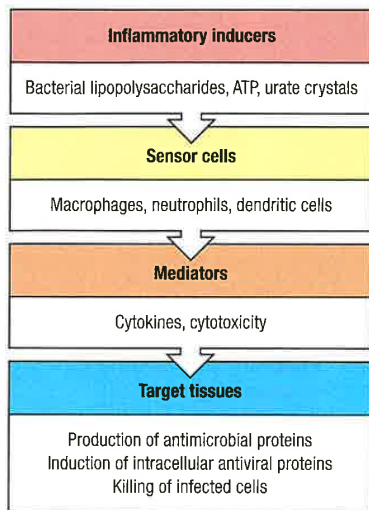


Fig. 1.6 Cell-mediated immunity proceeds in a series of steps.

Inflammatory inducers are chemical structures that indicate the presence of invading microbes or the cellular damage produced by them. Sensor cells detect these inducers by expressing various innate recognition receptors, and in response produce a variety of mediators that act directly in defense or that further propagate the immune response. Mediators include many cytokines, and they act on various target tissues, such as epithelial cells, to induce antimicrobial proteins and resist intracellular viral growth; or on other immune cells, such as ILCs that produce other cytokines that amplify the immune response.

1-3 The immune system is activated by inflammatory inducers that indicate the presence of pathogens or tissue damage.

A pathogen that breaches the host's anatomic and chemical barriers will encounter the cellular defenses of innate immunity. Cellular immune responses are initiated when **sensor cells** detect **inflammatory inducers** (Fig. 1.6). Sensor cells include many cell types that detect **inflammatory mediators** through expression of many **innate recognition receptors**, which are encoded by a relatively small number of genes that remain constant over an individual's lifetime. Inflammatory inducers that trigger these receptors include molecular components unique to bacteria or viruses, such as bacterial lipopolysaccharides, or molecules such as ATP, which is not normally found in the extracellular space. Triggering these receptors can activate innate immune cells to produce various mediators that either act directly to destroy invading microbes, or act on other cells to propagate the immune response. For example, macrophages can ingest microbes and produce toxic chemical mediators, such as degradative enzymes or reactive oxygen intermediates, to kill them. Dendritic cells may produce cytokine mediators, including many cytokines that activate target tissues, such as epithelial or other immune cells, to resist or kill invading microbes more efficiently. We will discuss these receptors and mediators briefly below and in much greater detail in Chapter 3.

Innate immune responses occur rapidly on exposure to an infectious organism (Fig. 1.7). In contrast, responses by the adaptive immune system take days rather than hours to develop. However, the adaptive immune system is capable of eliminating infections more efficiently because of exquisite specificity

Fig. 1.7 Phases of the immune response.

Phases of the immune response			
Response		Typical time after infection to start of response	Duration of response
Innate immune response	Inflammation, complement activation, phagocytosis, and destruction of pathogen	Minutes	Days
Adaptive immune response	Interaction between antigen-presenting dendritic cells and antigen-specific T cells: recognition of antigen, adhesion, co-stimulation, T-cell proliferation and differentiation	Hours	Days
	Activation of antigen-specific B cells	Hours	Days
	Formation of effector and memory T cells	Days	Weeks
	Interaction of T cells with B cells, formation of germinal centers. Formation of effector B cells (plasma cells) and memory B cells. Production of antibody	Days	Weeks
	Emigration of effector lymphocytes from peripheral lymphoid organs	A few days	Weeks
Immunological memory	Elimination of pathogen by effector cells and antibody	A few days	Weeks
	Maintenance of memory B cells and T cells and high serum or mucosal antibody levels. Protection against reinfection	Days to weeks	Can be lifelong

of antigen recognition by its lymphocytes. In contrast to a limited repertoire of receptors expressed by innate immune cells, lymphocytes express highly specialized **antigen receptors** that collectively possess a vast repertoire of specificity. This enables the adaptive immune system to respond to virtually any pathogen and effectively focus resources to eliminate pathogens that have evaded or overwhelmed innate immunity. But the adaptive immune system interacts with, and relies on, cells of the innate immune system for many of its functions. The next several sections will introduce the major components of the innate immune system and prepare us to consider adaptive immunity later in the chapter.

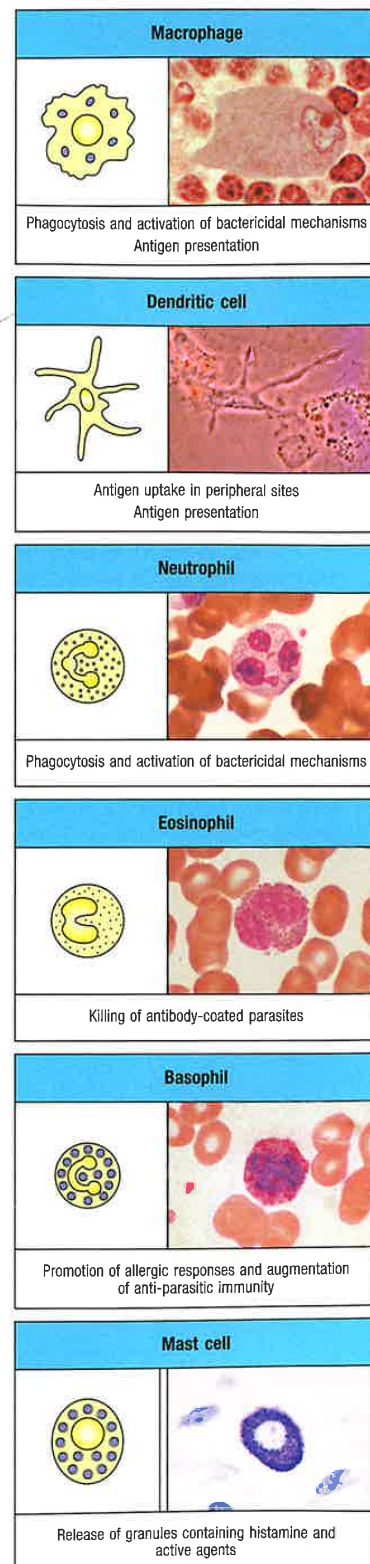
The myeloid lineage comprises most of the cells of the innate immune system.

The **common myeloid progenitor (CMP)** is the precursor of the macrophages, granulocytes (the collective term for the white blood cells called neutrophils, eosinophils, and basophils), mast cells, and dendritic cells of the innate immune system. Macrophages, granulocytes, and dendritic cells make up the three types of phagocytes in the immune system. The CMP also generates megakaryocytes and red blood cells, which we will not be concerned with here. The cells of the myeloid lineage are shown in [Fig. 1.8](#).

Macrophages are resident in almost all tissues. Many tissue-resident macrophages arise during embryonic development, but some macrophages that arise in the adult animal from the bone marrow are the mature form of **monocytes**, which circulate in the blood and continually migrate into tissues, where they differentiate. Macrophages are relatively long-lived cells and perform several different functions throughout the innate immune response and the subsequent adaptive immune response. One is to engulf and kill invading microorganisms. This phagocytic function provides a first defense in innate immunity. Macrophages also dispose of pathogens and infected cells targeted by an adaptive immune response. Both monocytes and macrophages are phagocytic, but most infections occur in the tissues, and so it is primarily macrophages that perform this important protective function. An additional and crucial role of macrophages is to orchestrate immune responses: they help induce inflammation, which, as we shall see, is a prerequisite to a successful immune response, and they produce many inflammatory mediators that activate other immune-system cells and recruit them into an immune response.

Local inflammation and the phagocytosis of invading bacteria can also be triggered by the activation of complement. Bacterial surfaces can activate the complement system, inducing a cascade of proteolytic reactions that coat the microbes with fragments of specific proteins of the complement system.

Fig. 1.8 Myeloid cells in innate and adaptive immunity. In the rest of the book, these cells will be represented in the schematic form shown on the left. A photomicrograph of each cell type is shown on the right. Macrophages and neutrophils are primarily phagocytic cells that engulf pathogens and destroy them in intracellular vesicles, a function they perform in both innate and adaptive immune responses. Dendritic cells are phagocytic when they are immature and can take up pathogens; after maturing, they function as specialized cells that present pathogen antigens to T lymphocytes in a form they can recognize, thus activating T lymphocytes and initiating adaptive immune responses. Macrophages can also present antigens to T lymphocytes and can activate them. The other myeloid cells are primarily secretory cells that release the contents of their prominent granules upon activation via antibody during an adaptive immune response. Eosinophils are thought to be involved in attacking large antibody-coated parasites such as worms; basophils are also thought to be involved in anti-parasite immunity. Mast cells are tissue cells that trigger a local inflammatory response to antigen by releasing substances that act on local blood vessels. Mast cells, eosinophils, and basophils are also important in allergic responses. Photographs courtesy of N. Rooney, R. Steinman, and D. Friend.



Microbes coated in this way are recognized by specific **complement receptors** on macrophages and neutrophils, taken up by phagocytosis, and destroyed. In addition to their specialized role in the immune system, macrophages act as general scavenger cells in the body, clearing it of dead cells and cell debris.

The **granulocytes** are named for the densely staining granules in their cytoplasm; they are also called **polymorphonuclear leukocytes** because of their oddly shaped nuclei. The three types of granulocytes—neutrophils, eosinophils, and basophils—are distinguished by the different staining properties of their granules, which serve distinct functions. Granulocytes are all relatively short-lived, surviving for only a few days. They mature in the bone marrow, and their production increases during immune responses, when they migrate to sites of infection or inflammation. The phagocytic **neutrophils** are the most numerous and important cells in innate immune responses: they take up a variety of microorganisms by phagocytosis and efficiently destroy them in intracellular vesicles by using degradative enzymes and other antimicrobial substances stored in their cytoplasmic granules. Hereditary deficiencies in neutrophil function open the way to overwhelming bacterial infection, which is fatal if untreated. Their role is discussed further in Chapter 3.

Eosinophils and **basophils** are less abundant than neutrophils, but like neutrophils, they have granules containing a variety of enzymes and toxic proteins, which are released when these cells are activated. Eosinophils and basophils are thought to be important chiefly in defense against parasites, which are too large to be ingested by macrophages or neutrophils. They can also contribute to allergic inflammatory reactions, in which their effects are damaging rather than protective.

Mast cells begin development in the bone marrow, but migrate as immature precursors that mature in peripheral tissues, especially skin, intestines, and airway mucosa. Their granules contain many inflammatory mediators, such as histamine and various proteases, which play a role in protecting the internal surfaces from pathogens, including parasitic worms. We cover eosinophils, basophils, and mast cells and their role in allergic inflammation further in Chapters 10 and 14.

Dendritic cells were discovered in the 1970s by **Ralph Steinman**, for which he received half the 2011 Nobel Prize. These cells form the third class of phagocytic cells of the immune system and include several related lineages whose distinct functions are still being clarified. Most dendritic cells have elaborate membranous processes, like the dendrites of nerve cells. Immature dendritic cells migrate through the bloodstream from the bone marrow to enter tissues. They take up particulate matter by phagocytosis and also continually ingest large amounts of the extracellular fluid and its contents by a process known as **macropinocytosis**. They degrade the pathogens that they take up, but their main role in the immune system is not the clearance of microorganisms. Instead, dendritic cells are a major class of sensor cells whose encounter with pathogens triggers them to produce mediators that activate other immune cells. Dendritic cells were discovered because of their role in activating a particular class of lymphocytes—T lymphocytes—of the adaptive immune system, and we will return to this activity when we discuss T-cell activation in Section 1-15. But dendritic cells and the mediators they produce also play a critical role in controlling responses of cells of the innate immune system.

1-5 Sensor cells express pattern recognition receptors that provide an initial discrimination between self and nonself.

Long before the mechanisms of innate recognition were discovered, it was recognized that purified antigens such as proteins often did not evoke an immune response in an experimental immunization—that is, they were not

immunogenic. Rather, the induction of strong immune responses against purified proteins required the inclusion of microbial constituents, such as killed bacteria or bacterial extracts, famously called the immunologist's 'dirty little secret' by **Charles Janeway** (see Appendix I, Sections A-1-A-4). This additional material was termed an **adjuvant**, because it helped intensify the response to the immunizing antigen (*adjuvare* is Latin for 'to help'). We know now that adjuvants are needed, at least in part, to activate innate receptors on various types of sensor cells to help activate T cells in the absence of an infection.

Macrophages, neutrophils, and dendritic cells are important classes of sensor cells that detect infection and initiate immune responses by producing inflammatory mediators, although other cells, even cells of the adaptive immune system, can serve in this function. As mentioned in Section 1-3, these cells express a limited number of invariant innate recognition receptors as a means of detecting pathogens or the damage induced by them. Also called **pattern recognition receptors (PRRs)**, they recognize simple molecules and regular patterns of molecular structure known as **pathogen-associated molecular patterns (PAMPs)** that are part of many microorganisms but not of the host body's own cells. Such structures include mannose-rich oligosaccharides, peptidoglycans, and lipopolysaccharides of the bacterial cell wall, as well as unmethylated CpG DNA common to many pathogens. All of these microbial elements have been conserved during evolution, making them excellent targets for recognition because they do not change (**Fig. 1.9**). Some PRRs are transmembrane proteins, such as the **Toll-like receptors (TLRs)** that detect PAMPs derived from extracellular bacteria or bacteria taken into vesicular pathways by phagocytosis. The role of the Toll receptor in immunity was discovered first in *Drosophila melanogaster* by **Jules Hoffman**, and later extended to homologous TLRs in mice by Janeway and **Bruce Beutler**. Hoffman and Beutler shared the remaining half of the 2011 Nobel Prize (see Section 1-4) for their work in the activation of innate immunity. Other PRRs are cytoplasmic proteins, such as the **NOD-like receptors (NLRs)** that sense intracellular bacterial invasion. Yet other cytoplasmic receptors detect viral infection based on differences in the structures and locations of the host mRNA and virally derived RNA species, and between host and microbial DNA. Some receptors expressed by sensor cells detect cellular damage induced by pathogens, rather than the pathogens themselves. Much of our knowledge of innate recognition has emerged only within the past 15 years and is still an active area of discovery. We describe these innate recognition systems further in Chapter 3, and how adjuvants are used as a component of vaccines in Chapter 16.

1-6 Sensor cells induce an inflammatory response by producing mediators such as chemokines and cytokines.

Activation of PRRs on sensor cells such as macrophages and neutrophils can directly induce effector functions in these cells, such as the phagocytosis and degradation of bacteria they encounter. But sensor cells serve to amplify the immune response by the production of inflammatory mediators. Two important categories of inflammatory mediators are the secreted proteins called **cytokines** and **chemokines**, which act in a manner similar to hormones to convey important signals to other immune cells.

'Cytokine' is a term for any protein secreted by immune cells that affects the behavior of nearby cells bearing appropriate receptors. There are more than 60 different cytokines; some are produced by many different cell types; others, by only a few specific cell types. Some cytokines influence many types of cells, while others influence only a few, through the expression pattern of each cytokine's specific receptor. The response that a cytokine induces in a target cell is typically related to amplifying an effector mechanism of the target cell, as illustrated in the next section.

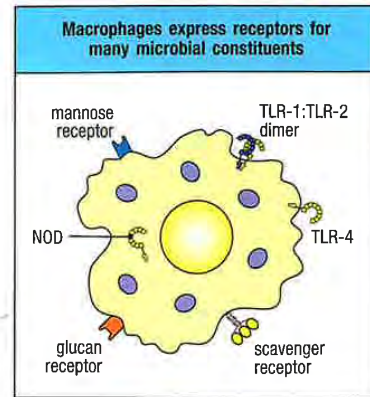
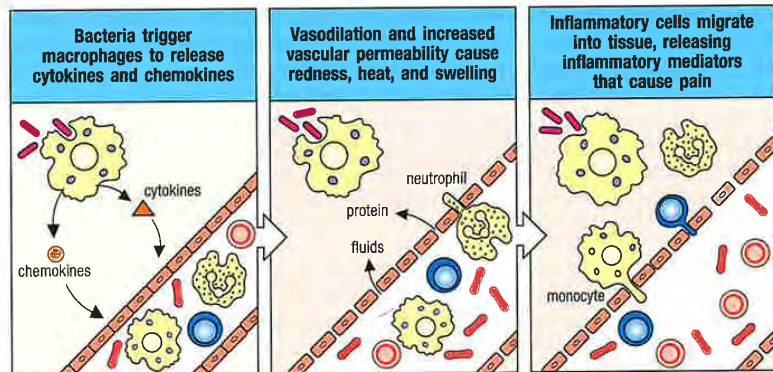


Fig. 1.9 Macrophages express a number of receptors that allow them to recognize different pathogens.

Macrophages express a variety of receptors, each of which is able to recognize specific components of microbes. Some, like the mannose and glucan receptors and the scavenger receptor, bind cell-wall carbohydrates of bacteria, yeast, and fungi. The Toll-like receptors (TLRs) are an important family of pattern recognition receptors present on macrophages, dendritic cells, and other immune cells. TLRs recognize different microbial components; for example, a heterodimer of TLR-1 and TLR-2 binds certain lipopeptides from pathogens such as Gram-positive bacteria, while TLR-4 binds both lipopolysaccharides from Gram-negative and lipoteichoic acids from Gram-positive bacteria.

Fig. 1.10 Infection triggers an inflammatory response. Macrophages encountering bacteria or other types of microorganisms in tissues are triggered to release cytokines (left panel) that increase the permeability of blood vessels, allowing fluid and proteins to pass into the tissues (center panel). Macrophages also produce chemokines, which direct the migration of neutrophils to the site of infection. The stickiness of the endothelial cells of the blood vessel wall is also changed, so that circulating cells of the immune system adhere to the wall and are able to crawl through it; first neutrophils and then monocytes are shown entering the tissue from a blood vessel (right panel). The accumulation of fluid and cells at the site of infection causes the redness, swelling, heat, and pain known collectively as inflammation. Neutrophils and macrophages are the principal inflammatory cells. Later in an immune response, activated lymphocytes can also contribute to inflammation.



Instead of presenting all the cytokines together all at once, we introduce each cytokine as it arises during our description of cellular and functional responses. We list the cytokines, their producer and target cells, and their general functions in Appendix III.

Chemokines are a specialized subgroup of secreted proteins that act as chemoattractants, attracting cells bearing chemokine receptors, such as neutrophils and monocytes, out of the bloodstream and into infected tissue (Fig. 1.10). Beyond this role, chemokines also help organize the various cells in lymphoid tissues into discrete regions where specialized responses can take place. There are on the order of 50 different chemokines, which are all related structurally but fall into two major classes. Appendix IV lists the chemokines, their target cells, and their general functions. We will discuss chemokines as the need arises during our descriptions of particular cellular immune processes.

The cytokines and chemokines released by activated macrophages act to recruit cells from the blood into infected tissues, a process, known as **inflammation**, that helps to destroy the pathogen. Inflammation increases the flow of lymph, which carries microbes or cells bearing their antigens from the infected tissue to nearby lymphoid tissues, where the adaptive immune response is initiated. Once adaptive immunity has been generated, inflammation also recruits these effector components to the site of infection.

Inflammation is described clinically by the Latin words *calor*, *dolor*, *rubor*, and *tumor*, meaning heat, pain, redness, and swelling. Each of these features reflects an effect of cytokines or other inflammatory mediators on the local blood vessels. Heat, redness, and swelling result from the dilation and increased permeability of blood vessels during inflammation, leading to increased local blood flow and leakage of fluid and blood proteins into the tissues. Cytokines and complement fragments have important effects on the **endothelium** that lines blood vessels; the **endothelial cells** themselves also produce cytokines in response to infection. These alter the adhesive properties of the endothelial cells and cause circulating leukocytes to stick to the endothelial cells and migrate between them into the site of infection, to which they are attracted by chemokines. The migration of cells into the tissue and their local actions account for the pain.

The main cell types seen in the initial phase of an inflammatory response are macrophages and neutrophils, the latter being recruited into the inflamed, infected tissue in large numbers. Macrophages and neutrophils are thus also known as **inflammatory cells**. The influx of neutrophils is followed a short time later by the increased entry of monocytes, which rapidly differentiate into macrophages, thus reinforcing and sustaining the innate immune response. Later, if the inflammation continues, eosinophils also migrate into inflamed tissues and contribute to the destruction of the invading microorganisms.

1-7 Innate lymphocytes and natural killer cells are effector cells that share similarities with lymphoid lineages of the adaptive immune system.

The **common lymphoid progenitor (CLP)** in the bone marrow gives rise both to antigen-specific lymphocytes of the adaptive immune system and to several innate lineages that lack antigen-specific receptors. Although the B and T lymphocytes of the adaptive immune system were recognized in the 1960s, the **natural killer (NK) cells** (Fig. 1.11) of the innate immune system were not discovered until the 1970s. NK cells are large lymphocyte-like cells with a distinctive granular cytoplasm that were identified because of their ability to recognize and kill certain tumor cells and cells infected with herpesviruses. Initially, the distinction between these cells and T lymphocytes was unclear, but we now recognize that NK cells are a distinct lineage of cells that arise from the CLP in the bone marrow. They lack the antigen-specific receptors of the adaptive immune system cells, but express members of various families of innate receptors that can respond to cellular stress and to infections by very specific viruses. NK cells play an important role in the early innate response to viral infections, before the adaptive immune response has developed.

More recently, additional lineages of cells related to NK cells have been identified. Collectively, these cells are called **innate lymphoid cells (ILCs)**. Arising from the CLP, ILCs reside in peripheral tissues, such as the intestine, where they function as the sources of mediators of inflammatory responses. The functions of NK cells and ILC cells are discussed in Chapter 3.

Summary.

Strategies of avoidance, resistance, and tolerance represent different ways to deal with pathogens. Anatomic barriers and various chemical barriers such as complement and antimicrobial proteins may be considered a primitive form of avoidance, and they are the first line of defense against entry of both commensal organisms and pathogens into host tissues. If these barriers are breached, the vertebrate immune response becomes largely focused on resistance. Inflammatory inducers, which may be either chemical structures unique to microbes (PAMPs) or the chemical signals of tissue damage, act on receptors expressed by sensor cells to inform the immune system of infection. Sensor cells are typically innate immune cells such as macrophages or dendritic cells. Sensor cells can either directly respond with effector activity or produce inflammatory mediators, typically cytokines and chemokines that act on other immune cells, such as the innate NK cells and ILCs. These cells then are recruited into target tissues to provide specific types of immune-response effector activities, such as cell killing or production of cytokines that have direct antiviral activity, all aimed to reduce or eliminate infection by pathogens. Responses by mediators in target tissues can induce several types of inflammatory cells that are specially suited for eliminating viruses, intracellular bacteria, extracellular pathogens, or parasites.

Principles of adaptive immunity.

We come now to the components of adaptive immunity, the antigen-specific lymphocytes. Unless indicated otherwise, we shall use the term lymphocyte to refer only to the antigen-specific lymphocytes. Lymphocytes allow responses against a vast array of antigens from various pathogens encountered during a person's lifetime and confer the important feature of immunological memory. Lymphocytes make this possible through the highly variable antigen receptors

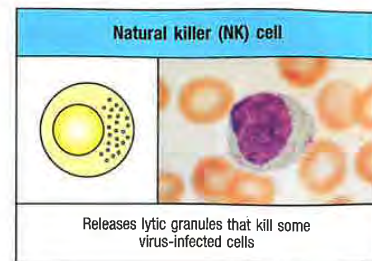


Fig. 1.11 Natural killer (NK) cells.

These are large, granular, lymphoid-like cells with important functions in innate immunity, especially against intracellular infections, being able to kill other cells. Unlike lymphocytes, they lack antigen-specific receptors. Photograph courtesy of B. Smith.

on their surface, by which they recognize and bind antigens. Each lymphocyte matures bearing a unique variant of a prototype antigen receptor, so that the population of lymphocytes expresses a huge repertoire of receptors that are highly diverse in their antigen-binding sites. Among the billion or so lymphocytes circulating in the body at any one time there will always be some that can recognize a given foreign antigen.

A unique feature of the adaptive immune system is that it is capable of generating **immunological memory**, so that having been exposed once to an infectious agent, a person will make an immediate and stronger response against any subsequent exposure to it; that is, the individual will have protective immunity against it. Finding ways of generating long-lasting immunity to pathogens that do not naturally provoke it is one of the greatest challenges facing immunologists today.

1-8 The interaction of antigens with antigen receptors induces lymphocytes to acquire effector and memory activity.

There are two major types of lymphocytes in the vertebrate immune system, the **B lymphocytes (B cells)** and **T lymphocytes (T cells)**. These express distinct types of antigen receptors and have quite different roles in the immune system, as was discovered in the 1960s. Most lymphocytes circulating in the body appear as rather unimpressive small cells with few cytoplasmic organelles and a condensed, inactive-appearing nuclear chromatin (Fig. 1.12). Lymphocytes manifest little functional activity until they encounter a specific antigen that interacts with an antigen receptor on their cell surface. Lymphocytes that have not yet been activated by antigen are known as **naïve lymphocytes**; those that have met their antigen, become activated, and have differentiated further into fully functional lymphocytes are known as **effector lymphocytes**.

B cells and T cells are distinguished by the structure of the antigen receptor that they express. The **B-cell antigen receptor**, or **B-cell receptor (BCR)**, is formed by the same genes that encode antibodies, a class of proteins also known as **immunoglobulins (Ig)** (Fig. 1.13). Thus, the antigen receptor of B lymphocytes is also known as **membrane immunoglobulin (mIg)** or **surface immunoglobulin (sIg)**. The **T-cell antigen receptor**, or **T-cell receptor (TCR)**, is related to the immunoglobulins but is quite distinct in its structure and recognition properties.

After antigen binds to a B-cell antigen receptor, or B-cell receptor (BCR), the B cell will proliferate and differentiate into **plasma cells**. These are the effector form of B lymphocytes, and they secrete antibodies that have the same antigen specificity as the plasma cell's B-cell receptor. Thus the antigen that activates a given B cell becomes the target of the antibodies produced by that B cell's progeny.

Fig. 1.12 Lymphocytes are mostly small and inactive cells. The left panel shows a light micrograph of a small lymphocyte in which the nucleus has been stained purple by hematoxylin and eosin dye, surrounded by red blood cells (which have no nuclei). Note the darker purple patches of condensed chromatin of the lymphocyte nucleus, indicating little transcriptional activity and the relative absence of cytoplasm. The right panel shows a transmission electron micrograph of a small lymphocyte. Again, note the evidence of functional inactivity: the condensed chromatin, the scanty cytoplasm, and the absence of rough endoplasmic reticulum. Photographs courtesy of N. Rooney.

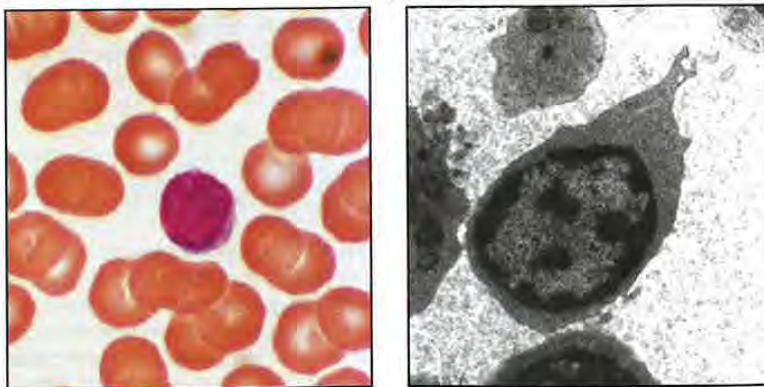
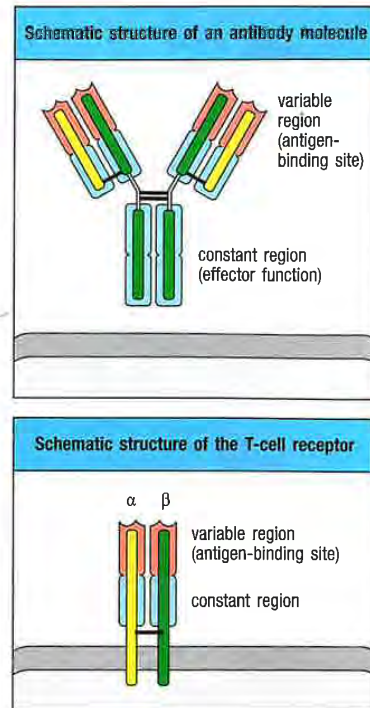


Fig. 1.13 Schematic structure of antigen receptors. Upper panel: an antibody molecule, which is secreted by activated B cells as an antigen-binding effector molecule. A membrane-bound version of this molecule acts as the B-cell antigen receptor (not shown). An antibody is composed of two identical heavy chains (green) and two identical light chains (yellow). Each chain has a constant part (shaded blue) and a variable part (shaded red). Each arm of the antibody molecule is formed by a light chain and a heavy chain, with the variable parts of the two chains coming together to create a variable region that contains the antigen-binding site. The stem is formed from the constant parts of the heavy chains and takes a limited number of forms. This constant region is involved in the elimination of the bound antigen. Lower panel: a T-cell antigen receptor. This is also composed of two chains, an α chain (yellow) and a β chain (green), each of which has a variable and a constant part. As with the antibody molecule, the variable parts of the two chains create a variable region, which forms the antigen-binding site. The T-cell receptor is not produced in a secreted form.



When a T cell first encounters an antigen that its receptor can bind, it proliferates and differentiates into one of several different functional types of **effector T lymphocytes**. When effector T cells subsequently detect antigen, they can manifest three broad classes of activity. **Cytotoxic T cells** kill other cells that are infected with viruses or other intracellular pathogens bearing the antigen. **Helper T cells** provide signals, often in the form of specific cytokines that activate the functions of other cells, such as B-cell production of antibody and macrophage killing of engulfed pathogens. **Regulatory T cells** suppress the activity of other lymphocytes and help to limit the possible damage of immune responses. We discuss the detailed functions of cytotoxic, helper, and regulatory T cells in Chapters 9, 11, 12, and 15.

Some of the B cells and T cells activated by antigen will differentiate into **memory cells**, the lymphocytes that are responsible for the long-lasting immunity that can follow exposure to disease or vaccination. Memory cells will readily differentiate into effector cells on a second exposure to their specific antigen. Immunological memory is described in Chapter 11.

1-9 Antibodies and T-cell receptors are composed of constant and variable regions that provide distinct functions.

Antibodies were studied by traditional biochemical techniques long before recombinant DNA technology allowed the study of the membrane-bound forms of the antigen receptors on B and T cells. These early studies found that antibody molecules are composed of two distinct regions. One is a **constant region**, also called the **fragment crystallizable region**, or **Fc region**, which takes one of only four or five biochemically distinguishable forms (see Fig. 1.13). The **variable region**, by contrast, can be composed of a vast number of different amino acid sequences that allow antibodies to recognize an equally vast variety of antigens. It was the uniformity of the Fc region relative to the variable region that allowed its early analysis by X-ray crystallography by **Gerald Edelman** and **Rodney Porter**, who shared the 1972 Nobel Prize for their work on the structure of antibodies.

The antibody molecule is composed of two identical **heavy chains** and two identical **light chains**. Heavy and light chains each have variable and constant regions. The variable regions of a heavy chain and a light chain combine to form an **antigen-binding site** that determines the antigen-binding specificity of the antibody. Thus, both heavy and light chains contribute to the antigen-binding specificity of the antibody molecule. Also, each antibody has two identical variable regions, and so has two identical antigen-binding sites. The constant region determines the effector function of the antibody, that is, how the antibody will interact with various immune cells to dispose of antigen once it is bound.

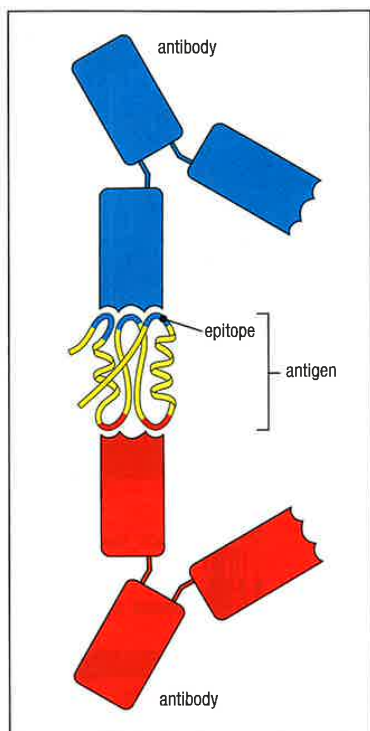


Fig. 1.14 Antigens are the molecules recognized by the immune response, while epitopes are sites within antigens to which antigen receptors bind.

Antigens can be complex macromolecules such as proteins, as shown in yellow. Most antigens are larger than the sites on the antibody or antigen receptor to which they bind, and the actual portion of the antigen that is bound is known as the antigenic determinant, or epitope, for that receptor. Large antigens such as proteins can contain more than one epitope (indicated in red and blue) and thus may bind different antibodies (shown here in the same color as the epitopes they bind). Antibodies generally recognize epitopes on the surface of the antigen.

The T-cell receptor shows many similarities to the B-cell receptor and antibody (see Fig. 1.13). It is composed of two chains, the TCR α and β chains, that are roughly equal in size and which span the T-cell membrane. Like antibody, each TCR chain has a variable region and a constant region, and the combination of the α - and β -chain variable regions creates a single site for binding antigen. The structures of both antibodies and T-cell receptors are described in detail in Chapter 4, and functional properties of antibody constant regions are discussed in Chapters 5 and 10.

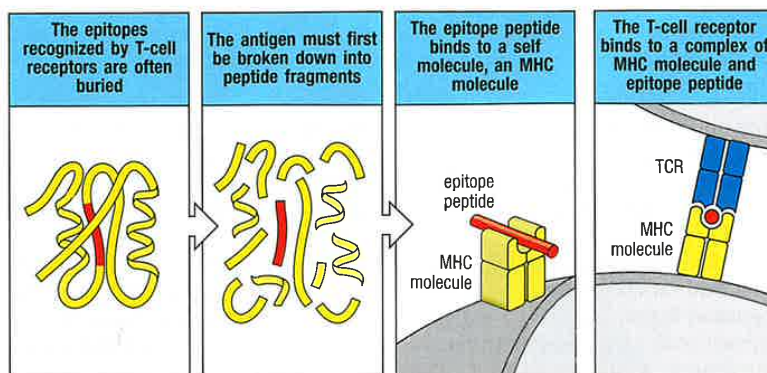
1-10 Antibodies and T-cell receptors recognize antigens by fundamentally different mechanisms.

In principle, almost any chemical structure can be recognized as an antigen by the adaptive immune system, but the usual antigens encountered in an infection are the proteins, glycoproteins, and polysaccharides of pathogens. An individual antigen receptor or antibody recognizes a small portion of the antigen's molecular structure, and the part recognized is known as an **antigenic determinant** or **epitope** (Fig. 1.14). Typically, proteins and glycoproteins have many different epitopes that can be recognized by different antigen receptors.

Antibodies and B-cell receptors directly recognize the epitopes of native antigen in the serum or the extracellular spaces. It is possible for different antibodies to simultaneously recognize an antigen by its different epitopes; such simultaneous recognition increases the efficiency of clearing or neutralizing the antigen.

Whereas antibodies can recognize nearly any type of chemical structure, T-cell receptors usually recognize protein antigens and do so very differently from antibodies. The T-cell receptor recognizes a peptide epitope derived from a partially degraded protein, but only if the peptide is bound to specialized cell-surface glycoproteins called **MHC molecules** (Fig. 1.15). The members of this large family of cell-surface glycoproteins are encoded in a cluster of genes called the **major histocompatibility complex (MHC)**. The antigens recognized by T cells can be derived from proteins arising from intracellular pathogens, such as a virus, or from extracellular pathogens. A further difference from the antibody molecule is that there is no secreted form of the T-cell receptor; the T-cell receptor functions solely to signal to the T cell that it has bound its antigen, and the subsequent immunological effects depend on the actions of the T cells themselves. We will further describe how epitopes from antigens are placed on MHC proteins in Chapter 6 and how T cells carry out their subsequent functions in Chapter 9.

Fig. 1.15 T-cell receptors bind a complex of an antigen fragment and a self molecule. Unlike most antibodies, T-cell receptors can recognize epitopes that are buried within antigens (first panel). These antigens must first be degraded by proteases (second panel) and the peptide epitope delivered to a self molecule, called an MHC molecule (third panel). It is in this form, as a complex of peptide and MHC molecule, that antigens are recognized by T-cell receptors (TCRs; fourth panel).



1-11 Antigen-receptor genes are assembled by somatic gene rearrangements of incomplete receptor gene segments.

The innate immune system detects inflammatory stimuli by means of a relatively limited number of sensors, such as the TLR and NOD proteins, numbering fewer than 100 different types of proteins. Antigen-specific receptors of adaptive immunity provide a seemingly infinite range of specificities, and yet are encoded by a finite number of genes. The basis for this extraordinary range of specificity was discovered in 1976 by **Susumu Tonegawa**, for which he was awarded the 1987 Nobel Prize. Immunoglobulin variable regions are inherited as sets of **gene segments**, each encoding a part of the variable region of one of the immunoglobulin polypeptide chains. During B-cell development in the bone marrow, these gene segments are irreversibly joined by a process of DNA recombination to form a stretch of DNA encoding a complete variable region. A similar process of antigen-receptor gene rearrangement takes place for the T-cell receptor genes during development of T cells in the thymus.

Just a few hundred different gene segments can combine in different ways to generate thousands of different receptor chains. This **combinatorial diversity** allows a small amount of genetic material to encode a truly staggering diversity of receptors. During this recombination process, the random addition or subtraction of nucleotides at the junctions of the gene segments creates additional diversity known as **junctional diversity**. Diversity is amplified further by the fact that each antigen receptor has two different variable chains, each encoded by distinct sets of gene segments. We will describe the gene rearrangement process that assembles complete antigen receptors from gene segments in Chapter 5.

1-12 Lymphocytes activated by antigen give rise to clones of antigen-specific effector cells that mediate adaptive immunity.

There are two critical features of lymphocyte development that distinguish adaptive immunity from innate immunity. First, the process described above that assembles antigen receptors from incomplete gene segments is carried out in a manner that ensures that each developing lymphocyte expresses only one receptor specificity. Whereas the cells of the innate immune system express many different pattern recognition receptors and recognize features shared by many pathogens, the antigen-receptor expression of lymphocytes is 'clonal,' so that each mature lymphocyte differs from others in the specificity of its antigen receptor. Second, because the gene rearrangement process irreversibly changes the lymphocyte's DNA, all its progeny inherit the same receptor specificity. Because this specificity is inherited by a cell's progeny, the proliferation of an individual lymphocyte forms a **clone** of cells with identical antigen receptors.

There are lymphocytes of at least 10^8 different specificities in an individual human at any one time, comprising the **lymphocyte receptor repertoire** of the individual. These lymphocytes are continually undergoing a process similar to natural selection: only those lymphocytes that encounter an antigen to which their receptor binds will be activated to proliferate and differentiate into effector cells. This selective mechanism was first proposed in the 1950s by **Macfarlane Burnet**, who postulated the preexistence in the body of many different potential antibody-producing cells, each displaying on its surface a membrane-bound version of the antibody that served as a receptor for the antigen. On binding antigen, the cell is activated to divide and to produce many identical progeny, a process known as **clonal expansion**; this clone of identical cells can now secrete **clonotypic** antibodies with a specificity identical to that of the surface receptor that first triggered activation and clonal expansion (Fig. 1.16). Burnet called this the **clonal selection theory**

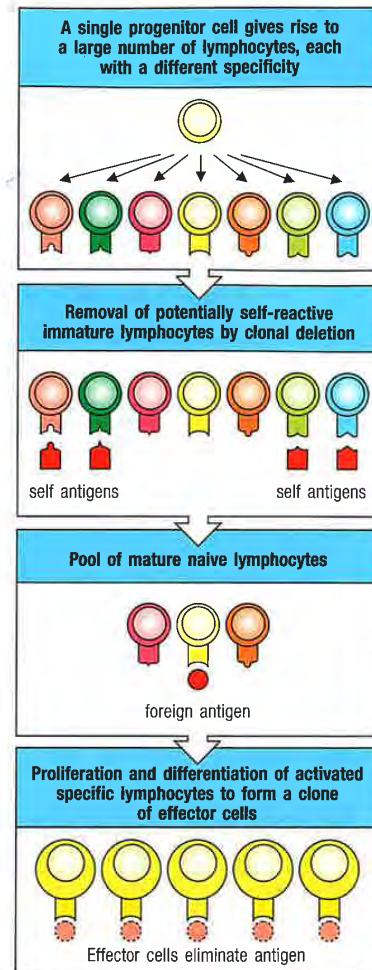


Fig. 1.16 Clonal selection. Each lymphoid progenitor gives rise to a large number of lymphocytes, each bearing a distinct antigen receptor. Lymphocytes with receptors that bind ubiquitous self antigens are eliminated before they become fully mature, ensuring tolerance to such self antigens. When a foreign antigen (red dot) interacts with the receptor on a mature naive lymphocyte, that cell is activated and starts to divide. It gives rise to a clone of identical progeny, all of whose receptors bind the same antigen. Antigen specificity is thus maintained as the progeny proliferate and differentiate into effector cells. Once antigen has been eliminated by these effector cells, the immune response ceases, although some lymphocytes are retained to mediate immunological memory.

Postulates of the clonal selection hypothesis
Each lymphocyte bears a single type of receptor with a unique specificity
Interaction between a foreign molecule and a lymphocyte receptor capable of binding that molecule with high affinity leads to lymphocyte activation
The differentiated effector cells derived from an activated lymphocyte will bear receptors of identical specificity to those of the parental cell from which that lymphocyte was derived
Lymphocytes bearing receptors specific for ubiquitous self molecules are deleted at an early stage in lymphoid cell development and are therefore absent from the repertoire of mature lymphocytes

Fig. 1.17 The four basic principles of clonal selection.

of antibody production; its four basic postulates are listed in Fig. 1.17. Clonal selection of lymphocytes is the single most important principle in adaptive immunity.

1-13 Lymphocytes with self-reactive receptors are normally eliminated during development or are functionally inactivated.

When Burnet formulated his theory, nothing was known of the antigen receptors or indeed the function of lymphocytes themselves. In the early 1960s, **James Gowans** discovered that removal of the small lymphocytes from rats resulted in the loss of all known adaptive immune responses, which were restored when the small lymphocytes were replaced. This led to the realization that lymphocytes must be the units of clonal selection, and their biology became the focus of the new field of **cellular immunology**.

Clonal selection of lymphocytes with diverse receptors elegantly explained adaptive immunity, but it raised one significant conceptual problem. With so many different antigen receptors being generated randomly during the lifetime of an individual, there is a possibility that some receptors might react against an individual's own **self antigens**. How are lymphocytes prevented from recognizing native antigens on the tissues of the body and attacking them? **Ray Owen** had shown in the late 1940s that genetically different twin calves with a common placenta, and thus a shared placental blood circulation, were immunologically unresponsive, or **tolerant**, to one another's tissues. **Peter Medawar** then showed in 1953 that exposure to foreign tissues during embryonic development caused mice to become immunologically tolerant to these tissues. Burnet proposed that developing lymphocytes that are potentially self-reactive are removed before they can mature, a process known as **clonal deletion**. Medawar and Burnet shared the 1960 Nobel Prize for their work on tolerance. This process was demonstrated to occur experimentally in the late 1980s. Some lymphocytes that receive either too much or too little signal through their antigen receptor during development are eliminated by a form of cell suicide called **apoptosis**—derived from a Greek word meaning the falling of leaves from trees—or **programmed cell death**. Other types of mechanisms of **immunological tolerance** have been identified since then that rely on the induction of an inactive state, called **anergy**, as well as mechanisms of active suppression of self-reactive lymphocytes. Chapter 8 will describe lymphocyte development and tolerance mechanisms that shape the lymphocyte receptor repertoire. Chapters 14 and 15 will discuss how immune tolerance mechanisms can sometimes fail.

1-14 Lymphocytes mature in the bone marrow or the thymus and then congregate in lymphoid tissues throughout the body.

Lymphocytes circulate in the blood and the lymph and are also found in large numbers in **lymphoid tissues** or **lymphoid organs**, which are organized aggregates of lymphocytes in a framework of nonlymphoid cells. Lymphoid organs can be divided broadly into the **central** or **primary lymphoid organs**, where lymphocytes are generated, and the **peripheral** or **secondary lymphoid organs**, where mature naive lymphocytes are maintained and adaptive immune responses are initiated. The central lymphoid organs are the bone marrow and the **thymus**, an organ in the upper chest. The peripheral lymphoid organs comprise the **lymph nodes**, the **spleen**, and the mucosal lymphoid tissues of the gut, the nasal and respiratory tract, the urogenital tract, and other mucosa. The locations of the main lymphoid tissues are shown schematically in Fig. 1.18; we describe the individual peripheral lymphoid organs in more detail later in the chapter. Lymph nodes are interconnected by a system of lymphatic vessels, which drain extracellular fluid from tissues, carry it through the lymph nodes, and deposit it back into the blood.

The progenitors that give rise to B and T lymphocytes originate in the bone marrow. B cells complete their development within the bone marrow. Although the 'B' in B lymphocytes originally stood for the **bursa of Fabricius**, a lymphoid organ in young chicks in which lymphocytes mature, it is a useful mnemonic for bone marrow. The immature precursors of T lymphocytes migrate to the thymus, from which they get their name, and complete their development there. Once they have completed maturation, both types of lymphocytes enter the bloodstream as mature naive lymphocytes and continuously circulate through the peripheral lymphoid tissues.

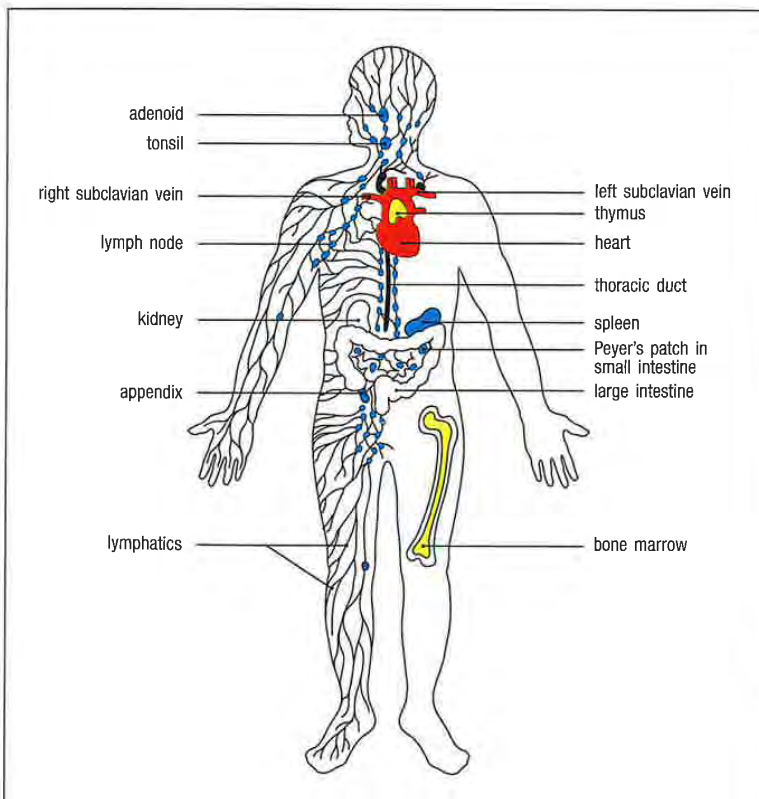
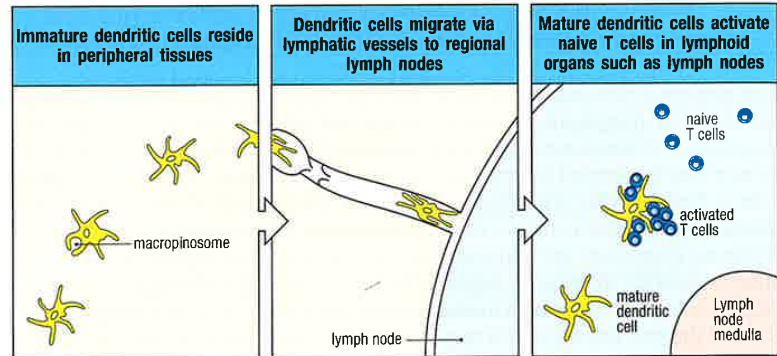


Fig. 1.18 The distribution of lymphoid tissues in the body. Lymphocytes arise from stem cells in bone marrow and differentiate in the central lymphoid organs (yellow)—B cells in the bone marrow and T cells in the thymus. They migrate from these tissues and are carried in the bloodstream to the peripheral lymphoid organs (blue). These include lymph nodes, spleen, and lymphoid tissues associated with mucosa, such as the gut-associated tonsils, Peyer's patches, and appendix. The peripheral lymphoid organs are the sites of lymphocyte activation by antigen, and lymphocytes recirculate between the blood and these organs until they encounter their specific antigen. Lymphatics drain extracellular fluid from the peripheral tissues, through the lymph nodes, and into the thoracic duct, which empties into the left subclavian vein. This fluid, known as lymph, carries antigen taken up by dendritic cells and macrophages to the lymph nodes, as well as recirculating lymphocytes from the lymph nodes back into the blood. Lymphoid tissue is also associated with other mucosa such as the bronchial linings (not shown).

Fig. 1.19 Dendritic cells initiate adaptive immune responses. Immature dendritic cells residing in a tissue take up pathogens and their antigens by macropinocytosis and by receptor-mediated endocytosis. They are stimulated by recognition of the presence of pathogens to migrate through the lymphatics to regional lymph nodes, where they arrive as fully mature nonphagocytic dendritic cells that express both antigen and the co-stimulatory molecules necessary to activate a naive T cell that recognizes the antigen. Thus the dendritic cells stimulate lymphocyte proliferation and differentiation.



1-15 Adaptive immune responses are initiated by antigen and antigen-presenting cells in secondary lymphoid tissues.

Adaptive immune responses are initiated when B or T lymphocytes encounter antigens for which their receptors have specific reactivity, provided that there are appropriate inflammatory signals to support activation. For T cells, this activation occurs via encounters with dendritic cells that have picked up antigens at sites of infection and migrated to secondary lymphoid organs. Activation of the dendritic cells' PRRs by PAMPs at the site of infection stimulates the dendritic cells in the tissues to engulf the pathogen and degrade it intracellularly. They also take up extracellular material, including virus particles and bacteria, by receptor-independent macropinocytosis. These processes lead to the display of peptide antigens on the MHC molecules of the dendritic cells, a display that activates the antigen receptors of lymphocytes. Activation of PRRs also triggers the dendritic cells to express cell-surface proteins called **co-stimulatory molecules**, which support the ability of the T lymphocyte to proliferate and differentiate into its final, fully functional form (Fig. 1.19). For these reasons dendritic cells are also called **antigen-presenting cells (APCs)**, and as such, they form a crucial link between the innate immune response and the adaptive immune response (Fig. 1.20). In certain situations, macrophages and B cells can also act as antigen-presenting cells, but dendritic cells are the cells that are specialized in initiating the adaptive immune response. Free antigens can also stimulate the antigen receptors of B cells, but most B cells require 'help' from activated helper T cells for optimal antibody responses. The activation of naive T lymphocytes is therefore an essential first stage in virtually all adaptive immune responses. Chapter 6 returns to dendritic cells to discuss how antigens are processed for presentation to T cells. Chapters 7 and 9 discuss co-stimulation and lymphocyte activation. Chapter 10 describes how T cells help in activating B cells.

MOVIE 1.1



Fig. 1.20 Dendritic cells form a key link between the innate immune system and the adaptive immune system. Like the other cells of innate immunity, dendritic cells recognize pathogens via invariant cell-surface receptors for pathogen molecules and are activated by these stimuli early in an infection. Dendritic cells in tissues are phagocytic; they are specialized to ingest a wide range of pathogens and to display their antigens at the dendritic cell surface in a form that can be recognized by T cells.

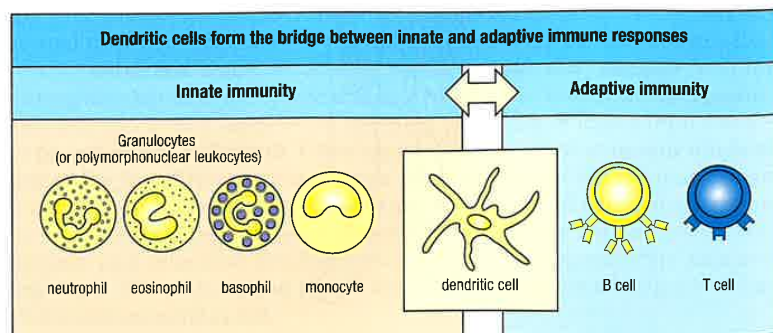


Fig. 1.21 Circulating lymphocytes encounter antigen in peripheral lymphoid organs. Naive lymphocytes recirculate constantly through peripheral lymphoid tissue, here illustrated as a popliteal lymph node—a lymph node situated behind the knee. In the case of an infection in the foot, this will be the draining lymph node, where lymphocytes may encounter their specific antigens and become activated. Both activated and nonactivated lymphocytes are returned to the bloodstream via the lymphatic system.

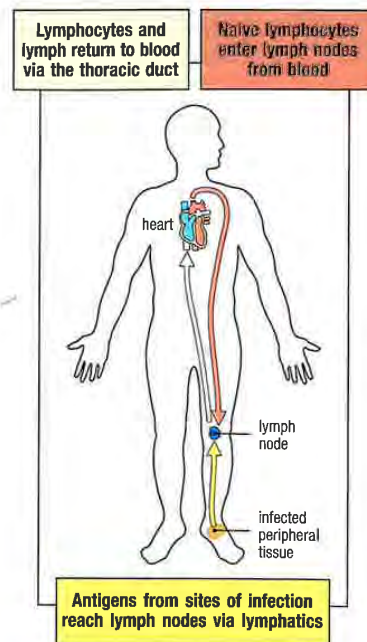
1-16 Lymphocytes encounter and respond to antigen in the peripheral lymphoid organs.

Antigen and lymphocytes eventually encounter each other in the peripheral lymphoid organs—the lymph nodes, spleen, and mucosal lymphoid tissues (see Fig. 1.18). Mature naive lymphocytes are continually recirculating through these tissues, to which pathogen antigens are carried from sites of infection, primarily by dendritic cells. The peripheral lymphoid organs are specialized to trap antigen-bearing dendritic cells and to facilitate the initiation of adaptive immune responses. Peripheral lymphoid tissues are composed of aggregations of lymphocytes in a framework of nonleukocyte stromal cells, which provide both the basic structural organization of the tissue and survival signals to help sustain the life of the lymphocytes. Besides lymphocytes, peripheral lymphoid organs also contain resident macrophages and dendritic cells.

When an infection occurs in a tissue such as the skin, free antigen and antigen-bearing dendritic cells travel from the site of infection through the afferent lymphatic vessels into the **draining lymph nodes** (Fig. 1.21)—peripheral lymphoid tissues where they activate antigen-specific lymphocytes. The activated lymphocytes then undergo a period of proliferation and differentiation, after which most leave the lymph nodes as effector cells via the efferent lymphatic vessel. This eventually returns them to the bloodstream (see Fig. 1.18), which then carries them to the tissues where they will act. This whole process takes about 4–6 days from the time that the antigen is recognized, which means that an adaptive immune response to an antigen that has not been encountered before does not become effective until about a week after infection (see Fig. 1.7). Naive lymphocytes that do not recognize their antigen also leave through the efferent lymphatic vessel and are returned to the blood, from which they continue to recirculate through lymphoid tissues until they recognize antigen or die.

The lymph nodes are highly organized lymphoid organs located at the points of convergence of vessels of the lymphatic system, which is the extensive system that collects extracellular fluid from the tissues and returns it to the blood (see Fig. 1.18). This extracellular fluid is produced continuously by filtration from the blood and is called **lymph**. Lymph flows away from the peripheral tissues under the pressure exerted by its continuous production, and is carried by **lymphatic vessels**, or **lymphatics**. One-way valves in the lymphatic vessels prevent a reverse flow, and the movements of one part of the body in relation to another are important in driving the lymph along.

As noted above, **afferent lymphatic vessels** drain fluid from the tissues and carry pathogens and antigen-bearing cells from infected tissues to the lymph nodes (Fig. 1.22). Free antigens simply diffuse through the extracellular fluid to the lymph node, while the dendritic cells actively migrate into the lymph node, attracted by chemokines. The same chemokines also attract lymphocytes from the blood, and these enter lymph nodes by squeezing through the walls of specialized blood vessels called **high endothelial venules (HEV)**, named for their thicker, more rounded appearance relative to flatter endothelial cells in other locations. In the lymph nodes, B lymphocytes are localized in **follicles**, which make up the outer **cortex** of the lymph node, with T cells more



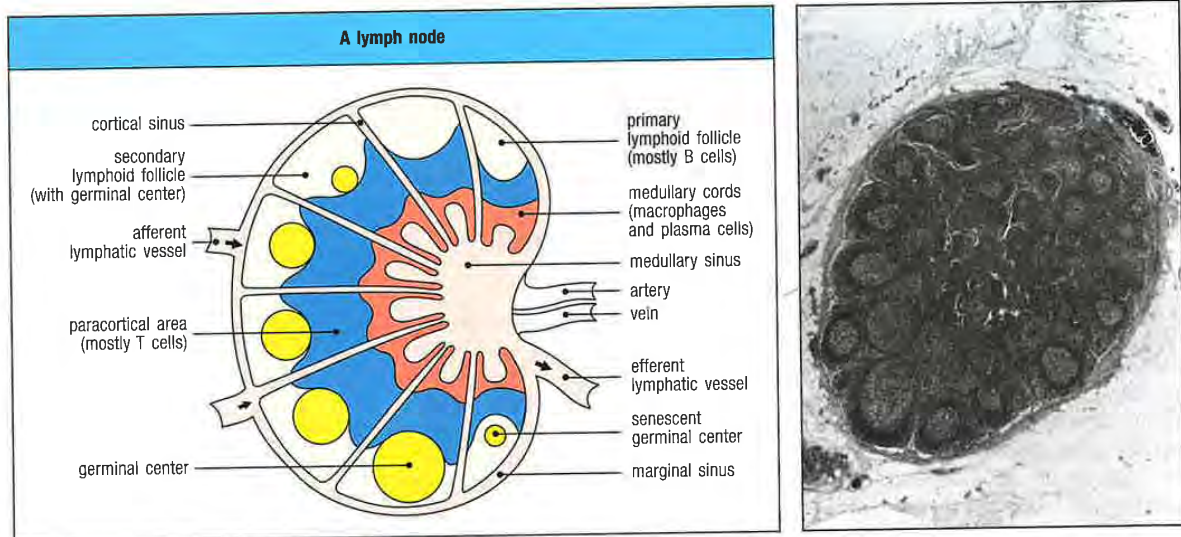


Fig. 1.22 Organization of a lymph node. As shown at left in the diagram of a lymph node in longitudinal section, a lymph node consists of an outermost cortex and an inner medulla. The cortex is composed of an outer cortex of B cells organized into lymphoid follicles and of adjacent, or paracortical, areas made up mainly of T cells and dendritic cells. When an immune response is under way, some of the follicles—known as secondary lymphoid follicles—contain central areas of intense B-cell proliferation called germinal centers. These reactions are very dramatic, but eventually die out as germinal centers become senescent. Lymph draining from the extracellular spaces of the body carries antigens in phagocytic

dendritic cells and phagocytic macrophages from the tissues to the lymph node via the afferent lymphatics. These migrate directly from the sinuses into the cellular parts of the node. Lymph leaves via the efferent lymphatics in the medulla. The medulla consists of strings of macrophages and antibody-secreting plasma cells known as the medullary cords. Naive lymphocytes enter the node from the bloodstream through specialized postcapillary venules (not shown) and leave with the lymph through the efferent lymphatic. The light micrograph (right) shows a transverse section through a lymph node, with prominent follicles containing germinal centers. Magnification $\times 7$. Photograph courtesy of N. Rooney.

diffusely distributed in the surrounding **paracortical areas**, also referred to as the **deep cortex** or **T-cell zones** (see Fig. 1.22). Lymphocytes migrating from the blood into lymph nodes enter the paracortical areas first, and because they are attracted by the same chemokines, antigen-presenting dendritic cells and macrophages also become localized there. Free antigen diffusing through the lymph node can become trapped on these dendritic cells and macrophages. This juxtaposition of antigen, antigen-presenting cells, and naive T cells in the T-cell zone creates an ideal environment in which naive T cells can bind their specific antigen and thus become activated.

As noted earlier, activation of B cells usually requires not only antigen, which binds to the B-cell receptor, but also the cooperation of activated helper T cells, a type of effector T cell. The location of B cells and T cells within the lymph node is dynamically regulated by their state of activation. When they become activated, T cells and B cells both move to the border of the follicle and T-cell zone, where T cells can first provide their helper function to B cells. Some of the B-cell follicles include **germinal centers**, where activated B cells are undergoing intense proliferation and differentiation into plasma cells. These mechanisms are described in detail in Chapter 10.

In humans, the spleen is a fist-sized organ situated just behind the stomach (see Fig. 1.18). It has no direct connection with the lymphatic system; instead, it collects antigen from the blood and is involved in immune responses to blood-borne pathogens. Lymphocytes enter and leave the spleen via blood vessels. The spleen also collects and disposes of senescent red blood cells. Its organization is shown schematically in Fig. 1.23. The bulk of the spleen is composed of **red pulp**, which is the site of red blood cell disposal. The lymphocytes surround the arterioles running through the spleen, forming isolated

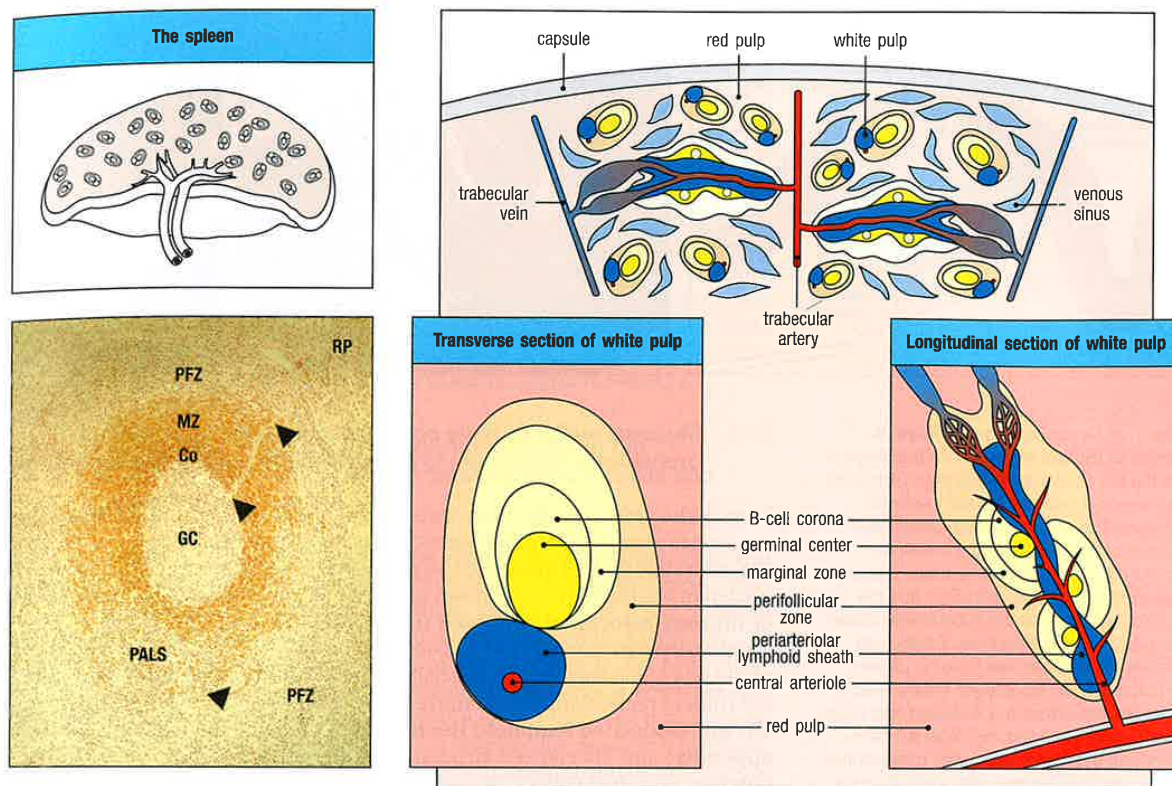


Fig. 1.23 Organization of the lymphoid tissues of the spleen.

The schematic at top left shows that the spleen consists of red pulp (pink areas), which is a site of red blood cell destruction, interspersed with the lymphoid white pulp. An enlargement of a small section of a human spleen (top right) shows the arrangement of discrete areas of white pulp (yellow and blue) around central arterioles. Most of the white pulp is shown in transverse section, with two portions in longitudinal section. The two schematics below this diagram show enlargements of a transverse section (bottom center) and longitudinal section (bottom right) of white pulp. Surrounding the central arteriole is the periarteriolar lymphoid sheath (PALS), made up of T cells. Lymphocytes and antigen-loaded dendritic cells come together here. The follicles consist mainly of B cells; in secondary follicles, a germinal center is surrounded by a B-cell corona. The follicles are surrounded by a so-called marginal zone

of lymphocytes. In each area of white pulp, blood carrying both lymphocytes and antigen flows from a trabecular artery into a central arteriole. From this arteriole smaller blood vessels fan out, eventually terminating in a specialized zone in the human spleen called the perfollicular zone (PFZ), which surrounds each marginal zone. Cells and antigen then pass into the white pulp through open blood-filled spaces in the perfollicular zone. The light micrograph at bottom left shows a transverse section of white pulp of human spleen immunostained for mature B cells. Both follicle and PALS are surrounded by the perfollicular zone. The follicular arteriole emerges in the PALS (arrowhead at bottom), traverses the follicle, goes through the marginal zone, and opens into the perfollicular zone (upper arrowheads). Co, follicular B-cell corona; GC, germinal center; MZ, marginal zone; RP, red pulp; arrowheads, central arteriole. Photograph courtesy of N. M. Milicevic.

areas of **white pulp**. The sheath of lymphocytes around an arteriole is called the **periarteriolar lymphoid sheath (PALS)** and contains mainly T cells. Lymphoid follicles occur at intervals along it, and these contain mainly B cells. An area called the **marginal zone** surrounds the follicle; it has few T cells, is rich in macrophages, and has a resident, noncirculating population of B cells known as **marginal zone B cells**. These B cells are poised to rapidly produce antibodies that have low affinity to bacterial capsular polysaccharides. These antibodies, which are discussed in Chapter 8, provide some degree of protection before the adaptive immune response is fully activated. Blood-borne microbes, soluble antigens, and antigen:antibody complexes are filtered from the blood by macrophages and immature dendritic cells within the marginal zone. Like the migration of immature dendritic cells from peripheral tissues to the T-cell areas of lymph nodes, dendritic cells in the marginal zones in the spleen migrate to the T-cell areas after taking up antigen and becoming activated; here they are able to present the antigens they carry to T cells.

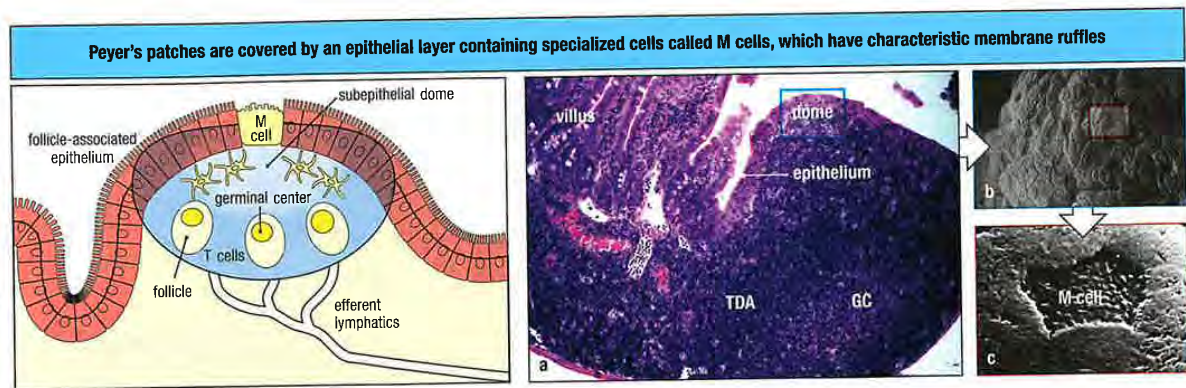


Fig. 1.24 Organization of a Peyer's patch in the gut mucosa. As the diagram on the left shows, a Peyer's patch contains numerous B-cell follicles with germinal centers. The areas between follicles are occupied by T cells and are therefore called the T-cell dependent areas. The layer between the surface epithelium and the follicles is known as the subepithelial dome, and is rich in dendritic cells, T cells, and B cells. Peyer's patches have no afferent lymphatics, and the antigen enters directly from the gut across a specialized epithelium made up of so-called microfold (M) cells. Although this tissue looks very different from other lymphoid organs, the basic divisions are maintained. As in the lymph nodes, lymphocytes enter Peyer's patches from the blood across the walls of high endothelial venules (not shown), and leave via the efferent lymphatic. The light micrograph in panel a shows a section through a Peyer's patch in the gut wall of a mouse. The Peyer's patch can be seen lying beneath the epithelial tissues. GC, germinal center; TDA, T-cell dependent area. Panel b, a scanning electron micrograph of the follicle-associated epithelium boxed in panel a, shows the M cells, which lack the microvilli and the mucus layer present on normal epithelial cells. Each M cell appears as a sunken area on the epithelial surface. Panel c, a higher-magnification view of the boxed area in panel b, shows the characteristic ruffled surface of an M cell. M cells are the portal of entry for many pathogens and other particles. Panel a, hematoxylin and eosin stain, magnification $\times 100$; panel b, $\times 5000$; panel c, $\times 23,000$.

1-17 Mucosal surfaces have specialized immune structures that orchestrate responses to environmental microbial encounters.

Most pathogens enter the body through mucosal surfaces, and these are also exposed to a vast load of other potential antigens from the air, food, and the natural microbial flora of the body. Mucosal surfaces are protected by an extensive system of lymphoid tissues known generally as the **mucosal immune system** or **mucosa-associated lymphoid tissues (MALT)**. Collectively, the mucosal immune system is estimated to contain as many lymphocytes as all the rest of the body, and they form a specialized set of cells obeying somewhat different rules of recirculation from those in the other peripheral lymphoid organs. The **gut-associated lymphoid tissues (GALT)** include the **tonsils**, **adenoids**, **appendix**, and specialized structures in the small intestine called **Peyer's patches**, and they collect antigen from the epithelial surfaces of the gastrointestinal tract. In Peyer's patches, which are the most important and highly organized of these tissues, the antigen is collected by specialized epithelial cells called **microfold** or **M cells** (Fig. 1.24). The lymphocytes form a follicle consisting of a large central dome of B lymphocytes surrounded by smaller numbers of T lymphocytes. Dendritic cells resident within the Peyer's patch present the antigen to T lymphocytes. Lymphocytes enter Peyer's patches from the blood and leave through efferent lymphatics. Effector lymphocytes generated in Peyer's patches travel through the lymphatic system and into the bloodstream, from where they are disseminated back into mucosal tissues to carry out their effector actions.

Similar but more diffuse aggregates of lymphocytes are present in the respiratory tract and other mucosa: **nasal-associated lymphoid tissue (NALT)** and **bronchus-associated lymphoid tissue (BALT)** are present in the respiratory tract. Like the Peyer's patches, these mucosal lymphoid tissues are also overlaid by M cells, through which inhaled microbes and antigens that become trapped in the mucous covering of the respiratory tract can pass. The mucosal immune system is discussed in Chapter 12.

Although very different in appearance, the lymph nodes, spleen, and mucosa-associated lymphoid tissues all share the same basic architecture. They all operate on the same principle, trapping antigens and antigen-presenting cells from sites of infection in order to present antigen to migratory small lymphocytes, thus inducing adaptive immune responses. The peripheral lymphoid tissues also provide sustaining signals to lymphocytes that do not encounter their specific antigen immediately, so that they survive and continue to recirculate.

Because they are involved in initiating adaptive immune responses, the peripheral lymphoid tissues are not static structures but vary quite markedly,

depending on whether or not infection is present. The diffuse mucosal lymphoid tissues may appear in response to infection and then disappear, whereas the architecture of the organized tissues changes in a more defined way during an infection. For example, the B-cell follicles of the lymph nodes expand as B lymphocytes proliferate to form germinal centers (see Fig. 1.22), and the entire lymph node enlarges, a phenomenon familiarly known as swollen glands.

Finally, specialized populations of lymphocytes and innate lymphoid cells can be found distributed throughout particular sites in the body rather than being found in organized lymphoid tissues. Such sites include the liver and the lamina propria of the gut, as well as the base of the epithelial lining of the gut, reproductive epithelia, and, in mice but not in humans, the epidermis. These lymphocyte populations seem to have an important role in protecting these tissues from infection, and are described further in Chapters 8 and 12.

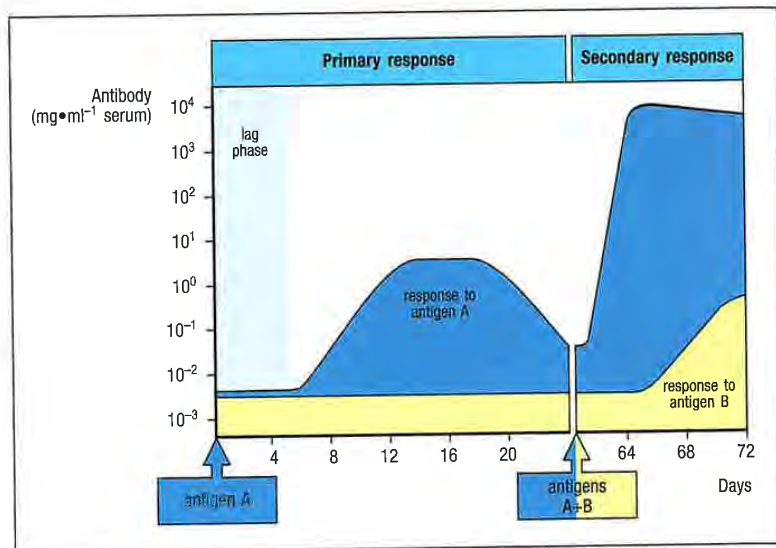
1-18 Lymphocytes activated by antigen proliferate in the peripheral lymphoid organs, generating effector cells and immunological memory.

