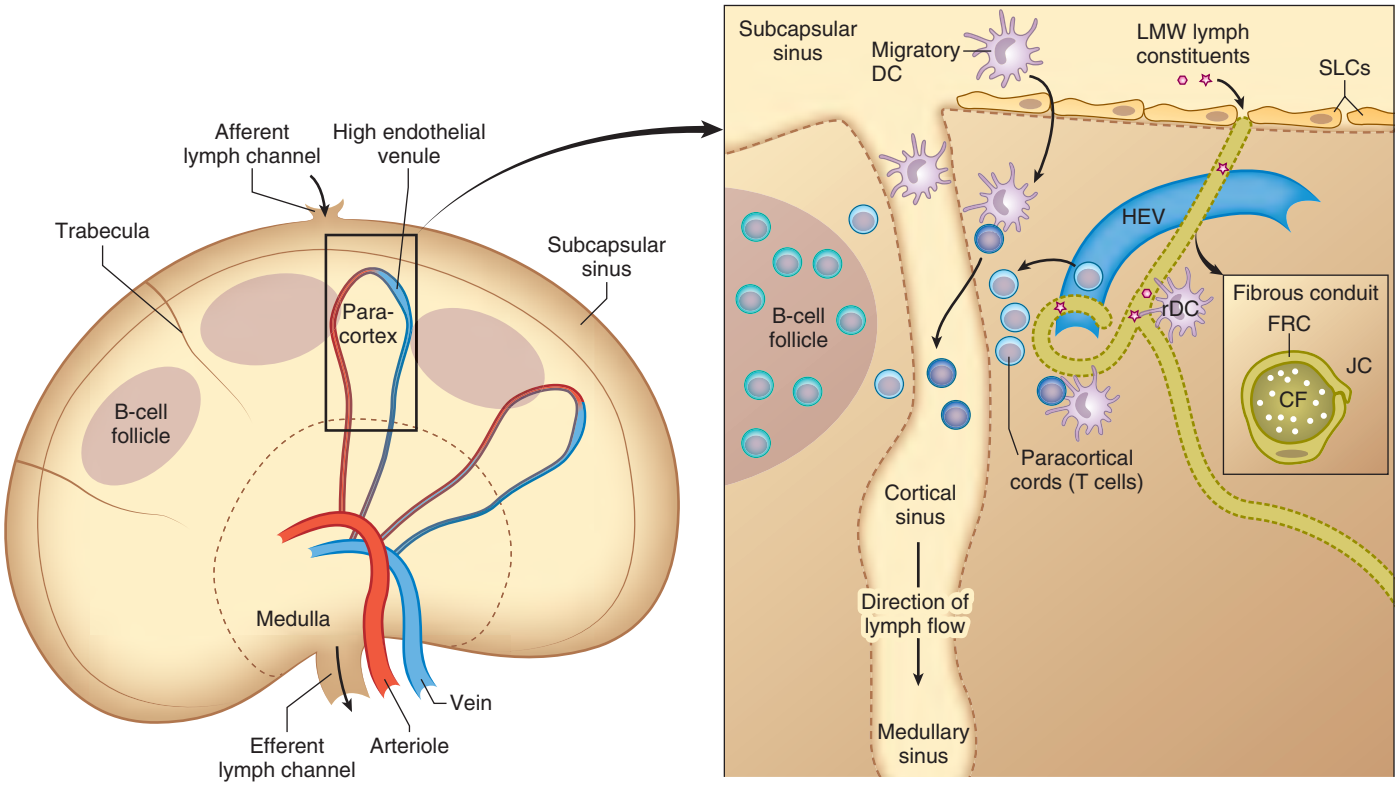

FIG. 6.4 Anatomy of lymph nodes. (A) Humans typically contain 500 to 600 bean-shaped lymph nodes that filter lymphatic fluid that is draining from peripheral tissues to the bloodstream via lymphatic vessels. Lymph nodes are encapsulated by dense, collagen-rich fibers that extend trabeculae into the lymph node substance. Afferent lymph fluid enters lymph nodes through channels that drain into subcapsular sinuses. Lymph fluid percolates through cortical and medullary sinuses before exiting the lymph node via efferent lymph channels. The outer region of the lymph node, termed the *cortex,* consists primarily of B cell–rich lymphoid follicles and T cell–rich paracortical aggregates. The inner region of the lymph node, termed the *medulla,* is far less cellular than the cortex, and this region contains coalescing lymph-filled sinuses. The blood supply to the lymph node enters and exits via the hilum. High endothelial venules represent the site of circulating leukocyte entry into the lymph node. Leukocytes exit the lymph node via the efferent lymphatic vessels and rejoin the circulation. (B) Lymph node microanatomy and network of conduits. This panel provides an outline of lymph node anatomy at a level that cannot be visualized by light microscopy, but that has been deduced by trafficking analyses of low-molecular-weight *(LMW)* markers that were experimentally introduced into lymphatic fluid and into blood that circulate into intact lymph nodes. Antigen-bearing migratory dendritic cells (*DC*s) can traverse the cellular lining of subcapsular and cortical sinuses to enter the paracortical cords to initiate antigen presentation to naïve T cells. Soluble inflammatory mediators and antigens are funneled into fibrous conduits that link lymphatic channels with high endothelial venules *(HEV)* and lymphoid follicles. Resident DCs can sample the antigenic content of fibrous conduits and present antigens to T cells that enter the paracortex through the HEV. Low-MW proteins, such as chemokines, can flow from the lymph node sinus through the conduit system to the HEV, where they can influence the recruitment of circulating lymphocytes. *CF,* Collagen fibers that provide structural support to the conduits and to the lymph node; *FRC,* fibroblastic reticular cells that surround the conduits; *JC,* junctional complexes that provide a tight barrier to the contents of the conduit; *rDC,* resident dendritic cells; *SLCs,* sinus-lining cells.

maximizes the likelihood that an individual T cell will encounter a DC that expresses a cognate peptide-loaded MHC.

Once a productive MHC-antigen-TCR interaction ensues, T cells remain in a complex stationary phase with the cognate DC for an estimate of 3 to 4 hours,[191] secrete IL-2 and IFN-γ, and then resume migratory behavior to enable additional interactions with DCs or T-cell subsets (e.g., CD4 T-cell help for CD8 T-cell activation), and ultimately lymph node exit. T cells exit the lymph node via the lymphatic sinuses and reenter the circulation via large lymphatic channels or the thoracic duct.[181]

The length of time that T lymphocytes remain in lymphoid tissues is regulated by a balance of retention signals, mediated primarily by chemokine receptor signaling via CCR7, and exit signals such as sphingosine-1-phosphate.[192] T-cell lymph node dwell times can be prolonged by expression of CD69, a C-type lectin that binds to and inhibits the activity of the sphingosine-1-phosphate receptor. CD69 expression is induced by inflammatory cues (e.g., type I interferon signaling) associated with microbial infections to increase the likelihood that a naïve T cell can productively interact with an antigen-bearing DC.[193]

Spleen

The spleen is a large secondary lymphoid organ that serves as an important filtration site to clear microbial pathogens from the bloodstream.[194] Although the spleen and lymph nodes share many T-cell, B-cell, and DC subsets and are similarly complex at the microanatomic level, they also differ in some important respects. The spleen is not a site for lymphatic drainage. Instead, antigens, pathogens, and circulating cells enter the spleen through the splenic artery that enters the hilum. The splenic artery branches into central arterioles (Fig. 6.5A), which course through the splenic cortex and are surrounded by lymphocytes in a structure called the *periarteriolar sheath.* T lymphocytes are most proximal to the central arteriole and are surrounded by aggregates of B lymphocytes in regions called *B-cell follicles* (Fig. 6.5B and 6.5C). The densely packed B and T lymphocytes constitute the splenic white pulp and are surrounded by the marginal zone (Fig. 6.5D), which separates white pulp from red pulp and contains marginal zone macrophages and marginal metallophilic macrophages. Both types of marginal zone macrophages have a role in trapping bloodborne particulate antigens.[195] Within the white pulp, CD8α$^+$ conventional DCs localize to the periarteriolar sheath and are involved in tolerance to self-antigens and highly active in antigen cross-presentation[196] (discussed under "MHC Class I Antigen-Processing Pathway" later). In contrast, CD8α$^-$ DCs reside primarily within the marginal zone and have a weaker cross-presentation ability and distinct cytokine responses to microbial stimuli compared to CD8α$^+$ conventional DCs.[197]

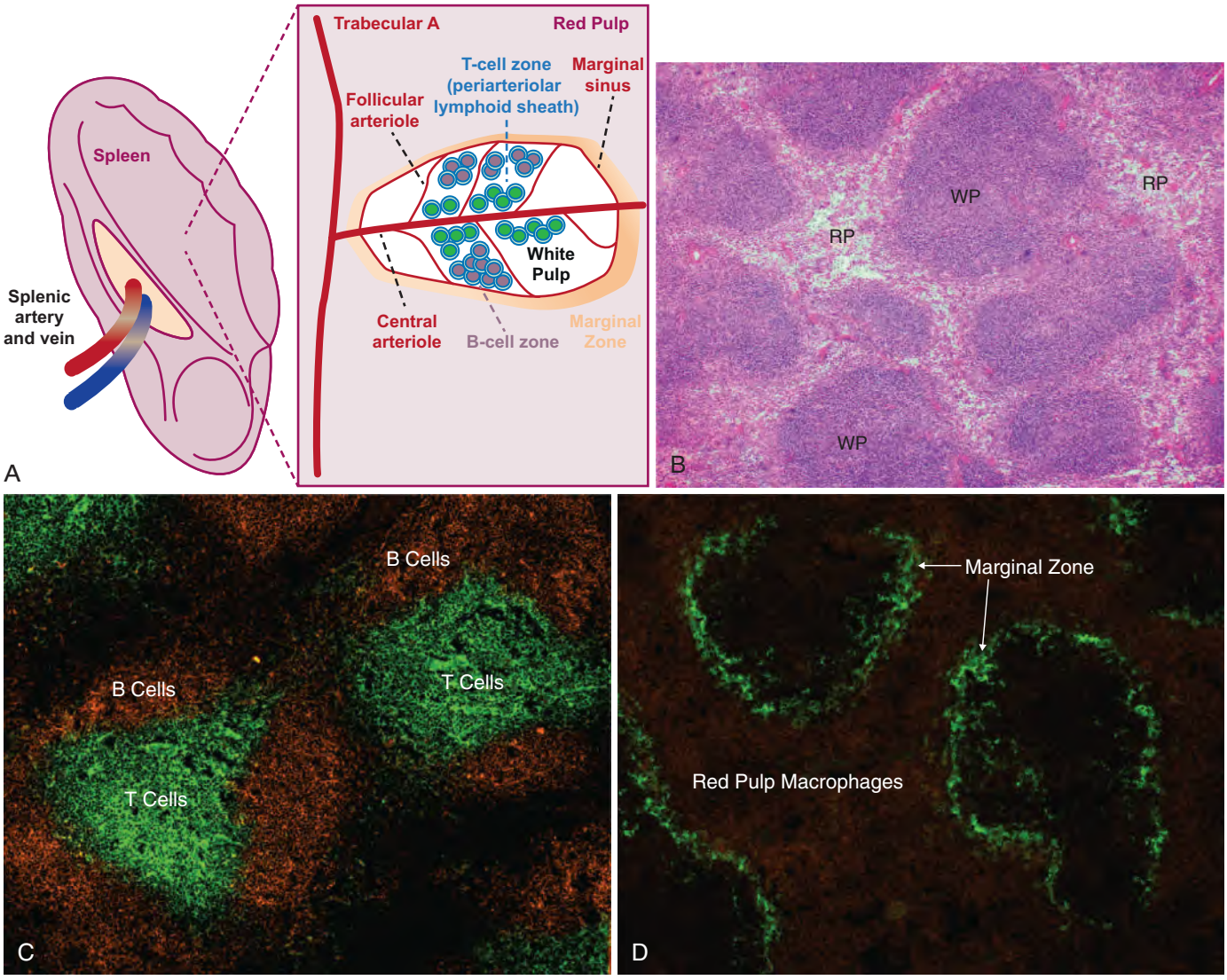

FIG. 6.5 Anatomy of the spleen. The spleen is a complex secondary lymphoid organ with compartmentalized cell populations. (A) Schematic diagram of spleen and a splenic arteriole that is surrounded by a periarteriolar lymphoid sheath (PALS) with a predominant T-cell population and a germinal follicle with a predominant B-cell population. The PALS and germinal follicles constitute the white pulp that is surrounded by a narrow marginal zone and the splenic red pulp. (B) Micrograph showing a hematoxylin-eosin–stained spleen section, demonstrating areas of white pulp *(WP)* separated by areas with more dispersed cell populations referred to as the red pulp *(RP).* (C) Micrograph showing a spleen section that has been stained with fluorescently labeled antibodies specific for B cells *(orange)* and T cells *(green),* demonstrating the distinct localization of B cells and T cells within the white pulp. (D) Micrograph showing staining for macrophages *(orange)* and the splenic marginal zone *(green),* demonstrating the density of macrophages and phagocytic cells in the red pulp and marginal zone.

The splenic red pulp is rich in macrophages (i.e., red pulp macrophages that clear senescent red blood cells) and contains many red blood cells, resulting from the percolating blood flow delivered by the termini of the splenic arterioles. The blood flow to the spleen predominantly terminates in the red pulp and the marginal zone. Most pathogens, on clearance from the bloodstream, first are localized to the marginal zone and red pulp of the spleen.[198] With respect to cellular trafficking into the spleen, the major entry point into the white pulp is from the marginal zone.[199] From here, activated APCs can enter the white pulp areas, most likely by traversing the marginal zone sinus and the metallophilic macrophage layer that forms a boundary between white pulp and marginal zone.

Access of protein antigens and other molecules into the splenic white pulp is highly restricted and, in many ways, similar to the system identified in lymph nodes. Specifically, fibroblastic reticular cells form small channels that surround collagen fibers that enter the T-cell and the B-cell zones of the spleen and deliver small molecules (i.e., generally <60 kDa) into the white pulp. The channels found in B-cell follicles bind chemokines associated with the recruitment of B cells, whereas the channels identified in T-cell zones bind chemokines associated with T-cell recruitment.

Mucosa-Associated Lymphoid Tissue

Mucosa-associated lymphoid tissue (MALT) is a network of lymphoid cell aggregates and tissue that is distributed in submucosal layers of the gastrointestinal, genital, respiratory, and urinary tracts, as well as in the eyes, skin, thyroid, breasts, tonsils, and salivary glands.[200] Cellular residents include T and B lymphocytes, as well as macrophages, all of which are positioned to trap and respond to antigens at mucosal portals of entry. Gut-associated lymphoid tissue (GALT), a MALT component, covers an estimated area of 300 m^2 in the human intestine. T lymphocytes are prevalent within the finger-like projections of the intestinal mucosa and can be found in the epithelial layer and in the underlying layer of connective tissue termed the *lamina propria.* GALT components include organized lymphoid tissues, such as Peyer's patches in the small intestine, and mesenteric lymph nodes.

T lymphocytes positioned in the epithelial layer at mucosal sites are a diverse population that, in aggregate, are called *intraepithelial lymphocytes*

(IELs).[180] Some IELs express the α- and β-chains of the coreceptor CD8 ($CD8\alpha^+\beta^+$), whereas others express exclusively α-chains ($CD8\alpha\alpha^+$) or no CD8 at all. With regard to TCR expression, γδ T cells are prominent cellular IEL constituents, as discussed earlier, though conventional CD8 T cells with αβ TCRs predominate in the intestinal lamina propria.[201,202] Nearly all IELs express adhesion molecules associated with an activated or memory phenotype and readily express effector functions, such as cytolytic activity and cytokine secretion.