The great diversity of lymphocyte receptor repertoire means that there will usually be some lymphocytes bearing a receptor for any given foreign antigen. Recent experiments suggest this number to be perhaps a few hundred per mouse, certainly not enough to mount a response against a pathogen. To generate sufficient antigen-specific effector lymphocytes to fight an infection, a lymphocyte with an appropriate receptor specificity is activated first to proliferate. Only when a large clone of identical cells has been produced do these finally differentiate into effector cells, a process that requires 4 to 5 days. This means that the adaptive immune response to a pathogen occurs several days after the initial infection has occurred and been detected by the innate immune system.

On recognizing its specific antigen on an activated antigen-presenting cell, a naive lymphocyte stops migrating, the volume of the nucleus and cytoplasm increases, and new mRNAs and new proteins are synthesized. Within a few hours, the cell looks completely different and is known as a **lymphoblast**. Dividing lymphoblasts are able to duplicate themselves two to four times every 24 hours for 3–5 days, so that a single naive lymphocyte can produce a clone of around 1000 daughter cells of identical specificity. These then differentiate into effector cells. In the case of B cells, the differentiated effector cells are the plasma cells, which secrete antibody. In the case of T cells, the effector cells are either cytotoxic T cells, which are able to destroy infected cells, or helper T cells, which activate other cells of the immune system (see Section 1-8).

Effector lymphocytes do not recirculate like naive lymphocytes. Some effector T cells detect sites of infection and migrate into them from the blood; others stay in the lymphoid tissues to activate B cells. Some antibody-secreting plasma cells remain in the peripheral lymphoid organs, but most plasma cells generated in the lymph nodes and spleen will migrate to the bone marrow and take up residence there, secreting large amounts of antibodies into the blood system. Effector cells generated in the mucosal immune system generally stay within the mucosal tissues. Most lymphocytes generated by clonal expansion in an immune response will eventually die. However, a significant number of activated antigen-specific B cells and T cells persist after antigen has been eliminated. These cells are known as **memory cells** and form the basis of immunological memory. They can be reactivated much more quickly than naive lymphocytes, which ensures a more rapid and effective response on a second encounter with a pathogen and thereby usually provides lasting protective immunity.

Fig. 1.25 The course of a typical antibody response. The first encounter with an antigen produces a primary response. Antigen A introduced at time zero encounters little specific antibody in the serum. After a lag phase (light blue), antibody against antigen A (dark blue) appears; its concentration rises to a plateau and then gradually declines, typical of a primary response. When the serum is tested for antibody against another antigen, B (yellow), there is little preset. When the animal is later challenged with a mixture of antigens A and B, a very rapid and intense antibody secondary response to A occurs, illustrating immunological memory. This is the main reason for giving booster injections after an initial vaccination. Note that the response to B resembles the primary response to A, as this is the first encounter with antigen B.



The characteristics of immunological memory are readily observed by comparing an individual's antibody response to a first or **primary immunization** with the response to a **secondary** or **booster immunization** with the same antigen. As shown in Fig. 1.25, the secondary antibody response occurs after a shorter lag phase and achieves a markedly higher level than in the primary response. During the secondary responses, antibodies can also acquire higher affinity, or strength of binding, for the antigen due to a process called **affinity maturation**, which takes place in the specialized germinal centers within B-cell follicles (see Section 1-16). Importantly, helper T cells are required for the process of affinity maturation, but T-cell receptors do not undergo affinity maturation. Compared with naive T cells, memory T cells show a lower threshold for activation, but as a result of changes in the responsiveness of the cell and not because of changes in the receptor. We describe the mechanisms of affinity maturation in Chapters 5 and 10.

The cellular basis of immunological memory is the clonal expansion and clonal differentiation of cells that have a specific attraction for the eliciting antigen, and the memory is therefore entirely antigen-specific. It is immunological memory that enables successful vaccination and prevents reinfection with pathogens that have been repelled successfully by an adaptive immune response. In Chapter 11, we will return to immunological memory, which is perhaps the most important biological consequence of adaptive immunity.

Summary.

While the innate immune system relies on invariant pattern recognition receptors to detect common microbial structures or the damage they cause, the adaptive immune system relies on a repertoire of antigen receptors to recognize structures that are specific to individual pathogens. This feature provides adaptive immunity with greater sensitivity and specificity. The clonal expansion of antigen-reactive lymphocytes also confers the property of immunological memory, which enhances protection against reinfection by the same pathogen.

Adaptive immunity relies on two major types of lymphocytes. B cells mature in the bone marrow and are the source of circulating antibodies. T cells mature in the thymus and recognize peptides from pathogens presented by MHC

molecules on infected cells or antigen-presenting cells. An adaptive response involves the selection and amplification of clones of lymphocytes bearing receptors that recognize the foreign antigen. This clonal selection provides the theoretical framework for understanding all the key features of an adaptive immune response.

Each lymphocyte carries cell-surface receptors of a single antigen specificity. These receptors are generated by the random recombination of variable receptor gene segments and the pairing of distinct variable protein chains: heavy and light chains in immunoglobulins, or the two chains of T-cell receptors. The large antigen-receptor repertoire of lymphocytes can recognize virtually any antigen. Adaptive immunity is initiated when an innate immune response fails to eliminate a new infection and activated antigen-presenting cells—typically dendritic cells that bear antigens from pathogens and co-stimulatory receptors—migrate to the draining lymphoid tissues.

Immune responses are initiated in several peripheral lymphoid tissues. The spleen serves as a filter for blood-borne infections. Lymph nodes draining various tissues and the mucosal and gut-associated lymphoid tissues (MALT and GALT) are organized into specific zones where T and B cells can be activated efficiently by antigen-presenting cells or helper T cells. When a recirculating lymphocyte encounters its corresponding antigen in these peripheral lymphoid tissues, it proliferates, and its clonal progeny differentiate into effector T and B lymphocytes that can eliminate the infectious agent. A subset of these proliferating lymphocytes differentiates into memory cells, ready to respond rapidly to the same pathogen if it is encountered again. The details of these processes of recognition, development, and differentiation form the main material of the central three parts of this book.

The effector mechanisms of immunity.

For activated innate and adaptive immune cells to destroy pathogens, they must employ an appropriate effector mechanism suited to each infecting agent. The different types of pathogens noted in Fig. 1.26 have different lifestyles and require different responses for both their recognition and their destruction. Perhaps it is not surprising, then, that defenses against different pathogen types are organized into **effector modules** suited for these different lifestyles. In this sense, an effector module is a collection of cell-mediated and humoral mechanisms, both innate and adaptive, that act together to achieve

The immune system protects against four classes of pathogens		
Type of pathogen	Examples	Diseases
Viruses (intracellular)	Variola Influenza Varicella	Smallpox Flu Chickenpox
Intracellular bacteria, protozoa, parasites	<i>Mycobacterium leprae</i> <i>Leishmania donovani</i> <i>Plasmodium falciparum</i> <i>Toxoplasma gondii</i>	Leprosy Leishmaniasis Malaria Toxoplasmosis
Extracellular bacteria, parasites, fungi	<i>Streptococcus pneumoniae</i> <i>Clostridium tetani</i> <i>Trypanosoma brucei</i> <i>Pneumocystis jirovecii</i>	Pneumonia Tetanus Sleeping sickness <i>Pneumocystis pneumonia</i>
Parasitic worms (extracellular)	<i>Ascaris</i> <i>Schistosoma</i>	Ascariasis Schistosomiasis

Fig. 1.26 The major types of pathogens confronting the immune system, and some of the diseases they cause.

elimination of a particular category of pathogen. For example, defense against extracellular pathogens can involve both phagocytic cells and B cells, which recognize extracellular antigens and become plasma cells that secrete antibody into the extracellular environment. Defense against intracellular pathogens involves T cells that can detect peptides generated inside the infected cell. Some effector T cells directly kill cells infected with intracellular pathogens such as viruses. Moreover, activated T cells differentiate into three major subsets of **helper T cells**, which produce different patterns of cytokines. These three subsets, discussed below, generally specialize in promoting defenses against pathogens having three major lifestyles: they can defend against intracellular infection, destroy extracellular bacteria and fungi, or provide barrier immunity directed at parasites. T cells also promote defense against extracellular pathogens by helping B cells make antibody.

Most of the other effector mechanisms used by an adaptive immune response to dispose of pathogens are the same as those of innate immunity and involve cells such as macrophages and neutrophils, and proteins such as complement. Indeed, it seems likely that the vertebrate adaptive immune response evolved by the addition of specific recognition properties to innate defense mechanisms already existing in invertebrates. This is supported by recent findings that the innate lymphoid cells—the ILCs—show similar patterns of differentiation into different cytokine-producing subsets to those of T cells.

We begin this section by outlining the effector actions of antibodies, which depend almost entirely on recruiting cells and molecules of the innate immune system.

1-19 Innate immune responses can select from several effector modules to protect against different types of pathogens.

As we mentioned in Section 1-7, the innate immune system contains several types of cells—NK cells and ILCs—that have similarities to lymphocytes, particularly T cells. NK cells lack the antigen-specific receptors of T cells, but can exhibit the cytotoxic capacity of T cells and produce some of the cytokines that effector T cells produce. ILCs develop from the same progenitor cells in the bone marrow as NK cells, and they also lack antigen-specific receptors. Very recent discoveries indicate that ILCs actually comprise several closely related lineages that differ in the specific cytokines that they will produce when activated. Remarkably, there is a striking similarity between the patterns of cytokines produced by ILC subsets and helper T-cell subsets, as mentioned above. It appears that subsets of ILCs are the innate homologs of their helper T-cell counterparts, and NK cells are the innate homolog of cytotoxic T cells.

As mentioned in Section 1-6, there are a large number of cytokines with different functions (see Appendix III). A convenient way to organize the effects of cytokines is by the effector module that each cytokine promotes. Some cytokines tend to promote immunity to intracellular pathogens. One such cytokine is **interferon- γ** , which acts both by activating phagocytes to more efficiently kill intracellular pathogens and by inducing target tissues to resist intracellular pathogens. This is called **type 1 immunity**. IFN- γ is produced by some but not all subsets of innate and adaptive lymphocytes, and the subset of ILC making IFN- γ is called **ILC1**. Other ILC subsets produce cytokines favoring effector modules called **type 2** and **type 3**, which coordinate defense against parasitic and extracellular pathogens, respectively. The modular nature of immune effector functions will be encountered frequently throughout this book. One principle seems to be that activated sensor cells from either the innate or the adaptive immune system can activate different subsets of innate or adaptive lymphocytes that are specialized for amplifying particular effector modules that are directed against different categories of pathogens (Fig. 1.27).

Effector module	Cell types, functions, and mechanisms
Cytotoxicity	NK cells, CD8 T cells
	Elimination of virally infected and metabolically stressed cells
Intracellular immunity (Type 1)	ILC1, T _H 1 cells
	Elimination of intracellular pathogens; activation of macrophages
Mucosal and barrier immunity (Type 2)	ILC2, T _H 2 cells
	Elimination and expulsion of parasites; recruitment of eosinophils, basophils, and mast cells
Extracellular immunity (Type 3)	ILC3, T _H 17 cells
	Elimination of extracellular bacteria and fungi; recruitment and activation of neutrophils

Fig. 1.27 Innate and adaptive lymphocyte cells share a variety of functions. The different effector modules are served by both innate and adaptive immune mechanisms. For each of the four major types of innate lymphocytes, there is a corresponding type of T cell with generally similar functional characteristics. Each set of innate lymphocyte and T cell exert an effector activity that is broadly directed at a distinct category of pathogen.

1-20 Antibodies protect against extracellular pathogens and their toxic products.

Antibodies are found in plasma—the fluid component of blood—and in extracellular fluids. Because body fluids were once known as humors, immunity mediated by antibodies is known as **humoral immunity**.

Antibodies are Y-shaped molecules with two identical antigen-binding sites and one constant, or Fc, region. As mentioned in Section 1-9, there are five forms of the constant region of an antibody, known as the antibody **classes** or **isotypes**. The constant region determines an antibody's functional properties—how it will engage with the effector mechanisms that dispose of antigen once it is recognized. Each class carries out its particular function by engaging a distinct set of effector mechanisms. We describe the antibody classes and their actions in Chapters 5 and 10.

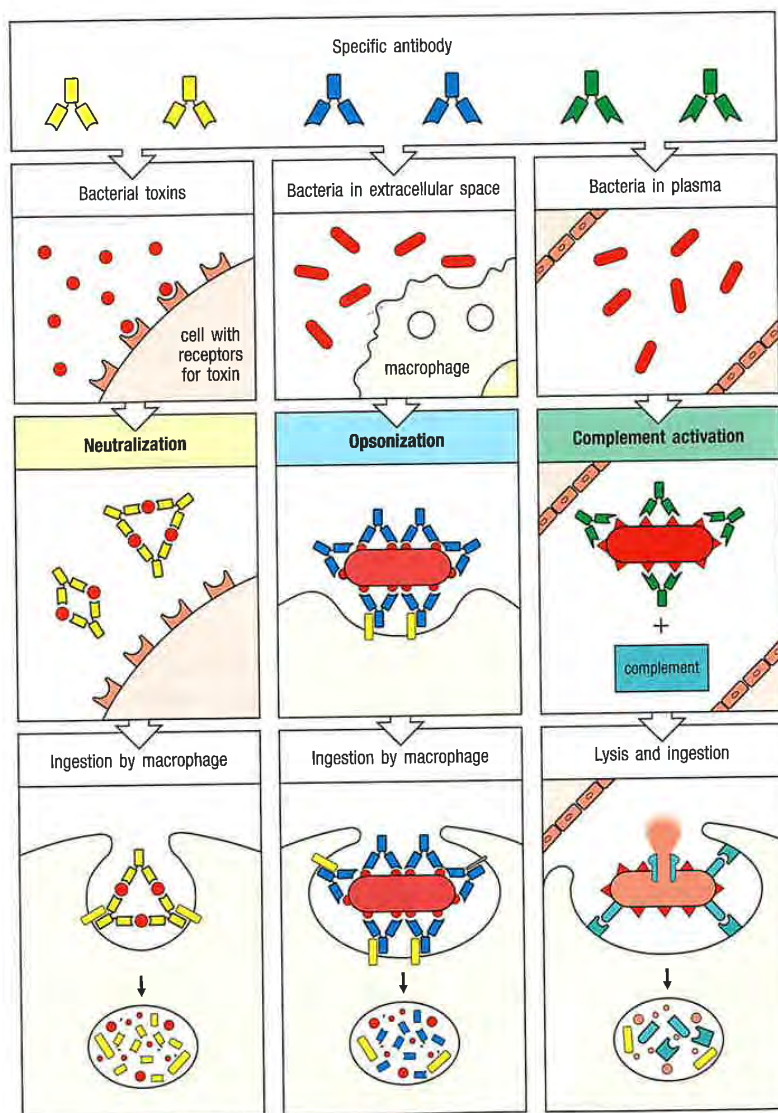
The first and most direct way in which antibodies can protect against pathogens or their products is by binding to them and thereby blocking their access to cells that they might infect or destroy (Fig. 1.28, left panels). This is known as **neutralization** and is important for protection against viruses, which become prevented from entering cells and replicating, and against bacterial toxin and is the form of immunity elicited by most vaccines.

For bacteria, however, binding by antibodies is not sufficient to stop their replication. In this case, the function of the antibody is to enable a phagocytic cell such as a macrophage or a neutrophil to ingest and destroy the bacterium. Many bacteria evade the innate immune system because they have an outer coat that is not recognized by the pattern recognition receptors of phagocytes. However, antigens in the coat can be recognized by antibodies, and phagocytes have receptors, called **Fc receptors**, that bind the constant region and facilitate phagocytosis of the bacterium (see Fig. 1.28, center panels). The coating of pathogens and foreign particles in this way is known as **opsonization**.

The third function of antibodies is **complement activation**. In Section 1-2 we briefly mentioned Bordet's discovery of complement as a serum factor that 'complements' the activities of antibodies. Complement can be activated by microbial surfaces even without the help of antibodies, which leads to the covalent deposition of certain complement proteins onto the bacterial surface. But when an antibody binds first to the bacterial surface, its constant region provides a platform that is much more efficient in complement activation than

Fig. 1.28 Antibodies can participate in host defense in three main ways.

The left panels show antibodies binding to and neutralizing a bacterial toxin, thus preventing it from interacting with host cells and causing pathology. Unbound toxin can react with receptors on the host cell, whereas the toxin:antibody complex cannot. Antibodies also neutralize complete virus particles and bacterial cells by binding and inactivating them. The antigen:antibody complex is eventually scavenged and degraded by macrophages. Antibodies coating an antigen render it recognizable as foreign by phagocytes (macrophages and neutrophils), which then ingest and destroy it; this is called opsonization. The center panels show activation of the complement system by antibodies coating a bacterial cell. Bound antibodies form a platform that activates the first protein in the complement system, which deposits complement proteins (blue) on the surface of the bacterium. This can lead in some cases to formation of a pore that lyses the bacterium directly. More generally, complement proteins on the bacterium can be recognized by complement receptors on phagocytes; this stimulates the phagocytes to ingest and destroy the bacterium. Thus, antibodies target pathogens and their toxic products for disposal by phagocytes.



microbial activation alone. Thus, once antibodies are produced, complement activation against a pathogen can be substantially increased.

Certain complement components that are deposited on the bacterial surface can directly lyse the membranes of some bacteria, and this is important in a few bacterial infections (see Fig. 1.28, right panels). The major function of complement, however, is to enable phagocytes to engulf and destroy bacteria that the phagocytes would not otherwise recognize. Most phagocytes express receptors that bind certain complement proteins; called **complement receptors**, these receptors bind to the complement proteins deposited onto the bacterial surface and thus facilitate bacterial phagocytosis. Certain other complement proteins also enhance the phagocytes' bactericidal capacity. The end result is that all pathogens and free molecules bound by antibody are eventually delivered to phagocytes for ingestion, degradation, and removal from the body (see Fig. 1.28, bottom panels). The complement system and the phagocytes that antibodies recruit are not themselves antigen-specific; they depend upon antibody molecules to mark the particles as foreign.

1-21 T cells orchestrate cell-mediated immunity and regulate B-cell responses to most antigens.

Some bacteria and parasites, and all viruses, replicate inside cells, where they cannot be detected by antibodies, which access only the blood and extracellular space. The destruction of intracellular invaders is the function of the T lymphocytes, which are responsible for the **cell-mediated immune responses** of adaptive immunity. But T lymphocytes participate in responses to a wide variety of pathogens, including extracellular organisms, and so must exert a wide variety of effector activities.

T lymphocytes, of which there are several types, develop in the thymus. They are characterized by the type of T-cell receptors they express and by the expression of certain markers. The two main classes of T cells express either a cell-surface protein called **CD8** or another called **CD4**. These are not just random markers, but are important for a T cell's function, because they help to determine the interactions between the T cell and other cells. Recall from Section 1-10 that T cells detect peptides derived from foreign antigens that are displayed by MHC molecules on a cell's surface. CD8 and CD4 function in antigen recognition by recognizing different regions of MHC molecules and by being involved in the signaling of the T-cell receptor that is engaged with its antigen. Thus, CD4 and CD8 are known as **co-receptors** and they provide a functional difference between CD8 and CD4 T cells,

Importantly, there are two main types of MHC molecules, called **MHC class I** and **MHC class II**. These have slightly different structures, but both have an elongated groove on the outer surface that can bind a peptide (Fig. 1.29). The peptide becomes trapped in this groove during the synthesis and assembly of the MHC molecule inside the cell, and the peptide:MHC complex is then transported to the cell surface and displayed to T cells (Fig. 1.30). Because CD8 recognizes a region of the MHC class I protein while CD4 recognizes a region of MHC class II protein, the two co-receptors functionally distinguish T cells. Therefore, CD8 T cells selectively recognize peptides that are bound to MHC class I molecules, while CD4 T cells recognize peptides presented by MHC class II.

The most direct action of T cells is cytotoxicity. Cytotoxic T cells are effector T cells that act against cells infected with viruses. Antigens derived from the virus multiplying inside the infected cell are displayed on the cell's surface, where they are recognized by the antigen receptors of cytotoxic T cells. These T cells can then control the infection by directly killing the infected cell before viral replication is complete and new viruses are released (Fig. 1.31). Cytotoxic T cells carry CD8, and so recognize antigen presented by MHC class I molecules. Because MHC class I molecules are expressed on most cells of the body, they serve as an important mechanism to defend against viral infections.

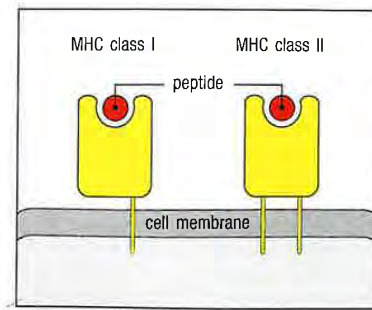


Fig. 1.29 MHC molecules on the cell surface display peptide fragments of antigens. MHC molecules are membrane proteins whose outer extracellular domains form a cleft in which a peptide fragment is bound. These fragments are derived from proteins degraded inside the cell and include both self and foreign protein antigens. The peptides are bound by the newly synthesized MHC molecule before it reaches the cell surface. There are two kinds of MHC molecules, MHC class I and MHC class II; they have related but distinct structures and functions. Although not shown here for simplicity, both MHC class I and MHC class II molecules are trimers of two protein chains and the bound self or nonself peptide.

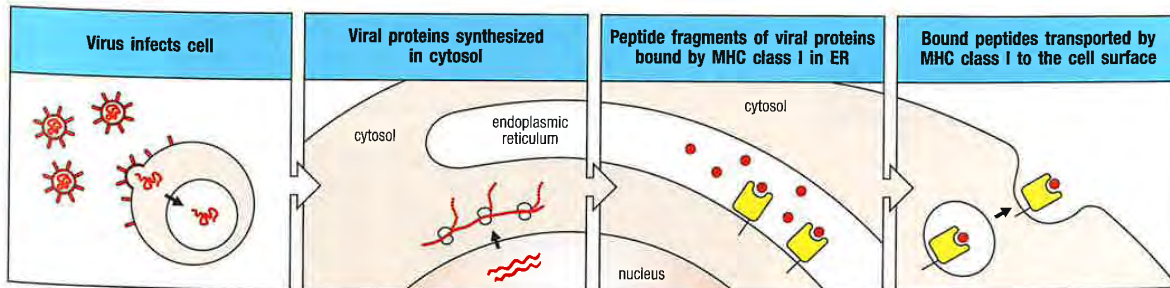
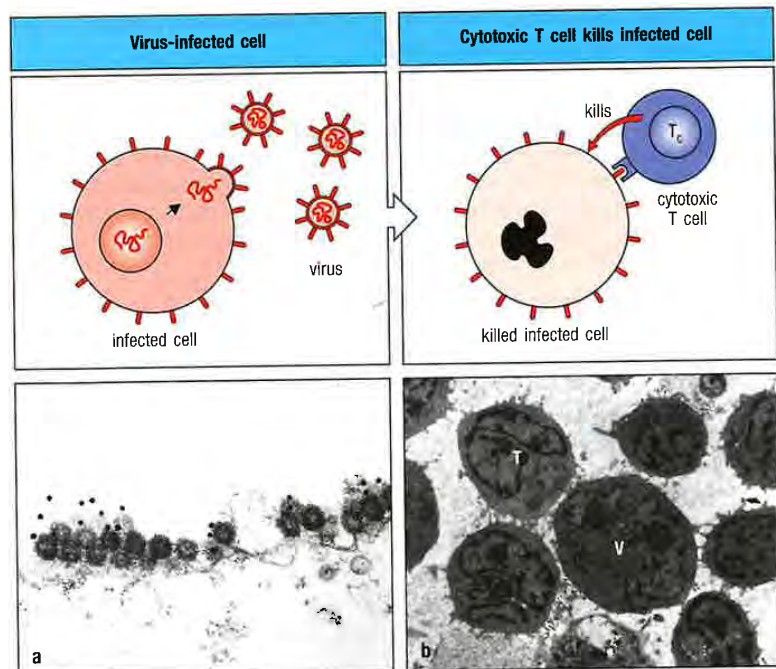


Fig. 1.30 MHC class I molecules present antigen derived from proteins in the cytosol. In cells infected with viruses, viral proteins are synthesized in the cytosol. Peptide fragments of viral proteins are transported into the endoplasmic reticulum (ER), where they are bound by MHC class I molecules, which then deliver the peptides to the cell surface.

Fig. 1.31 Mechanism of host defense against intracellular infection by viruses.

Cells infected by viruses are recognized by specialized T cells called cytotoxic T cells, which kill the infected cells directly. The killing mechanism involves the activation of enzymes known as caspases, which contain cysteine in their active site and cleave target proteins at aspartic acid. The caspases in turn activate a cytosolic nuclease that cleaves host and viral DNA in the infected cell. Panel a is a transmission electron micrograph showing the plasma membrane of a cultured CHO cell (the Chinese hamster ovary cell line) infected with influenza virus. Many virus particles can be seen budding from the cell surface. Some of these have been labeled with a monoclonal antibody that is specific for a viral protein and is coupled to gold particles, which appear as the solid black dots in the micrograph. Panel b is a transmission electron micrograph of a virus-infected cell (V) surrounded by cytotoxic T lymphocytes. Note the close apposition of the membranes of the virus-infected cell and the T cell (T) in the upper left corner of the micrograph, and the clustering of the cytoplasmic organelles in the T cell between its nucleus and the point of contact with the infected cell. Panel a courtesy of M. Bui and A. Helenius; panel b courtesy of N. Rooney.



MHC class I molecules bearing viral peptides are recognized by CD8-bearing cytotoxic T cells, which then kill the infected cell (Fig. 1.32).

CD4 T cells recognize antigen presented by MHC class II proteins, which are expressed by the predominant antigen-presenting cells of the immune system: dendritic cells, macrophages, and B cells (Fig. 1.33). Thus CD4 T cells tend to recognize antigens taken up by phagocytosis from the extracellular environment. CD4 T cells are the helper T cells mentioned earlier in the chapter. They develop into a variety of different effector subsets, called T_H1 (for T helper type 1), T_H2 , T_H17 , and so on, and they produce cytokines in patterns similar to the subsets of ILCs mentioned earlier that activate effector modules protective against different pathogens. These subsets act primarily at sites of infection or injury in peripheral tissues. In the lymphoid tissues, a subset of CD4 T cells, called the **T follicular helper** (T_{FH}) cell, interacts with B cells to regulate antibody production during the immune response. The various T helper subsets are described later, in Chapter 9.

For example, the T_H1 subset of CD4 T cells helps to control certain bacteria that take up residence in membrane-enclosed vesicles inside macrophages. They produce the same cytokine as ILC1 cells, IFN- γ , which activates macrophages to increase their intracellular killing power and destroy these bacteria. Important infections that are controlled by this function are tuberculosis and leprosy, which are caused by the bacteria *Mycobacterium tuberculosis* and *M. leprae*, respectively. Mycobacteria survive intracellularly because they prevent the vesicles they occupy from fusing with lysosomes, which contain a variety of degradative enzymes and antimicrobial substances (Fig. 1.34). However, on its surface, the infected macrophage presents mycobacteria-derived antigens that can be recognized by activated antigen-specific T_H1 cells, which in turn secrete particular cytokines that induce the macrophage to

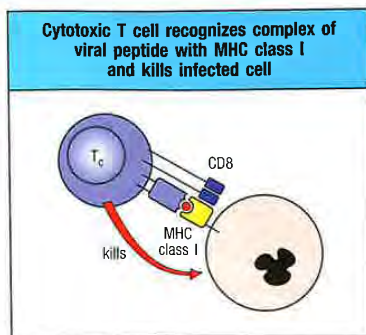


Fig. 1.32 Cytotoxic CD8 T cells recognize antigen presented by MHC class I molecules and kill the cell. The peptide:MHC class I complex on virus-infected cells is detected by antigen-specific cytotoxic T cells. Cytotoxic T cells are preprogrammed to kill the cells they recognize.

Fig. 1.33 CD4 T cells recognize antigen presented by MHC class II molecules.

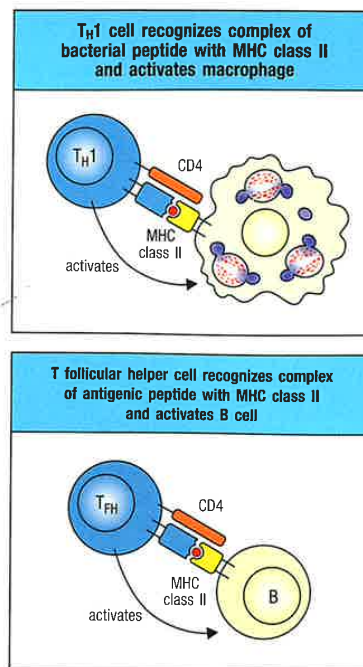
On recognition of their specific antigen on infected macrophages, T_H1 cells activate the macrophage, leading to the destruction of the intracellular bacteria (top panel). When T follicular helper (T_{FH}) cells recognize antigen on B cells (bottom panel), they activate these cells to proliferate and differentiate into antibody-producing plasma cells (not shown).

overcome the block on vesicle fusion. T_H2 and T_H17 subsets produce cytokines that are specialized for promoting responses against parasites or extracellular bacteria and fungi, respectively. CD4 T cells, and their specialized subsets, play a pervasive role in adaptive immunity, and we will be returning to them many times in this book, including in Chapters 8, 9, 11 and 12.

1-22 Inherited and acquired defects in the immune system result in increased susceptibility to infection.

We tend to take for granted the ability of our immune systems to free our bodies of infection and prevent its recurrence. In some people, however, parts of the immune system fail. In the most severe of these **immunodeficiency diseases**, adaptive immunity is completely absent, and death occurs in infancy from overwhelming infection unless heroic measures are taken. Other less catastrophic failures lead to recurrent infections with particular types of pathogens, depending on the particular deficiency. Much has been learned about the functions of the different components of the human immune system through the study of these immunodeficiencies, many of which are caused by inherited genetic defects. Because understanding the features of immunodeficiencies requires a detailed knowledge of normal immune mechanisms, we have postponed discussion of most of these diseases until Chapter 13, where they can be considered together.

More than 30 years ago, a devastating form of immunodeficiency appeared, the **acquired immune deficiency syndrome**, or **AIDS**, which is caused by an infectious agent, the human immunodeficiency viruses HIV-1 and HIV-2. This disease destroys T cells, dendritic cells, and macrophages bearing CD4, leading to infections caused by intracellular bacteria and other pathogens



Acquired Immune Deficiency Syndrome (AIDS)

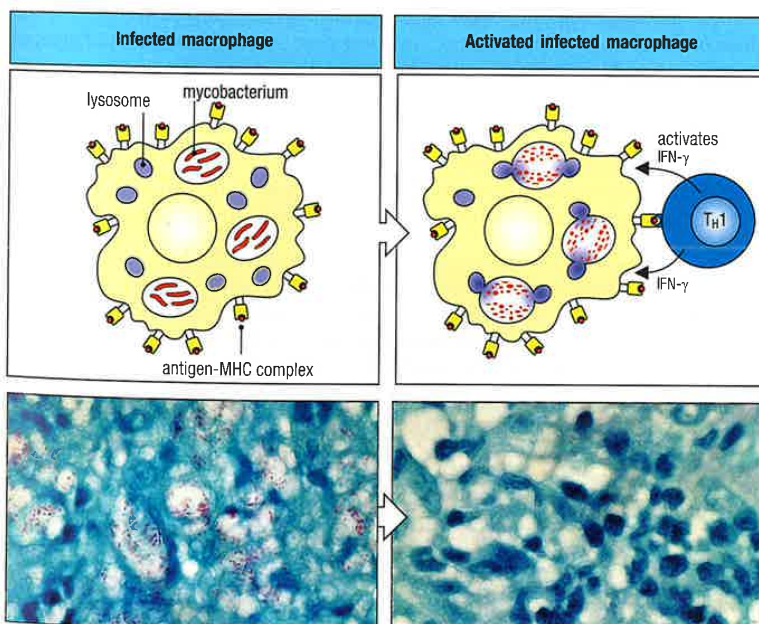


Fig. 1.34 Mechanism of host defense against intracellular infection by mycobacteria.

Mycobacteria are engulfed by macrophages but resist being destroyed by preventing the intracellular vesicles in which they reside from fusing with lysosomes containing bactericidal agents. Thus the bacteria are protected from being killed. In resting macrophages, mycobacteria persist and replicate in these vesicles. When the phagocyte is recognized and activated by a T_H1 cell, however, the phagocytic vesicles fuse with lysosomes, and the bacteria can be killed. Macrophage activation is controlled by T_H1 cells, both to avoid tissue damage and to save energy. The light micrographs (bottom row) show resting (left) and activated (right) macrophages infected with mycobacteria. The cells have been stained with an acid-fast red dye to reveal mycobacteria. These are prominent as red-staining rods in the resting macrophages but have been eliminated from the activated macrophages. Photographs courtesy of G. Kaplan.

normally controlled by such cells. These infections are the major cause of death from this increasingly prevalent immunodeficiency disease, which is discussed fully in Chapter 13 together with the inherited immunodeficiencies.

1-23 Understanding adaptive immune responses is important for the control of allergies, autoimmune disease, and the rejection of transplanted organs.

The main function of our immune system is to protect the human host from infectious agents. However, many medically important diseases are associated with a normal immune response directed against an inappropriate antigen, often in the absence of infectious disease. Immune responses directed at noninfectious antigens occur in **allergy**, in which the antigen is an innocuous foreign substance; in **autoimmune disease**, in which the response is to a self antigen; and in **graft rejection**, in which the antigen is borne by a transplanted foreign cell (discussed in Chapter 15). The major antigens provoking graft rejection are, in fact, the MHC molecules, as each of these is present in many different versions in the human population—that is, they are highly **polymorphic**—and most unrelated people differ in the set of MHC molecules they express, a property commonly known as their ‘tissue type.’ The MHC was originally recognized by the work of Peter Goren in the 1930s as a gene locus in mice, the **H-2 locus**, that controlled the acceptance or rejection of transplanted tumors, and later by **George Snell**, who examined their role in tissue transplantation by developing mouse strains differing only at these histocompatibility loci. The human MHC molecules were first discovered during the Second World War, when attempts were made to use skin grafts from donors to repair badly burned pilots and bomb victims. The patients rejected the grafts, which were recognized by their immune systems as being ‘foreign.’ What we call a successful immune response or a failure, and whether the response is considered harmful or beneficial to the host, depends not on the response itself but rather on the nature of the antigen and the circumstances in which the response occurs (Fig. 1.35). Snell was awarded the 1980 Nobel Prize for his work on MHC, together with **Baruj Benacerraf** and **Jean Dausset**.

Allergic diseases, which include asthma, are an increasingly common cause of disability in the developed world. Autoimmunity is also now recognized as the cause of many important diseases. An autoimmune response directed against pancreatic β cells is the leading cause of diabetes in the young. In allergies and autoimmune diseases, the powerful protective mechanisms of the adaptive immune response cause serious damage to the patient.

Immune responses to harmless antigens, to body tissues, or to organ grafts are, like all other immune responses, highly specific. At present, the usual way to treat these responses is with immunosuppressive drugs, which inhibit all

Fig. 1.35 Immune responses can be beneficial or harmful, depending on the nature of the antigen. Beneficial responses are shown in white, harmful responses in red shaded boxes. Where the response is beneficial, its absence is harmful.

Antigen	Effect of response to antigen	
	Normal response	Deficient response
Infectious agent	Protective immunity	Recurrent infection
Innocuous substance	Allergy	No response
Grafted organ	Rejection	Acceptance
Self organ	Autoimmunity	Self tolerance
Tumor	Tumor immunity	Cancer

immune responses, desirable and undesirable alike. If it were possible to suppress only those lymphocyte clones responsible for the unwanted response, the disease could be cured or the grafted organ protected without impeding protective immune responses. At present, antigen-specific immunoregulation is outside the reach of clinical treatment. But as we shall see in Chapter 16, many new drugs have been developed recently that offer more selective immune suppression to control autoimmune and other unwanted immune responses. Among these, therapies using highly specific **monoclonal antibodies** were made possible by **Georges Köhler** and **César Milstein**, who shared the 1984 Nobel Prize for the discovery of their production. We shall discuss the present state of understanding of allergies, autoimmune disease, graft rejection, and immunosuppressive drugs and monoclonal antibodies in Chapters 14–16, and we shall see in Chapter 15 how the mechanisms of immune regulation are beginning to emerge from a better understanding of the functional subsets of lymphocytes and the cytokines that control them.

1-24 Vaccination is the most effective means of controlling infectious diseases.

The deliberate stimulation of an immune response by immunization, or vaccination, has achieved many successes in the two centuries since Jenner's pioneering experiment. Mass immunization programs have led to the virtual eradication of several diseases that used to be associated with significant morbidity (illness) and mortality (Fig. 1.36). Immunization is considered so safe

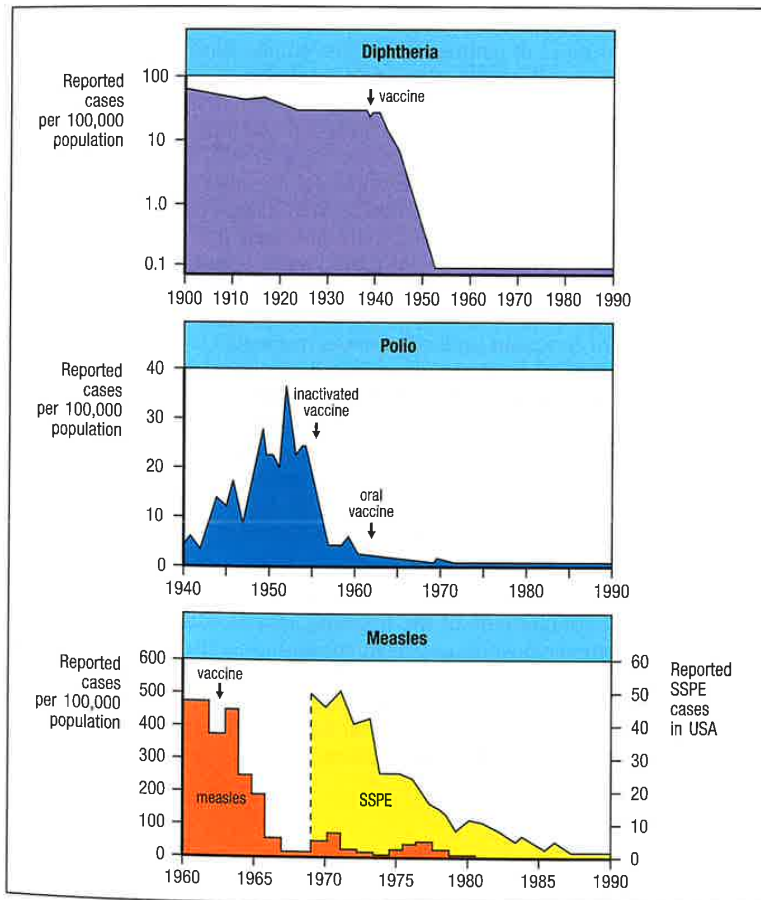


Fig. 1.36 Successful vaccination campaigns. Diphtheria, polio, and measles and their consequences have been virtually eliminated in the United States, as shown in these three graphs. SSPE stands for subacute sclerosing panencephalitis, a brain disease that is a late consequence of measles infection in a few patients. When measles was prevented, SSPE disappeared 15–20 years later. However, because these diseases have not been eradicated worldwide, immunization must be maintained in a very high percentage of the population to prevent their reappearance.

and so important that most states in the United States require children to be immunized against up to seven common childhood diseases. Impressive as these accomplishments are, there are still many diseases for which we lack effective vaccines. And even where vaccines for diseases such as measles can be used effectively in developed countries, technical and economic problems can prevent their widespread use in developing countries, where mortality from these diseases is still high.

The tools of modern immunology and molecular biology are being applied to develop new vaccines and improve old ones, and we discuss these advances in Chapter 16. The prospect of controlling these important diseases is tremendously exciting. The guarantee of good health is a critical step toward population control and economic development. At a cost of pennies per person, great hardship and suffering can be alleviated.

Many serious pathogens have resisted efforts to develop vaccines against them, often because they can evade or subvert the protective mechanisms of an adaptive immune response. We examine some of the evasive strategies used by successful pathogens in Chapter 13. The conquest of many of the world's leading diseases, including malaria and diarrheal diseases (the leading killers of children) as well as the more recent threat from AIDS, depends on a better understanding of the pathogens that cause them and their interactions with the cells of the immune system.

Summary.

The responses to infection can be organized into several effector modules that target the various types of pathogen lifestyles. Innate sensor cells that detect infection generate mediators that activate innate lymphoid cells (ILCs) and T cells, which amplify the immune response and also activate various effector modules. Innate lymphoid cells include subsets that produce different cytokines and activate distinct effector modules. T cells fall into two major classes that are based on the expression of the co-receptors CD8 and CD4; these T cells recognize antigen presented by MHC class I or MHC class II proteins, respectively. These subsets of T cells, like their ILC counterparts, also promote the actions of distinct effector modules. NK cells and CD8 T cells can exert cytotoxic activity to target intracellular infections such as viruses. Other subsets of innate lymphoid and helper T cells can secrete mediators that activate other effector functions, ones that target intracellular bacteria, extracellular bacteria and fungi, and parasites. T cells also provide signals that help regulate B cells and stimulate them to produce antibodies. Specific antibodies mediate the clearance and elimination of soluble toxins and extracellular pathogens. They interact not only with the toxins or the antigens on microbes, but also with the Fc region of specific receptors that are expressed by many types of phagocytes. Phagocytes also express receptors for complement proteins that are deposited on microbial surfaces, particularly in the presence of antibody.

Failures of immunity can be caused by genetic defects or by infections that target important components of the immune system. Misdirected immune responses can damage host tissues, as in autoimmune diseases or allergy, or lead to the failure of transplanted organs. While vaccination is still the greatest tool of immunology to fight diseases, modern approaches have added new tools, such as monoclonal antibodies, that have become progressively more important in the clinic over the past two decades.

Summary to Chapter 1.

The immune system defends the host against infection. Innate immunity serves as a first line of defense but lacks the ability to recognize certain pathogens and

to provide the specific protective immunity that prevents reinfection. Adaptive immunity is based on clonal selection from a repertoire of lymphocytes bearing highly diverse antigen-specific receptors that enable the immune system to recognize any foreign antigen. In the adaptive immune response, antigen-specific lymphocytes proliferate and differentiate into clones of effector lymphocytes that eliminate the pathogen. Figure 1.7 summarizes the phases of the immune response and their approximate timings. Host defense requires different recognition systems and a wide variety of effector mechanisms to seek out and destroy the wide variety of pathogens in their various habitats within the body and at its external and internal surfaces. Not only can the adaptive immune response eliminate a pathogen, but, in the process, it also generates increased numbers of differentiated memory lymphocytes through clonal selection, and this allows a more rapid and effective response upon reinfection. The regulation of immune responses, whether to suppress them when unwanted or to stimulate them in the prevention of infectious disease, is the major medical goal of research in immunology.

Questions.

- 1.1 Multiple Choice:** Which of the following examples can be considered an illustration of vaccination?
- Inoculating an individual with cowpox in order to protect that individual against smallpox
 - Administering the serum of animals immune to diphtheria to protect against the effect of diphtheria toxin in an exposed individual
 - A bacterial infection that results in complement activation and destruction of the pathogen
 - An individual that becomes ill with chickenpox, but does not develop it again due to the development of immunologic memory
- 1.2 Multiple Choice:** Which of the following is an appropriate definition for immunological memory?
- The mechanism by which an organism prevents the development of an immune response against the host's own tissues
 - The mechanism by which an organism prevents exposure to microbes
 - The persistence of pathogen-specific antibodies and lymphocytes after the original infection has been eliminated so that reinfection can be prevented
 - The process of reducing or eliminating a pathogen
- 1.3 True or False:** Toll-like receptors (TLRs) recognize intracellular bacteria, while NOD-like receptors (NLRs) recognize extracellular bacteria.
- 1.4 Matching:** Classify the following as lymphoid or myeloid in origin:
- Eosinophils
 - B cells
 - Neutrophils
 - NK cells
 - Mast cells
 - Macrophages
 - Red blood cells
- 1.5 Multiple Choice:** The immunologist's 'dirty little secret' involves the addition of microbial constituents in order to stimulate a strong immune response against the desired protein antigen of interest. Which of the following is not a receptor or receptor family that can recognize microbial products in order to achieve a potent immune response?
- Toll-like receptors (TLRs)
 - T-cell antigen receptor (TCR)
 - NOD-like receptors (NLRs)
 - Pattern recognition receptors (PRRs)
- 1.6 True or False:** Hematopoietic stem cells can develop into any cell type in the body.
- 1.7 Matching:** Match each of the following terms to the numbered phrase that describes it best:
- | | |
|--|---|
| A. Allergy ____ | 1. Immunological response to an antigen present on a transplanted foreign cell |
| B. Immunological tolerance ____ | 2. Immunological response to an antigen that is an innocuous foreign substance |
| C. Autoimmune disease ____ | 3. Immunological process that prevents an immune response to self antigens |
| D. Graft rejection ____ | 4. Immunological response to a self antigen |
- 1.8 Multiple Choice:** Which of the following processes is not a mechanism of maintaining immunologic tolerance?
- Clonal deletion
 - Anergy
 - Clonal expansion
 - Suppression of self-reactive lymphocytes

- 1.9 Matching:** Classify each of the following as a central/primary or peripheral/secondary lymphoid organ:
- Bone marrow
 - Lymph node
 - Spleen
 - Thymus
 - Appendix
- 1.10 Matching:** Match the following region, structure, or subcompartments with the numbered organ they are present in:
- Lymph node ____
 - Spleen ____
 - Mucosa of the small intestine ____
- Periarteriolar lymphatic sheath (PALS)
 - Peyer's patches
 - High endothelial venules
- 1.11 Multiple Choice:** Which of the following events do not occur during inflammation?
- Cytokine secretion
 - Chemokine secretion
 - Recruitment of innate immune cells
 - Constriction of blood vessels
- 1.12 Fill-in-the-Blanks:** _____ T cells are able to kill infected cells, while _____ T cells activate other cells of the immune system.
- 1.13 True or False:** Both T-cell and B-cell receptors undergo the process of affinity maturation in order to acquire progressively higher affinity for an antigen during an immune response.
- 1.14 True or False:** Each lymphocyte carries cell-surface receptors with multiple antigen specificity.
- 1.15 Multiple Choice:** Which cell type forms an important link between the innate immune response and the adaptive immune response?
- Dendritic cell
 - Neutrophil
 - B cell
 - Innate lymphoid cell (ILC)
- 1.16 Multiple Choice:** Which of the following options is not a mechanism by which an antibody can protect against a pathogen?
- Neutralization
 - Co-stimulation of T cells
 - Opsonization
 - Complement activation/deposition
- 1.17 True or False:** T_H2 cells do not possess MHC class I molecules.

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PART II

THE RECOGNITION OF ANTIGEN

- 4 Antigen Recognition by B-cell and T-cell Receptors
- 5 The Generation of Lymphocyte Antigen Receptors
- 6 Antigen Presentation to T Lymphocytes

Antigen Recognition by B-cell and T-cell Receptors

4

Innate immune responses are the body's initial defense against infection, but these work only to control pathogens that have certain molecular patterns or that induce interferons and other nonspecific defenses. To effectively fight the wide range of pathogens an individual will encounter, the lymphocytes of the adaptive immune system have evolved to recognize a great variety of different **antigens** from bacteria, viruses, and other disease-causing organisms. An antigen is any molecule or part of a molecule that is specifically recognized by the highly specialized recognition proteins of lymphocytes. On B cells these proteins are the **immunoglobulins (Igs)**, which these cells produce in a vast range of antigen specificities, each B cell producing immunoglobulins of a single specificity (see Section 1-12). A membrane-bound form of immunoglobulin on the B-cell surface serves as the cell's receptor for antigen, and is known as the **B-cell receptor (BCR)**. A secreted form of immunoglobulin of the same antigen specificity is the **antibody** produced by terminally differentiated B cells—plasmablasts and plasma cells. The secretion of antibodies, which bind pathogens or their toxic products in the extracellular spaces of the body (see Fig. 1.25), is the main effector function of B cells in adaptive immunity.

Antibodies were the first proteins involved in specific immune recognition to be characterized, and are understood in great detail. The antibody molecule has two separate functions: one is to bind specifically to the pathogen or its products that have elicited the immune response; the other is to recruit other cells and molecules to destroy the pathogen once antibody has bound. For example, binding by antibodies can neutralize viruses and mark pathogens for destruction by phagocytes and complement, as described in Chapters 2 and 3. Recognition and effector functions are structurally separated in the antibody molecule, one part of which specifically binds to the antigen whereas the other engages the elimination mechanisms. The antigen-binding region varies extensively between antibody molecules and is known as the **variable region** or **V region**. The variability of antibody molecules allows each antibody to bind a different specific antigen, and the total repertoire of antibodies made by a single individual is large enough to ensure that virtually any structure can be recognized. The region of the antibody molecule that engages the effector functions of the immune system does not vary in the same way and

IN THIS CHAPTER

The structure of a typical antibody molecule.

The interaction of the antibody molecule with specific antigen.

Antigen recognition by T cells.

is known as the **constant region** or **C region**. It comes in five main forms, called **isotypes**, each of which is specialized for activating different effector mechanisms. The membrane-bound B-cell receptor does not have these effector functions, because the C region remains inserted in the membrane of the B cell. The function of the B-cell receptor is to recognize and bind antigen via the V regions exposed on the surface of the cell, thus transmitting a signal that activates the B cell, leading to clonal expansion and antibody production. To this end, the B-cell receptor is associated with a set of intracellular signaling proteins, which will be described in Chapter 7. Antibodies have become an important class of drug due to their highly specific activities, and we return to discuss their therapeutic uses in Chapter 16.

The antigen-recognition molecules of T cells are made solely as membrane-bound proteins, which are associated with an intracellular signaling complex and function only to signal T cells for activation. These **T-cell receptors (TCRs)** are related to immunoglobulins both in their protein structure—having both V and C regions—and in the genetic mechanism that produces their great variability, which is discussed in Chapter 5. The T-cell receptor differs from the B-cell receptor in an important way, however: it does not recognize and bind antigen by itself, but instead recognizes short peptide fragments of protein antigens that are presented to them by proteins known as **MHC molecules** on the surface of host cells.

The MHC molecules are transmembrane glycoproteins encoded in the large cluster of genes known as the **major histocompatibility complex (MHC)**. The most striking structural feature of MHC molecules is a cleft in the extracellular face of the molecule in which peptides can be bound. MHC molecules are highly **polymorphic**—each type of MHC molecule occurs in many different versions—within the population. These are encoded by slightly different versions of individual genes called **alleles**. Most people are therefore heterozygous for the MHC molecules; that is, they express two different alleles for each type of MHC molecule, thus increasing the range of pathogen-derived peptides and self-peptides that can be bound. T-cell receptors recognize features of both the peptide antigen and the MHC molecule to which it is bound. This introduces an extra dimension to antigen recognition by T cells, known as **MHC restriction** because any given T-cell receptor is specific for a particular peptide bound to a particular MHC molecule.

In this chapter we focus on the structure and antigen-binding properties of immunoglobulins and T-cell receptors. Although B cells and T cells recognize foreign molecules in separate distinct fashions, the receptor molecules they use for this task are very similar in structure. We will see how this basic structure can accommodate great variability in antigen specificity, and how it enables immunoglobulins and T-cell receptors to perform their functions as the antigen-recognition molecules of the adaptive immune response. With this foundation, we will return to discuss the impact of MHC polymorphism on T-cell antigen recognition and T-cell development in Chapters 6 and 8, respectively.

The structure of a typical antibody molecule.

Antibodies are the secreted form of the B-cell receptor. Because they are soluble and secreted into the blood in large quantities, antibodies are easily obtained and easily studied. For this reason, most of what we know about the B-cell receptor comes from the study of antibodies.

Antibody molecules are roughly Y-shaped, as represented in Fig. 4.1 using three different schematic styles. This part of the chapter will explain how this structure is formed and allows the antibody molecule to perform its dual tasks of binding to a wide variety of antigens while also binding to effector molecules

Fig. 4.1 Structure of an antibody molecule. In panel a, the X-ray crystallographic structure of an IgG antibody is illustrated as a ribbon diagram of the backbones of the polypeptide chains. The two heavy chains are shown in yellow and purple. The two light chains are both shown in red. Three globular regions form an approximate Y shape. The two antigen-binding sites are at the tips of the arms, which are tethered at their other end to the trunk of the Y by a flexible hinge region. The light-chain variable (V_L) and constant region (C_L) are indicated. The heavy-chain variable region (V_H) and V_L together form the antigen-binding site of the antibody. In panel b, a schematic representation of the same structure denotes each immunoglobulin domain as a separate rectangle. The hinge that tethers each heavy chain's first constant domain (C_{H1}) to its second (C_{H2}) is illustrated by a thin purple or yellow line, respectively. The antibody-binding sites are indicated by concave regions in V_L and V_H . Positions of carbohydrate modifications and disulfide linkages are indicated. In panel c, a more simplified schematic is shown that will be used throughout this book with the variable region in red and the constant region in blue. C terminus, carboxy terminus; N terminus, amino terminus. Structure courtesy of R.L. Stanfield and I.A. Wilson.

and to cells that destroy the antigen. Each of these tasks is performed by different parts of the molecule. The ends of the two arms of the Y—the V regions—are involved in antigen binding, and they vary in their detailed structure between different antibody molecules. The stem of the Y—the C region—is far less variable and is the part that interacts with effector molecules and cells. There are five different **classes** of immunoglobulins, distinguished in their being constructed from C regions that have different structures and properties. These are known as **immunoglobulin M (IgM)**, **immunoglobulin D (IgD)**, **immunoglobulin G (IgG)**, **immunoglobulin A (IgA)**, and **immunoglobulin E (IgE)**.

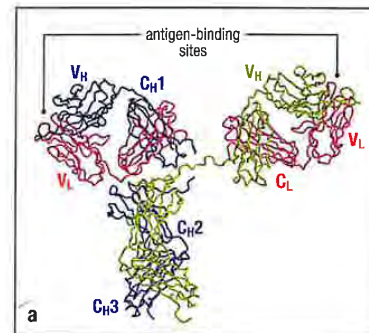
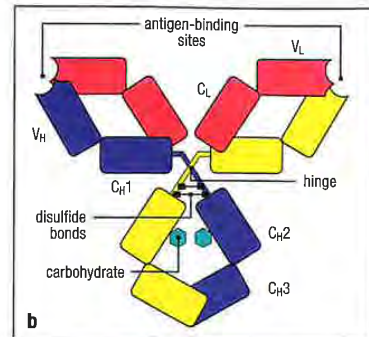
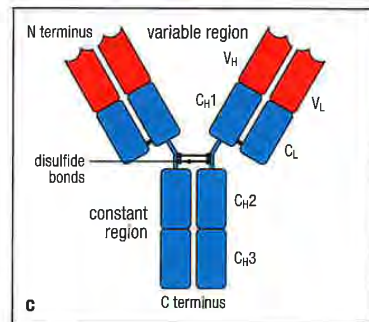
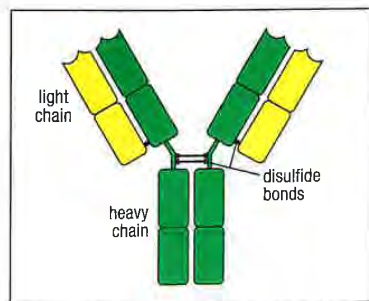
All antibodies are constructed in the same way from paired heavy and light polypeptide chains, and the generic term immunoglobulin is used for all such proteins. More subtle differences confined to the V region account for the specificity of antigen binding. We will use the IgG antibody molecule as an example to describe the general structural features of immunoglobulins.

4-1 IgG antibodies consist of four polypeptide chains.

IgG antibodies are large molecules with a molecular weight of approximately 150 kDa and are composed of two different kinds of polypeptide chains. One, of approximately 50 kDa, is called the **heavy** or **H chain**, and the other, of 25 kDa, is the **light** or **L chain** (Fig. 4.2). Each IgG molecule consists of two heavy chains and two light chains. The two heavy chains are linked to each other by disulfide bonds, and each heavy chain is linked to a light chain by a disulfide bond. In any given immunoglobulin molecule, the two heavy chains and the two light chains are identical, giving an antibody molecule two identical antigen-binding sites. This gives the antibody the ability to bind simultaneously to two identical antigens on a surface, thereby increasing the total strength of the interaction, which is called its **avidity**. The strength of the interaction between a single antigen-binding site and its antigen is called its **affinity**.

Two types of light chains, **lambda (λ)** and **kappa (κ)**, are found in antibodies. A given immunoglobulin has either κ chains or λ chains, never one of each. No functional difference has been found between antibodies having λ or κ light chains, and either type of light chain can be found in antibodies of any of the five major classes. The ratio of the two types of light chains varies from species to species. In mice, the average κ to λ ratio is 20:1, whereas in humans it is 2:1 and in cattle it is 1:20. The reason for this variation is unknown. Distortions of this ratio can sometimes be used to detect the abnormal proliferation of a

Fig. 4.2 Immunoglobulin molecules are composed of two types of protein chains: heavy chains and light chains. Each immunoglobulin molecule is made up of two hinged heavy chains (green) and two light chains (yellow) joined by disulfide bonds so that each heavy chain is linked to a light chain and the two heavy chains are linked together.



B-cell clone, since all progeny of a particular B cell will express an identical light chain. For example, an abnormally high level of λ light chains in a person might indicate the presence of a B-cell tumor that is producing λ chains.

The class, and thus the effector function, of an antibody is defined by the structure of its heavy chain. There are five main heavy-chain classes, or **isotypes**, some of which have several subtypes, and these determine the functional activity of an antibody molecule. The five major classes of immunoglobulin are IgM, IgD, IgG, IgA, and IgE, and their heavy chains are denoted by the lower-case Greek letters μ , δ , γ , α , and ϵ , respectively. For example, the constant region of IgM is denoted by C_{μ} . IgG is by far the most abundant immunoglobulin in serum and has several subclasses (IgG1, 2, 3, and 4 in humans). The distinctive functional properties of the different classes and subclasses of antibodies are conferred by the carboxy-terminal part of the heavy chain, where this chain is not associated with the light chain. The general structural features of all the isotypes are similar, particularly with respect to antigen binding. Here we will consider IgG as a typical antibody molecule, and we will return to discuss the structural and functional properties of the different heavy-chain isotypes in Chapter 5.

The structure of a B-cell receptor is identical to that of its corresponding antibody except for a small portion of the carboxy terminus of the heavy-chain C region. In the B-cell receptor, the carboxy terminus is a hydrophobic amino acid sequence that anchors the molecule in the membrane, and in the antibody it is a hydrophilic sequence that allows secretion.

4-2 Immunoglobulin heavy and light chains are composed of constant and variable regions.

The amino acid sequences of many immunoglobulin heavy and light chains have been determined and reveal two important features of antibody molecules. First, each chain consists of a series of similar, although not identical, sequences, each about 110 amino acids long. Each of these repeats corresponds to a discrete, compactly folded region of protein known as an **immunoglobulin domain**, or **Ig domain**. The light chain consists of two Ig domains, whereas the heavy chain of the IgG antibody contains four Ig domains (see Fig. 4.2). This suggests that the immunoglobulin chains have evolved by repeated duplication of ancestral gene segments corresponding to a single Ig domain.

The second important feature is that the amino-terminal amino acid sequences of the heavy and light chains vary greatly between different antibodies. The variability is limited to approximately the first 110 amino acids, corresponding to the first Ig domain, whereas the remaining domains are constant between immunoglobulin chains of the same isotype. The amino-terminal **variable Ig domains (V domains)** of the heavy and light chains (V_H and V_L , respectively) together make up the V region of the antibody and determine its antigen-binding specificity, whereas the **constant Ig domains (C domains)** of the heavy and light chains (C_H and C_L , respectively) make up the C region (see Fig. 4.1). The multiple heavy-chain C domains are numbered from the amino-terminal end to the carboxy terminus, for example, C_{H1} , C_{H2} , and so on.

4-3 The domains of an immunoglobulin molecule have similar structures.

Immunoglobulin heavy and light chains are composed of a series of Ig domains that have a similar overall structure. Within this basic structure, there are distinct differences between V and C domains that are illustrated for the light chain in Fig. 4.3. Each V or C domain is constructed from two **β sheets**. A β sheet is

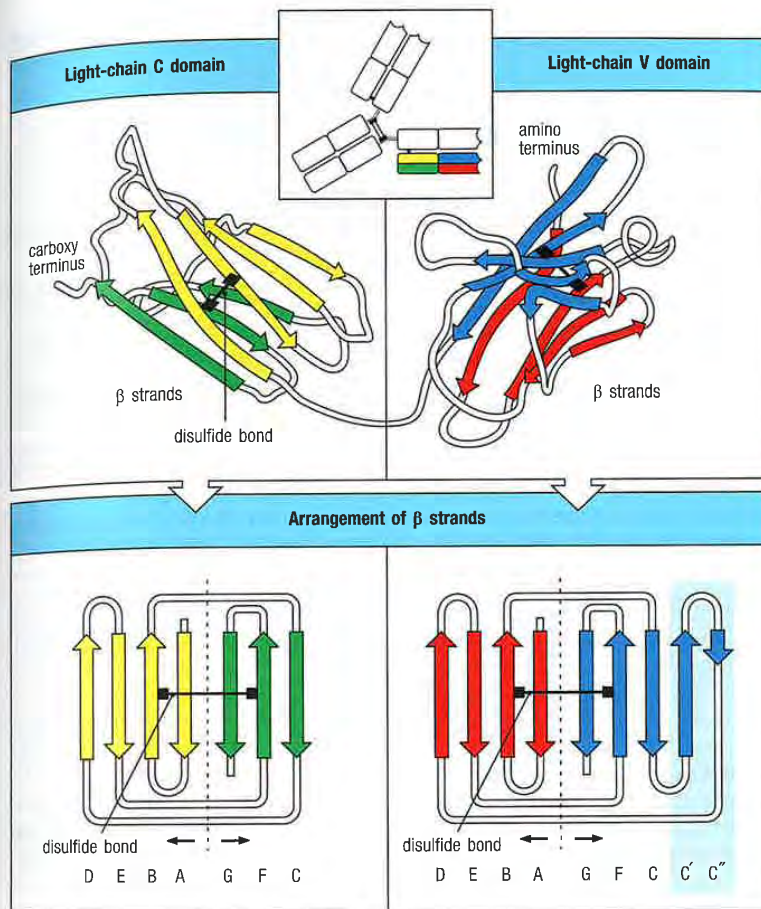
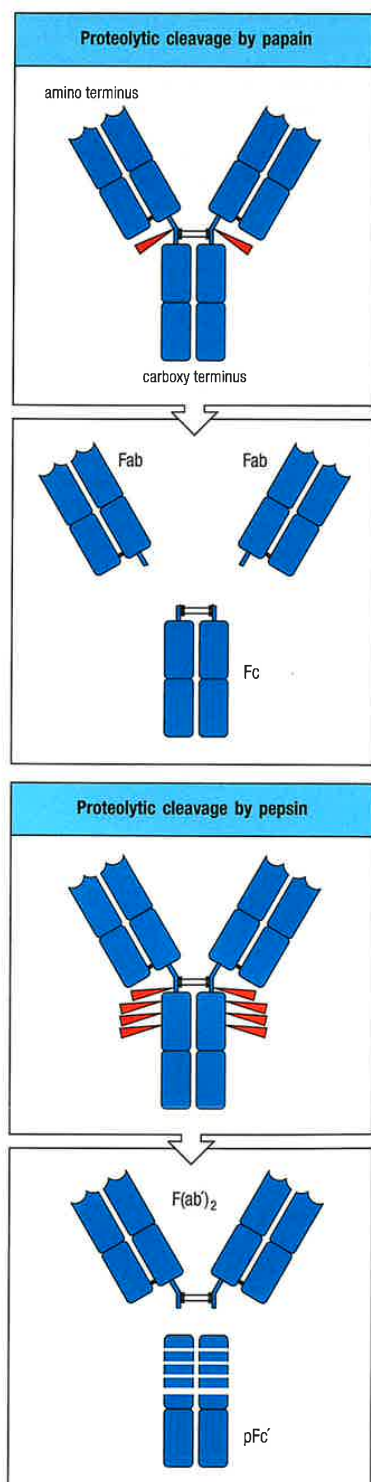


Fig. 4.3 The structure of immunoglobulin constant and variable domains. The upper panels show schematically the folding pattern of the constant (C) and variable (V) domains of an immunoglobulin light chain. Each domain is a barrel-shaped structure in which strands of polypeptide chain (β strands) running in opposite directions (antiparallel) pack together to form two β sheets (shown in yellow and green for the C domain and red and blue for the V domain), which are held together by a disulfide bond. The way in which the polypeptide chain folds to give the final structure can be seen more clearly when the sheets are opened out, as shown in the lower panels. The β strands are lettered sequentially with respect to the order of their occurrence in the amino acid sequence of the domains; the order in each β sheet is characteristic of immunoglobulin domains. The β strands C' and C'' that are found in the V domains but not in the C domains are indicated by a blue-shaded background. The characteristic four-strand plus three-strand (C-region type domain) or four-strand plus five-strand (V-region type domain) arrangements are typical immunoglobulin superfamily domain building blocks, found in a whole range of other proteins as well as antibodies and T-cell receptors.

built from several **β strands**, which are regions of protein where several consecutive polypeptides have their peptide backbone bonds arranged in an extended, or flat, conformation. β strands in proteins are sometimes shown as 'ribbons with an arrow' to indicate the direction of the polypeptide backbone (see Fig. 4.3). β strands can pack together in a side-by-side manner, being stabilized laterally by two or three backbone hydrogen bonds between adjacent strands. This arrangement is called a **β sheet**. The Ig domain has two β sheets that are folded onto each other, like two pieces of bread, into a structure called a **β sandwich**, and are covalently linked by a disulfide bond between cysteine residues from each β sheet. This distinctive structure is known as the **immunoglobulin fold**.

The similarities and differences between V and C domains can be seen in the bottom panels of Fig. 4.3. Here, the Ig domains have been opened out to show how their respective polypeptide chains fold to create each of the β sheets and how each polypeptide chain forms flexible loops between adjacent β strands as it turns to change direction. The main difference between the V and C domains is that the V domain is larger and contains extra β strands, called C' and C''. In the V domain, the flexible loops formed between some of the β strands contribute to the antigen-binding site of the immunoglobulin molecule.

Many of the amino acids that are common to the C and V domains are present in the core of the immunoglobulin fold and are essential for its stability. Other proteins with sequences similar to those of immunoglobulins have been



found to have domains with a similar structure, called **immunoglobulin-like domains (Ig-like domains)**. These domains are present in many proteins of the immune system, such as the KIRs expressed by NK cells described in Chapter 3. They are also frequently involved in cell-cell recognition and adhesion, and together with the immunoglobulins and the T-cell receptors, these proteins make up the extensive **immunoglobulin superfamily**.

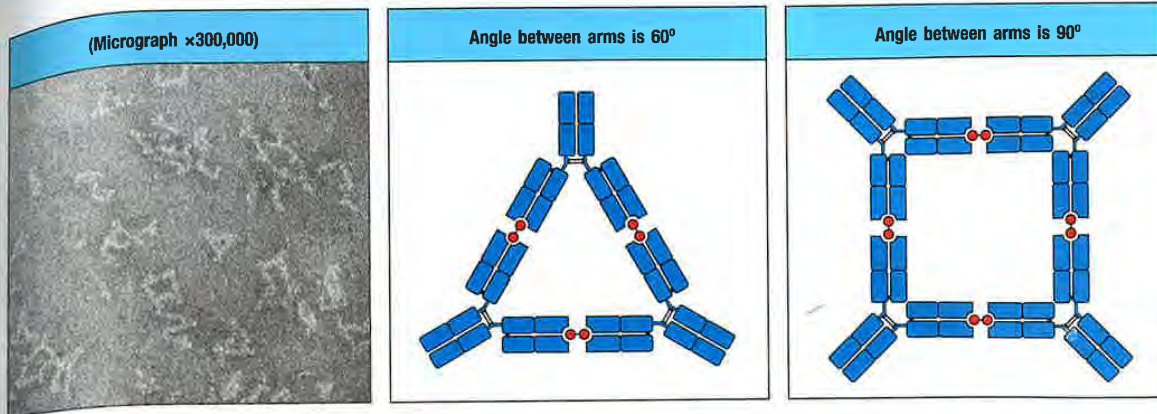
4-4 The antibody molecule can readily be cleaved into functionally distinct fragments.

When fully assembled, an antibody molecule comprises three equal-sized globular portions, with its two arms joined to its trunk by a flexible stretch of polypeptide chain known as the **hinge region** (see Fig. 4.1b). Each arm of this Y-shaped structure is formed by the association of a light chain with the amino-terminal half of a heavy chain; the V_H domain is paired with the V_L domain, and the C_H1 domain is paired with the C_L domain. The two antigen-binding sites are formed by the paired V_H and V_L domains at the ends of the two arms of the Y (see Fig. 4.1b). The trunk of the Y is formed by the pairing of the carboxy-terminal halves of the two heavy chains. The C_H3 domains pair with each other but the C_H2 domains do not interact. Carbohydrate side chains attached to the C_H2 domains lie between the two heavy chains.

Proteolytic enzymes (proteases) were an important tool in early studies of antibody structure, and it is valuable to review the terminology they generated. Limited digestion with the protease papain cleaves antibody molecules into three fragments (Fig. 4.4). **Papain** cuts the antibody molecule on the amino-terminal side of the disulfide bonds that link the two heavy chains, releasing the two arms of the antibody molecule as two identical fragments that contain the antigen-binding activity. These are called the **Fab fragments**, for fragment antigen binding. The other fragment contains no antigen-binding activity, but because it crystallized readily, it was named the **Fc fragment** (fragment crystallizable). It corresponds to the paired C_H2 and C_H3 domains. The Fc fragment is the part of the antibody molecule that does not interact with antigen, but rather interacts with effector molecules and cells, and it differs between heavy-chain isotypes. Another protease, **pepsin**, cuts on the carboxy-terminal side of the disulfide bonds (see Fig. 4.4). This produces a fragment, the **F(ab')₂ fragment**, in which the two antigen-binding arms of the antibody molecule remain linked. Pepsin cuts the remaining part of the heavy chain into several small fragments. The F(ab')₂ fragment has exactly the same antigen-binding characteristics as the original antibody but is unable to interact with any effector molecule, such as C1q or Fc receptors, and can be used experimentally to separate the antigen-binding functions from the antibody's other effector functions.

Many antibody-related molecules can be constructed using genetic engineering techniques, and many antibodies and antibody-related molecules are being used therapeutically to treat a variety of diseases. We will return to this topic in Chapter 16, where we discuss the various therapeutic uses of antibodies that have been developed over the last two decades.

Fig. 4.4 The Y-shaped immunoglobulin molecule can be dissected by partial digestion with proteases. Upper panels: papain cleaves the immunoglobulin molecule into three pieces, two Fab fragments and one Fc fragment. The Fab fragment contains the V regions and binds antigen. The Fc fragment is crystallizable and contains C regions. Lower panels: pepsin cleaves immunoglobulin to yield one F(ab')₂ fragment and many small pieces of the Fc fragment, the largest of which is called the pFc' fragment. F(ab')₂ is written with a prime because it contains a few more amino acids than Fab, including the cysteines that form the disulfide bonds.



4-5 The hinge region of the immunoglobulin molecule allows flexibility in binding to multiple antigens.

The hinge region between the Fc and Fab portions of the IgG molecule allows for some degree of independent movement of the two Fab arms. For example, in the antibody molecule shown in Fig. 4.1a, not only are the two hinge regions clearly bent differently, but the angle between the V and C domains in each of the two Fab arms is also different. This range of motion has led to the junction between the V and C domains being referred to as a 'molecular ball-and-socket joint.' This flexibility can be revealed by studies of antibodies bound to small antigens known as **haptens**. These are molecules of various types that are typically about the size of a tyrosine side chain. Although haptens are specifically recognized by antibody, they can stimulate the production of anti-hapten antibodies only when linked to a protein (see Appendix I, Section A-1). Two identical hapten molecules joined by a short flexible region can link two or more anti-hapten antibodies, forming dimers, trimers, tetramers, and so on, which can be seen by electron microscopy (Fig. 4.5). The shapes formed by these complexes show that antibody molecules are flexible at the hinge region. Some flexibility is also found at the junction between the V and C domains, allowing bending and rotation of the V domain relative to the C domain. Flexibility at both the hinge and the V-C junction enables the two arms of an antibody molecule to bind to sites some distance apart, such as the repeating sites on bacterial cell-wall polysaccharides. Flexibility at the hinge also enables antibodies to interact with the antibody-binding proteins that mediate immune effector mechanisms.

Summary.

The IgG antibody molecule is made up of four polypeptide chains, comprising two identical light chains and two identical heavy chains, and can be thought of as forming a flexible Y-shaped structure. Each of the four chains has a variable (V) region at its amino terminus, which contributes to the antigen-binding site, and a constant (C) region. The light chains are bound to the heavy chains by many noncovalent interactions and by disulfide bonds, and the V regions of the heavy and light chains pair in each arm of the Y to generate two identical antigen-binding sites, which lie at the tips of the arms of the Y. The possession of two antigen-binding sites allows antibody molecules to cross-link antigens and to bind them much more stably and with higher avidity. The trunk of the Y, also called the Fc fragment, is composed of the carboxy-terminal domains of the heavy chains, and it is these domains that determine the antibody's isotype. Joining the arms of the Y to the trunk are the flexible hinge regions. The Fc fragment and hinge regions differ in antibodies of different isotypes.

Fig. 4.5 Antibody arms are joined by a flexible hinge. An antigen consisting of two hapten molecules (red balls in diagrams) that can cross-link two antigen-binding sites is used to create antigen:antibody complexes, which can be seen in the electron micrograph. Linear, triangular, and square forms are seen, with short projections or spikes. Limited pepsin digestion removes these spikes (not shown in the figure), which therefore correspond to the Fc portion of the antibody; the $F(ab')_2$ pieces remain cross-linked by antigen. The interpretation of some of the complexes is shown in the diagrams. The angle between the arms of the antibody molecules varies. In the triangular forms, this angle is 60° , whereas it is 90° in the square forms, showing that the connections between the arms are flexible. Photograph courtesy of N.M. Green.

Different isotypes have different properties and therefore differ in their interactions with effector molecules and cell types. However, the overall organization of the domains is similar in all isotypes.

The interaction of the antibody molecule with specific antigen.

In this part of the chapter we look at the antigen-binding site of an immunoglobulin molecule in more detail. We discuss the different ways in which antigens can bind to antibody, and address the question of how variation in the sequences of the antibody V domains determines the specificity for antigen.

4-6 Localized regions of hypervariable sequence form the antigen-binding site.

The V regions of any given antibody molecule differ from those of every other. Sequence variability is not, however, distributed evenly throughout the V region but is concentrated in certain segments, as is clearly seen in a **variability plot** (Fig. 4.6), in which the amino acid sequences of many different antibody V regions are compared. Three particularly variable segments can be identified in both the V_H and V_L domains. They are designated **hypervariable regions** and are denoted HV1, HV2, and HV3. In the heavy chains they are located at residues 30 to 36, 49 to 65, and 95 to 103, respectively, while in the light chains they are located at residues 28 to 35, 49 to 59, and 92 to 103, respectively. The most variable part of the domain is in the HV3 region. The regions between the hypervariable regions comprise the rest of the V domain; they show less variability and are termed the **framework regions**. There are four such regions in each V domain, designated FR1, FR2, FR3, and FR4.

The framework regions form the β sheets that provide the structural framework of the immunoglobulin domain. The hypervariable sequences correspond to three loops and are positioned near one another in the folded domain at the outer edge of the β sandwich (Fig. 4.7). Thus, not only is diversity concentrated in particular parts of the V domain sequence, but it is also localized to a particular

Fig. 4.6 There are discrete regions of hypervariability in V domains.

The hypervariability regions of both the heavy and the light chain contribute to antigen binding of an antibody molecule. A variability plot derived from comparison of the amino acid sequences of several dozen heavy-chain and light-chain V domains is shown. At each amino acid position, the degree of variability is the ratio of the number of different amino acids seen in all of the sequences together to the frequency of the most common amino acid. Three hypervariable regions (HV1, HV2, and HV3) are indicated in red. They are flanked by less variable framework regions (FR1, FR2, FR3, and FR4, shown in blue or yellow).

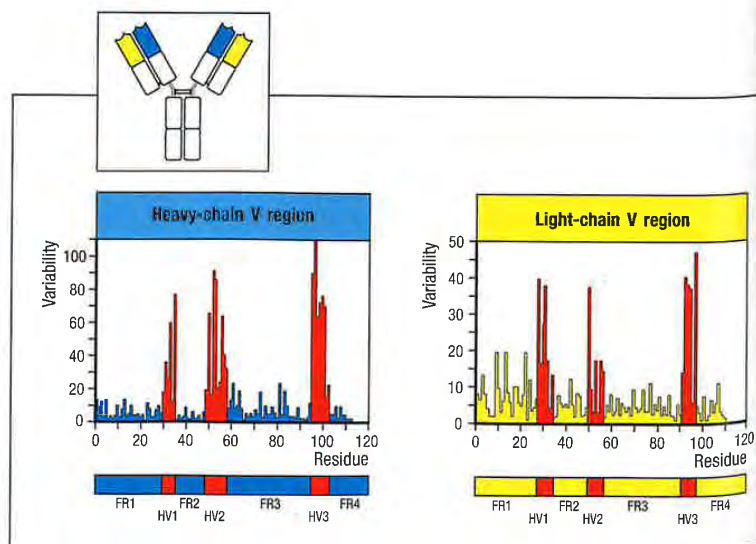


Fig. 4.7 The hypervariable regions lie in discrete loops of the folded structure.

First panel: the hypervariable regions (red) are positioned on the structure of a map of the coding region of the V domain. Second panel: when shown as a flattened ribbon diagram, hypervariable regions are seen to occur in loops (red) that join particular β strands. Third panel: in the folded structure of the V domain, these loops (red) are brought together to form antigen-binding regions. Fourth panel: in a complete antibody molecule, the pairing of a heavy chain and a light chain brings together the hypervariable loops from each chain to create a single hypervariable surface, which forms the antigen-binding site at the tip of each arm. Because they are complementary to the antigen surface, the hypervariable regions are also commonly known as the complementarity-determining regions (CDRs). C, carboxy terminus; N, amino terminus.

region on the surface of the molecule. When the V_H and V_L immunoglobulin domains are paired in the antibody molecule, the three hypervariable loops from each domain are brought together, creating a single hypervariable site at the tip of each arm of the molecule. This is the **antigen-binding site**, or **antibody-combining site**, which determines the antigen specificity of the antibody. These six hypervariable loops are more commonly termed the **complementarity-determining regions**, or **CDRs**, because the surface they form is complementary to that of the antigen they bind. There are three CDRs from each of the heavy and light chains, namely, CDR1, CDR2, and CDR3. In most cases, CDRs from both V_H and V_L domains contribute to the antigen-binding site; thus it is the combination of the heavy and the light chain that usually determines the final antigen specificity (see Fig. 4.6). However, there are some Fab crystal structures that show antigen interaction with just the heavy chain; for example, in one influenza Fab, antigen interaction involves binding mostly to the V_H CDR3, and only minor contacts with other CDRs. Thus, one way in which the immune system is able to generate antibodies of different specificities is by generating different combinations of heavy-chain and light-chain V regions. This is known as **combinatorial diversity**; we will encounter a second form of **combinatorial diversity** in Chapter 5 when we consider how the genes encoding the heavy-chain and light-chain V regions are created from smaller segments of DNA during the development of B cells in the bone marrow.

4-7 Antibodies bind antigens via contacts in CDRs that are complementary to the size and shape of the antigen.

In early investigations of antigen binding to antibodies, the only available sources of large quantities of a single type of antibody molecule were tumors of antibody-secreting cells. The antigen specificities of these antibodies were unknown, and therefore many compounds had to be screened to identify ligands that could be used to study antigen binding. In general, the substances found to bind to these antibodies were haptens (see Section 4-5) such as phosphocholine or vitamin K_1 . Structural analysis of complexes of antibodies with their hapten ligands provided the first direct evidence that the hypervariable regions form the antigen-binding site, and demonstrated the structural basis of specificity for the hapten. Subsequently, with the discovery of methods of generating **monoclonal antibodies** (see Appendix I, Section A-7), it became possible to make large amounts of pure antibody specific for a given antigen. This has provided a more general picture of how antibodies interact with their antigens, confirming and extending the view of antibody-antigen interactions derived from the study of haptens.

The surface of the antibody molecule formed by the juxtaposition of the CDRs of the heavy and light chains is the site to which an antigen binds. The amino acid sequences of the CDRs are different in different antibodies, and so too are the shapes and properties of the surfaces created by these CDRs. As a general principle, antibodies bind ligands whose surfaces are complementary to that of the antigen-binding site. A small antigen, such as a hapten or a short

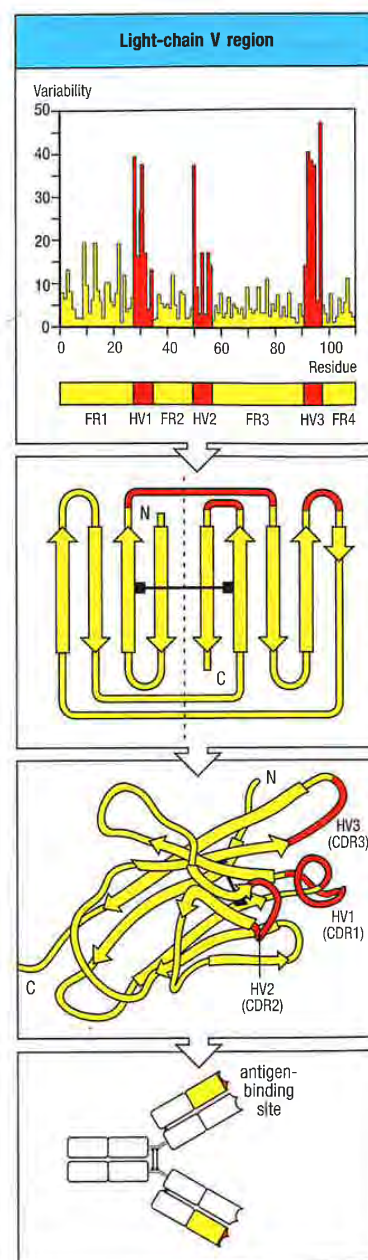
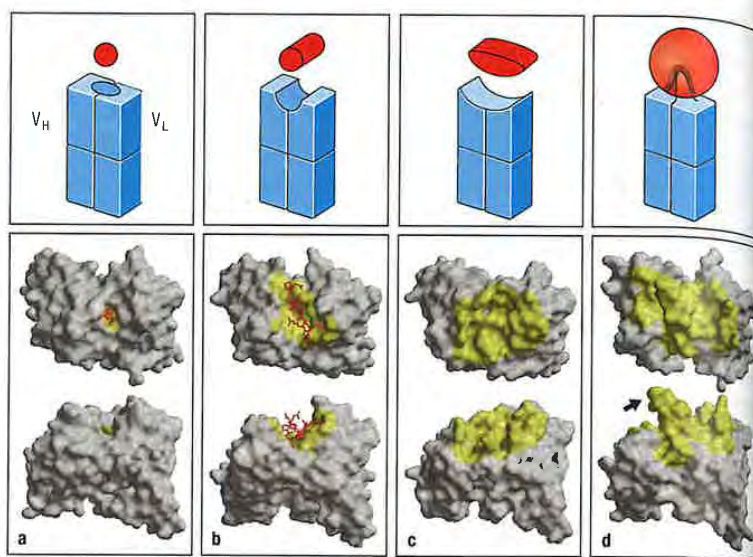


Fig. 4.8 Antigens can bind in pockets, or grooves, or on extended surfaces in the binding sites of antibodies.

The panels in the top row show schematic representations of the different types of binding sites in a Fab fragment of an antibody: first panel, pocket; second panel, groove; third panel, extended surface; and fourth panel, protruding surface. Below are examples of each type. Panel a: the top image shows the molecular surface of the interaction of a small hapten with the complementarity-determining regions (CDRs) of a Fab fragment as viewed looking into the antigen-binding site. The ferrocene hapten, shown in red, is bound into the antigen-binding pocket (yellow). In the bottom image (and in those of panels b, c, and d), the molecule has been rotated by about 90° to give a side-on view of the binding site. Panel b: in a complex of an antibody with a peptide from the human immunodeficiency virus (HIV), the peptide (red) binds along a groove (yellow) formed between the heavy-chain and light-chain V domains. Panel c: shown is a complex between hen egg-white lysozyme and the Fab fragment of its corresponding antibody (HyHel5). The surface on the antibody that comes into contact with the lysozyme is colored yellow. All six CDRs of the antigen-binding site are involved in the binding. Panel d: an antibody molecule against the HIV gp120 antigen has an elongated CDR3 loop (arrow) that protrudes into a recess on the side of the antigen. The structure of the complex between this antibody and gp120 has been solved, and in this case only the heavy chain interacts with gp120. Structures courtesy of R.L. Stanfield and I.A. Wilson.



peptide, generally binds in a pocket or groove lying between the heavy-chain and light-chain V domains (Fig. 4.8a and b). Some antigens, such as proteins, can be the same size as, or larger than, the antibody itself. In these cases, the interface between antigen and antibody is often an extended surface that involves all the CDRs and, in some cases, part of the framework region as well (see Fig. 4.8c). This surface need not be concave but can be flat, undulating, or even convex. In some cases, antibody molecules with elongated CDR3 loops can protrude a 'finger' into recesses in the surface of the antigen, as shown in Fig. 4.8d, where an antibody binding to the HIV gp120 antigen projects a long loop into its target.

4-8 Antibodies bind to conformational shapes on the surfaces of antigens using a variety of noncovalent forces.

The biological function of antibodies is to bind to pathogens and their products, and to facilitate their removal from the body. An antibody generally recognizes only a small region on the surface of a large molecule such as a polysaccharide or protein. The structure recognized by an antibody is called an **antigenic determinant** or **epitope**. Some of the most important pathogens have polysaccharide coats, and antibodies that recognize epitopes formed by the sugar subunits of these molecules are essential in providing immune protection against such pathogens. In many cases, however, the antigens that provoke an immune response are proteins. For example, many protective antibodies against viruses recognize viral coat proteins. In all such cases, the structures recognized by the antibody are located on the surface of the protein. Such sites are likely to be composed of amino acids from different parts of the polypeptide chain that have been brought together by protein folding. Antigenic determinants of this kind are known as **conformational** or **discontinuous epitopes** because the structure recognized is composed of segments of the protein that are discontinuous in the amino acid sequence of the antigen but are brought together in the three-dimensional structure. In contrast, an epitope composed of a single segment of polypeptide chain is termed a **continuous** or **linear epitope**. Although most antibodies raised against intact, fully folded proteins recognize discontinuous epitopes, some will bind to peptide fragments of the protein. Conversely, antibodies raised against peptides of a protein or against synthetic peptides corresponding to part of its sequence

are occasionally found to bind to the natural folded protein. This makes it possible, in some cases, to use synthetic peptides in vaccines that aim to raise antibodies against a pathogen's protein.

The interaction between an antibody and its antigen can be disrupted by high salt concentrations, by extremes of pH, by detergents, and sometimes by competition with high concentrations of the pure epitope itself. The binding is therefore a reversible noncovalent interaction. The forces, or bonds, involved in these noncovalent interactions are outlined in Fig. 4.9. **Electrostatic interactions** occur between charged amino acid side chains, as in salt bridges. Most antibody-antigen interactions involve at least one electrostatic interaction. Interactions also occur between electric dipoles, as in hydrogen bonds, or can involve short-range van der Waals forces. High salt concentrations and extremes of pH disrupt antigen-antibody binding by weakening electrostatic interactions and/or hydrogen bonds. This principle is employed in the purification of antigens by using affinity columns of immobilized antibodies (or in the purification of antibody by using antigens in a like manner) (see Appendix I, Section A-3). **Hydrophobic interactions** occur when two hydrophobic surfaces come together to exclude water. The strength of a hydrophobic interaction is proportional to the surface area that is hidden from water, and for some antigens, hydrophobic interactions probably account for most of the binding energy. In some cases, water molecules are trapped in pockets in the interface between antigen and antibody. These trapped water molecules, especially those between polar amino acid residues, may also contribute to binding and hence to the specificity of the antibody.

The contribution of each of these forces to the overall interaction depends on the particular antibody and antigen involved. A striking difference between antibody interactions with protein antigens and most other natural protein-protein interactions is that antibodies often have many aromatic amino acids

Noncovalent forces	Origin	
Electrostatic forces	Attraction between opposite charges	$\text{—NH}_3^+ \quad \text{OOC}^-$
Hydrogen bonds	Hydrogen shared between electronegative atoms (N, O)	$\text{>N} \cdots \text{H} \cdots \text{O}=\text{C}<$ $\delta^- \quad \delta^+ \quad \delta^-$
Van der Waals forces	Fluctuations in electron clouds around molecules polarize neighboring atoms oppositely	$\delta^+ \cdots \delta^-$ $\delta^- \cdots \delta^+$
Hydrophobic forces	Hydrophobic groups interact unfavorably with water and tend to pack together to exclude water molecules. The attraction also involves van der Waals forces	$\text{H} \cdots \text{O} \cdots \text{H}$ $\delta^+ \quad \delta^- \quad \delta^+$ $\delta^- \quad \delta^+ \quad \delta^-$ $\text{H} \cdots \text{O} \cdots \text{H}$
Cation- π interaction	Non-covalent interaction between a cation and an electron cloud of a nearby aromatic group	Na^+ $\delta^- \quad \delta^- \quad \delta^-$ $\delta^+ \quad \delta^+ \quad \delta^+$

Fig. 4.9 The noncovalent forces that hold together the antigen:antibody complex. Partial charges found in electric dipoles are shown as δ^+ or δ^- . Electrostatic forces diminish as the inverse square of the distance separating the charges, whereas van der Waals forces, which are more numerous in most antigen-antibody contacts, fall off as the sixth power of the separation and therefore operate only over very short ranges. Covalent bonds never occur between antigens and naturally produced antibodies.

in their antigen-binding sites. These amino acids participate mainly in van der Waals and hydrophobic interactions, and sometimes in hydrogen bonds and **pi-cation interactions**. Tyrosine, for example, can take part in both hydrogen bonding and hydrophobic interactions; it is therefore particularly suitable for providing diversity in antigen recognition and is overrepresented in antigen-binding sites. In general, the hydrophobic and van der Waals forces operate over very short ranges and serve to pull together two surfaces that are complementary in shape: hills on one surface must fit into valleys on the other for good binding to occur. In contrast, electrostatic interactions between charged side chains, and hydrogen bonds bridging oxygen and/or nitrogen atoms, accommodate more specific chemical interactions while strengthening the interaction overall. The side chains of aromatic amino acids such as tyrosine can interact noncovalently through their pi-electron system with nearby cations, including nitrogen-containing side chains that may be in a protonated cationic state.

4-9 Antibody interaction with intact antigens is influenced by steric constraints.

An example of an antibody-antigen interaction involving a specific amino acid in the antigen can be seen in the complex of hen egg-white lysozyme with the antibody D1.3 (Fig. 4.10). In this structure, strong hydrogen bonds are formed between the antibody and a particular glutamine in the lysozyme molecule that protrudes between the V_H and V_L domains. Lysozymes from partridge and turkey have another amino acid in place of the glutamine and do not bind to this antibody. In the high-affinity complex of hen egg-white lysozyme with another antibody, HyHel5 (see Fig. 4.8c), two salt bridges between two basic arginines on the surface of the lysozyme interact with two glutamic acids, one each from the V_H CDR1 and CDR2 loops. Lysozymes that lack one of the two arginine residues show a 1000-fold decrease in affinity for HyHel5. Overall surface complementarity must have an important role in antigen-antibody interactions, but in most antibodies that have been studied at this level of detail, only a few residues make a major contribution to the binding energy and hence to the final specificity of the antibody. Although many antibodies naturally bind their ligands with high affinity, in the nanomolar range, genetic engineering by site-directed mutagenesis can tailor an antibody to bind even more strongly to its epitope.

Even when antibodies have high affinity for antigens on a larger structure, such as an intact viral particle, antibody binding may be prevented by their particular arrangement. For example, the intact West Nile virion is built from an icosahedral scaffold that has 90 homodimers of a membrane-anchored envelope glycoprotein, E, which has three domains, DI, DII, and DIII. The DIII domain has four polypeptide loops that protrude outward from the viral particle. A neutralizing antibody against West Nile virus, E16, recognizes these loops of DIII, as shown in Fig. 4.11. In theory, there should be 180 possible antigen-binding sites for the E16 antibody on the West Nile viral particle. However, a combination of crystallographic and electron micrographic studies show that even with an excess of the E16 Fab fragment, only about 120 of the total 180 DIII domains of E are able to bind E16 Fab fragment (see Fig. 4.11).

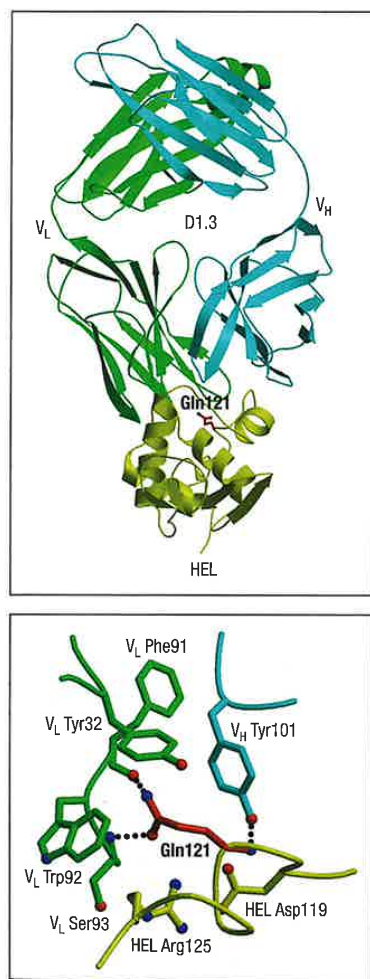


Fig. 4.10 The complex of lysozyme with the antibody D1.3. Top panel: The interaction of the Fab fragment of D1.3 with hen egg-white lysozyme (HEL) is shown. HEL is depicted in yellow, the heavy chain (V_H) in turquoise, and the light chain (V_L) in green. Bottom panel: A glutamine residue (Gln121) that protrudes from HEL (yellow) extends its side chain (shown in red) between the V_L (green) and V_H (turquoise) domains of the antigen-binding site and makes hydrogen bonds with the hydroxyl group (red dots) of the indicated amino acids of both domains. These hydrogen bonds are important to the antigen-antibody binding. Courtesy of R. Mariuzza and R.J. Poljak.

Fig. 4.11 Steric hindrance occludes the binding of antibody to native antigen in the intact West Nile viral particle. Top panel: the monoclonal antibody E16 recognizes the intact West Nile virus glycoprotein E. Shown is a DIII, one of the three structural domains in the West Nile virus glycoprotein E. Shown is a crystal structure of the E16 Fab bound to the DIII epitope. Bottom left panel: a computer model was used to dock E16 Fab to the mature West Nile virion. E16 Fabs were able to bind 120 of the 180 DIII epitopes. Sixty of the five-fold clustered DIII epitopes are sterically hindered by the binding of Fab to four nearby DIII epitopes. An example of an occluded epitope is the blue area indicated by the arrow. Bottom right panel: cryogenic electron microscopic reconstruction of saturating E16 Fab bound to West Nile virion confirmed the predicted steric hindrance. The vertices of the triangle shown in the figure indicate the icosahedral symmetry axes.

This appears to result from steric hindrance, with the presence of one Fab blocking the ability of another Fab to bind to some nearby E protein sites. Presumably, such steric hindrance would become more severe with intact antibody than is evident with the smaller Fab fragment. This study also showed that the Fab bound to the DIII region using only one of its antigen-binding arms, indicating that antibodies may not always bind to antigens with both antigen-binding sites, depending on the orientation of the antigens being recognized. These constraints will impact the ability of antibodies to neutralize their targets.

4-10 Some species generate antibodies with alternative structures.

Our focus in this chapter has been on the structure of antibodies produced by humans, which is generally similar in most mammalian species, including mice, an important model system for immunology research. However, some mammals have the ability to produce an alternative form of antibody that is based on the ability of a single V_H domain to interact with antigen in the absence of a V_L domain (Fig. 4.12). It has been known for some time that the serum of camels contained abundant immunoglobulin-like material composed of heavy-chain dimers that lack associated light chains but retain the capacity to bind antigens. These antibodies are called **heavy-chain-only IgGs (hcIgGs)**. This property is shared by other camelids, including llamas and alpacas. These species have retained the genes for the immunoglobulin light chains, and some IgG-like material in their sera remains associated with light chains, and so it is unclear what led to this particular adaptation during their evolution. In camelids, the ability to produce hcIgGs arises from mutations that allow the alternative splicing of the heavy-chain mRNA, with loss of the C_H1 exon and thus the joining of the V_H directly to the C_H2 domain in the protein. Other mutations stabilize this structure in the absence of V_L domains.

Cartilaginous fish, in particular the shark, also have an antibody molecule that differs substantially from human or murine antibodies (see Fig. 4.12). Like camelids, the shark also has genes encoding both immunoglobulin heavy and light chains, and does produce immunoglobulins containing both

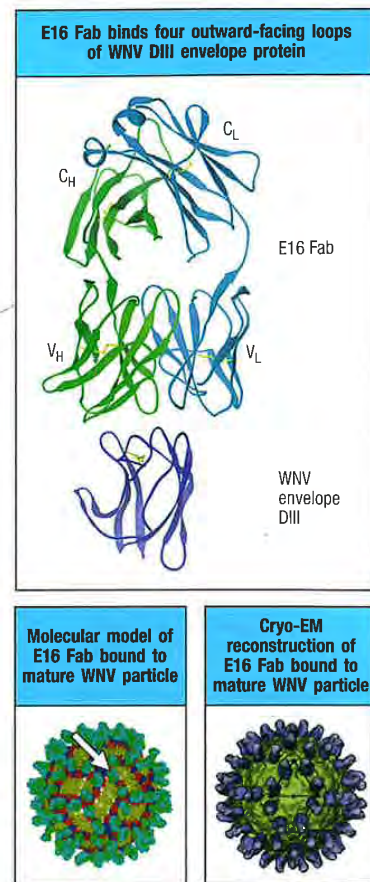
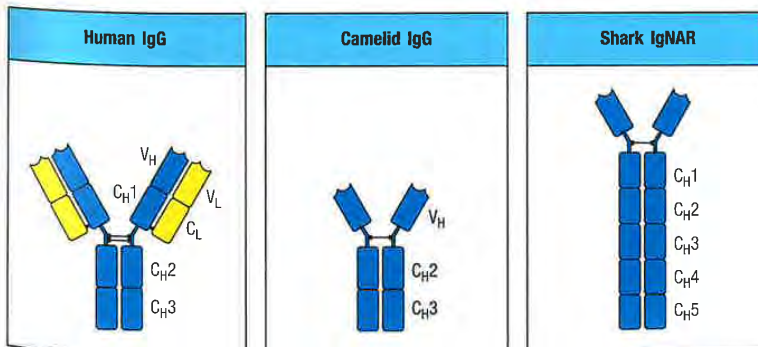


Fig. 4.12 Camelid and shark antibody can consist of heavy chain only.

In the camelid heavy-chain-only antibody, a splicing event of the mature heavy chain can delete the exon encoding the C_H1 region and thereby create an in-frame hinge region linking the V_H1 to the C_H2 region. In the shark, the heavy-chain-only Ig molecule retains the C_H1 region, suggesting that this form of antibody may predate the evolution of light chains. For both, the repertoire of antigen-binding sites involves extensive variations in long CDR3 regions of the V_H domain relative to other types of antibody.

heavy and light chains. But sharks also produce an **immunoglobulin new antigen receptor (IgNAR)**, with heavy-chain-only antibody in which the V_H is spliced to the C_H1 exon, rather than the C_H1 exon being spliced out as in camelids. These differences suggest that hIgG production by camelids and sharks represents an event of convergent evolution. The ability of camelid V_H domains to interact efficiently with antigens is the basis for producing so-called **single-chain antibody**. The simplification of using only a single domain for antigen recognition has prompted recent interest in single-chain monoclonal antibodies as an alternative to standard monoclonal antibodies, which we will discuss more in Chapter 16.

Summary.

X-ray crystallographic analyses of antigen:antibody complexes have shown that the hypervariable loops (complementarity-determining regions, CDRs) of immunoglobulin V regions determine the binding specificity of an antibody. Contact between an antibody molecule and a protein antigen usually occurs over a broad area of the antibody surface that is **complementary** to the surface recognized on the antigen. Electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic and pi-cation interactions can all contribute to binding. Depending on the size of the antigen, amino acid side chains in most or all of the CDRs make contact with antigen and determine both the specificity and the affinity of the interaction. Other parts of the V region normally play little part in the direct contact with the antigen, but they provide a stable structural framework for the CDRs and help to determine their position and conformation. Antibodies raised against intact proteins usually bind to the surface of the protein and make contact with residues that are discontinuous in the primary structure of the molecule; they may, however, occasionally bind peptide fragments of the protein, and antibodies raised against peptides derived from a protein can sometimes be used to detect the native protein molecule. Peptides binding to antibodies usually bind in a cleft or pocket between the V regions of the heavy and light chains, where they make specific contact with some, but not necessarily all, of the CDRs. This is also the usual mode of binding for carbohydrate antigens and small molecules such as haptens.

Antigen recognition by T cells.

In contrast to the immunoglobulins, which interact with pathogens and their toxic products in the extracellular spaces of the body, T cells recognize foreign antigens only when they are displayed on the surface of the body's own cells. These antigens can derive from pathogens such as viruses or intracellular bacteria, which replicate within cells, or from pathogens or their products that have been internalized by endocytosis from the extracellular fluid.

T cells detect the presence of an intracellular pathogen because the infected cells display peptide fragments of the pathogen's proteins on their surface. These foreign peptides are delivered to the cell surface by specialized host-cell glycoproteins—the MHC molecules. These are encoded in a large cluster of genes that were first identified by their powerful effects on the immune response to transplanted tissues. For that reason, the gene complex was called the major histocompatibility complex (MHC), and the peptide-binding glycoproteins are known as MHC molecules. The recognition of antigen as a small peptide fragment bound to an MHC molecule and displayed at the cell surface is one of the most distinctive features of T cells, and will be the focus of this part of the chapter. How the peptide fragments of antigen are generated and become associated with MHC molecules will be described in Chapter 6.

We describe here the structure and properties of the T-cell receptor (TCR). As might be expected from the T-cell receptors' function as highly variable antigen-recognition structures, the genes for TCRs are closely related to those for immunoglobulins. There are, however, important differences between T-cell receptors and immunoglobulins, and these differences reflect the special features of antigen recognition by T cells.

4-11 The TCR α : β heterodimer is very similar to a Fab fragment of immunoglobulin.

T-cell receptors were first identified by using monoclonal antibodies that bound to a single cloned T-cell line: such antibodies either specifically inhibit antigen recognition by the clone or specifically activate it by mimicking the antigen (see Appendix I, Section A-20). These **clonotypic** antibodies were then used to show that each T cell bears about 30,000 identical antigen receptors on its surface, each receptor consisting of two different polypeptide chains, termed the **T-cell receptor α (TCR α)** and **β (TCR β)** chains. Each chain of the **α : β heterodimer** is composed of two Ig domains, and the two chains are linked by a disulfide bond, similar to the structure of the Fab fragment of an immunoglobulin molecule (Fig. 4.13). α : β heterodimers account for antigen recognition by most T cells. A minority of T cells bear an alternative, but structurally similar, receptor made up of a different pair of polypeptide chains designated γ and δ . The **γ : δ T-cell receptors** seem to have different antigen-recognition properties from the **α : β T-cell receptors**, and the functions of γ : δ T cells in immune responses are still being clarified as the various ligands they recognize are identified (see Section 6-20). In the rest of this chapter and elsewhere in the book we use the term T-cell receptor to mean the α : β receptor, except where specified otherwise. Both types of T-cell receptors differ from the membrane-bound immunoglobulin that serves as the B-cell receptor in two main ways. A T-cell receptor has only one antigen-binding site, whereas a B-cell receptor has two, and T-cell receptors are never secreted, whereas immunoglobulins can be secreted as antibodies.

Further insights into the structure and function of the α : β T-cell receptor came from studies of cloned cDNA encoding the receptor chains. The amino acid sequences predicted from the cDNA showed that both chains of the T-cell receptor have an amino-terminal variable (V) region with sequence homology to an immunoglobulin V domain, a constant (C) region with homology to an immunoglobulin C domain, and a short stalk segment containing a cysteine residue that forms the interchain disulfide bond (Fig. 4.14). Each chain spans the lipid bilayer by a hydrophobic transmembrane domain, and ends in a short cytoplasmic tail. These close similarities of T-cell receptor chains to the heavy and light immunoglobulin chains first enabled prediction of the structural resemblance of the T-cell receptor heterodimer to a Fab fragment of immunoglobulin.

The three-dimensional structure of the T-cell receptor determined by X-ray crystallography in Fig. 4.15a shows that T-cell receptor chains fold in much the same way as the regions comprising the Fab fragment in Fig. 4.1a.

Fig. 4.14 Structure of the T-cell receptor. The T-cell receptor heterodimer is composed of two transmembrane glycoprotein chains, α and β . The extracellular portion of each chain consists of two domains, resembling immunoglobulin V and C domains, respectively. Both chains have carbohydrate side chains attached to each domain. A short stalk segment, analogous to an immunoglobulin hinge region, connects the Ig-like domains to the membrane and contains the cysteine residue that forms the interchain disulfide bond. The transmembrane helices of both chains are unusual in containing positively charged (basic) residues within the hydrophobic transmembrane segment. The α chain carries two such residues; the β chain has one.

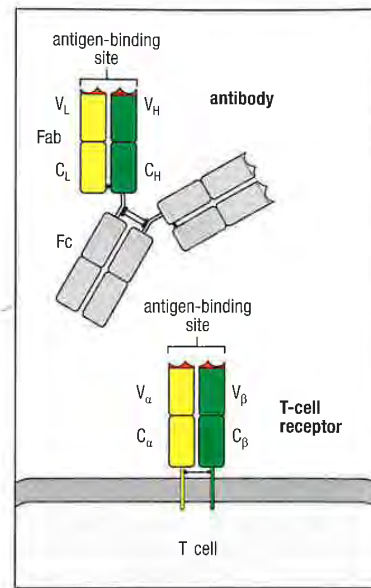
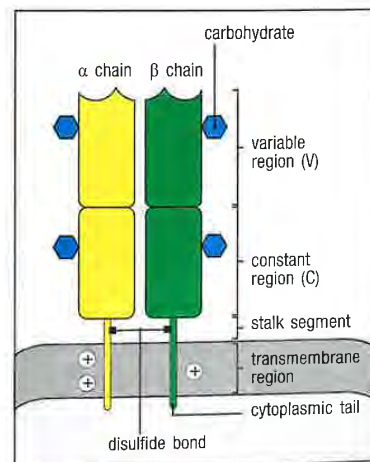


Fig. 4.13 The T-cell receptor resembles a membrane-bound Fab fragment. The Fab fragment of an antibody molecule is a disulfide-linked heterodimer, each chain of which contains one immunoglobulin C domain and one V domain; the juxtaposition of the V domains forms the antigen-binding site (see Section 4-6). The T-cell receptor is also a disulfide-linked heterodimer, with each chain containing an immunoglobulin C-like domain and an immunoglobulin V-like domain. As in the Fab fragment, the juxtaposition of the V domains forms the site for antigen recognition.



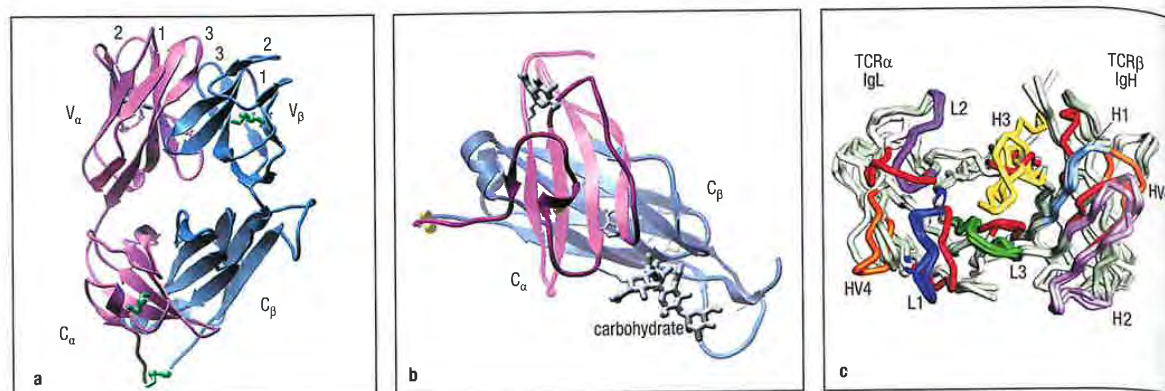


Fig. 4.15 The crystal structure of an $\alpha:\beta$ T-cell receptor resolved at 0.25 nm. In panels a and b, the α chain is shown in pink and the β chain in blue. Disulfide bonds are shown in green. In panel a, the T-cell receptor is viewed from the side as it would sit on a cell surface, with the CDR loops that form the antigen-binding site (labeled 1, 2, and 3) arrayed across its relatively flat top. In panel b, the C α and C β domains are shown. The C α domain does not fold into a typical Ig-like domain; the face of the domain away from the C β domain is mainly composed of irregular strands of polypeptide rather than β sheet. The intramolecular disulfide bond (far left) joins a β strand to this segment of α helix. The interaction between the C α and C β domains is assisted by carbohydrate (colored gray and labeled), with a sugar group from the C α domain making hydrogen bonds to the C β domain. In panel c, the T-cell

receptor is shown aligned with the antigen-binding sites from three different antibodies. This view is looking down into the binding site. The V α domain of the T-cell receptor is aligned with the V L domains of the antigen-binding sites of the antibodies, and the V β domain is aligned with the V H domains. The CDRs of the T-cell receptor and immunoglobulin molecules are colored, with CDRs 1, 2, and 3 of the TCR shown in red and the HV4 loop in orange. For the immunoglobulin V domains, the CDR1 loops of the heavy chain (H1) and light chain (L1) are shown in light and dark blue, respectively, and the CDR2 loops (H2, L2) in light and dark purple, respectively. The heavy-chain CDR3 loops (H3) are in yellow; the light-chain CDR3s (L3) are in bright green. The HV4 loops of the TCR (orange) have no hypervariable counterparts in immunoglobulins. Model structures courtesy of I.A. Wilson.

There are, however, some distinct structural differences between T-cell receptors and Fab fragments. The most striking is in the C α domain, where the fold is unlike that of any other Ig-like domain. The half of the C α domain that is juxtaposed with the C β domain forms a β sheet similar to that found in other Ig-like domains, but the other half of the domain is formed of loosely packed strands and a short segment of α helix (see Fig. 4.15b). In a C α domain the intramolecular disulfide bond, which in Ig-like domains normally joins two β strands, joins a β strand to this segment of α helix.

There are also differences in the way in which the domains interact. The interface between the V and C domains of both T-cell receptor chains is more extensive than in most antibodies. The interaction between the C α and C β domains is distinctive, as it might be assisted by carbohydrates, with a sugar group from the C α domain making a number of hydrogen bonds to the C β domain (see Fig. 4.15b). Finally, a comparison of the variable binding sites shows that, although the CDR loops align fairly closely with those of antibody molecules, there is some relative displacement (see Fig. 4.15c). This is particularly marked in the V α CDR2 loop, which is oriented at roughly right angles to the equivalent loop in antibody V domains, as a result of a shift in the β strand that anchors one end of the loop from one face of the domain to the other. A strand displacement also causes a change in the orientation of the V β CDR2 loop in some V β domains. These differences with antibodies influence the ability of the T-cell receptor to recognize their specific ligands, as we will discuss in the next section. In addition to the three hypervariable regions shared with immunoglobulins, the T-cell receptor has a fourth hypervariability region, HV4, in both of its chains (see Fig. 4.15c). These regions occur away from the antigen-binding face of the receptor, and have been implicated in other functions of the TCR, such as superantigen binding, which we will describe in Section 6-14.

4-12 A T-cell receptor recognizes antigen in the form of a complex of a foreign peptide bound to an MHC molecule.

Antigen recognition by T-cell receptors clearly differs from recognition by B-cell receptors and antibodies. The immunoglobulin on B cells binds directly to the intact antigen, and, as discussed in Section 4-8, antibodies typically bind to the surface of protein antigens, contacting amino acids that are discontinuous in the primary structure but are brought together in the folded protein. In contrast, $\alpha\beta$ T cells respond to short, continuous amino acid sequences. As we described in Section 1-10, these peptide sequences are often buried within the native structure of the protein. Thus, antigens cannot be recognized directly by T-cell receptors unless the protein is unfolded and processed into peptide fragments (Fig. 4.16), and then presented by an MHC molecule (see Fig. 1.15). We will return to the issue of how this process occurs in Chapter 6.

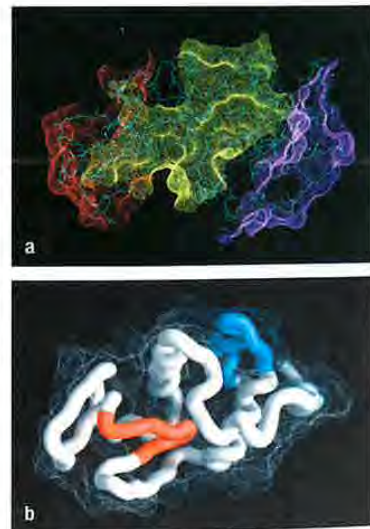
The nature of the antigen recognized by T cells became clear with the realization that the peptides that stimulate T cells are recognized only when bound to an MHC molecule. The ligand recognized by the T cell is thus a complex of peptide and MHC molecule. The evidence for involvement of the MHC in T-cell recognition of antigen was at first indirect, but it has been proven conclusively by stimulating T cells with purified peptide:MHC complexes. The T-cell receptor interacts with this ligand by making contacts with both the MHC molecule and the antigen peptide.

4-13 There are two classes of MHC molecules with distinct subunit compositions but similar three-dimensional structures.

There are two classes of MHC molecules—**MHC class I** and **MHC class II**—and they differ in both their structure and their expression pattern in the tissues of the body. As shown in Figs. 4.17 and 4.18, MHC class I and MHC class II molecules are closely related in overall structure but differ in their subunit composition. In both classes, the two paired protein domains nearest to the membrane resemble immunoglobulin domains, whereas the two domains furthest away from the membrane fold together to create a long cleft, or groove, which is the site at which a peptide binds. Purified peptide:MHC class I and peptide:MHC class II complexes have been characterized structurally, allowing us to describe in detail both the MHC molecules themselves and the way in which they bind peptides.

MHC class I molecules (see Fig. 4.17) consist of two polypeptide chains. One chain, the α chain, is encoded in the MHC (on chromosome 6 in humans) and is noncovalently associated with a smaller chain, β_2 -microglobulin, which is encoded on a different chromosome—chromosome 15 in humans. Only the class I α chain spans the membrane. The complete MHC class I molecule has four domains, three formed from the MHC-encoded α chain, and one contributed by β_2 -microglobulin. The α_3 domain and β_2 -microglobulin closely resemble Ig-like domains in their folded structure. The folded α_1 and α_2

Fig. 4.16 Differences in the recognition of hen egg-white lysozyme by immunoglobulins and T-cell receptors. Antibodies can be shown by X-ray crystallography to bind epitopes on the surface of proteins, as shown in panel a, where the epitopes for three antibodies are shown in different colors on the surface of hen egg-white lysozyme (see also Fig. 4.10). In contrast, the epitopes recognized by T-cell receptors need not lie on the surface of the molecule, because the T-cell receptor recognizes not the antigenic protein itself but a peptide fragment of the protein. The peptides corresponding to two T-cell epitopes of lysozyme are shown in panel b. One epitope, shown in blue, lies on the surface of the protein, but a second, shown in red, lies mostly within the core and is inaccessible in the folded protein. This implies that T-cell receptors do not recognize their epitopes in the context of the native protein. Panel a courtesy of S. Sheriff.



domains form the walls of a cleft on the surface of the molecule; because this is where the peptide binds, this part of the MHC molecule is known as the **peptide-binding cleft** or **peptide-binding groove**. The MHC molecules are highly polymorphic, and the major differences between the different allelic

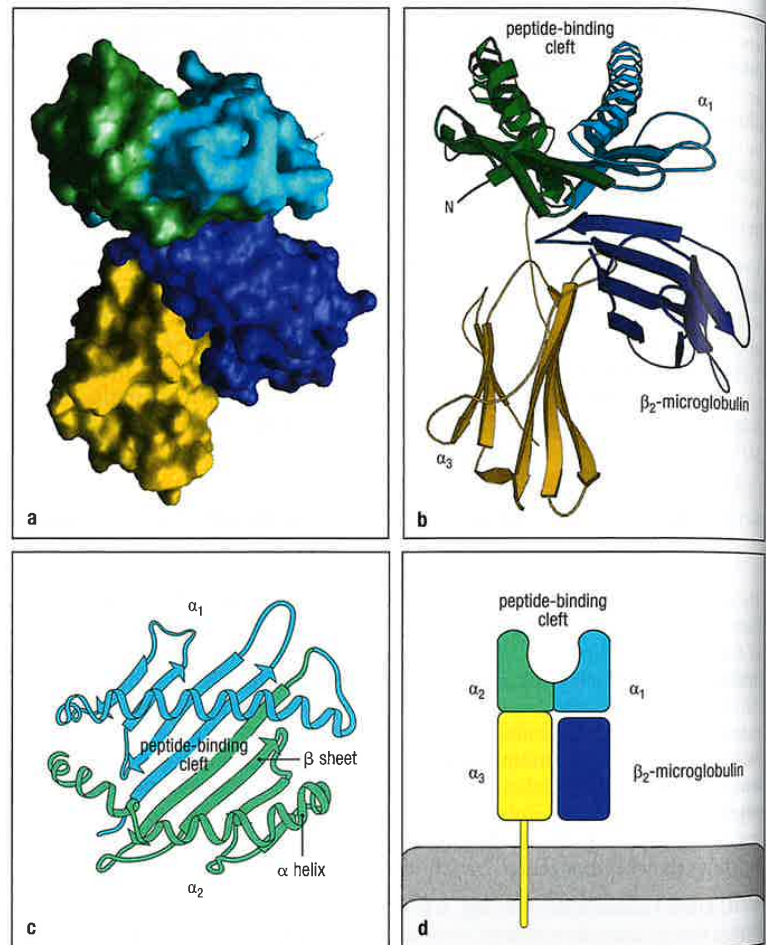


Fig. 4.17 The structure of an MHC class I molecule determined by X-ray crystallography. Panel a shows a computer graphic representation of a human MHC class I molecule, HLA-A2, which has been cleaved from the cell surface by the enzyme papain. The surface of the molecule is shown, colored according to the domains shown in panels b–d and described below. Panels b and c show a ribbon diagram of that structure. Shown schematically in panel d, the MHC class I molecule is a heterodimer of a membrane-spanning α chain (molecular weight 43 kDa) bound noncovalently to β_2 -microglobulin (12 kDa), which does not span the membrane. The α chain folds into three domains: α_1 , α_2 , and α_3 . The α_3 domain and β_2 -microglobulin show similarities in amino acid sequence to immunoglobulin C domains and have similar folded structures, whereas the α_1 and α_2 domains are part of the same polypeptide and fold together into a single structure consisting of two separated α helices lying on a sheet of eight antiparallel β strands. The folding of the α_1 and α_2 domains creates a long cleft or groove, which is the site at which peptide antigens bind to the MHC molecules. For class I molecules, this groove is open at only one end. The transmembrane region and the short stretch of peptide that connects the external domains to the cell surface are not seen in panels a and b because they have been removed by the digestion with papain. As can be seen in panel c, looking down on the molecule from above, the sides of the cleft are formed from the inner faces of the two α helices; the β pleated sheet formed by the pairing of the α_1 and α_2 domains creates the floor of the cleft.

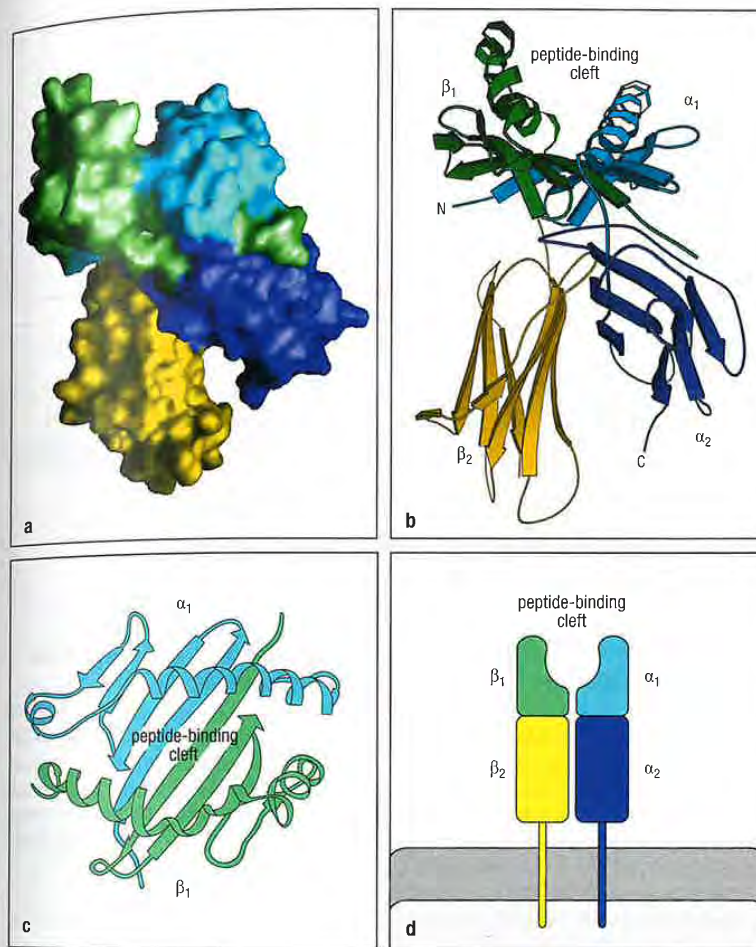


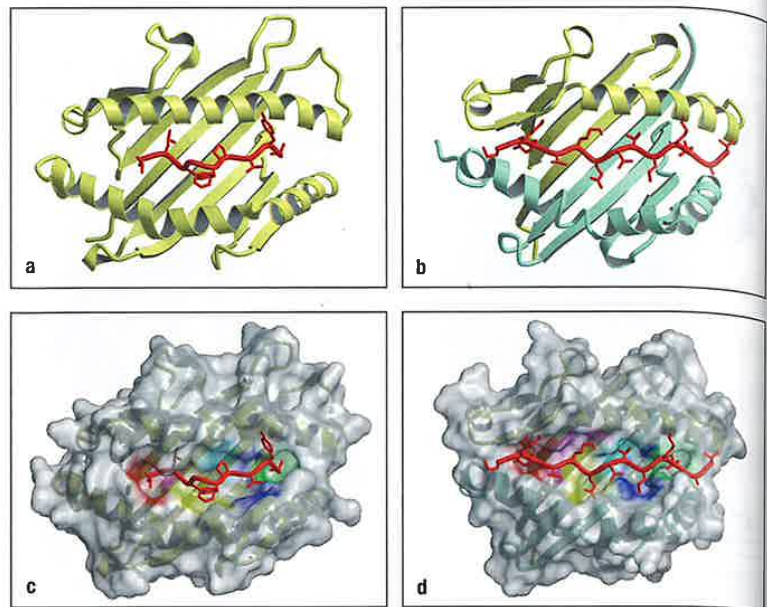
Fig. 4.18 MHC class II molecules resemble MHC class I molecules in overall structure.

The MHC class II molecule is composed of two transmembrane glycoprotein chains, α (34 kDa) and β (29 kDa), as shown schematically in panel d. Each chain has two domains, and the two chains together form a compact four-domain structure similar to that of the MHC class I molecule (compare with panel d of Fig. 4.17). Panel a shows a computer graphic representation of the surface of the MHC class II molecule, in this case the human protein HLA-DR1, and panel b shows the equivalent ribbon diagram. N, amino terminus; C, carboxy terminus. The α_2 and β_2 domains, like the α_3 and β_2 -microglobulin domains of the MHC class I molecule, have amino acid sequence and structural similarities to immunoglobulin C domains; in the MHC class II molecule the two domains forming the peptide-binding cleft are contributed by different chains and are therefore not joined by a covalent bond (see panels c and d). Another important difference, not apparent in this diagram, is that the peptide-binding groove of the MHC class II molecule is open at both ends.

forms are located in the peptide-binding cleft, influencing which peptides will bind and thus the specificity of the dual antigen presented to T cells. By contrast, β_2 -microglobulin, which does not contribute directly to peptide binding, is not polymorphic.

An MHC class II molecule consists of a noncovalent complex of two chains, α and β , both of which span the membrane (see Fig. 4.18). The MHC class II α chain is a different protein from the class I α chain. The MHC class II α and β chains are both encoded within the MHC. The crystallographic structure of the MHC class II molecule shows that it is folded very much like the MHC class I molecule, but the peptide-binding cleft is formed by two domains from different chains, the α_1 and β_1 domains. The major differences lie at the ends of the peptide-binding cleft, which are more open in MHC class II than in MHC class I molecules. Consequently, the ends of a peptide bound to an MHC class I molecule are substantially buried within the molecule, whereas the ends of peptides bound to MHC class II molecules are not. In both MHC class I and class II molecules, bound peptides are sandwiched between the two α -helical segments of the MHC molecule (Fig. 4.19). The T-cell receptor interacts with this compound ligand, making contacts with both the MHC molecule and the peptide antigen. As in the case of MHC class I molecules, the sites of major polymorphism in MHC class II molecules are located in the peptide-binding cleft.

Fig. 4.19 MHC molecules bind peptides tightly within the cleft. When MHC molecules are crystallized with a single synthetic peptide antigen, the details of peptide binding are revealed. In MHC class I molecules (panels a and c), the peptide is bound in an elongated conformation with both ends tightly bound at either end of the cleft. In MHC class II molecules (panels b and d), the peptide is also bound in an elongated conformation but the ends of the peptide are not tightly bound and the peptide extends beyond the cleft. The upper surface of the peptide:MHC complex is recognized by T cells, and is composed of residues of the MHC molecule and the peptide. The amino acid side chains of the peptide insert into pockets in the peptide-binding groove of the MHC molecule; these pockets are lined with residues that are polymorphic within the MHC. In representations c and d, the surfaces of the different pockets for the different amino acids are depicted as areas of different colors. Structures courtesy of R.L. Stanfield and I.A. Wilson.



4-14 Peptides are stably bound to MHC molecules, and also serve to stabilize the MHC molecule on the cell surface.

An individual can be infected by a wide variety of pathogens, whose proteins will not generally have peptide sequences in common. For T cells to be able to detect the widest possible array of infections, the MHC molecules (both class I and class II) of an individual should be able to bind stably to many different peptides. This behavior is quite distinct from that of other peptide-binding receptors, such as those for peptide hormones, which usually bind only a single type of peptide. The crystal structures of peptide:MHC complexes have helped to show how a single binding site can bind a peptide with high affinity while retaining the ability to bind a wide variety of different peptides.

An important feature of the binding of peptides to MHC molecules is that the peptide is bound as an integral part of the MHC molecule's structure, and MHC molecules are unstable when peptides are not bound. This dependence on bound peptide applies to both MHC class I and MHC class II molecules. Stable peptide binding is important, because otherwise peptide exchanges occurring at the cell surface would prevent peptide:MHC complexes from being reliable indicators of infection or of uptake of a specific antigen. When MHC molecules are purified from cells, their stably bound peptides co-purify with them, and this fact has enabled the peptides bound by particular MHC molecules to be analyzed. Peptides are released from the MHC molecules by denaturing the complex in acid, and they are then purified and sequenced. Pure synthetic peptides can also be incorporated into empty MHC molecules and the structure of the complex determined, revealing details of the contacts between the MHC molecule and the peptide. From such studies a detailed picture of the binding interactions has been built up. We first discuss the peptide-binding properties of MHC class I molecules.

4-15 MHC class I molecules bind short peptides of 8–10 amino acids by both ends.

Binding of a peptide to an MHC class I molecule is stabilized at both ends of the peptide-binding cleft by contacts between atoms in the free amino and carboxy termini of the peptide and invariant sites that are found at each end

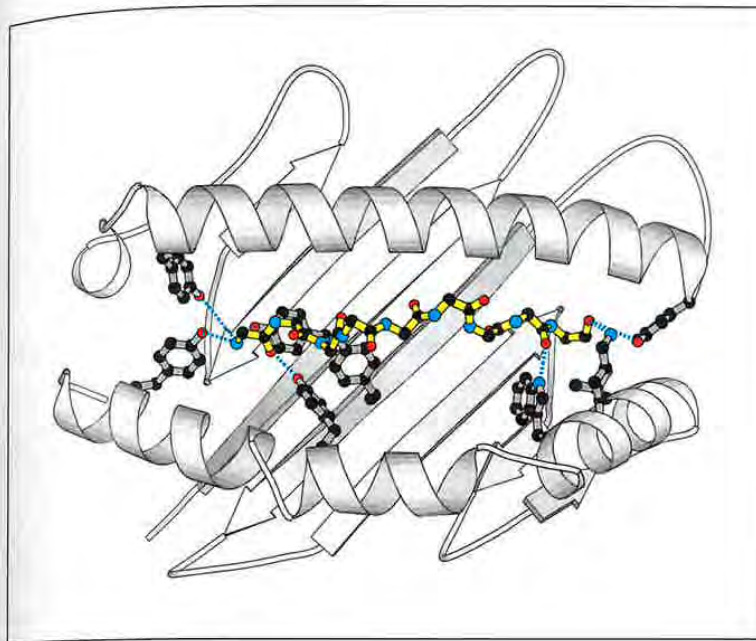


Fig. 4.20 Peptides are bound to MHC class I molecules by their ends.

MHC class I molecules interact with the backbone of a bound peptide (shown in yellow) through a series of hydrogen bonds and ionic interactions (shown as dotted blue lines) at each end of the peptide. The amino terminus of the peptide is to the left, the carboxy terminus to the right. Black circles are carbon atoms; red are oxygen; blue are nitrogen. The amino acid residues in the MHC molecule that form these bonds are common to all MHC class I molecules, and their side chains are shown in full (in gray) on a ribbon diagram of the MHC class I groove. A cluster of tyrosine residues common to all MHC class I molecules forms hydrogen bonds to the amino terminus of the bound peptide, while a second cluster of residues forms hydrogen bonds and ionic interactions with the peptide backbone at the carboxy terminus and with the carboxy terminus itself.

of the cleft in all MHC class I molecules (Fig. 4.20). These are thought to be the main stabilizing contacts for peptide:MHC class I complexes, because synthetic peptide analogs lacking terminal amino and carboxyl groups fail to bind stably to MHC class I molecules. Other residues in the peptide serve as additional anchors. Peptides that bind to MHC class I molecules are usually 8–10 amino acids long. Longer peptides are thought to bind, however, particularly if they can bind at their carboxy terminus, but they are subsequently shortened to 8–10 amino acids through cleavage by exopeptidases present in the endoplasmic reticulum, which is where MHC class I molecules bind peptides. The peptide lies in an elongated conformation along the cleft; variations in peptide length seem to be accommodated, in most cases, by a kinking in the peptide backbone. However, in some cases, length variation can also be accommodated in MHC class I molecules by allowing the peptide to extend out of the cleft at the carboxy terminus.

These interactions give MHC class I molecules a broad peptide-binding specificity. In addition, MHC molecules are highly polymorphic. As mentioned earlier, MHC genes are highly polymorphic and there are hundreds of different allelic variations of the MHC class I genes in the human population. Each individual carries only a small selection of these variants. The main differences between allelic MHC variants are found at certain sites in the peptide-binding cleft, resulting in different amino acids in key peptide-interaction sites. Because of this, different MHC variants preferentially bind different peptides. The peptides that can bind to a given MHC variant have the same or very similar amino acid residues at two or three particular positions along the peptide sequence. The amino acid side chains at these positions insert into pockets in the MHC molecule that are lined by the polymorphic amino acids. Because this binding anchors the peptide to the MHC molecule, the peptide residues involved are called the **anchor residues**, as illustrated in Fig. 4.21. Both the position and identity of these anchor residues can vary, depending on the particular MHC class I variant that is binding the peptide. However, most peptides that bind to MHC class I molecules have a hydrophobic (or sometimes basic) residue at the carboxy terminus that also serves to anchor the peptide in the groove. Whereas changing an anchor residue will in most cases prevent the peptide from binding, not every synthetic peptide of suitable length that

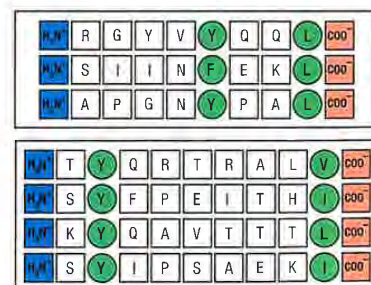


Fig. 4.21 Peptides bind to MHC molecules through structurally related anchor residues.

Peptides eluted from two different MHC class I molecules are shown in the upper and lower panels, respectively. The anchor residues (green) differ for peptides that bind different allelic variants of MHC class I molecules but are similar for all peptides that bind to the same MHC molecule. The anchor residues that bind a particular MHC molecule need not be identical, but are always related: for example, phenylalanine (F) and tyrosine (Y) are both aromatic amino acids, whereas valine (V), leucine (L), and isoleucine (I) are all large hydrophobic amino acids. Peptides also bind to MHC class I molecules through their amino (blue) and carboxy (red) termini.

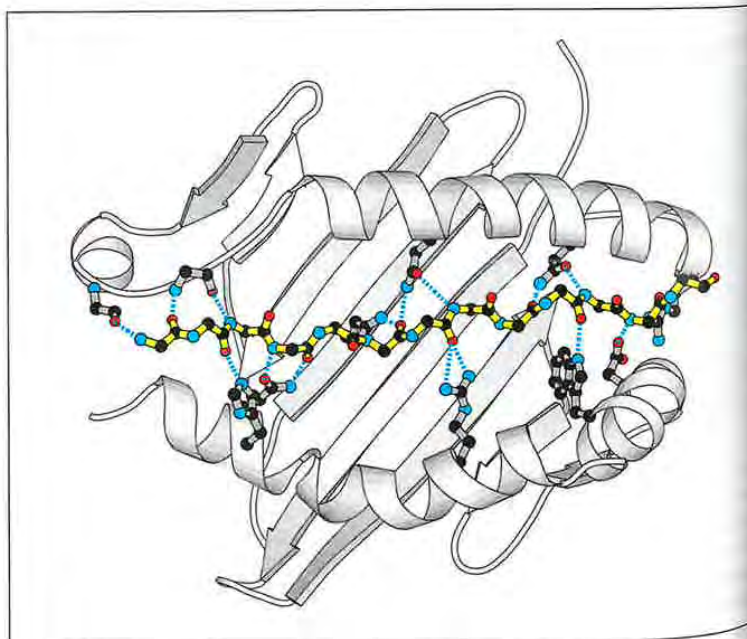
contains these anchor residues will bind the appropriate MHC class I molecule, and so the overall binding must also depend on the nature of the amino acids at other positions in the peptide. In some cases, particular amino acids are preferred in certain positions, whereas in others the presence of particular amino acids prevents binding. These additional amino acid positions are called 'secondary anchors.' These features of peptide binding enable an individual MHC class I molecule to bind a wide variety of different peptides, yet allow different MHC class I allelic variants to bind different sets of peptides. As we will see in Chapter 15, MHC polymorphisms also impact the binding of peptides derived from self-proteins and can influence the susceptibility of an individual to various autoimmune diseases.

4-16 The length of the peptides bound by MHC class II molecules is not constrained.

Like MHC class I molecules, MHC class II molecules that lack bound peptide are unstable. Peptide binding to MHC class II molecules has also been analyzed by elution of bound peptides and by X-ray crystallography, and differs in several ways from peptide binding to MHC class I molecules. Natural peptides that bind to MHC class II molecules are at least 13 amino acids long and can be much longer. The clusters of conserved residues that bind the two ends of a peptide in MHC class I molecules are not found in MHC class II molecules, and the ends of the peptide are not bound. Instead, the peptide lies in an extended conformation along the peptide-binding cleft. It is held there both by peptide side chains that protrude into shallow and deep pockets lined by polymorphic residues, and by interactions between the peptide backbone and the side chains of conserved amino acids that line the peptide-binding cleft in all MHC class II molecules (Fig. 4.22). Structural data show that amino acid side chains at residues 1, 4, 6, and 9 of an MHC class II-bound peptide can be held in these binding pockets.

The binding pockets of MHC class II molecules accommodate a greater variety of side chains than those of MHC class I molecules, making it more difficult to define anchor residues and to predict which peptides will be able to bind

Fig. 4.22 Peptides bind to MHC class II molecules by interactions along the length of the binding groove. A peptide (yellow; shown as the peptide backbone only, with the amino terminus to the left and the carboxy terminus to the right) is bound by an MHC class II molecule through a series of hydrogen bonds (dotted blue lines) that are distributed along the length of the peptide. The hydrogen bonds toward the amino terminus of the peptide are made with the backbone of the MHC class II polypeptide chain, whereas throughout the peptide's length bonds are made with residues that are highly conserved in MHC class II molecules. The side chains of these residues are shown in gray on the ribbon diagram of the MHC class II groove.



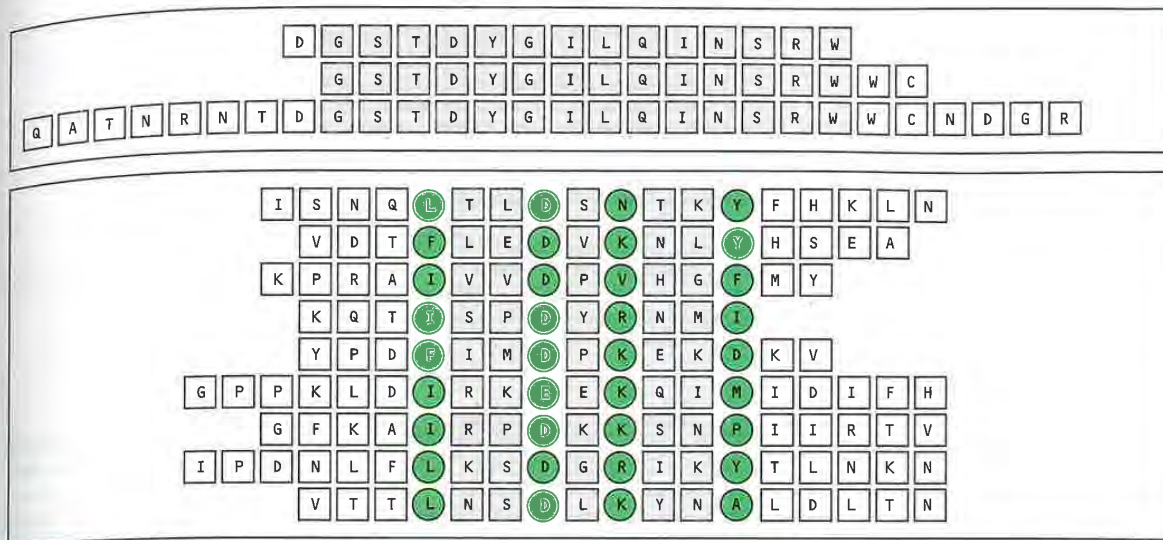


Fig. 4.23 Peptides that bind MHC class II molecules are variable in length and their anchor residues lie at various distances from the ends of the peptide. The sequences of a set of peptides that bind to the mouse MHC class II A^k allele are shown in the upper panel. All contain the same core sequence (shaded) but differ in length. In the lower panel, different peptides binding to the human MHC class II allele HLA-DR3 are shown. Anchor residues are shown as green circles. The lengths of these

peptides can vary, and so by convention the first anchor residue is denoted as residue 1. Note that all of the peptides share a hydrophobic residue in position 1, a negatively charged residue [aspartic acid (D) or glutamic acid (E)] in position 4, and a tendency to have a basic residue [lysine (K), arginine (R), histidine (H), glutamine (Q), or asparagine (N)] in position 6 and a hydrophobic residue [for example, tyrosine (Y), leucine (L), phenylalanine (F)] in position 9.

a particular MHC class II variant (Fig. 4.23). Nevertheless, by comparing the sequences of known binding peptides it is usually possible to detect patterns of amino acids that permit binding to different MHC class II variants, and to model how the amino acids of this peptide sequence motif will interact with the amino acids of the peptide-binding cleft. Because the peptide is bound by its backbone and allowed to emerge from both ends of the binding groove, there is, in principle, no upper limit to the length of peptides that could bind to MHC class II molecules. An example of this is the protein known as **invariant chain**, part of which lies entirely across the peptide-binding groove of nascent MHC class II molecules during their synthesis in the endoplasmic reticulum. We will return in Chapter 6 to the role of the invariant chain in the loading of peptides onto MHC class II molecules. In most cases, long peptides bound to MHC class II molecules are trimmed by peptidases to a length of around 13–17 amino acids.

4-17 The crystal structures of several peptide:MHC:T-cell receptor complexes show a similar orientation of the T-cell receptor over the peptide:MHC complex.

At the time that the first X-ray crystallographic structure of a T-cell receptor was published, a structure of the same T-cell receptor bound to a peptide:MHC class I ligand was also produced. The orientation revealed by these structures showed that the T-cell receptor is aligned diagonally over the peptide and the peptide-binding cleft (Fig. 4.24). The TCR α chain lies over the α_2 domain of the MHC molecule at the amino-terminal end of the bound peptide, as seen from the side view shown in Fig. 4.24a. The TCR β chain lies over the MHC molecule's α_1 domain, closer to the carboxy-terminal end of the peptide. Figure 4.24b shows a view of this structure as if looking down through a transparent T-cell receptor to indicate where it contacts the MHC molecule. The CDR3 loops of both TCR α and TCR β chains come together and lie over

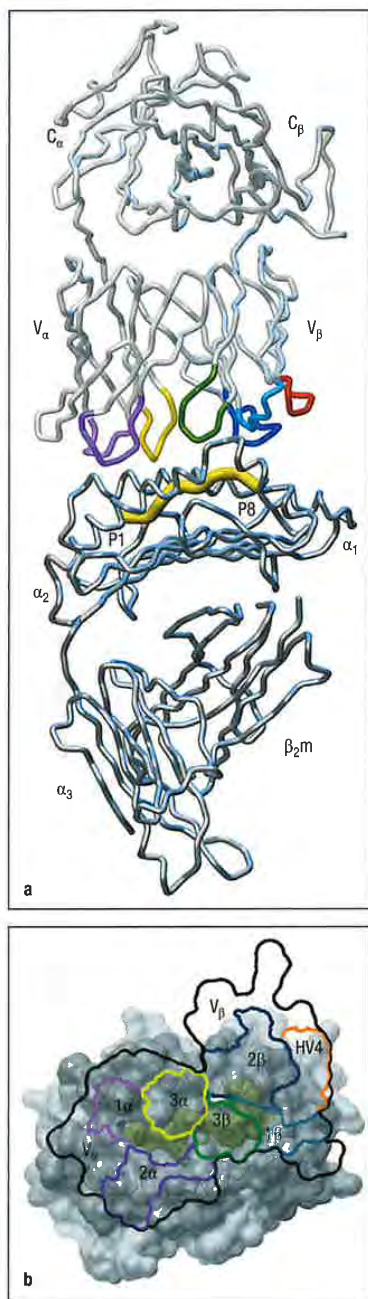


Fig. 4.24 The T-cell receptor binds to the peptide:MHC complex. Panel a: the T-cell receptor binds to the top of a peptide:MHC class I molecule, touching both the α_1 and α_2 domain helices. The CDRs of the T-cell receptor are shown in color: the CDR1 and CDR2 loops of the β chain are light and dark blue, respectively; and the CDR1 and CDR2 loops of the α chain are light and dark purple, respectively. The α -chain CDR3 loop is yellow, and the β -chain CDR3 loop is green. The β -chain HV4 loop is in red. The thick yellow line P1–P8 is the bound peptide. Panel b: the outline of the T-cell receptor's antigen-binding site (thick black line) is superimposed over the top surface of the peptide:MHC complex (the peptide is shaded dull yellow). The T-cell receptor lies at a somewhat diagonal angle across the peptide:MHC complex, with the α and β CDR3 loops of the T-cell receptor (3α , 3β , yellow and green, respectively) contacting the center of the peptide. The α -chain CDR1 and CDR2 loops (1α , 2α , light and dark purple, respectively) contact the MHC helices at the amino terminus of the bound peptide, whereas the β -chain CDR1 and CDR2 loops (1β , 2β , light and dark blue, respectively) make contact with the helices at the carboxy terminus of the bound peptide. Courtesy of I.A. Wilson.

central amino acids of the peptide. The T-cell receptor is threaded through a valley between the two high points on the two surrounding α helices that form the walls of the peptide-binding cleft. This can be seen in Fig. 4.25, which shows a view from the end of the peptide-binding groove of a peptide:MHC class II:T-cell receptor complex. Comparison of various peptide:MHC:T-cell receptor complexes shows that the axis of the TCR as it binds the surface of the MHC molecule is rotated somewhat relative to the peptide-binding groove of the MHC molecule (see Fig. 4.24b). In this orientation, the V_α domain makes contact primarily with the amino-terminal half of the bound peptide, whereas the V_β domain contacts primarily the carboxy-terminal half. Both chains also interact with the α helices of the MHC class I molecule (see Fig. 4.24). The T-cell receptor contacts are not symmetrically distributed over the MHC molecule: whereas the V_α CDR1 and CDR2 loops are in close contact with the helices of the peptide:MHC complex around the amino terminus of the bound peptide, the β -chain CDR1 and CDR2 loops, which interact with the complex at the carboxy terminus of the bound peptide, have variable contributions to the binding.

Comparison of the three-dimensional structure of an unliganded T-cell receptor and the same T-cell receptor complexed to its peptide:MHC ligand shows that the binding results in some degree of conformational change, or 'induced fit,' particularly within the V_α CDR3 loop. Subtle variations at amino acids that contact the T-cell receptor can have strikingly different effects on the recognition of an otherwise identical peptide:MHC ligand by the same T cell. The flexibility in the CDR3 loop demonstrated by these two structures helps to explain how the T-cell receptor can adopt conformations that recognize related, but different, peptide ligands.

The specificity of T-cell recognition involves both the peptide and its presenting MHC molecule. Kinetic analysis of T-cell receptor binding to peptide:MHC ligands suggests that the interactions with MHC molecules might predominate at the start of the contact, but that subsequent interactions with the peptide as well as the MHC molecule dictate the final outcome—binding or dissociation. As with antibody-antigen interactions, only a few amino acids at the interface might provide the essential contacts that determine the specificity and strength of binding. Simply changing a leucine to isoleucine in the peptide, for example, is sufficient to alter a T-cell response from strong killing to no response at all. Mutations of single residues in the presenting MHC molecules can have the same effect. This dual specificity for T-cell recognition of antigen underlies the MHC restriction of T-cell responses, a phenomenon that was observed long before the peptide-binding properties of MHC molecules were known. Another consequence of this dual specificity is a need for T-cell receptors to exhibit some inherent specificity for MHC molecules in order to be able to interact appropriately with the antigen-presenting surface of MHC

Fig. 4.25 The T-cell receptor interacts with MHC class I and MHC class II molecules in a similar fashion. Shown is the structure of a T-cell receptor, specific for a peptide derived from chicken cytochrome c, bound to an MHC class II molecule. This T-cell receptor's binding is at a site and orientation similar to that of the T-cell receptor bound to the MHC class I molecule shown in Fig. 4.24. The α and β chains of the T-cell receptor are colored in light and dark blue, respectively. The cytochrome c peptide is light orange. The T-cell receptor sits in a shallow saddle formed between the α -helical regions of the MHC class II α (brown) and β (yellow) chains at roughly 90° to the long axis of the MHC class II molecule and the bound peptide. Structure derived from PDB 3QIB. Courtesy of K.C. Garcia.

molecules. We will return to these issues in Chapter 6, where we recount the discovery of MHC restriction in the context of T-cell recognition and MHC polymorphisms, and in Chapter 8, where we discuss the impact of these phenomena on T-cell development in the thymus.