Within the intestine, epithelial cells and DCs are postulated to present antigen to T lymphocytes.[203–205] DCs sample intestinal contents by transiently traversing the tight junctions of the intestinal epithelial cell layer.[206–208] After engulfing bacterial pathogens, such as *S. typhimurium,* DCs migrate to secondary lymphoid tissues to activate naïve T lymphocytes. However, a study proposed that CD8 effector memory cell precursors can be selected in the periphery by *thymus leukemia antigen,* an MHC class I molecule expressed by epithelial cells and DCs. In this model, thymus leukemia antigen preferentially binds to high-affinity $CD8\alpha\alpha^+$ or $\alpha\beta^+$ effector memory cell precursors and promotes affinity maturation of resident mucosal $CD8\alpha\beta^+$ T_{EM} cells for long-term persistence at mucosal sites of surveillance.[209]

Studies in mice have shown that intestinal infection with viral or bacterial pathogens induces robust expansion of pathogen-specific T lymphocytes in the small intestinal epithelium and lamina propria.[201,202,210] Systemic infection with viruses, such as vesicular stomatitis virus, or the intracellular bacterium *L. monocytogenes* also induces marked increases in the frequency of pathogen-specific CD8 T cells in the gut, suggesting that these T cells traffic to the intestine during systemic immune responses. The concept that immune responses to infectious pathogens result in the distribution of pathogen-specific T lymphocytes throughout the body is supported by two studies that measured whole-animal immunity.[211,212] In both studies, although T-cell priming occurred in secondary lymphoid tissues, after priming, antigen-specific T lymphocytes were found at higher frequencies in nonlymphoid tissues, such as liver, lamina propria, and adipose tissues.

T-CELL ACTIVATION

T-lymphocyte activation begins when the TCR binds to cognate MHC-peptide complexes, triggering a complex signaling cascade.[213,214] CD8 T cells bind to MHC class I complexes, while CD4 T cells bind to MHC class II complexes.

Beyond the first signal of MHC-TCR engagement, a second signal involves *costimulation,* and is mediated by the T-cell coreceptors CD28 and CD80/B7.1 and CD86/B7.2 on the APC. This interaction induces T-cell IL-2 production, which promotes their proliferation. Upon activation, T lymphocytes express CTLA-4 (CD152), which also binds CD80 and CD86, but instead of stimulating proliferation, this signaling molecule inhibits proliferation and acts as a brake on expansion. Studies have identified many other related B7 molecules that play a role in T-cell activation in the periphery.[215] One of these molecules, B7H2 (CD275), interacts with the inducible molecule ICOS on the surface of activated T cells and promotes their differentiation toward a Th2 phenotype.

Many TNF receptor superfamily members play a role in T-cell activation and differentiation.[216] Among these, CD40 and its agonist CD40L play a critical role in T-cell and B-cell immunity, since genetic defects in CD40 and CD40L underlie hyperimmunoglobulin M syndrome.[217] Affected individuals have defects in antibody isotype switching, accounting for high circulating immunoglobulin M levels, and are susceptible to infections with *Pneumocystis jirovecii,* indicating defective T-cell–mediated immunity (see Table 6.1). In mice deficient for CD40 or CD40L, T-cell activation and maintenance of memory T-cell populations are defective, accounting for increased susceptibility to some infections.[218,219] TNF receptor superfamily member 9 (CD137; 4-1BB) and TNF receptor superfamily member 4 (CD134; OX40) have also been implicated in the generation of pathogen-specific T-cell responses. OX40-dependent signals promote T-cell clonal expansion by inducing survivin, a protein that blocks T-cell apoptosis.[220]

The interface between T lymphocytes and APCs is a highly organized structure called the *immunologic synapse.*[221] This structure, also referred to as the supramolecular activation complex (SMAC), contains TCR and MHC contacts in the central portion and lymphocyte function–associated molecule 1 (CD18/CD11a; β_2/αL integrin) and intercellular adhesion molecule 1 (CD54) contacts in the periphery. The SMAC contains costimulatory (e.g., CD28) and signaling molecules as well. Synapse formation occurs when an antigen-specific T cell encounters an APC, enabling these cells to engage each other for several hours. The role of the SMAC in T-cell activation remains controversial, since T-cell signaling can precede synapse development.[222] Researchers have hypothesized that SMAC formation may play a more significant role in the response to infrequent peptide-MHC complexes or perhaps to lower affinity ligands for the T-cell receptor.[223]

Organization of the Major Histocompatibility Complex

The MHC, so named because its discovery resulted from studies of tissue transplant rejection, contains many of the genes associated with cell-mediated immune defenses.[224] The MHC complex encodes the α-chains of the MHC class I molecules human leukocyte antigen (HLA)-A, HLA-B, and HLA-C and the α- and β-chains of the MHC class II molecules HLA-DR, HLA-DP, and HLA-DQ, all of which are expressed in a co-dominant fashion.

MHC class I is expressed by all nucleated cells and platelets in jawed vertebrates, although the amount on the cell surface varies among cell types and under different inflammatory conditions.[225] The nucleotide-binding oligomerization domain (NOD)–like receptor (NLR) family member NLRC5 can regulate MHC class I expression at the transcriptional level, in response to viral and bacterial infections and to type I IFN and IFN-γ.[226] Consistent with this role, *nlrc5*-deficient mice exhibit a reduced CD8 T-cell response to systemic listeriosis, resulting in defective bacterial clearance.[227]

In contrast to MHC class I molecules, MHC class II expression is generally restricted to DCs, macrophages, B lymphocytes, innate lymphoid cells, and thymic epithelial cells under homeostatic conditions. However, MHC class II expression can be induced by various inflammatory signals, in particular IFN-γ. Regulation of MHC class II expression is mediated in part by the class II transactivator (CIITA) protein, a transcription factor that enhances expression of MHC class II molecules, the associated invariant chain, and other molecules associated with MHC class II antigen processing.[228] In humans, four promoters (i.e., pI–pIV) with different *cis*-regulatory elements regulate CIITA expression in specific cell types.[229] For example, pIV drives constitutive expression of MHC class II in thymic epithelial cells as well as IFN-γ-induced CIITA expression in endothelial cells, epithelial cells, fibroblasts, and astrocytes. Thus, during tissue inflammation, nonconventional myeloid cell populations that include neutrophils, basophils, and eosinophils, as well as certain epithelial, endothelial, and stromal cell populations can upregulate class II expression. Because most of these observations have been conducted in mice, it remains unclear whether these atypical class II–expressing cells contribute significantly to T cell activation and function in humans.[230]

Mutations in the *CIITA* gene give rise to bare lymphocyte syndrome type II, an important human primary immunodeficiency syndrome associated with profound immunosuppression, since circulating peripheral blood mononuclear cells do not express surface MHC class II molecules (see Table 6.1).[231] Other causes of bare lymphocyte syndrome type II include mutations in *RFX5, RFXANK,* and *RFXAP* (see Table 6.1).[232,233]

The MHC locus also contains genes associated with the antigen-processing pathways. These include genes that encode several proteasome subunits (low-molecular-mass polypeptides 2 and 7 [LMP-2, LMP-7] and multicatalytic endopeptidase complex–like 1 [MECL-1]), the peptide transporter associated with antigen processing 1 and 2 (TAP1 and TAP2) proteins, and the MHC class II processing–associated HLA-DM and HLA-DO proteins (Fig. 6.6).

A cardinal feature of the MHC is the polymorphism of some of its genes, particularly MHC class I and class II. For the MHC class I HLA-B gene, there are more than 2000 known alleles.[224] Although some of these alleles bind similar peptides, most, by virtue of their morphologically distinct peptide binding grooves, bind distinct families of peptides. An evolutionary force driving the diversity of MHC alleles comes from the microbial world and its ability to undergo antigenic variation.[234,235]

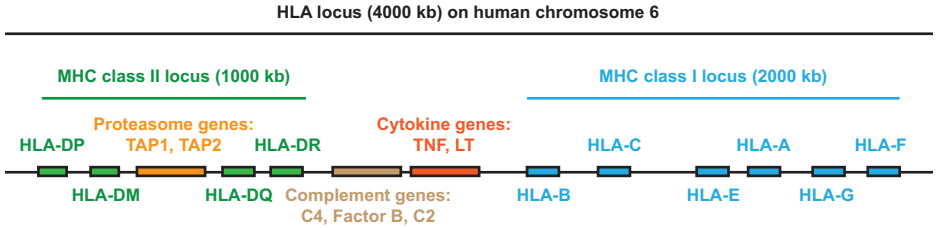

FIG. 6.6 Genes of the major histocompatibility complex *(MHC)* locus. Schematic map of the 4000-kb human MHC on chromosome 6, termed the *human leukocyte antigen (HLA) complex,* and major genes that encode proteins involved in antigen presentation, processing, and immune responses (drawing is not to scale). The MHC class II genes encode distinct α- and β-chains. *LT,* Lymphotoxin; *TAP,* transporter associated with antigen processing; *TNF,* tumor necrosis factor.

Human population studies in malaria-endemic regions demonstrated that certain HLA alleles are associated with a greater ability to survive malaria.[236] Conversely, malaria has a contravailing ability to alter protein sequences bound by HLA molecules, allowing parasite populations to evolve that are invisible to individuals with certain HLA molecules.[237] This escape strategy has been described for viral pathogens,[238,239] including HIV.[240]

The sequencing of the human genome, the advent of microarray-based, high-throughput genotyping technology, and the completion of the international HapMap project have facilitated numerous genome-wide association studies for human susceptibility to infectious agents.[23] Genome-wide association studies have identified significant associations of polymorphisms in MHC class I genes with HIV control, HIV viral load at set-point, and long-term nonprogression,[241] and in MHC class II genes with susceptibility to leprosy[242] and hepatitis B.[243]

The MHC complex also encodes proteins for MHC class I molecules that, unlike HLA-A, HLA-B, and HLA-C, are not highly polymorphic and generally highly conserved among individuals. These molecules also are called *MHC class Ib* molecules and in humans consist of HLA-E, HLA-F, and HLA-G (see Fig. 6.6). HLA-G is highly expressed in the placenta, regulates fetomaternal tolerance, and may protect cells from NK-cell–mediated lysis.[244] HLA-E binds the signal sequences of conventional MHC class I molecules, providing a readout of cellular synthesis of MHC class I molecules. HLA-E also binds to two NK-cell receptors, CD94/NKG2B and CD94/NKG2C, that inhibit NK-cell–mediated cytolysis.[245,246]

Antigen Presentation to T Lymphocytes via MHC Class I

Classic studies revealed that T lymphocytes recognize antigens in the context of MHC haplotype-matched APCs that degrade internalized pathogen-derived proteins and transport microbial antigens to the cell surface.[247,248] T cells respond to peptide fragments of pathogen-derived proteins, a process that can be recapitulated with corresponding short synthetic peptides.[249,250]

The precise nature of MHC involvement in antigen presentation became clear with the crystallization of HLA-A2, which showed a globular protein with a central groove that precisely accommodates a solitary peptide.[251,252] The identification of the TCR was the other key development that allowed a complete picture of the T-cell recognition process.[253] The TCR, a heterodimeric membrane-spanning receptor, provides diversity and specificity by a gene recombination process that is mechanistically similar to the generation of antibody diversity in B cells. The following sections describe the structure of MHC molecules and the process by which pathogen-derived peptides are presented to TCRs expressed by CD4 and CD8 T cells.[225]

MHC Class I Structure

MHC class I molecules present peptide antigens to CD8 T lymphocytes. MHC class I molecules are transmembrane proteins that consist of a single α-chain and associate with β_2-microglobulin for proper folding and trafficking to the cell surface. The characteristic structural features of MHC class I molecules are the α1, α2, and α3 domains. These create a globular protein in which a β-pleated sheet forms the floor of the peptide binding groove and two helical regions form the sides of the groove.[251,252] An important feature of the MHC class I groove is the restricted size of the peptide, typically 9 amino acids in length, that can be accommodated since the groove is closed at both ends[254] (Fig. 6.7).

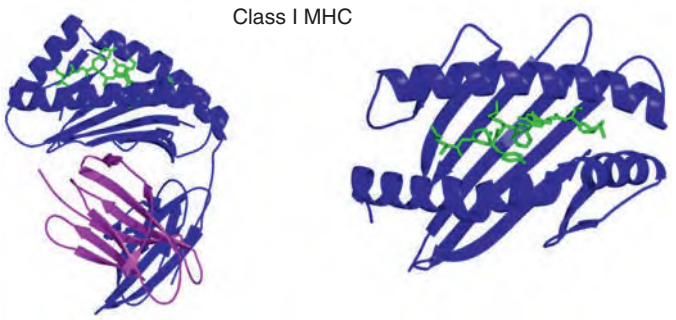

FIG. 6.7 Structure of class I major histocompatibility complex *(MHC)* with bound peptide. MHC class I molecules bind short antigenic peptides in a central groove formed by two α-helices of the heavy chain. The T-cell receptor binds the peptide and also associates with the MHC class I molecule in order to transmit an activation signal to the T lymphocyte. The depicted structures represent a murine H2-K^b MHC class I molecule *(purple)* that is binding a peptide derived from vesicular stomatitis virus *(green).* β_2-Microglobulin is shown in pink. The left panel shows a side view of the complex while the right panel shows a top view of the complex as it would be detected by a T-cell receptor on a CD8 T cell. *(Courtesy Dr. Chris Garcia, Stanford University, Palo Alto, CA.)*

Although binding of peptides and interaction with TCRs is mediated entirely by the MHC class I protein, stable surface expression of peptide–MHC class I complexes requires the association with β_2-microglobulin. In the absence of β_2-microglobulin, most MHC class I molecules fold improperly and are destroyed before leaving the endoplasmic reticulum (ER).[255]

MHC Class I Antigen-Processing Pathway

Antigens presented by MHC class I and class II molecules generally derive from different cellular compartments. The MHC class I antigen-processing pathway begins in the cytosol with the degradation of a protein that, in most cases, is an endogenous self-protein, or in the setting of infection, a microbial-encoded protein (Fig. 6.8).[225] In addition, exogenous proteins internalized into membrane-bound compartments by endocytosis, phagocytosis, or autophagy can enter the MHC class I processing pathway by a mechanism termed *cross-presentation.*[196]

In the cytosol, protein turnover and degradation is primarily mediated by the *ubiquitin-proteasome* pathway. Proteasomes are barrel-shaped multicomponent proteases that consist of four stacked rings with a heptameric structure; they hydrolyze cellular proteins in the cytosol. In activated cells or after exposure to IFN-γ, the proteasome composition can change, with some components replaced by subunits that enhance the generation of antigenic peptides and other components added to the ends of the barrel to alter the efficiency and specificity of protein degradation.[225] Proteasomes target endogenous and exogenous proteins for degradation by a variety of mechanisms. Misfolded proteins and