4-18 The CD4 and CD8 cell-surface proteins of T cells directly contact MHC molecules and are required to make an effective response to antigen.

As we introduced in Section 1-21, T cells fall into two major classes distinguished by the expression of the cell-surface proteins **CD4** and **CD8**. CD8 is expressed by cytotoxic T cells, while CD4 is expressed by T cells whose function is to activate other cells. CD4 and CD8 were known as markers for these functional sets for some time before it became clear that the distinction was based on the ability of T cells to recognize different classes of MHC molecules. We now know that CD8 recognizes MHC class I molecules and CD4 recognizes MHC class II. During antigen recognition, CD4 or CD8 (depending on the type of T cell) associates with the T-cell receptor on the T-cell surface and binds to invariant sites on the MHC portion of the composite peptide:MHC ligand, away from the peptide-binding site. This binding contributes to the overall effectiveness of the T-cell response, and so CD4 and CD8 are called **co-receptors**.

CD4 is a single-chain protein composed of four Ig-like domains (Fig. 4.26). The first two domains (D1 and D2) are packed tightly together to form a rigid

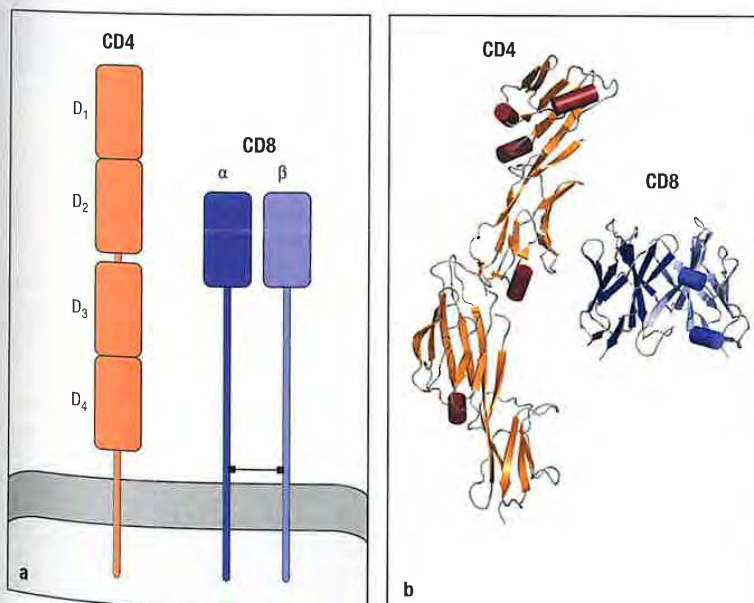
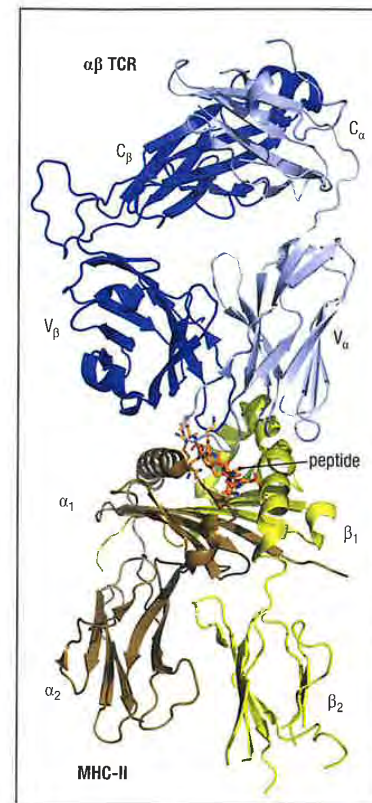


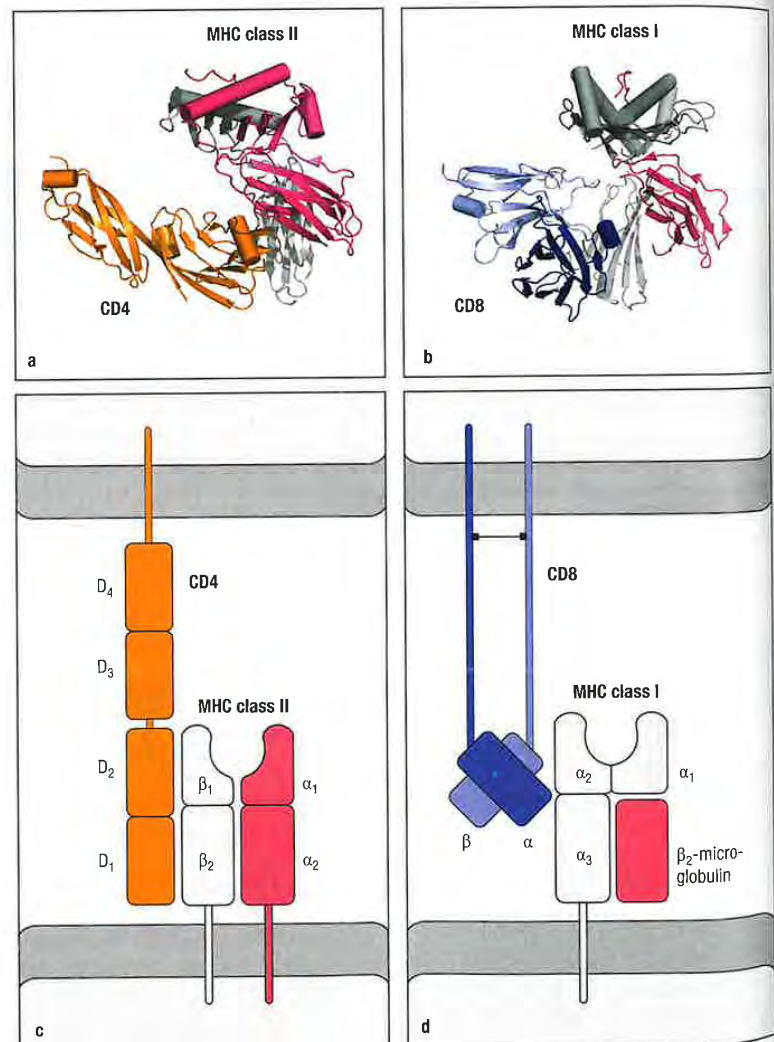
Fig. 4.26 The structures of the CD4 and CD8 co-receptor molecules. The CD4 molecule contains four Ig-like domains, shown in schematic form in panel a and as a ribbon diagram from the crystal structure in panel b. The amino-terminal domain, D1, is similar in structure to an immunoglobulin V domain. The second domain, D2, although related to an immunoglobulin domain, is different from both V and C domains and has been termed a C2 domain. The first two domains of CD4 form a rigid rodlike structure that is linked to the two carboxy-terminal domains by a flexible link. The binding site for MHC class II molecules involves mainly the D1 domain. The CD8 molecule is a heterodimer of an α and a β chain covalently linked by a disulfide bond. An alternative form of CD8 exists as a homodimer of α chains. The heterodimer is depicted in panel a, whereas the ribbon diagram in panel b is of the homodimer. CD8 α and CD8 β chains have very similar structures, each having a single domain resembling an immunoglobulin V domain and a stretch of polypeptide chain, believed to be in a relatively extended conformation, that anchors the V-like domain to the cell membrane.

rod about 6 nm long, which is joined by a flexible hinge to a similar rod formed by the third and fourth domains (D3 and D4). The MHC-binding region on CD4 is located mainly on a lateral face of the D1 domain, and CD4 binds to a hydrophobic crevice formed at the junction of the α_2 and β_2 domains of the MHC class II molecule (Fig. 4.27a). This site is well away from the site where the T-cell receptor binds, as shown by the complete crystal structure of a T-cell receptor bound to peptide:MHC class II with bound CD4 (Fig. 4.28). This structure demonstrates that the CD4 molecule and the T-cell receptor can bind simultaneously to the same peptide:MHC class II complex. CD4 enhances sensitivity to antigen, as the T cell is about 100-fold more sensitive to the antigen when CD4 is present. The enhancement process results from the ability of the intracellular portion of CD4 to bind to a cytoplasmic tyrosine kinase called **Lck**. As we will discuss in detail Chapter 7, bringing Lck into proximity with the T-cell receptor complex helps activate the signaling cascade induced by antigen recognition.

The structure of CD8 is quite different. It is a disulfide-linked dimer of two different chains, called α and β , each containing a single Ig-like domain linked to the membrane by a segment of extended polypeptide (see Fig. 4.26). This segment

Fig. 4.27 The binding sites for CD4 and CD8 on MHC class II and class I molecules lie in the Ig-like domains.

The binding sites for CD4 and CD8 on the MHC class II and class I molecules, respectively, lie in the Ig-like domains nearest to the membrane and distant from the peptide-binding cleft. The binding of CD4 to an MHC class II molecule is shown as a ribbon structure in panel a and schematically in panel c. The α chain of the MHC class II molecule is shown in pink, and the β chain in white, while CD4 is in gold. Only the D₁ and D₂ domains of the CD4 molecule are shown in panel a. The binding site for CD4 lies at the base of the β_2 domain of an MHC class II molecule, in the hydrophobic crevice between the β_2 and α_2 domains. The binding of CD8 $\alpha\beta$ to an MHC class I molecule is shown in panel b and schematically in panel d. The class I heavy chain and β_2 -microglobulin are shown in white and pink, respectively, and the two chains of the CD8 dimer are shown in light (CD8 β) and dark (CD8 α) purple. The binding site for CD8 on the MHC class I molecule lies in a similar position to that of CD4 in the MHC class II molecule, but CD8 binding also involves the base of the α_1 and α_2 domains, and thus the binding of CD8 to MHC class I is not completely equivalent to the binding of CD4 to MHC class II. Structures derived from PDB 3S4S (CD4/MHC class II) and PDB 3DMM (CD8 $\alpha\beta$ /MHC class I). Courtesy of K.C. Garcia.



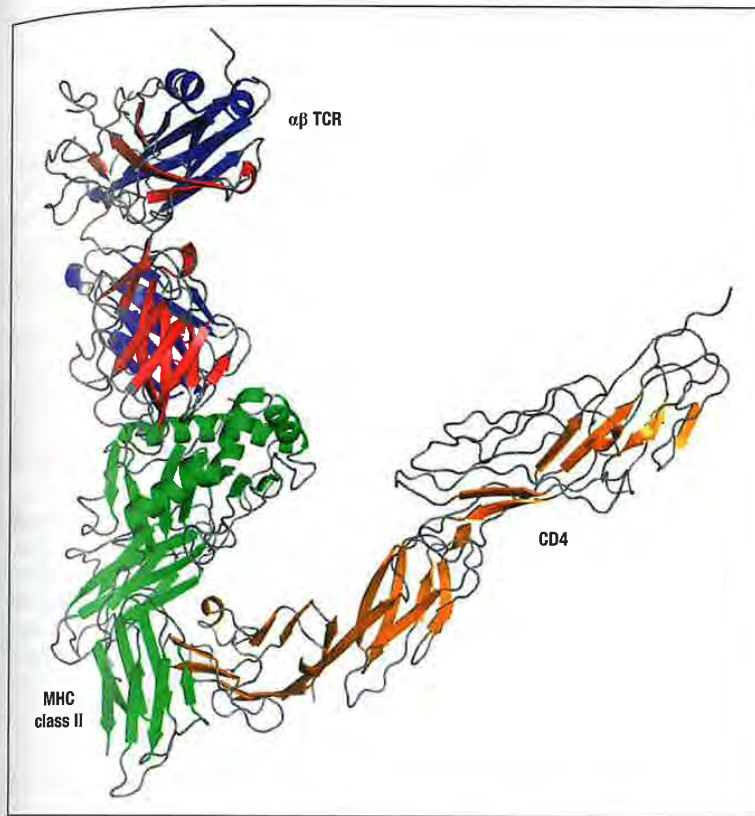


Fig. 4.28 CD4 and the T-cell receptor bind to distinct regions of the MHC class II molecule. A ribbon diagram is shown from a crystal structure of a complete $\alpha\beta$ TCR:peptide-MHC:CD4 ternary complex. The α and β chains of the T-cell receptor (TCR) are blue and red, respectively. The MHC class II molecule is green, with the bound peptide shown in gray. CD4 is shown in orange. Structure derived from PDB 3T0E. Courtesy of K.C. Garcia.

is extensively glycosylated, which is thought to maintain it in an extended conformation and protect it from cleavage by proteases. CD8 α chains can form homodimers, although these are usually not found when CD8 β is expressed. Naive T cells express CD8 $\alpha\beta$, but the CD8 $\alpha\alpha$ homodimer can be expressed by highly activated effector and memory T cells. CD8 $\alpha\alpha$ is also expressed by a population of intraepithelial lymphocytes known as **mucosal associated invariant T cells (MAIT cells)**; these cells recognize metabolites of folic acid that are produced by bacteria in association with the nonclassical MHC class I molecule **MR1**, which we will describe in Chapter 6.

CD8 $\alpha\beta$ binds weakly to an invariant site in the α_3 domain of an MHC class I molecule (see Fig. 4.27b). The CD8 β chain interacts with residues in the base of the α_2 domain of the MHC class I molecule, while the α chain is in a lower position interacting with the α_3 domain of the MHC class I molecule. The strength of binding of CD8 to the MHC class I molecule is influenced by the glycosylation state of the CD8 molecule; increasing the number of sialic acid residues on CD8 carbohydrate structures decreases the strength of the interaction. The pattern of sialylation of CD8 changes during the maturation of T cells and also on activation, and this likely has a role in modulating antigen recognition.

Like the interactions with MHC class II molecules, the T-cell receptor and CD8 can interact simultaneously with one MHC class I molecule (Fig. 4.29). Like CD4, CD8 binds Lck through the cytoplasmic tail of the α chain, and CD8 $\alpha\beta$ increases the sensitivity of T cells to antigen presented by MHC class I molecules about 100-fold. Although the molecular details are unclear, the CD8 $\alpha\alpha$ homodimer appears to function less efficiently than CD8 $\alpha\beta$ as a co-receptor, and may negatively regulate activation. In contrast to CD8, CD4 is not thought to dimerize.

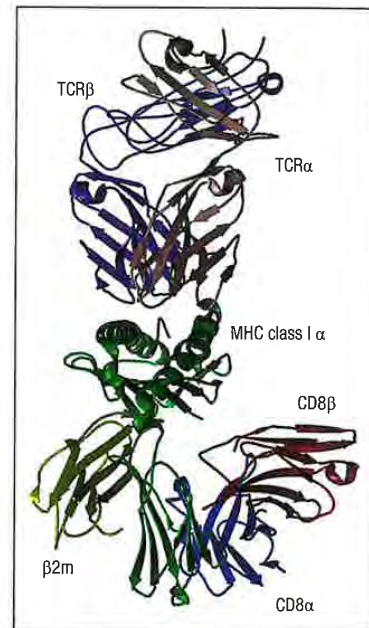


Fig. 4.29 CD8 binds to a site on MHC class I molecules that is distant from where the T-cell receptor binds. The relative binding positions of both the T-cell receptor and CD8 molecules can be seen in this hypothetical reconstruction of their interaction with MHC class I (α chain in dark green and β_2 -microglobulin in light green). The α and β chains of the T-cell receptor are shown in brown and purple, respectively. The CD8 $\alpha\beta$ heterodimer is shown bound to the MHC class I α_3 domain. The CD8 α chain is in blue, and the CD8 β chain is in red. Courtesy of Chris Nelson and David Fremont.

Tissue	MHC class I	MHC class II
Lymphoid tissues		
T cells	+++	+
B cells	+++	+++
Macrophages	+++	++
Dendritic cells	+++	+++
Epithelial cells of thymus	+	+++
Other nucleated cells		
Neutrophils	+++	—
Hepatocytes	+	—
Kidney	+	—
Brain	+	—†
Nonnucleated cells		
Red blood cells	—	—

Fig. 4.30 The expression of MHC molecules differs among tissues.

MHC class I molecules are expressed on all nucleated cells, although they are most highly expressed in hematopoietic cells. MHC class II molecules are normally expressed only by a subset of hematopoietic cells and by thymic stromal cells, although they may be expressed by other cell types on exposure to the inflammatory cytokine IFN- γ . *In humans, activated T cells express MHC class II molecules, whereas in mice all T cells are MHC class II-negative. †In the brain, most cell types are MHC class II-negative, but microglia, which are related to macrophages, are MHC class II-positive.

4-19 The two classes of MHC molecules are expressed differentially on cells.

MHC class I and MHC class II molecules have distinct distributions among cells, and these reflect the different effector functions of the T cells that recognize them (Fig. 4.30). MHC class I molecules present peptides from pathogens, commonly viruses, to CD8 cytotoxic T cells, which are specialized to kill any cell that they specifically recognize. Because viruses can infect any nucleated cell, almost all such cells express MHC class I molecules, although the level of constitutive expression varies from one cell type to the next. For example, cells of the immune system express abundant MHC class I on their surface, whereas liver cells (hepatocytes) express relatively low levels (see Fig. 4.30). Nonnucleated cells, such as mammalian red blood cells, express little or no MHC class I, and thus the interior of red blood cells is a site in which an infection can go undetected by cytotoxic T cells. Because red blood cells cannot support viral replication, this is of no great consequence for viral infection, but it might be the absence of MHC class I that allows the *Plasmodium* parasites that cause malaria to live in this privileged site.

In contrast, a major function of the CD4 T cells that recognize MHC class II molecules is to activate other effector cells of the immune system. Thus, MHC class II molecules are normally found on dendritic cells, B lymphocytes, and macrophages—antigen-presenting cells that participate in immune responses—but not on other tissue cells (see Fig. 4.30). The peptides presented by MHC class II molecules expressed by dendritic cells can function to activate naive CD4 T cells. When previously activated CD4 T cells recognize peptides bound to MHC class II molecules on B cells, the T cells secrete cytokines that can influence the isotype of antibody that those B cells will choose to produce. Upon recognizing peptides bound to MHC class II molecules on macrophages, CD4 T cells activate these cells, again in part through cytokines, to destroy the pathogens in their vesicles.

The expression of both MHC class I and MHC class II molecules is regulated by cytokines, in particular, interferons, released in the course of an immune response. Interferon- α (IFN- α) and IFN- β increase the expression of MHC class I molecules on all types of cells, whereas IFN- γ increases the expression of both MHC class I and MHC class II molecules, and can induce the expression of MHC class II molecules on certain cell types that do not normally express them. Interferons also enhance the antigen-presenting function of MHC class I molecules by inducing the expression of key components of the intracellular machinery that enables peptides to be loaded onto the MHC molecules.

4-20 A distinct subset of T cells bears an alternative receptor made up of γ and δ chains.

During the search for the gene for the TCR α chain, another T-cell receptor-like gene was unexpectedly discovered. This gene was named TCR γ , and its discovery led to a search for further T-cell receptor genes. Another receptor chain was identified by using antibody against the predicted sequence of the γ chain and was called the δ chain. It was soon discovered that a minority population of T cells bore a distinct type of T-cell receptor made up of γ : δ heterodimers rather than α : β heterodimers. The development of these cells is described in Sections 8-11 and 8-12.

Like α : β T cells, γ : δ T cells can be found in the lymphoid tissues of all vertebrates, but they are also prominent as populations of intraepithelial lymphocytes, particularly in the skin and female reproductive tracts, where their receptors display very limited diversity. Unlike α : β T cells, γ : δ T cells do not generally recognize antigen as peptides presented by MHC molecules, and γ : δ

T-cell receptors are not restricted by the 'classical' MHC class I and class II molecules that function in binding and presenting peptides to T cells. Instead, $\gamma\delta$ T-cell receptors seem to recognize their target antigens directly and thus probably are able to recognize and respond rapidly to molecules expressed by many different cell types. Their ligands have been difficult to identify, but several have now been described and seem to indicate that $\gamma\delta$ T cells play an intermediate, or transitional, role between wholly innate and fully adaptive immune responses.

Like NK-cell receptor ligands, such as the proteins MIC and RAET1 (see Section 3-27), many of the ligands seen by $\gamma\delta$ T cells are induced by cellular stress or damage. $\gamma\delta$ T cells may also bind antigens presented by 'nonclassical' **MHC class Ib molecules**, which we will discuss in Chapter 6. These proteins are related structurally to the MHC proteins we have already discussed, but have functions besides binding peptides for presentation to T cells. Additional ligands may include heat-shock proteins and nonpeptide ligands such as phosphorylated ligands or mycobacterial lipid antigens. $\gamma\delta$ T cells can also respond to unorthodox nucleotides and phospholipids. Recognition of molecules expressed as a consequence of infection, rather than recognition of pathogen-specific antigens themselves, distinguishes intraepithelial $\gamma\delta$ T cells from other lymphocytes, and this would place them in the innate-like class. For these reasons, the term '**transitional immunity**' has been proposed to clarify the role of $\gamma\delta$ T cells, since the function of these cells seems to be someplace between innate and adaptive responses.

The crystallographic structure of a $\gamma\delta$ T-cell receptor reveals that, as expected, it is similar in shape to $\alpha\beta$ T-cell receptors. **Figure 4.31** shows a crystal structure of a $\gamma\delta$ T-cell receptor complex bound to one of the nonclassical MHC class I molecules mentioned above, called **T22**. This structure shows that the overall orientation of the $\gamma\delta$ T-cell receptor with the MHC molecule is strikingly different from that of an $\alpha\beta$ T-cell receptor, in that it interacts primarily with one end of the T22 molecule. However, the CDR3 regions of the $\gamma\delta$ T-cell receptor still play a critical role in recognition, similar to that of antibodies and of $\alpha\beta$ T-cell receptors. Further, the CDR3 of the $\gamma\delta$ T-cell receptor is longer than either of these other two antigen receptors, and this could have implications toward the type of ligand that the $\gamma\delta$ T-cell receptor recognizes, as there is enormous combinatorial diversity from the CDR3 within the $\gamma\delta$ T-cell receptor repertoire. We will return to discuss further the ligands and the development of $\gamma\delta$ T cells in Chapters 6 and 8.

Summary.

The receptor for antigen on most T cells, the $\alpha\beta$ T-cell receptor, is composed of two protein chains, TCR α and TCR β , and resembles in many respects a single Fab fragment of immunoglobulin. $\alpha\beta$ T-cell receptors are always membrane-bound and recognize a composite ligand of a peptide antigen bound to an MHC molecule. Each MHC molecule binds a wide variety of different peptides, but the different variants of MHC each preferentially recognize sets of peptides with particular sequences and physical features. The peptide antigen is generated intracellularly, and is bound stably in a peptide-binding cleft on the surface of the MHC molecule. There are two classes of MHC molecules, and these are bound in their nonpolymorphic domains by CD8 and CD4 molecules that distinguish two different functional classes of $\alpha\beta$ T cells. CD8 binds MHC class I molecules and can bind simultaneously to the same peptide:MHC class I complex being recognized by a T-cell receptor, thus acting as a co-receptor and enhancing the T-cell response; CD4 binds MHC class II molecules and acts as a co-receptor for T-cell receptors that recognize peptide:MHC class II ligands. A T-cell receptor interacts directly both with the antigenic peptide

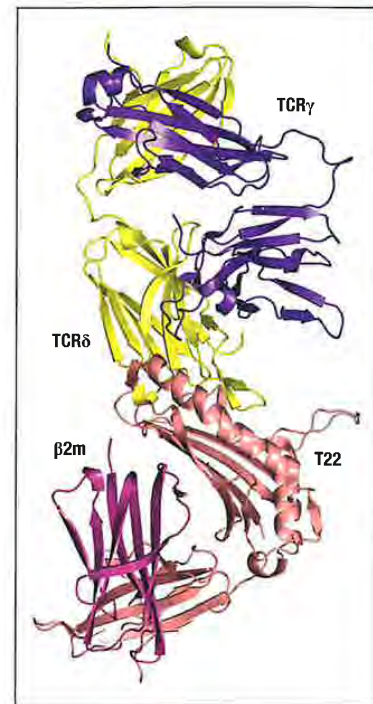


Fig. 4.31 Structures of $\gamma\delta$ T-cell receptor bound to the nonclassical MHC class I molecule T22. The $\gamma\delta$ T-cell receptor has a similar overall structure to the $\alpha\beta$ T-cell receptor and the Fab fragment of an immunoglobulin. The C δ domain is more like an immunoglobulin domain than is the corresponding C α domain of the $\alpha\beta$ T-cell receptor. In this structure, the overall orientation of the $\gamma\delta$ T-cell receptor with respect to the nonclassical MHC molecule T22 is very different from the orientation of an $\alpha\beta$ T-cell receptor with either MHC class I or class II molecules. Rather than lying directly over the peptide-binding groove, the $\gamma\delta$ T-cell receptor is engaged with one end much more than the other; this is consistent with a lack of peptide contact and absence of MHC-restricted recognition.

and with polymorphic features of the MHC molecule that displays it, and this dual specificity underlies the MHC restriction of T-cell responses. A second type of T-cell receptor, composed of a γ and a δ chain, is structurally similar to the $\alpha\beta$ T-cell receptor, but it binds to different ligands, including nonpeptide ligands, nonpolymorphic nonclassical MHC molecules, and certain lipids. The receptor is thought not to be MHC-restricted and is found on a minority population of lymphoid and intraepithelial T cells, the $\gamma\delta$ T cells.

Summary to Chapter 4.

B cells and T cells use different, but structurally similar, molecules to recognize antigen. The antigen-recognition molecules of B cells are immunoglobulins, and are made both as a membrane-bound receptor for antigen, the B-cell receptor, and as secreted antibodies that bind antigens and elicit humoral effector functions. The antigen-recognition molecules of T cells, in contrast, are made only as cell-surface receptors and so elicit only cellular effector functions. Immunoglobulins and T-cell receptors are highly variable molecules, with the variability concentrated in that part of the molecule—the variable (V) region—that binds to antigen. Immunoglobulins bind a wide variety of chemically different antigens, whereas the major type of T-cell receptor, the $\alpha\beta$ T-cell receptor, predominantly recognizes peptide fragments of foreign proteins bound to MHC molecules, which are ubiquitous on cell surfaces.

Binding of antigen by immunoglobulins has chiefly been studied with antibodies. The binding of antibody to its antigen is highly specific, and is determined by the shape and physicochemical properties of the antigen-binding site. Located at the other end of the antibody from the antigen-binding site is the constant, or Fc, region, which influences the types of effector function the antibody can elicit. There are five main functional classes of antibodies, each encoded by a different type of constant region. As we will see in Chapter 10, these interact with different components of the immune system to incite an inflammatory response and eliminate the antigen.

T-cell receptors differ in several respects from the B-cell immunoglobulins. One is the absence of a secreted form of T-cell receptor, reflecting the functional differences between T cells and B cells. B cells deal with pathogens and their protein products circulating within the body; secretion of a soluble antigen-recognition molecule enables the B cell to act in the clearance of antigen effectively throughout the extracellular spaces of the body. T cells, in contrast, are specialized for active surveillance of pathogens, and T-cell recognition does not involve a soluble, secreted receptor. Some, such as CD8 T cells, are able to detect intracellular infections and are able to kill infected cells that bear foreign antigenic peptides on their surface. Others, such as CD4 T cells, interact with cells of the immune system that have taken up foreign antigen and are displaying it on the cell surface.

T-cell receptors also recognize a composite ligand made up of the foreign peptide bound to a self MHC molecule, and not intact antigen. This means that T cells can interact only with a body cell displaying the antigen, not with the intact pathogen or protein. Each T-cell receptor is specific for a particular combination of peptide and a self MHC molecule. MHC molecules are encoded by a family of highly polymorphic genes. Expression of multiple variant MHC molecules, each with a different peptide-binding repertoire, helps to ensure that T cells from an individual will be able to recognize at least some peptides generated from nearly every pathogen.

Questions.

- 4.1 **True or False:** An antibody proteolytically cleaved by papain yields a fragment with higher avidity to the cognate antigen than an antibody cleaved by pepsin.
- 4.2 **Short Answer:** How is CD4 and CD8 co-receptor binding to MHC important for T-cell receptor signaling?
- 4.3 **Short Answer:** Why and how is it advantageous to have heterozygosity in the MHC locus?
- 4.4 **Matching:** Match the term to the best description:
- | | |
|---|--|
| A. Antigenic determinant | i. The structure recognized by an antibody (that is, the epitope) |
| B. Conformational/discontinuous epitope | ii. Regions of the V region that have significant sequence variation |
| C. Continuous/linear epitope | iii. An epitope composed of a single segment of a polypeptide chain |
| D. Hypervariable region | iv. An epitope composed of amino acids from different parts of a polypeptide chain brought together by protein folding |
- 4.5 **Fill-in-the-Blanks:** Most vertebrates, including humans and mice, produce antibodies composed of _____ and _____ chains. These bear _____ regions that recognize the antigen and _____ regions that dictate the antibody class and isotype. Camelids and cartilaginous fish, however, produce _____ and _____, respectively, which are forming the basis for single-chain antibody production for clinical applications.
- 4.6 **Multiple Choice:** Which of the following statements is *not* true?
- T-cell receptor α and β chains pair together, but the α chain can be switched out for a γ or a δ chain.
 - Electrostatic interactions (for example, a salt bridge) occur between charged amino acids.
 - Hydrophobic interactions occur between two hydrophobic surfaces and exclude water.
 - Antibodies often have many aromatic amino acids such as tyrosine in their antigen-binding sites.
 - MHC restriction is the phenomenon where T cells will recognize a unique set of peptides bound to a particular MHC molecule.
- 4.7 **Multiple Choice:** Which of the following is the most abundant immunoglobulin class in healthy adult humans and mice?
- IgA
 - IgD
 - IgE
 - IgG
 - IgM
- 4.8 **Multiple Choice:** Which of the following describes the structure of an immunoglobulin fold?
- Two antiparallel β sheets with an α -helical linker and a disulfide bond link
 - Two β strands linked by a disulfide bond
 - Four α helices linked by two disulfide bonds
 - Seven antiparallel α helices in series
 - One β sandwich of two β sheets folded together and linked by a disulfide bond
- 4.9 **Multiple Choice:** Antibodies have flexibility at various points in the molecule, particularly the hinge region between the Fc and Fab portion and, to some extent, the junction between the V and C regions. Which of the following properties of an antibody are not affected by its flexibility?
- Binding to small antigens (haptens)
 - Avidity to antigen
 - Affinity to antigen
 - Interaction with antibody-binding proteins
 - Binding to distantly spaced antigens
- 4.10 **Multiple Choice:** Which region of the antigen receptor of B cells and T cells is most critical in antigen recognition and specificity?
- FR1
 - CDR1
 - FR2
 - CDR2
 - FR3
 - CDR3
 - FR4

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PART III

THE DEVELOPMENT OF MATURE LYMPHOCYTE RECEPTOR REPERTOIRES

- 7 Lymphocyte Receptor Signaling
- 8 The Development of B and T Lymphocytes

Lymphocyte Receptor Signaling

7

T and B lymphocytes are cells of the adaptive immune system, each of which expresses a unique antigen receptor. These cells circulate between the blood, the lymph, and, most importantly, the secondary lymphoid organs, where they survey antigen-presenting cells for their specific antigen. Once that antigen is encountered, signals from the antigen receptor activate several downstream pathways that convert quiescent naive lymphocytes into metabolically active cells that are reorganizing their actin cytoskeleton, activating transcription factors, and synthesizing a wide range of new proteins. As a result of these events, naive T and B cells undergo rapid cell division and differentiate into armed effector cells, thus expanding lymphocyte populations during an immune response and equipping these cells with the machinery to combat infections.

We begin by discussing some general principles of intracellular signaling, and then outline the pathways activated when a naive lymphocyte encounters its specific antigen. Next, we briefly discuss the co-stimulatory signaling that is necessary to activate naive T cells and, in most cases, naive B cells. In the last part of the chapter we focus on inhibitory receptors, and their roles in down-regulating signaling pathways in T and B cells.

General principles of signal transduction and propagation.

In this part of the chapter we review briefly some general principles of receptor action and signal transduction that are common to many of the pathways discussed here. All cell-surface receptors that have a signaling function either are transmembrane proteins themselves or form parts of protein complexes that link the exterior and interior of the cell. Different classes of receptors transduce extracellular signals in a variety of ways. A common theme among the receptors covered in this chapter is that ligand binding results in the activation of an intracellular enzymatic activity.

IN THIS CHAPTER

General principles of signal transduction and propagation.

Antigen receptor signaling and lymphocyte activation.

Co-stimulatory and inhibitory receptors modulate antigen receptor signaling in T and B lymphocytes.

7-1 Transmembrane receptors convert extracellular signals into intracellular biochemical events.

The enzymes most commonly associated with receptor activation are the **protein kinases**. This large group of enzymes catalyzes the covalent attachment of a phosphate group to a protein, a reversible process called **phosphorylation**. For receptors that use protein kinases, the binding of ligand to the extracellular part of the receptor allows the receptor-associated protein kinase to become 'active'—that is, to phosphorylate its intracellular substrate—and thus to propagate the signal. As we shall see, receptor-associated kinases can become activated in various ways, such as by undergoing modifications to the kinase itself that alter its intrinsic catalytic efficiency or by changes in subcellular localization that increase access to its biochemical substrates.

In animals, protein kinases phosphorylate proteins on three amino acids—tyrosine, serine, or threonine. Most of the enzyme-linked receptors we discuss in detail in this chapter activate **tyrosine protein kinases**. Tyrosine kinases are specific for tyrosine residues, whereas serine/threonine kinases phosphorylate serine and threonine residues; less common are dual-specificity kinases that phosphorylate both tyrosine and serine/threonine residues in their substrates. Protein tyrosine phosphorylation is much less common than serine/threonine phosphorylation in general, and is employed mainly in signaling pathways. One large group of receptors—the so-called **receptor tyrosine kinases**—carry a kinase activity within the cytoplasmic region of the receptor itself (Fig. 7.1, top panel). This group contains receptors for many growth factors; lymphocyte receptors of this type include Kit and FLT3, which are expressed on developing lymphocytes in addition to other hematopoietic progenitor cells and are discussed in Chapter 8. The receptor for transforming growth factor- β (TGF- β), an important regulatory cytokine produced by many cells, is a **receptor serine/threonine kinase**.

Even more important to the function of mature lymphocytes are receptors that have no intrinsic enzymatic activity themselves but associate with intracellular tyrosine kinases. The antigen receptors on B lymphocytes and T lymphocytes are of this type, as are the receptors for some types of cytokines. Ligand binding to the extracellular domain of such receptors causes particular amino acid residues in their cytoplasmic domains to become phosphorylated by specific cytoplasmic tyrosine kinases (see Fig. 7.1, bottom panel). These **nonreceptor kinases** either can be constitutively associated with the cytoplasmic domains of the receptors, as with many cytokine receptors, or may become associated with the receptors when they bind their ligands, as is the case for the antigen receptors.

For many cytokine receptors, ligand binding causes dimerization or clustering of individual receptor molecules, bringing the associated kinases together and enabling them to phosphorylate the cytoplasmic tail of adjacent receptors—thus initiating an intracellular signal. In the case of the lymphocyte antigen receptors, association with cytoplasmic tyrosine kinases occurs after ligand binding but is unlikely to be due to a simple clustering mechanism. Instead, the actions of co-receptors are required: these bring cytoplasmic tyrosine kinases into proximity with the cytoplasmic regions of the antigen receptor, a complex process that we will describe later.

Signaling is usually not a simple 'on or off' switch. Depending on the affinity of the receptor for the ligand, the abundance of the ligand, the concentrations of intracellular signaling components, and a complex network of positive- and negative-feedback pathways, receptor activation and downstream signaling occur when a minimum threshold determined by all of these factors is exceeded. These features are often merged into the simple term 'signal

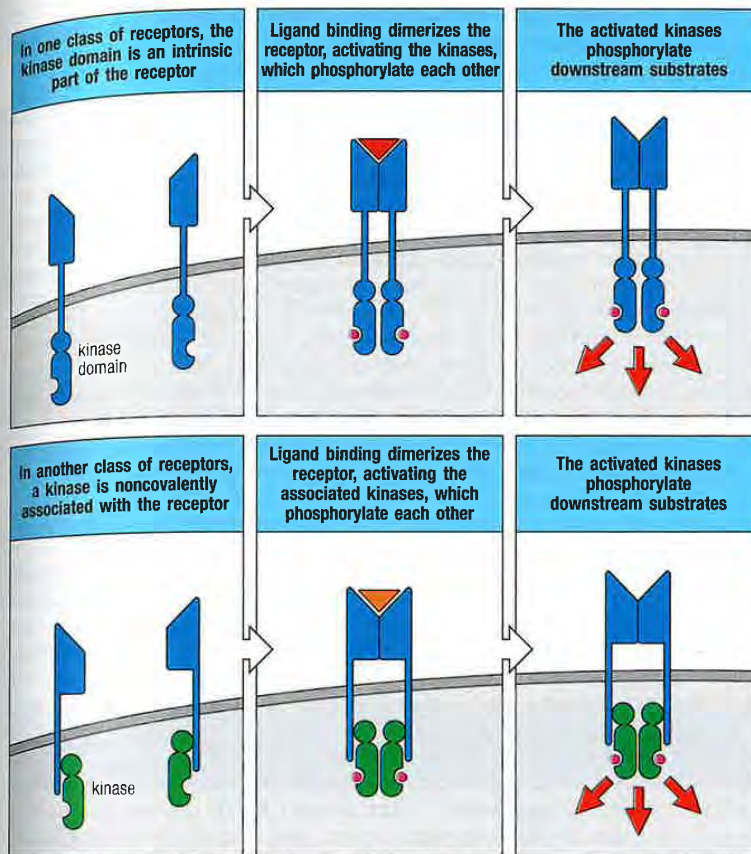


Fig. 7.1 Enzyme-associated receptors of the immune system can use intrinsic or associated protein kinases to signal.

These receptors activate a protein kinase on the cytoplasmic side of the membrane to convey the information that a ligand has bound to their extracellular portion. Receptor tyrosine kinases (top panels) contain the kinase activity as part of the receptor itself. Ligand binding results in clustering of the receptor, activation of catalytic activity, and the consequent phosphorylation (denoted by red dots) of the receptor tails and other substrates, transmitting the signal onward. Receptors that lack intrinsic kinase activity associate with nonreceptor kinases (bottom panels). Receptor dimerization or clustering after ligand binding activates the associated enzyme. In all receptors of these types encountered in this chapter, the enzyme is a tyrosine kinase.

strength.' It is important to keep in mind that variations in signal strength will determine the magnitude of cellular responses—some will be all-or-nothing, whereas others will increase as the strength of signaling increases.

The role of protein kinases in cell signaling is not confined to receptor activation, as they act at many different stages in intracellular signaling pathways. Protein kinases figure largely in cell signaling because phosphorylation and **dephosphorylation**—the removal of a phosphate group—are the means of regulating the activity of many enzymes, transcription factors, and other proteins. Equally important to the workings of signaling pathways is the fact that phosphorylation generates sites on proteins to which other signaling proteins can bind.

Phosphate groups are removed from proteins by a large class of enzymes called **protein phosphatases**. Different classes of protein phosphatases remove phosphate groups from phosphotyrosine or from phosphoserine/phosphothreonine, or both (as in dual-specificity phosphatases). Specific dephosphorylation by phosphatases is one important means of regulating signaling pathways by resetting a protein to its original state and thus switching signaling off. Dephosphorylation does not always inhibit a protein's activity. In many instances the removal of a particular phosphate group by a specific phosphatase is needed to activate an enzyme. In other cases, the extent of phosphorylation of an enzyme determines its activity, and represents a balance between the activity of kinases and phosphatases.

7-2 Intracellular signal propagation is mediated by large multiprotein signaling complexes.

As we learned in Chapter 3, binding of a ligand to its receptor can initiate a cascade of events involving intracellular proteins that sequentially convey signaling information onward. The unique enzymes and other components assembled into a particular multiprotein receptor complex will determine the character of the signal it generates. These components may be shared by several receptor pathways, or they may be exclusive to one receptor pathway, thus allowing distinct signaling pathways to be built up from a relatively limited number of components. The assembly of multisubunit signaling complexes involves specific interactions of a number of distinct types of **protein-interaction domains**, or **protein-interaction modules**, carried by the signaling proteins. **Figure 7.2** gives a few examples of such domains. Signaling proteins in general contain at least one such protein-interaction domain, but many contain multiple domains. These protein modules cooperate with each other, for example, to organize signaling proteins into the correct subcellular localizations, to enable specific binding between protein partners, and to modify enzymatic activity.

For the pathways considered in this chapter, the most important mechanism underlying the formation of signaling complexes is the phosphorylation of protein tyrosine residues. Phosphotyrosines are binding sites for a number of protein-interaction domains, including the **SH2 (Src homology 2) domain** (see Fig. 7.2). SH2 domains, built from approximately 100 amino acids, are present in many intracellular signaling proteins, where they are frequently linked to other types of enzymatic or other functional domains. SH2 domains recognize the phosphorylated tyrosine (pY) and, typically, the amino acid three positions away (pYXXZ, where X is any amino acid and Z is a specific amino acid); they bind in a sequence-specific fashion, with different SH2 domains preferring different combinations of amino acids. In this way, the unique SH2 domain of a signaling molecule can act as a 'key' that allows inducible and specific association with a protein containing the appropriate pY-containing amino acid sequence.

Tyrosine kinase-associated receptors can assemble multiprotein signaling complexes by using proteins called **scaffolds** and **adaptors**. Scaffolds and adaptors lack enzymatic activity, and they function by recruiting other proteins into a signaling complex so that interactions among these proteins can take place.

Fig. 7.2 Signaling proteins interact with each other and with lipid signaling molecules via modular protein domains. A few of the most common protein domains used by immune-system signaling proteins are listed, together with some proteins that contain them and the general class of ligand bound by the interaction domain. The right-hand column lists specific examples of a protein motif (in single-letter amino acid code) or, for the phosphoinositide-binding domains, the particular phosphoinositide that they bind. All these domains are used in many other nonimmune signaling pathways as well.

Protein domain	Found in	Ligand class	Example of ligand
SH2	Lck, ZAP-70, Fyn, Src, Grb2, PLC- γ , STAT, Cbl, Btk, Itk, SHIP, Vav, SAP, PI3K	phosphotyrosine	pYXXZ
SH3	Lck, Fyn, Src, Grb2, Btk, Itk, Tec, Fyb, Nck, Gads	proline	PXXP
PH	Tec, PLC- γ , Akt, Btk, Itk, Sos	phosphoinositides	PIP ₃
PX	P40 ^{phox} , P47 ^{phox} , PLD	phosphoinositides	PI(3)P
PDZ	CARMA1	C termini of proteins	IESDV, VETDV
C1	RasGRP, PKC- θ	membrane lipid	diacylglycerol (DAG) phorbol ester
NZF	TAB2	polyubiquitin (K63-linked)	polyubiquitinated RIP, TRAF-6, or NEMO

Scaffolds are relatively large proteins that can, for example, become tyrosine phosphorylated on multiple sites in order to recruit many different proteins (Fig. 7.3, top panel). By specifying which proteins are recruited, scaffolds can define the character of a particular signaling response. This is accomplished by several mechanisms. For example, scaffolds can regulate the specificity of a recruited enzyme by recruiting one of the enzyme's substrates. Binding to a scaffold can also change the conformation of a recruited protein, thereby revealing sites for protein modifications, such as phosphorylation or ubiquitination, or for protein-protein interactions. Finally, scaffolds can function to promote membrane localization of the signaling complex.

Adaptors are membrane-anchored or cytoplasmic proteins containing several signaling modules that serve to link two or more proteins together. The adaptor proteins Grb2 and Gads, for example, each contain an SH2 domain and two copies of another module called the SH3 domain (see Fig. 7.2). This arrangement of modules can be used to link tyrosine phosphorylation of a receptor to molecules acting in the next stage of signaling. For example, the SH2 domain of Grb2 binds to a phosphotyrosine residue on a receptor or a scaffold protein, while its two SH3 domains bind to proline-rich motifs on other signaling proteins (see Fig. 7.3, bottom panel), such as Sos, which we discuss in the next section.

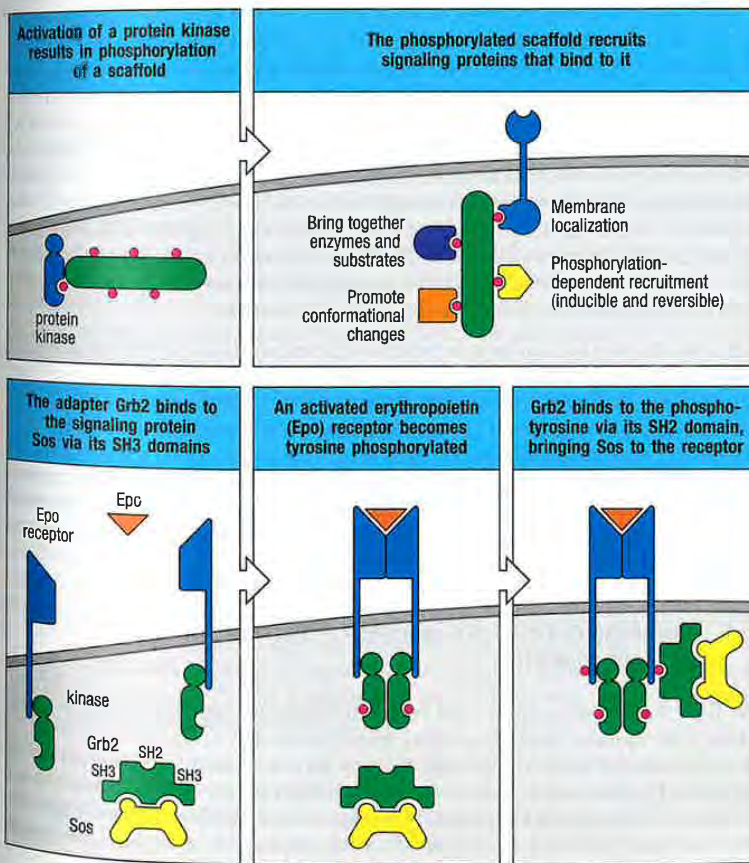


Fig. 7.3 Assembly of signaling complexes is mediated by scaffold and adaptor proteins.

Assembly of signaling complexes is an important aspect of signal transduction. This is often achieved through scaffold and adaptor proteins. In general, scaffolds have numerous sites of phosphorylation that function to bring many different signaling proteins together (top panel). Scaffolds may also function to promote membrane localization, to bring enzymes into close proximity with their substrates, and to induce conformational changes in proteins that regulate their functions. An adaptor protein functions to bring two different proteins together (bottom panel). When erythropoietin (Epo) binds to its receptor, associated tyrosine kinases phosphorylate (red dots) sites on the Epo receptor cytoplasmic domain, generating binding sites for the SH2 domain of an adaptor protein. The adaptor protein (green) shown here contains two SH3 domains in addition to an SH2 domain. With the SH3 domains it can, for example, bind proline-rich sites on an intracellular signaling molecule (yellow).

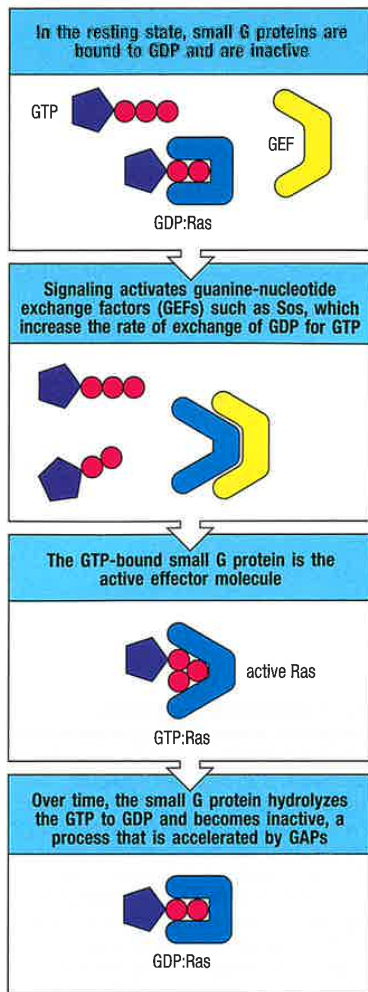


Fig. 7.4 Small G proteins are switched from inactive to active states by guanine-nucleotide exchange factors (GEFs) and the binding of GTP. Ras is a small GTP-binding protein with intrinsic GTPase activity. In its resting state, Ras is bound to GDP. Receptor signaling activates guanine-nucleotide exchange factors (GEFs), such as Sos, which can bind to small G proteins such as Ras and increase the rate of exchange of GDP for GTP (center panels). The GTP-bound form of Ras can then bind to a large number of effectors, recruiting them to the membrane. Over time, the intrinsic GTPase activity of Ras will result in the hydrolysis of GTP to GDP. GTPase-activating proteins (GAPs) can accelerate the hydrolysis of GTP to GDP, thus shutting off the signal more rapidly.

7-3 Small G proteins act as molecular switches in many different signaling pathways.

Monomeric GTP-binding proteins known as **small G proteins** or **small GTPases** are important in the signaling pathways leading from many tyrosine kinase-associated receptors. The small GTPases are distinct from the larger heterotrimeric G proteins associated with G-protein-coupled receptors such as the chemokine receptors discussed in Chapter 3. The superfamily of small GTPases comprises more than 100 different proteins, and many are important in lymphocyte signaling. One of these, **Ras**, is involved in many pathways leading to cell proliferation. Other small GTPases include Rac, Rho, and Cdc42, which control changes in the actin cytoskeleton caused by signals received through the T-cell receptor or B-cell receptor. We will describe their actions in more detail in Section 7-19.

Small GTPases exist in two states, depending on whether they are bound to GTP or to GDP. The GDP-bound form is inactive but is converted into the active form by exchange of the GDP for GTP. This reaction is mediated by proteins known as **guanine-nucleotide exchange factors**, or **GEFs**, which cause the GTPase to release GDP and to bind the more abundant GTP (Fig. 7.4). Sos, which is recruited to signaling pathways by the adaptor Grb2 (see Section 7-2), is one of the GEFs for Ras. The binding of GTP induces a conformational change in the small GTPase that enables it to bind to and induce effector activity in a variety of target proteins. Thus, GTP binding functions as an on/off switch for small GTPases.

This GTP-bound form does not remain permanently active but is eventually converted into the inactive GDP-bound form by the intrinsic GTPase activity in the G protein, which removes a phosphate group from the bound GTP. Regulatory cofactors known as **GTPase-activating proteins (GAPs)** accelerate the conversion of GTP to GDP, thus rapidly downregulating the activity of the small GTPase. Because of GAP activity, small GTPases are usually present in the inactive GDP-bound state and are activated only transiently in response to a signal from an activated receptor. *RAS* is frequently mutated in cancer cells, and the mutated Ras protein is thought to be a significant contributor to the cancerous state. The importance of GAPs in signaling regulation is indicated by the fact that some mutations in Ras found in cancer act by preventing GAP from enhancing the intrinsic GTPase activity of Ras, thus prolonging the duration for which Ras exists in the active GTP-bound state.

GEFs are the key to G-protein activation and are recruited to the site of receptor activation at the cell membrane by binding to adaptor proteins or to lipid metabolites produced by receptor activation. Once recruited, they are able to activate Ras or other small G proteins, which are localized to the inner surface of the plasma membrane via fatty acids that are attached to the G protein post-translationally. Thus, G proteins act as molecular switches, becoming switched on when a cell-surface receptor is activated and then being switched off. Each G protein has its own specific GEFs and GAPs, which help to confer specificity on the pathway.

7-4 Signaling proteins are recruited to the membrane by a variety of mechanisms.

We have seen how receptors can recruit intracellular signaling proteins to the plasma membrane through tyrosine phosphorylation of the receptor itself or of an associated scaffold, followed by recruitment of SH2-domain-containing signaling proteins or adaptors, such as Grb2 (Fig. 7.5). A second mechanism for membrane recruitment of signaling proteins is via binding to small GTPases such as Ras, following their activation. As described in Section 7-3, small GTPases are constitutively bound to the cytoplasmic surface of the plasma

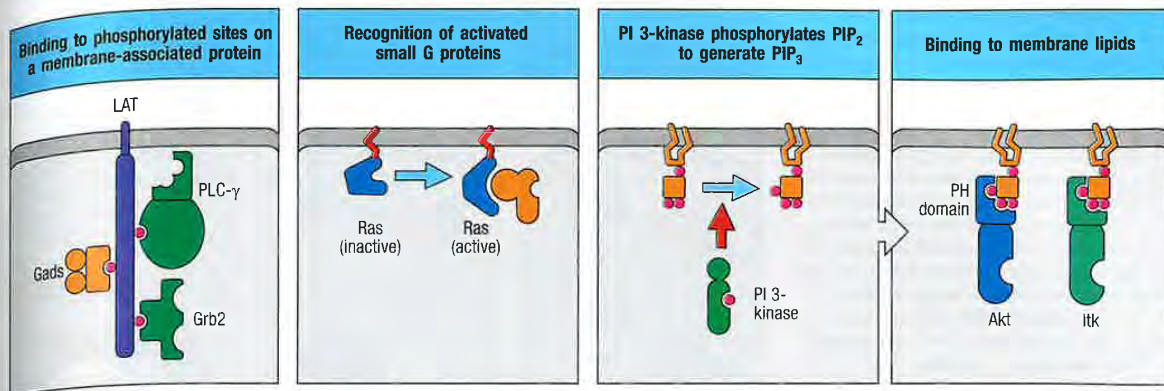


Fig. 7.5 Signaling proteins can be recruited to the membrane in a variety of ways. Recruitment of signaling proteins to the plasma membrane is important in signal propagation because this is where receptors are usually located. Left panel: tyrosine phosphorylation of membrane-associated proteins, such as the scaffold LAT, recruits phosphotyrosine-binding proteins. This can also protect the scaffold from dephosphorylation by tyrosine phosphatases, which inhibit signaling. Second panel: small G proteins such as Ras can associate with the membrane by having lipid modifications (shown in red). When activated, they can bind a variety of signaling proteins. Right two panels: modifications to the membrane itself that result from receptor activation can recruit signaling proteins. In this example the membrane lipid PIP_3 has been produced in the inner leaflet of the plasma membrane by the phosphorylation of PIP_2 by PI 3-kinase. PIP_3 is recognized by the PH domains of signaling proteins such as the protein kinases Akt and Itk.

membrane due to their fatty acid modifications. Once activated by exchange of GDP for GTP, the activated GTPases bind to signaling proteins such as Sos, relocalizing the bound proteins to the plasma membrane (see Fig. 7.5).

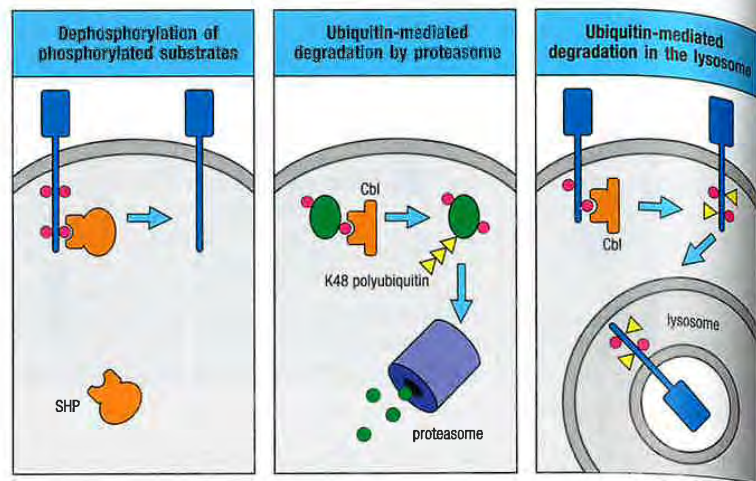
Another way in which receptors can recruit signaling molecules to the plasma membrane is by the local production of modified membrane lipids. These lipids are produced by phosphorylation of the membrane phospholipid phosphatidylinositol by enzymes known as **phosphatidylinositol kinases**, which are activated as a result of receptor signaling. The inositol headgroup of phosphatidylinositol is a carbohydrate ring that can be phosphorylated at one or more positions to generate a wide variety of derivatives. The ones that we will focus on in this chapter are phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3), the latter of which is generated from PIP_2 by the enzyme **phosphatidylinositol 3-kinase (PI 3-kinase)** (see Fig. 7.5). PI 3-kinase is often recruited by binding of the SH2 domain of its regulatory subunit to phosphotyrosines in a receptor tail, bringing its catalytic subunit into proximity with inositol phospholipid substrates in the membrane. In this way, membrane phosphoinositides such as PIP_3 are rapidly produced after receptor activation. This, combined with their short life span, makes them ideal signaling molecules. PIP_3 is recognized specifically by proteins containing a pleckstrin homology (PH) domain or, less commonly, a PX domain (see Fig. 7.2), and one of its functions is to recruit such proteins to the membrane and in some cases contribute to enzyme activation.

7-5 Post-translational modifications of proteins can both activate and inhibit signaling responses.

Protein phosphorylation is a common mechanism for transducing signals from cellular receptors to downstream pathways. These signals are terminated by the action of protein phosphatases, which dephosphorylate signaling intermediates (Fig. 7.6). The importance of protein phosphatases in terminating signaling is underscored by the existence of diseases, such as autoimmunity and cancer, which may result from absent or deficient protein phosphatase activity. However, protein dephosphorylation can also function as a mechanism of activation. Dephosphorylation can regulate protein-protein interactions, protein subcellular localization, or nucleic acid binding, thereby promoting downstream signaling events.

Another general mechanism of protein regulation by post-translational modification is the covalent attachment of one or more molecules of the small protein **ubiquitin**. Ubiquitination is a potent means of signal termination, as it often leads to protein degradation. Ubiquitin is attached by its carboxy-terminal glycine to lysine residues of target proteins in a multi-step process.

Fig. 7.6 Signaling must be turned off as well as turned on. The inability to terminate a signaling pathway can result in serious diseases such as autoimmunity or cancer. As a significant proportion of signaling events depend on protein phosphorylation, protein phosphatases, such as SHP, have an important part in shutting down signaling pathways (left panel). Another common mechanism for terminating signaling is regulated protein degradation (center and right panels). Phosphorylated proteins recruit ubiquitin ligases, such as Cbl, that add the small protein ubiquitin to proteins, thus targeting them for degradation. Cytoplasmic proteins are targeted for destruction in the proteasome by the addition of polyubiquitin chains, linked through lysine 48 (K48) of ubiquitin (center panel). Membrane receptors that become ubiquitinated by individual ubiquitin molecules or di-ubiquitin are internalized and transported to the lysosome for destruction (right panel).



First, an E1 ubiquitin-activating enzyme promotes attachment of ubiquitin to an E2 ubiquitin-conjugating enzyme. The ubiquitin is then transferred to the protein substrate by an enzyme known as an E3 **ubiquitin ligase**. Ubiquitin ligases can continue to add ubiquitin molecules to form polyubiquitin. Importantly, different ubiquitin ligases add the carboxy terminus of one ubiquitin molecule to different lysine residues of the conjugated ubiquitin, typically either lysine 48 (K48) or lysine 63 (K63). These different forms of polyubiquitin produce divergent outcomes for signaling pathways.

When polyubiquitin chains are formed using K48 linkages, the outcome is to target the protein for degradation by the proteasome (see Fig 7.6). An important ubiquitin ligase of this kind in lymphocytes is Cbl, which selects its targets via its SH2 domain. Cbl can thus bind to specific tyrosine-phosphorylated targets, causing them to become ubiquitinated via K48 linkages. Proteins that recognize this form of polyubiquitin then target the ubiquitinated proteins to degradative pathways via the proteasome. Membrane proteins such as receptors can be tagged by single ubiquitin molecules or by di-ubiquitin. These are not recognized by the proteasome, but instead, are recognized by specific ubiquitin-binding proteins that target proteins for degradation in lysosomes (see Fig. 7.6). Thus, ubiquitination of proteins can inhibit signaling. Unlike phosphatases, where the mechanism of inhibition is reversible, inhibition by ubiquitin-mediated protein degradation is a more permanent means of terminating signaling.

Ubiquitination can also be used to activate signaling pathways. We have already discussed this aspect in Section 3-7 in connection with the NF κ B signaling pathway from TLRs. There, the ubiquitin ligase TRAF-6 produces K63-linked polyubiquitin chains on TRAF-6 and NEMO. In lymphocytes, K63-linked polyubiquitination is a key step in signaling through tumor necrosis factor (TNF) receptor family members, as will be discussed in Section 7-23 (and Fig. 7.31). This form of polyubiquitin is recognized by specific domains in signaling proteins that recruit additional signaling molecules to the pathway (see Fig. 3.15).

7-6 The activation of some receptors generates small-molecule second messengers.

In many cases, intracellular signaling involves the activation of enzymes that produce small-molecule biochemical mediators known as **second messengers** (Fig. 7.7). These mediators can diffuse throughout the cell, enabling the

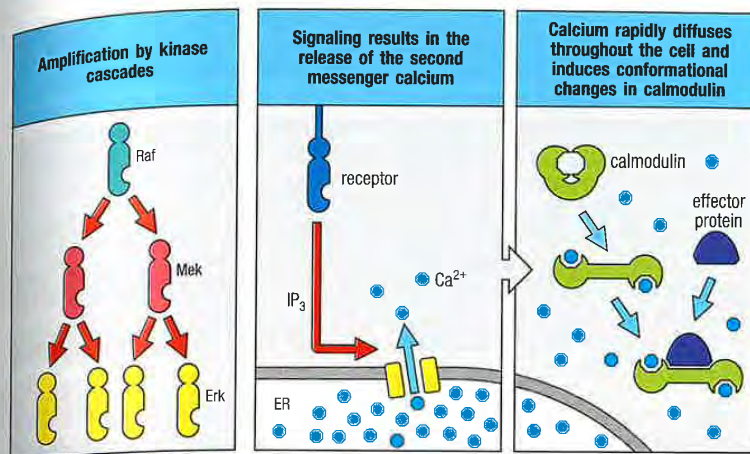


Fig. 7.7 Signaling pathways amplify the initial signal. Amplification of the initial signal is an important element of most signal transduction pathways. One means of amplification is a kinase cascade (left panel), in which protein kinases successively phosphorylate and activate each other. In this example, taken from a commonly used kinase cascade (see Fig. 7.19), activation of the kinase Raf results in the phosphorylation and activation of a second kinase, Mek, that phosphorylates yet another kinase, Erk. As each kinase can phosphorylate many different substrate molecules, the signal is amplified at each step, resulting in a huge amplification of the initial signal. Another method of signal amplification is the generation of second messengers (center and right panels). In this example, signaling results in the release of the second messenger calcium (Ca^{2+}) from intracellular stores into the cytosol or its influx from the extracellular environment. Ca^{2+} release from the endoplasmic reticulum (ER) is shown here. The sharp increase in free Ca^{2+} in the cytoplasm can potentially activate many downstream signaling molecules, such as the calcium-binding protein calmodulin. Calcium binding induces a conformational change in calmodulin, which allows it to bind to and regulate a variety of effector proteins.

signal to activate a variety of target proteins. The enzymatic production of second messengers also serves the dual purpose of achieving concentrations of them sufficient to activate the next stage of the pathway and of amplifying the signaling cascade. The second messengers generated by receptors that signal via tyrosine kinases include calcium ions (Ca^{2+}) and a variety of membrane lipids and their soluble derivatives. Although some of these lipid messengers are confined to membranes, they can move within them. A second messenger binding to its target protein typically induces a conformational change that allows the protein to be activated.

Summary.

Cell-surface receptors serve as the front line of a cell's interaction with its environment; they sense extracellular events and convert them into biochemical signals for the cell. As most receptors sit in the plasma membrane, a critical step in the transduction of extracellular signals to the interior of the cell is recruitment of intracellular proteins to the membrane and changes in the composition of the membrane surrounding the receptor. Many immune receptors operate by activating tyrosine kinases to transmit their signals onward, often using scaffolds and adaptors to form large multiprotein signaling complexes. Both the qualitative and quantitative changes that take place in the composition of these signaling complexes determine the character of the response and biological outcomes. Formation of signaling complexes is mediated by a wide variety of protein-interaction domains, or modules, including the SH2, SH3, and PH domains found in proteins. In many cases, the increase in enzymatically produced small-molecule signaling intermediates called second messengers regulates and amplifies the signaling cascade. Termination of signaling involves protein dephosphorylation as well as ubiquitin-mediated protein degradation.

Antigen receptor signaling and lymphocyte activation.

The ability of T cells and B cells to recognize and respond to their specific antigen is central to adaptive immunity. As described in Chapters 4 and 5, the B-cell antigen receptor (BCR) and the T-cell antigen receptor (TCR) are made up of antigen-binding chains—the heavy and light immunoglobulin chains

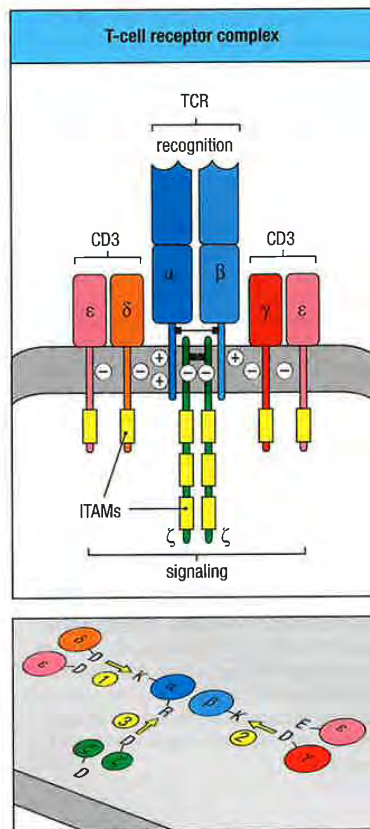


Fig. 7.8 The T-cell receptor complex is made up of variable antigen-recognition proteins and invariant signaling proteins. Upper panel: the functional T-cell receptor (TCR) complex is composed of the antigen-binding TCR α : β heterodimer associated with six signaling chains: two ϵ , one δ , and one γ collectively called CD3, and a homodimer of ζ . Cell-surface expression of the antigen-binding chains requires assembly of TCR α : β with the signaling subunits. Each CD3 chain has one immunoreceptor tyrosine-based activation motif (ITAM), shown as a yellow segment, whereas each ζ chain has three. The transmembrane regions of each chain have unusual acidic or basic residues as shown. Lower panel: the transmembrane regions of the various TCR subunits are represented in cross-section. It is thought that one of the positive charges, from a lysine (K) of the α chain, interacts with the two negative charges of aspartic acid (D) of the CD3 δ : ϵ dimer, while the other positive charge, of arginine (R), interacts with aspartic acids of the ζ homodimer. The positive arginine (K) charge of the β chain interacts with the negative charges of aspartic acid and glutamic acid (E) in the CD3 γ : ϵ dimer.

in the B-cell receptor, and the TCR α and TCR β chains in the T-cell receptor. These variable chains have exquisite specificity for antigen, allowing each lymphocyte to detect the presence of one type of pathogen. However, binding of antigen to the antigen receptor is not sufficient for a lymphocyte to respond—the information that antigen receptor engagement has occurred also needs to be transduced into the intracellular compartment of the lymphocyte. Thus, the fully functional antigen receptor complex must include proteins that can transduce a signal across the plasma membrane. For the B-cell antigen receptor and the T-cell antigen receptor, this function is mediated by invariant accessory proteins that initiate signaling when the receptors bind antigen. Assembly with these accessory proteins is also essential for transport of the receptor to the cell surface. In this part of the chapter we describe the structure of the antigen receptor complexes on T cells and B cells, and the signaling pathways that lead from them.

7-7 Antigen receptors consist of variable antigen-binding chains associated with invariant chains that carry out the signaling function of the receptor.

In T cells, the highly variable TCR α : β heterodimer (see Chapter 5) is not sufficient on its own to make up a complete cell-surface antigen receptor. When cells were transfected with cDNAs encoding the TCR α and TCR β chains, the heterodimers formed were degraded and did not appear on the cell surface. This implied that other molecules are required for the T-cell receptor to be expressed on the cell surface. In the T-cell receptor, the other required molecules are the CD3 γ , CD3 δ , and CD3 ϵ chains, which together form the CD3 complex; and the ζ chain, which is present as a disulfide-linked homodimer (Fig. 7.8). The CD3 proteins have an extracellular immunoglobulin-like domain, whereas the ζ chain contains only a short extracellular domain. Throughout the remainder of the chapter, we will use the term T-cell receptor to refer to the entire T-cell receptor complex, including these associated signaling subunits.

Although the exact stoichiometry of the complete T-cell receptor is not definitively established, it is thought that the receptor α chain interacts with one CD3 δ :CD3 ϵ dimer and one ζ dimer, while the receptor β chain interacts with one CD3 γ :CD3 ϵ dimer (see Fig. 7.8). These interactions are mediated by reciprocal charge interactions between basic and acidic intramembrane amino acids of the receptor subunits. There are two positive charges in the TCR α transmembrane region and one in the TCR β transmembrane domain. Negative charges in the CD3 and ζ transmembrane domains interact with the positive charges in α and β . Assembly of CD3 and a ζ dimer with the α : β heterodimer stabilizes the α and β dimer during its production in the endoplasmic reticulum and allows the complex to be transported to the plasma membrane. These associations ensure that all T-cell receptors present on the plasma membrane are properly assembled.

Signaling from the T-cell receptor is initiated by tyrosine phosphorylation within cytoplasmic regions called **immunoreceptor tyrosine-based activation motifs (ITAMs)** in the CD3 ϵ , γ , δ , and ζ chains. CD3 γ , δ , and ϵ each contain one ITAM, and each ζ chain contains three, giving the T-cell receptor a total of 10 ITAMs. This motif is also present in the signaling chains of the B-cell receptor and in the NK-cell receptors described in Chapter 3, as well as in the receptors for the immunoglobulin constant region (Fc receptors) that are present on mast cells, macrophages, monocytes, neutrophils, and NK cells (see Section 7-11 below).

Each ITAM contains two tyrosine residues that become phosphorylated by specific protein tyrosine kinases when the receptor binds its ligand, providing

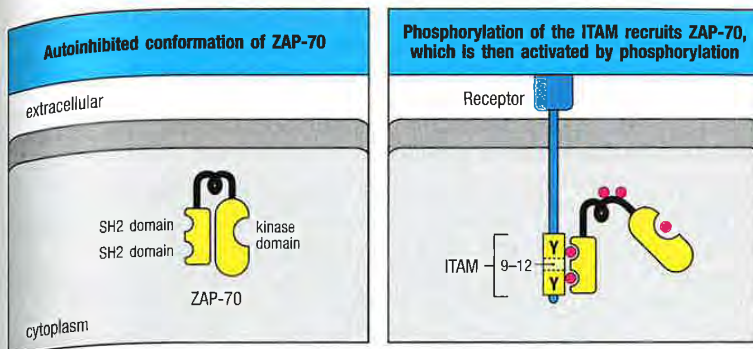


Fig. 7.9 ITAMs recruit signaling proteins that have tandem SH2 domains.

The ITAMs of the T-cell receptor (TCR) and B-cell receptor (BCR) contain tyrosine residues within the motif ...YXX[L/I]X₆₋₉YXX[L/I]... The spacing between the tyrosines is important in binding to tandem SH2-containing proteins such as Syk and ZAP-70. Left panel: prior to TCR or BCR stimulation, these kinases are in an inactive conformation, known as the autoinhibited conformation. The autoinhibited conformation is stabilized by interactions between the tandem SH2 domain-kinase domain linker region and the kinase domain that hold the enzyme in a catalytically inactive state.

Right panel: after phosphorylation of both tyrosines within one ITAM (here depicted as two tyrosines separated by 9–12 amino acids), the tandem SH2 domains of Syk or ZAP-70 can dock cooperatively to both phosphotyrosines, as shown here for ZAP-70. By being recruited into the active signaling complex, ZAP-70 can itself be phosphorylated, so that it becomes an active kinase that can then phosphorylate its substrates. This final step of ZAP-70 activation requires phosphorylation on two tyrosines in the linker region between the tandem SH2 domains and the kinase domain, together with phosphorylation of a tyrosine in the catalytic site of the kinase domain.

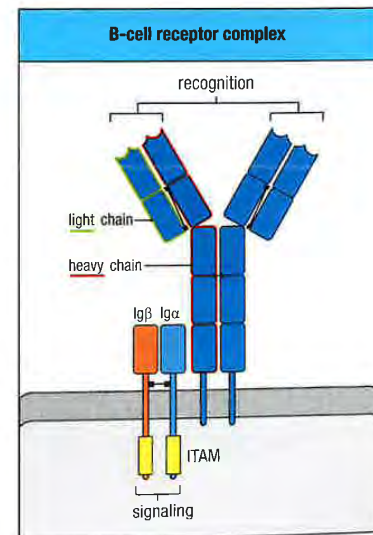
sites for the recruitment of the SH2 domains of signaling proteins as described earlier in the chapter. Two YXXL/I motifs are separated by about six to nine amino acids within each ITAM, so the canonical ITAM sequence is ...YXX[L/I]X₆₋₉YXX[L/I]..., where Y is tyrosine, L is leucine, I is isoleucine, and X represents any amino acid. The two tyrosines of the ITAM make it particularly efficient in recruiting signaling proteins that contain two tandem SH2 domains (Fig. 7.9). When both tyrosines of the ITAM are phosphorylated, tandem SH2 domain-containing proteins such as Syk or ZAP-70 are recruited. This leads to the phosphorylation of Syk or ZAP-70, an essential step in the activation of these kinases, as will be discussed further below (see Section 7-10).

The antigen-binding immunoglobulin on the B-cell surface is also associated with invariant protein chains that mediate signaling. These two polypeptides, called Igα and Igβ, are required for transport of the receptor to the surface and for B-cell receptor signaling (Fig. 7.10). Igα and Igβ are single-chain proteins composed of an extracellular immunoglobulin-like domain connected by a transmembrane domain to a cytoplasmic tail. They form a disulfide-linked heterodimer that becomes associated with immunoglobulin heavy chains and enables their transport to the cell surface. The Igα:Igβ dimer associates with the B-cell receptor through hydrophilic rather than charge interactions between their transmembrane regions. The complete B-cell receptor is thought to be a complex of six chains—two identical light chains, two identical heavy chains, and one associated heterodimer of Igα and Igβ. Like CD3 and the ζ chains of the T-cell receptor, Igα and Igβ have ITAMs, but just one each, and these are essential for the ability of the B-cell receptor to signal.

7-8 Antigen recognition by the T-cell receptor and its co-receptors transduces a signal across the plasma membrane to initiate signaling.

To make an effective immune response, T cells and B cells must be able to respond to their specific antigen even when it is present at extremely low levels. This is especially important for T cells, as an antigen-presenting cell will display on its surface many different peptides from both self and foreign proteins, and the number of peptide:MHC complexes specific for a particular T-cell receptor is likely to be very low. Some estimates suggest that a naive CD4 T

Fig. 7.10 The B-cell receptor complex is made up of cell-surface immunoglobulin with one each of the invariant signaling proteins Igα and Igβ. The immunoglobulin recognizes and binds antigen but cannot itself generate a signal. It is associated with antigen-nonspecific signaling molecules—Igα and Igβ. These each have a single ITAM (yellow segment) in their cytosolic tails that enables them to signal when the B-cell receptor is ligated with antigen. Igα and Igβ form a disulfide-linked heterodimer that is non-covalently associated with the heavy chains.



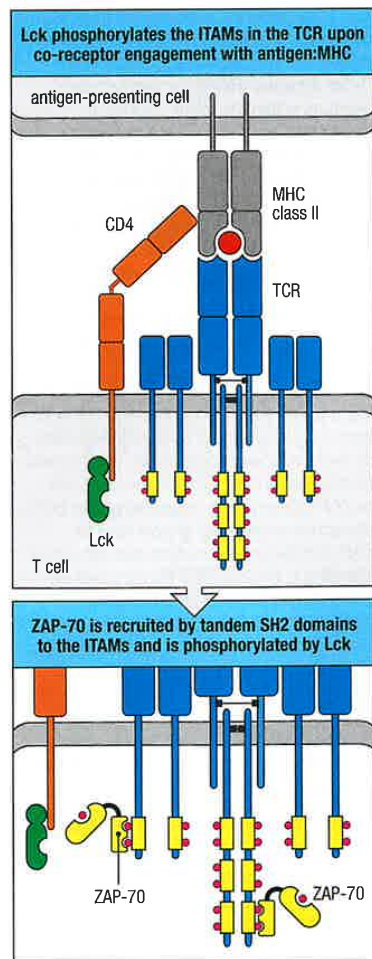


Fig. 7.11 Engagement of co-receptors with the T-cell receptor enhances phosphorylation of the ITAMs. Upper panel: for simplicity, we show the CD4 co-receptor engaging the same MHC molecule as the T-cell receptor (TCR), although signaling within receptor microclusters may differ from this arrangement. When T-cell receptors and co-receptors are brought together by binding to peptide:MHC complexes on the surface of an antigen-presenting cell, recruitment of the co-receptor-associated kinase Lck leads to phosphorylation of ITAMs in CD3 γ , δ , and ϵ , and in the ζ chains. Lower panel: the tyrosine kinase ZAP-70 binds to phosphorylated ITAMs through its SH2 domains, enabling ZAP-70 to be phosphorylated and activated by Lck. ZAP-70 then phosphorylates other intracellular signaling molecules.

cell can become activated by fewer than 50 antigenic peptide:MHC complexes displayed by an antigen-presenting cell, and effector CD8 cytotoxic T cells may be even more sensitive. B cells become activated when about 20 B-cell receptors are engaged. These estimates based on *in vitro* studies may not be precise for cells *in vivo*, but it is clear that the antigen receptors on T cells and B cells confer remarkable sensitivity to antigen.

For a peptide:MHC complex to activate T cells, it must bind directly to the T-cell receptor (Fig. 7.11, upper panel, and see Fig. 4.22). However, it remains unclear precisely how this extracellular recognition event is transmitted across the T-cell membrane to initiate signaling. Questions remain as to the stoichiometry and physical arrangement of T-cell receptors and peptide:MHC complexes required to initiate the signaling cascade. We will discuss this area of active research only briefly before moving on to explain well-understood intracellular events that occur after antigen recognition.

One suggestion is that signaling is initiated by T-cell receptor dimerization through formation of 'pseudo-dimeric' peptide:MHC complexes containing one antigen peptide:MHC molecule and one self peptide:MHC molecule on the surface of the antigen-presenting cell. This model relies on a weak interaction occurring between the T-cell receptor and self peptide:MHC complexes, stabilized by the CD4 or CD8 co-receptor interactions with the self peptide:MHC complexes, but it would explain signaling induced by low densities of antigenic peptides. An additional possibility is that the antigenic peptide:MHC complex induces a conformational change in the T-cell receptor, or its associated CD3 and ζ chains, that promotes receptor phosphorylation; however, direct structural evidence supporting this model is still lacking.

It has also been suggested that signaling might involve receptor oligomerization, or clustering, as antibodies that bind to and cross-link T-cell receptors can activate T cells. Since antigenic peptides are vastly outnumbered by other peptides displayed on the surface of the antigen-presenting cell, it is unlikely that physiologic amounts of antigen induce conventional oligomerization as observed with antibodies. However, assemblies of small numbers of T-cell receptors called **microclusters** have been observed in the zone of contact between the T cell and the antigen-presenting cell. These microclusters form rapidly following TCR stimulation, and quickly merge with microclusters containing downstream signaling components, such as scaffolds and adaptors. Current evidence indicates that signaling is initiated in these microclusters. One popular model proposes that signal initiation takes place when inhibitory signaling proteins are excluded from these complexes. A key component of this model is that prior to TCR signaling, activating and inhibitory enzymes are in a balanced equilibrium; the initiation of signaling occurs when this equilibrium is perturbed in favor of the activating modifications.

7-9 Antigen recognition by the T-cell receptor and its co-receptors leads to phosphorylation of ITAMs by Src-family kinases, generating the first intracellular signal in a signaling cascade.

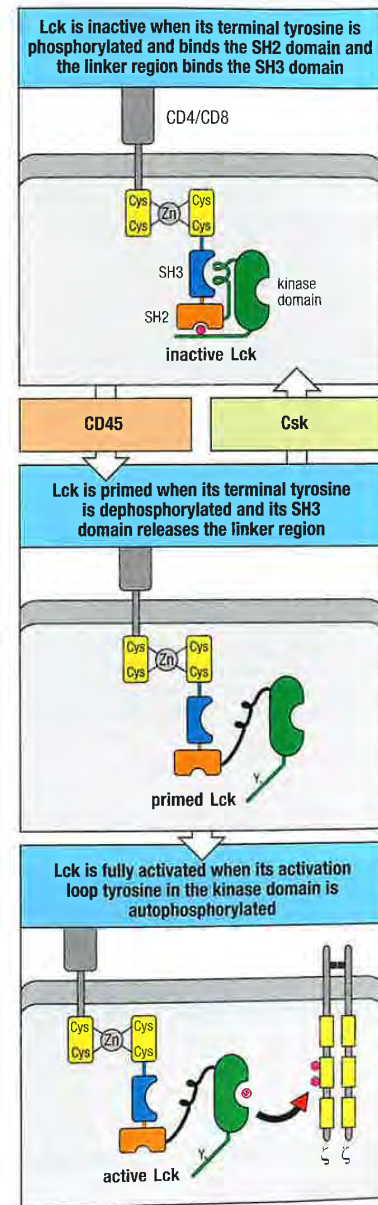
The first intracellular signal generated after the T cell has detected its specific antigen is the phosphorylation of both tyrosines in the ITAMs of the T-cell receptor. This signal is initiated with the help of the CD4 and CD8 co-receptors, which bind to MHC class II molecules and class I molecules, respectively, via their extracellular domains (see Section 4-18), and associate with nonreceptor kinases via their intracellular domains. The Src-family kinase **Lck** is constitutively associated with the cytoplasmic domains of CD4 and CD8 and is thought to be the kinase primarily responsible for phosphorylation of the ITAMs of the T-cell receptor (see Fig. 7.11). Evidence suggests that binding of the co-receptor to the peptide:MHC complex that binds the T-cell receptor

enhances the recruitment of Lck to the engaged T-cell receptor, leading to more efficient phosphorylation of the T-cell receptor ITAMs. The importance of this event is demonstrated by the profound reduction in T-cell development in Lck-deficient mice. This indicates the essential role of Lck in T-cell receptor signaling during the selection of developing T cells in the thymus (discussed in Chapter 8). Lck is important for T-cell receptor signaling in naive T cells and effector T cells, but is less important for the activation or maintenance of memory CD8 T cells by their specific antigen. A related tyrosine kinase, Fyn, is weakly associated with the ITAMs of the T-cell receptor and may have some role in signaling. Whereas mice lacking Fyn develop normal CD4 and CD8 T cells that respond in essentially normal fashion to antigen, mice lacking both Lck and Fyn show a more complete loss of T-cell development than mice lacking Lck alone.

Another role of the co-receptors in T-cell receptor signaling may be to stabilize interactions between the receptor and the peptide:MHC complex. Affinities of individual receptors for their specific peptide:MHC complexes are in the micromolar range, which means that the T-cell receptor:peptide:MHC complexes have half-lives of less than 1 second and dissociate rapidly. The additional binding of a co-receptor to the MHC molecule is thought to stabilize the interaction by increasing its duration, thereby providing time for an intracellular signal to be generated.

The Lck bound to the cytoplasmic tails of CD4 or CD8 is brought near its substrate ITAMs in the T-cell receptor when the co-receptor binds the receptor:peptide:MHC complex (see Fig. 7.11). Lck's activity is also regulated allosterically by phosphorylation of a tyrosine in its carboxy terminus by the C-terminal Src kinase (Csk). The resulting phosphotyrosine interacts with Lck's SH2 domain and helps maintain Lck in a closed conformation, resulting in a catalytically inactive state (Fig. 7.12). The absence of Csk during T-cell development causes T cells to mature autonomously in the thymus without needing to bind peptide:MHC, presumably as a result of abnormal activation of TCR signaling by hyperactive Lck in Csk-deficient thymocytes. This suggests that Csk normally acts to reduce Lck activity and to attenuate TCR signaling. Dephosphorylation of the tyrosine or engagement of the SH2 or SH3 domains by their ligands releases Lck from its inactive conformation, resulting in a primed, but not fully active, Lck kinase (see Fig. 7.12). Full activation of catalytic activity also requires Lck autophosphorylation of a tyrosine in its kinase domain. In nonstimulated lymphocytes, phosphorylation of Lck is counteracted by the tyrosine phosphatase CD45, which can dephosphorylate both of the Lck tyrosine phosphorylation sites. Prior to TCR stimulation, multiple phosphorylated species of Lck are found in T cells, but antigen receptor stimulation is required to stabilize the activated form of Lck and lead to ITAM phosphorylation.

Fig. 7.12 Lck activity is regulated by tyrosine phosphorylation and dephosphorylation. Src kinases such as Lck contain SH3 (blue) and SH2 (orange) domains preceding the kinase domain (green). Lck also contains a unique amino-terminal motif (yellow) with two cysteine residues that bind a Zn ion that is also bound to a similar motif in the cytoplasmic domain of CD4 or CD8. Upper panel: In inactive Lck, the two lobes of the kinase domain are constrained by interactions with both the SH2 and SH3 domains. The SH2 domain interacts with a phosphorylated tyrosine (red) at the carboxy-terminal end of the kinase domain. The SH3 domain interacts with a proline-rich sequence in the linker between the SH2 domain and the kinase domain. Middle panel: dephosphorylation of the carboxy-terminal tyrosine by the phosphatase CD45 (not shown) releases the SH2 domain. Binding of other ligands to the SH3 region can facilitate release of the linker region (not shown). In this state, Lck is considered primed, but not fully activated. Lower panel: full activation of Lck catalytic activity requires autophosphorylation on the activation loop in the kinase domain. Active Lck can then phosphorylate ITAMs in the signaling chains of the nearby T-cell receptor. Rephosphorylation of the carboxy-terminal tyrosine by the C-terminal Src kinase (Csk) or loss of the SH3 ligand returns Lck to the inactive state.



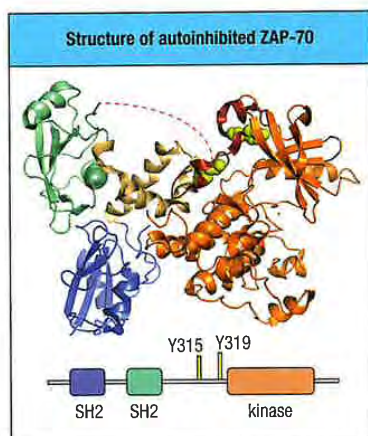


Fig. 7.13 Structure of the autoinhibited ZAP-70 kinase. The structure of the inactive autoinhibited conformation of ZAP-70 is shown with the protein domains color-coded according to the domain map shown at the bottom; the dashed red line indicates a region of the protein that was not detected in the structural analysis. Prior to T-cell receptor stimulation, the ZAP-70 kinase is in this inactive conformation, based on interactions between the tandem SH2 domain-kinase domain linker region (thick red line) and the kinase domain. This interaction stabilizes ZAP-70 in a catalytically inactive state, referred to as the autoinhibited form of ZAP-70, by locking the kinase domain in an inactive conformation. Following T-cell receptor stimulation, Lck phosphorylates two tyrosine residues in this linker region, Y315 and Y319, shown in yellow. Lck also phosphorylates a tyrosine residue in the catalytic (kinase) domain. When Y315 and Y319 are phosphorylated, the linker region no longer binds to the kinase domain, allowing the phosphorylated kinase domain to adopt an active conformation. Courtesy of Arthur Weiss.

7-10 Phosphorylated ITAMs recruit and activate the tyrosine kinase ZAP-70.

The precise spacing of the two YXXL/I motifs in an ITAM suggests that the ITAM is a binding site for a signaling protein with two SH2 domains. In the case of the T-cell receptor, this protein is the tyrosine kinase **ZAP-70** (**ζ-chain-associated protein**), which carries the activation signal onward. ZAP-70 has two tandem SH2 domains that can be simultaneously engaged by the two phosphorylated tyrosines in an ITAM (see Fig. 7.9). The affinity of the phosphorylated YXXL sequence for a single SH2 domain is low; binding of both SH2 domains to the ITAM is significantly stronger and confers specificity on ZAP-70 binding. Thus, when Lck has sufficiently phosphorylated an ITAM in the T-cell receptor, ZAP-70 binds to it. Once bound, ZAP-70 is phosphorylated by Lck at three tyrosine residues, two in the linker region between the tandem SH2 domains and the kinase domain, and a third residue in the catalytic domain. Together these phosphorylations activate ZAP-70 by disrupting the autoinhibited form of inactive ZAP-70, allowing the ZAP-70 kinase domain to adopt the active conformation (Fig. 7.13). ZAP-70 can also be activated by autophosphorylation.

7-11 ITAMs are also found in other receptors on leukocytes that signal for cell activation.

The signaling subunits of the T-cell receptor and the B-cell receptor each contain ITAMs, which are essential for T-cell receptor and B-cell receptor signaling. Phosphorylation of both tyrosines in the ITAM functions to recruit a tyrosine kinase with tandem SH2 domains—ZAP-70 in the case of T cells, and a closely related kinase, Syk, in B cells. Other immune-system receptors also use ITAM-containing accessory chains to transduce activating signals (Fig. 7.14). One example is **FcγRIII** (CD16); this is a receptor for IgG that triggers antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells, which we consider in Chapter 11; CD16 is also found on macrophages and neutrophils, where it facilitates the uptake and destruction of antibody-bound pathogens. To signal, FcγRIII must associate either with the ζ chain found also in the T-cell receptor or with another member of the same protein family known as the Fcγ chain. The Fcγ chain is also the signaling component of another Fc receptor—the Fcε receptor I (FcεRI) on mast cells. As we discuss in Chapter 14, this receptor binds IgE antibodies, and on cross-linking by allergens, it triggers the degranulation of mast cells. Last, many activating receptors on NK cells are associated with DAP12, another ITAM-containing protein (see Section 3-26). Each of these additional ITAM-containing signaling chains becomes tyrosine phosphorylated following stimulation of its associated receptor, leading to the recruitment of a tyrosine kinase, either Syk or ZAP-70. With the exception of

Receptors other than antigen receptors also associate with ITAM-containing chains that deliver activating signals		
NK cells Macrophages Neutrophils	NK cells	Mast cells Basophils
FcγRIII (CD16) FcγRIV	NKG2C, D, E (CD94)	FcεRI
γ or ζ	DAP12	α β

Fig. 7.14 Other receptors that pair with ITAM-containing chains can deliver activating signals. Cells other than B and T cells have receptors that pair with accessory chains containing ITAMs, which are phosphorylated when the receptor is cross-linked. These receptors deliver activating signals. The Fcγ receptor III (FcγRIII, or CD16) is found on NK cells, macrophages, and neutrophils. Binding of IgG to this receptor activates the killing function of the NK cell, leading to the process known as antibody-dependent cell-mediated cytotoxicity (ADCC). Activating receptors on NK cells, such as NKG2C, NKG2D, and NKG2E, also associate with ITAM-containing signaling chains. The Fcε receptor (FcεRI) is found on mast cells and basophils. The α subunit binds to IgE antibodies with very high affinity. The β subunit is a four-spanning transmembrane protein. When antigen subsequently binds to the IgE, the mast cell is triggered to release granules containing inflammatory mediators. The γ chain associated with the Fcγ receptors, and the DAP12 chain that associates with the NK killer-activating receptors, also contain one ITAM per chain and are present as homodimers.

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T cells, Syk is broadly expressed in all leukocyte subsets; in contrast, ZAP-70 has been found only in T cells and NK cells.

Several viral pathogens seem to have acquired ITAM-containing receptors from their hosts. These include the Epstein-Barr virus (EBV), whose *LMP2A* gene encodes a membrane protein with a cytoplasmic tail containing an ITAM. This enables EBV to trigger B-cell proliferation by the signaling pathways discussed in Section 7-20, an important step in the development of EBV-induced malignancies. Another virus that expresses an ITAM-containing protein is the Kaposi sarcoma herpesvirus (KSHV or HHV8), which causes malignant transformation and proliferation of the cells it infects.

7-12 Activated ZAP-70 phosphorylates scaffold proteins and promotes PI 3-kinase activation.

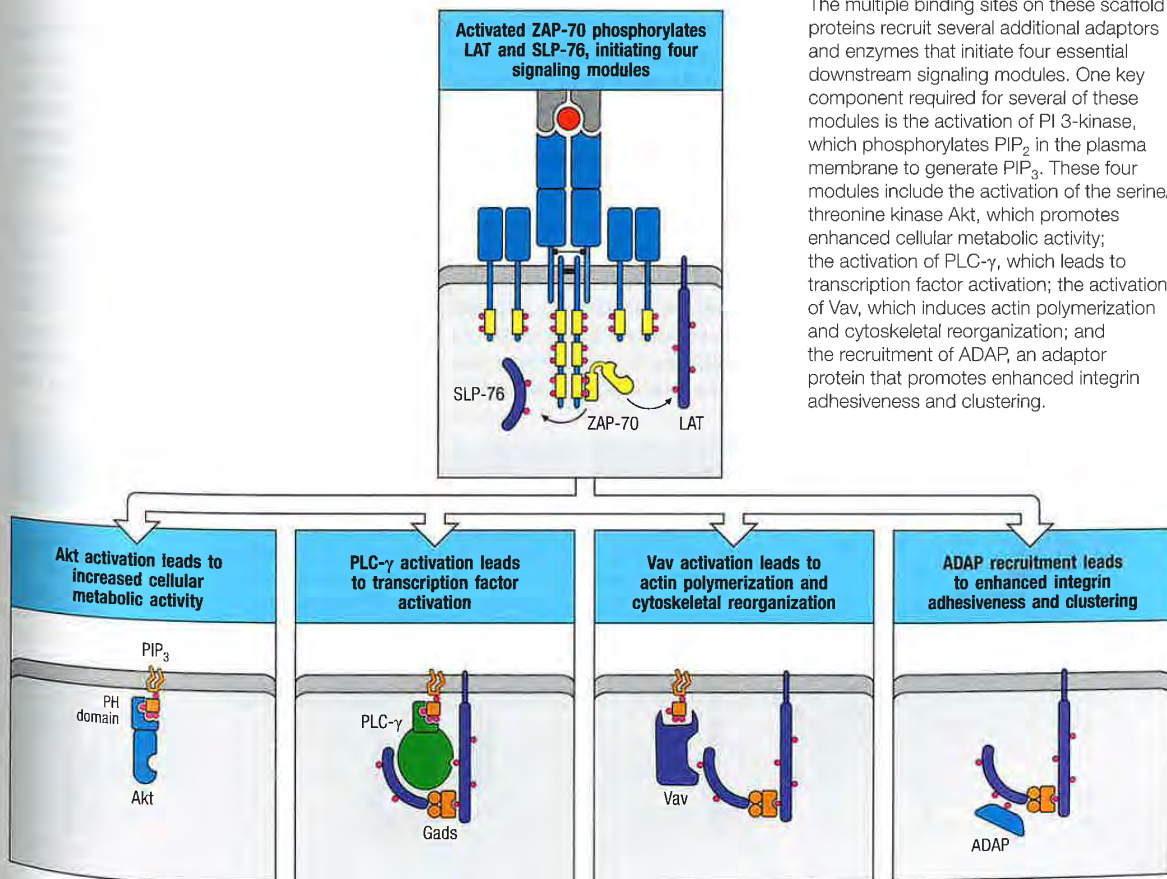
As described in Section 7-10, phosphorylation of the tyrosines in the T-cell receptor ITAMs leads to the recruitment and activation of ZAP-70. This provides ZAP-70 proximity to the cell membrane, where it phosphorylates the scaffold protein LAT (linker for activated T cells), a transmembrane protein with a large cytoplasmic domain (Fig. 7.15). ZAP-70 also phosphorylates another adaptor protein, SLP-76. LAT and SLP-76 can be linked by the adaptor protein Gads; this three-protein complex, referred to as the LAT:Gads:SLP-76 complex, plays a central role in T-cell activation. This is illustrated by the profound TCR signaling and T-cell development defects seen in mice lacking any



MOVIE 7.1

Fig. 7.15 ZAP-70 phosphorylates LAT and SLP-76, initiating four downstream signaling modules.

Activated ZAP-70 phosphorylates the scaffold proteins LAT and SLP-76 and recruits them to the activated T-cell receptor (TCR) complex. An adaptor protein, Gads, holds the tyrosine-phosphorylated LAT and SLP-76 together. The multiple binding sites on these scaffold proteins recruit several additional adaptors and enzymes that initiate four essential downstream signaling modules. One key component required for several of these modules is the activation of PI 3-kinase, which phosphorylates PIP_2 in the plasma membrane to generate PIP_3 . These four modules include the activation of the serine/threonine kinase Akt, which promotes enhanced cellular metabolic activity; the activation of PLC- γ , which leads to transcription factor activation; the activation of Vav, which induces actin polymerization and cytoskeletal reorganization; and the recruitment of ADAP, an adaptor protein that promotes enhanced integrin adhesiveness and clustering.



T Cell Signaling Defects



Fig. 7.16 The recruitment of phospholipase C- γ by LAT and SLP-76, and its phosphorylation and activation by the protein kinase Itk, are crucial steps in T-cell activation. ZAP-70 phosphorylates the scaffold proteins LAT and SLP-76, which are brought together to form a complex at the activated T-cell receptor by the adaptor protein Gads. This complex also promotes the activation of PI 3-kinase, leading to the production of PIP_3 (formed by the phosphorylation of PIP_2 by PI 3-kinase). Phospholipase C- γ (PLC- γ) is recruited to the membrane by its PH domain binding to PIP_3 and then binds to phosphorylated sites in LAT and to the proline-rich domain of SLP-76. To be activated, PLC- γ must be phosphorylated by the Tec-family kinase Itk. Itk is recruited to the membrane by interaction of its PH domain with PIP_3 , and by interactions with phosphorylated SLP-76. Once phosphorylated by Itk, phospholipase C- γ is active.

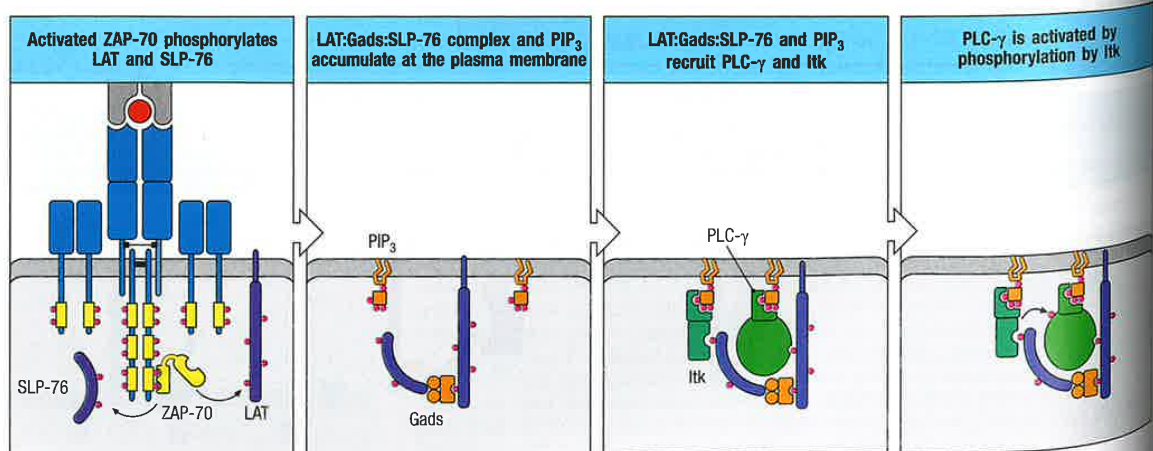
one of these components, and in humans lacking ZAP-70. A second essential event that occurs rapidly following ZAP-70 activation is the recruitment and activation of the enzyme PI 3-kinase (see Section 7.4); while the detailed mechanism linking PI 3-kinase activation to T-cell receptor stimulation is not well understood, current evidence suggests a role for the small GTPase Ras. In this case, Ras may be activated by recruitment of the Ras-GEF Sos to LAT via Sos binding to the small adaptor protein Grb2, forming a second three-protein complex containing LAT and Sos, bridged by Grb2.

Following formation of the LAT:Gads:SLP-76 complex and the activation of PI 3-kinase, the T-cell receptor signaling pathway branches into several downstream modules, each of which induces cellular changes that contribute to optimal T-cell activation (see Figure 7.15). Each module is initiated by the recruitment of a key intermediate to the active signaling complexes, either via binding to the LAT:Gads:SLP-76 complex, to the PIP_3 generated by PI 3-kinase, or to both. In brief, these modules lead to the activation of phospholipase C- γ (PLC- γ), which affects transcription; the activation of the serine/threonine kinase Akt, which affects metabolism, among other things; the recruitment of the adaptor protein ADAP, which upregulates cell adhesion; and the activation of the protein Vav, which initiates actin polymerization. Each of these modules will be described in detail in the sections below.

7-13 Activated PLC- γ generates the second messengers diacylglycerol and inositol trisphosphate that lead to transcription factor activation.

One important module of T-cell receptor signaling is the activation of the enzyme **phospholipase C- γ (PLC- γ)**. First, PLC- γ is brought to the inner face of the plasma membrane by the binding of its PH domain to the PIP_3 that has been formed by the phosphorylation of PIP_2 by PI 3-kinase; it then binds to phosphorylated LAT and SLP-76. The actions of PLC- γ produce two second messengers that activate three distinct terminal branches of the T-cell receptor pathway leading to transcription factor activation.

Due to this crucial role in T-cell activation, PLC- γ activation is controlled at several different levels. Recruitment to the membrane, while necessary, is not sufficient to activate PLC- γ . PLC- γ activation requires phosphorylation by Itk, a member of the Tec family of cytoplasmic tyrosine kinases. Like PLC- γ , Tec kinases contain PH, SH2, and SH3 domains and are recruited to the plasma membrane by interactions via these domains; specifically, the PH domain interacts with PIP_3 on the inner face of the membrane (Fig. 7.16), and the SH2



and SH3 domains interact with SLP-76. These interactions serve to localize Itk in close proximity to its substrate, PLC- γ .

Once PLC- γ has been recruited to the inner face of the plasma membrane and has been activated, it can catalyze the breakdown of the membrane lipid PIP₂ (see Section 7-4 and Fig. 7.5) to generate two products, the membrane lipid **diacylglycerol (DAG)** and the diffusible second messenger **inositol 1,4,5-trisphosphate (IP₃)** (not to be confused with the membrane lipid PIP₃) (Fig. 7.17). DAG is confined to the membrane, but diffuses in the plane of the membrane and serves as a molecular target that recruits other signaling molecules to the membrane. IP₃ diffuses into the cytosol and binds to IP₃ receptors on the endoplasmic reticulum (ER) membrane. These receptors are Ca²⁺ channels, which open and release calcium stored in the ER into the cytosol. The consequent low levels of calcium in the ER then cause a conformational change in the transmembrane protein **STIM1**, promoting its clustering within the ER membrane. The STIM1 oligomers bind to the plasma membrane, where they interact directly with ORAI1, the plasma membrane calcium channel (also known as the **CRAC channel**: calcium release-activated calcium channel). Binding of STIM1 to ORAI1 triggers calcium channel opening, allowing extracellular calcium to flow into the cell to activate further signaling pathways and to replenish ER calcium stores.

The activation of PLC- γ marks an important step in T-cell activation, because after this point the PLC- γ signaling module splits into three distinct branches—the stimulation of Ca²⁺ entry, the activation of Ras, and the activation of **protein kinase C- θ (PKC- θ)**—each of which ends in the activation of a different transcription factor. These signaling pathways are utilized by many cell types, in addition to lymphocytes. Their importance in T-cell activation is shown by the observation that treatment of T cells with phorbol myristate acetate (an analog of DAG) and ionomycin (a pore-forming drug that allows extracellular calcium to flow into the cell) can reconstitute many of the effects of T-cell receptor stimulation. Additionally, deficiencies in several of these signaling components, including Lck, Zap-70, Itk, CD45, Carma1, and ORAI1, have been found to be mutated in cases of **severe combined immunodeficiency (SCID)**.

7-14 Ca²⁺ entry activates the transcription factor NFAT.

One of the three signaling pathways leading from PLC- γ results in an influx of calcium ions into the cytosol. An important outcome of the increased cytosolic Ca²⁺ resulting from T-cell receptor signaling via PLC- γ is the activation of a family of transcription factors called **NFAT (nuclear factor of activated T cells)**. NFAT is something of a misnomer, because the five members of this family are expressed in many different tissues. In the absence of activating

Fig. 7.17 Phospholipase C- γ cleaves inositol phospholipids to generate two important signaling molecules. Top panel: phosphatidylinositol bisphosphate (PIP₂) is a component of the inner leaflet of the plasma membrane. When PLC- γ is activated by phosphorylation, it cleaves PIP₂ into two parts: inositol trisphosphate (IP₃), which diffuses away from the membrane into the cytosol, and diacylglycerol (DAG), which stays in the membrane. Both of these molecules are important in signaling. Middle panel: there are two phases of calcium release. IP₃ binds to a receptor in the endoplasmic reticulum (ER) membrane, opening calcium channels (yellow) and allowing the early phase of calcium ions (Ca²⁺) to enter the cytosol from the ER. The depletion of Ca²⁺ stores in the ER causes the aggregation of an ER calcium sensor, STIM1. Bottom panel: aggregated STIM1 stimulates the second phase of calcium entry by binding to and opening calcium channels called ORAI1 in the plasma membrane. This further increases cytosolic calcium and restores ER Ca²⁺ stores. DAG binds and recruits signaling proteins to the membrane, most importantly the Ras-GEF called RasGRP and a serine/threonine kinase called protein kinase C- θ (PKC- θ). Recruitment of RasGRP to the plasma membrane activates Ras, and PKC- θ activation results in the activation of the transcription factor NFATB.

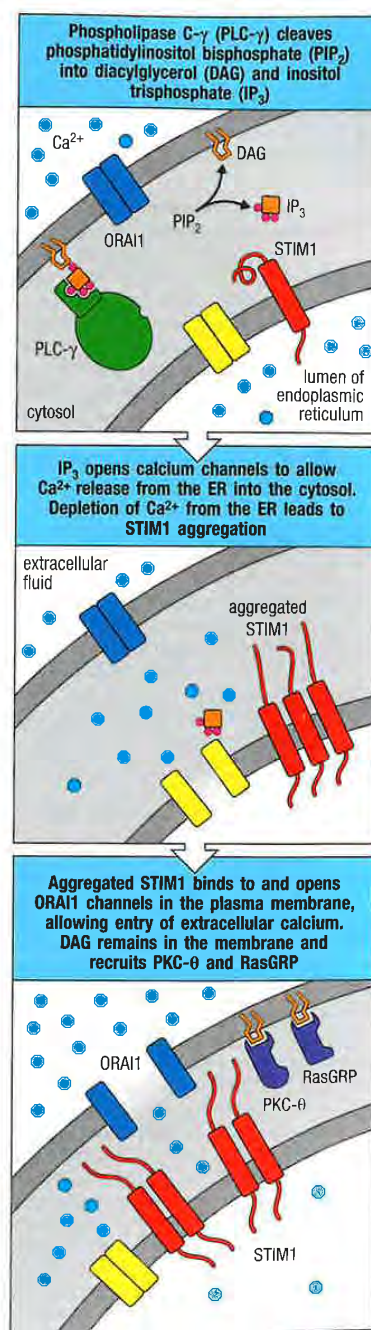
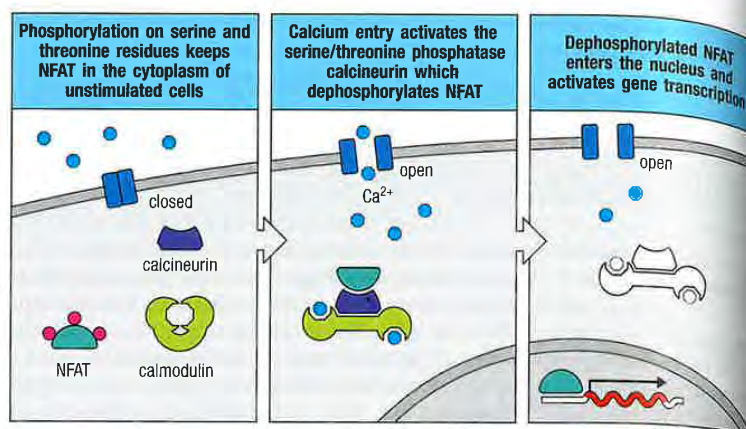


Fig. 7.18 The transcription factor NFAT is regulated by calcium signaling.

Left panel: NFAT is maintained in the cytoplasm by phosphorylation on serine and threonine residues. Center panel: after antigen receptor stimulation, calcium enters the cytosol, first from the endoplasmic reticulum (not shown; see Fig. 7.17) and later from the extracellular space (shown). After entering the cytosol, calcium binds to calmodulin, and the Ca^{2+} :calmodulin complex binds to the serine/threonine phosphatase calcineurin, activating it to dephosphorylate NFAT. Right panel: once dephosphorylated, NFAT moves into the nucleus, where it binds to promoter elements and activates the transcription of various genes.



signals, NFAT is kept in the cytosol of resting T cells by phosphorylation on serine/threonine residues. This phosphorylation is mediated by serine/threonine kinases, including glycogen synthase kinase 3 (GSK3) and casein kinase 2 (CK2). When phosphorylated, the nuclear localization sequence of NFAT is not recognized by nuclear transporters, and NFAT is unable to enter into the nucleus (Fig. 7.18).

The cytoplasmic Ca^{2+} resulting from T-cell receptor signaling binds to and induces a conformational change in a protein called **calmodulin**, which is then able to bind to and activate a wide range of different target enzymes. In T cells, an important target of calmodulin is **calcineurin**, a protein phosphatase that acts on NFAT. Dephosphorylation of NFAT by calcineurin allows the nuclear localization sequence to be recognized by nuclear transporters, and NFAT enters the nucleus (see Fig. 7.18). There it participates in turning on many of the genes crucial for T-cell activation, such as the gene for the cytokine interleukin-2 (IL-2).

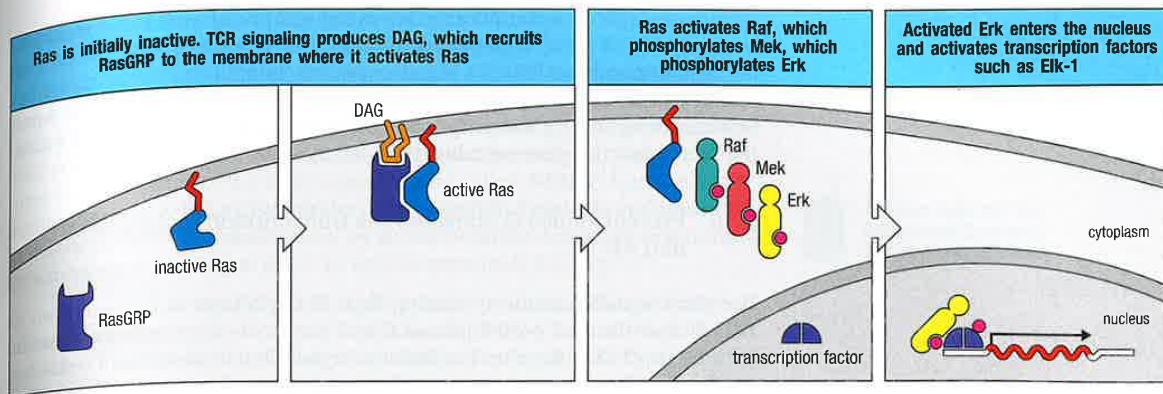
The importance of NFAT in T-cell activation is illustrated by the effects of selective inhibitors of calcineurin called **cyclosporin A (CsA)** and **tacrolimus** (also known as **FK506**). CsA forms a complex with the protein cyclophilin A, and this complex inhibits calcineurin. Tacrolimus binds a different protein, FK-binding protein (FKBP), making a complex that similarly inhibits calcineurin. By inhibiting calcineurin, these drugs prevent the formation of active NFAT. T cells express low levels of calcineurin, so they are more sensitive to inhibition of this pathway than are many other cell types. Both cyclosporin A and tacrolimus thus act as effective immunosuppressants, and are widely used to prevent the rejection of organ transplants (see Chapter 16, Section 16-3).

7-15 Ras activation stimulates the mitogen-activated protein kinase (MAPK) relay and induces expression of the transcription factor AP-1.

MOVIE 7.2



A second branch of the PLC- γ signaling module is the activation of the small GTPase Ras. This can occur by various means. The most efficient mechanism for Ras activation in T cells is via the DAG generated by PLC- γ , which diffuses in the plasma membrane and activates a variety of proteins. One of these is the protein RasGRP, which is a guanine-nucleotide exchange factor that specifically activates Ras. RasGRP contains a protein-interaction module called a C1 domain that binds to DAG. This interaction recruits RasGRP to the membrane near active signaling complexes (Fig. 7.19), where it activates Ras by promoting the exchange of GDP for GTP. Ras is also activated in the T-cell receptor



signaling pathway by the guanine-exchange factor Sos, which is recruited by the adaptor protein Grb2 (see Sections 7-2 and 7-3), which has itself been recruited by binding to phosphorylated LAT and SLP-76.

Activated Ras then triggers a three-kinase relay that ends in the activation of a serine/threonine kinase known as a **mitogen-activated protein kinase or MAP kinase (MAPK)** (see Fig. 7.19). In the case of antigen receptor signaling, the first member of the relay is a MAPK kinase kinase (MAP3K) called **Raf**. Raf is a serine/threonine kinase that phosphorylates the next member of the series, a MAPK kinase (MAP2K) called **MEK1**. MEK1 is a dual-specificity protein kinase that phosphorylates a tyrosine and a threonine residue on the last member of the relay, a MAPK which in T cells and B cells is **Erk (extracellular signal-related kinase)**.

Signaling by MAPK cascades is facilitated by specialized scaffold proteins that bind to all three kinases in a particular MAPK relay, thereby accelerating their interactions. The scaffold protein **kinase suppressor of Ras (KSR)** functions in the Raf/MEK1/Erk pathway. During T-cell receptor signaling, KSR associates with Raf, MEK1, and Erk and localizes itself and its cargo to the membrane. In that location, activated Ras can engage with the Raf bound to KSR and trigger the kinase relay (see Fig. 7.19).

An important function of MAPKs is to phosphorylate and activate transcription factors that can then induce new gene expression. Erk acts indirectly to generate the transcription factor **AP-1**, which is a heterodimer composed of one monomer each from the Fos and Jun families of transcription factors (Fig. 7.20). Active Erk phosphorylates the transcription factor Elk-1, which

Fig. 7.19 DAG activates MAPK cascades, leading to transcription factor activation. All MAPK cascades are initiated by the activation of small G proteins, such as Ras in this example. Ras is switched from an inactive state (first panel) to an active state (second panel) by a guanine-nucleotide exchange factor (GEF), RasGRP, which is recruited to the membrane by DAG. Ras activates the first enzyme of the cascade, a protein kinase called Raf, a MAPK kinase kinase (MAP3K) (third panel). Raf phosphorylates Mek, a MAP2K, which in turn phosphorylates and activates Erk, a MAPK. The scaffold protein KSR associates with Raf, Mek, and Erk to facilitate their efficient interactions (not shown). Phosphorylation and activation of Erk releases it from the complex so that it can diffuse within the cell and enter the nucleus (fourth panel). Phosphorylation of transcription factors by Erk results in new gene transcription.

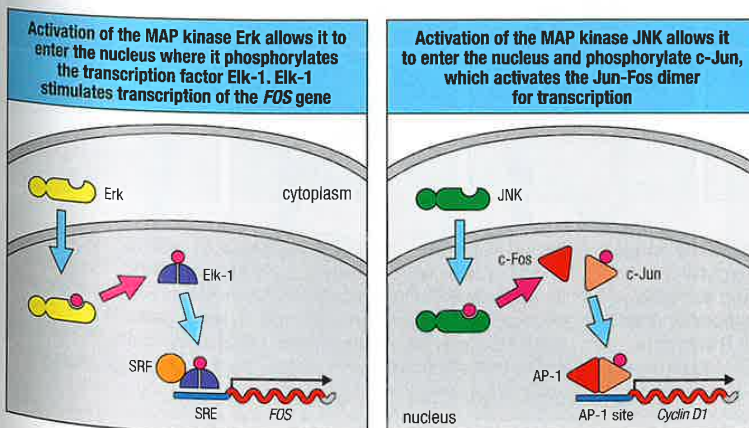


Fig. 7.20 The transcription factor AP-1 is formed as a result of the Ras/MAPK signaling pathway. Left panel: phosphorylation of the MAPK Erk activated as a result of the Ras-MAPK cascade allows Erk to enter the nucleus, where it phosphorylates the transcription factor Elk-1. Elk-1, along with serum response factor (SRF), binds to the serum response element (SRE) in the promoter of the gene (*FOS*) for the transcription factor c-Fos, stimulating its transcription. Right panel: the protein kinase PKC- θ can induce the phosphorylation of another MAPK called Jun kinase (JNK). This enables JNK to enter the nucleus and phosphorylate the transcription factor c-Jun, which forms a dimer with c-Fos. The phosphorylated c-Jun/Fos dimer is an active AP-1 transcription factor that binds to AP-1 sites and promotes transcription of many target genes.

cooperates with a transcription factor called serum response factor to initiate transcription of the *FOS* gene. Fos protein then associates with Jun to form the AP-1 heterodimer, but this remains transcriptionally inactive until another MAPK called **Jun kinase (JNK)** phosphorylates Jun. Similar to NFAT, AP-1 participates in turning on transcription of many genes important for T-cell activation, including the gene encoding the cytokine IL-2.

7-16 Protein kinase C activates the transcription factors NF κ B and AP-1.

The third signaling pathway leading from PLC- γ results in the activation of PKC- θ , an isoform of protein kinase C that is restricted to T cells and muscle. Mice lacking PKC- θ develop T cells in the thymus, but their mature T cells have a defect in the activation of two crucial transcription factors, NF κ B and AP-1, in response to signaling by the T-cell receptor and CD28. These transcription factors also participate in turning on genes required for T-cell activation. For example, transcription of the gene for IL-2 requires NF κ B in addition to NFAT and AP-1, making PKC- θ activation an important component of T-cell activation.

PKC- θ has a C1 domain and is recruited to the membrane when DAG is generated by activated PLC- γ (see Fig. 7.17). In this location, the kinase activity of PKC- θ initiates a series of steps that results in the activation of NF κ B (Fig. 7.21). PKC- θ phosphorylates the large membrane-localized scaffold protein CARMA1, causing it to oligomerize and form a multisubunit complex with other proteins. This complex recruits and activates TRAF-6, the same protein that we encountered in Chapter 3 in its role in activating NF κ B in the TLR signaling pathway (see Fig. 3.13).

NF κ B is the general name for a member of a family of homo- and heterodimeric transcription factors made up of the Rel family of proteins. The most common NF κ B activated in lymphocytes is a heterodimer of p50:p65Rel.

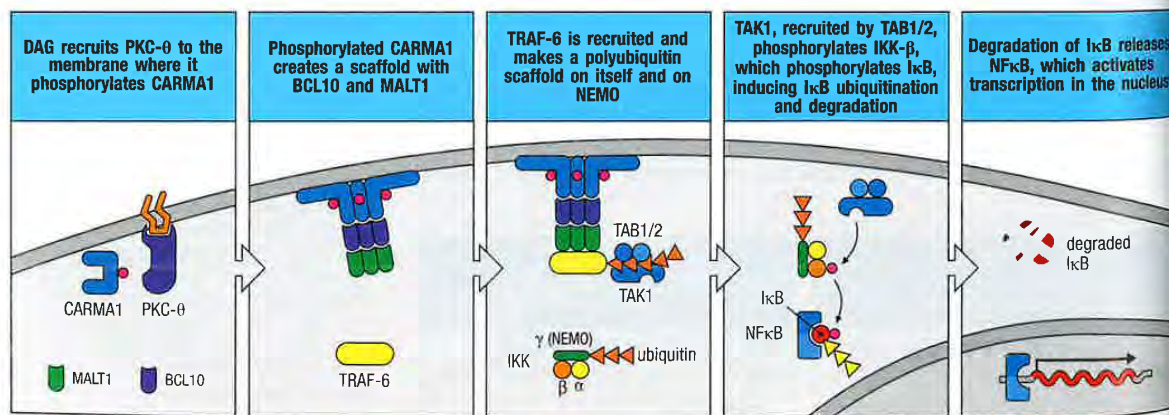


Fig. 7.21 Activation of the transcription factor NF κ B by antigen receptors is mediated by protein kinase C. Diacylglycerol (DAG), produced as a result of T-cell receptor signaling activating PLC- γ , recruits a protein kinase C (PKC- θ) to the membrane, where it phosphorylates a scaffold protein called CARMA1. This forms a complex with BCL10 and MALT1 that recruits the E3 ubiquitin ligase TRAF-6. As described in Fig. 3.13, the kinase TAK1 is recruited by the polyubiquitin scaffold produced by TRAF-6 and phosphorylates

the I κ B kinase (IKK) complex [IKK α :IKK β :IKK γ (NEMO)]. IKK phosphorylates I κ B, stimulating its ubiquitination, which targets I κ B for degradation by the proteasome. This releases NF κ B to enter the nucleus and stimulate transcription of its target genes. A defect in NEMO that prevents NF κ B activation causes immunodeficiency that results in susceptibility to extracellular bacterial infections, along with a skin disease known as ectodermal dysplasia.

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The dimer is held in an inactive state in the cytoplasm by binding to an inhibitory protein called inhibitor of κB (IκB). As described for TLR signaling (see Fig. 3.13), TRAF-6 stimulates the degradation of IκB by first activating the kinase TAK1, which activates a complex of serine kinases, IκB kinase (IKK). IKK phosphorylates IκB, causing its ubiquitination and subsequent degradation, and the consequent release and entry into the nucleus of active NFκB. Inherited deficiency of the IKKγ subunit (also called NEMO) leads to a syndrome known as **X-linked hypohidrotic ectodermal dysplasia and immunodeficiency**, which is characterized by developmental defects in ectodermal structures such as skin and teeth, as well as immunodeficiency.

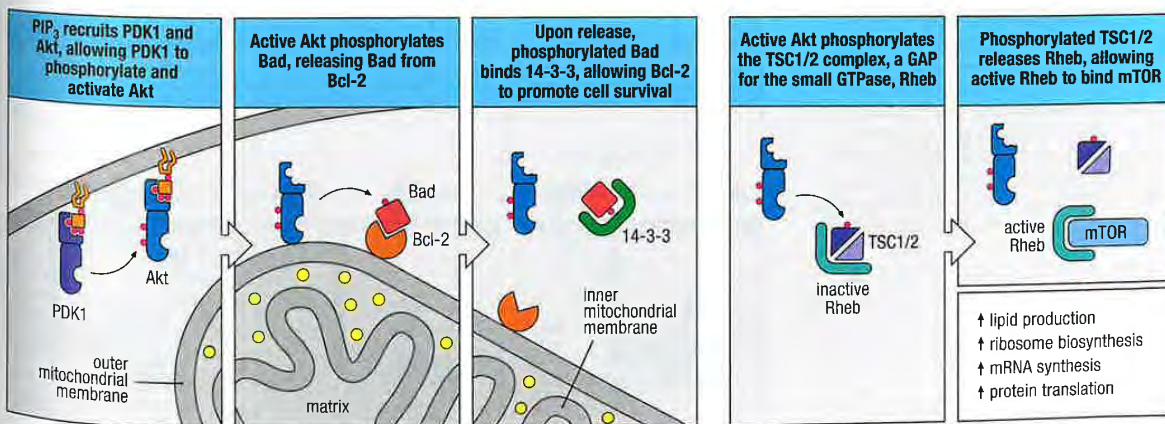
PKC-θ can also activate JNK, and might be able to activate the transcription factor AP-1 by this route. However, T cells lacking PKC-θ have a defect in AP-1 activation in addition to their defect in NFκB activation, but no defect in JNK activation, indicating that our understanding of this pathway is still incomplete.

7-17 PI 3-kinase activation upregulates cellular metabolic pathways via the serine/threonine kinase Akt.

While the activation of transcription factors is an important outcome of antigen receptor signaling, a productive T-cell response also requires substantial changes in cellular metabolism, needed to accommodate the energetic and macromolecular demands of rapidly dividing cells. The PI 3-kinase pathway plays a central role in this response via the recruitment and activation of the second important signaling module, initiated by the serine/threonine kinase Akt (also known as protein kinase B). Akt, via its PH domain, binds to PIP₃ in the membrane, which is generated by PI 3-kinase (Fig. 7.22; see also Fig. 7.5). In that location, Akt is phosphorylated by PDK1, and once activated, phosphorylates a variety of downstream proteins. One of its effects is to promote cell survival by inhibiting cell death via multiple mechanisms. A major mechanism is the phosphorylation of the pro-apoptotic protein Bad. When phosphorylated, Bad can no longer bind to and inhibit the anti-apoptotic (pro-survival) protein Bcl-2 (see Fig. 7.22). Another effect of activated Akt is to regulate the expression of homing and adhesion receptors that orchestrate the migratory properties of activated T cells (discussed in detail in Chapters 9 and 11). Activated Akt also functions to stimulate the cell's metabolism by increasing the utilization of glucose; this is mediated by increasing the activity of glycolytic enzymes and by inducing the upregulation of nutrient transporters on the T-cell membrane.



Fig. 7.22 The serine/threonine kinase Akt is activated by TCR signaling and promotes cell survival and enhanced metabolic activity via mTOR. Panel one: T-cell receptor (TCR) signaling activates PI 3-kinase (not shown), generating PIP₃ in the plasma membrane; PIP₃ recruits and activates the kinase PDK1. Akt, a second serine/threonine kinase, binds to PIP₃ via its PH domain, and is phosphorylated and activated by PDK1. Panel two: active Akt phosphorylates the pro-apoptotic protein Bad, which is binding to and inhibiting the anti-apoptotic protein Bcl-2 at the mitochondrial membrane. Panel three: phosphorylated Bad binds to 14-3-3, releasing Bcl-2 to promote cell survival. Panel four: a second function of active Akt is to phosphorylate the TSC1/2 complex, a GAP for the small GTPase Rheb. Panel five: When TSC1/2 is phosphorylated, it releases the inactive Rheb protein, leading to Rheb activation. Active Rheb binds to and activates mTOR (mammalian target of rapamycin). Once activated, mTOR acts on multiple pathways that lead to increased lipid production, ribosome biosynthesis, mRNA synthesis, and protein translation.

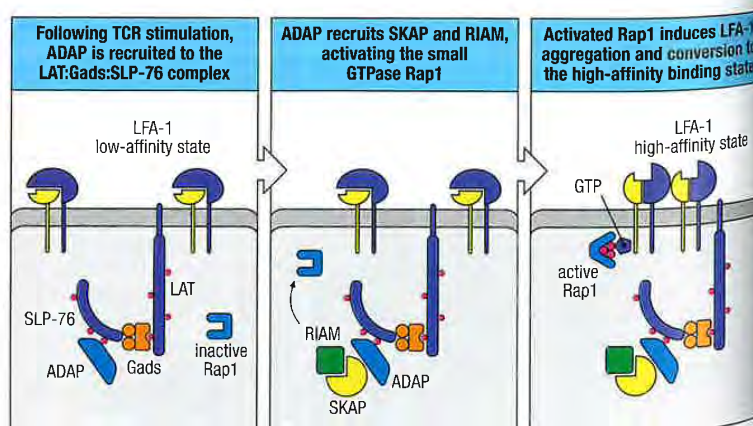


Yet another important function of activated Akt is to stimulate the mTOR (mammalian target of rapamycin) pathway, a key regulator of macromolecular biosynthesis (see Fig. 7.22). In this case, Akt phosphorylates and inactivates the TSC complex, a GAP for the small GTPase Rheb. This leads to Rheb activation, and in turn, to the activation of mTOR. The mTOR pathway has multiple effects on cellular metabolism; collectively, these changes are essential to provide the raw materials needed to carry out the increased gene expression, protein production, and cell division that accompany T-cell activation. Specifically, mTOR activation leads to increased lipid production, ribosome biosynthesis, mRNA synthesis, and protein translation.

7-18 T-cell receptor signaling leads to enhanced integrin-mediated cell adhesion.

The third signaling module induced by TCR stimulation leads to enhanced integrin adhesion. Together with cytoskeletal changes (discussed in the next section), this process promotes stability of the T cell-APC interaction and localizes active signaling complexes into a structure known as the 'immune synapse' described below in Section 7-19 (and see Fig. 7.25). The immune synapse, the region of the T-cell membrane that is in direct and stable contact with the APC or target cell, is formed within minutes of T-cell receptor recognition of MHC/peptide ligands. One important component of this is increased adhesiveness of the T-cell integrin LFA-1. On nonstimulated T cells, LFA-1 resides in a low-affinity state and is well dispersed on the T-cell membrane, resulting in weak binding to its ligand, ICAM-1. Following T-cell receptor stimulation, LFA-1 molecules aggregate at the synapse, and also undergo a conformational change that converts each LFA-1 molecule into a high-affinity binding partner for ICAM-1. Together, these changes lead to enhanced adhesion between the T cell and the APC, and to stabilization of this cell-cell interaction. These effects on LFA-1 are induced by the recruitment of the adaptor protein ADAP to the LAT:Gads:SLP-76 scaffold complex (Fig. 7.23). In turn, ADAP recruits two additional proteins, SKAP55 and RIAM. The ADAP:SKAP55:RIAM complex binds to the small GTPase Rap1, activating Rap1 at the site of T-cell receptor signaling. GTP-bound Rap1 then promotes LFA-1 aggregation and the conformational change that converts LFA-1 into a high-affinity binding partner for ICAM-1. The importance of this pathway is underscored by the finding that ADAP-deficient T cells show impaired proliferation and cytokine production following T-cell receptor stimulation.

Fig. 7.23 Recruitment of ADAP to the LAT:Gads:SLP-76 complex activates integrin adhesion and aggregation. Left panel: prior to T-cell receptor (TCR) signaling, the integrin LFA-1 is present on the T-cell membrane in a low-affinity conformation that binds weakly to ICAM-1 on antigen-presenting-cells. Middle panel: following TCR signaling, the adaptor protein ADAP is recruited to the LAT:Gads:SLP-76 complex by an interaction between tyrosine-phosphorylated ADAP and the SH2 domain of SLP-76. ADAP then recruits a complex of SKAP and RIAM (Rap1-GTP-interacting adaptor molecule), activating the small GTPase Rap1. Right panel: active Rap1 induces aggregation of LFA-1 and a conformational change in LFA-1 that leads to high-affinity binding to ICAM-1.



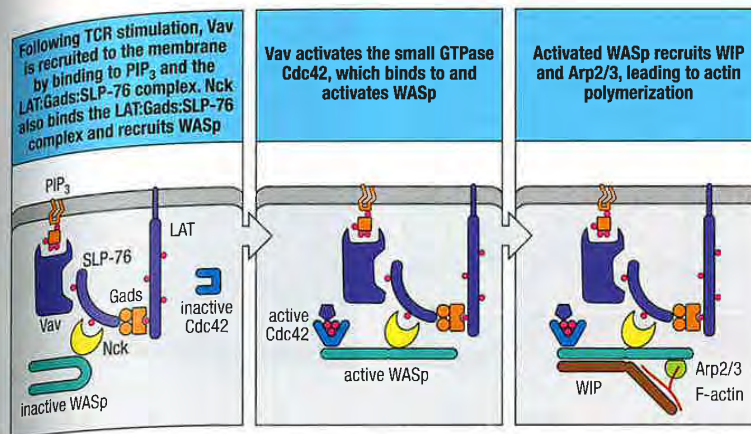


Fig. 7.24 Recruitment of Vav to the LAT:Gads:SLP-76 complex induces activation of Cdc42, leading to actin polymerization. Left panel: Vav, a guanine-nucleotide exchange factor (GEF) for the small GTPase Cdc42, is recruited to the activated T-cell receptor (TCR) complex by binding via its PH domain to PIP₃ in the membrane and by binding to phosphorylated SLP-76. The small adaptor protein Nck binds to an adjacent phosphorylated tyrosine on SLP-76 and recruits the inactive form of the protein WASp. Middle panel: Vav activates Cdc42, which binds to and activates WASp. Right panel: active WASp binds to WIP, recruiting Arp2/3 and inducing actin polymerization. The importance of this pathway is illustrated by the discovery of WASp as the protein encoded by the gene responsible for the human immunodeficiency disease *Wiskott-Aldrich syndrome*.

7-19 T-cell receptor signaling induces cytoskeletal reorganization by activating the small GTPase Cdc42.

The fourth TCR signaling module, also involved in the formation of a stable immune synapse, leads to reorganization of the actin cytoskeleton. Without this process, integrin aggregation would not occur, interactions between the T cell and the APC would not stabilize, and in fact, T-cell activation would completely fail. A major component of this T-cell receptor signal is transduced by Vav, a GEF that activates Rho-family GTPases, including Cdc42. Like PLC- γ and Itk, Vav is recruited to the site of receptor activation by interactions of the Vav PH domain with PIP₃ and of the Vav SH2 domain with the LAT:Gads:SLP-76 scaffold complex (Fig. 7.24). When Cdc42 is activated by Vav, the GTP-bound Cdc42 induces a conformational change in the protein **WASp** (**Wiskott-Aldrich syndrome** protein), which is also recruited to the LAT:Gads:SLP-76 scaffold complex by binding to the small adaptor protein Nck. This active form of WASp binds to WIP, and together, these proteins recruit Arp2/3, leading to actin polymerization. The importance of this pathway is underscored by the fact that defects in WASp are the basis for the immunodeficiency disease Wiskott-Aldrich syndrome. Due to the widespread expression of WASp, individuals suffering from this disease have impairments in multiple immune cell types, all of which depend on WASp-dependent actin polymerization for their functions. One major defect in this disease is in T cell-dependent antibody responses, due to the requirement for actin polymerization to ensure effective interactions between CD4 T cells and B cells. Thus, the failure of WASp-deficient T cells to provide adequate 'help' to B cells most likely results from a defect in the formation of the immune synapse, which is normally required to ensure directed secretion of cytokines from the T cell onto the B-cell membrane (Fig. 7.25).



Wiskott-Aldrich Syndrome

7-20 The logic of B-cell receptor signaling is similar to that of T-cell receptor signaling, but some of the signaling components are specific to B cells.

There are many similarities between signaling from T-cell receptors and signaling from B-cell receptors. As with the T-cell receptor, the B-cell receptor is composed of antigen-specific chains associated with ITAM-containing signaling chains, in this case Ig α and Ig β (see Fig. 7.10). In B cells, three protein tyrosine kinases of the Src family—Fyn, Blk, and Lyn—are thought to be responsible for phosphorylation of the ITAMs (Fig. 7.26). These kinases associate with

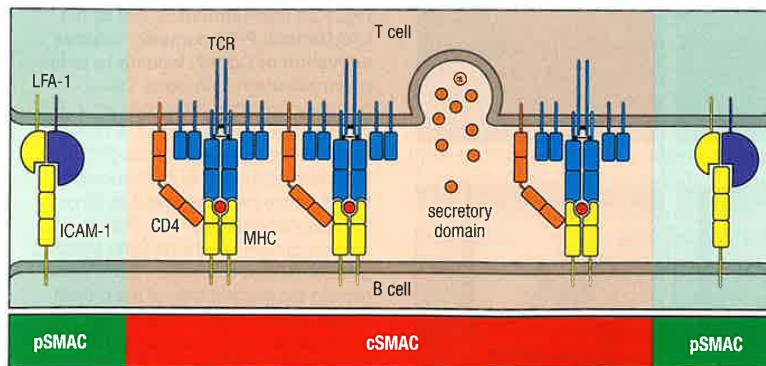
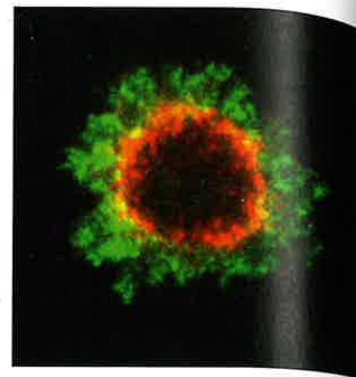
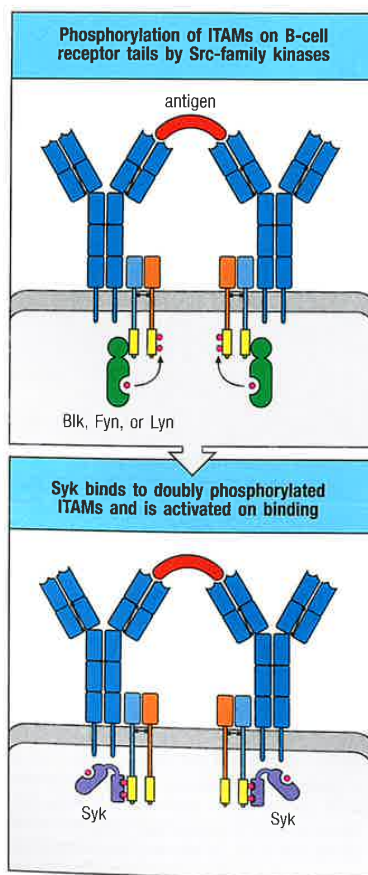


Fig. 7.25 The immune synapse provides a structure for directed secretion of T-cell cytokines. When the T-cell receptors (TCRs) on a T cell recognize peptide:MHC on an antigen-presenting cell, a process of receptor reorganization takes place on the plasma membranes of the two interacting cells. Left panel: when a CD4⁺ T cell recognizes its peptide:MHC ligand on a B cell, the immune synapse functions to direct T-cell-secreted cytokines onto the B-cell surface at the site of closest contact between the plasma membranes of the two cells. Right panel: confocal microscopy

images of the TCR/peptide:MHC (red) and the LFA-1/ICAM-1 (green) proteins 30 minutes after the initiation of signaling show a central accumulation of the TCR/peptide:MHC complexes and a peripheral ring of the LFA-1/ICAM-1 complexes. These structures have been called the central supermolecular activation complex (cSMAC, red) and the peripheral supermolecular activation complex (pSMAC, green). The combined structure is known as the immune synapse. Photograph courtesy of Y. Kaizuka.



resting receptors via a low-affinity interaction with unphosphorylated ITAMs in Ig α and Ig β . After the receptors have bound a multivalent antigen, which cross-links them, the receptor-associated kinases are activated and phosphorylate the tyrosine residues in the ITAMs. B cells do not express ZAP-70; instead, a closely related tyrosine kinase, **Syk**, containing two SH2 domains, is recruited to the phosphorylated ITAM. In contrast to ZAP-70, which requires additional Lck phosphorylation for activation, Syk is activated simply by its binding to the phosphorylated site.

For B cells, the co-receptor and co-stimulatory receptor functions are combined into one accessory receptor that is a complex of cell-surface proteins—**CD19**, **CD21**, and **CD81**—often referred to as the **B-cell co-receptor** (Fig. 7.27). As with T cells, antigen-dependent signaling from the B-cell receptor is enhanced if the B-cell co-receptor is simultaneously bound by its ligand and clusters with the antigen receptor. CD21 (also known as complement receptor 2, CR2) is a receptor for the C3dg fragment of complement. This means that antigens such as bacterial pathogens on which C3dg is bound (see Fig. 7.27) can cross-link the B-cell receptor with the CD21:CD19:CD81 complex. This induces phosphorylation of the cytoplasmic tail of CD19 by B-cell receptor-associated tyrosine kinases, which in turn leads to the binding of additional Src-family kinases, the augmentation of signaling through the B-cell receptor itself, and the recruitment of PI 3-kinase (see Section 7-4). PI 3-kinase

Fig. 7.26 Src-family kinases are associated with B-cell receptors and phosphorylate the tyrosines in ITAMs to create binding sites for Syk and Syk activation via transphosphorylation. The membrane-bound Src-family kinases Fyn, Blk, and Lyn associate with the B-cell antigen receptor by binding to ITAMs, either (as shown in the figure) through their amino-terminal domains or by binding a single phosphorylated tyrosine through their SH2 domains. After ligand binding and receptor clustering, the associated kinases phosphorylate tyrosines in the ITAMs on the cytoplasmic tails of Ig α and Ig β . Subsequently, Syk binds to the phosphorylated ITAMs of the Ig β chain. Because there are at least two receptor complexes in each cluster, Syk molecules become bound in close proximity and can activate each other by transphosphorylation, thus initiating further signaling.

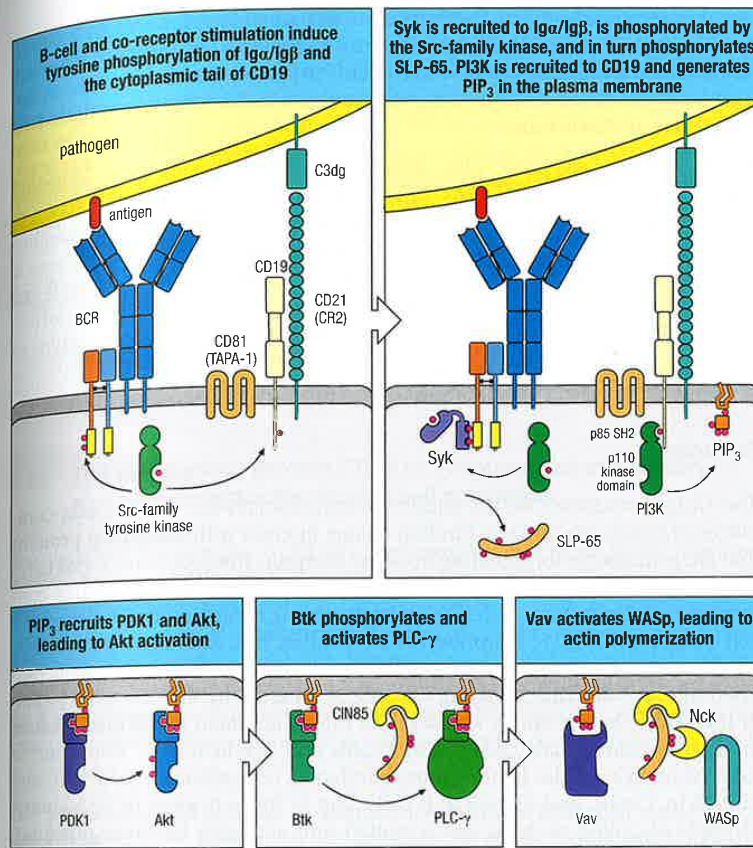


Fig. 7.27 B-cell antigen receptor plus co-receptor engagement activate downstream signaling modules leading to activation of Akt, PLC-γ, and WASp.

B-cell receptor (BCR) signaling is greatly enhanced when the antigen is tagged by complement fragments, engaging the B-cell co-receptor together with the B-cell antigen receptor. Cleavage of the antigen-bound complement component C3 to C3dg (see Fig. 2.30) allows the tagged antigen to bind to the cell-surface protein CD21 (complement receptor 2, CR2), a component of the B-cell co-receptor complex, which also includes CD19 and CD81 (TAPA-1). Cross-linking and clustering of the co-receptor with the antigen receptor result in the phosphorylation of tyrosine residues in the ITAM sequences of the cytoplasmic domains of the BCR signaling subunits, Igα and Igβ. The Src-family kinase also phosphorylates tyrosine residues in the cytoplasmic domain of CD19. The phosphorylated ITAMs in Igα and Igβ recruit and activate the tyrosine kinase Syk, which functions similarly to ZAP-70 in T cells. The phosphorylated tail of CD19 recruits PI 3-kinase, leading to PIP₃ generation in the plasma membrane. Activated Syk phosphorylates the membrane-associated scaffold protein SLP-65, which associates with the plasma membrane by binding CIN85. PIP₃ recruits PDK1 and Akt, leading to Akt activation. Phosphorylated SLP-65 and PIP₃ recruit the Tec-family tyrosine kinase Btk and PLC-γ, leading to Btk phosphorylation and activation of PLC-γ. Phosphorylated SLP-65 and PIP₃ also recruit Vav, Nck, and inactive WASp. Vav activates small GTPases that activate WASp, leading to actin polymerization; the activated GTPases also induce integrin aggregation and conversion of LFA-1 to the high-affinity binding state.



X-linked Agammaglobulinemia

initiates an additional signaling pathway leading from the B-cell receptor (see Fig. 7.27). Thus, the B-cell co-receptor serves to strengthen the signal resulting from antigen recognition. The role of the third component of the B-cell receptor complex, CD81 (TAPA-1), is as yet unknown.

Once activated, Syk phosphorylates the scaffold protein **SLP-65** (also known as **BLNK**). Like LAT and SLP-76 in T cells, SLP-65 functions as a composite of these two proteins, providing multiple sites for tyrosine phosphorylation and recruiting a variety of SH2-containing proteins, including enzymes and adaptor proteins, to form several distinct multiprotein signaling complexes that can act in concert. As in T cells, a key signaling protein is the phospholipase PLC-γ, which is activated with the aid of the B cell-specific Tec kinase **Bruton's tyrosine kinase (Btk)** and hydrolyzes PIP₂ to form DAG and IP₃ (see Fig. 7.27). As discussed for the T-cell receptor, signaling by calcium and DAG leads to the activation of downstream transcription factors. A deficiency in Btk (which is encoded by a gene on the X chromosome) prevents the development and functioning of B cells, resulting in the disease **X-linked agammaglobulinemia**, which is characterized by a lack of antibodies. Besides Btk, mutations in other signaling molecules in B cells, including receptor chains and SLP-65, have been linked to B-cell immunodeficiencies (see Chapter 8).

Several other downstream pathways described for TCR signaling are also shared with BCR signaling, and are dependent on the adaptor protein SLP-65. These include the Vav-dependent induction of actin polymerization by Cdc42 and WASp, and the recruitment and activation of small GTPases that promote integrin adhesion (see Fig. 7.27). In the case of B-cell recognition of

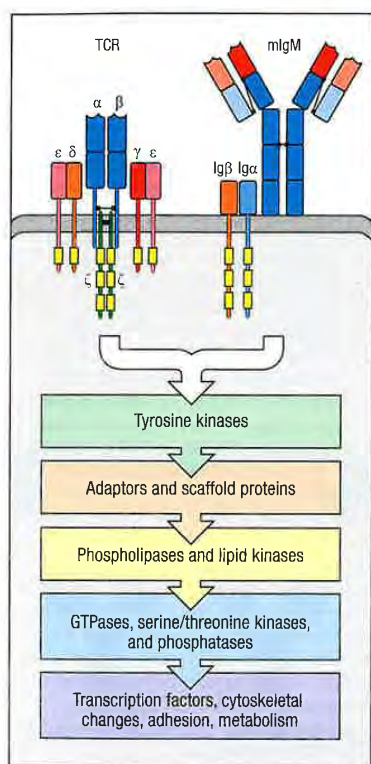


Fig. 7.28 Summary of antigen receptor signaling pathways. As outlined in this section, the signal transduction pathways downstream of the T-cell and B-cell receptors occur in an orchestrated series of stages involving many categories of proteins that produce widespread changes in the cells. The first detectable events following antigen receptor stimulation are the activation of tyrosine kinases. Following this, adaptor proteins and scaffolds are modified, recruiting phospholipases and lipid kinases to the activated receptor complexes. The next level of signaling amplifies these earlier stages by activating multiple small GTPases, serine/threonine kinases, and protein phosphatases. Together, these lead to transcription factor activation, cytoskeletal changes, and increases in cellular adhesion and metabolism, all of which contribute to T- and B-cell activation.

membrane-bound antigen, B-cell receptor signaling also produces an immune synapse that localizes signaling complexes to the cell-cell interface. One key function of the immune synapse in B cells is to promote antigen uptake by the B cell, a prerequisite to presenting that antigen in the form of peptide:MHC complexes to CD4 T cells.