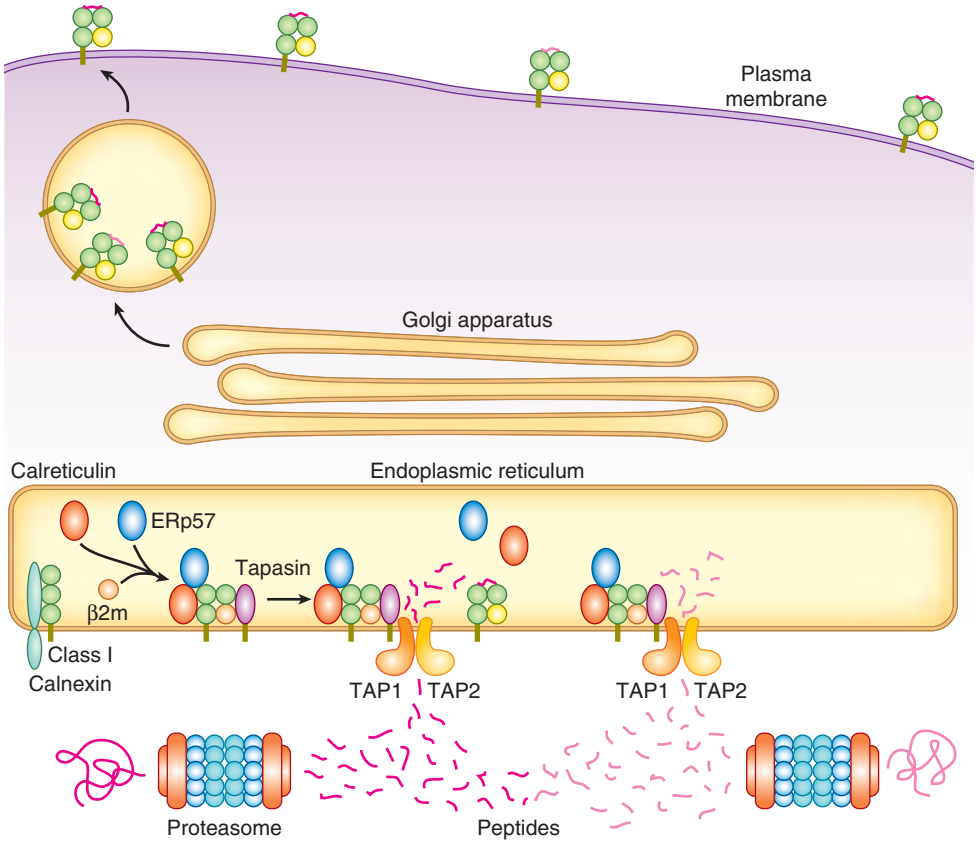

FIG. 6.8 The major histocompatibility complex (MHC) class I antigen-processing pathway. The MHC class I antigen-processing pathway begins in the cytoplasm with the degradation of proteins by proteasomes. Peptides are transported into the endoplasmic reticulum *(ER)* lumen, where MHC class I molecules bind and transport peptides to the cell surface. *β2m,* β_2-Macroglobulin; *ERp57,* endoplasmic reticulum protein 57; *TAP,* transporter associated with antigen processing. *(Courtesy Anne Ackerman and Peter Cresswell of Yale University, New Haven, CT.)*

defective ribosomal products, both of which fail to assume native conformation states, may reveal peptide sequences that are recognized by proteasomes, leading to their rapid degradation. Alternatively, many proteins targeted for rapid proteasomal degradation undergo conjugation to ubiquitin by enzymes that recognize the phosphorylation of specific amino acid residues.

In the setting of infection, it is unclear whether pathogen-derived polypeptides and proteins are degraded selectively and targeted for presentation by MHC class I molecules. Bacterial proteins in the cytosol are degraded rapidly because they express unique amino-terminal amino acids[256] or contain internal amino acid sequences that promote rapid degradation.[257] In most circumstances, however, pathogen-derived antigens probably are degraded nonselectively with endogenous proteins and must compete with far more prevalent endogenous peptides for a place in an MHC class I peptide-binding groove.

Proteasomes generate peptides that, by virtue of the length of the proteasome channel, are 9 to 12 amino acids in length. Proteasome-generated peptides are bound to the peptide transporter TAP. This heterodimeric, ATP-dependent transporter efficiently moves peptides from the cytosol into the lumen of the ER. TAP is the major peptide transporter involved in the generation of peptide–MHC class I complexes; mice with genetic deletions of TAP have markedly decreased levels of surface MHC class I and markedly diminished numbers of CD8 T cells.[258] TAP deficiency has been identified rarely in humans; it underlies bare lymphocyte syndrome type I, and is associated with markedly decreased numbers of circulating CD8 T cells and modest immunodeficiency (see Table 6.1).[259,260]

The TAP1 and TAP2 molecules each contain seven transmembrane regions and an ATP binding site and together transport peptides from the cytosol into the ER lumen.[261] Newly synthesized MHC class I molecules associate with TAP in the ER, and the recruitment of several other ER resident proteins and chaperones leads to the formation of the *peptide loading complex* (PLC). The PLC includes peptide-receptive MHC class I–/β_2-microglobulin complexes that are recruited by the chaperone calreticulin to the TAP channel that translocates antigenic precursor peptides into the ER. Tapasin and the thiol reductase ERp57 serve as an editing module to enable the ER resident protease ERAP (humans) or ERAAP (mice) to trim the peptide precursors before their final integration into the MHC class I groove.[262–264] The key role of β_2-microglobulin and of the PLC is to maintain the MHC class I binding groove in a conformation that favors the binding of high-affinity peptides. If the affinity of the peptide–MHC class I interaction is sufficiently high, the MHC class I–β_2-microglobulin–peptide complex is released from the PLC, allowing the complex to traffic via the Golgi complex to the cell surface. Low-affinity peptide–MHC class I interactions result in the reglucosylation of an *N*-linked glycan on the MHC class I heavy chain,[265] a reaction that redirects MHC class I molecules into the PLC for peptide exchange.

In the setting of microbial infection and inflammation, the MHC class I antigen-processing pathway is enhanced, allowing more efficient presentation of pathogen-derived peptides to CD8 T cells. IFN-γ, in particular, impacts the MHC class I pathway at multiple levels. First, IFN-γ enhances the transcription of many components of the MHC class I pathway, including MHC class I molecules, TAP, tapasin, and several components of the proteasome. Specifically, three subunits of the proteasome—LMP-2, LMP-7, and MECL—are induced and replace three subunits of the core proteasome complex.[225] IFN-γ induces additional accessory proteins (e.g., PA-28) that can increase the efficiency with which virus-derived, MHC class I–restricted epitopes are presented to CD8 T cells.[266]

Viral Interactions With the MHC Class I Antigen-Processing Pathway

CD8 T lymphocytes and the MHC class I antigen-processing pathway are involved principally in defense against viral infection. Viruses have evolved multiple strategies to evade MHC class I–dependent presentation, illustrating the importance of this process in antiviral defense. Broadly speaking, viruses can interfere with the peptide loading process, induce MHC class I–peptide complex retention in the ER, stimulate MHC class I–peptide complex degradation, and reroute MHC class I–peptide complex for concealment.[267]

To interfere with the peptide loading process, herpes simplex virus ICP47 blocks human TAP by plugging the peptide transport channel from the cytosolic side.[268,269] The human cytomegalovirus (CMV)–encoded protein US6 blocks TAP transport by obstructing the peptide transporter from the ER luminal side.[270,271] CMV also encodes a microRNA (i.e., miR-US4-1) that downregulates ERAP messenger RNA and prevents proteolytic processing of antigenic peptides in the PLC.[272]

The CMV US3 protein binds MHC class I molecules in the ER and prevents their trafficking to the cell surface.[273,274] A similar strategy is used by adenoviruses, which encode the type I membrane protein E3-19K.[275,276] This protein binds MHC class I molecules in the ER lumen and prevents their egress from the ER by expressing an ER retention motif on its cytoplasmic tail.

Another strategy for downregulating surface MHC class I retention is to displace ER-resident MHC class I molecules into the cytoplasm, where they are ubiquitinated rapidly by E3 ligases and targeted for proteasomal degradation. Human CMV encodes two proteins, US2 and US11, that mediate this process.[276,277] Remarkably, transport of proteins from the ER lumen back to the cytosol via the Sec61 translocon is a normal process that usually is restricted to misfolded or otherwise nonfunctional proteins. US2 and US11 seem to accelerate this process selectively for MHC class I molecules.

The Kaposi sarcoma herpesvirus also downregulates surface MHC class I expression, but by another mechanism. Two Kaposi sarcoma herpesvirus–encoded proteins, K3 and K5, function as ubiquitin ligases that selectively conjugate ubiquitin to the cytoplasmic tail of MHC class I and CD86/B7.2 molecules.[278,279] On ubiquitination, surface MHC class I molecules are internalized rapidly and targeted for lysosomal degradation. HIV also has evolved mechanisms to downregulate surface expression of MHC class I molecules.[280–282] In this case, the retrovirally encoded Nef protein selectively downregulates the expression of HLA-A and HLA-B molecules by associating with the clathrin adaptor complex.

A consequence of MHC class I downregulation is that affected cells become susceptible to NK cell–mediated lysis. NK cells express receptors that inhibit NK cell activation on contact with MHC class I molecules. To prevent NK cell–mediated lysis of virally infected cells, CMV encodes an MHC class I–like molecule, UL18,[283,284] that acts as a decoy for the NK cell inhibitory receptor LIR-1, providing yet another level of camouflage to the viral pathogen.

MHC Class I Cross-Presentation

The MHC class I antigen-processing pathway performs two principal functions. First, it presents antigens to naïve CD8 T cells in a manner that promotes their activation, proliferation, and differentiation. Second, it presents antigens to activated CD8 T cells as a signal of cellular infection. The first function is predominantly, if not exclusively, mediated by DCs.[285] Any MHC class I–expressing cell that becomes infected can perform the second function. The rules of antigen processing differ in these two circumstances. The conventional MHC class I antigen-processing pathway, as described in the preceding sections, applies to the second function.

Because they often are not infected directly, the major route for CD8 T-cell priming involves uptake of debris from infected cells by DCs, and re-presentation of pathogen-derived peptides[286] by an antigen-processing pathway that involves endocytosis and TAP-mediated transport of antigen into the ER.[287] The $CD8^+$ subset of DCs is particularly efficient at taking up and delivering exogenous antigens for cross-presentation by the MHC class I antigen-processing pathway. Antigen-containing phagosomes in DCs fuse with ER membranes, resulting in the recruitment of a retrotranslocation machinery that shuttles misfolded proteins or antigens from the phagosome lumen into the cytosol, where proteins are degraded by proteasomes and enter the conventional MHC class I processing pathway.[288]

A second route of cross-presentation involves a vacuolar pathway that does not require proteasomes or TAP. In this route, peptides are generated by cathepsin- or lysosomal protease–mediated hydrolysis in the endosome and loaded in situ on MHC class I complexes that traffic to the phagosome in a Sec22-dependent manner.[196,289]

Antigen Presentation to T Lymphocytes via MHC Class II

MHC Class II Structure

MHC class II molecules present peptide antigens to CD4 T lymphocytes. The folded MHC class II molecule consists of two transmembrane proteins, termed the α-chain and the β-chain. The α1 and β1 domains of each chain together form an open-ended peptide-binding groove.[290] The remaining α2 and β2 domains of each chain form an extracellular immunoglobulin–like domain. The open-endedness of the MHC class II groove accounts for the binding of substantially longer peptides than seen with MHC class I molecules.[291] Peptides bound by MHC class II molecules typically are longer than 10 amino acids, and occasionally are more than 20 amino acids in length. The structure of an MHC class II molecule binding an antigenic peptide is shown in Fig. 6.9.

MHC Class II Antigen-Processing Pathway

Although similarities to the MHC class I antigen-processing pathway exist, there are some key distinctions. First, most peptides presented by MHC class II molecules derive from extracellular proteins that have been endocytosed by MHC class II–expressing cells.[292] MHC class II molecules also present peptides from membrane or secretory proteins that are degraded in endosomal compartments during transport to the cell surface (Fig. 6.10). The MHC class II antigen-processing pathway has been implicated principally in the response to extracellular pathogens and pathogens that reside within vacuolar compartments, such as *S. typhimurium* and *M. tuberculosis*.

The first step in the MHC class II antigen-processing pathway is the translocation and assembly of MHC class α- and β-chains in the ER, a reaction mediated by a dedicated chaperone, the invariant chain. The α- and β-chains fold and use the invariant chain, a membrane-bound protein, as a substitute for a peptide. A portion of the invariant chain occupies the MHC class II groove as the complex exits the ER and traffics to the endosomal compartments. When endosomal compartments acidify, proteases (e.g., cathepsin B and D) are activated and hydrolyze the invariant chain except for the portion protected by the MHC class II groove.[293]

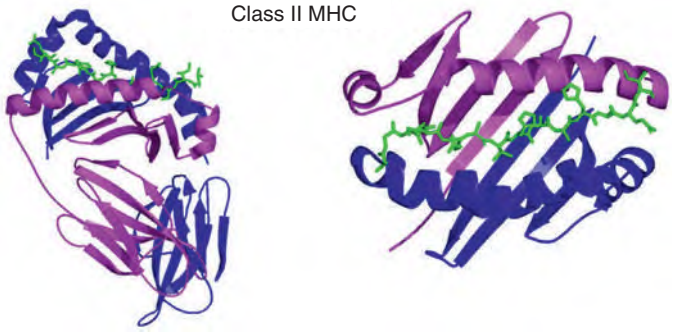

FIG. 6.9 Structure of class II major histocompatibility complex *(MHC)* with bound peptide. MHC class II molecules also bind antigenic peptides in a groove formed by the α- and β-chains. In contrast to peptides bound by MHC class I molecules, which are of defined length, the MHC class II structure accommodates peptides of various lengths. The MHC class II α- and β-chains are shown in pink and purple, while the antigenic peptide is shown in green. The left panel shows a side view of the complex while the right panel shows a top view of the complex as it would be detected by a T-cell receptor on a CD4 T cell. *(Courtesy Dr. Chris Garcia, Stanford University, Palo Alto, CA.)*

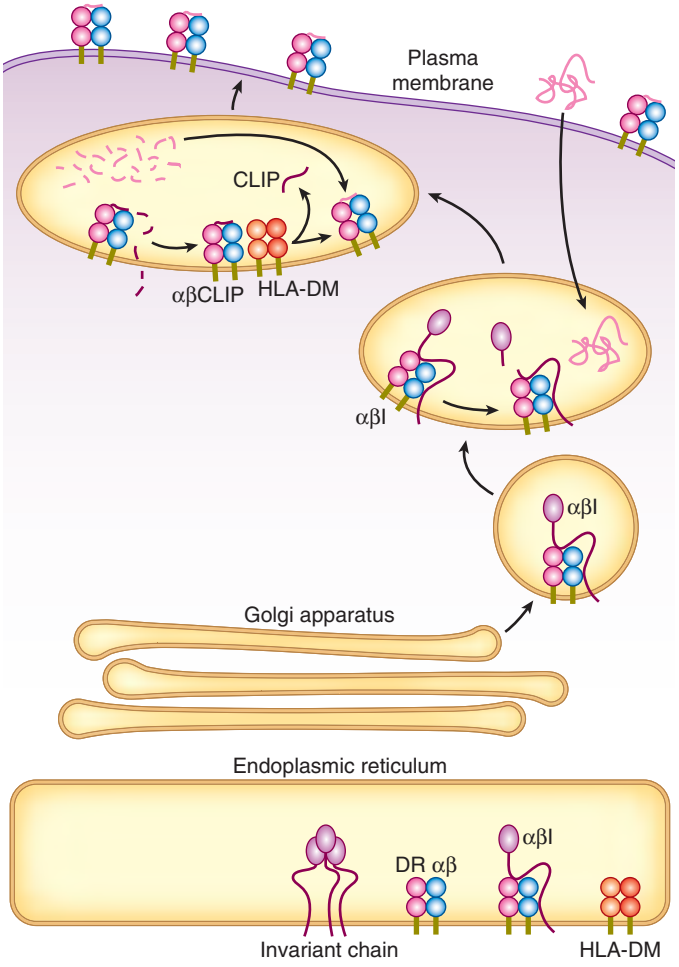

FIG. 6.10 The major histocompatibility complex (MHC) class II antigen-processing pathway. The MHC class II antigen-processing pathway presents peptides derived from extracellular proteins to $CD4^+$ T lymphocytes. Peptides are generated in an endosomal compartment. The peptide editor human leukocyte antigen DM *(HLA-DM)* promotes dissociation of the placeholder class II–associated invariant chain peptide *(CLIP)* from MHC class II molecules, allowing the exogenous peptide to associate with MHC class II. The MHC class II–peptide complexes are then transported to the cell surface. *(Courtesy Anne Ackerman and Peter Cresswell of Yale University, New Haven, CT.)*

In the acidified endosome, in a compartment called the *MCH class II–positive compartment (MIIC),* MHC class II molecules can interact with endocytosed antigens in the process of degradation.[294,295] An MHC class II–like molecule, HLA-DM, resides in MIICs and catalyzes the extraction of the invariant chain peptide fragment from the MHC class II molecule. Crystallographic studies reveal that HLA-DM stabilizes empty MHC class II proteins in a conformation that promotes the insertion of a high-affinity peptide.[296] In addition to proteases, a thiol reductase called *γ-interferon–inducible lysosomal thiol reductase (GILT)* is involved in the denaturation of some antigens before their degradation and presentation by MHC class II molecules.[297] GILT activates the major secreted virulence factor listeriolysin O, of the bacterial pathogen *L. monocytogenes,* providing an example of microbial exploitation of the MHC class II antigen-processing pathway.[298] Following peptide binding in the MIIC, MHC class II molecules traffic to the cell surface, where the MHC class II–peptide complex can be detected by CD4 T cells. MHC class II molecules are subsequently reinternalized, recycle to the endosomal compartments, and acquire new peptides before returning to the cell surface.

CD1 and Antigen Presentation

The CD1 family comprises antigen-presenting molecules that resemble MHC class I molecules in their general structure and association with β_2-microglobulin.[299] The human CD1 locus on chromosome 1 contains five distinct genes that encode for proteins designated CD1a through CD1e. In contrast to humans, mice lack CD1a, CD1b, and CD1c and have a duplicated CD1d gene. The extracellular portion of CD1 is composed of three domains that mediate antigen binding and form an antigen-binding groove that is structurally analogous to the peptide-binding groove of the MHC. The three-dimensional structure of mouse CD1[300] and human CD1 isoforms a through d[301] reveals a complicated network of hydrophobic channels that can accommodate diverse lipids of varying aliphatic chain length.[302]

The CD1 isoforms are enriched in different intracellular compartments, suggesting that each isoform of CD1 has evolved to survey the microbial antigens that appear in distinct parts of the endosomal-lysosomal network. All CD1 isoforms are found at the cell surface and internalized during endocytosis. CD1a is found predominantly in early endosomes, CD1c in late endosomes, and CD1b and CD1d in late endosomes and lysosomes. Specific amino acid residues in their short intracellular tails target CD1 isoforms to these various compartments by binding cytosolic adaptor molecules that mediate organelle trafficking.[303–305]

CD1b presents glycosylated and free mycolic acids and lipoarabinomannan, two major lipid and glycolipid components of the *M. tuberculosis* cell envelope. CD1-presented antigens have expanded to include other microbial glycolipids, including diacylglycerols from *Streptococcus pneumoniae*[306] and the fungal glycosphingolipid asperamide B.[302,307] Thus CD1 isoforms facilitate cell-mediated defense against infection by presenting a wide range of microbial lipidic antigens to CD1-restricted T cells.

INNATE IMMUNE RECOGNITION: SETTING THE STAGE FOR T-CELL RESPONSES

The adaptive immune system consists of B and T lymphocytes that express distinct, somatically recombined receptors with exquisite specificity, at the cost of low frequency. In contrast, the innate immune leukocytes (i.e., neutrophils, monocytes, macrophages, and DCs) express families of germline-encoded receptors that survey the cell surface, the cytosol, and endosomal or phagosomal compartments, and recognize a wide array of microbial-derived molecules (Fig. 6.11). It is clear from studies on the genetic basis of human susceptibility to infectious diseases and from studies in mice that innate immune responses to microbial molecules, by promoting the expression of costimulatory molecules and the secretion of cytokines, form the foundation for adaptive immune responses (see Table 6.1).

The molecular basis for antigen-specific responses derives from combinatorial receptors (i.e., αβ TCRs) that have almost infinitely diverse specificity. This system ensures that a great diversity of pathogenic antigens can be recognized, but, because pathogen-specific cells are infrequent, it requires time for expansion of these cells to numbers that are sufficient to combat the infection. In the hours after a pathogen breach of an anatomic barrier, the most rapid recognition events are mediated by cells bearing innate immune receptors. These receptors are not combinatorial, but recognize conserved structural elements in broad classes of microbial molecules that serve as a general signal of infection. This recognition event, rapid but relatively nonspecific, plays an essential role in the generation of pathogen-specific immunity through the generation of cytokines and chemokines that recruit APCs to the site of infection and shape the ensuing adaptive immune response. This section details the major known receptors of the innate immune system and links the function of these receptors to antigen-specific cellular immunity.

Molecular Recognition of Microbial Products

Several outstanding reviews provide a comprehensive overview on the molecular recognition of microbial products and its role on human susceptibility to infectious diseases.[308–314]

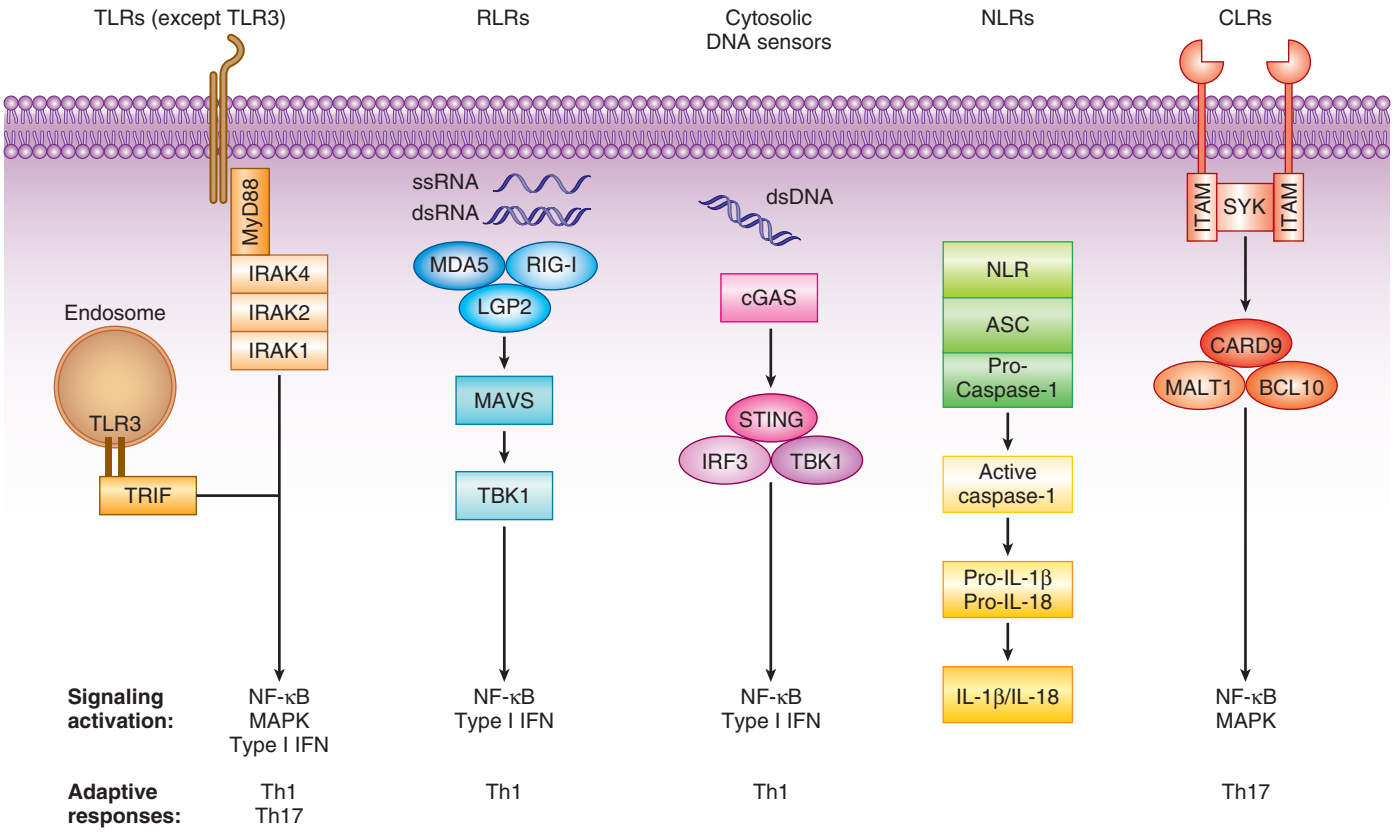

FIG. 6.11 Cell surface and intracellular classes of pattern recognition receptors (PRRs) and associated signaling pathways and adaptive responses. Toll-like receptors *(TLRs)* are present on the cell surface or in the endosome (TLR3, TLR7, TLR9). Most TLRs utilize the MyD88 protein, with the exception of TLR3, which utilizes TRIF, and TLR4, which utilizes both MyD88 and TRIF, as adaptor proteins for downstream signaling. TLR engagement induces NF-κB, MAPK, or type I IFN signaling, or a combination of these, and helper T cell Th1 or Th17 responses, depending on the pathogen. The RLR family of PRRs recognizes RNA ligands and consists of MDA5, RIG-I, and LGP2. RLRs signal through MAVS, an adaptor on the mitochondrial surface, and TBK1 to induce NF-κB and type I IFN signaling and Th1 responses. The cytosolic DNA sensor cGAS signals via the ER adaptor protein STING, which associates with TBK1 and IRF3 to induce NF-κB and type I IFN signaling and Th1 responses. NLRs are cytosolic PRRs, several of which are members of the inflammasome complex, which includes the adaptor protein ASC and pro-caspase 1. Inflammasome activation results in the activation of caspase 1, which leads to IL-1β and IL-18 release. CLRs are surface ITAM-bearing receptors that bind carbohydrates and signal via SYK and the CARD9–MALT1–BCL10 complex to induce NF-κB and MAPK signaling and Th17 responses. *ASC,* apoptosis-associated speck-like protein containing CARD; *BCL10,* B-cell lymphoma/leukemia 10; *CARD9,* caspase recruitment domain–containing protein 9; *cGAS,* cyclic GMP-AMP synthase; *CLR,* C-type lectin receptor; *dsRNA,* double-stranded RNA; *ER,* endoplasmic reticulum; *IFN,* interferon; *IL,* interleukin; *IRAK,* interleukin-1 receptor–associated kinase; *IRF3,* interferon regulatory factor 3; *ITAM,* immunoreceptor tyrosine-based activation motif; *LGP2,* laboratory of genetics and physiology 2; *MALT1,* mucosa-associated lymphoid tissue lymphoma translocation protein 1; *MAPK,* mitogen-activated protein kinase; *MAVS,* mitochondrial antiviral-signaling protein; *MDA5,* melanoma differentiation–associated gene 5; *MyD88,* myeloid differentiation primary response gene 88; *NF-κB,* nuclear factor kappa-light-chain-enhancer of activated B cells; *NLR,* nucleotide-binding oligomerization domain–like receptor; *RIG-I,* retinoic acid–inducible gene 1; *RLR,* RIG-I–like receptor; *ssRNA,* single-stranded RNA; *STING,* stimulator of interferon genes; *SYK,* spleen tyrosine kinase; *TBK1,* TANK-binding kinase 1; *TRIF,* TIR domain–containing adapter inducing interferon-β.

Toll-Like Receptors

Toll-like receptors (TLRs) are a family at least 10 distinct transmembrane proteins that mediate the recognition of extracellular (i.e., TLR1, TLR2, TLR4, TLR5, TLR6) and endosomal (i.e., TLR3, TLR7, TLR8, TLR9) microbial products. The TLR ectodomains contain leucine-rich repeats that bind to a broad range of microbial products, and an intracellular Toll–IL-1 (TIR) domain that activates downstream signal adaptors, transducers, and kinases, including the IL-1 receptor–associated kinase (IRAK) complex, typically resulting in the activation of nuclear factor kappa B (NF-κB), a transcription factor that promotes the expression of genes associated with immune defense.[308] TLR-mediated signals induce the secretion of proinflammatory cytokines, such as TNF and IL-12, and induce the maturation of DCs, enabling them to activate naïve, pathogen-specific T lymphocytes. In addition, stimulation of TLRs can directly stimulate antimicrobial effector mechanisms of the host cell, limiting pathogen replication until adaptive immune cells are recruited to the site of infection.[315]

Each TLR recognizes specific microbial products, and well-defined ligands include bacterial lipoproteins (TLR1, TLR2, TLR6), double-stranded RNA (TLR3), lipopolysaccharide (TLR4), the bacterial peptide flagellin (TLR5), single-stranded RNA (TLR7 and TLR8), and CpG motifs within DNA (TLR9). TLR heterodimerization can mediate novel antigen-binding functions and increase the microbial specificity of individual TLR family members. For example, TLR2 heterodimerizes with TLR1 or TLR6 to recognize triacylated or diacylated lipoproteins, respectively.[316] Structural studies have demonstrated how microbial ligands associate with different TLR proteins, revealing a common pattern of TLR dimerization induced by association with microbial ligands.[317,318]

With the exception of endosomal TLR3, which utilizes the alternative pathway adaptor molecule TIR domain–containing adapter inducing IFN-β (TRIF),[319] most TLRs signal through the adaptor protein myeloid differentiation primary response gene 88 (MyD88); TLR4 uses both MyD88 and TRIF. Children who have autosomal-recessive defects in MyD88-mediated signal transduction due to mutations in *MYD88* or *IRAK4*, two very rare primary immunodeficiencies, lack input from most TLRs and from IL-1 receptor family members and develop severe and life-threatening pyogenic bacterial infections, particularly invasive streptococcal, staphylococcal, and pseudomonal infections (see Table 6.1).[320,321] More commonly, polymorphisms in TLR coding sequences and regulatory elements can confer susceptibility to infectious diseases,[322] as illustrated by the association of a common TLR5 stop codon polymorphism with susceptibility to the flagellated bacterium *Legionella pneumophila.*[323]

Patients with deficiency in TLR3 itself or the ER protein UNC-93B, the adaptor TRIF, the TNF receptor–associated factor 3 (TRAF3), or the TRAF-associated NF-κB activator (TANK)–binding kinase 1 (TBK1), all of which are involved in TLR3 activation,[324–326] develop selective susceptibility to herpes simplex encephalitis (see Table 6.1). The distinct infection susceptibility pattern of MYD88/IRAK4 (bacterial) versus TLR3-pathway (herpetic) defects illustrates the pathogen specificity of and host tissue requirements[327] for individual TLRs in host defense. Elegant recent studies using induced pluripotent stem cell–derived neurons and oligodendrocytes from TLR3- and UNC-93B–deficient individuals revealed that impaired IFN-α/β–dependent intrinsic immunity to HSV-1 by these cells underlies the pathogenesis of herpes simplex encephalitis in children with TLR3 pathway deficiencies.[327]

NOD-Like Receptors, RIG-I–Like Receptors, the Cyclic GMP-AMP Synthase–Stimulator of Interferon Genes Sensing Pathway, and the Inflammasome

While TLRs predominantly respond to extracellular or to endosomal microbial ligands, NLR proteins detect microbial ligands in the cytosol.[309] Among the best characterized of these, NOD1 and NOD2 respond to fragments of bacterial peptidoglycan, while NLRC4 responds to bacterial flagellin in the cytosol.[328,329] NLRs activate NF-κB signaling, and assemble into multicomponent structures called inflammasomes that consist of an NLR, an adaptor protein, and caspase subunits.[330] Inflammasomes activate a host cell apoptotic pathway and the expression of IL-1 and IL-18 by activating caspase-1 or caspase-8.[164] Macrophage caspase-1 activity is also important for phagosomal maturation.[331] Thus detection of microbial molecules in the cytosol induces an inflammatory response that has consequences for adaptive Th1 and Th2 cell responses.[332]

The RIG-I–like receptors (RLRs) retinoic acid–inducible gene 1 (RIG-I), melanoma differentiation–associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) are members of a family of molecules that detect cytosolic nucleic acids from RNA viruses (e.g., measles virus, influenza virus, hepatitis C virus, and West Nile virus).[311] RLRs signal via the adaptor protein mitochondrial antiviral-signaling protein (MAVS, also known as IPS1/VISA/Cardif) on the mitochondrial surface and collaborate with other pattern recognition receptors to induce antiviral responses.[333–337] For example, inherited MDA5 deficiency is linked to recurrent rhinovirus, influenza, and respiratory syncytial virus infections (see Table 6.1).[338] Influenza and other viruses can subvert cytosolic detection systems by inhibiting RLR signal transduction and targeting components of this pathway for degradation.