B-cell receptor signaling also induces metabolic changes in activated B cells. As is the case for T cells, this response is dependent on the action of PI 3-kinase, whose activation leads to formation of the membrane phosphoinositide $PI(3,4,5)P_3$ at the site of the activated B-cell receptor. This response is augmented by combined signaling through the B-cell receptor plus the B-cell co-receptor complex of CD19/CD21/CD81. $PI(3,4,5)P_3$ recruits Akt, which is then phosphorylated and activated, leading to downstream activation of mTOR, as well as additional Akt-dependent pathways promoting cell survival and proliferation (see Fig. 7.27).

Summary.

The antigen receptors on the surface of lymphocytes are multiprotein complexes in which the antigen-binding chains interact with additional proteins that are responsible for signaling from the receptor. These protein chains carry tyrosine-containing signaling motifs known as ITAMs. Signaling chains containing ITAM motifs are widely used by activating receptors in many immune cell types in addition to lymphocytes. In lymphocytes, activation of the receptors by antigen results in a series of biochemical events that are broadly outlined in Fig. 7.28. This signaling cascade is initiated by the phosphorylation of the ITAMs by Src-family kinases. The phosphorylated ITAM then recruits another tyrosine kinase, ZAP-70 in T cells and Syk in B cells. Activation of ZAP-70 or Syk results in the phosphorylation of scaffolds called LAT and SLP-76 in T cells, and SLP-65 in B cells, and in the activation of PI 3-kinase. Multiple signaling proteins are recruited and activated by these phosphorylated scaffolds, including phospholipase C- γ , ADAP, and Vav, whereas Akt is recruited and activated by the action of PI 3-kinase generating PIP_3 at the plasma membrane. PLC- γ generates inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 has an important role in inducing changes in intracellular calcium concentrations, and DAG is involved in activating protein kinase C- θ and the small G protein Ras. In T cells, these pathways ultimately result in the activation of three transcription factors, namely, AP-1, NFAT, and NF- κ B; together, these transcription factors induce transcription of the cytokine IL-2, which is essential for the proliferation and further differentiation of the activated lymphocyte. In addition to transcription factor activation, antigen receptor signaling in both T cells and B cells leads to enhanced cell survival, metabolic activity, adhesiveness, and cytoskeletal reorganization. Signaling by antigen receptors is facilitated by co-receptors that become engaged as a result of receptor-antigen binding. These co-receptors are the MHC-binding CD4 and CD8 transmembrane proteins in T cells and the complement-binding B-cell co-receptor complex containing CD19 in B cells.

Co-stimulatory and inhibitory receptors modulate antigen receptor signaling in T and B lymphocytes.

Signals initiated by the T-cell and B-cell antigen receptors are essential for lymphocyte activation, and determine the specificity of the adaptive immune response that is initiated. However, signaling from the antigen receptor is

not on its own sufficient to activate a naive T cell or B cell. These naive lymphocytes require additional signals to achieve full activation. Receptors on T cells and B cells that can provide this necessary second signal are called **co-stimulatory receptors**, and are members of either the CD28 family of proteins or of the TNF receptor superfamily. While naive T cells primarily utilize CD28 as the co-stimulatory receptor, naive B cells use the TNF receptor family member CD40. The overall function of signaling through these co-stimulatory receptors is to enhance the antigen receptor signals that induce transcription factor activation and PI 3-kinase activation, thereby ensuring activation of the T cell or B cell. In contrast to these activating co-stimulatory receptor signals, other cell-surface receptors on T cells and B cells function to downregulate activation signals. These inhibitory receptors are important in preventing excessive immune responses that can lead to destructive inflammatory or autoimmune conditions, particularly in the case of chronic infections that are inefficiently controlled by the immune system.

7-21 The cell-surface protein CD28 is a required co-stimulatory signaling receptor for naive T-cell activation.

The signaling through the T-cell receptor complex described in the previous sections is not by itself sufficient to activate a naive T cell. As noted in Chapter 1, antigen-presenting cells that can activate naive T cells bear cell-surface proteins known as **co-stimulatory molecules** or co-stimulatory ligands. These interact with cell-surface receptors, known as co-stimulatory receptors, on the naive T cell to transmit a signal that is required, along with antigen stimulation, for T-cell activation—this signal is sometimes called ‘signal 2.’ We discuss the immunological consequences of this requirement for co-stimulation in detail in Chapter 9.

The best understood of these co-stimulatory receptors is the cell-surface protein **CD28**. CD28 is present on the surface of all naive T cells and binds the co-stimulatory ligands **B7.1 (CD80)** and **B7.2 (CD86)**, which are expressed mainly on specialized antigen-presenting cells such as dendritic cells. To become activated, the naive lymphocyte must engage both antigen and a co-stimulatory ligand on the same antigen-presenting cell. CD28 signaling aids antigen-dependent T-cell activation mainly by promoting T-cell proliferation, cytokine production, and cell survival. All these effects are mediated by signaling motifs present in the cytoplasmic domain of CD28.

After engagement by B7 molecules, CD28 becomes tyrosine phosphorylated by Lck in its cytoplasmic domain on tyrosine residues in a YXN motif that can recruit the adaptor protein Grb2, and in a non-ITAM motif YMN. The cytoplasmic tail of CD28 also carries a proline-rich motif (PXXP) that binds the SH3 domains of Lck and Itk. Although the details are uncertain, a major effect of CD28 phosphorylation is to activate PI 3-kinase to generate PIP₃ (Fig. 7.29). By this mechanism, the co-stimulatory signal induced by CD28 cooperates with the T-cell receptor signal to ensure maximal activation of three of the four T-cell receptor signaling modules described above. Specifically, a high concentration of PIP₃ recruits Itk to the membrane, where Lck can phosphorylate it, thereby enhancing PLC-γ activation. PIP₃ also functions to recruit and activate Akt, which promotes cell survival and increased cellular metabolism (see Section 7.17). An additional function of Akt is to phosphorylate the RNA-binding protein NF-90; when phosphorylated, NF-90 translocates from the nucleus to the cytoplasm and binds to and stabilizes the IL-2 mRNA, leading to increased IL-2 synthesis. Finally, PIP₃ recruits Vav, leading to cytoskeletal reorganization (see Section 7.19). Thus, co-stimulatory signaling through CD28 functions to amplify most of the downstream responses to T-cell receptor stimulation (see Fig. 7.29).



MOVIE 7.3

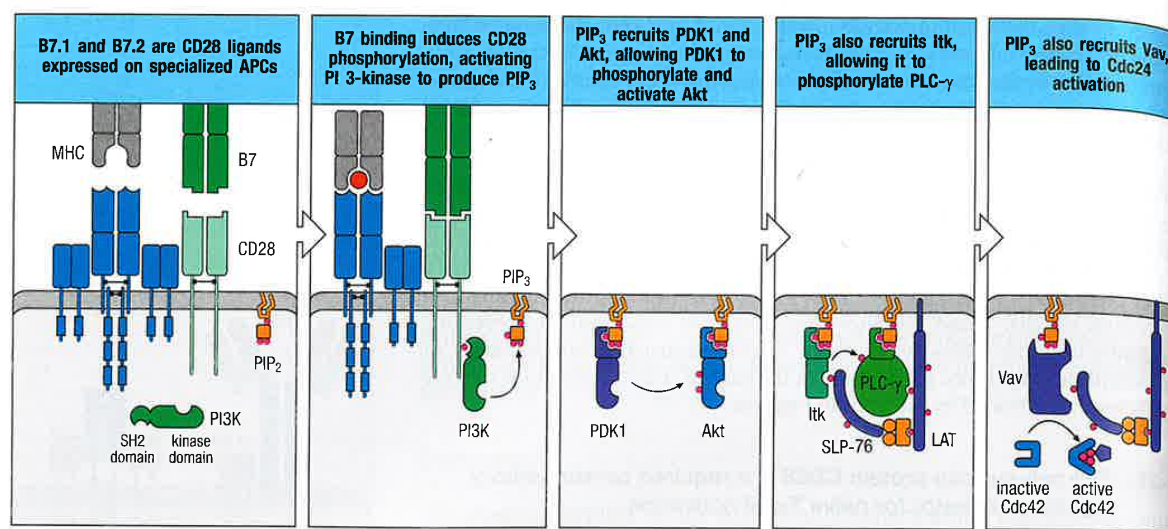


Fig. 7.29 The T-cell co-stimulatory protein CD28 transduces signals that enhance antigen receptor signaling pathways. The ligands for CD28, namely B7.1 and B7.2, are expressed only on specialized antigen-presenting cells (APCs) such as dendritic cells (first panel). Engagement of CD28 induces its tyrosine phosphorylation, which activates PI 3-kinase (PI3K), with subsequent production of PIP_3 that recruits several enzymes via their PH domains, thus bringing them together with their substrates in the membrane. The protein kinase Akt, which becomes phosphorylated by phosphoinositide-dependent protein kinase-1 (PDK1), is activated and enhances cell survival and upregulates cell metabolism (see Fig. 7.22). Recruitment of the kinase Itk to the membrane is critical for the full activation of PLC- γ (see Fig. 7.16). PIP_3 also recruits Vav, leading to Cdc42 activation and inducing actin polymerization (see Fig. 7.24).

7-22 Maximal activation of PLC- γ , which is important for transcription factor activation, requires a co-stimulatory signal induced by CD28.

One important function of co-stimulatory signaling through CD28 is to promote the maximal activation of PLC- γ via the local production of PIP_3 . This recruits Itk by its PH domain, enhancing Itk phosphorylation by Lck. Activated Itk is then recruited to the phosphorylated LAT:Gads:SLP-76 complex by its SH2 and SH3 domains binding to SLP-76, where it phosphorylates and activates PLC- γ (see Fig. 7.16). Activated PLC- γ cleaves PIP_2 to produce the two second messengers DAG and IP_3 , ultimately leading to the activation of transcription factors NFAT, AP-1, and NF κ B. Thus, the full activation of PLC- γ leading to transcription factor activation requires signals emanating from both the T-cell receptor and CD28.

In T cells, one of the major functions of NFAT, AP-1, and NF κ B is to act together to stimulate expression of the gene for the cytokine IL-2, which is essential for promoting T-cell proliferation and differentiation into effector cells. The promoter for the *IL-2* gene contains multiple regulatory elements that must be bound by transcription factors to initiate *IL-2* expression. Some control sites are already bound by transcription factors, such as Oct1, that are produced constitutively in lymphocytes, but this is not sufficient to switch on *IL-2*. Only when AP-1, NFAT, and NF κ B are all activated and are bound to their control sites in the *IL-2* promoter is the gene expressed. NFAT and AP-1 bind to the promoter cooperatively and with higher affinity by forming a heterotrimer of NFAT, Jun, and Fos. In addition, CD28 co-stimulation further enhances *IL-2* transcription by increasing NF κ B activation. Thus, the *IL-2* promoter integrates signals from both the T-cell receptor and CD28 signaling pathways to ensure that IL-2 is produced only in appropriate circumstances (Fig. 7.30). Together with the CD28-induced phosphorylation of NF-90 leading to increased IL-2 mRNA stability, CD28 co-stimulation leads to substantially increased production of IL-2 by activated T cells.

7-23 TNF receptor superfamily members augment T-cell and B-cell activation.

While naive T- and B-cell activation requires signaling through the antigen receptors on these cells, T-cell receptor or B-cell receptor signaling

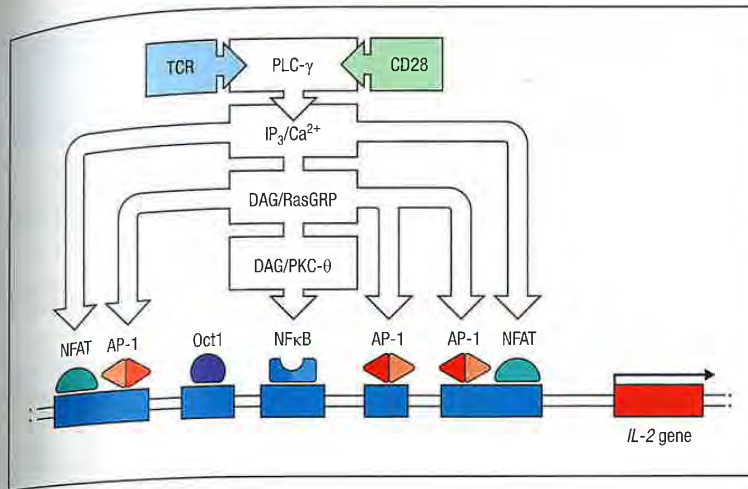


Fig. 7.30 Simplified scheme depicting multiple signaling pathways that converge on the *IL-2* promoter.

AP-1, NFAT, and NFκB binding to the promoter of the *IL-2* gene integrate multiple signaling pathways emanating from the T-cell receptor (TCR) and CD28 into a single output, the production of the cytokine *IL-2*. The MAPK pathway activates AP-1; calcium activates NFAT; and protein kinase C activates NFκB. All three pathways are required to stimulate *IL-2* transcription. Activation of the gene requires both the binding of NFAT and AP-1 to a specific promoter element, and the additional binding of AP-1 on its own to another site. Oct1 is a transcription factor that is required for *IL-2* transcription. Unlike the other transcription factors, it is constitutively bound to the promoter and is therefore not regulated by T-cell receptor or CD28 signaling.

respectively, is not sufficient to activate the cells. For naive T cells, an additional co-stimulatory signal is required and, as discussed above (see Sections 7-21 and 7-22), is frequently provided by the CD28 receptor. For naive B cells, the additional activation signal can be contributed by direct interactions between the pathogen and an innate pattern recognition receptor (PRR), such as a TLR, on the B cell. However, for more effective B-cell activation leading to the production of all classes of antibodies and to the formation of memory B cells, additional B-cell activation signals are contributed by CD4 T cells. One component of this is the production of T-cell cytokines, which bind to and stimulate their receptors on the B-cell surface (see Chapter 10). The second and more essential component provided by the CD4 T cells is the stimulation of CD40 on the B cell by the CD40 ligand expressed on the T cell. The importance of the CD40-CD40-ligand interaction for B-cell responses to protein antigens is highlighted by the discovery that a severe immunodeficiency disease resulting from impaired antibody responses is caused by the absence of CD40 ligand expression on a patient's CD4 T cells.

CD40 is a member of the large TNF receptor superfamily, which consists of more than 20 members. While some members of this family, such as Fas (see Chapter 11), are specialized to induce cell death, the majority of TNF receptor superfamily members, including CD40, activate both the NFκB and the PI 3-kinase pathways following receptor stimulation (Fig. 7.31). While NFκB activation leads to enhanced cell survival, the PI 3-kinase pathway has widespread and pleiotropic effects on B-cell physiology, and is a central feature of CD40 signaling. The major mediator of the PI 3-kinase signal is the serine/threonine kinase Akt, which is recruited and activated following the generation of PI(3,4,5)P₃ at the B-cell membrane. Akt then stimulates multiple downstream pathways that induce cell survival, cell cycle progression, glucose uptake and metabolism, and mTOR activation, all of which are essential for the productive response of the activated B cell. In general terms, CD40 on the B cell functions in a manner analogous to CD28 on the T cell, as both receptors serve to enhance the levels of Akt activation induced by the B-cell receptor or T-cell receptor signaling pathways, respectively.

TNF receptor superfamily members, including CD40, signal by a mechanism distinct from that of antigen receptors, as it does not involve the activation of tyrosine kinases. Instead, stimulation of TNF receptors recruits adaptor proteins known as TRAFs (TNF receptor-associated factors). In addition to serving as simple adaptors that promote the assembly of multiprotein complexes, five



CD40 Ligand Deficiency

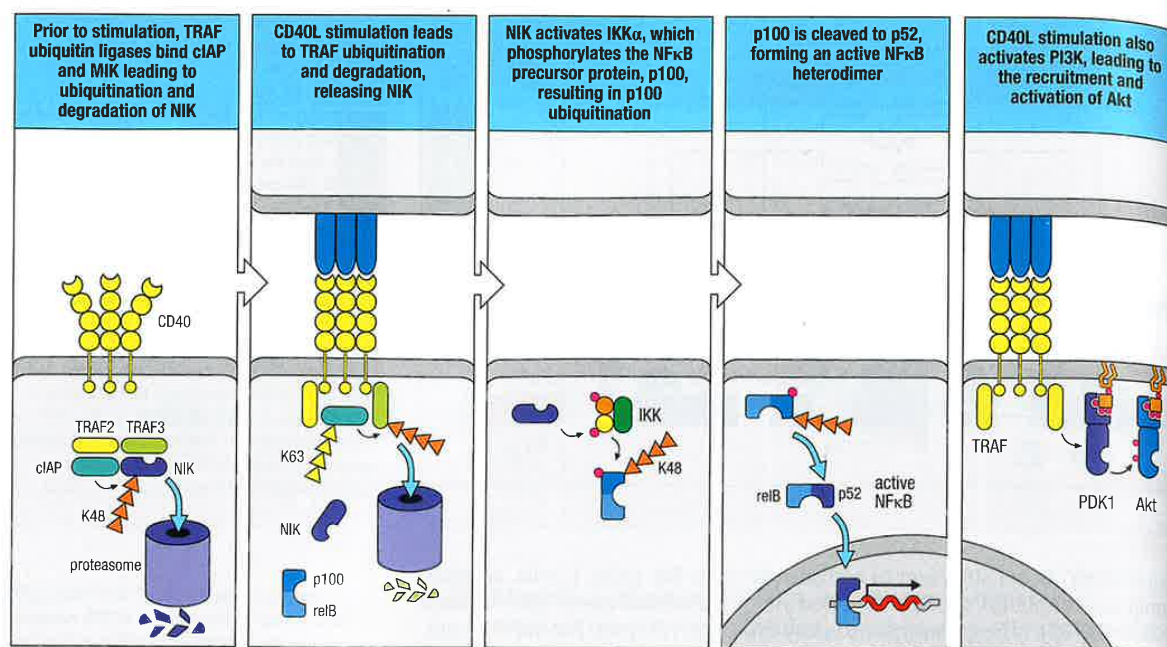


Fig. 7.31 The TNF receptor superfamily member CD40 is an important co-stimulatory molecule on B cells. Several members of the TNF receptor superfamily are expressed on T cells and B cells. A key function of these receptors is the activation of NF κ B, which occurs by a pathway distinct from the one initiated by antigen receptor stimulation and is often referred to as the non-canonical NF κ B pathway. TNF receptor superfamily members also activate PI 3-kinase signaling pathways. One important TNF receptor superfamily member on B cells is CD40. Prior to stimulation, TRAF molecules, which are ubiquitin ligases, are associated with cIAP, another ubiquitin ligase, and the NF κ B-inducing kinase NIK. Under these steady-state conditions, TRAF binding promotes ubiquitination and degradation of NIK. When CD40 is stimulated by binding to CD40L, this complex is recruited to the intracellular domain of CD40. TRAF2 catalyzes K63-linked ubiquitination of cIAP, leading to cIAP-mediated K48-linked ubiquitination of TRAF3. This leads to TRAF3 degradation, releasing NIK, and allowing NIK to phosphorylate and activate I κ B kinase α (IKK α). IKK α phosphorylates the NF κ B precursor protein p100, inducing its cleavage to form the active p52 subunit, which binds to relB to form the active NF κ B transcription factor. CD40L stimulation of CD40 also activates PI 3-kinase, which leads to the activation of Akt by PDK1.

of the six known TRAFs also function as E3 ubiquitin ligases. This activity contributes to the ability of most TNF receptor superfamily members to activate the NF κ B pathway, using a pathway distinct from the one initiated by antigen receptor stimulation and often referred to as the **non-canonical NF κ B pathway** (see Fig. 7.31). In contrast, the precise biochemical mechanism by which TNF receptors and TRAFs induce PI 3-kinase activation is not yet known.

CD40 is constitutively expressed on B cells and functions during B-cell activation in response to antigen recognition by the B-cell receptor. Additional TNF receptor superfamily members are also expressed on B cells, and each of them is important in B-cell survival at a particular stage of B-cell maturation, including B cells that have differentiated into antibody-secreting cells or memory cells. Similarly, TNF receptor superfamily members are expressed on T cells, many of which are upregulated following T-cell activation. These molecules, such as OX40, 4-1BB, CD30, and CD27, contribute important survival signals and function to enhance cellular metabolism at later stages of the T-cell response to infection (see Chapter 11).

7-24 Inhibitory receptors on lymphocytes downregulate immune responses by interfering with co-stimulatory signaling pathways.

CD28 belongs to a family of structurally related receptors that are expressed by lymphocytes and bind B7-family ligands. Some, such as the receptor ICOS, which is discussed in Chapter 9, act as activating receptors, but others inhibit signaling by the antigen receptors, can stimulate apoptosis, and are important in regulating the immune response. Inhibitory receptors related to CD28 and expressed by T cells include **CTLA-4** (CD152) and **PD-1** (**programmed death-1**), while the **B and T lymphocyte attenuator** (BTLA) is expressed by both T cells and B cells. Of these, CTLA-4 seems to be the most important: mice lacking CTLA-4 die at a young age from an uncontrolled proliferation of T cells in multiple organs, whereas loss of PD-1 or BTLA causes less marked changes

that alter the magnitude of responses following lymphocyte activation, rather than causing widespread spontaneous lymphoproliferation. Both CTLA-4 and PD-1 have been targeted for the development of protein-based therapeutics that function to block the activities of these receptors. The goal of these therapeutics is to enhance T-cell responses by inhibiting these inhibitory receptors, a therapeutic strategy referred to as **checkpoint blockade** (see Chapter 16). Recent clinical trials demonstrate that both CTLA-4 and PD-1 blockade have remarkable efficacy in the treatment of cancer by enhancing the patient's own antitumor T-cell responses.

CTLA-4 is induced on activated T cells and binds to the same co-stimulatory ligands (B7.1 and B7.2) as CD28, but CTLA-4 engagement is inhibitory for T-cell activation, rather than enhancing (Fig. 7.32). The function of CTLA-4 is controlled largely by regulation of its surface expression. Initially, CTLA-4 resides on intracellular membranes but moves to the cell surface after T-cell receptor signaling. The surface expression of CTLA-4 is controlled by phosphorylation of the tyrosine-based motif GVYVKM in its cytoplasmic tail. When this motif is not phosphorylated, it is able to bind to the clathrin adaptor molecule AP-2, which removes CTLA-4 from the surface. When it is phosphorylated, this motif cannot bind AP-2, and CTLA-4 remains in the membrane, where it can bind B7 molecules on antigen-presenting cells.

CTLA-4 has a higher affinity for its B7 ligands than does CD28, and, apparently of importance for its inhibitory function, it engages B7 molecules in a different orientation. CD28, CTLA-4, and B7.1 are all expressed as homodimers. A CD28 dimer engages one B7.1 dimer in a direct one-to-one correspondence, but a CTLA-4 dimer engages two different B7 dimers in a configuration that allows for extended cross-linkages that confer high avidity to the interaction (see Fig. 7.32). CTLA-4 was once presumed to act by recruiting inhibitory phosphatases, like some of the other inhibitory receptors described later, but this is no longer thought to be so. It is still not clear whether CTLA-4 directly activates inhibitory signaling pathways. Instead, its actions may result in part from blocking the binding of CD28 to B7, thereby reducing CD28-dependent co-stimulation.

CTLA-4-expressing T cells can also exert an inhibitory effect on the activation of other T cells. How they do this is not yet clear, but it might result from CTLA-4 binding to B7 molecules on antigen-presenting cells, in effect stealing the ligand for CD28 required by the other T cells. Direct actions of CTLA-4 on T cells have not been excluded, however. Notably, the regulatory T cells that are needed to suppress autoimmunity express high levels of CTLA-4 on their surface, and they require CTLA-4 to function normally. Regulatory cells are described in detail in Chapter 9.

7-25 Inhibitory receptors on lymphocytes downregulate immune responses by recruiting protein or lipid phosphatases.

Some other receptors that can inhibit lymphocyte activation possess motifs in their cytoplasmic regions that are known as the **immunoreceptor tyrosine-based inhibitory motif (ITIM)**, consensus sequence [I/V]XYXX[L/I], where X is any amino acid (Fig. 7.33) or the related **immunoreceptor tyrosine-based switch motif (ITSM)**, consensus sequence TXYXX[V/I]). When the tyrosine in an ITIM or ITSM is phosphorylated, it can recruit either of two inhibitory phosphatases, called **SHP** (SH2-containing phosphatase) and **SHIP** (SH2-containing inositol phosphatase), via their SH2 domains. SHP is a protein tyrosine phosphatase that removes phosphate groups added by tyrosine kinases to a variety of proteins. SHIP is an inositol phosphatase and removes the phosphate from PIP_3 to generate PIP_2 , thus reversing the recruitment of proteins such as Tec kinases and Akt to the cell membrane and thereby inhibiting signaling.

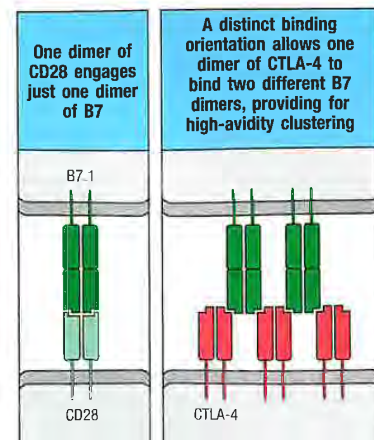


Fig. 7.32 CTLA-4 has a higher affinity than CD28 for B7 and engages it in a multivalent orientation. CD28 and CTLA-4 are both expressed as dimers on the cell surface and both bind to two ligands of B7.1, which is a dimer, and B7.2, which is not. However, the orientations of the B7 binding of CD28 and of CTLA-4 differ in a way that contributes to the inhibitory action of CTLA-4. One dimer of CD28 engages just one dimer of B7.1. But one dimer of CTLA-4 binds in such a way that two different dimers of B7.1 are engaged at once, allowing these molecules to cluster into complexes of high avidity. This, and the higher affinity of CTLA-4 for B7 molecules, may give it an advantage in competing for available B7 molecules on an antigen-presenting cell, providing one mechanism by which it could block the co-stimulation of T cells.

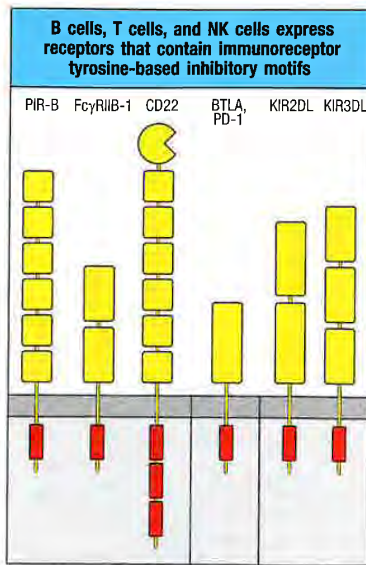


Fig. 7.33 Some lymphocyte cell-surface receptors contain motifs involved in downregulating activation. Several receptors that transduce signals that inhibit lymphocyte or NK-cell activation contain one or more ITIMs (immunoreceptor tyrosine-based inhibitory motifs) in their cytoplasmic tails (red rectangles). ITIMs bind to various phosphatases that, when activated, inhibit signals derived from ITAM-containing receptors.

One ITIM-containing receptor is PD-1 (see Fig. 7.33), which is induced transiently on activated T cells, B cells, and myeloid cells. It can bind to the B7-family ligands **PD-L1** (programmed death ligand-1, B7-H1) and **PD-L2** (programmed death ligand-2, B7-DC). Despite their names, we now understand that these proteins function as ligands for the inhibitory receptor PD-1, rather than acting directly in cell death. PD-L1 is constitutively expressed by a wide variety of cells, whereas PD-L2 expression is induced on antigen-presenting cells during inflammation. Because PD-L1 is expressed constitutively, regulation of PD-1 expression could have a critical role in controlling T-cell responses. For example, signaling by pro-inflammatory cytokines can repress PD-1, thus enhancing the T-cell response. Mice lacking PD-1 gradually develop autoimmunity, presumably because of an inability to regulate T-cell activation. In chronic infections, the widespread expression of PD-1 reduces the effector activity of T cells; this helps to limit potential damage to bystander cells, but at the expense of pathogen clearance.

BTLA contains an ITIM and an ITSM and is expressed on activated T cells and B cells, as well as on some cells of the innate immune system. Unlike other CD28-family members, however, BTLA does not interact with B7 ligands but binds a member of the TNF receptor family; called the **herpesvirus entry molecule (HVEM)**, this receptor is highly expressed on resting T cells and immature dendritic cells. When BTLA and HVEM are co-expressed on the same cell, BTLA utilizes a second mechanism that further inhibits lymphocyte activation. In this configuration, BTLA binds to HVEM and prevents HVEM from binding to alternative partners that would stimulate NFκB-dependent pro-survival signaling pathways downstream of HVEM. Alternatively, when BTLA and HVEM are expressed on different cells, the interaction of these two receptors functions to stimulate the positive pro-survival signal in the HVEM-expressing cell.

Other receptors on B cells and T cells also contain ITIMs and can inhibit cell activation when ligated along with the antigen receptors. One example is the receptor **FcγRIIB-1** on B cells, which binds the Fc region of IgG antibodies. As a result, antigens present as immune complexes containing IgG antibodies are poor at activating naive B cells, due to the co-engagement of the B-cell receptor with this inhibitory Fc receptor. The ITIM in FcγRIIB-1 recruits SHIP into a complex with the B-cell receptor to block the actions of PI 3-kinase (Fig. 7.34). Another inhibitory receptor on B cells is **CD22**, a transmembrane protein that recognizes sialic acid-modified glycoproteins commonly found on mammalian cells but rarely on microbial pathogen surfaces. CD22 contains an ITIM that interacts with SHP, a phosphatase that can dephosphorylate adaptors such as SLP-65 that associate with CD22, thereby inhibiting signaling from the B-cell receptor.

The ITIM motif is also an important motif in signaling by receptors on NK cells that inhibit the killer activity of these cells (see Section 3-26). These inhibitory receptors recognize MHC class I molecules and transmit signals that inhibit the release of the NK cell's cytotoxic granules when the NK cell recognizes a healthy uninfected cell. In NK cells, ITIM-containing receptors play an important role in setting the threshold for NK cell activation by balancing positive signals from ITAM-containing receptors.

Summary.

Signaling through the antigen receptors on T cells and B cells is essential for the activation of these cells. However, for naive T and B cells, the signal through the T-cell receptor or B-cell receptor, respectively, is not sufficient to initiate a response. In addition to the antigen receptor signals, these cells require signals through accessory receptors that serve to monitor the environment of the cell to ensure the presence of an infection. An important secondary signaling

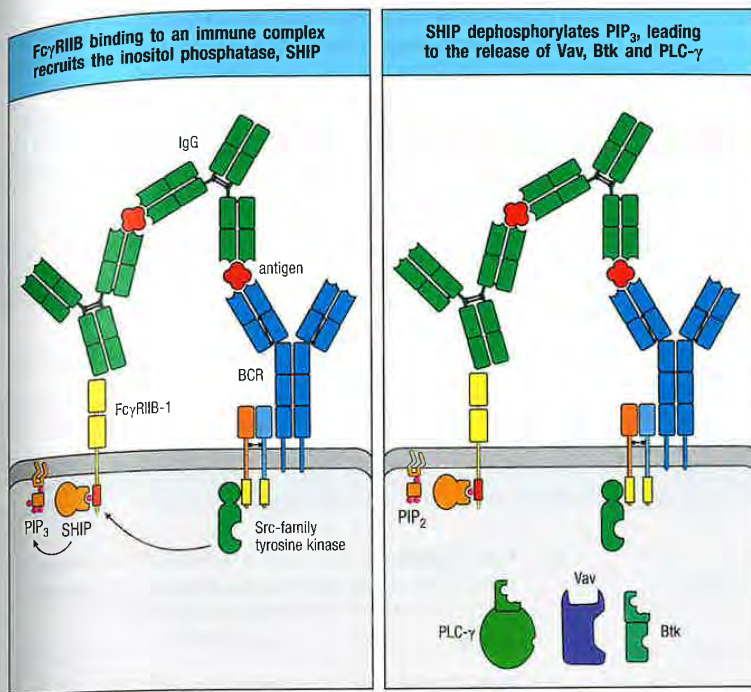


Fig. 7.34 The ITIM-containing Fc receptor inhibits B-cell receptor signaling by recruiting the inositol phosphatase SHIP. When the B-cell receptor binds an antigen that is already present in immune complexes with IgG, the ITIM-containing Fc receptor FcγRIIB is engaged at the same time as the B-cell receptor. The Src-family kinase present at the B-cell receptor (BCR) phosphorylates the ITIM motif of FcγRIIB, which then recruits the SH2 domain-containing inositol phosphatase SHIP. SHIP dephosphorylates PIP₃ in the plasma membrane, generating PIP₂. PH domain-containing enzymes, such as Vav, Btk, and PLC-γ, depend on their PH domain binding to PIP₃ for their stable recruitment to the activated B-cell receptor complex. The loss of PIP₃ terminates the recruitment of these enzymes and inhibits B-cell receptor signaling.

system in naive T cells is provided by the CD28 family of co-stimulatory proteins, which bind members of the B7 family of proteins. Activating members of the CD28 family provide co-stimulatory signals that amplify the signal from the T-cell receptor and are important in ensuring the activation of naive T cells by the appropriate target cell. In B cells, these secondary signals are provided by members of the TNF receptor superfamily, such as CD40. Inhibitory members of the CD28 and other receptor families function to attenuate or completely block signaling by activating receptors. The regulated expression of activating and inhibitory receptors and their ligands generates a sophisticated level of control of immune responses that is only beginning to be understood.

Summary to Chapter 7.

Signaling by cell-surface receptors of many different sorts is crucial to the ability of the immune system to respond appropriately to foreign pathogens. The importance of these signaling pathways is demonstrated by the many diseases that are due to aberrant signaling, which include both immunodeficiency diseases and autoimmune diseases. Common features of many signaling pathways are the generation of second messengers such as calcium and phosphoinositides and the activation of both serine/threonine and tyrosine kinases. An important concept in the initiation of signaling pathways by receptor proteins is the recruitment of signaling proteins to the plasma membrane and the assembly of multiprotein signaling complexes. In many cases, signal transduction leads to the activation of transcription factors that lead directly or indirectly to the proliferation, differentiation, and effector function of activated lymphocytes. Other roles of signal transduction are to mediate changes in the cytoskeleton that are important for cell functions such as migration and shape changes. These steps of the T-cell and B-cell receptor signaling pathways are summarized in Fig. 7.28.

While we are beginning to understand the basic circuitry of signal transduction pathways, it is important to keep in mind that we do not yet understand why these pathways are so complex. One reason might be that the signaling pathways have roles in properties such as amplification, robustness, diversity, and efficiency of signaling responses. An important goal for the future will be to understand how the design of each signaling pathway contributes to the particular quality and sensitivity needed for specific signaling responses.

Questions.

- 7.1 True or False:** Antigen receptors bear intrinsic kinase activity that allows for phosphorylation of cytoplasmic proteins and subsequent downstream signaling events.
- 7.2 Matching:** Indicate whether the following receptors are receptor tyrosine kinases (**RTKs**), are receptor serine/threonine kinases (**RSTKs**), or have no intrinsic enzymatic activity (null).
- A. ___ Kit
B. ___ B-cell receptor
C. ___ FLT3
D. ___ TGF- β receptor
- 7.3 Short Answer:** How can scaffolds and adaptors modulate signaling responses if they have no intrinsic enzymatic activity?
- 7.4 Multiple Choice:** Which of the following alterations would result in increased activity of Ras (one or more may apply):
- A. A mutation in Ras that enhances its GTPase activity
B. Overexpression of GEFs
C. Depletion of GTP in the cytoplasm
D. Overexpression of GAPs
E. A mutation in Ras that renders it unsusceptible to the activities of GAPs
- 7.5 Matching:** Order (by numbering 1–5) the downstream signaling events that occur immediately after T-cell receptor engagement:
- ___ LAT and SLP-76, scaffold proteins linked by Gads, are phosphorylated
___ ZAP-70, a tandem SH2 domain containing kinase, binds to ITAMs
___ Recruitment and activation of SH2, PH, and PX domain-containing proteins
___ PI 3-kinase is activated and produces PIP_3
___ Phosphorylation of ITAMs by Lck, a Src-family kinase

- 7.6 Fill-in-the-Blanks:** For each of the following sentences, fill in the blanks with the best word selected from the list below. Each word should be used only once.

PH/PX	PLC- γ
SH2	ADAP
Vav	PI 3-kinase
LAT:Gads:SLP-76	Akt

Antigen receptor signaling leads to many downstream events that branch out into many signaling pathways or modules. These can be activated by the scaffold complex _____, the generation of PIP_3 from PIP_2 by the enzyme _____, or both. Phosphorylated tyrosine residues on the scaffold recruit proteins containing _____ domains, while PIP_3 recruits proteins containing _____ domains. These four modules are the activation of (1) _____, which cleaves PIP_2 to produce DAG and IP_3 , (2) _____, which binds to PIP_3 and activates the mTOR pathway by phosphorylating and inactivating the TSC complex, (3) _____, an adaptor that recruits SKAP55 and RIAM, and (4) _____, a GEF that leads to activation of WASp. These pathways ultimately lead to increased transcription of key genes, increased cellular metabolism, increased cellular adhesion, and actin polymerization, respectively.

- 7.7 Matching:** Match the small G protein (GTPase) to its function.
- A. ___ Ras
B. ___ Cdc42 (Rho family)
C. ___ Rap1
D. ___ Rheb
- i. WASp; actin polymerization
ii. mTOR; cellular metabolism
iii. LFA-1 aggregation; cellular adhesion
iv. MAPK pathway; cellular proliferation

- 7.8 **Multiple Choice:** Which of the following statements is false?
- A. K63 polyubiquitination leads to downstream cellular signaling.
 - B. K48 polyubiquitination leads to degradation by the proteasome.
 - C. Out of the three families of enzymes involved in ubiquitination—E1 (ubiquitin-activating) enzymes, E2 (ubiquitin-conjugating) enzymes, and E3 enzymes (ubiquitin ligases)—Cbl is an E3 enzyme that selects its target via its SH2 domain.
 - D. Mono- or di-ubiquitination of surface receptors leads to degradation by the proteasome.

- 7.9 **Matching:** Match the human disease with the gene that is defective:

- | | |
|---|----------|
| A. ____ X-linked agammaglobulinemia | i. ORAI1 |
| B. ____ Wiscott-Aldrich syndrome | ii. NEMO |
| C. ____ Severe combined immunodeficiency | iii. Btk |
| D. ____ X-linked hypohidrotic ectodermal dysplasia and immunodeficiency | iv. WASp |

- 7.10 **Fill-in-the-Blanks:** Name the corresponding receptor or signaling component in its respective T/B cell counterpart:

- | T cell | B cell |
|--|----------------|
| CD3ε:CD3δ:(CD3γ) ₂ :(CD3ζ) ₂ | A. _____ |
| B. _____ | CD21:CD19:CD81 |
| CD28 | C. _____ |
| D. _____ | Fyn, Blk, Lyn |
| E. _____ | Syk |
| LAT:Gads:SLP-76 | F. _____ |

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- 7.11 **True or False:** CTLA-4 and PD-1 are both ITIM-containing inhibitory receptors that interfere with co-stimulatory signaling pathways by activating intracellular protein and/or lipid phosphatases.

- 7.12 **Multiple Choice:** Intravenous administration of exogenous immunoglobulin is a widely used therapy for autoimmune disorders that involve the production of autoantibodies (antibodies against self antigens). Researchers have discovered that the presence of sialic acids on the infused immunoglobulins is critical for the inhibition of autoantibody production in the patient's own B cells. Which of the following receptors could potentially be responsible for the inhibition of antibody production by B cells, given this finding?

- A. FcγRIIB
- B. CD22
- C. PD-1
- D. CD40
- E. BTLA

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APPENDICES

- I The Immunologist's Toolbox
- II CD antigens
- III Cytokines and their Receptors
- IV Chemokines and their Receptors

The Immunologist's Toolbox

A-1. Immunization.

Natural immune responses are normally directed at antigens borne by pathogenic microorganisms. The immune system can also be induced to respond to simple nonliving antigens, and experimental immunologists have focused on the responses to these simple antigens in developing our understanding of the immune response. The deliberate induction of an immune response is known as **immunization**. Experimental immunizations are routinely carried out by injecting the test antigen into the animal or human subject. The route, dose, and form in which antigen is administered can profoundly affect whether a response occurs and the type of response that is produced. The induction of **protective immune responses against common microbial pathogens in humans is often called vaccination**, although this term originally referred to the induction of immune responses against smallpox by immunizing with the cross-reactive virus, vaccinia.

To determine whether an immune response has occurred and to follow its course, the immunized individual is monitored for the appearance of immune reactants. Immune responses to most antigens include soluble factors, such as cytokines and specific antibodies, and cellular responses, such as the generation of specific effector T cells. Monitoring the cytokine and antibody responses usually involves the analysis of relatively crude preparations of **antisera** (plural: **antisera**). **Serum** is the fluid phase of clotted blood, which, if taken from an individual immunized against a particular antigen, is called **antisera**. To study immune responses mediated by T cells, blood lymphocytes or cells from lymphoid organs such as the spleen are tested; T-cell responses are more commonly studied in experimental animals than in humans.

Antisera generated by immunization with even the simplest antigen will contain many different antibody molecules that bind to the immunogen in slightly different ways. In addition, antisera contain many antibodies that do not bind at all to the immunizing antigen, because they were present in the individual prior to immunization. These nonspecific antibodies often lead to technical

difficulties in using antisera for detecting an immunogen. To circumvent this problem, antibodies that bind to the immunogen can be purified by affinity chromatography using immobilized antigen (see Section A-3). Alternatively, these problems can be avoided by making monoclonal antibodies (see Section A-7).

Any substance that can elicit an immune response is said to be **immunogenic** and is called an **immunogen**. There is a clear operational distinction between an immunogen and an antigen. Immunogens are substances that elicit an adaptive immune response, whereas an antigen is defined as any substance that can bind to a specific antibody. All antigens therefore have the potential to elicit specific antibodies; however, not all antigens are immunogenic. An example of this distinction is evident when considering protein antigens. In spite of the fact that antibodies against proteins are of enormous utility in experimental biology and medicine, purified proteins are not generally immunogenic. This is because purified proteins lack microbial-associated molecular patterns (MAMPs), and therefore do not elicit an innate immune response. To provoke an immune response to a purified protein, the protein must be administered together with an adjuvant (see below).

Certain properties of antigens that favor the initiation of an adaptive immune response have been defined by studying antibody responses to simple natural proteins such as hen egg-white lysozyme, to synthetic polypeptide antigens, and to small organic molecules of simple structure. The study of antibody responses to small organic molecules, such as phenyl arsonates and nitrophenyls, was essential in defining early immunological principles. These molecules do not provoke antibodies when injected by themselves. However, antibodies can be raised against them if the molecule is attached covalently, by simple chemical reactions, to a protein carrier. Such small molecules were termed **haptens** (from the Greek *haptein*, to fasten) by the immunologist **Karl Landsteiner**, who first studied them in the early 20th century. He found that animals immunized with a hapten-carrier conjugate produced three distinct sets of antibodies (Fig. A.1). One set comprised hapten-specific antibodies that reacted with the same hapten on any carrier, as well as with free hapten. The second set of antibodies was specific for the carrier protein, as shown by their ability to bind both the hapten-modified and unmodified carrier protein. Finally, some antibodies reacted only with the specific conjugate of hapten and carrier used for immunization. Landsteiner's studies focused primarily on the antibody response to the hapten, as these small molecules could be synthesized in many closely related forms. He observed that antibodies raised against a particular hapten bind that hapten but, in general, fail to bind even very closely related chemical structures. The binding of haptens by anti-hapten antibodies has played an important part in defining the precision of antigen binding by antibody molecules. Anti-hapten antibodies are also important medically because they mediate allergic reactions to penicillin and other compounds that elicit antibody responses when they attach to self proteins (see Section 14-10).

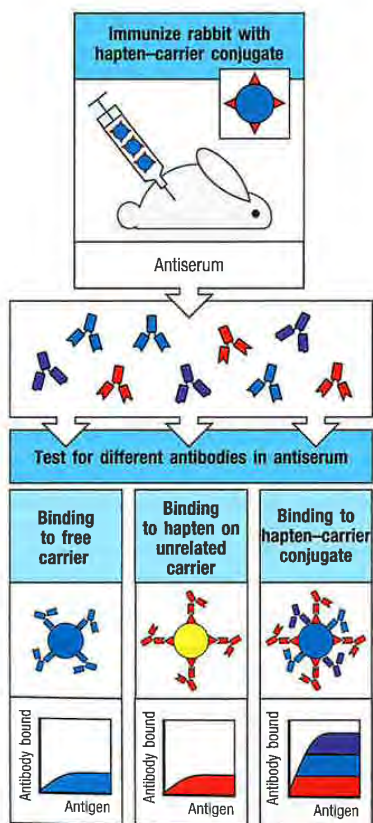


Fig. A.1 Antibodies can be elicited by small chemical groups called haptens only when the hapten is linked to an immunogenic protein carrier. Following immunization with a hapten-carrier conjugate, three types of antibodies are produced. One set (blue) binds the carrier protein alone and is called carrier-specific. One set (red) binds to the hapten on any carrier or to free hapten in solution and is called hapten-specific. One set (purple) binds only the specific conjugate of hapten and carrier used for immunization, apparently binding to sites at which the hapten joins the carrier, and is called conjugate-specific. The amount of antibody of each type in this serum is shown schematically in the graphs at the bottom; note that the original antigen binds more antibody than the sum of anti-hapten and anti-carrier antibodies as a result of the additional binding of conjugate-specific antibody.

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The route by which antigen is administered affects both the magnitude and the type of response obtained. The most common routes by which antigen is introduced experimentally or as a vaccine into the body are into tissue by **subcutaneous (s.c.)** injection into the fatty layer just below the dermis or by **intra-dermal (i.d.)** or **intra-muscular (i.m.)** injection; directly into the blood-stream by **intra-venous (i.v.)** injection or transfusion; into the gastrointestinal tract by oral administration; and into the respiratory tract by **intra-nasal (i.n.)** administration or inhalation.

Antigens injected subcutaneously generally elicit strong responses, most probably because the antigen is taken up by resident dendritic cells in the skin and efficiently presented in local lymph nodes, and so this is the method most commonly used when the object of the experiment is to elicit specific antibodies or T cells against a given antigen. Antigens injected or transfused directly into the bloodstream tend to induce immune unresponsiveness or tolerance unless they bind to host cells or are in the form of aggregates that are readily taken up by antigen-presenting cells.

Antigen administration via the gastrointestinal tract is used mostly in the study of allergy. It has distinctive effects, frequently eliciting a local antibody response in the intestinal lamina propria, while producing a systemic state of tolerance that manifests as a diminished response to the same antigen if subsequently administered in immunogenic form elsewhere in the body (see Chapter 12). This 'split tolerance' may be important in avoiding allergy to antigens in food, because the local response prevents food antigens from entering the body, while the inhibition of systemic immunity helps to prevent the formation of IgE antibodies, which are the cause of such allergies (see Chapter 14).

The immune response to an antigen is also influenced by the dose of immunogen administered. Below a certain threshold dose, most proteins do not elicit any immune response. Above the threshold dose, there is a gradual increase in the response as the dose of antigen is increased, until a broad plateau level is reached, followed by a decline at very high antigen doses (Fig. A.2). In general, secondary and subsequent immune responses occur at lower antigen doses and achieve higher plateau values, which is a sign of immunological memory.

Most proteins are poorly immunogenic or nonimmunogenic when administered by themselves. Strong adaptive immune responses to protein antigens almost always require that the antigen be injected in a mixture known as an **adjuvant**. An adjuvant is any substance that enhances the immunogenicity of substances mixed with it. Commonly used adjuvants are listed in Fig. A.3.

Adjuvants generally enhance immunogenicity in two different ways. First, adjuvants convert soluble protein antigens into particulate material, which is more readily ingested by phagocytic antigen-presenting cells such as macrophages and dendritic cells. For example, the antigen can be adsorbed on particles of the adjuvant (such as alum), be made particulate by emulsification in mineral oils, or be incorporated into the colloidal particles of immune stimulatory complexes (ISCOMs). Second, and more important, adjuvants contain PAMPs that elicit a strong innate immune response. When taken up by phagocytic cells, the PAMPs in the adjuvant stimulate inflammatory cytokine production and induce the activation of the antigen-presenting cell. The activated antigen-presenting cells upregulate abundant levels of co-stimulatory molecules that are important for activating T cells. Activated antigen-presenting cells also upregulate high levels of MHC class I and class II proteins, plus many of the proteins important for efficient antigen processing and presentation (see Section 3-12). Due to the strong local inflammatory responses induced by adjuvants that contain PAMPs, most of the adjuvants commonly used in experimental animals are not approved for use in humans.

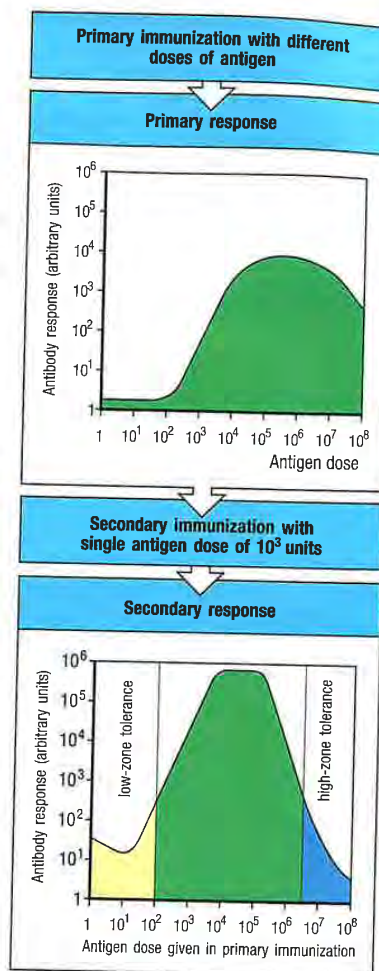


Fig. A.2 The dose of antigen used in an initial immunization affects the primary and the secondary antibody response.

The typical antigen dose-response curve shown here illustrates both the influence of dose on a primary antibody response (amounts of antibody produced expressed in arbitrary units) and the effect of the dose used for priming on a secondary antibody response elicited by a dose of antigen of 10^3 arbitrary mass units. Very low doses of antigen do not cause an immune response at all. Slightly higher doses seem to inhibit specific antibody production, an effect known as low-zone tolerance. Above these doses there is a steady increase in the response with antigen dose until an optimum response is reached; this response persists across a broad range of doses. Very high doses of antigen also inhibit immune responsiveness to a subsequent challenge, a phenomenon known as high-zone tolerance.

Fig. A.3 Common adjuvants and their use. Adjuvants are mixed with the antigen and usually render it particulate, which helps to retain the antigen in the body and promotes uptake by macrophages. Most adjuvants include bacteria or bacterial components that stimulate macrophages and dendritic cells, aiding in the induction of the immune response. ISCOMs are small micelles of the detergent Quil A; when viral proteins are placed in these micelles, they apparently fuse with the antigen-presenting cell, allowing the antigen to enter the cytosol. Thus, the antigen-presenting cell can stimulate a response to the viral protein, much as a virus infecting these cells would stimulate an antiviral response. Vaccines designed to elicit responses to purified proteins often include compounds that stimulate pattern recognition receptors, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), or C-type lectin receptors.

Adjuvants that enhance immune responses		
Adjuvant name	Composition	Mechanism of action
Incomplete Freund's adjuvant	Oil-in-water emulsion	Delayed release of antigen; enhanced uptake by macrophages
Complete Freund's adjuvant	Oil-in-water emulsion with dead mycobacteria that stimulate C-type lectin receptors	Delayed release of antigen; enhanced uptake by macrophages; induction of co-stimulators in macrophages
Freund's adjuvant with MDP	Oil-in-water emulsion with muramyl dipeptide (MDP), a constituent of mycobacteria that stimulates NOD-like receptors	Similar to complete Freund's adjuvant
Alum (aluminum hydroxide)	Aluminum hydroxide gel	Delayed release of antigen; enhanced macrophage uptake
Alum plus <i>Bordetella pertussis</i>	Aluminum hydroxide gel with killed <i>B. pertussis</i>	Delayed release of antigen; enhanced uptake by macrophages; induction of co-stimulators
Immune stimulatory complexes (ISCOMs)	Matrix of Quil A containing viral proteins	Delivers antigen to cytosol; allows induction of cytotoxic T cells
TLR agonists	Lipopolysaccharide, flagellin, lipopeptides, ds-RNA, unmethylated DNA	Inflammatory cytokine production, induction of co-stimulators, enhanced antigen presentation to T cells
NOD-like receptor (NLR) agonists	Muramyl dipeptide (bacterial cell wall constituent)	Inflammatory cytokine production, induction of co-stimulators, enhanced antigen presentation to T cells
C-type lectin receptor agonists	Mycobacterial cell wall component trehalose-6,6'-dimycolate	Inflammatory cytokine production

Nevertheless, some human vaccines naturally contain microbial antigens that can also act as effective adjuvants. For example, purified constituents of the bacterium *Bordetella pertussis*, which is the causative agent of whooping cough, are used as both antigen and adjuvant in the triplex DPT (diphtheria, pertussis, tetanus) vaccine. In addition, modified TLR ligands, such as monophosphoryl lipid A, a derivative of LPS, or poly(I):poly(C12U), a derivative of poly(I):C, are currently included as components of several human vaccines.

A-2 Antibody responses.

B cells contribute to adaptive immunity by secreting antibodies, and the response of B cells to an injected immunogen is usually measured by analyzing the specific antibody produced in a **humoral immune response**. This is most conveniently achieved by assaying the antibody that accumulates in the fluid phase of the blood, or **plasma**; such antibodies are known as circulating antibodies. Circulating antibody is usually measured by collecting blood, allowing it to clot, and then isolating the serum from the clotted blood. The amount and characteristics of the antibody in the resulting antiserum are then determined using the assays described below. Because assays for antibody were originally conducted using antisera from immune individuals, they are commonly referred to as **serological assays**, and the use of antibodies in such testing is often called **serology**.

The most important characteristics of an antibody response are the specificity, amount, isotype or class, and affinity of the antibodies produced. The **specificity** determines the ability of the antibody to distinguish the immunogen

from other antigens. The amount of antibody can be determined in many different ways and is a function of the number of responding B cells, their rate of antibody synthesis, and the persistence of the antibody after production. The persistence of an antibody in the plasma and extracellular fluid bathing the tissues is determined mainly by its isotype or class (see Sections 5-12 and 10-14); each isotype has a different half-life *in vivo*. The isotypic composition of an antibody response also determines the biological functions these antibodies can perform and the sites in which antibody will be found. Finally, the strength of binding of the antibody to its antigen in terms of a single antigen-binding site binding to a monovalent antigen is termed its **affinity**; the total binding strength of a molecule with more than one binding site is called its **avidity**. Binding strength is important: the higher the affinity of the antibody for its antigen, the less antibody is required to eliminate the antigen, because antibodies with higher affinity will bind at lower antigen concentrations. All these parameters of the humoral immune response help to determine the capacity of that response to protect the host from infection.

A-3 Affinity chromatography.

The specificity of antigen:antibody binding interactions can be exploited for the purification of a specific antigen from a complex mixture, or alternatively, for the purification of specific antibodies from antiserum containing a mixture of different antibodies. The technique employed is called **affinity chromatography** (Fig. A.4). For purification of an antigen, antigen-specific antibodies are bound, often covalently, to small, chemically reactive beads, which are loaded into a column. The antigen mixture is allowed to pass over the beads. The specific antigen binds; all the other components in the mixture can then be washed away. The specific antigen is then eluted, typically by lowering the pH to 2.5 or raising it to greater than 11. Antibodies bind stably under physiological conditions of salt concentration, temperature, and pH, but the binding is reversible because the bonds are noncovalent. Affinity chromatography can also be used to purify antibodies from complex antisera by using beads coated with specific antigen. The technique is known as affinity chromatography because it separates molecules on the basis of their affinity for one another.

A-4 Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and competitive inhibition assay.

Radioimmunoassay (RIA) and **enzyme-linked immunosorbent assay (ELISA)** are direct binding assays for antibody (or antigen); both work on the same principle, but the means of detecting specific binding is different. Radioimmunoassays are commonly used to measure the levels of hormones in blood and tissue fluids, while ELISA assays are frequently used in viral diagnostics, for example, in detecting cases of infection with the human immunodeficiency virus (HIV), which is the cause of AIDS. For both of these methods, one needs a pure preparation of a known antigen or antibody, or both, in order to standardize the assay. We will describe the assay that is used to determine the amount of a specific antigen in a sample, for instance, the amount of HIV p24 protein in a patient's serum. For this, a preparation of pure antibody specific for the antigen is required. One can also use RIA or ELISA to determine the amount of specific antibody in a mixture, such as serum; in this case, a preparation of pure antigen is needed as a starting point.

For the determination of antigen concentration using RIA, pure antibody against the antigen is radioactively labeled, usually with ^{125}I ; for ELISA, an enzyme is linked chemically to the antibody. The unlabeled component, which in this case would be the solution containing an unknown amount of antigen, is attached to a solid support, such as the wells of a plastic multiwell plate, which will adsorb a certain amount of any protein. Following this, the labeled antibody

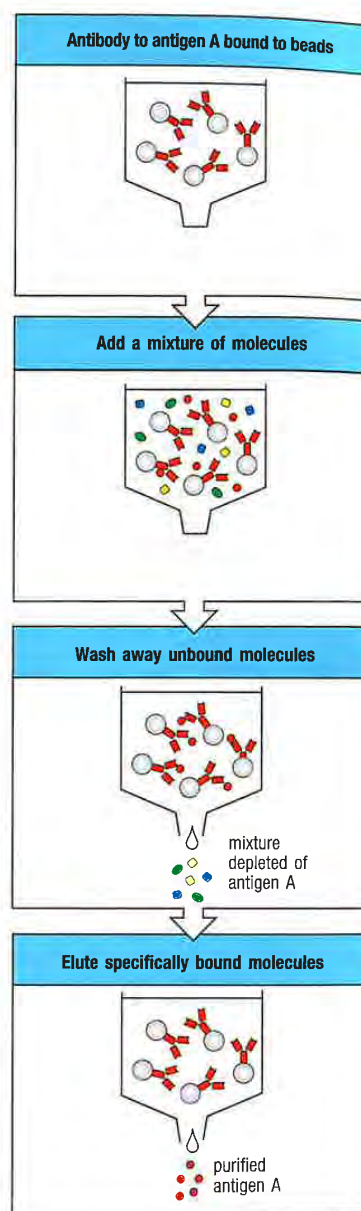


Fig. A.4 Affinity chromatography uses antigen-antibody binding to purify antigens or antibodies. To purify a specific antigen from a complex mixture of molecules, a monoclonal antibody is attached to an insoluble matrix, such as chromatography beads, and the mixture of molecules is passed over the matrix. The specific antibody binds the antigen of interest; other molecules are washed away. Specific antigen is then eluted by altering the pH, which can usually disrupt antibody-antigen bonds. Antibodies can be purified in the same way on beads coupled to antigen (not shown).

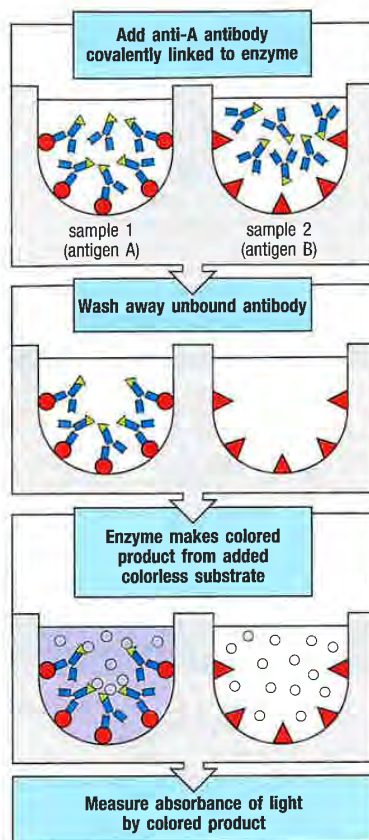


Fig. A.5 The principle of the enzyme-linked immunosorbent assay (ELISA).

To detect antigen A, purified antibody specific for antigen A is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic wells, to which they bind nonspecifically; residual sticky sites on the plastic are blocked by adding irrelevant proteins (not shown). The labeled antibody is then added to the wells under conditions that prevent nonspecific binding, so that only binding to antigen A causes the labeled antibody to be retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction. This assay allows arrays of wells known as microtiter plates to be read in fiberoptic multichannel spectrometers, greatly speeding the assay. Modifications of this basic assay allow antibody or antigen in unknown samples to be measured as shown in Figs. A.6 and A.25.

is added to the well and allowed to bind to the unlabeled antigen under conditions in which nonspecific adsorption is blocked, and any unbound antibody and other proteins are washed away. Antibody binding in RIA is measured directly in terms of the amount of radioactivity retained by the coated wells, whereas in ELISA the binding is detected by a reaction that converts a substrate into a reaction product of a different color (Fig. A.5). The color change can be read directly in the reaction tray, making data collection very easy and providing a quantitative measurement of reaction product concentration; furthermore, ELISA also avoids the hazards of radioactivity. This makes ELISA the preferred method for most direct-binding assays. In a variation of this assay, labeled anti-immunoglobulin antibodies can also be used in RIA or ELISA as a second layer, following the binding of unlabeled antibody to unlabeled antigen-coated plates. The use of such a second layer amplifies the signal, because at least two molecules of the labeled anti-immunoglobulin antibody are able to bind to each unlabeled antibody. As mentioned above, RIA and ELISA can also be carried out in reverse when the goal is to determine the amount of antibody in a solution; in this case, unlabeled antibody is adhered to the plates, labeled antigen is added, and the amount of labeled antigen bound after washing is measured.

A modification of ELISA known as a **capture** or **sandwich ELISA** (or more generally as an **antigen-capture assay**) is commonly used to detect secreted products such as cytokines. Rather than the antigen being directly attached to a plastic plate, antigen-specific antibodies are bound to the plate. These are able to bind antigen with high affinity, and thus concentrate it on the surface of the plate, even with antigens that are present in very low concentrations in the initial mixture. A separate labeled antibody that recognizes a different epitope from that recognized by the immobilized first antibody is then used to detect the bound antigen.

Another variant of the antigen-capture assay, often referred to as a **multiplex** assay, has been developed to allow quantitation of multiple antigens in a single sample. This technique is often utilized to examine the levels of multiple cytokines in clinical serum samples, or in sera from experimental animals, cases in which it is not feasible to assess each cytokine of interest individually. For this type of assay, small microspheres are differentially labeled with fluorescent dyes that can be distinguished based on their distinct emission spectra. Microspheres labeled with a given fluorescent dye are conjugated to antibodies specific for one antigen, for instance, a single cytokine. The microspheres—up to 100 different microspheres with unique identifiers—are added to the sample to capture the antigen. Bound antigen is then detected using a second antibody that binds the antigen at a distinct site. This second antibody is conjugated to a different fluorescent dye, and the magnitude of its fluorescence is a measure of the quantity of bound antigen. The machine that performs this multiplex analysis, the Luminex® analyzer, then measures the amount of fluorescence associated with each differentially labeled microsphere.

These assays illustrate two crucial aspects of all serological assays. First, at least one of the reagents must be available in a pure, detectable form in order to obtain quantitative information. Second, there must be a means of separating the bound fraction of the labeled reagent from the unbound, free fraction so that the percentage of specific binding can be determined. Normally, this separation is achieved by having the unlabeled partner trapped on a solid support. Labeled molecules that do not bind can then be washed away, leaving just the labeled partner that has bound. In Fig. A.5, the unlabeled antigen is attached to the well and the labeled antibody is trapped by binding to it. The separation of bound reagent from the free fraction is an essential step in every assay that uses antibodies.

RIA and ELISA do not allow one to measure directly the amount of antigen or antibody in a sample of unknown composition, because both depend on the binding of a pure labeled antigen or antibody. There are various ways around

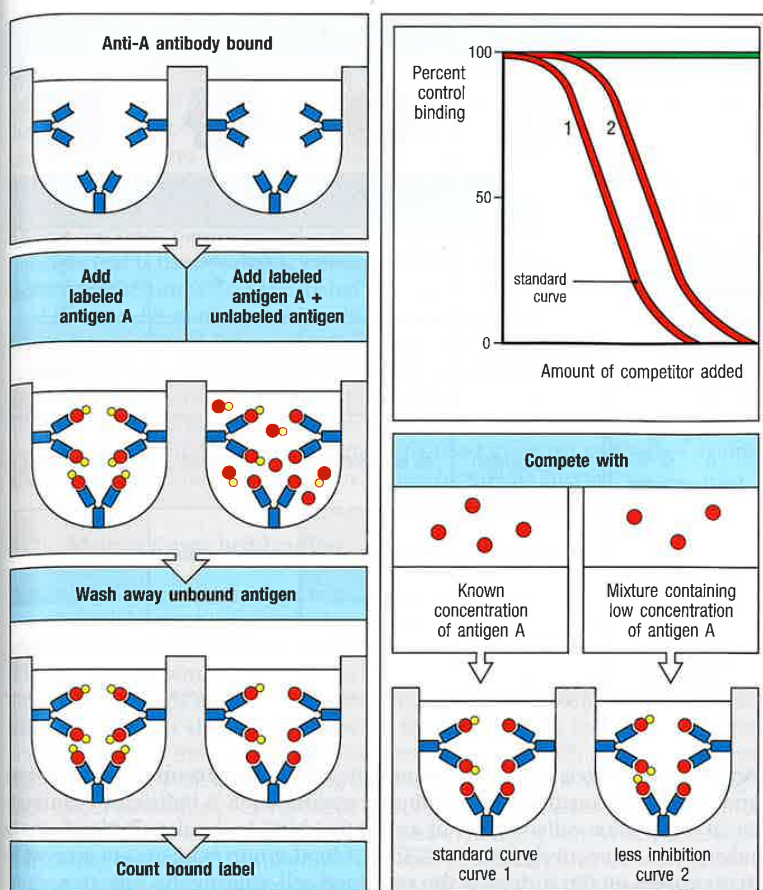


Fig. A.6 Competitive inhibition assay for antigen in unknown samples.

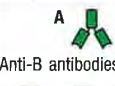
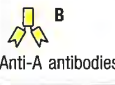
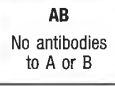
A fixed amount of unlabeled antibody is attached to a set of wells, and a standard reference preparation of a labeled antigen is bound to it. Unlabeled standard or test samples are then added in various amounts and the displacement of labeled antigen is measured, generating characteristic inhibition curves. A standard curve is obtained by using known amounts of unlabeled antigen identical to that used as the labeled species, and comparison with this curve allows the amount of antigen in unknown samples to be calculated. The green line on the graph represents a sample lacking any substance that reacts with anti-A antibodies.

this problem, one of which is to use a **competitive inhibition assay**, as shown in Fig. A.6. In this type of assay, the presence and amount of a particular antigen in an unknown sample are determined by the antigen's ability to compete with a labeled reference antigen for binding to an antibody attached to a plastic well. A standard curve is first constructed by adding various amounts of a known, unlabeled standard preparation; the assay can then measure the amount of antigen in unknown samples by comparison with the standard. The competitive binding assay can also be used for measuring antibody in a sample of unknown composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labeled specific antibody.

A-5 Hemagglutination and blood typing.

The direct measurement of antibody binding to antigen is used in most quantitative serological assays. However, some important assays are based on the ability of antibody binding to alter the physical state of the antigen to which the antibody binds. These secondary interactions can be detected in a variety of ways. For instance, when the antigen is displayed on the surface of a large particle such as a bacterium, antibodies can cause the bacteria to clump, or **agglutinate**. The same principle applies to the reactions used in blood typing, only here the target antigens are on the surface of red blood cells and the clumping reaction caused by antibodies against them is called **hemagglutination** (from the Greek *haima*, blood).

Fig. A.7 Hemagglutination is used to type blood groups and match compatible donors and recipients for blood transfusion. Common gut bacteria bear antigens that are similar or identical to blood-group antigens, and these stimulate the formation of antibodies against these antigens in individuals who do not bear the corresponding antigen on their own red blood cells (left column); thus, type O individuals, who lack A and B, have both anti-A and anti-B antibodies, whereas type AB individuals have neither. The pattern of agglutination of the red blood cells of a transfusion donor or recipient with anti-A and anti-B antibodies reveals the individual's ABO blood group. Before transfusion, the serum of the recipient is also tested for antibodies that agglutinate the red blood cells of the donor, and vice versa, a procedure called a cross-match, which may detect potentially harmful antibodies against other blood groups that are not part of the ABO system.

Serum from individuals of type	Red blood cells from individuals of type			
				
	Express the carbohydrate structures			
	R - GlcNAc - Gal Fuc	R - GlcNAc - Gal - GalNAc Fuc	R - GlcNAc - Gal - Gal Fuc	R - GlcNAc - Gal - GalNAc Fuc + R - GlcNAc - Gal - Gal Fuc
 Anti-A and anti-B antibodies	no agglutination	agglutination	agglutination	agglutination
 Anti-B antibodies	no agglutination	no agglutination	agglutination	agglutination
 Anti-A antibodies	no agglutination	agglutination	no agglutination	agglutination
 No antibodies to A or B	no agglutination	no agglutination	no agglutination	no agglutination

Hemagglutination is used to determine the **ABO blood group** of blood donors and transfusion recipients. Clumping or agglutination is induced by antibodies or agglutinins called anti-A or anti-B that bind to the A or B blood-group substances, respectively (Fig. A.7). These blood-group antigens are arrayed in many copies on the surface of the red blood cell, causing the cells to agglutinate when cross-linked by antibodies. Because hemagglutination involves the cross-linking of blood cells by the simultaneous binding of antibody molecules to identical antigens on different cells, this reaction also demonstrates that each antibody molecule must have at least two identical antigen-binding sites.

A-6 Coombs tests and the detection of rhesus incompatibility.

Coombs tests use anti-immunoglobulin antibodies to detect the antibodies that cause **hemolytic disease of the newborn**, or **erythroblastosis fetalis**. Anti-immunoglobulin antibodies were first developed by **Robin Coombs**, and the test for this disease is still called the Coombs test. Hemolytic disease of the newborn occurs when a mother makes IgG antibodies specific for the **rhesus** or **Rh blood-group antigen** expressed on the red blood cells of her fetus. Rh-negative mothers make these antibodies when they are exposed at delivery to Rh-positive fetal red blood cells bearing the paternally inherited Rh antigen. During subsequent pregnancies, these antibodies are transported across the placenta to the fetus. This normal process is generally beneficial, as it protects newborn infants against infection. However, IgG anti-Rh antibodies coat the fetal red blood cells, which are then destroyed by phagocytic cells in the liver, causing a hemolytic anemia in the fetus and newborn infant.

Rh antigens are widely spaced on the red blood cell surface, and so the IgG anti-Rh antibodies do not bind in the correct conformation to fix complement and so do not cause lysis of red blood cells *in vitro*. Furthermore, for reasons that are not fully understood, antibodies against Rh antigens do not

agglutinate red blood cells, unlike antibodies against the ABO blood-group antigens. Thus, detecting anti-Rh antibodies was difficult until anti-human immunoglobulin antibodies were developed. With these, maternal IgG antibodies bound to the fetal red blood cells can be detected after washing the cells to remove unbound immunoglobulin that is present in the fetal serum. Adding anti-human immunoglobulin antibodies against the washed fetal red blood cells agglutinates any cells to which maternal antibodies are bound. This is the **direct Coombs test** (Fig. A.8), so called because it directly detects antibody bound to the surface of the fetal red blood cells. An **indirect Coombs test** is used to detect nonagglutinating anti-Rh antibody in maternal serum: the serum is first incubated with Rh-positive red blood cells, which bind the anti-Rh antibody, after which the antibody-coated cells are washed to remove unbound immunoglobulin and are then agglutinated with anti-immunoglobulin antibody (see Fig. A.8). The indirect Coombs test allows Rh incompatibilities that might lead to hemolytic disease of the newborn to be detected, and this knowledge allows the disease to be prevented (see Section 15-10). The Coombs test is also commonly used to detect antibodies against drugs that bind to red blood cells and cause hemolytic anemia.

A-7 Monoclonal antibodies.

The antibodies generated in a natural immune response or after immunization in the laboratory are a mixture of molecules of different specificities and affinities. Some of this heterogeneity results from the production of antibodies that bind to different epitopes on the immunizing antigen, but even antibodies directed at a single antigenic determinant such as a hapten can be markedly heterogeneous, as shown by **isoelectric focusing**. In this technique, proteins are separated on the basis of their isoelectric point, the pH at which their net charge is zero. By electrophoresing proteins in a pH gradient for long enough, each molecule migrates along the pH gradient until it reaches the pH at which it is neutral and is thus concentrated (focused) at that point. When antiserum containing anti-hapten antibodies is treated in this way and then transferred to a solid support such as nitrocellulose paper, the anti-hapten antibodies can be detected by their ability to bind labeled hapten. The binding of antibodies of various isoelectric points to the hapten shows that even antibodies that bind the same antigenic determinant can be heterogeneous.

Antisera are valuable for many biological purposes but they have certain inherent disadvantages that relate to the heterogeneity of the antibodies they contain. First, each antiserum is different from all other antisera, even if raised in a genetically identical animal by using the identical preparation of antigen and the same immunization protocol. Second, antisera can be produced in only limited volumes, and thus it is impossible to use the identical serological reagent in a long or complex series of experiments or clinical tests. Finally, even antibodies purified by affinity chromatography (see Section A-3) can include minor populations of antibodies that give unexpected cross-reactions, which confound the analysis of experiments. To avoid these problems, and to harness the full potential of antibodies, it was necessary to develop a way of making an unlimited supply of antibody molecules of homogeneous structure and known specificity. This has been achieved through the production of monoclonal antibodies from cultures of hybrid antibody-forming cells or, more recently, by genetic engineering.