Detection of cytosolic DNA, specifically to cyclic dinucleotides, occurs via receptors that signal through the endoplasmic reticulum adaptor molecule stimulator of interferon genes (STING).[339] A central regulator of cytosolic DNA sensing is cyclic GMP-AMP synthase (cGAS), an interferon-inducible nucleotidyl transferase that has broad antiviral activity against HSV-1, vaccinia virus, and Kaposi sarcoma–associated herpesvirus (see Fig. 6.11).[340–343] Notably, these and other viruses can evade cytosolic sensing by disrupting DNA binding to cGAS or interfering with downstream STING phosphorylation, or both.[313,314] RLR- and cGAS/STING-dependent signaling events, either alone or via their cross talk, turn on NF-κB and type I interferon signaling in both infected and bystander cells.[313,314] The role of cytosolic sensors of viral nucleic acids in shaping B- and T-cell responses remains less well defined, although emerging evidence implicates the cGAS-STING pathway in DC maturation, development of antigen-specific Th1 cells, and immunoglobulin G2c production.[344]

C-Type Lectin Receptors

C-type lectin receptors (CLRs) bind to carbohydrate moieties of endogenous and exogenous origin, trigger phagocytic responses, and activate innate immune cells, particularly of myeloid origin.[345,346] The binding specificity for some of these receptors has been elucidated and includes fungal, bacterial, mycobacterial, viral, and parasitic microorganisms.

Many CLRs signal via tyrosine-based motifs that are embedded in their intracellular domain or that are present in adaptor molecules that associate with CLRs. Other important components of this signaling pathway are spleen tyrosine kinase and CARD9, which forms a complex with mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) and B-cell lymphoma/leukemia 10 (BCL10) (see Fig. 6.11).[49] This pathway is central to the recognition of fungal β-glucans (via the CLR Dectin-1/Clec7a),[347] fungal mannans (via the CLRs Dectin-2/Clec4n in mice or Dectin-2/Clec6a in humans and Dectin-3/MCL/CLECSF8/Clec4d),[348,349] and ligands of *M. tuberculosis* (via Dectin-3),[350] *Malassezia* (via Dectin-2 and the CLR Mincle/Clec4e),[351] *B. dermatiditis* (via Dectin-2),[352] and *Fonsecaea pedrosoi* (via Mincle), the agent of chromoblastomycosis.[353] Dectin-1 and Dectin-2 ligation promotes macrophage and DC cytokine and chemokine release and favors the development of Th17 responses.[354]

Inborn errors of CLR-dependent signaling result in susceptibility to fungal infections (see Table 6.1). Biallelic mutations in *CLEC7A*, which encodes Dectin-1, are associated with chronic mucocutaneous candidiasis. CARD9 deficiency leads to greater fungal infection susceptibility to both mucosal and invasive mycoses with a unique predilection for central nervous system candidiasis, extrapulmonary aspergillosis, and subcutaneous and central nervous system phaeohyphomycoses. In these patients, mucosal fungal susceptibility correlates with impaired Th17 responses, whereas deep-seated fungal susceptibility is associated with impaired neutrophil recruitment to the site of infection.[355–358] In contrast to mutations in *CARD9* that result in fungal-specific infection susceptibility, deficiencies in its partners MALT1 or BCL10 cause mucosal candidiasis but also have far broader immune implications, and affected patients manifest with a severe combined immunodeficiency phenotype because these molecules are downstream components of T- and B-lymphocyte signaling cascades (see Table 6.1).[359]

Links Between Innate Immune Recognition and Adaptive Immune Responses

Antibody and T-cell responses to inoculation of protein antigens are minimal unless an innate inflammatory response is elicited concurrently. Thus adjuvants that induce innate inflammatory responses are coadministered with some vaccines.[360] Adjuvants can facilitate slow release of antigens at depot sites, promote the release of proinflammatory cytokines and chemokines, and facilitate the recruitment of APCs that mature by the induction of TLR, CLR, NLR, RLR, and inflammasome signaling pathways. This process enhances antigen processing and presentation capacity by the MHC class II pathway and facilitates the interactions with antigen-specific T and B lymphocytes to promote potent humoral and cellular responses that characterize vaccine immunity. In support of this model, early studies demonstrated that immunization with model antigens and Freund adjuvant led to impaired T-cell responses and abnormal isotype-specific antibody production in MyD88-deficient mice, suggesting that TLR recognition is essential for proper Th1 adjuvant effects.[361] DCs that directly engage in TLR signaling following stimulation with microbial products can promote the proliferation and differentiation of naïve T cells. In contrast, DCs that undergo indirect activation by exposure to cytokines elicited by TLR signaling only support T-cell proliferation, but not differentiation.[362,363]

More recent studies indicate that non-TLR signaling pathways can provide adjuvant effects and support T-cell priming and antibody generation. Alum, a commonly used adjuvant for human vaccines, enhances antibody production by stimulating the NLRP3 component of inflammasomes to produce IL-1 and IL-18.[364] The CLRs Mincle and Dectin-2 can drive adjuvant effects that result in the development of antigen-specific Th1 and Th17 cells following vaccination against mycobacterial or fungal pathogens.[352,365]

Dendritic Cells

Although other immune and nonimmune cells have antigen-presenting capacity, DCs are the major immune cell type that presents antigens to naïve T lymphocytes and promotes their proliferation and differentiation. In vivo elimination of DCs, as has been performed experimentally in mice, abrogates priming of naïve T cells that respond to microbial infections.[285] High surface levels of MHC class I and class II and the expression of an array of costimulatory molecules enable DCs to stimulate naïve T cells effectively. DCs are a highly complex and heterogeneous

population of cells that reside both in lymphoid and nonlymphoid tissues and share phenotypic and functional characteristics that distinguish these cells from other classes of myeloid cells.[366]

DCs derive from progressively committed pluripotent bone marrow precursors. In mice, a common DC precursor gives rise to circulating plasmacytoid DCs and pre-DCs. Plasmacytoid DCs express high levels of TLR7 and TLR9 and secrete very high levels of type I interferons on stimulation; these are critical to antiviral defense.[367,368] Pre-DCs give rise to CD8α$^+$ and CD11b$^+$ DC populations in lymphoid organs and to CD11b$^+$ and CD103$^+$ DCs in peripheral nonlymphoid tissues. A combination of transcriptomic and functional studies revealed that murine lymphoid tissue–resident CD8α$^+$ DCs and nonlymphoid tissue–resident CD103$^+$ DCs are the likely counterparts of human CD141$^+$ DCs, all of which are highly effective at antigen cross-presentation following viral, bacterial, and parasitic, but not fungal, infections.[369–374] Murine CD11b$^+$ DCs in non-lymphoid tissues are the likely homologue of human CD1c$^+$ DCs and mediate conserved mucosal Th17 responses across species.[375]

The role of DCs in antimicrobial defense extends beyond stimulation of T cells. In mice and humans, a common monocyte precursor gives rise to circulating monocytes that can differentiate into monocyte-derived DCs or macrophages at portals of infection.[376–382] Monocyte-derived DCs, often termed inflammatory DCs, express a range of TLRs, CLRs, and NLRs that induce their activation and result in their production of cytokines that can orchestrate helper T cell differentiation.[354,377,383,384] Furthermore, monocyte-derived DCs exert direct microbicidal activity at portals of infection, as has been demonstrated during pulmonary *A. fumigatus*, *B. dermatiditis*, and *K. pneumoniae* infection.[54,385–388]

DCs are also primarily infected in some settings. For example, CD11b$^+$ DCs and monocyte-derived DCs are major infected cell populations in murine tuberculosis and leishmaniasis.[377,389] In pulmonary aspergillosis and tuberculosis and in a vaccine model of blastomycosis, monocyte-derived DCs and CD11b$^+$ DCs played an important role in transporting microbial antigen to draining lymph nodes.[14,378,390,391] Trafficking of activated DCs into lymph nodes involves CCR7 upregulation on DCs, enabling them to respond to CCL19 and CCL21, which are expressed in the lymph node paracortex.[392] DC trafficking to draining lymph nodes has also been demonstrated following respiratory[393] and cutaneous viral infection,[394] as has trafficking from the marginal zone to the T-cell zone of the spleen following systemic bacterial infection.[395] With respect to systemic bacterial infections, DCs may play an important role in localizing bacteria to the spleen.[396,397]

In humans, autosomal-recessive or autosomal-dominant deficiency in interferon regulatory factor (IRF) 8, a transcription factor critical in the regulation of the myeloid lineage commitment, results in marked or selective depletion of the APC compartment, respectively (see Table 6.1). The recessive severe form of the disease results in complete absence of CD14$^+$ and CD16$^+$ monocytes and all DC subsets and manifests with mucosal candidiasis and nontuberculous mycobacterial infections.[398] The dominant milder form of the disease predisposes to nontuberculous mycobacterial infections and leads to selective absence of CD11c$^+$CD1c$^+$ DCs, which are major producers of IL-12 for development of protective Th1 responses against mycobacterial disease.[398]

MICROBIAL PATHOGENESIS AND THE CELLULAR IMMUNE SYSTEM

The diverse properties of microbial pathogens provide a challenge to the cellular immune system. Although the details of cellular immune responses to different pathogens vary substantially, some broad generalizations regarding cellular immune responses to microbial pathogens can be made. In most cases, the subcellular anatomic location of a pathogen (extracellular, endosomal/vacuolar, cytoplasmic) predicts the arm of the cellular immune response that is necessary to contain infection by that particular pathogen. In many cases, the most definitive information about the important arms of antimicrobial defense for a particular pathogen has come from inherited or acquired immunodeficiency states, in particular, defects in immune receptors or signaling molecules. In addition, because different classes of pathogens are associated with a particular type of cellular immune response, many pathogens have developed sophisticated molecular countermeasures to evade elimination.

Viral Infections

In general, viruses use the host cell for protein synthesis and thus viral proteins are ready substrates for the MHC class I antigen-processing pathway and CD8 T-cell activation. Due to intracellular viral replication, the cellular immune system eliminates viruses by cytolytic destruction of the infected host cell by virus-specific lymphocytes or by inhibition of viral replication in host cells.

There are several reports of congenital deficiency of CD8 T cells or MHC class I function caused by mutations in *CD8A, TAP1, TAP2, B2M,* or *ZAP70* (see Table 6.1)[259,260,399–401] These patients have reduced MHC class I surface expression or CD8 T cells, or both. In contrast to the canonical function presented for CD8 T cells in antiviral defense, these patients collectively do not manifest increased susceptibility to viral infections. Instead, they present with recurrent pulmonary bacterial infections and bronchiectasis in the presence or absence of prior pulmonary infections, and approximately 50% of them develop ulcerative granulomatous skin lesions infiltrated by activated NK cells.[402] These findings support the notion that NK cells may provide functional redundancy in the context of diminished CD8 T-cell–dependent cytolytic activity, yet cause tissue damage. Indeed, patients with inherited NK deficiency caused by mutations in *MCM4, GATA2, RTEL1,* or *FCGR3A* manifest with severe infections by herpesviruses, including HSV-1, varicella-zoster virus, CMV, and Epstein-Barr virus (EBV) (see Table 6.1).[403] Although congenital CD8 or MHC class I deficiency may be compensated by NK cells for effective viral surveillance, studies of MHC class I polymorphisms in HIV-infected patients support a role for CD8 T cells/MHC class I in the efficiency of viral infection control in humans.[241]

Some viruses become transcriptionally inactive after host cell infection (e.g., human herpesviruses) and represent a particular challenge for the cellular immune system. Although primary active infection with these viruses is controlled, herpesviruses establish clinical latency and can cause intermittent disease or disease in the setting of impaired immunity. Transcriptionally inactive proviral DNA is invisible to host T cells because viral proteins are not presented by MHC class I molecules and therefore these viruses cannot be detected by antigen-specific T lymphocytes. Although immunologic elimination of the infection is often impossible, immunologic control of intermittent reactivation is the rule.[404]

Beyond interfering with the MHC class I antigen-processing pathway, many viral pathogens interfere with cytokine signaling or chemokine-mediated cell recruitment. EBV contains a gene for an IL-10 homologue that binds the IL-10 receptor and induces immunosuppressive effects of this pleiotropic cytokine. Poxviruses express a high-affinity IL-18–binding protein that interferes with early IFN-γ–mediated inflammatory responses. Poxviruses and herpesviruses also produce virally encoded -C-, -CC-, and -CXC- chemokines or chemokine receptors, or a combination of these, that help evade the cellular immune response by one or more of three mechanisms: (1) disrupting the recruitment of monocytes, NK cells, and activated T cells to sites of viral infection, (2) promoting the recruitment of immune cells that support viral spread or latency, and (3) activating certain chemokine receptor axes that skew the cellular response from an antiviral Th1 state to a proviral Th2 state.[405]

Infections With Intracellular Bacteria and Fungi

Many important human pathogens have evolved mechanisms to escape antibody-, complement-, and neutrophil-mediated immune defense. One of the most effective escape mechanisms involves entering host cells, often phagocytic cells. In this circumstance, the host cell becomes a protective barrier from extracellular microbicidal defenses. Bacterial pathogens employ many strategies to exploit the interior of host cells to their benefit, by manipulating intracellular trafficking pathways or by targeting specific intracellular niches.[406,407] The challenge facing the cell-mediated immune system is to detect and eliminate these pathogens.

Phagosomal Pathogens

Multiple bacterial and fungal pathogens parasitize phagocytic cells, such as macrophages, and reside within the endosomal-phagosomal network.