Starting in the 1950s, biochemists in search of a homogeneous preparation of antibody that they could subject to detailed chemical analysis turned to proteins produced by patients with multiple myeloma, a common tumor of plasma cells. It was known that antibodies are normally produced by plasma cells, and because this disease is associated with the presence of large amounts of a homogeneous gamma globulin called a **myeloma protein** in the patient's serum, it seemed likely that myeloma proteins would serve as models for

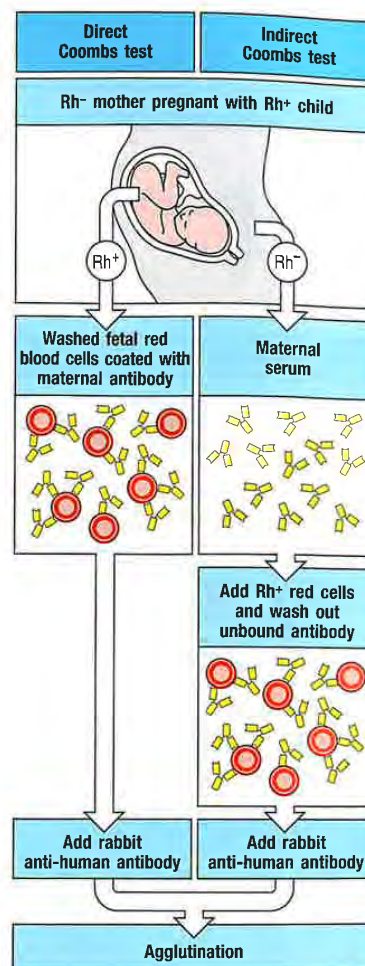


Fig. A.8 The Coombs direct and indirect anti-globulin tests for antibody against red blood cell antigens. An Rh⁻ mother of an Rh⁺ fetus can become immunized to fetal red blood cells that enter the maternal circulation at the time of delivery. In a subsequent pregnancy with an Rh⁺ fetus, IgG anti-Rh antibodies can cross the placenta and damage the fetal red blood cells. In contrast to anti-Rh antibodies, maternal anti-ABO antibodies are of the IgM isotype and cannot cross the placenta, and so do not cause harm. Anti-Rh antibodies do not agglutinate red blood cells, but their presence on the fetal red blood cell surface can be shown by washing away unbound immunoglobulin and then adding antibody against human immunoglobulin, which agglutinates the antibody-coated cells. Anti-Rh antibodies can be detected in the mother's serum in an indirect Coombs test; the serum is incubated with Rh⁺ red blood cells, and once the antibody has bound, the red blood cells are treated as in the direct Coombs test.

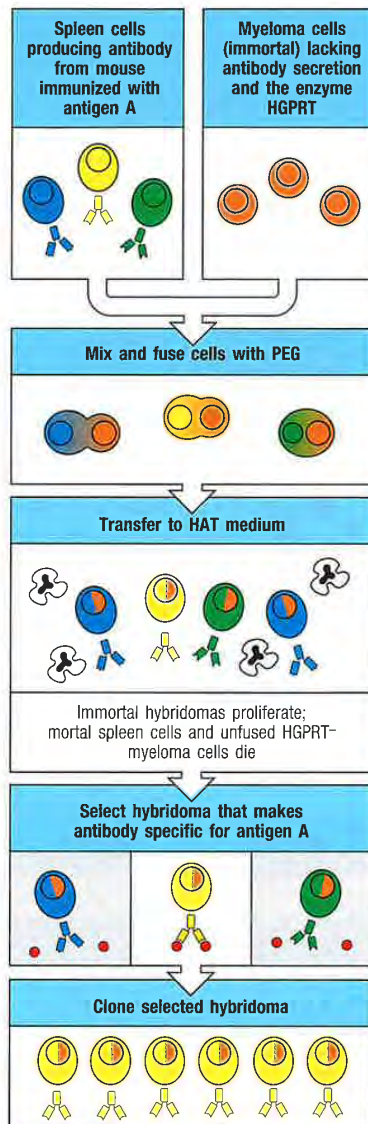


Fig. A.9 The production of monoclonal antibodies. Mice are immunized with antigen A and given an intravenous booster immunization 3 days before they are killed, in order to produce a large population of spleen cells secreting specific antibody. Spleen cells die after a few days in culture. To produce a continuous source of antibody they are fused with immortal myeloma cells by using polyethylene glycol (PEG) to produce a hybrid cell line called a hybridoma. The myeloma cells are selected beforehand to ensure that they are not secreting antibody themselves and that they lack the enzyme hypoxanthine:guanine phosphoribosyl transferase (HGPRT); without this enzyme, unfused myeloma cells are sensitive to the hypoxanthine-aminopterin-thymidine (HAT) medium, which is used to select hybrid cells. The HGPRT gene contributed by the spleen cell allows hybrid cells to survive in the HAT medium, and only hybrid cells can grow continuously in culture, because of the malignant potential contributed by the myeloma cells combined with the finite life-span of unfused spleen cells. Unfused myeloma cells and unfused spleen cells therefore die in the HAT medium, as shown here by cells with dark, irregular nuclei. Individual hybridomas are obtained by single cell dilution and then screened for antibody production, and single clones that make antibody of the desired specificity can be isolated and grown. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. As each hybridoma is descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule, which is thus called a monoclonal antibody.

normal antibody molecules. Thus, much of the early knowledge of antibody structure came from studies on myeloma proteins. These studies showed that monoclonal antibodies could be obtained from immortalized plasma cells. However, the antigen specificity of most myeloma proteins was unknown, which limited their usefulness as objects of study or as immunological tools.

This problem was solved by **Georges Köhler** and **César Milstein**, who devised a technique for producing a homogeneous population of antibodies of known antigenic specificity. They did this by fusing spleen cells from an immunized mouse to cells of a mouse myeloma to produce hybrid cells that both proliferated indefinitely and secreted antibody specific for the antigen used to immunize the spleen cell donor. The spleen cell provides the ability to make specific antibody, while the myeloma cell provides the ability to grow indefinitely in culture and secrete immunoglobulin continuously. By using a myeloma cell partner that produces no antibody proteins itself, the antibody produced by the hybrid cells comes only from the immune spleen cell partner. After fusion, the hybrid cells are selected using drugs that kill the myeloma parental cell, while the unfused parental spleen cells have a limited life-span and soon die, so that only hybrid myeloma cell lines, or **hybridomas**, survive. Those hybridomas producing antibody of the desired specificity are then identified and cloned by regrowing the cultures from single cells (Fig. A.9). Because each hybridoma is a clone derived from fusion with a single B cell, all the antibody molecules it produces are identical in structure, including their antigen-binding site and isotype. Such antibodies are called **monoclonal antibodies**. This technology has revolutionized the use of antibodies by providing a limitless supply of antibody of a single and known specificity. Monoclonal antibodies are now used in most serological assays, as diagnostic probes, and as therapeutic agents. So far, however, only mouse monoclonals are routinely produced in this way, and efforts to use the same approach to make human monoclonal antibodies have met with limited success. 'Fully human' therapeutic monoclonal antibodies are currently made by using phage display technology (described in Section A-8), by using recombinant DNA technology to clone and express antibody genes from human plasma cells (see Section A-9), or by immunizing transgenic mice (see Section A-34) carrying human antibody genes.

A-8 Phage display libraries for antibody V-region production.

In a technique for producing antibody-like molecules, gene segments encoding the antigen-binding variable, or V, domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophages

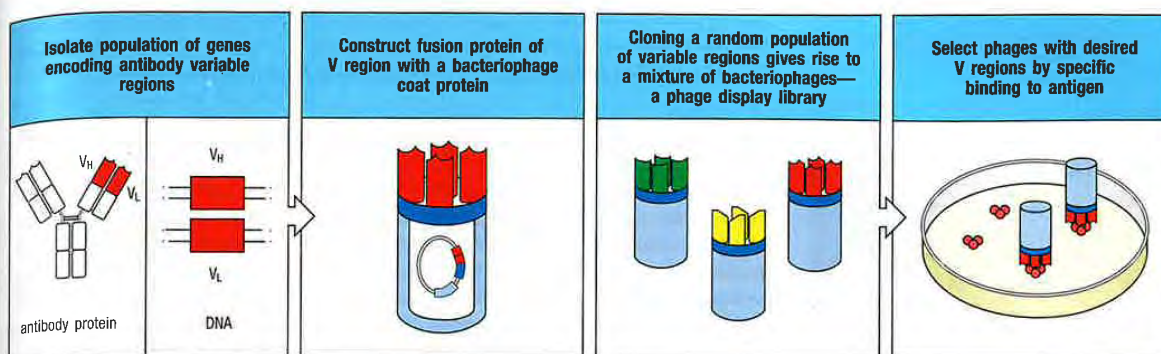


Fig. A.10 The production of antibodies by genetic engineering.

Short primers to consensus sequences in heavy- and light-chain variable (V) regions of immunoglobulin genes are used to generate a library of heavy- and light-chain V-region DNAs by PCR, with spleen DNA as the starting material. These heavy- and light-chain V-region genes are cloned randomly into filamentous phages such that each phage expresses one heavy-chain and one light-chain V region as a surface fusion protein with antibody-like properties. The resulting phage display library is multiplied in bacteria, and the phages are

then bound to a surface coated with antigen. The unbound phages are washed away; the bound phages are recovered, multiplied in bacteria, and again bound to antigen. After a few cycles, only specific high-affinity antigen-binding phages are left. These can be used like antibody molecules, or their V genes can be recovered and engineered into antibody genes to produce genetically engineered antibody molecules (not shown). This technology may replace the hybridoma technology for producing monoclonal antibodies, and has the advantage that humans can be used as the source of DNA.

containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophages. A collection of recombinant phages, each displaying a different antigen-binding domain on the surface, is known as a **phage display library**. In much the same way that antibodies specific for a particular antigen can be isolated from a complex mixture by affinity chromatography (see Section A-3), phages expressing antigen-binding domains specific for a particular antigen can be isolated by selecting the phages in the library for binding to that antigen. The phage particles that bind are recovered and used to infect fresh bacteria. Each phage isolated in this way will produce a monoclonal antigen-binding particle analogous to a monoclonal antibody (Fig. A.10). The genes encoding the antigen-binding site, which are unique to each phage, can then be recovered from the phage DNA and used to construct genes for a complete antibody molecule by joining them to parts of immunoglobulin genes that encode the invariant parts of an antibody. When these reconstructed antibody genes are introduced into a suitable host-cell line, such as the non-antibody-producing myeloma cells used for hybridomas, the transfected cells can secrete antibodies with all the desirable characteristics of monoclonal antibodies produced from hybridomas.

A-9 Generation of human monoclonal antibodies from vaccinated individuals.

In some cases, human monoclonal antibodies can be made by cloning the rearranged antibody heavy- and light-chain gene sequences from plasma cells isolated from vaccinated individuals. Based on the expression of cell-surface molecules such as CD27 and CD38, human plasma cells can be isolated from the peripheral blood of individuals who were immunized approximately 1 week earlier. Individual plasma cells are sorted into wells of microtiter plates, and the antibody heavy- and light-chain variable-region sequences are cloned from each cell by PCR. These sequences are then inserted into constructs that recreate the full-length antibody heavy- and light-chain genes, and the paired heavy- and light-chain vectors are introduced into an immortalized human

Excitation and emission wavelengths of some commonly used fluorochromes		
Probe	Excitation (nm)	Emission (nm)
R-phycoerythrin (PE)	480; 565	578
Fluorescein	495	519
PerCP	490	675
Texas Red	589	615
Rhodamine	550	573

Fig. A.11 Excitation and emission wavelengths for common fluorochromes.

cell line. Human cells are then screened to identify those secreting antibody proteins that bind to the immunizing antigen. These immortalized cells become a permanent source of the specific human antibody protein.

A-10 Microscopy and imaging using fluorescent dyes.

Because antibodies bind stably and specifically to their corresponding antigen, they are invaluable as probes for identifying a particular molecule in cells, tissues, or biological fluids. Antibody molecules can be used to locate their target molecules accurately in single cells or in tissue sections by a variety of different labeling techniques. When the antibody itself, or the anti-immunoglobulin antibody used to detect it, is labeled with a fluorescent dye (a fluorochrome or fluorophore) and then detected by microscopy, the technique is known as **immunofluorescence microscopy**. As in all serological techniques, the antibody binds stably to its antigen, allowing unbound antibody to be removed by thorough washing. Because antibodies against proteins recognize the surface features of the native, folded protein, the native structure of the protein being sought usually needs to be **preserved**, either by using only the most gentle chemical fixation techniques or by using frozen tissue sections that are fixed only after the antibody reaction has been performed. Some antibodies, however, bind proteins even if they are denatured, and such antibodies will bind specifically even to protein in fixed tissue sections.

The fluorescent dye can be covalently attached directly to the specific antibody; however, the bound antibody is more commonly detected by fluorescently labeled anti-immunoglobulin, a technique known as **indirect immunofluorescence**. The dyes chosen for immunofluorescence are excited by light of one wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum. The most commonly used fluorochromes are fluorescein, which emits green light; Texas Red and peridinin chlorophyll protein (PerCP), which emit red light; and rhodamine and phycoerythrin (PE), which emit orange/red light (Fig. A.11). By using selective filters, only the light coming from the fluorochrome used is detected in the fluorescence microscope (Fig. A.12). Although Albert Coons first devised this technique to

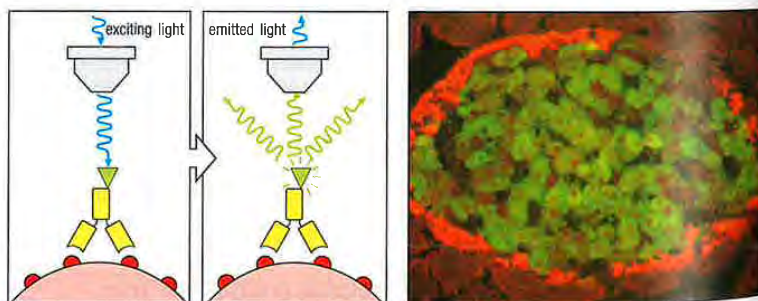


Fig. A.12 Immunofluorescence microscopy. Antibodies labeled with a fluorescent dye such as fluorescein (green triangle) are used to reveal the presence of their corresponding antigens in cells or tissues. The stained cells are examined using a microscope that exposes them to blue or green light to excite the fluorescent dye. The excited dye emits light at a characteristic wavelength, which is captured by viewing the sample through a selective filter. This technique is applied widely in biology to determine the location of molecules in cells and tissues. Different antigens can be detected in tissue sections by labeling antibodies with dyes of distinctive color. Here, antibodies against the protein glutamic acid decarboxylase (GAD) coupled to a green dye are shown to stain the β cells of pancreatic islets of Langerhans. The α cells do not make this enzyme and are labeled with antibodies against the hormone glucagon coupled with an orange fluorescent dye. GAD is an important antigen in type 1 diabetes, a disease in which the insulin-secreting β cells of the islets of Langerhans are destroyed by an immune attack on self tissues (see Chapter 15). Photograph courtesy of M. Solimena and P. De Camilli.

identify the plasma cell as the source of antibody, it can be used to detect the distribution of any protein. By attaching different dyes to different antibodies, the distribution of two or more molecules can be determined in the same cell or tissue section (see Fig. A.12).

The development of the **confocal fluorescent microscope**, which uses computer-aided techniques to produce ultrathin optical sections of a cell or tissue, gives very high resolution (sub-micrometer) fluorescence microscopy without the need for elaborate sample preparation. The light source for excitation (a laser) is focused onto a particular plane in the specimen, and the emitted light is refocused through a 'pinhole' so that only light from the desired plane reaches the detector, thus **removing out-of-focus emissions** from above or below the plane. This gives a **sharper image than conventional** fluorescence microscopy, and a three-dimensional picture can be built up from successive optical sections taken along the 'vertical' axis. Confocal microscopy can be used on fixed cells stained with fluorescently tagged antibodies or on living cells expressing proteins tagged with naturally fluorescent proteins. The first of these fluorescent proteins to come into wide use was green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*. The list of fluorescent proteins in routine use now includes those emitting red, blue, cyan, or yellow fluorescence. By using cells transfected with genes encoding different fusion proteins, it has been possible to visualize the redistribution of T-cell receptors, co-receptors, adhesion molecules, and other signaling molecules, such as CD45, that takes place when a T cell makes contact with a target cell (see Fig. 9.37).

Confocal microscopy, however, can penetrate only around 80 μm into a tissue, and at the wavelengths typically used for excitation, the source light will soon bleach the fluorescent label and damage the specimen. This means that the technique is not suitable for imaging a live specimen over a period of time sufficient, for example, to track the movements of cells in a tissue. The more recently developed technique of **two-photon scanning fluorescence microscopy** overcomes some of these limitations. In this technology, ultrashort pulses of laser light of much longer wavelength (and thus with photons of lower energy) are used for excitation, and two of these lower-energy photons arriving nearly simultaneously are required to excite the fluorophore. Excitation will therefore occur in only a very small region at the focus of the microscope, where the beam of light is most intense, and so fluorescence emission will be restricted to the plane of focus, producing a sharp, high-contrast image. The longer-wavelength light (typically in the near infrared) is also less damaging to living tissue than the blue and ultraviolet wavelengths typically used in confocal microscopy, and so imaging can be carried out over a longer period. More of the emitted light is collected than in confocal microscopy, and because single photons scattering within the tissue cannot cause fluorescence and consequent background haze, imaging to greater depths (several hundred micrometers) is possible. Like confocal microscopy, two-photon microscopy produces thin optical sections from which a three-dimensional image can be built up.

To track the movements of molecules or cells over time, confocal or two-photon microscopy is combined with **time-lapse video imaging** using sensitive digital cameras. In immunology, time-lapse two-photon fluorescence imaging has been particularly valuable for tracking the movements of individual T cells and B cells expressing fluorescent proteins in intact lymphoid organs and observing where they interact (see Chapter 10).

A-11 Immunoelectron microscopy.

Antibodies can be used to detect the intracellular location of structures or particular proteins at high resolution by electron microscopy, a technique known as immunoelectron microscopy. Antibodies against the required antigen are labeled with gold particles and then applied to ultrathin sections, which are

then examined in the transmission electron microscope. Antibodies labeled with gold particles of different diameters enable two or more proteins to be studied simultaneously (see Fig. 6.12). The difficulty with this technique is in staining the ultrathin section adequately, because few molecules of antigen are present in each section.

A-12 Immunohistochemistry.

An alternative to immunofluorescence (see Section A-10) for detecting a protein in tissue sections is **immunohistochemistry**, in which the specific antibody is chemically coupled to an enzyme that converts a colorless substrate into a colored reaction product *in situ*. The localized deposition of the colored product where antibody has bound can be directly observed under a light microscope. The antibody binds stably to its antigen, allowing unbound antibody to be removed by thorough washing. This method of detecting bound antibody is analogous to ELISA (see Section A-4) and frequently uses the same coupled enzymes, the difference in detection being primarily that in immunohistochemistry the colored products are insoluble and precipitate at the site where they are formed. Horseradish peroxidase and alkaline phosphatase are the two enzymes most commonly used in these applications. Horseradish peroxidase oxidizes the substrate diaminobenzidine to produce a brown precipitate, while alkaline phosphatase can produce red or blue dyes depending on the substrates used; a common substrate is 5-bromo-4-chloro-3-indolyl phosphate plus nitroblue tetrazolium (BCIP/NBT), which gives rise to a dark blue or purple stain. As with immunofluorescence, the native structure of the protein being sought usually needs to be preserved so that it will be recognized by the antibody. Tissues are fixed by the most gentle chemical fixation techniques, or frozen tissue sections are used that are fixed only after the antibody reaction has been performed.

A-13 Immunoprecipitation and co-immunoprecipitation.

To raise antibodies against membrane proteins and other cellular structures that are difficult to purify, mice are often immunized with whole cells or crude cell extracts. Antibodies against the individual molecules are then obtained by using these immunized mice to produce hybridomas making monoclonal antibodies (see Section A-7) that bind to the cell type used for immunization. To characterize the molecules identified by the antibodies, cells of the same type are labeled with radioisotopes and dissolved in nonionic detergents that disrupt cell membranes but do not interfere with antigen-antibody interactions. This allows the labeled protein to be isolated by binding to the antibody in a reaction known as **immunoprecipitation**. The antibody is usually attached to a solid support, such as the beads that are used in affinity chromatography (see Section A-3), or to Protein A, a protein derived from the cell wall of *Staphylococcus aureus* that binds tightly to the Fc region of IgG antibodies. Cells can be labeled in two main ways for immunoprecipitation analysis. All the proteins in a cell can be labeled metabolically by growing the cell in a medium containing radioactive amino acids that are then incorporated into cellular proteins (Fig. A.13). Alternatively, one can label only the cell-surface proteins by radioiodination under conditions that prevent iodine from crossing the plasma membrane and labeling proteins inside the cell, or by a reaction that labels only membrane proteins with biotin, a small molecule that is detected readily by labeled avidin, a protein found in egg whites that binds biotin with very high affinity.

Once the labeled proteins have been isolated by the antibody, they can be characterized in several ways. The most common is polyacrylamide gel electrophoresis (PAGE) of the proteins after they have been dissociated from antibody in the strong ionic detergent sodium dodecyl sulfate (SDS), a technique

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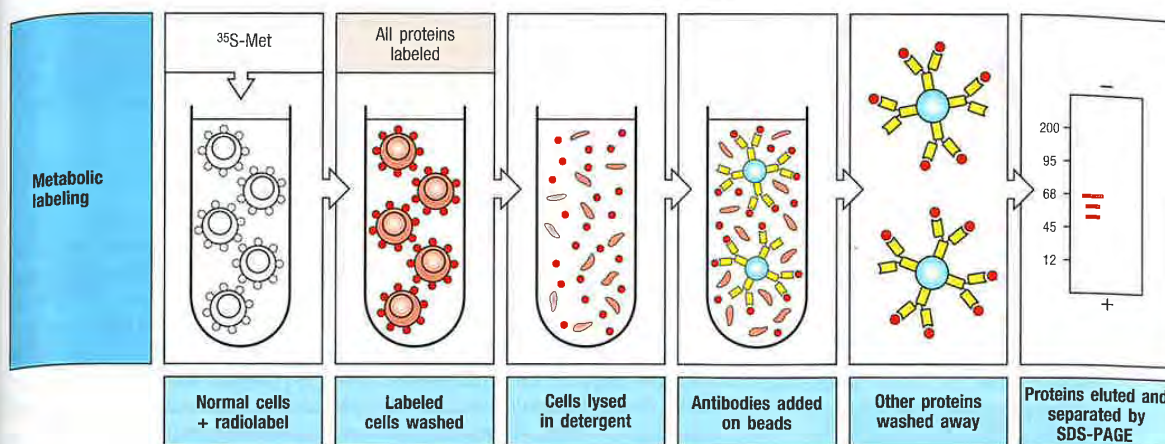


Fig. A.13 Cellular proteins reacting with an antibody can be characterized by immunoprecipitation of labeled cell lysates.

All actively synthesized cellular proteins can be labeled metabolically by incubating cells with radioactive amino acids (shown here for methionine; ^{35}S -Met), or one can label just the cell-surface proteins by using radioactive iodine in a form that cannot cross the cell membrane or by a reaction with the small molecule biotin, detected by its reaction with labeled avidin (not shown). Cells are lysed with detergent and individual labeled cell-associated proteins can be precipitated with a monoclonal antibody attached to beads. After unbound proteins have been washed away, the bound protein is eluted in the detergent sodium dodecyl sulfate (SDS), which

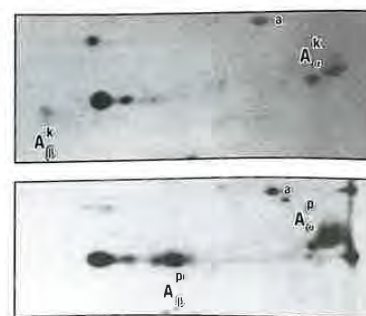
dissociates it from the antibody and also coats the protein with a strong negative charge, allowing it to migrate according to its size in polyacrylamide gel electrophoresis (PAGE). The positions of the labeled proteins are determined by autoradiography using X-ray film. This technique of SDS-PAGE can be used to determine the molecular weight and subunit composition of a protein. Patterns of protein bands observed with metabolic labeling are usually more complex than those revealed by radioiodination, owing to the presence of precursor forms of the protein (right panel). The mature form of a surface protein can be identified as being the same size as that detected by surface iodination or biotinylation (not shown).

generally abbreviated as **SDS-PAGE**. SDS binds relatively homogeneously to proteins, conferring a charge that allows the electrophoretic field to drive protein migration through the gel. The rate of migration is controlled mainly by protein size (see Fig. A.13). Proteins of differing charges can be separated using isoelectric focusing (see Section A-7). This technique can be combined with SDS-PAGE in a procedure known as **two-dimensional gel electrophoresis**. For this, the immunoprecipitated protein is eluted in urea, a nonionic solubilizing agent, and run on an isoelectric focusing gel in a narrow tube of polyacrylamide. This first-dimensional isoelectric focusing gel is then placed across the top of an SDS-PAGE slab gel, which is then run vertically to separate the proteins by molecular weight (Fig. A.14). Two-dimensional gel electrophoresis is a powerful technique that allows many hundreds of proteins in a complex mixture to be distinguished from one another.

Immunoprecipitation and the related technique of immunoblotting (see Section A-14) are useful for determining the molecular weight and isoelectric point of a protein as well as its abundance, distribution, and whether, for example, it undergoes changes in molecular weight and isoelectric point as a result of processing within the cell.

Co-immunoprecipitation is an extension of the immunoprecipitation technique and is used to determine whether a given protein interacts physically

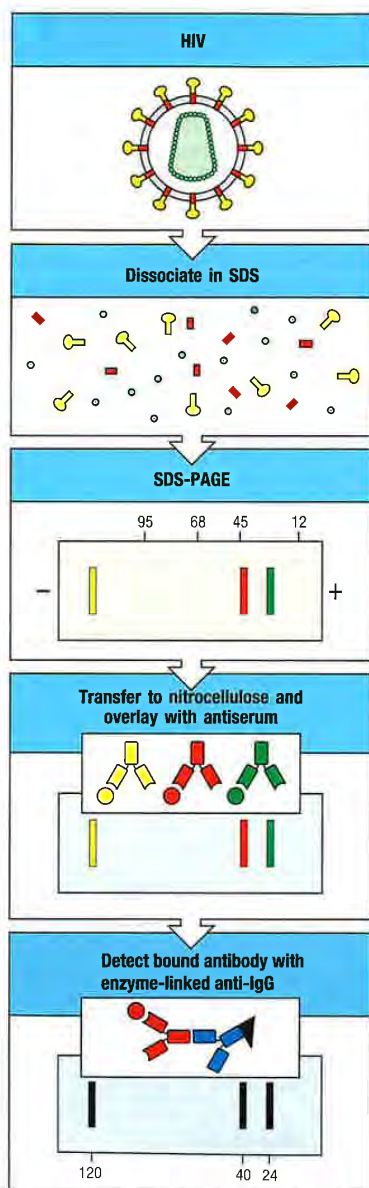
Fig. A.14 Two-dimensional gel electrophoresis of MHC class II molecules. Proteins in mouse spleen cells have been labeled metabolically (see Fig. A.13), precipitated with a monoclonal antibody against the mouse MHC class II molecule H2-A, and separated by isoelectric focusing in one direction and SDS-PAGE in a second direction at right angles to the first (hence the term two-dimensional gel electrophoresis). This allows one to distinguish molecules of the same molecular weight on the basis of their charge. The separated proteins are detected by autoradiography. The MHC class II molecules are composed of two chains, α and β , and in the different MHC class II molecules these have different isoelectric points (compare upper and lower panels). The MHC genotype of mice is indicated by lowercase superscripts (k, p). Actin, a common contaminant, is marked a. Photographs courtesy of J.F. Babich.



with another given protein. Cell extracts containing the presumed interaction complex are first immunoprecipitated with antibody against one of the proteins. The material isolated by this means is then tested for the presence of the other protein by immunoblotting with a specific antibody against the second protein.

A-14 Immunoblotting (Western blotting).

Like immunoprecipitation (see Section A-13), **immunoblotting** is used for identifying the presence of a given protein in a cell lysate, but it avoids the problem of having to label large quantities of cells with radioisotopes. Unlabeled cells are placed in detergent to solubilize all cell proteins, and the lysate is run on SDS-PAGE to separate the proteins (see Section A-13). The size-separated proteins are then transferred from the gel to a stable support such as a nitrocellulose membrane. Specific proteins are detected by treatment with antibodies able to react with SDS-solubilized proteins (mainly those that react with denatured sequences); the bound antibodies are revealed by anti-immunoglobulin antibodies labeled with an enzyme. The term **Western blotting** as a synonym for immunoblotting arose because the comparable technique for detecting specific DNA sequences is known as Southern blotting, after Edwin Southern, who devised it, which in turn provoked the name 'Northern' for blots of size-separated RNA, and 'Western' for blots of size-separated proteins. Western blots have many applications in basic research and clinical diagnosis. They are often used to test sera for the presence of antibodies against specific proteins, for example, to detect antibodies against different constituents of HIV (Fig. A.15).



A-15 Use of antibodies in the isolation and characterization of multiprotein complexes by mass spectrometry.

Many of the proteins that function in immune cells are components of multiprotein complexes. This is the case for cell-surface receptors, such as the T-cell and B-cell antigen receptors and most cytokine receptors, as well as intracellular proteins involved in signal transduction, gene expression, and cell death. Antibodies that bind to one member of such a complex can be used to identify the other members of the complex by a process of co-immunoprecipitation followed by Western blotting or **mass spectrometry**.

A mass spectrometer can provide extremely precise measurements of the masses of constituents in a preparation of molecules. To identify unknown proteins in a sample, such as that acquired by co-immunoprecipitation, the sample often is first subjected to one-dimensional SDS-PAGE or two-dimensional gel electrophoresis (see Section A-13) to separate the proteins in the complex for individual analysis. Gel slices are excised and treated with a proteolytic enzyme, such as trypsin, to digest the protein into a series of peptides that can be easily eluted from the gel. The peptide mixture is then introduced into the mass spectrometer, which ionizes the peptides, transfers them to the gas phase, and then separates them under high vacuum by subjecting them to a magnetic field. The separation is based on the mass/charge (m/z)

Fig. A.15 Western blotting is used to identify antibodies against the human immunodeficiency virus (HIV) in serum from infected individuals. The virus is dissociated into its constituent proteins by treatment with the detergent SDS, and its proteins are separated using SDS-PAGE. The separated proteins are transferred to a nitrocellulose sheet and reacted with the test serum. Anti-HIV antibodies in the serum bind to the various HIV proteins and are detected with enzyme-linked anti-human immunoglobulin, which deposits colored material from a colorless substrate. This general methodology will detect any combination of antibody and antigen and is used widely, although the denaturing effect of SDS means that the technique works most reliably with antibodies that recognize the antigen when it is denatured.

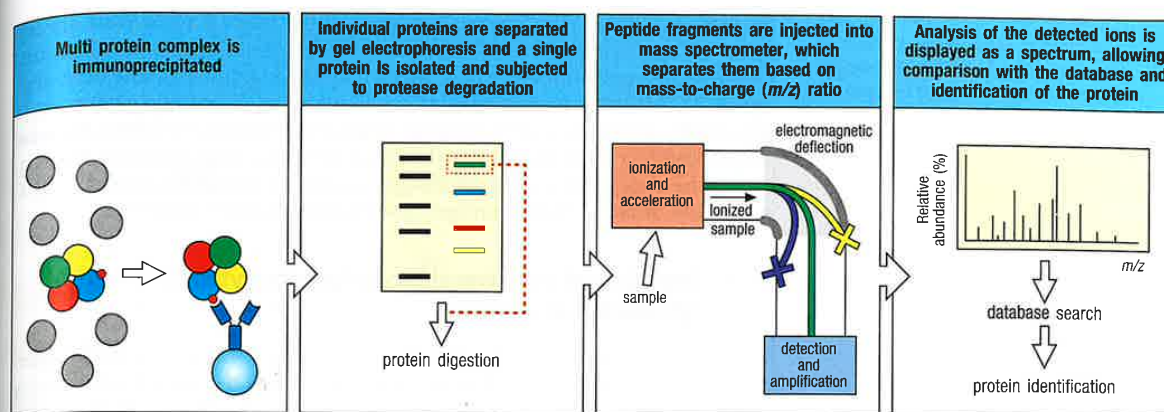


Fig. A.16 Characterization of multiprotein complexes by mass spectrometry. Following immunoprecipitation of a multiprotein complex using antibodies specific for one component of the complex, the individual proteins are separated by gel electrophoresis. An individual band representing one protein is isolated and digested with a protease such as trypsin. The digested protein sample is injected into the mass spectrometer, which ionizes the peptides, transfers them to the gas phase, and then separates them based

on differences in their mass to charge (m/z) ratio by subjecting them under high vacuum to a magnetic field. A detector collects information on the signal intensity for each peptide ion and displays the information as a histogram. This histogram, usually referred to as a spectrum, is compared with a database containing potential cleavage sites for the proteolytic enzyme used in all known protein sequences, allowing for identification (ID) of the protein in the sample.

ratio of each ion, and a detector collects information on the signal intensity for each ion and displays the data as a histogram (Fig. A.16). This histogram, usually referred to as a spectrum, can be compared with a database containing potential cleavage sites (for the proteolytic enzyme used) in all known protein sequences. Due to the precision of these measurements, and the information derived from multiple peptides comprising the initial protein, the spectrum can often be unambiguously assigned to a unique protein in the database.

Modern multidimensional mass spectrometers (MS/MS) allow peptide ions to be sequenced as well as analyzed by their mass. In these instruments, peptide ions separated in one sector are fragmented in a second sector by collision with other molecules (often an inert gas such as N_2), with the resultant fragments separated in a third section (Fig. A.17). Fragmentation occurs primarily in the

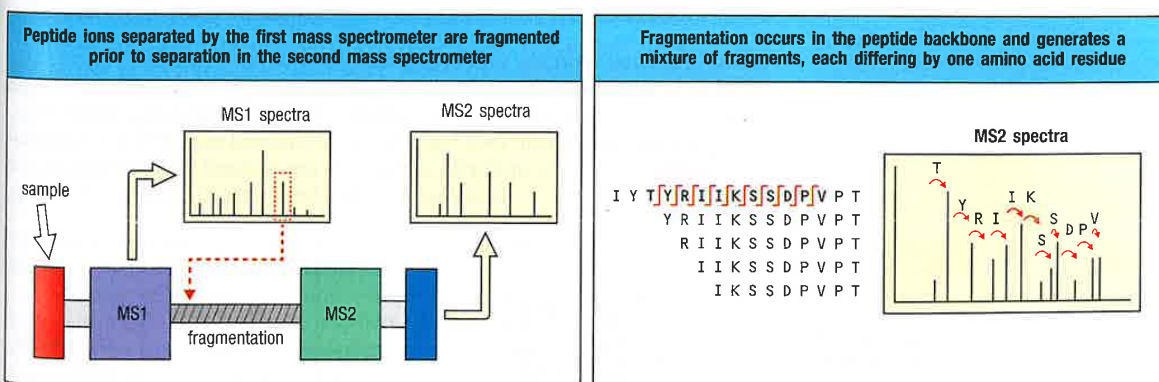


Fig. A.17 Determining the amino acid sequence of a peptide by multidimensional mass spectrometry (MS/MS). Multidimensional mass spectrometers (MS/MS) consist of two mass spectrometers linked in tandem but with an interceding middle sector that fragments ions. In the first sector, the first mass spectrometer separates peptide ions, as shown in Fig. A.16. Each peptide ion from this first separation is then fragmented in the middle sector of the apparatus by collision with other molecules (often an inert gas such as N_2). Since fragmentation occurs primarily in the peptide backbone,

a mixture of fragments is generated in which the fragments each differ by one amino acid residue. The resultant fragments are then separated in the second mass spectrometer, the final sector. The sequence of the peptide can be read directly from the second mass spectrum. The order of amino acid residues in the peptide can be deduced because of the exquisite precision of the measurements of each ion together with knowledge of the exact mass of each possible amino acid residue.

peptide backbone, allowing the sequence of the peptide to be read directly from the mass spectrum of the mixture of fragments. In place of gel electrophoresis, a liquid chromatograph can be employed upstream of the mass analyzers (LC-MS/MS), to provide additional separation of peptides prior to mass separation and allow a very complex mixture of thousands of peptides to be sequenced in a single run. It was this latter technique that played an essential role in the early studies that identified the repertoire of peptides bound to MHC molecules on the surface of antigen-presenting cells (see Chapter 6).

A-16 Isolation of peripheral blood lymphocytes by density-gradient fractionation.

The first step in studying lymphocytes is to isolate them so that their behavior can be analyzed *in vitro*. Human lymphocytes can be isolated most readily from peripheral blood by density centrifugation over a step gradient consisting of a mixture of the carbohydrate polymer Ficoll-Hypaque™ and the dense iodine-containing compound metrizamide. A step gradient is made by preparing a solution of Ficoll-Hypaque at a precise density (1.077 g/liter for human cells) and placing a layer of this solution at the bottom of a centrifuge tube. A sample of heparinized blood mixed with saline (heparin prevents clotting) is carefully layered on top of the Ficoll-Hypaque solution. Following centrifugation for about 30 minutes, the components of the blood have separated based on their densities. The upper layer contains the blood plasma and platelets, which remain in the top layer during the short centrifugation. Red blood cells and granulocytes have a higher density than the Ficoll-Hypaque solution and collect at the bottom of the tube. The resulting population, called **peripheral blood mononuclear cells (PBMCs)**, collects at the interface between the blood and the Ficoll-Hypaque layers and consists mainly of lymphocytes and monocytes (Fig. A.18). Although this population is readily accessible, it is not necessarily representative of the lymphoid system, because only recirculating lymphocytes can be isolated from blood.

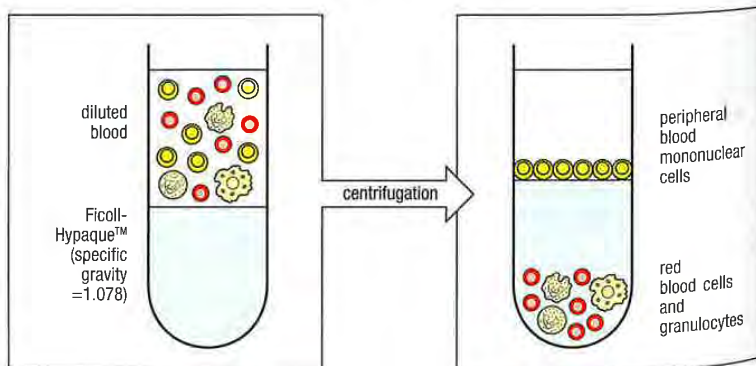
The 'normal' ranges in the numbers of the different types of white blood cells in blood, along with the normal ranges in concentrations of the various antibody classes, are given in Fig. A.19.

A-17 Isolation of lymphocytes from tissues other than blood.

In experimental animals, and occasionally in humans, lymphocytes are isolated from lymphoid organs, such as spleen, thymus, bone marrow, lymph nodes, or mucosal-associated lymphoid tissues—in humans, most commonly the palatine tonsils (see Fig. 12.6). A specialized population of lymphocytes resides in surface epithelia; these cells are isolated by fractionating the

Fig. A.18 Peripheral blood mononuclear cells can be isolated from whole blood by Ficoll-Hypaque™ centrifugation.

Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque™ and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are denser and travel through the Ficoll-Hypaque™, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface (right panel).



Evaluation of the cellular components of the human immune system					
	B cells		T cells		Phagocytes
Normal numbers ($\times 10^9$ per liter of blood)	Approximately 0.3		Total 1.0–2.5 CD4 0.5–1.6 CD8 0.3–0.9		Monocytes 0.15–0.6 Polymorphonuclear leukocytes: Neutrophils 3.00–5.5 Eosinophils 0.05–0.25 Basophils 0.02
Measurement of function <i>in vivo</i>	Serum Ig levels Specific antibody levels		Skin test		—
Measurement of function <i>in vitro</i>	Induced antibody production in response to pokeweed mitogen		T-cell proliferation in response to phytohemagglutinin or to tetanus toxoid		Phagocytosis Nitroblue tetrazolium uptake Intracellular killing of bacteria
	Evaluation of the humoral components of the human immune system				
	Immunoglobulins				Complement
Component	IgG	IgM	IgA	IgE	
Normal levels	600–1400 $\text{mg} \cdot \text{dl}^{-1}$	40–345 $\text{mg} \cdot \text{dl}^{-1}$	60–380 $\text{mg} \cdot \text{dl}^{-1}$	0–200 $\text{IU} \cdot \text{ml}^{-1}$	CH_{50} of 125–300 $\text{IU} \cdot \text{ml}^{-1}$

epithelial layer after its detachment from the basement membrane. Finally, in situations where local immune responses are prominent, lymphocytes can be isolated from the site of the response itself. For example, in order to study the autoimmune reaction that is thought to be responsible for rheumatoid arthritis, an inflammatory response in joints, lymphocytes are isolated from the fluid aspirated from the inflamed joint space.

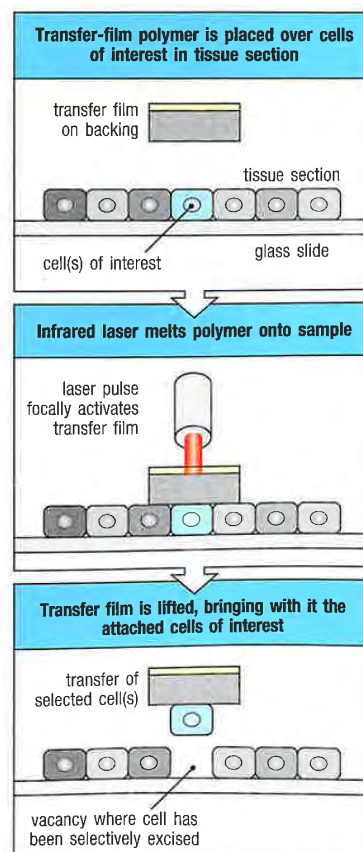
Laser-capture microdissection is a technique used to isolate specific populations of cells from an intact tissue sample or histological specimen after visualizing the cells by light microscopy. The cells of interest can be 'captured' by placing a polymer over the sample on the microscope slide, and using an infrared laser to melt the polymer onto the sample in discrete locations. Once completed, the polymer-cell composite can be lifted and DNA, RNA, or proteins can be isolated from the dissected cells (Fig. A.20). A variant of this approach makes use of an ultraviolet (UV) laser, rather than infrared. In this case, the UV laser acts as a molecular cutting tool, and can be used to cut away or even ablate the unwanted portions of the tissue, leaving intact the area of interest.

A-18 Flow cytometry and FACS analysis.

An immensely powerful tool for defining and enumerating populations of immune cells is the flow cytometer, which detects and counts individual cells passing in a stream through a laser beam. A flow cytometer equipped to separate the identified cells is called a **fluorescence-activated cell sorter (FACS)**. These instruments are used to study the properties of cell subsets that are identified by using monoclonal antibodies against cell-surface or intracellular proteins. Individual cells within a mixed population are first labeled by

Fig. A.20 Laser-capture microdissection. Specific populations of cells from an intact tissue sample or histological specimen can be isolated after visualizing the cells by light microscopy. A polymer called the transfer film is placed over the sample on the microscope slide, and an infrared laser is used to melt the polymer onto the sample in discrete locations. The polymer-cell composite is then lifted and the cells of interest are isolated. DNA, RNA, or proteins can be prepared from the dissected cells.

Fig. A.19 The major cellular and humoral components of human blood. Human blood contains B cells, T cells, and myeloid cells, as well as high concentrations of antibodies and complement proteins.



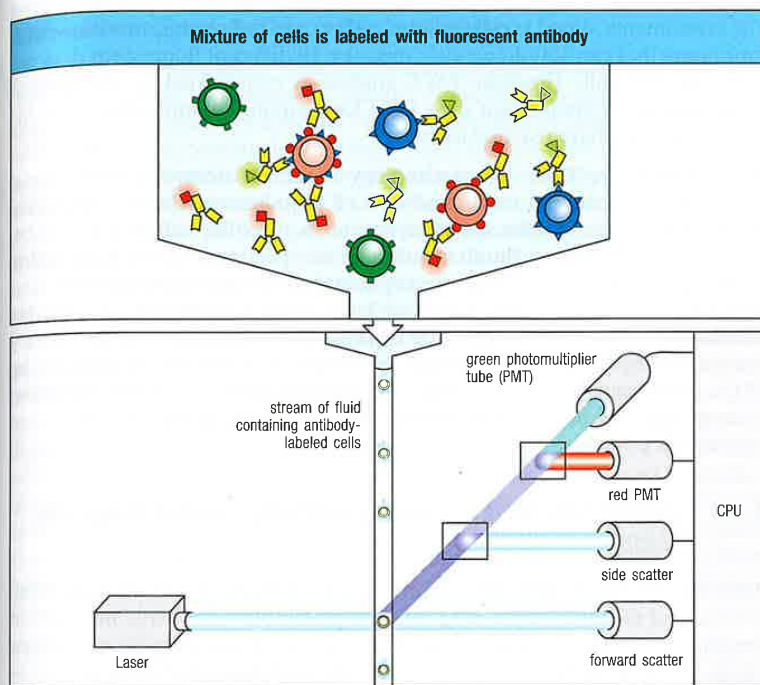
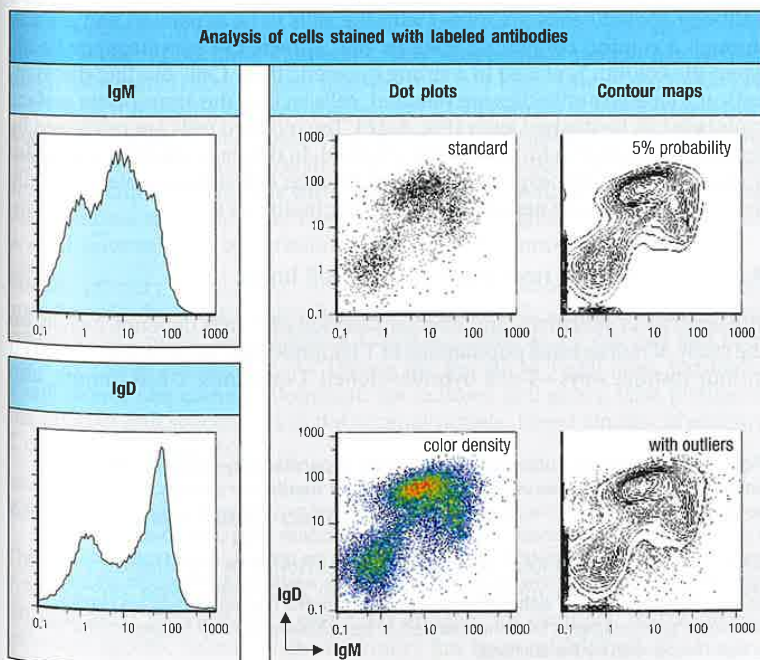
treatment with specific monoclonal antibodies coupled to fluorescent dyes, or by specific antibodies followed by fluorescently tagged anti-immunoglobulin antibodies. The mixture of labeled cells is then suspended in a much larger volume of saline and forced through a small nozzle, creating a fine stream of liquid composed of droplets, each containing a single cell. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell are excited and fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information about the size and granularity of the cell, and the fluorescence emissions, which give information about the binding of the labeled monoclonal antibodies and hence about the expression of cell-surface or intracellular proteins by each cell (Fig. A.21).

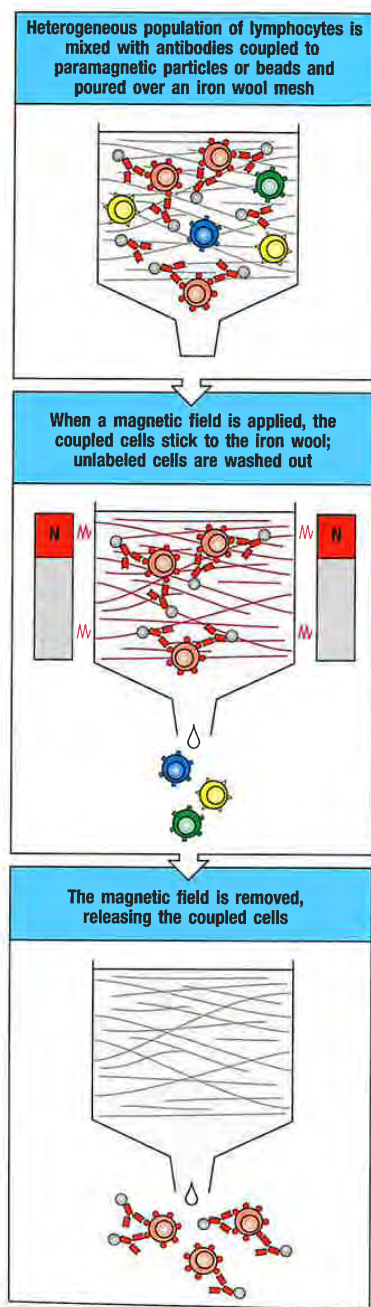
In the cell sorter, the signals passed back to the computer are used to generate an electric charge, which is passed from the nozzle through the liquid stream at the precise time that the stream breaks up into droplets, each containing no more than a single cell; droplets containing a charge can then be deflected from the main stream of droplets as they pass between plates of opposite charge, so that positively charged droplets are attracted to a negatively charged plate, and vice versa. Once deflected, droplets containing cells are collected in tubes. In this way, specific subpopulations of cells, distinguished by the binding of the labeled antibody, can be purified from a mixed population of cells. Alternatively, to deplete a population of cells, the same fluorochrome can be used to label different antibodies directed at marker proteins expressed by the various undesired cell types. The cell sorter can be used to direct labeled cells to a waste channel, retaining only the unlabeled cells.

When cells are labeled with a single fluorescent antibody, the data from a flow cytometer are usually displayed in the form of a one-dimensional histogram of fluorescence intensity versus cell numbers. If two or more antibodies are used, each coupled to a different fluorescent dye, then the data are usually

Fig. A.21 Flow cytometers allow individual cells to be identified by their cell-surface antigens and to be sorted. Cells to be analyzed by flow cytometry are first labeled with fluorescent dyes (top panel). Direct labeling uses dye-coupled antibodies specific for cell-surface antigens (as shown here), while indirect labeling uses a dye-coupled immunoglobulin to detect unlabeled cell-bound antibody. The cells are forced through a nozzle in a single-cell stream that passes through a laser beam (second panel). Photomultiplier tubes (PMTs) detect the scattering of light, which is a sign of cell size and granularity, as well as emissions from the different fluorescent dyes. This information is analyzed by computer (CPU). By examining a large number of cells, the proportion of cells with a specific set of characteristics can be determined and levels of expression of various molecules on these cells can be measured. The lower part of the figure shows how these data can be represented, the example in this case being the expression of two surface immunoglobulins, IgM and IgD, on a sample of B cells from a mouse spleen. The two immunoglobulins have been labeled with different-colored dyes. When the expression of just one type of molecule is to be analyzed (IgM or IgD), the data are usually displayed as a histogram, as in the left-hand panels. Histograms display the distribution of cells expressing a single measured parameter (for example, size, granularity, fluorescence intensity). When two or more parameters are measured for each cell (IgM and IgD), various types of two-dimensional plots can be used to display the data, as shown in the right-hand panel. All four plots represent the same data, and in each case, the horizontal axis represents intensity of IgM fluorescence, and the vertical axis the intensity of IgD fluorescence. Two-color plots provide more information than histograms; they allow recognition, for example, of cells that are 'bright' for both colors, 'dull' for one and bright for the other, dull for both, negative for both, and so on. For example, the cluster of dots in the extreme lower left portions of the plots represents cells that do not express either immunoglobulin, and are mostly T cells. The standard dot plot (upper left) places a single dot for each cell whose fluorescence is measured. This format works well for identifying cells that lie outside the main groups, but tends to saturate in areas containing a large number of cells of the same type. A second means of presenting these data is the color dot plot (lower left), which uses color density to indicate high-density areas. A contour plot (upper right) draws 5% 'probability' contours, with contour lines drawn to indicate each successive 5% of the population; this format provides the best monochrome visualization of regions of high and low density. The lower right plot is a 5% probability contour map, which also shows outlying cells as dots.

displayed in the form of a two-dimensional scatter diagram or as a contour diagram, where the fluorescence of one dye-labeled antibody is plotted against that of a second, with the result that a population of cells labeled with one antibody can be further subdivided by its labeling with the second antibody (see Fig. A.21). By examining large numbers of cells, flow cytometry can give quantitative data on the percentage of cells bearing different





proteins, such as surface immunoglobulin, which characterizes B cells; the T-cell receptor-associated molecules known as CD3; and the CD4 and CD8 co-receptor proteins that distinguish the major T-cell subsets. Likewise, FACS analysis has been instrumental in defining stages in the early development of B and T cells. This technology also played a vital role in the early identification of AIDS as a disease in which T cells bearing CD4 are depleted selectively (see Chapter 13). Advances in FACS technology permit progressively more antibodies labeled with distinct fluorescent dyes to be used at the same time. For experiments aimed at cell analysis, rather than cell sorting, machines with four lasers that can simultaneously measure 18 different fluorescent dyes are currently available. However, FACS analysis is constrained by the spectral properties of the fluorescent dyes used for coupling to antibodies, and this technology may have reached its limit.

An alternative to FACS is a technology based on detecting heavy-metal atoms that are coupled to antibodies. Cell populations labeled with heavy metal-coupled antibodies are analyzed on a machine called a CyTOF™, which combines liquid fluidics with a mass spectrometer. As each cell is analyzed, the quantity of each heavy metal associated with that cell, and thus the abundance of the target of each antibody, is measured. In total, this technology is estimated to have the capability to measure 100 distinct heavy metals, greatly expanding the range of analysis that is currently possible by FACS. However, with this technique, the cells are destroyed by the ionization process required for the mass spectrometry analysis, so the CyTOF cannot function as a cell sorter.

A-19 Lymphocyte isolation using antibody-coated magnetic beads.

Although FACS is superb for isolating small numbers of cells in pure form, mechanical means of separating cells are preferable when large numbers of lymphocytes must be prepared quickly. A powerful and efficient way of isolating lymphocyte populations is to couple paramagnetic beads to monoclonal antibodies that recognize distinguishing cell-surface molecules. These antibody-coated beads are mixed with the cells to be separated and are run through a column containing material that attracts the paramagnetic beads when the column is placed in a strong magnetic field. Cells binding the magnetically labeled antibodies are retained; cells lacking the appropriate surface molecule can be washed away (Fig. A.22). The retained cells are recovered by removing the column from the magnetic field. In this case, the bound cells are positively selected for expression of the particular cell-surface molecule, while the unbound cells are negatively selected for its absence.

A-20 Isolation of homogeneous T-cell lines.

The analysis of specificity and effector function of T cells depends heavily on the study of monoclonal populations of T lymphocytes. These can be obtained in four distinct ways—T-cell hybrids, cloned T-cell lines, T-cell tumors, and

Fig. A.22 Lymphocyte subpopulations can be separated physically by using antibodies coupled to paramagnetic particles or beads. A mouse monoclonal antibody specific for a particular cell-surface molecule is coupled to paramagnetic particles or beads. It is mixed with a heterogeneous population of lymphocytes and poured over an iron wool mesh in a column. A magnetic field is applied so that the antibody-bound cells stick to the iron wool while cells that have not bound antibody are washed out; these cells are said to be negatively selected for lack of the molecule in question. The bound cells are released by removing the magnetic field; they are said to be positively selected for presence of the antigen recognized by the antibody.

limiting-dilution culture. By analogy with B-cell hybridomas (see Section A-7), normal T cells proliferating in response to specific antigen can be fused to malignant T-cell lymphoma lines to generate **T-cell hybrids**. The hybrids express the receptor of the normal T cell, but proliferate indefinitely owing to the cancerous state of the lymphoma parent. T-cell hybrids can be cloned to yield a population of cells all having the same T-cell receptor. When stimulated by their specific antigen, these cells release cytokines such as the T-cell growth factor interleukin-2 (IL-2), and the production of cytokines is used as an assay to assess the antigen specificity of the T-cell hybrid.

T-cell hybrids are excellent tools for the analysis of T-cell specificity, because they grow readily in suspension culture. However, they cannot be used to analyze the regulation of specific T-cell proliferation in response to antigen because they are continually dividing. T-cell hybrids also cannot be transferred into an animal to test for function *in vivo* because they would give rise to tumors. Functional analysis of T-cell hybrids is also confounded by the fact that the malignant partner cell affects their behavior in functional assays. Therefore, the regulation of T-cell growth and the effector functions of T cells must be studied using **T-cell clones**. These are clonal cell lines of a single T-cell type and antigen specificity, which are derived from cultures of heterogeneous T cells, called **T-cell lines**, whose growth is dependent on periodic restimulation with specific antigen and, frequently, on the addition of T-cell growth factors (Fig. A.23). T-cell clones also require periodic restimulation with antigen and are more tedious to grow than T-cell hybrids, but because their growth depends on specific antigen recognition, they maintain antigen specificity, which is often lost in T-cell hybrids. Cloned T-cell lines can be used for studies of effector function both *in vitro* and *in vivo*. In addition, the proliferation of T cells, a critical aspect of clonal selection, can be characterized only in cloned T-cell lines, where such growth is dependent on antigen recognition. Thus, both types of monoclonal T-cell lines, T-cell hybrids and antigen-dependent T-cell clones, have valuable applications in experimental studies.

Studies of human T cells have relied largely on T-cell clones because a suitable fusion partner for making T-cell hybrids has not been identified. However, a human T-cell lymphoma line, called Jurkat, has been characterized extensively because it secretes IL-2 when its antigen receptor is cross-linked with anti-receptor monoclonal antibodies. This simple assay system has yielded much information about signal transduction in T cells. One of the Jurkat cell line's most interesting features, shared with T-cell hybrids, is that it stops growing when its antigen receptor is cross-linked. This has allowed mutants lacking the receptor or having defects in signal transduction pathways to be selected simply by culturing the cells with anti-receptor antibody and selecting those that continue to grow. Thus, T-cell tumors, T-cell hybrids, and cloned T-cell lines all have valuable applications in experimental immunology.

Finally, primary T cells from any source can be isolated as single, antigen-specific cells by limiting dilution (see Section A-21) rather than by first establishing a mixed population of T cells in culture as a T-cell line and then deriving clonal subpopulations. During the growth of T-cell lines, particular T-cell clones can come to dominate the cultures and give a false picture of the number and specificities in the original sample. Direct cloning of primary T cells avoids this artifact.

A-21 Limiting-dilution culture.

On many occasions it is important to know the frequency of antigen-specific lymphocytes, especially T cells, in order to measure the efficiency with which an individual responds to a particular antigen, for example, or the degree to which specific immunological memory has been established. There are a

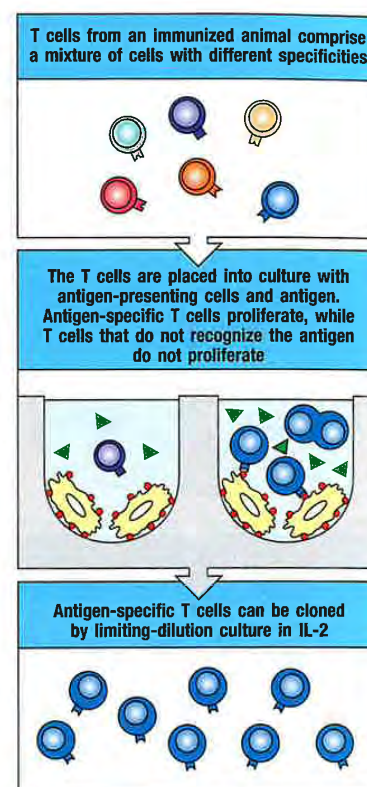


Fig. A.23 Production of cloned T-cell lines. T cells from an immunized donor, comprising a mixture of cells with different specificities, are activated with antigen and antigen-presenting cells. Single responding cells are cultured by limiting dilution (see Section A-21) in the T-cell growth factor IL-2, which selectively stimulates the responding cells to proliferate. From these single cells, cloned lines specific for antigen are identified and can be propagated by culture with antigen, antigen-presenting cells, and IL-2.

number of methods for doing this, either by detecting the cells directly by the specificity of their receptor, or by detecting activation of the cells to provide some particular function, such as cytokine secretion or cytotoxicity.

The response of a lymphocyte population is a measure of the overall response, but the frequency of lymphocytes able to respond to a given antigen can be determined by **limiting-dilution culture**. This assay makes use of the Poisson distribution, a statistical function that describes how objects are distributed at random. For instance, when a sample of heterogeneous T cells is distributed equally into a series of culture wells, some wells will receive no T cells specific for a given antigen, some will receive one specific T cell, some two, and so on. The T cells in the wells are activated with specific antigen, antigen-presenting cells, and growth factors. After allowing several days for their growth and differentiation, the cells in each well are tested for a response to antigen, such as cytokine release or the ability to kill specific target cells (Fig. A.24). The assay is replicated with different numbers of T cells in the samples. The logarithm of the proportion of wells in which there is no response is plotted against the number of cells initially added to each well. If cells of one type, typically antigen-specific T cells because of their rarity, are the only limiting factor for obtaining a response, then a straight line is obtained. From the Poisson distribution, it is known that there is, on average, one antigen-specific cell per well when the proportion of negative wells is 37%. Thus, the frequency of antigen-specific cells in the population equals the reciprocal of the number of cells added to each well when 37% of the wells are negative. After priming, the frequency of specific cells goes up substantially, reflecting the antigen-driven proliferation of antigen-specific cells. The limiting-dilution assay can also be used to measure the frequency of B cells that can make antibody against a given antigen.

Fig. A.24 The frequency of specific lymphocytes can be determined using limiting-dilution assay. Various numbers of lymphoid cells from normal or immunized mice are added to individual culture wells and stimulated with antigen and antigen-presenting cells (APCs) or polyclonal mitogen and added growth factors. After several days, the wells are tested for a specific response to antigen, such as cytotoxic killing of target cells. Each well that initially contained a specific T cell will make a response to its target, and from the Poisson distribution one can determine that when 37% of the wells are negative, each well contained, on average, one specific T cell at the beginning of the culture. In the example shown, for the unimmunized mouse 37% of the wells are negative when 160,000 T cells have been added to each well; thus the frequency of antigen-specific T cells is 1 in 160,000. When the mouse is immunized, 37% of the wells are negative when only 1100 T cells have been added; hence the frequency of specific T cells after immunization is 1 in 1100, an increase in responsive cells of 150-fold.

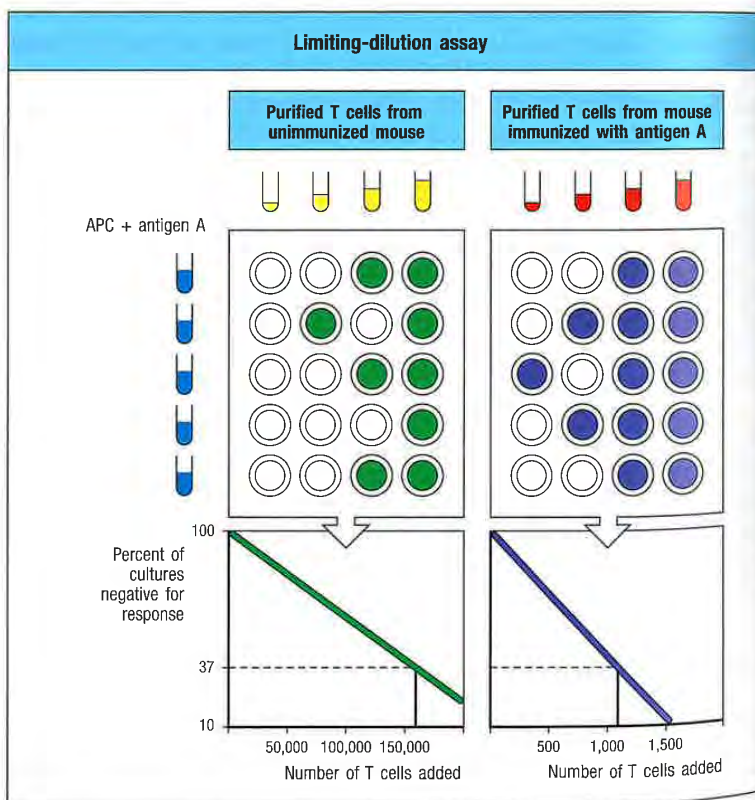


Fig. A.25 The frequency of cytokine-secreting T cells can be determined by the ELISPOT assay. The ELISPOT assay is a variant of the ELISA assay in which antibodies bound to a plastic surface are used to capture cytokines secreted by individual T cells. Usually, cytokine-specific antibodies are bound to the surface of a plastic tissue-culture well and the unbound antibodies are removed (top panel). Activated T cells are then added to the well and settle onto the antibody-coated surface (second panel). If a T cell is secreting the appropriate cytokine, this will then be captured by the antibody molecules on the plate surrounding the T cell (third panel). After a period of time the T cells are removed, and the presence of the specific cytokine is detected using an enzyme-labeled second antibody specific for the same cytokine. Where this antibody binds, a colored reaction product can be formed (fourth panel). Each T cell that originally secreted the cytokine gives rise to a single spot of color, hence the name of the assay. The results of such an ELISPOT assay for T cells secreting IFN- γ in response to different stimuli are shown in the last panel. In this example, T cells from a stem-cell transplant recipient were treated with a control peptide (top two panels) or a peptide from cytomegalovirus (bottom two panels). As can be seen, there are a greater number of spots in the bottom two panels, a clear indication that the patient's T cells are able to respond to the viral peptide and produce IFN- γ . Photographs courtesy of S. Nowack.

A-22 ELISPOT assay.

A modification of the ELISA antigen-capture assay (see Section A-4), called the **ELISPOT assay**, is a powerful tool for measuring the frequency of T-cell responses and also provides information about the cytokines produced. Populations of T cells are stimulated with the antigen of interest, and are then allowed to settle onto a plastic plate coated with antibodies against the cytokine that is to be assayed (Fig. A.25). If an activated T cell is secreting that cytokine, the cytokine is captured by the antibody on the plastic plate. After a period of time the cells are removed, and a second antibody against the cytokine is added to the plate to reveal a circle ('spot') of bound cytokine surrounding the position of each activated T cell; it is these circles that give the ELISPOT assay its name. By counting each spot and knowing the number of T cells originally added to the plate, one can easily calculate the frequency of T cells secreting that particular cytokine. ELISPOT can also be used to detect specific antibody secretion by B cells, in this case by using antigen-coated surfaces to trap specific antibody and labeled anti-immunoglobulin to detect the bound antibody.

A-23 Identification of functional subsets of T cells based on cytokine production or transcription factor expression.

One problem with the detection of cytokine production on a single-cell level is that the cytokines are secreted by the T cells into the surrounding medium, and any association with the originating cell is lost. Three methods have been devised that allow the cytokine profile produced by individual cells to be determined. The first, that of **intracellular cytokine staining** (Fig. A.26), relies on the use of metabolic poisons that inhibit protein export from the cell. The cytokine thus accumulates within the endoplasmic reticulum and vesicular network of the cell. If the cells are subsequently fixed and rendered permeable by the use of mild detergents, antibodies can gain access to these intracellular compartments and detect the cytokine. The T cells can be stained for other markers simultaneously, and thus the frequency of IL-10-producing CD25⁺ CD4 T cells, for example, can be easily obtained.

A second method, which has the advantage that the cells being analyzed are not killed in the process, is called cytokine capture. This technique uses hybrid antibodies, in which the two separate heavy- and light-chain pairs from different antibodies are combined to give a mixed antibody molecule in which

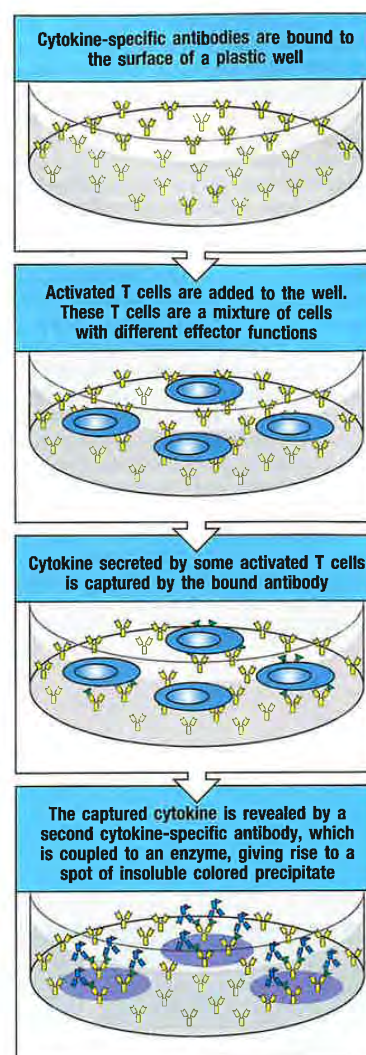
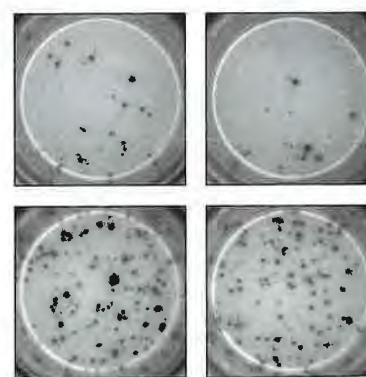
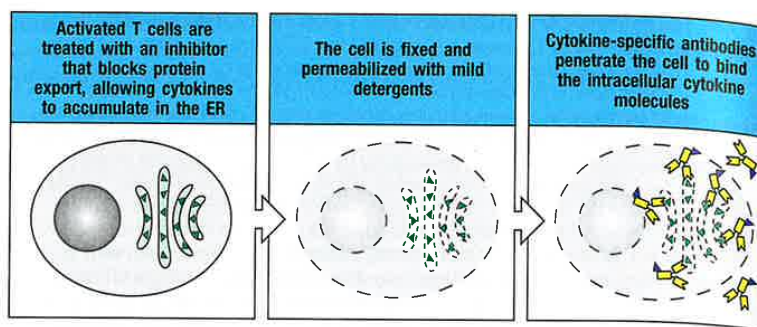


Fig. A.26 Cytokine-secreting cells can be identified by intracellular cytokine staining. Fluorochrome-labeled antibodies can be used to detect the cytokines secreted by activated T cells after the cytokine molecules have been allowed to accumulate inside the cell. The accumulation of cytokine molecules to a high enough concentration for efficient detection is achieved by treating the activated T cells with inhibitors of protein export. In such treated cells, proteins destined to be secreted are instead retained within the endoplasmic reticulum (left panel). These treated cells are then fixed, to cross-link the proteins inside the cell and in the cell membranes, so that they are not lost when the cell is permeabilized by dissolving the cell membrane in a mild detergent (center panel). Fluorochrome-labeled antibodies can now enter the permeabilized cell and bind to the cytokines inside the cell (right panel). Cells labeled in this way can also be labeled with antibodies that bind to cell-surface proteins to determine which subsets of T cells are secreting particular cytokines.



the two antigen-binding sites recognize different ligands (Fig. A.27). In the bispecific antibodies used to detect cytokine production, one of the antigen-binding sites is specific for a T-cell surface marker, while the other is specific for the cytokine in question. The bispecific antibody binds to the T cells through the binding site for the cell-surface marker, leaving the cytokine-binding site free. If that T cell is secreting the particular cytokine, it is captured by the bound antibody before it diffuses away from the surface of the cell. It can then be detected by adding to the cells a fluorochrome-labeled second antibody specific for the cytokine.

A third method for identifying which T cells in a population produce a particular cytokine utilizes cytokine gene reporter mice. In these lines of mice, a cDNA clone encoding a readily detectable protein (the 'reporter' protein) is inserted into the 3' untranslated region of the targeted cytokine gene downstream of a sequence known as an internal ribosome entry site (IRES). The IRES sequence allows translation of the reporter protein from the same mRNA as that encoding the cytokine; thus, the reporter protein is produced only when the cytokine mRNA is expressed (Fig. A.28). Common reporter proteins for this application are fluorescent proteins, such as green fluorescent protein (GFP). In fact, the GFP commonly used for this purpose contains

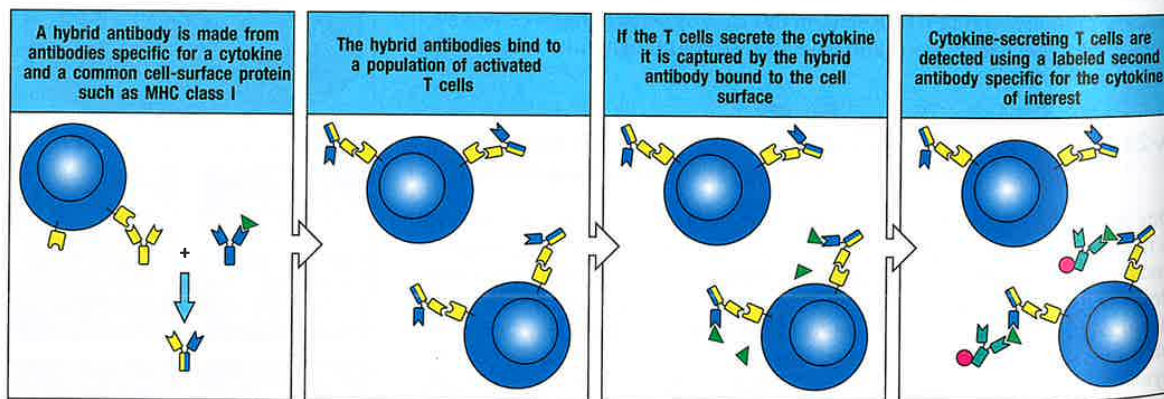


Fig. A.27 Hybrid antibodies containing cell-specific and cytokine-specific binding sites can be used to assay cytokine secretion by living cells and to purify cells secreting particular cytokines. Hybrid antibodies can be made by mixing together heavy- and light-chain pairs from antibodies of different specificities, for example, an antibody against an MHC class I molecule and an antibody specific for a cytokine such as IL-4 (first panel). The hybrid antibodies are then added to a population of activated T cells, and bind to each cell via the MHC class I binding arm (second panel). If some of the cells in the population are secreting the appropriate

cytokine, IL-4, this is captured by the cytokine-specific arm of the hybrid antibody (third panel). The presence of the cytokine can then be revealed, for example, by using a fluorochrome-labeled second antibody specific for the same cytokine but binding to a different site from the one used by the hybrid antibody (last panel). The labeled cells are analyzed by flow cytometry or are isolated using a fluorescence-activated cell sorter (FACS). Alternatively, the second cytokine-specific antibody can be coupled to magnetic beads, and the cytokine-producing cells isolated magnetically.