These pathogens often are accessible to the MHC class II antigen presentation network and can colocalize with the antimicrobial effector molecules of macrophages that are delivered to the phagolysosome. Prototypical pathogens of this type include mycobacteria, endemic dimorphic fungi, and *Salmonella* spp. Control of these pathogens depends mostly on IL-12–dependent CD4 T-cell activation that results in IFN-γ release. In turn, IFN-γ activates macrophage effector mechanisms to kill pathogens through oxidative and nonoxidative mechanisms. The latter mechanisms, which remain poorly understood, are critical in humans since patients with chronic granulomatous disease who lack phagocytic oxidative burst are not susceptible to infections by these intracellular pathogens.[20] Mice deficient in IFN-γ are highly susceptible to intracellular pathogens, including *M. tuberculosis,* nontuberculous mycobacteria, *Histoplasma, Coccidioides, Cryptococcus, Toxoplasma,* and *Salmonella,* providing support for the importance of the Th1-mediated stimulation of antimicrobial killing of phagosomal pathogens.

In many parts of the world, infants receive bacillus Calmette-Guérin (BCG) vaccination. Although highly attenuated compared to its parental *M. bovis* strain, BCG can replicate within human hosts with impaired IL-12/IFN-γ–mediated immunity. This worldwide cohort of BCG-exposed infants has uncovered inherited defects in IL-12/IFN-γ–mediated immunity. The clinical syndrome is called *mendelian susceptibility to mycobacterial disease* (MSMD)[408] and includes patients with disseminated infection by BCG or other low-pathogenicity nontuberculous mycobacteria. These patients also show increased susceptibility to disseminated infections by endemic dimorphic fungi, *Cryptococcus* and *Salmonella*. Mutations that cause MSMD via impairing the IL-12/IFN-γ signaling axis involve the IFN-γ receptors 1 and 2, STAT1, IL-12p40 and its IL-12 receptor subunit β1, NF-κB essential modulator (NEMO), GATA2, IRF8, and interferon-stimulated gene 15 (ISG15) (see Table 6.1).[21,22,409–412] In agreement with these inherited defects, patients with neutralizing autoantibodies against IFN-γ exhibit an acquired immunodeficiency marked by disseminated infections with intracellular pathogens.[413] Similarly, the administration of TNF-α inhibitors results in susceptibility to disseminated mycobacterial disease and histoplasmosis.[414]

The data presented here collectively document the central role of the Th1 immunity and the CD4 T cell–macrophage cross talk in protection from and control of phagosomal pathogens. Notably, such pathogens have evolved countermeasures to dampen or subvert effective host immunity. For example, *M. tuberculosis* infection of macrophages renders these cells resistant to activation with IFN-γ.[415] *M. tuberculosis* also prevents acidification of vacuoles by excluding the proton–adenosine triphosphatase complex from the endosomes that it occupies. One possible outcome of diminished vacuolar acidification is decreased antigen degradation, resulting in diminished presentation of mycobacterial peptides by MHC class II molecules. Other pathogens, such as *Legionella pneumophila,* segregate themselves in an endosomal compartment that does not communicate with the MHC class II antigen-processing pathway.[416] Intracellular fungi such as *Cryptococcus, Coccidioides,* and *Histoplasma* can evade phagosomal destruction via generation of urease that creates a pH-neutral phagosomal environment, or by producing virulence factors that scavenge iron or counteract reactive oxygen species, or via lytic or nonlytic escape from the macrophage, or by a combination of these.[417]

Cytoplasmic Pathogens

Some bacterial pathogens have evolved a different intracellular survival strategy by escaping the phagocytic vacuole and replicating in the cytoplasm of host cells (e.g., *L. monocytogenes, Shigella flexneri,* and *Rickettsia* spp.).[407] These pathogens secrete proteins that are essential for virulence and that destroy the vacuolar membrane, providing direct access to the host cell cytosol. In terms of the cellular immune response, these pathogens are similar to viruses because defense against these agents predominantly depends on the MHC class I/CD8 T-cell axis. Because of their cytoplasmic location, the antimicrobial effector mechanisms of phagocytic cells cannot be localized spatially to the cytoplasmic site of infection, necessitating killing of the infected cell by cytolytic T cells to eliminate the infection. Extensive evidence from animal models of *L. monocytogenes* supports the role of CD8 T cells in protective immunity against cytoplasmic bacterial pathogens.

Infections With Extracellular Bacteria and Fungi

Bacteria that replicate extracellularly are accessible to antibody-mediated neutralization or killing by externalized microbicidal products of leukocytes. Defense against pyogenic bacteria (e.g., *S. aureus* and *S. pneumoniae*) depends on adequate humoral immunity and intact neutrophil function. To the extent that adequate specific and high-affinity antibody production depends on CD4 helper T cell function, patients with impaired CD4 T-cell function are susceptible to these pathogens. Th17 cells have been implicated in defense against *Klebsiella* infections in the mouse lung.[52,418] A role for CD8 T-cell responses in defense against extracellular bacteria has been postulated in anaerobic abscesses. In this setting, CD8 T cells recognize carbohydrate antigens of *Bacteroides fragilis.*

Host defense against extracellular fungi depends on IL-17 signaling at mucosal barrier surfaces. IL-17 signaling–deficient mice and humans with inherited mutations in *IL17F, IL17RA, IL17RC,* or *ACT1* develop chronic mucocutaneous candidiasis (see Table 6.1).[20,50,419] Patients with *IL17RA* and *ACT1* mutations also develop skin staphylococcal and pulmonary bacterial infections, underscoring the importance of this pathway in extracellular pathogen host defense at barrier sites.[420] Mechanistically, IL-17 produced by Th17 cells, γδ T cells, and type 3 ILCs promotes *Candida* control via the production of anti-*Candida* antimicrobial peptides by epithelial cells.[50] Consistent with the contribution of IL-17 signaling to mucosal antifungal host defense, patients with psoriasis treated with biologics that target the IL-17/IL-23 signaling axis are susceptible to mucosal candidiasis.[421,422]

Other inborn errors of IL-17 immunity that result in chronic mucocutaneous candidiasis include patients with Job syndrome due to *STAT3* mutations who have absent Th17 cells and are also susceptible to staphylococcal infections.[53] Mutations in *RORC, AIRE, CARD9, IRF8, STK4, DOCK8,* and *STAT1* (gain of function), as well as the various forms of severe combined immunodeficiency disorder, give rise to inborn errors of IL-17 immunity and are reviewed in detail elsewhere (see Table 6.1).[20]

PRIMER ON BASIC IMMUNOLOGIC TECHNIQUES

Rapid evolution of immunologic techniques has facilitated the increasingly sophisticated view of the mammalian immune system. Understanding current immunologic techniques is important for the practicing infectious diseases specialist for two reasons. First, these techniques form the basis on which we formulate our understanding of protective immunity. Second, immunologic techniques, such as flow cytometry, intracellular cytokine and MHC tetramer staining, and ELISPOT assays, increasingly are used in the clinical setting to evaluate immunologic function. We briefly review some of the more recently developed immunologic techniques.

Characterizing and Measuring Pathogen-Specific Immunity

Flow cytometry has transformed immunologic analysis by allowing rapid and efficient analysis of complex lymphocyte and nonlymphocyte cell populations. A flow cytometer analyzes single cells at a rate of up to 10^5 cells per second for the presence of typically 10 to 20 fluorescently labeled monoclonal antibodies, dyes, genetically encoded fluorescent proteins, and scatter parameters and allows each cell in a complex population to be scored for these cellular markers.[423] This powerful technique provides a detailed picture of mixed cell populations, such as lymph node, spleen, or peripheral blood cells (Fig. 6.12A-D). With the steady introduction of new monoclonal antibodies specific for novel surface or intracellular proteins, flow cytometry continues to uncover increasingly greater complexity among cell populations that previously were assumed to be homogeneous. A recurring theme in immunologic studies is the discovery that a cell subset, on the basis of a new marker, can be divided into two or three distinct cell populations. Flow cytometry is used routinely in the clinical arena for CD4 and CD8 T-cell quantitation in HIV-infected and in other immunocompromised patients.

Technical innovations that have affected cellular immunology studies involve the precise quantitation of antigen-specific T lymphocytes. Three

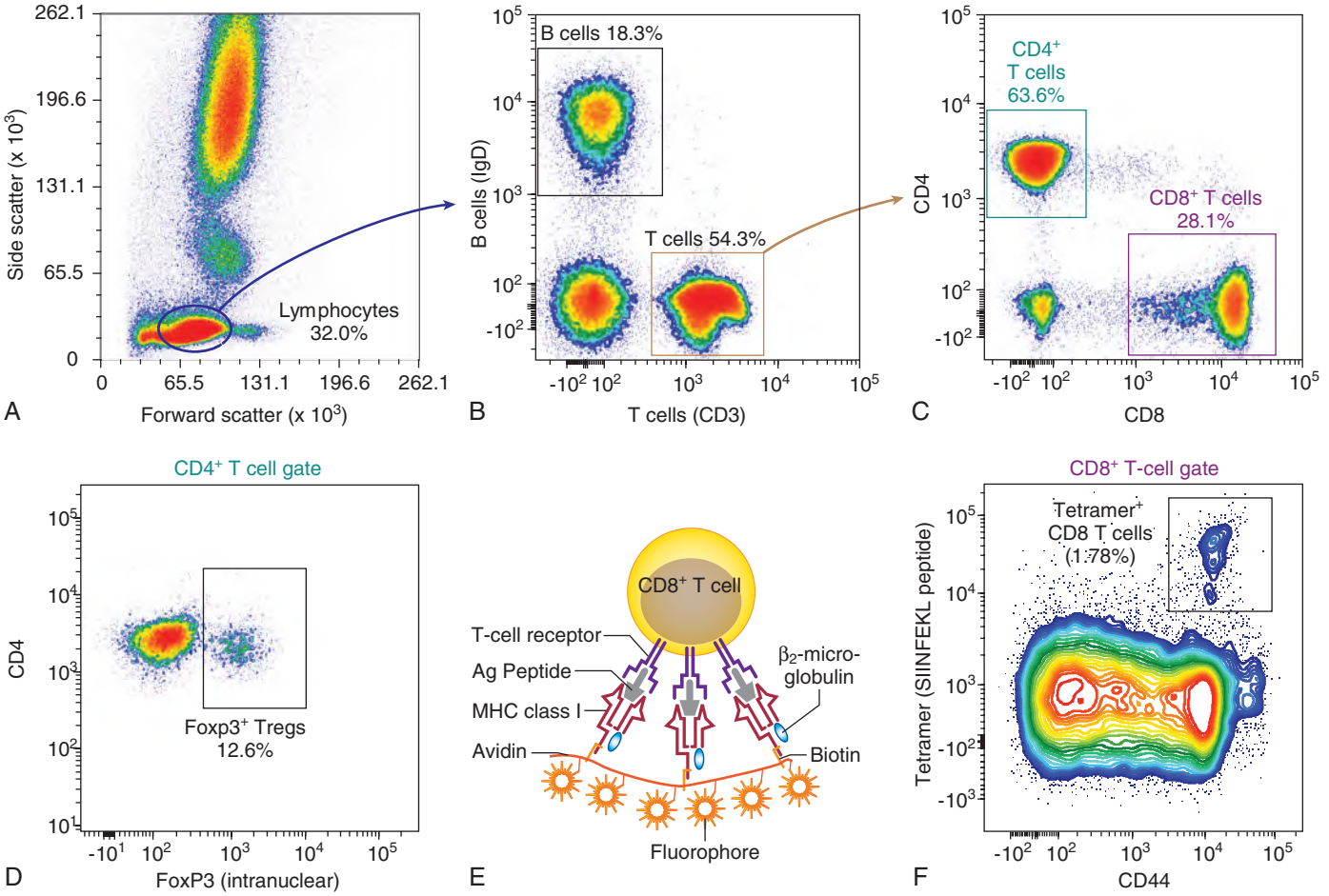

FIG. 6.12 Flow cytometry (A-D) and major histocompatibility complex *(MHC)* tetramer staining (E and F) can be used to identify and characterize distinct immune cell populations within a complex mixture of cells. (A) Plot of the forward scatter versus the side scatter of light that emanates from individual cells. These parameters group cells based on relative size and granularity. The region enclosed by a dark blue oval (gate) contains a cell population that is enriched for lymphocytes. The frequency of cells within the lymphocyte gate is indicated. (B) The cells within the lymphocyte gate are further divided into B cells (anti-IgD; vertical axis) and T cells (anti-CD3; horizontal axis) on the basis of staining with specific monoclonal antibodies that are coupled to different fluorochromes. The brown gate surrounds the B-cell population and the tan gate the T-cell population. (C) The gated T-cell population is analyzed for surface expression of CD4 (vertical axis) or CD8 (horizontal axis) using monoclonal antibodies that fluoresce at two additional wavelengths. (D) CD4 T cells are analyzed further for intranuclear Foxp3 expression by permeabilizing the nuclear membrane with a detergent and staining the cells with a fluorochrome-coupled anti-Foxp3 antibody. The CD4 T-cell subset in the *blue gate* represents Foxp3⁺ regulatory CD4 T cells. (E) Schematic showing a fluorochrome-coupled MHC tetramer that can simultaneously interact with multiple antigen-specific T-cell receptors. In most studies, MHC tetramers are generated by biotinylation of the carboxyl terminus of a soluble MHC molecule that contain a specific antigenic (Ag) peptide, followed by complex formation with a fluorescently labeled avidin molecule, which contains four biotin-binding sites. (F) MHC tetramer staining of CD8 T cells using a tetramer complexed with a SIINFEKL peptide. In this example, CD8 T cells are analyzed for CD44 staining as well. The black gate indicates the frequency of antigen-specific CD8 T cells that bind and recognize the MHC tetramer–peptide complex. *(Flow cytometry plots courtesy Dr. Rui Gardner, Memorial Sloan Kettering Cancer Center, New York, NY.)*

methods—enzyme-linked immunosorbent spot (ELISPOT) assays, intracellular cytokine staining, and MHC tetramer staining—have revolutionized the study of pathogen-specific T-cell responses. The ELISPOT assay is relatively simple and does not require a flow cytometer. ELISPOT assays provide accurate, quantitative data and can be performed with complex mixtures of cells, such as peripheral blood mononuclear cells or lymph node or spleen cells.[424] To perform this assay, complex mixtures of cells are stimulated with antigen on a membrane coated with a monoclonal antibody specific for a cytokine, such as IFN-γ, TNF, or IL-4. Antigen-specific T cells in the mixture, on stimulation, release cytokines that are captured by membrane-bound antibodies directly adjacent to the stimulated cell. Bound cytokines are detected with a secondary, enzyme-conjugated monoclonal antibody in a fashion identical to a standard sandwich enzyme-linked immunosorbent assay. Each stimulated cell leaves a "spot" on the membrane, and the number of spots is quantified.

Intracellular cytokine staining is similar to the ELISPOT assay in that complex cell populations are stimulated with an antigen, but in the presence of either Brefeldin A or monensin, drugs that inhibit cellular secretion of cytokines. During this incubation, cytokines are produced by antigen-specific T cells, but instead of being secreted, they accumulate within the cell. After stimulation, cells are fixed and permeabilized, then stained with cytokine-specific, fluorescently tagged antibodies. Permeabilization with a dilute detergent is necessary to provide antibody access to the accumulated intracellular cytokine. Stained cells are examined by flow cytometry, and antigen-specific T cells are identified and quantified on the basis of cytokine production. This technique can be modified to measure the levels of T-cell lineage–specific transcription factors in lymphocyte populations.[425] Although technically more demanding, intracellular cytokine staining is more informative than the ELISPOT assay.

Another direct method for quantifying antigen-specific T cells involves the use of MHC tetramers.[426] Because the interaction between TCRs and MHC-peptide complexes is of low affinity, attempts to identify antigen-specific T cells with soluble MHC-peptide complexes was not possible. Generation of tetrameric forms of MHC and peptide complexed

with a fluorophore readily enabled antigen-specific T cells to be stained and identified by flow cytometry (Fig. 6.12E and F). In addition, tetramer staining can be used to isolate viable, pathogen-specific T lymphocytes or NKT cells.[108,427] An advantage of MHC tetramer staining over either intracellular cytokine staining or ELISPOT assays is that T-cell or NKT-cell detection does not depend on cytokine production, which in turn depends on the T-cell phenotype.

In many cases, use of these quantitative assays radically revised prior estimates of pathogen-specific T-cell frequencies.[428–430] In some infections, such as primary EBV infection, the frequency of virus-specific $CD8^+$ T cells approaches 70%.[431,432] Although EBV is arguably an extreme example, in other infections, such as those caused by HIV, HSV, influenza virus, and *L. monocytogenes,* pathogen-specific T-cell frequencies are astonishingly large, generally ranging from 2% to 25%.[429,430,433,434]

These techniques enable researchers to combine measurements of pathogen-specific T-cell frequencies with T-cell functionality. An example of this application relates to understanding the expansion and effector cytokines produced by pathogen-specific T cells during immune reconstitution inflammatory syndrome (IRIS) in patients who receive highly active antiretroviral therapy for HIV. In longitudinal analyses, preexisiting CD4 cytokine-producing T cells that specifically targeted antigens of underlying coinfections (e.g., due to CMV, *Cryptococcus, Histoplasma, M. tuberculosis*) were increased in magnitude during IRIS flares. In contrast, T-cell responses against HIV or non-IRIS–associated infections were not enhanced during IRIS episodes or in non-IRIS patients, indicating that IRIS does not represent a general T-cell dysfunction, but rather a dysregulated CD4 T-cell response against a residual IRIS-associated opportunistic infection antigen.[435]

In clinical practice, the development of experimental procedures to generate pluripotent viral-specific T cells, even from third-party immune individuals with common HLA polymorphisms, has emerged as a major breakthrough in the management of allogeneic hematopoietic stem cell transplant recipients who are at high risk for developing life-threatening infections of CMV, EBV, and adenovirus.[436]

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The complete reference list is available online at Expert Consult.

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7 Mucosal Immunity

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SHORT VIEW SUMMARY

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ROLE OF MUCOSAL IMMUNITY BEYOND THE MUCOSA
- Role of the microbiota in homeostatic networks
- Mucosal immune responses discriminate between molecular signals that reflect a threat to the host versus signals that are benign or even enhance health.
- This balance between local mucosal immune responses and the microbiota appears to become established in infancy.
- The host-microbial interactions are shaped by millions of ligands in the local environment that are presented in an endocrine, paracrine, or autocrine manner.
- Although most of these ligands represent environmental antigens, they also include cytokines, chemokines, growth factors, integrins, metabolites, hormones, and neurotransmitters.
- The balance struck between mucosal immunity and local microbial communities is recognized for its impact on the health of tissues well beyond the site where the original encounter occurs.

SPECIALIZATION OF HOST RESPONSES IN MUCOSAL TISSUES

The Challenge

Mucosal tissues provide a barrier between the host and the microbial world, and these very delicate structures perform important physiologic functions as well. For example, to maximize air exchange in the respiratory tract or nutrient absorption in the gut, these tissues have numerous morphologic adaptations that expand their surface area. This vast surface is exposed to myriad environmental antigens including toxins, allergens, food antigens, and microbes. Furthermore, humans alter their habits and lifestyle, and these stimuli vary as humans change their location and diet and encounter new microbial challenges. Other challenges facing mucosal immune responses include the need to reach luminal "offshore" antigens and to protect against infection without compromising physiologic functions. Thus, immune and inflammatory cells in mucosal tissues adapt to changes in stimuli to acquire a pattern of differentiation that provides protection without disrupting adjacent tissues. Increasing evidence discussed in this chapter suggests this equilibrium is achieved by sensing the local microbial communities and stimulating local immune responses that contribute to homeostatic or pathologic responses.

The gastrointestinal tract provides an informative model to illustrate the unique properties of mucosal immunity. Although estimates vary, there are more bacteria, viruses, fungi, parasites, and archaea than nucleated host cells throughout the entire body, and the microbiome entails several hundred–fold more genes than the human genome. In response to this microbial burden, the gut has as many lymphocytes, in both diffuse (e.g., lamina propria) and organized (e.g., gut-associated lymphoid tissue) tissues, as found in the spleen. Moreover, the gut secretes antibody (e.g., secretory immunoglobulin A [SIgA]) that accounts for approximately 75% of the total amount of immunoglobulin produced by the entire body every day. Other mucosal tissues share similar challenges and require many comparable adaptations in their immune responses to accommodate the physiologic uniqueness of different tissues (e.g., upper and lower respiratory tracts, reproductive tissues, eye, middle ear).

Adaptation of Mucosal Immune Responses

The concept of a common mucosal immune system emerged based on the related interface separating mucosal tissues from luminal stimuli. Furthermore, the presence of polymeric immunoglobulin A (pIgA) is an adaptation found in most mucosal secretions. Humans differ from mice in that they have immunoglobulin A1 (IgA1) and IgA2 isotypes that are represented to varying degrees in different sites. As described in Chapter 5, the structure of IgA reflects its adaptation to the mucosal environment, as it is actively transported across the epithelium via the pIgA receptor, and the secreted complex of the two molecules comprises SIgA. IgA resists proteolysis as well as acid hydrolysis and serves to prevent the binding of pathogens or toxins without efficiently activating complement and its associated inflammatory effects. Furthermore, IgA antibodies have been shown to shape the symbiosis with commensal bacteria, as approximately 70% of these microbes are coated with IgA antibodies.[1,2] Paradoxically, IgA nephropathy is an IgA-mediated disease that is driven by the deposition of IgA1 that leads to the impairment of glomerular function.[3] Thus even with adapted systems, responses can go awry and contribute to disease in addition to protection.

After the discovery of IgA, Ogra and colleagues[4] demonstrated that oral immunization with a live, attenuated polio vaccine induced both IgG and IgA antibody responses in the serum and IgA in secretions, whereas systemic immunization with an inactivated virus had little effect on mucosal immunity. Evidence for the exchange of immunologic information among different mucosal tissues emerged from studies demonstrating that when antigens were administered orally, for example, antigen-specific SIgA was detected in other secretions such as tears. The detection of antibody in multiple tissues is attributable to the selective

circulation of antigen-specific B cells acquiring mucosal imprinting molecules (e.g., CCR9, CCR10, α4β7) allowing them to traffic from one mucosal tissue to another.[5]

Immunity includes many responses, some of which neutralize the intended target, but some may also mediate collateral damage to host tissues. In mucosal sites, the antigen burden requires a very delicate homeostatic balance. A major contribution to mucosal homeostasis is the acquired tolerance to antigens that persist in the lumen of either the digestive tract (oral tolerance)[6] or the airway.[7,8] This response allows the host to avoid adverse, immune-mediated diseases to environmental and nutritional antigens as well as to tolerate the microbiota that persist in the lumen. The ability of local host responses to adapt to changes in luminal content due to major changes in diet or microbial communities exemplifies the flexibility in immune regulation that is needed to prevent overly exuberant responses to benign, local antigens.

Scientists studying mucosal immune/inflammatory cells directly have gained an appreciation of the mucosal cell phenotype. One approach has employed lavage of the airway or reproductive tracts as a window to view the lineage and function of cells in these sites. Although many investigators have studied mucosal cells directly in the gastrointestinal tract, cells such as neutrophils are rarely isolated from these tissues due to technical challenges, whereas cells in the urogenital tract, salivary glands, and eye await more extensive characterization. As creative techniques to investigate these cells are developed, novel insights are expected to emerge that will be important to consider in a comprehensive understanding of mucosal immunity in different aerodigestive, reproductive, visual, and hearing tissues.

Although a multitude of factors shape mucosal immune responses, the selective accumulation of cells with a preferred phenotype for mucosal homeostasis occurs due to a limited array of biologic processes.[9] The selection of a preferred phenotype can be achieved by deletion of cells with an unwanted phenotype, selective homing of cells with the preferred phenotype to the mucosae, differentiation of cells toward the preferred phenotype, or expansion of the desired cells through proliferation (Fig. 7.1). With respect to proliferation, not all daughter cells are "identical twins." Proliferation in lymphocytes is often asymmetric; that is, the engagement of receptors in the immunologic synapse leads to the migration of receptors and their associated signaling molecules toward one pole. When cell division begins, the signaling molecules inherited by the daughter cells are asymmetrically distributed, and this necessarily changes the pattern of gene expression.[10–12] These processes contribute to the phenotype of antigen-presenting cells (APCs), mast cells, and T and B cells found in mucosal tissues. One can assume that in immune-mediated disease, the events change, and increased recruitment of immune/inflammatory cells differentiate toward a more responsive phenotype rather than acquiring an anergic tone.[13] Understanding the relative contribution of the signals and cell biology that contribute to immune cell phenotype in health or disease will enable the ability to promote mucosal health.

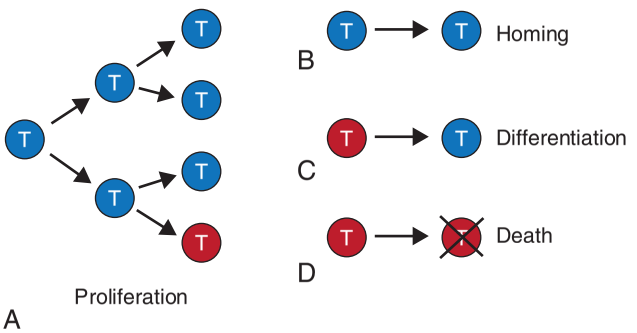

FIG. 7.1 Mechanisms contributing to selection of the immunologic phenotype in mucosal tissues. (A) The selection of a preferred phenotype (illustrated in blue) in mucosal tissues can be achieved by proliferation that expands the cells with the preferred traits. With respect to proliferation, not all daughter cells are "identical twins." Proliferation in lymphocytes is often asymmetric giving rise to daughter cells that are not always identical (red cell). (B–D) Accumulation of cells with the desired phenotype can also be achieved by homing of cells with the preferred phenotype to the mucosae (B), differentiation of cells toward the preferred phenotype (C), and deletion of cells with an unwanted phenotype (D). Targeting these pathways to direct the differentiation of an ideal mucosal immune response could be achieved with adjuvants or biologic response modifiers.

Microbial Communities and Development of Mucosal Immune Responses

The role of the microbial community in an individual's health continues to generate tremendous interest. Through endeavors such as the American Gut Project (www.americangut.org), vast amounts of data are accumulating about the factors that impact the intestinal microbiota and their potential impact on health. For example, it is becoming apparent that by approximately 3 years of age the microbiota takes on many of the characteristics of that in adults, and its composition may portend many aspects of wellness.[14] In general, the microbiota is influenced by factors such as the microbial composition of the mother, vaginal versus cesarean birth, breastfeeding versus formula feeding, diet, antibiotic exposures, genetics, aging, and immune status. These factors influence species diversity, which consists of the number of microbial species (richness), the genetic relationship between species (taxonomic diversity), and the relative abundance of one to another (evenness). The signature of diversity within an individual can impact immune cell phenotype or the metabolome, which often begins in mucosal sites but affects immunologic surveillance throughout the body. For example, animal models of autism are associated with a distinct intestinal microbiota that, in turn, can be offset by other, "protective" bacteria.[15]

Mucosal immune homeostasis reflects an equilibrium in which host responses and microbes influence each other and permit persistent infections.[16] As the immune system influences local immunity, microbes affect host responses. The loss of species diversity may be one phenomenon that accounts, at least partially, for the hygiene hypothesis (see "Good Bugs, Bad Bugs, and the Hygiene Hypothesis") that ascribes an increased risk of allergic and other immune-mediated diseases to the lack of diversity in childhood microbial communities. Using the middle of the 20th century as a key point in time, one can trace the increased use of antibiotics, convenience foods (processed foods, infant formula), increased rates of cesarean section versus vaginal birth, and an increasing emphasis on germaphobia as factors that have impacted diversity.[14,17,18] Furthermore, loss of diversity is passed between generations, resulting in a successive attenuation in microbial diversity[17–19] that may have an adverse effect on susceptibility to disease.

Although stool has been a convenient and informative source to sample, evidence points to the importance of microbes in specific tissues and sites including the mucosa (vs. the lumen) as well as even being embedded within lymphoid tissues. For example, the segmented filamentous bacteria found in the epithelial-mucus niche in mice can favor the development of Th17 responses that have also been associated with an exacerbation of immune-mediated disease.[20,21] The predominate juxtaposition of *Helicobacter pylori* to gastric epithelial cells and their impact on health may be another example of microbes within a limited niche.[22] Mucosal lymphoid tissue–resident bacteria (e.g., *Alcaligenes* spp.) are just beginning to be appreciated for their ability to shape mucosal immunity.[23] Bacteria in these sites have been shown to favor IgA production and induce protective interleukin-10 (IL-10)–producing responses that limit damage following mucosal provocation.[24,25] Most of these findings have been identified in mice, and it remains to be determined where such niches may be found in humans and how they could impact health.

INDUCTION OF MUCOSAL IMMUNE RESPONSES

Epithelial Cell Responses and Immunophysiology

Although single factors rarely explain complex biology, the interactions in mucosal immunity benefit from some insight into the individual elements. First, one should consider the interface between the host and its microbial milieu. In mucosal tissues, this is invariably the epithelial

barrier, its mucus coating, and a variety of secreted host factors (e.g., antimicrobial peptides, SIgA) that modify the microbial niche.[26–30] Mucosal epithelial cells provide a physical barrier that limits the translocation of microbes or their metabolites into the underlying layers. M cells are a specialized epithelial cell overlying the Peyer patches that serve as preferential sampling sites for the uptake of luminal antigens.[31] Many microorganisms are preferentially taken up by M cells.[32,33] Similar secondary lymphoid structures can also be found in the nasal-associated lymphoid tissue of the airway. Cells resembling M cells are scattered throughout the epithelium of the gastrointestinal tract[34] suggesting that their enhanced ability to sample is more widespread than the Peyer patches. In other studies, goblet cells have been shown to pass luminal antigens on to dendritic cells.[35]

Epithelial cells express receptors for many microbes, both pathogens and commensals, including human immunodeficiency virus (HIV),[36] respiratory syncytial virus,[37] *H. pylori,*[38–40] and *Salmonella* spp.[41] These interactions favor colonization and invasion that together lead to signaling events and the induction of mucosal immune responses.[27] Epithelial cells in the airway,[42–44] digestive tract,[45–47] and urogenital tract[48,49] respond to infection, toxins, and inflammation with the release of cytokines that not only alert the host of potential damage or danger but also focus the responses to the site of injury based on a gradient of chemokines and other factors.[50] Thus epithelial cells are able to sense a danger signal[51] and transduce responses that recruit and activate other immune or inflammatory cells.

Some epithelial cells are highly specialized to produce antimicrobial factors or to express pIgA receptor to mediate the transfer of pIgA into the lumen. The ability to transport IgA is shared by most epithelial cells and increased during inflammation. However, the secretion of antimicrobial factors tends to be more localized. For example, saliva contains secretory leukocyte protease inhibitor[52]; gastric parietal cells produce acid; and other epithelial cells produce a range of factors including lysozyme, lactoferrin, defensins,[28] and antibacterial lectins[26,53] that interfere directly with microbial growth or modify physiologic function to protect the host. One example of altered physiology in host defense is the role played by secretions to create a pressure gradient that impairs colonization in the deep regions of mucosal glands. Furthermore, highly adapted processes such as the mucociliary apparatus in the lungs aid in the elimination of environmental contaminants and microbes.