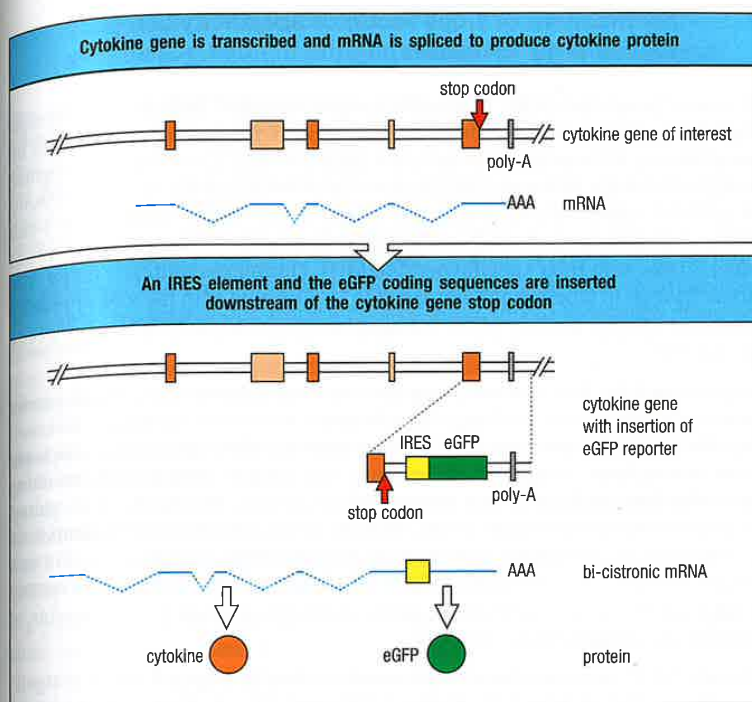
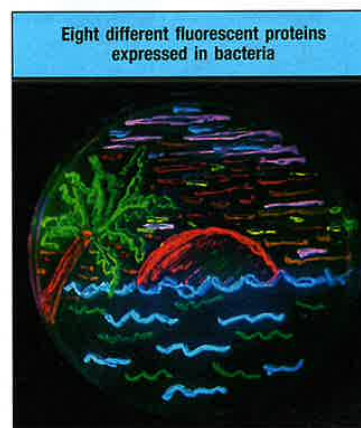


Fig. A.28 Cytokine-expressing cells can be tracked *in vivo* using cytokine gene knock-in reporter mice. To identify cells expressing a specific cytokine in intact animals, the locus encoding the cytokine is modified by homologous recombination (see Fig. A.44 and Section A-35). An internal ribosome entry site (IRES) and the gene for a fluorescent protein such as eGFP are inserted 3' of the last exon of the cytokine gene, downstream of the cytokine protein stop codon and upstream of the mRNA transcription termination and polyadenylation signal (the poly-A site). The IRES element allows the ribosome to initiate translation of a second protein-coding sequence at an internal site on the mRNA. When the modified locus is transcribed and spliced to form the mature mRNA, both the intact cytokine protein and the fluorescent reporter protein (e.g., eGFP) are produced from the same transcript. This allows the identification and characterization of cytokine-expressing cells, such as by flow cytometry, based on the detection of eGFP.

a point mutation that greatly improves its spectral properties for experimental purposes. This version of GFP is commonly referred to as 'enhanced GFP' or 'eGFP' for short. eGFP can be detected by FACS or by fluorescent microscopy using the settings designed to detect the commonly used fluorescent dye FITC. Due to broad utility of these fluorescent proteins, a host of GFP derivatives have been developed by genetic engineering of the original GFP protein. Each derivative has distinct fluorescent properties and therefore can be uniquely identified, allowing these proteins to be used in combination with each other to provide information about multiple cytokines at the same time (Fig. A.29).

Several of these techniques for measuring cytokine expression by T-cell subsets have been adapted to examine the transcription factors expressed by T cells and other lymphocytes, providing an alternative method for identifying functional lymphocyte subsets. In one approach, antibodies specific for lineage-defining transcription factors are used to label permeabilized cells. As was described above for the intracellular cytokine staining assay, the cells can then be examined by flow cytometry or immunofluorescence microscopy. Lines of reporter mice have also been generated, in which the gene locus encoding a transcription factor is modified to express a fluorescent protein, such as eGFP. For both of these approaches, the advantage of using transcription factors to identify lymphocyte subsets is that there is no need to stimulate the cells prior to antibody staining or to assessing reporter protein expression, as lineage-defining transcription factors are constitutively expressed by the cells. Therefore, this approach is more widely used for the identification of T-cell and other lymphocyte subsets in intact tissues by microscopy.

Fig. A.29 Fluorescent proteins are available in a rainbow of colors. Derivatives of GFP and a red-fluorescent coral protein can generate eight different fluorescent colors. A beach scene is drawn with bacterial strains expressing each fluorescent protein. Courtesy of Roger Tsien.



A-24 Identification of T-cell receptor specificity using peptide:MHC tetramers.

For many years, the ability to identify antigen-specific T cells directly through their receptor specificity eluded immunologists. Foreign antigen could not be used directly to identify T cells because, unlike B cells, T cells do not recognize antigen alone but rather the complexes of peptide fragments of antigen bound to self MHC molecules. Moreover, the affinity of interaction between the T-cell receptor and the peptide:MHC complex is in practice so low that attempts to label T cells with their specific peptide:MHC complexes routinely failed. The breakthrough in labeling antigen-specific T cells came with the idea of making multimers of the peptide:MHC complex, so as to increase the avidity of the interaction.

Peptides can be biotinylated using the bacterial enzyme BirA, which recognizes a specific amino acid sequence. Recombinant MHC molecules containing this target sequence are used to make peptide:MHC complexes, which are then biotinylated. Avidin, or its bacterial counterpart streptavidin, contains four sites that bind biotin with extremely high affinity. Mixing the biotinylated peptide:MHC complex with avidin or streptavidin results in the formation of a **peptide:MHC tetramer**—four specific peptide:MHC complexes bound to a single molecule of streptavidin (Fig. A.30). Routinely, the streptavidin moiety is labeled with a fluorochrome to allow detection of those T cells capable of binding the peptide:MHC tetramer.

Peptide:MHC tetramers have been used to identify populations of antigen-specific T cells in, for example, patients with acute Epstein-Barr virus infections (infectious mononucleosis), showing that up to 80% of the peripheral T cells in infected individuals can be specific for a single peptide:MHC complex. They have also been used to follow responses over timescales of years in individuals with HIV or, in the example we show, cytomegalovirus infections. These reagents have also been important in identifying the cells responding, for example, to nonclassical MHC class I molecules such as HLA-E or HLA-G, in both cases showing that these nonclassical molecules are recognized by subsets of NK receptors.

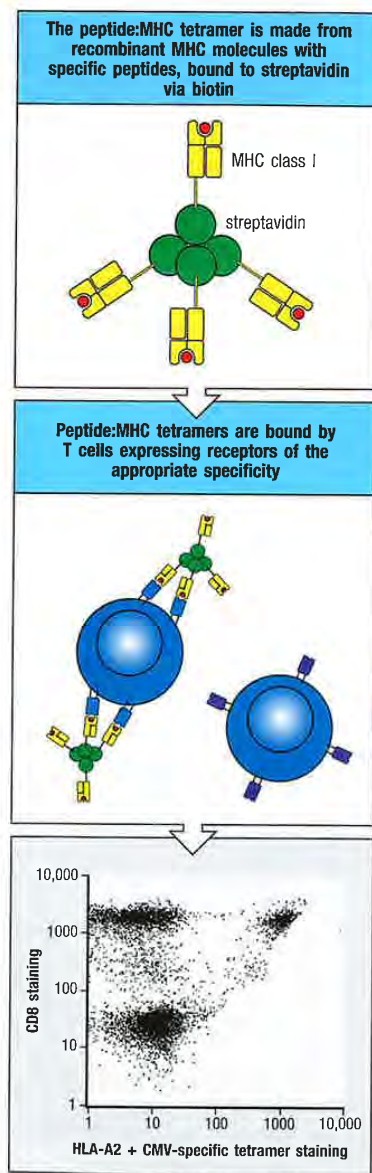


Fig. A.30 Peptide:MHC complexes coupled to streptavidin to form tetramers are able to stain antigen-specific T cells. Peptide:MHC tetramers are formed from recombinant refolded peptide:MHC complexes containing a single defined peptide epitope. MHC molecules that contain biotin can be chemically synthesized, but usually the recombinant MHC heavy chain is linked to a bacterial biotinylation sequence, a target for the *Escherichia coli* enzyme BirA, which is used to add a single biotin group to the MHC molecule. Streptavidin is a tetramer, each subunit having a single binding site for biotin; hence the streptavidin:peptide:MHC complex creates a tetramer of peptide:MHC complexes (top panel). Although the affinity between the T-cell receptor and its peptide:MHC ligand is too low for a single complex to bind stably to a T cell, the tetramer, by being able to make a more avid interaction with multiple peptide:MHC complexes binding simultaneously, is able to bind to T cells whose receptors are specific for the particular peptide:MHC complex (middle panel). Routinely, the streptavidin molecules are coupled to a fluorochrome, so that the binding to T cells can be monitored by flow cytometry. In the example shown in the bottom panel, T cells have been stained simultaneously with antibodies specific for CD3 and CD8, and with a tetramer of HLA-A2 molecules containing a cytomegalovirus peptide. Only the CD3⁺ cells are shown, with the staining of CD8 displayed on the vertical axis and the tetramer staining displayed along the horizontal axis. The CD8⁺ cells (mostly CD4⁺), on the bottom left of the figure, show no specific tetramer staining, while the bulk of the CD8⁺ cells, on the top left, likewise show no tetramer staining. However, a discrete population of tetramer positive CD8⁺ cells, at the top right of the panel, comprising some 5% of the total CD8⁺ cells, can clearly be seen. Data courtesy of G. Aubert.

A-25 Biosensor assays for measuring the rates of association and dissociation of antigen receptors for their ligands.

Two of the important questions that are always asked of any receptor-ligand interaction are, What is the strength of binding, or affinity, of the interaction, and, What are the rates of association and dissociation? These parameters are generally assessed using purified preparations of proteins. For receptors that are integral membrane proteins in their native state, soluble forms of the proteins are prepared, usually by truncating the proteins to eliminate their membrane-spanning domains. With these purified proteins, binding rates can be measured by following the binding of ligands to receptors immobilized on gold-plated glass slides, using a phenomenon known as **surface plasmon resonance (SPR)** to detect the binding (Fig. A.31). A full explanation of surface plasmon resonance is beyond the scope of this textbook, as it is based on advanced physical and quantum mechanical principles. In brief, it relies on the total internal reflection of a beam of light from the surface of a gold-coated glass slide. As the light is reflected, some of its energy excites electrons in the gold coating and these excited electrons are affected by the electric field of any molecules binding to the surface of the glass coating. The more molecules that bind to the surface, the greater is the effect on the excited electrons, and this in turn affects the reflected light beam. The reflected light thus becomes a sensitive measure of the number of atoms bound to the gold surface of the slide.

If a purified receptor is immobilized on the surface of the gold-coated glass slide, to make a biosensor 'chip,' and a solution containing the ligand is streamed over that surface, the binding of ligand to the receptor can be followed until it reaches equilibrium (see Fig. A.31). If the ligand is then washed out, dissociation of ligand from the receptor can easily be followed and the

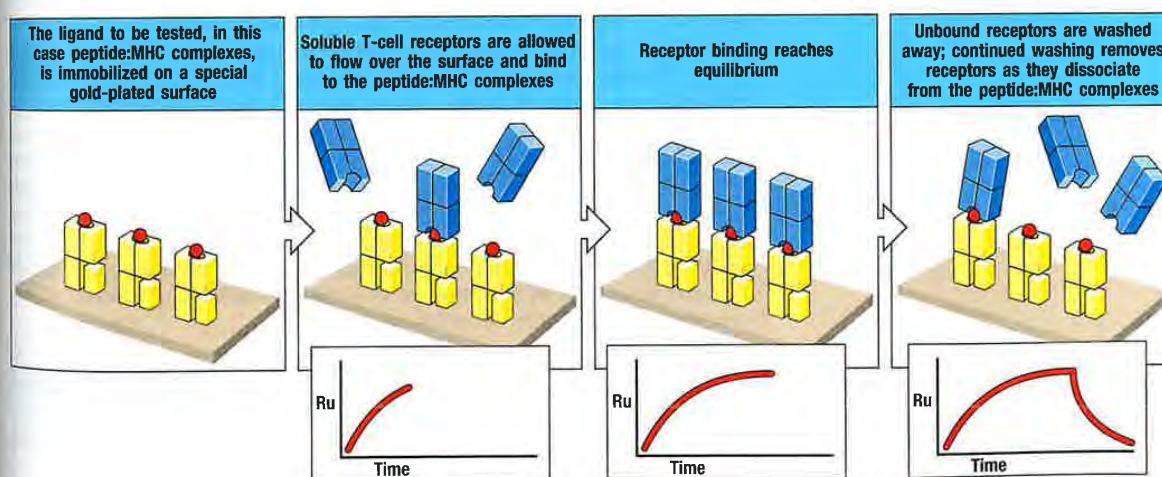


Fig. A.31 Measurement of receptor-ligand interactions can be made in real time using a biosensor. Biosensors are able to measure the binding of molecules on the surface of gold-plated glass chips through the indirect effects of the binding on the total internal reflection of a beam of polarized light at the surface of the chip. Changes in the angle and intensity of the reflected beam are measured in 'resonance units' (Ru) and plotted against time in what is termed a 'sensorgram.' Depending on the exact nature of the receptor-ligand pair to be analyzed, either the receptor or the ligand can be immobilized on the surface of the chip. In the example shown, peptide:MHC complexes are immobilized on such a surface

(first panel). T-cell receptors in solution are now allowed to flow over the surface, and to bind to the immobilized peptide:MHC complexes (second panel). As the T-cell receptors bind, the sensorgram (inset panel below the main panel) reflects the increasing amount of protein bound. As the binding reaches either saturation or equilibrium (third panel), the sensorgram shows a plateau, as no more protein binds. At this point, unbound receptors can be washed away. With continued washing, bound receptors now start to dissociate and are removed in the flow of the washing solution (last panel). The sensorgram now shows a declining curve, reflecting the rate at which receptor and ligand dissociation occurs.

Mitogen	Responding cells
Phytohemagglutinin (PHA) (red kidney bean)	T cells
Concanavalin (ConA) (jack bean)	T cells
Pokeweed mitogen (PWM) (pokeweed)	T and B cells
Lipopolysaccharide (LPS) (<i>Escherichia coli</i>)	B cells (mouse)

Fig. A.32 Polyclonal mitogens, many of plant origin, stimulate lymphocyte proliferation in tissue culture. Many of these mitogens are used to test the ability of lymphocytes in human peripheral blood to proliferate.

dissociation rate calculated. A new solution of the ligand at a different concentration can then be streamed over the chip and the binding once again measured. The affinity of binding can be calculated in a number of ways in this type of assay. Most simply, the ratio of the rates of association and dissociation will give an estimate of the affinity, but more accurate estimates can be obtained from the measurements of the binding at different concentrations of ligand. From measurements of binding at equilibrium, a Scatchard plot will give a measurement of the affinity of the receptor-ligand interaction.

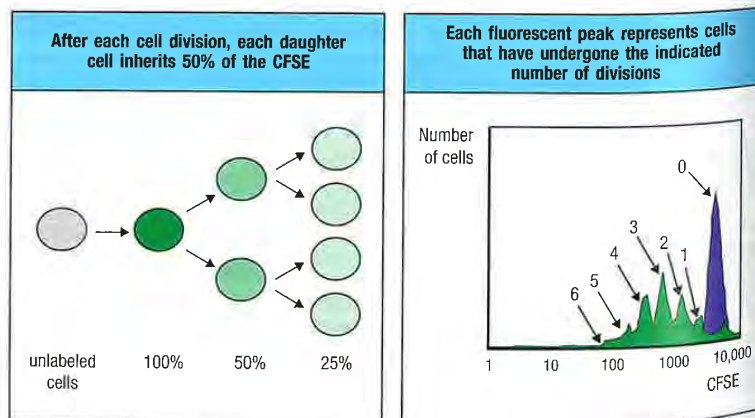
A-26 Assays of lymphocyte proliferation.

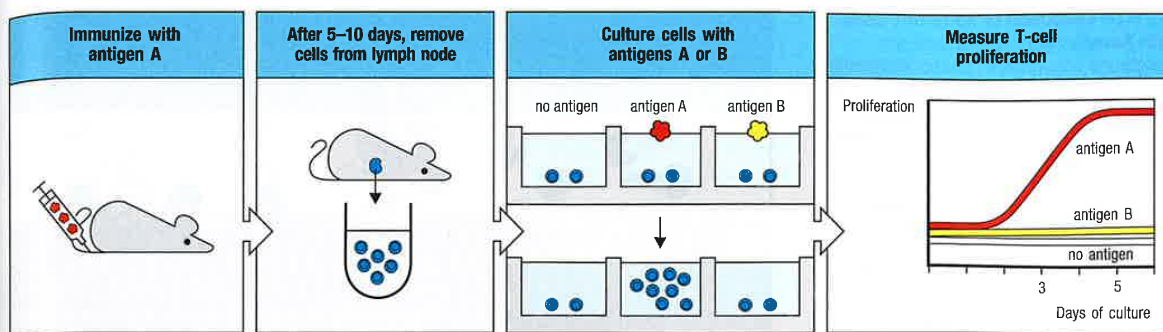
To function in adaptive immunity, rare antigen-specific lymphocytes must proliferate extensively before they differentiate into functional effector cells in order to generate sufficient numbers of effector cells of a particular specificity. Thus, the analysis of induced lymphocyte proliferation is a central issue in their study. It is, however, difficult to detect the proliferation of normal lymphocytes in response to specific antigen because only a minute proportion of cells will be stimulated to divide. Great impetus was given to the field of lymphocyte culture by the finding that certain substances induce many or all lymphocytes of a given type to proliferate. These substances are referred to collectively as **polyclonal mitogens** because they induce mitosis in lymphocytes of many different specificities or clonal origins. T and B lymphocytes are stimulated by different polyclonal mitogens (Fig. A.32). Polyclonal mitogens seem to trigger essentially the same growth response mechanisms as antigen. Lymphocytes normally exist as resting cells in the G_0 phase of the cell cycle. When stimulated with polyclonal mitogens, they rapidly enter the G_1 phase and progress through the cell cycle. In most studies, lymphocyte proliferation is most simply measured by the incorporation of ^3H -thymidine into DNA. This assay is used clinically for assessing the ability of lymphocytes from patients with suspected immunodeficiencies to proliferate in response to a nonspecific stimulus.

An alternative to the use of a radioisotope to measure lymphocyte proliferation is to use a fluorescent assay that can be performed by FACS. For this assay, the lymphocytes are incubated with a fluorescent dye such as carboxyfluorescein succinimidyl ester (CFSE). This dye enters the cell and, once in the cytosol, becomes covalently coupled to lysine residues on cellular proteins. Each time the cell divides, the amount of CFSE is cut in half, as each daughter cell inherits one-half of the CFSE-labeled proteins. When a population of dividing cells is analyzed by FACS, peaks of CFSE fluorescence can be detected, each of which represents cells that have undergone a fixed number of divisions (Fig. A.33). This assay is capable of detecting up to 7–8 cell divisions, after which CFSE fluorescence can no longer be measured.

Fig. A.33 Flow cytometric assay for cell proliferation based on CFSE dilution.

Cells are first incubated with a fluorescent dye such as carboxyfluorescein succinimidyl ester (CFSE). This dye enters the cell and, once in the cytosol, becomes covalently coupled to lysine residues on cellular proteins. Each time the cell divides, the amount of CFSE is diluted by one-half, as each daughter cell inherits one-half of the CFSE-labeled proteins. Cell division can then be analyzed by flow cytometry, where a histogram of CFSE fluorescence displays a series of peaks, each of which represents cells that have undergone a fixed number of divisions. Under optimal conditions, this assay is capable of detecting up to 7–8 cell divisions, after which CFSE fluorescence can no longer be measured.





Once lymphocyte culture had been optimized using the proliferative response to polyclonal mitogens as an assay, it became possible to detect antigen-specific T-cell proliferation in culture by measuring ^3H -thymidine uptake in response to an antigen to which the T-cell donor had previously been immunized (Fig. A.34). This is the assay most commonly used for assessing T-cell responses after immunization, but it reveals little about the functional capabilities of the responding T cells. These must be ascertained by functional assays, as outlined in Sections A-28 and A-29.

A-27 Measurements of apoptosis.

Apoptotic cells can be detected by a procedure known as **TUNEL (TdT-dependent dUTP-biotin nick end labeling) staining**. In this technique, the 3' ends of the DNA fragments generated in apoptotic cells are labeled with biotin-coupled uridine by using the enzyme terminal deoxynucleotidyl transferase (TdT). The biotin label is then detected with enzyme-tagged streptavidin, which binds to biotin. When the colorless substrate of the enzyme is added to a tissue section or cell culture, it produces a colored precipitate only in cells that have undergone apoptosis (Fig. A.35).

Additional methods are often used to detect apoptosis of cells in experimental animals. One simple method is to incubate cells with a fluorescently labeled preparation of the protein **Annexin V**. This protein has a high affinity for a specific membrane phospholipid, phosphatidylserine (PS). In healthy cells, PS is found exclusively on the inner leaflet of the plasma membrane, and is therefore inaccessible to extracellular incubation with Annexin V. When cells are undergoing apoptosis, the PS is transported to the outer cell surface, where it can be bound by the fluorescently labeled Annexin V, which is then detected

Fig. A.34 Antigen-specific T-cell proliferation is used frequently as an assay for T-cell responses. T cells from mice or humans that have been immunized with an antigen (A) proliferate when they are exposed to antigen A and antigen-presenting cells but not when cultured with unrelated antigens to which the hosts have not been immunized (antigen B). Proliferation can be measured by incorporation of ^3H -thymidine into the DNA of actively dividing cells. Antigen-specific proliferation is a hallmark of specific CD4 T-cell immunity.

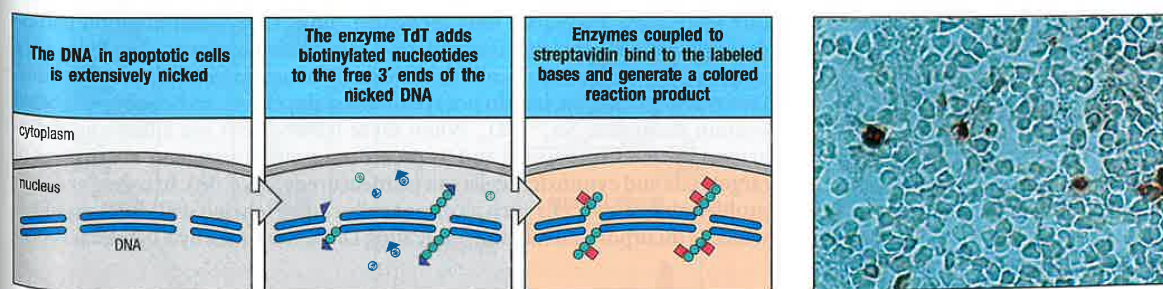
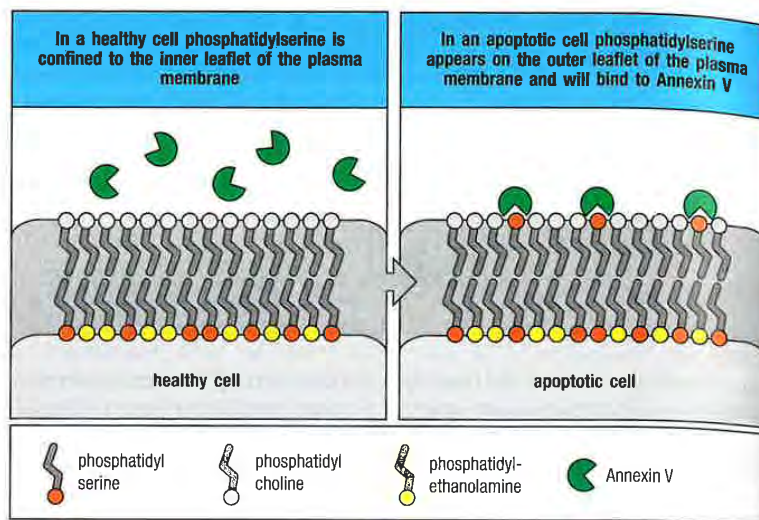


Fig. A.35 In the TUNEL assay, fragmented DNA is labeled by terminal deoxynucleotidyl transferase (TdT) to reveal apoptotic cells. When cells undergo programmed cell death, or apoptosis, their DNA becomes fragmented (first panel). The enzyme TdT is able to add nucleotides to the ends of DNA fragments; most commonly in this assay, biotin-labeled nucleotides (usually dUTP)

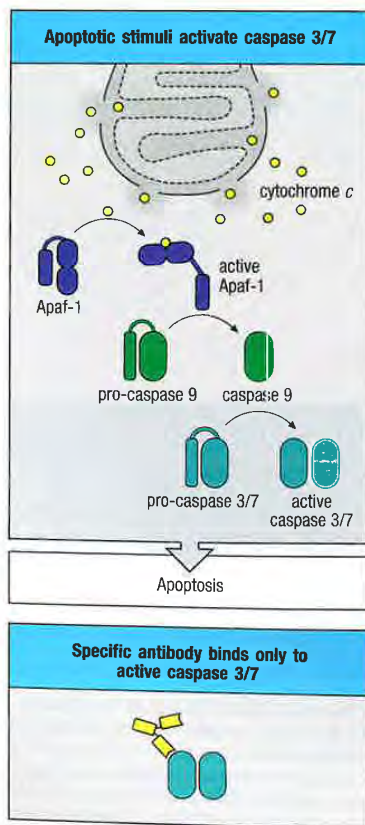
are added (second panel). The biotinylated DNA can be detected by using streptavidin, which binds to biotin, coupled to enzymes that convert a colorless substrate into a colored insoluble product (third panel). Cells stained in this way can be detected by light microscopy, as shown in the photograph of apoptotic cells (stained red) in the thymic cortex. Photograph courtesy of R. Budd and J. Russell.

Fig. A.36 Detection of apoptotic cells with Annexin V. In healthy cells, the membrane phospholipid phosphatidylserine is oriented with its polar headgroup facing the cytosolic face of the plasma membrane. When cells undergo apoptosis, the enzyme responsible for maintaining phosphatidylserine polarity, called flippase, is no longer active. As a result, phosphatidylserine becomes randomly oriented, with many molecules exposing their polar head groups on the extracellular face of the plasma membrane. The protein Annexin V binds tightly to the exposed phosphatidylserine and, if fluorescently labeled, can be used to detect apoptotic cells by FACS.



by FACS (Fig. A.36). Annexin V staining is often combined with a viability dye such as propidium iodide (PI) or 7-aminoactinomycin D (7-AAD). These two dyes fluoresce when bound to DNA, but are unable to enter viable or apoptotic cells prior to their loss of membrane integrity. Therefore, when combined with Annexin V, cells in the early stages of apoptosis can be identified as Annexin V-positive but PI/7-AAD-negative, whereas cells in the late stages of apoptosis are both Annexin V-positive and PI/7-AAD-positive.

An additional assay that provides a sensitive means of detecting apoptotic cells by FACS analysis is based on the detection of activated caspase 3, a cysteine protease that functions in the execution phase of the apoptotic cell death program. Caspase 3 is initially synthesized by cells in an inactive precursor form called a pro-caspase. When cells are undergoing apoptosis, pro-caspase 3 is cleaved into two subunits that dimerize to form the active enzyme. Antibodies have been generated that detect the active form of caspase 3, but not pro-caspase 3, and fluorescently coupled versions of these antibodies can be used to detect apoptotic cells that have been fixed and permeabilized (Fig A.37).



A-28 Assays for cytotoxic T cells.

Activated CD8 T cells generally kill any cells that display the specific peptide:MHC class I complex they recognize. Thus, CD8 T-cell function can be determined using the simplest and most rapid T-cell bioassay—the killing of a target cell by a cytotoxic T cell. This is usually detected in a ^{51}Cr -release assay. Live cells will take up, but do not spontaneously release, radioactively labeled sodium chromate, $\text{Na}_2^{51}\text{CrO}_4$. When these labeled cells are killed, the radioactive chromate is released and its presence in the supernatant of mixtures of target cells and cytotoxic T cells can be measured (Fig. A.38). In a similar assay, proliferating target cells such as tumor cells can be labeled with ^3H -thymidine, which is incorporated into the replicating DNA. On attack by a cytotoxic T cell,

Fig. A.37 Detection of apoptotic cells by intracellular staining for active caspases. An early event in the apoptotic process is the release of cytochrome c from mitochondria. Cytochrome c binds to Apaf-1, leading to the cleavage of pro-caspase 9 into active caspase 9. Caspase 9 then cleaves pro-caspase 3 and pro-caspase 7 to yield their active forms, 'executioner' caspases that promote cell death. Antibodies that recognize the active caspase 3 or caspase 7, but not the pro-caspase forms of these enzymes, will detect permeabilized cells undergoing apoptosis.

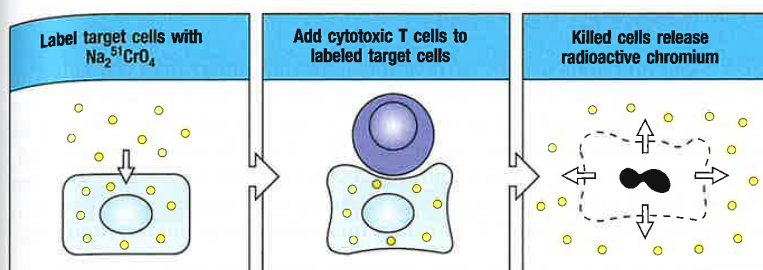


Fig. A.38 Cytotoxic T-cell activity is often assessed by chromium release from labeled target cells. Target cells are labeled with radioactive chromium as $\text{Na}_2^{51}\text{CrO}_4$, washed to remove excess radioactivity, and exposed to cytotoxic T cells. Cell destruction is measured by the release of radioactive chromium into the medium, detectable within 4 hours of mixing target cells with T cells.

the DNA of the target cells is rapidly fragmented and retained in the filtrate, while large, unfragmented DNA is collected on a filter, and one can measure either the release of these fragments or the retention of ^3H -thymidine in chromosomal DNA. These assays provide a rapid, sensitive, and specific measure of the activity of cytotoxic T cells.

An alternative to these *in vitro* cytotoxicity assays is to measure target-cell killing by cytotoxic T cells in intact experimental animals. This assay is generally performed with mice that have been infected with a pathogen known to induce a strong cytotoxic T-cell response, such as a virus. Target cells are incubated with the antigenic peptide, which will bind to MHC class I on the target-cell surface. These cells are then incubated with a low concentration of the fluorescent dye CFSE (see Section A-26). A control population of cells that is not given the antigenic peptide is incubated with a high concentration of CFSE, allowing these cells to be distinguished from the antigen-bearing target cells. The two cell populations are mixed 1:1 and injected into the experimental animals. Four hours later, spleen cells are recovered from the animals and analyzed by FACS, allowing the specific target-cell lysis to be calculated from the ratio of the two CFSE-labeled cell populations (Fig. A.39).

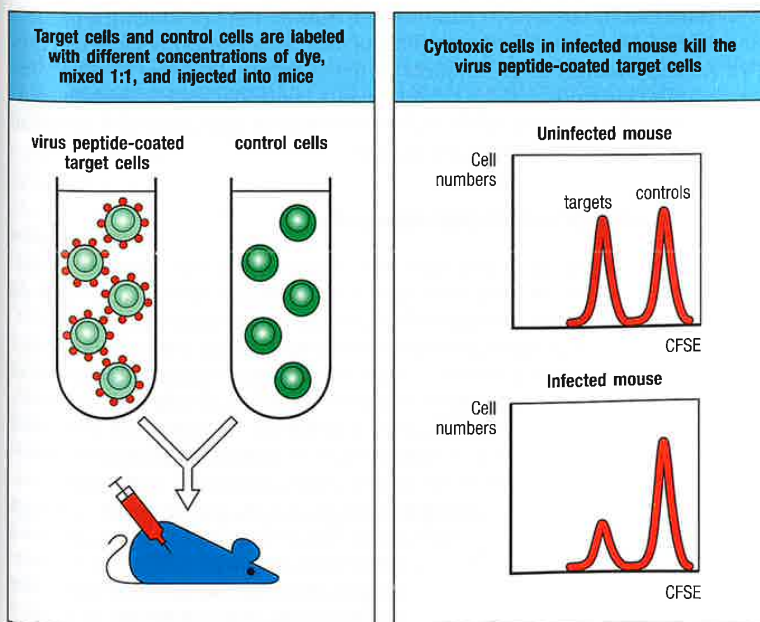


Fig. A.39 Assay for cytotoxic T-cell activity using CFSE-labeled target cells. To detect cytotoxic T-cell activity in intact experimental animals, mice that have been infected with a virus are injected with a mixture of target cells labeled with the fluorescent dye CFSE. One group of target cells is pre-incubated with a viral peptide that binds to MHC class I on the target cells; this group of cells is labeled with a low concentration of CFSE. A second group of cells is incubated with a control (nonviral) peptide and is labeled with a high concentration of CFSE. The two groups of cells are mixed together in a 1:1 ratio, and injected into the infected mice. After 4 hours, the mice are sacrificed and the target cells are recovered and analyzed by flow cytometry. Examination of the ratio of the two target-cell populations provides a measure of specific lysis of viral peptide-coated target cells.

A-29 Assays for CD4 T cells.

CD4 T-cell functions usually involve the activation rather than the killing of cells bearing specific antigen. The activating effects of CD4 T cells on B cells or macrophages are mediated in large part by cytokines, which are released by the T cell when it recognizes antigen. Thus, CD4 T-cell function is usually studied by measuring the type and amount of cytokine released. Because different effector T cells release different amounts and types of cytokines, one can learn about the effector potential of a T cell by measuring the proteins it produces.

Cytokines can be detected by their activity in biological assays of cell growth, where the cytokines serve either as growth factors or as growth inhibitors. A more specific assay is a modification of ELISA known as a capture or sandwich ELISA (see Section A-4). In this assay, the cytokine is characterized by its ability to act as a bridge between two monoclonal antibodies reacting with different epitopes on the cytokine molecule. Cytokine-secreting cells can also be detected by ELISPOT (see Section A-22).

Sandwich ELISA and ELISPOT avoid a major problem of cytokine bioassays, namely, the ability of different cytokines to stimulate the same response in a bioassay. Bioassays must always be confirmed by inhibition of the response with neutralizing monoclonal antibodies specific for the cytokine. Another way of identifying cells actively producing a given cytokine is to stain them with a fluorescently tagged anti-cytokine monoclonal antibody, and then identify and count them by FACS (see Section A-23).

A quite different approach to detecting cytokine production is to determine the presence and amount of the relevant cytokine mRNA in stimulated T cells. This can be done for single cells by *in situ* hybridization and for cell populations by the **reverse transcriptase-polymerase chain reaction (RT-PCR)**. Reverse transcriptase is an enzyme used by certain RNA viruses, such as HIV, to convert an RNA genome into a DNA copy, or cDNA. In RT-PCR, mRNA is isolated from cells and cDNA copies are made *in vitro* using reverse transcriptase. The desired cDNA is then selectively amplified by PCR by using sequence-specific primers. When the products of the reaction are subjected to electrophoresis on an agarose gel, the amplified DNA can be visualized as a band of a specific size. The amount of amplified cDNA sequence will be proportional to its representation in the mRNA; stimulated T cells actively producing a particular cytokine will produce large amounts of that particular mRNA and will thus give correspondingly large amounts of the selected cDNA on RT-PCR. The level of cytokine mRNA in the original tissue is usually determined by comparison with the outcome of RT-PCR on the mRNA produced by a so-called 'housekeeping gene' expressed by all cells.

A-30 Transfer of protective immunity.

Protective immunity to a pathogen may involve humoral immunity, cell-mediated immunity, or both. For studies in experimental animals such as inbred mice, the nature of protective immunity can be determined by transferring serum or lymphoid cells from an immunized donor animal to an unimmunized syngeneic recipient (that is, a genetically identical animal of the same inbred strain). If protection against infection can be conferred by the transfer of serum, the immunity is provided by circulating antibodies and is called **humoral immunity**. Transfer of immunity by antiserum or purified antibodies provides immediate protection against many pathogens and against toxins such as those of tetanus and snake venom (Fig. A.40). However, although protection is immediate, it is temporary, lasting only so long as the transferred antibodies remain active in the recipient's body. This type of transfer is therefore called **passive immunization**, to distinguish it from **active immunization** with antigen, which can provide lasting immunity. A disadvantage of passive

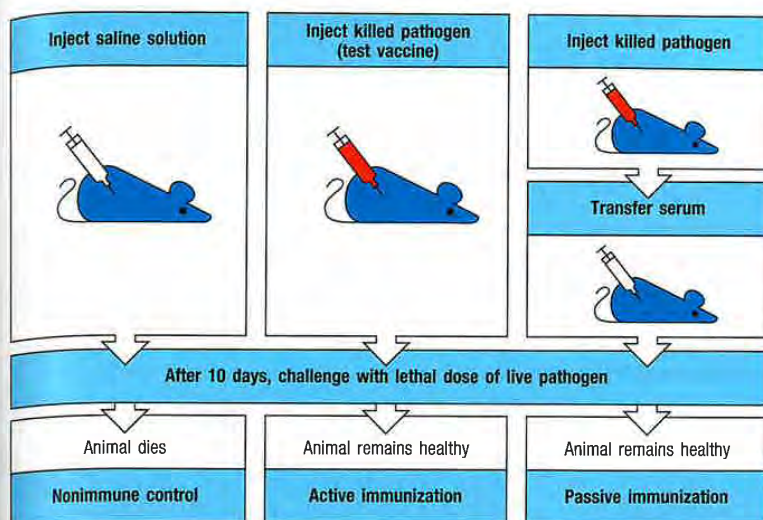


Fig. A.40 In vivo assay for the presence of protective immunity after vaccination in animals. Mice are injected with the test vaccine, such as a heat-killed pathogen, or a control such as saline solution. Different groups are then challenged with lethal or pathogenic doses of the test pathogen or with an unrelated pathogen as a specificity control (not shown). Unimmunized animals die or become severely infected (left panel). Successful vaccination is seen as specific protection of immunized mice against infection with the test pathogen. This is called active immunity, and the process is called active immunization (middle panel). If this immune protection can be transferred to a normal syngeneic recipient with serum from an immune donor, then immunity is mediated by antibodies; such immunity is called humoral immunity and the process is called passive immunization (right panel). If immunity can be transferred only by infusing lymphoid cells from the immune donor into a normal syngeneic recipient, then the immunity is called cell-mediated immunity and the transfer process is called adoptive transfer or adoptive immunization (not shown). Passive immunity is short-lived, because antibody is eventually catabolized, but adoptively transferred immunity is mediated by immune cells, which can survive and provide longer-lasting immunity.

immunization is that the recipient may become immunized to the antiserum used to transfer immunity. Horse or sheep sera are the usual sources of antiserum. Snake venoms used in humans, and repeated administration can lead either to serum sickness (see Section 14-5) or, if the recipient becomes allergic to the foreign serum, to anaphylaxis (see Section 14-10).

Protection against many diseases cannot be transferred with serum but can be transferred by lymphoid cells from immunized donors. The transfer of lymphoid cells from an immune donor to a normal syngeneic recipient is called **adoptive transfer** or **adoptive immunization**, and the immunity transferred is called **adoptive immunity**. Immunity that can be transferred only with lymphoid cells is called **cell-mediated immunity**. Such cell transfers must be between genetically identical donors and recipients, such as members of the same inbred strain of mouse, so that the donor lymphocytes are not rejected by the recipient and do not attack the recipient's tissues. Adoptive transfer of immunity is used clinically in humans in experimental approaches to cancer therapy or as an adjunct to bone marrow transplantation; in these cases, the patient's own T cells, or the T cells of the bone marrow donor, are given.

A-31 Adoptive transfer of lymphocytes.

Ionizing radiation from X-ray or gamma-ray sources kills lymphoid and other immune cells at doses that spare the other tissues of the body. This makes it possible to eliminate immune function in a recipient animal before

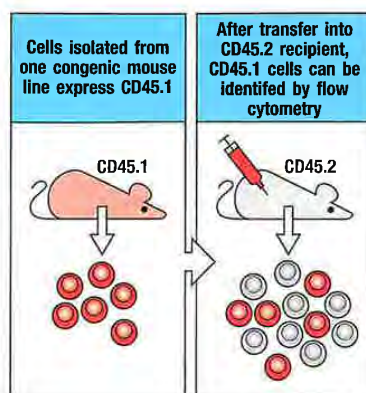


Fig. A.41 Adoptive transfer of congenically marked cells. Hematopoietic cells can be transferred between genetically identical (or nearly identical) mice. The transferred cells, usually a minority population in the recipient, are identified based on expression of an allelic variant of an abundant cell-surface receptor. One common receptor used for this purpose is CD45, which has two alleles that can be distinguished by allele-specific antibodies. When cells from a CD45.1⁺ mouse are transferred into mice of the identical strain (save for their expression of CD45.2), the donor-cell population can easily be identified by antibody staining followed by flow cytometry or immunofluorescence microscopy.

attempting to restore immune function by adoptive transfer, and allows the effect of the adoptively transferred cells to be studied in the absence of other lymphoid cells. **James Gowans** originally used this technique to prove the role of the lymphocyte in immune responses. He showed that all active immune responses could be transferred to irradiated recipients by small lymphocytes from immunized donors.

One common use of adoptive transfer assays takes advantage of the availability of T-cell receptor or B-cell receptor transgenic mice (see Section A-34). In this case, the adoptively transferred lymphocytes are a homogeneous population with a fixed antigen specificity. These cells can be transferred into unmanipulated recipient animals of the same inbred strain without the need to deplete the host immune system, and their ability to respond to immunization or challenge by infection can be monitored. One advantage of this experimental strategy is that relatively small numbers of antigen-specific T cells or B cells can be transferred; after dilution by the recipient's lymphocyte population, the responses of these cells can be examined in the environment of a normal immune response carried out by the host's immune system. Commonly, the transferred cells are 'marked' with an allelic variant of an abundant cell-surface receptor, such as CD45 (Fig. A.41). When the donor lymphocytes express one allelic variant of CD45 and the recipient cells express a different variant, the transferred cells can easily be distinguished from the host cells by staining with an antibody that binds to one variant of CD45, but not the other. When two strains of mice are genetically identical with the exception of a single gene, they are said to be **congenic**. In the example above, the donor strain and the recipient strain are referred to as 'CD45 congenics'; it should be noted, however, that in the case where one strain is a T-cell receptor or B-cell receptor transgenic line, this terminology is not completely accurate, as the presence of the transgenic DNA as a genetic difference is conveniently ignored. Such adoptive transfer studies are a cornerstone in the study of the intact immune system. They have provided a rapid and convenient means of determining the effects of many gene deficiencies, such as those in cell-surface receptors, transcription factors, cytokines, and cell survival/cell death genes, on the ability of T cells or B cells to mount protective immune responses.

A-32 Hematopoietic stem-cell transfers.

All cells of hematopoietic origin can be eliminated by treatment with high doses of γ radiation or X rays, allowing replacement of the entire hematopoietic system, including lymphocytes, by transfusion of donor bone marrow or purified hematopoietic stem cells from another animal. The resulting animals are called **radiation bone marrow chimeras**, from the Greek word *chimera*, a mythical animal that had the head of a lion, the tail of a serpent, and the body of a goat. This technique is used experimentally to examine the development of immune-cell lineages, as opposed to their effector functions, and has been particularly important in studying T-cell development. Essentially the same technique is used in humans to replace the hematopoietic system when it fails, as in aplastic anemia or after nuclear accidents, or to eradicate the bone marrow and replace it with normal marrow in the treatment of certain cancers. In humans, bone marrow is the main source of hematopoietic stem cells, but they are increasingly being obtained from peripheral blood after the donor

has been treated with hematopoietic growth factors such as GM-CSF, or from umbilical cord blood, which is rich in such stem cells (see Chapter 15).

A-33 *In vivo* administration of antibodies.

Antibodies administered to intact experimental animals, or to humans, provide a potent means of manipulating the immune system. Depending on the target molecule recognized by the antibody, and the intrinsic properties of each antibody, *in vivo* antibody administration can either inhibit the function of the target molecule or, in some cases, lead to the elimination of a cell population that expresses the target molecule.

In animal models, antibodies targeting an individual cytokine have been used to inhibit that cytokine's function during an otherwise intact immune response. Experiments of this type provided some of the first evidence that the cytokine IL-12 provides an important signal in polarizing CD4⁺ T cells into the T_H1 lineage following infection with an intracellular protozoan. This approach has also been used with great success in humans. One of the most common treatments for the inflammatory autoimmune disease rheumatoid arthritis (see Chapter 16) is the administration of an antibody that binds to the cytokine TNF- α ; in this case, the inhibition of TNF- α activity provides patients with relief from the symptoms of joint inflammation. The great utility of this antibody therapy has led to the development of related strategies for inhibiting cytokine actions *in vivo*. One successful approach has been to create a hybrid protein that has the ligand-binding domain of the cytokine's receptor fused to the constant-region domains (Fc) of an antibody heavy chain (Fig. A.42). This Fc-fusion protein acquires the stability and long half-life of an antibody, but has the binding properties of the cytokine receptor. When given *in vivo*, the Fc-fusion protein binds to the cytokine, thereby interfering with the cytokine's ability to stimulate its receptor on immune cells. As an example, the Fc-fusion protein containing the TNF-receptor ligand-binding domain has also been used as an effective treatment for patients with rheumatoid arthritis.

Antibody administration can also be used to augment the immune response by interfering with T-cell surface receptors, such as CTLA-4 or PD-1. When engaged by their ligands, these receptors normally function to downregulate the immune response. From experiments in mice, antibodies that bind to and inhibit these receptors have been found to enhance immune responses to tumors, leading in some cases to tumor eradication. Currently, these strategies are being tested in humans for a variety of tumor types, and the initial results have shown great promise.

In vivo administration of antibodies can also be used to deplete specific cell populations. The efficiency with which a given antibody functions for *in vivo* depletion is quite variable, as the mechanism relies on a process known as **antibody-dependent cell-mediated cytotoxicity (ADCC)** (see Section 10-23 and Fig. 10.36). When a cell is coated with antibodies, it becomes a target of natural killer (NK) cells that express the Fc receptor known as CD16 or Fc γ RIII. The cross-linking of Fc γ RIII induces the NK cell to kill the antibody-coated target cell. While Fc γ RIII is a receptor for IgG, it does not bind with equal affinity to all IgG subtypes; thus the efficiency of ADCC after administration of a given antibody is determined by its ability to cross-link Fc γ RIII and induce NK cell killing. Common uses for this technique include the depletion of CD4⁺ T cells with an antibody to CD4, or the depletion of CD8⁺ T cells with an antibody to CD8. In human patients undergoing organ transplantation, T cells are transiently depleted by administering an antibody to the CD3 component of the T-cell receptor complex. This produces a severe, but temporary, state of immunosuppression during the early stages of post-transplantation. As with all *in vivo* antibody depletion regimens, the depleted cell population gradually returns as cells of that subset are replenished by ongoing lymphocyte development.

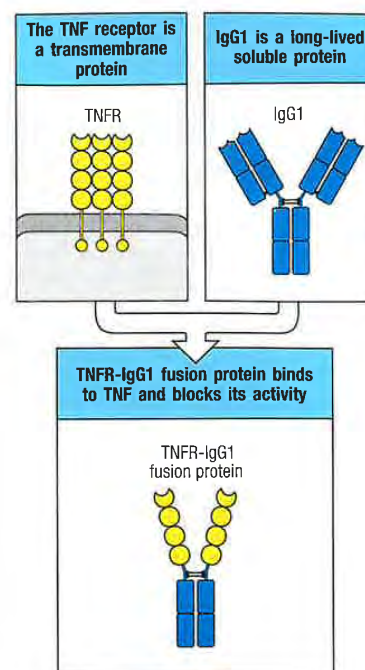


Fig. A.42 *In vivo* administration of antibodies is an effective therapeutic.

The cytokine TNF- α contributes to chronic inflammation in a range of conditions, including rheumatoid arthritis, by binding to and triggering signaling of the TNF receptor (TNFR). To treat these conditions, a fusion protein consisting of the constant-region domains of human IgG1 is fused to the extracellular portion of the TNFR, creating a therapeutic known as etanercept. When administered to patients, this fusion protein is effective at binding TNF- α and preventing it from triggering TNFR signaling, thereby reducing inflammation.

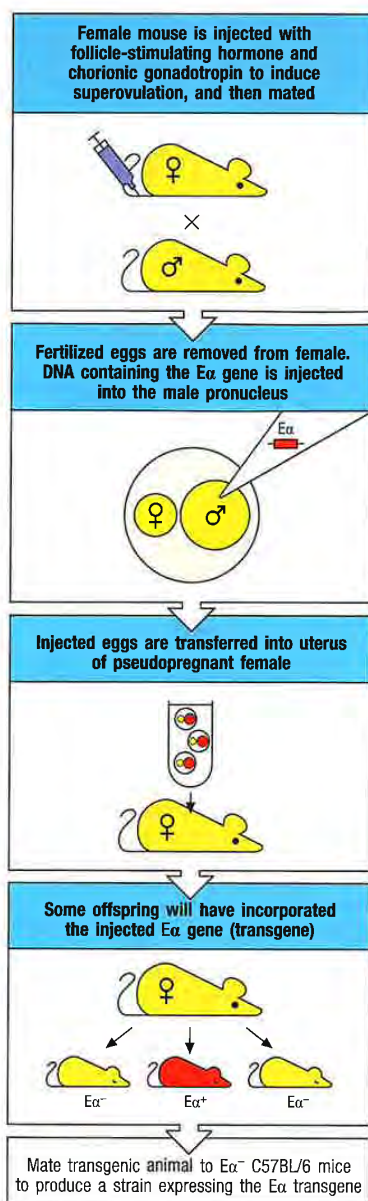


Fig. A.43 The function and expression of genes can be studied *in vivo* by using **transgenic mice**. DNA encoding a protein of interest, here the mouse MHC class II protein $E\alpha$, is purified and microinjected into the male pronuclei of fertilized eggs. The eggs are then implanted into pseudopregnant female mice. The resulting offspring are screened for the presence of the transgene in their cells, and positive mice are used as founders that transmit the transgene to their offspring, establishing a line of transgenic mice that carry one or more extra genes. The function of the $E\alpha$ gene used here is tested by breeding the transgene into C57BL/6 mice that carry an inactivating mutation in their endogenous $E\alpha$ gene.

A-34 Transgenic mice.

The function of genes has traditionally been studied by observing the effects of spontaneous mutations in whole organisms and, more recently, by analyzing the effects of targeted mutations in cultured cells. The advent of gene cloning and *in vitro* mutagenesis now makes it possible to produce specific mutations in whole animals. Mice with extra copies or altered copies of a gene in their genome can be generated by **transgenesis**, which is now a well-established procedure. To produce **transgenic mice**, a cloned gene is introduced into the mouse genome by microinjection into the male pronucleus of a fertilized egg, which is then implanted into the uterus of a pseudopregnant female mouse. In some of the eggs, the injected DNA becomes integrated randomly into the genome, giving rise to a mouse that has an extra genetic element of known structure, the transgene (Fig. A.43).

This technique allows one to study the impact of a newly discovered gene on development, to identify the regulatory regions of a gene required for its normal tissue-specific expression, to determine the effects of its overexpression or its expression in inappropriate tissues, and to find out the impact of mutations on gene function. Transgenic mice have been particularly useful in studying the role of T-cell and B-cell receptors in lymphocyte development, as described in Chapter 8, and in providing a source of primary T and B lymphocytes of known antigen specificity for adoptive transfer studies (see Section A-31). This utility is largely due to the fact that expression of the transgene-encoded T-cell and B-cell receptors preempts the rearrangement and expression of the endogenous antigen receptor genes during T-cell and B-cell development, respectively, thereby generating homogeneous populations of cells bearing a unique antigen receptor of known specificity.

A-35 Gene knockout by targeted disruption.

In many cases, the functions of a particular gene can be fully understood only if a mutant animal that does not express the gene can be obtained. Whereas genes used to be discovered through the identification of mutant phenotypes, it is now far more common to discover and isolate the normal gene and then determine its function by replacing it *in vivo* with a defective copy. This procedure is known as **gene knockout**, and it has been made possible by two developments: a powerful strategy to select for targeted mutation by homologous recombination, and the development of continuously growing lines of **embryonic stem cells (ES cells)**. These are embryonic cells that, on implantation into a blastocyst, can give rise to all cell lineages in a chimeric mouse.

The technique of **gene targeting** takes advantage of the phenomenon known as **homologous recombination** (Fig. A.44). Cloned copies of the target gene are altered to make them nonfunctional and are then introduced into the ES cell, where they recombine with the homologous gene in the cell's genome, replacing the normal gene with a nonfunctional copy. Homologous recombination is a rare event in mammalian cells, and thus a powerful selection strategy is required to detect those cells in which it has occurred. Most commonly, the introduced gene construct has its sequence disrupted by an inserted antibiotic-resistance gene such as that for neomycin resistance (*neo*^r). If this

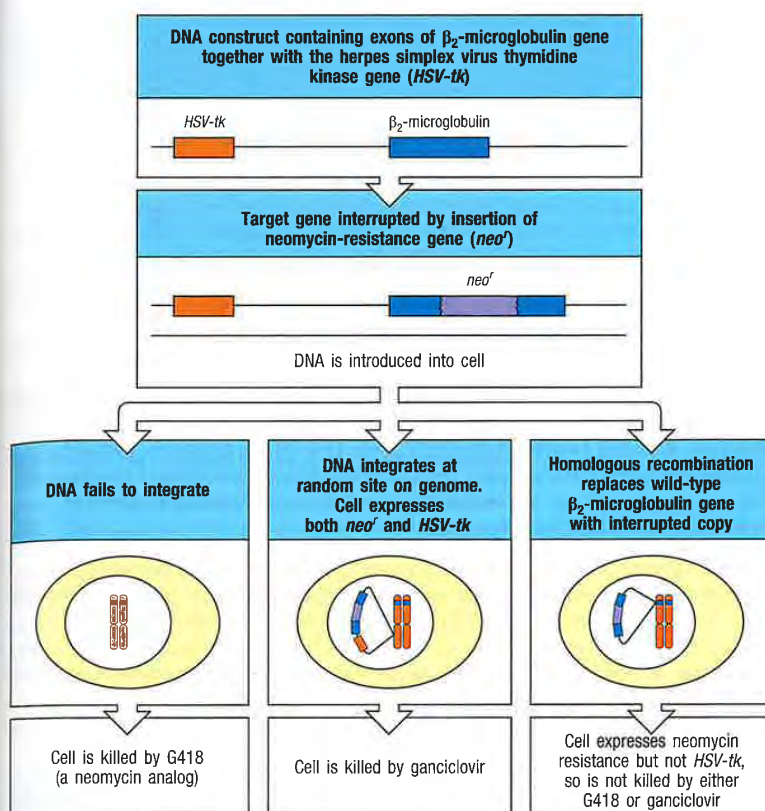


Fig. A.44 The deletion of specific genes can be accomplished by homologous recombination. When pieces of DNA are introduced into cells, they can integrate into cellular DNA in two different ways. If they randomly insert into sites of DNA breaks, the whole piece is usually integrated, often in several copies. However, extrachromosomal DNA can also undergo homologous recombination with the cellular copy of the gene, in which case only the central, homologous region is incorporated into cellular DNA. Inserting a selectable marker gene such as resistance to neomycin (*neo^r*) into the coding region of a gene does not prevent homologous recombination, and it achieves two goals. First, any cell that has integrated the injected DNA is protected from the neomycin-like antibiotic G418. Second, when the gene recombines with homologous cellular DNA, the *neo^r* gene disrupts the coding sequence of the modified cellular gene. Homologous recombinants can be discriminated from random insertions if the gene encoding herpes simplex virus thymidine kinase (*HSV-tk*) is placed at one or both ends of the DNA construct, which is often known as a 'targeting construct' because it targets the cellular gene. In random DNA integrations, *HSV-tk* is retained. *HSV-tk* renders the cell sensitive to the antiviral agent ganciclovir. However, as *HSV-tk* is not homologous to the target DNA, it is lost from homologous recombinants. Thus, cells that have undergone homologous recombination are uniquely resistant to both G418 and ganciclovir, and survive in a mixture of the two antibiotics. The presence of the disrupted gene has to be confirmed by Southern blotting or by PCR using primers in the *neo^r* gene and in cellular DNA lying outside the region used in the targeting construct. By using two different resistance genes, one can disrupt the two cellular copies of a gene, making a deletion mutant (not shown).

construct undergoes homologous recombination with the endogenous copy of the gene, the endogenous gene is disrupted but the antibiotic-resistance gene remains functional, allowing cells that have incorporated the gene to be selected in culture for resistance to the neomycin-like drug G418. However, antibiotic resistance on its own shows only that the cells have taken up and integrated the neomycin-resistance gene. To be able to select for those cells in which homologous recombination has occurred, the ends of the construct usually carry the thymidine kinase gene from the herpes simplex virus (*HSV-tk*). Cells that incorporate DNA randomly usually retain the entire DNA construct including *HSV-tk*, whereas homologous recombination between the construct and cellular DNA, the desired result, involves the exchange of homologous DNA sequences so that the nonhomologous *HSV-tk* genes at the ends of the construct are eliminated. Cells carrying *HSV-tk* are killed by the antiviral drug ganciclovir, and so cells with homologous recombinations have the unique feature of being resistant to both neomycin and ganciclovir, allowing them to be selected efficiently when these drugs are added to the cultures (see Fig. A.44).

To knock out a gene *in vivo*, it is necessary only to disrupt one copy of the cellular gene in an ES cell. These ES cells are then injected into a blastocyst, which is reimplanted into the uterus. The cells carrying the disrupted gene become incorporated into the developing embryo and contribute to all tissues of the resulting chimeric offspring, including those of the germline. The mutated gene can therefore be transmitted to some of the offspring of the original chimera, and further breeding to homozygosity of the mutant gene produces mice that completely lack the expression of that particular gene product (Fig. A.45). The effects of the absence of the gene's function can then be

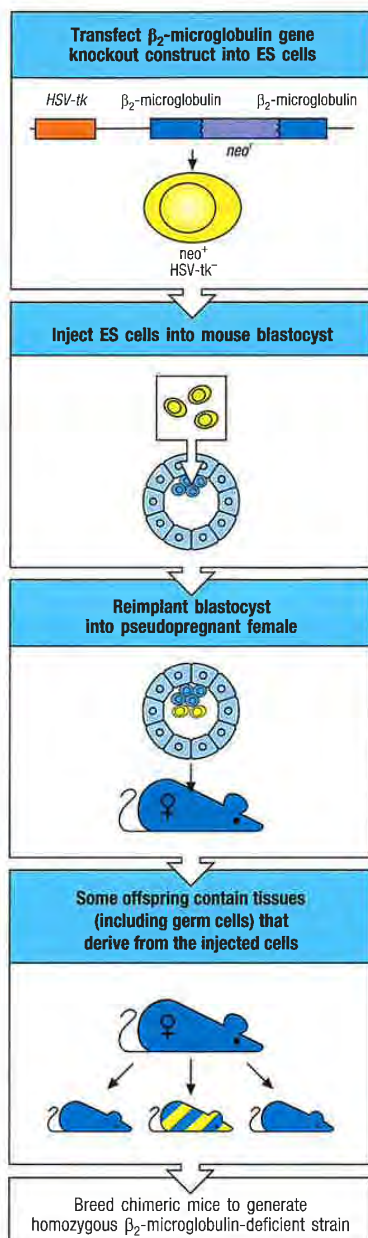


Fig. A.45 Gene knockout in embryonic stem cells enables mutant mice to be produced. Specific genes can be inactivated by homologous recombination in cultures of embryonic stem cells (ES cells). Homologous recombination is performed as described in Fig. A.44. In this example, the gene encoding β_2 -microglobulin in ES cells is disrupted by homologous recombination with a targeting construct. Only a single copy of the gene needs to be disrupted. ES cells in which homologous recombination has taken place are injected into mouse blastocysts. If the mutant ES cells give rise to germ cells in the resulting chimeric mice (striped in the figure), the mutant gene can be transferred to their offspring. By breeding the mutant gene to homozygosity, offspring can be tested to determine if a mutant phenotype is generated. In this case, the homozygous mutant mice lack MHC class I molecules on their cells, because MHC class I molecules have to pair with β_2 -microglobulin for surface expression. The β_2 -microglobulin-deficient mice can then be bred with mice transgenic for subtler mutants of the deleted gene, allowing the effect of such mutants to be tested *in vivo*.

studied. In addition, the parts of the gene that are essential for its function can be identified by determining whether function can be restored by introducing different mutated copies of the gene back into the genome by transgenesis. The manipulation of the mouse genome by gene knockout and transgenesis has revolutionized our understanding of the role of individual genes in lymphocyte development and function.

Because the most commonly used ES cells are derived from a poorly characterized strain of mice known as strain 129, the analysis of the function of a gene knockout often requires extensive back-crossing to another strain. One can track the presence of the mutant copy of the gene by the presence of the *neo* $^+$ gene. After sufficient back-crossing, the mice are intercrossed to produce mutants on a stable genetic background.

A problem with gene knockouts arises when the function of the gene is essential for the survival of the animal; in such cases the gene is termed a **recessive lethal gene**, and homozygous animals cannot be produced. To study the function of such a gene, tissue-specific or developmentally regulated gene deletion can be employed. This strategy makes use of the DNA sequences and enzymes used by bacteriophage P1 to excise itself from a host cell's genome. Integrated bacteriophage P1 DNA is flanked by recombination signal sequences called *loxP* sites. A recombinase, Cre, recognizes these sites, cuts the DNA, and joins the two ends, thus excising the intervening DNA in the form of a circle. This mechanism can be adapted to allow the deletion of specific genes in a transgenic animal only in certain tissues or at certain times in development. First, *loxP* sites flanking a gene, or perhaps flanking just a single exon, are introduced by homologous recombination (Fig. A.46). Usually, the introduction of these sequences into flanking or intronic DNA does not disrupt the normal function of the gene. Next, mice containing such *loxP* mutant genes are mated with mice made transgenic for Cre recombinase that has been placed under the control of a tissue-specific or inducible promoter. When the Cre recombinase is active, either in the appropriate tissue or when induced, it excises the DNA between the inserted *loxP* sites, thus inactivating the gene or exon. Thus, for example, using a T-cell-specific promoter to drive expression of the Cre recombinase, a gene can be deleted only in T cells while remaining functional in all other cells of the animal. This extremely powerful genetic technique was used to demonstrate the importance of B-cell receptors in B-cell survival.

Recently, a new technology has been developed for inducing specific gene disruptions in mice; it is known as the CRISPR/Cas9 system. This technique is adapted from a bacterial system that uses an RNA-based strategy to generate double-stranded DNA breaks in the genomes of invading pathogens or plasmids, a form of bacterial immunity. The Cas9 gene encodes an endonuclease; this has been modified for use in eukaryotic cells by incorporating a nuclear localization signal into the protein-coding sequence of the enzyme. To target mutations to a particular gene, a synthetic guide RNA is produced that incorporates a short sequence (~20 nucleotides) homologous to the gene

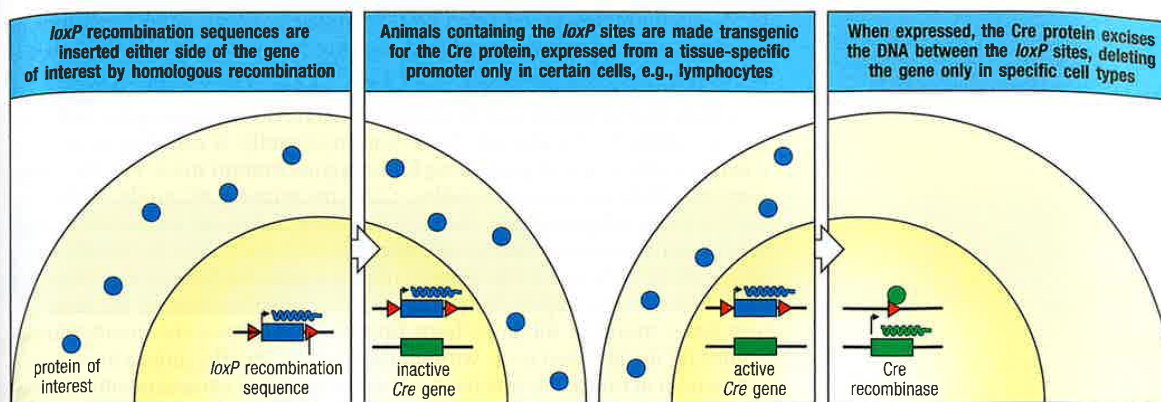


Fig. A.46 The P1 bacteriophage recombination system can be used to eliminate genes in particular cell lineages. The P1 bacteriophage protein Cre excises DNA that is bounded by recombination signal sequences called *loxP* sequences. These sequences can be introduced at either end of a gene by homologous recombination (left panel). Animals carrying genes flanked by *loxP* can also be made transgenic for the gene encoding the Cre protein, which is placed under the control of a tissue-specific promoter so that it is expressed only in certain cells or only at certain

times during development (center panel). In the cells in which the Cre protein is expressed, it recognizes the *loxP* sequences and excises the DNA lying between them (right panel). Thus, individual genes can be deleted only in certain cell types or only at certain times. In this way, genes that are essential for the normal development of a mouse can be analyzed for their function in the developed animal and/or in specific cell types. Genes are shown as boxes, RNA as squiggles, and proteins as colored balls.

being targeted along with sequences that bind the Cas9 enzyme. The guide RNA recruits Cas9 to the genomic location, where the endonuclease will produce a double-stranded DNA break (Fig. A.47). When this break is repaired

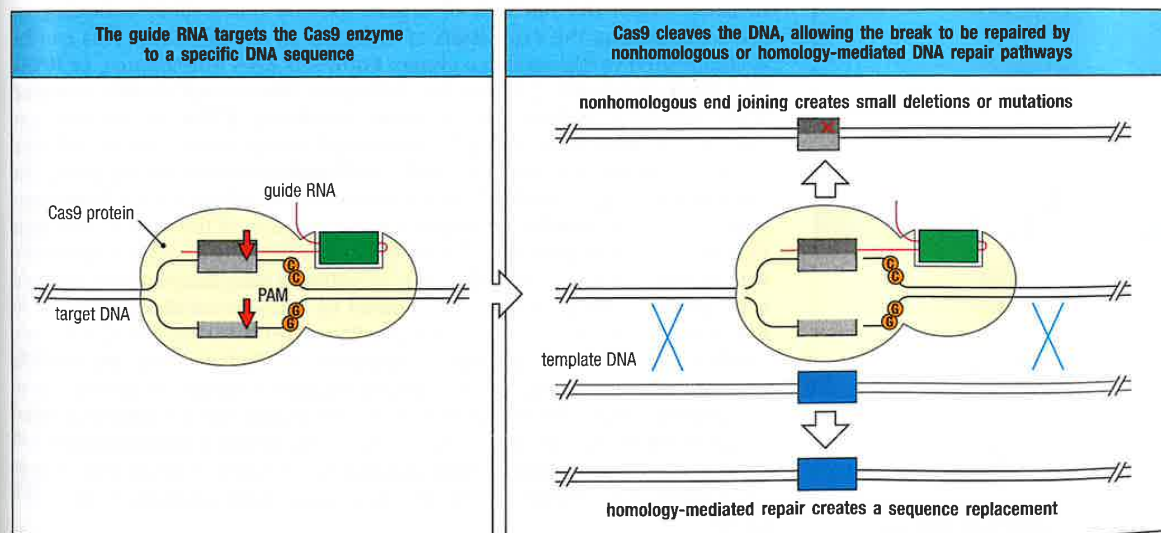
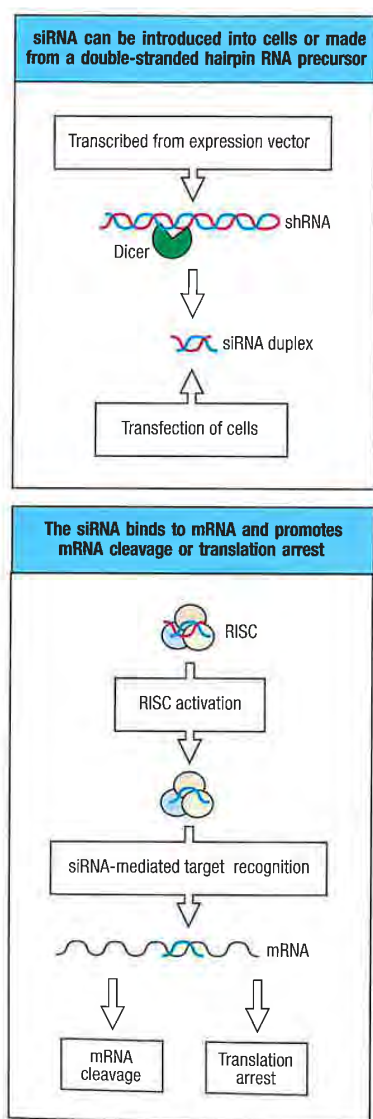


Fig. A.47 Genetic engineering using the bacterial CRISPR/Cas9 system. Genetic engineering can be targeted to a specific gene locus in cells by using two components, the bacterial Cas9 enzyme and a guide RNA (left panel). The guide RNA is a single-stranded RNA that contains two regions of sequence in tandem, the first with homology to the gene being targeted and a second recognized by the Cas9 enzyme. The guide RNA targets the enzyme to the homologous genomic region, promoting double-stranded DNA cleavage by the Cas9 endonuclease 3–4 nucleotides upstream of the protospacer adjacent motif (PAM) sequence (right panel). The PAM sequence required by the Cas9 endonuclease is the dinucleotide GG (CC on the other strand). When the double-

stranded DNA break is repaired by the nonhomologous end-joining pathway, small deletions and/or point mutations are introduced into the target gene, often leading to loss of gene function. To induce a specific sequence replacement in the target gene, cells are provided with a template DNA, in addition to Cas9 and the guide RNA. This template is a double-stranded DNA sequence homologous to the target gene, but containing specific nucleotide changes. In the presence of this template, cells will repair the Cas9-mediated double-stranded cleavage using homologous recombination, rather than nonhomologous end-joining, thereby replacing the original sequence with the sequence provided in the template DNA.



by the nonhomologous end-joining DNA repair pathway, small insertions or deletions are commonly introduced, leading to a disruption of the original sequence.

This powerful technique can be used to generate homozygous gene deficiencies in cultured cells and cell lines, but importantly, it can also be used as a single-step means of generating homozygous mutant mice. For this latter purpose, RNA molecules encoding Cas9 are mixed with guide RNAs and injected into single-cell mouse zygotes, using the same technique as that used to generate transgenic mouse lines (see Fig. A.43). Due to the efficiency of the CRISPR/Cas9 system, these embryos frequently harbor mutations on both alleles of the targeted gene. Thus, after transplantation of the embryos into foster mothers, the pups born from these embryos are already homozygous for the targeted gene, without the need for lengthy mouse breeding. A refinement of this technique has been developed that allows specific nucleotide changes to be introduced into the targeted gene, rather than the random changes resulting from nonhomologous end-joining. This is accomplished by introducing a DNA oligonucleotide into the fertilized mouse zygotes along with the Cas9 and guide RNAs. The oligonucleotide contains the desired nucleotide changes flanked by sequences homologous to the targeted gene. When this oligonucleotide is present, the double-strand DNA break introduced by Cas9 is preferentially repaired by a homology-directed process that replaces the damaged DNA with the sequences from the oligonucleotide (see Fig. A.47).

A-36 Knockdown of gene expression by RNA interference (RNAi).

In some cases, the function of a gene can be assessed by reducing, or even eliminating, the expression of that gene in specific cells. This can be accomplished by harnessing a system known as RNA interference, or RNAi, that is present in many eukaryotic cell types. When small double-stranded RNA molecules (referred to as small interfering RNAs, or siRNAs) are introduced into cells, the two RNA strands will be separated and one will bind to an enzyme complex known as RISC (RNA-induced silencing complex). The bound siRNA targets the RISC complex to the mRNA to which it has homology, leading either to translation arrest or to degradation of the mRNA, and thus to silencing of the gene (Fig. A.48). For cells that are not easily transfected with siRNA molecules directly, such as primary lymphocytes and myeloid cells, gene silencing can be implemented by using recombinant viruses. In this case, genes encoding small hairpin RNAs (shRNAs) are introduced into viral vectors that can be packaged into infectious viral particles. The shRNAs encode small RNAs that form a double-stranded hairpin structure; these hairpins are processed by enzymes in the cell to generate the siRNAs needed for gene silencing (see Fig. A.48). Since many primary hematopoietic cell types are readily transduced with recombinant viruses, such as retroviruses and lentiviruses, shRNAs can be effectively used to silence genes in these cell types.

Fig. A.48 Knockdown of gene expression using the RNAi pathway. Small double-stranded RNA molecules with homology to an mRNA transcript will target the mRNA for degradation or translation arrest. This pathway is initiated by expression of a short hairpin RNA (shRNA), which can be produced from an expression vector that is introduced into cells, or by the direct transfection of cells with small double-stranded RNA molecules called siRNA. shRNA molecules are processed by the enzyme Dicer to generate the siRNAs needed for gene silencing (see Fig. A.48). Since many primary hematopoietic cell types are readily transduced with recombinant viruses, such as retroviruses and lentiviruses, shRNAs can be effectively used to silence genes in these cell types.