In the gut, motility partners with secretions to create a catharsis that contributes to the clearance of infections. This concept was illustrated in studies of immunity to nematodes. These organisms induce a robust Th2 response associated with the accumulation of IgE and mucosal mast cells.[54] Degranulation of mast cells releases mediators that contribute to epithelial cell secretion and an increase in motility, which, when impaired, decreases the clearance of several nematode species.[55–57] These physiologic responses are regulated by neuroendocrine cells and often triggered by microbial products[58,59] as well as cytokine responses that contribute collectively to mucosal immunity, as they facilitate the clearance of infections. Neuroendocrine factors also regulate immune cell function. In short the protective or pathogenic effects of mucosal immune and inflammatory cells cannot be separated from the other cells and mediators within the tissue. This raises the possibility that drugs impairing motility may counter integrated responses that normally help in clearance of some pathogens.

Antigen-Presenting Cells

The mucosal tissues have a full complement of macrophages and dendritic cells. These cells are found throughout the lamina propria; some are enriched in secondary lymphoid structures including the Peyer patches in the gut, similar subepithelial aggregates in the airway (e.g., nasopharyngeal-associated lymphoid tissue), or other tissues (e.g., tear duct–associated lymphoid tissue) as well as in the draining lymph nodes. It has been reported that some dendritic cells extend their dendrites into the lumen and sample antigens directly.[60,61] In addition to this occurring across the tight junction of absorptive epithelial cells,[60] some dendritic cells extend dendrites through M cell–specific transcellular pores for antigen sampling.[62] Phagocytes have also been shown to engulf apoptotic epithelial cells,[63–65] which, if infected, could carry microbes and create another mechanism of antigen sampling.[66,67]

It is increasingly clear that many factors within the mucosa select for unique APCs[68] capable of facilitating the accumulation of helper T (Th) cells that favor the induction of tolerance or mucosal IgA or both. Thus there is a feedforward as cells involved in the recognition and processing of antigen acquire a mucosal phenotype that, in turn, favors the expansion of Th cells that select for effector mechanisms such as IgA or mucosal mast cells that culminate in an ideal mucosal immune response. For example, initial engagement of nucleotide oligomerization domain 2 (NOD2) expressed by APCs with muramyl dipeptide administered intranasally stimulates the production of thymic stromal lymphopoietin and the induction of Th2 cells in the lung.[69] Similarly, initial stimulation of NOD2 in the gut or in the context of ocular toxoplasmosis[70] induces proinflammatory responses. However, repeated stimulation with NOD2 ligands renders APCs nonresponsive to ligands for NOD2 or Toll-like receptors (TLRs)[71,72] as evidenced by decreased production of IL-1β, IL-8, and tumor necrosis factor-α (TNF-α).[72] Mutations in the *NOD2* gene are associated with Crohn disease,[73] possibly by impairing the ability of APCs to adapt to these repeated signals from microbial ligands. Mouse models have shown that *NOD2* is important to stimulate homeostasis to protective bacteria, so mutations in this gene associated with immune-mediated disease could reflect a loss of homeostatic regulatory T (Treg) cell induction.[74]

Innate Lymphoid Cells

Complementing the transition from innate to adaptive responses is a population of innate lymphoid cells (ILCs). These cells are non-B, non-T cells with a lymphoid morphology.[75–77] Historically ILCs included natural killer (NK) cells and lymphoid tissue inducer cells. At the present time, it is clear that there are other subsets that are closely related to most, if not all, Th cell subsets, as they express similar transcription factors and patterns of cytokine production. To clarify this area, a new nomenclature has been applied in which ILCs are divided into subsets referred to as ILC1, ILC2, and ILC3.[78] ILC1 and ILC2 express transcription factors and cytokines that resemble Th1 and Th2 cells, respectively, whereas ILC3 includes cells resembling Th17 or Th22 cells as well as lymphoid tissue inducer cells (Fig. 7.2),[78] thus giving a functional homologue of Th cell subsets to innate immunity.

ILCs arise in the bone marrow from a common lymphoid precursor that distinguishes itself from T-cell and B-cell lineages by the expression of T cell factor-1. Subsequently a transcriptional program is engaged involving inhibitor of DNA binding 2 (Id2) that leads to ILC commitment and the subsequent transcription factor induction associated with the various ILC subsets.[79] Relatively little is known about the timing and mechanisms that lead to the tissue distribution of ILCs; however, they reside in the tissue constitutively and become activated by some of the cytokines released by APCs, epithelial cells, and T cells. In turn, ILCs express the transcription factors and cytokines that are appropriate to amplify the desired host response for the current antigenic signal. Given that adaptive immunity takes several days to be induced, ILCs complement the role played by macrophages and other innate cells in limiting infections as well as enhancing the milieu to select for the appropriate T-cell and B-cell responses that eventually confer adaptive immunity and memory.

Further evidence of the integration between ILCs and Th cells is found in the response of ILCs to Th-derived cytokines. For example, IL-17A or IL-22 from Th17 cells has been reported to activate NK cells and enhance immunity to fungi in mucosal sites such as the kidney[80] and oral cavity.[81] Other Th cell cytokines activate cells involved in acute/innate inflammatory response in different ways. For example, Th2 cells enhance the accumulation of mast cells, basophils, and eosinophils as well as increase IgE antibodies that enable IgE binding cells (e.g., mast cells and basophils) to recognize antigen and release their mediators that contribute to clearance of nematodes.[54]

Sensing Mucosal Microbiota

The importance of the microbiota in regulating host responses begins with the microbes having access to the host and the subsequent recognition of their molecular structures.[82–84] The sensing of microorganisms is mediated through pathogen-associated molecular patterns (PAMPs).[85] PAMPs are detected by pattern recognition receptors (PRRs) expressed

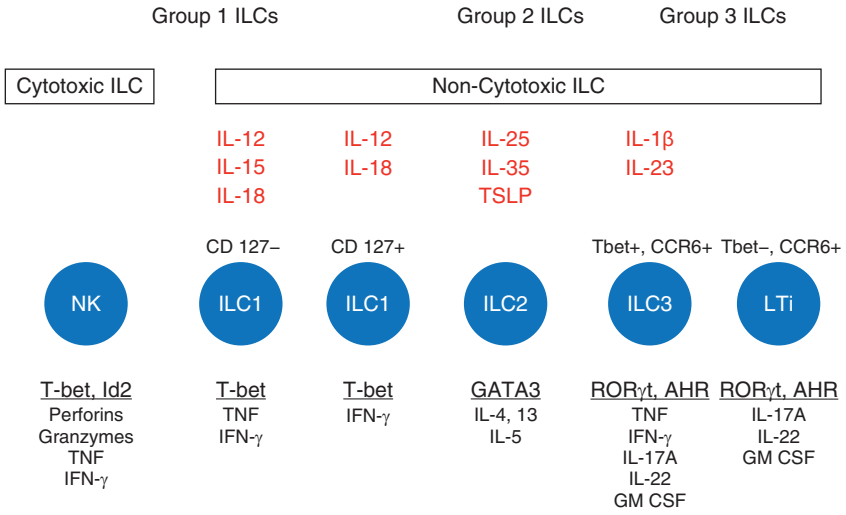

FIG. 7.2 Properties of innate lymphoid cells (ILCs). ILCs are believed to be derived from a common precursor and differentiate into their respective subsets based on the selectivity imparted by the milieu and their respective transcription factors. In response to specific cytokines (in red), transcription factors *(underlined)* are induced that control the selection of gene expression. In general, the pattern of transcription factor usage and cytokine gene induction in ILC1, ILC2, and ILC3 resemble the same control of differentiation observed in Th1, Th2, and Th17 cells. As reviewed elsewhere,[78] the stimuli of ILC1 include IL-12, IL-15, and IL-18; ILC2 is driven by IL-25, IL-33, and TSLP; and ILC3 require IL-1β and IL-23. ILC1 are often associated with cancer, immune-mediated diseases, and intracellular infections, typically viruses. ILC2 are more often seen in the context of nematode infections and allergic disorders. ILC3 are induced in response to different bacterial challenges in mucosal tissues and contribute to induction of secondary lymphoid tissue by the LTi subset. *AHR,* Aryl hydrocarbon receptor; *CCR6,* C-C chemokine receptor 6; *GATA3,* transcription factor binding to DNA sequence guanine-adenine-thymine-adenine; *GM CSF,* granulocyte macrophage colony-stimulating factor; *Id2,* inhibitor of DNA binding 2; *IFN-γ,* interferon-γ; *IL,* interleukin; *Lti,* lymphoid tissue inducer; *NK,* natural killer; *RORγt,* retinoic acid–related orphan receptor gamma; *T-bet,* T-box expressed in T cells; *TNF,* tumor necrosis factor; *TSLP,* thymic stromal lymphopoietin.

on the surface of several cell lineages. There are several classes of PRRs including TLRs[85]; NOD-like receptors (NLR) including the NOD family[86]; Rig-like helicases (RLH)[87]; Dectin-1 and Dectin-2[88,89]; and several proteins containing thrombospondin repeats such as thrombospondin 1,[90] mindin,[91] and brain angiogenesis inhibitor 1 (BAI1).[41] In addition to bacterial factors, tissue damage, including debris from cells dying of apoptosis or necrosis, provides an array of molecules referred to as alarmins or danger-associated molecular patterns (DAMPs).[92] Furthermore, the release of adenosine triphosphate (ATP) from dead cells in damaged tissue is important for additional phagocyte recruitment[93,94] and the regulation of inflammation that contributes to tissue repair.

Many PRRs engage their ligand on the cell surface, whereas others are activated in phagosomes or elsewhere in the intracellular space following translocation of the PAMPs. Soluble PAMPs in the blood such as endotoxin can activate surface receptors directly, although concentrations of these ligands rarely reach the thresholds needed to activate leukocytes ex vivo. Microbes, exemplified by *H. pylori,* remain primarily on the cell surface of (gastric) epithelial cells where surface PRRs may be engaged. Additional activation occurs subsequent to the translocation of bacterial effector molecules into the cytoplasm where peptidoglycan can induce IL-8 responses after sensing by NOD1.[95] Other organisms such as enteroinvasive *Escherichia coli* or various *Salmonella* spp. enter a cell by means of invasion as well as engulfment.[96] The internalization process creates phagosomes that can juxtapose PAMPs with PRRs within the phagosome where they activate their respective responses.

Following the engagement of PRRs, signaling pathways are stimulated leading to the activation of transcription factors and the production of various host response molecules. Perhaps best studied is the role of myeloid differentiation primary response protein (MyD88) as an integral part of the signaling for several TLRs.[97,98] The fact that several PRRs share a signaling pathway creates a synergy that may augment the potential stimulation from relatively small concentrations of individual PAMPs. Accumulating PAMPs within a phagosome would further enhance their concentration and potential for signaling.

Several distinct host responses are induced following sensing by PRRs. One of the first is the production of chemokines that recruit and activate other immune cells to the site of damage. Other responses include changes in the expression of accessory molecules on APCs that contribute to T-cell activation; oxidative burst; and production of reactive nitrogen species, various vasoactive mediators, or arachidonic acid metabolites.

An important function of innate cells is to clear microbes or damaged tissue by phagocytosis. Engulfment of sterile debris or benign microbes leads primarily to the production of antiinflammatory cytokines such as IL-10 or transforming growth factor-β1 (TGF-β1).[99,100] In contrast, clearance of pathogens usually leads to innate antimicrobial responses (e.g., reactive oxygen species) as well as production of cytokines that activate appropriate Th cell subsets. Another consequence of internalization of antigen into dendritic cells is presentation of antigen to $CD4^+$ or $CD8^+$ T cells leading to T-cell activation in a major histocompatibility complex (MHC) class II–dependent or MHC class I–dependent manner, respectively. Epithelial cells often express molecules associated with T-cell activation including molecules recognized by invariant NK-T cells[101] as well as T cells.[102,103] An interesting nonmicrobial example is the rapid uptake of gliadin by duodenal epithelial cells.[104] Subsequent to this uptake, gliadin is processed by tissue transglutaminase to create a peptide that fits well into the DQ2 peptide–binding or DQ8 peptide–binding pocket and leads to Th cell activation and celiac disease.[105] Microbial antigen uptake and processing by epithelial cells or APCs by similar or distinct mechanisms would enhance adaptive immunity to an organism.

Sensing Metabolites

PAMPs and DAMPs recognized by the host represent a fraction of the possible signals that shape mucosal immune responses. The broad collection of microbial and host metabolites constitutes a much larger pool of ligands that is just beginning to be appreciated. The value in understanding metabolomics can be illustrated with a few examples.

Several leukocyte lineages including dendritic cells and Th cells express a receptor that binds different ligands including polycyclic aromatic hydrocarbons, suggesting that environmental toxins modulate host responses.[106] The aryl hydrocarbon receptor is a ligand-activated transcription factor that regulates expression of many immune genes. In addition to toxins, natural ligands can be generated from dietary tryptophan by the initial enzymatic activity of indoleamine

2,3-deoxygenase to yield kynurenine. Kynurenine can accumulate in the placenta and induce Treg cells that may help prevent rejection of the semiallogeneic fetus.[107] Furthermore, catabolism of dietary tryptophan in the gut by bacteria or fungi to other aryl hydrocarbon receptor ligands can assist in the induction of IL-22, which binds to epithelial cells and stimulates the release of antimicrobial peptides.[108,109] Thus microbiota and the host compete for the same substrates to generate different metabolic products that may modify mucosal immune responses toward Treg or Th22 cells.[110]

Purine metabolism is another pathway in which bacteria and the host compete for the same substrate.[111] In brief the accumulation of ATP, from bacteria[112] or from dying cells,[93] can be metabolized to adenosine diphosphate, 5′-adenosine monophosphate (AMP), and then to adenosine.[111,113] ATP can be a proinflammatory signal that favors the accumulation of Th17 cells in the mucosa,[114] whereas the accumulation of adenosine depletes this stimulus and usually confers an antiinflammatory activity mediated by the A_{2A} adenosine receptor.[111] Although Treg cells can generate adenosine as a mediator of suppression, bacteria can also achieve this to suppress innate responses to favor colonization.[115]

Another example of the role for metabolism in regulating host responses is the conversion of vitamin A to retinoic acid by dendritic cells expressing retinol dehydrogenase.[116] This process plays an important role in conditioning leukocytes with a mucosal phenotype[117,118] and imparting the ability for these cells to home to mucosal tissues.[116,119] Furthermore, APCs exposed to retinoic acid assume the capacity to direct Th cell differentiation away from Th17 cells and toward Th2 and Treg cells[120,121] that subsequently increase IgA production along with anergic conditions that enable the survival of symbionts.[74,122,123] One can only imagine how new data emerging from studies of the microbiome will identify other metabolic reactions that contribute to the molecular environment shaping mucosal immune response and the microbial niche.

Discrimination Between a Commensal and a Pathogen

The crux of the matter for mucosal immune responses in health and disease is defined by the equilibrium of host-microbial interactions and knowing when and how to respond. For years, microbes have been described as being commensals or pathogens, even though these categories represent opposite ends of a spectrum that defines the interrelationship between the host and its microbial communities. However, many microbes exist as *amphibionts,* a term coined by Rosebury[124] to describe microbes that have a symbiotic relationship that can be either a benefit or a detriment to the host or microbe. As discussed later, this relationship is illustrated by organisms that can exist without harming the host, but in the context of a particular host response, they can assume a biologic role that is decidedly disadvantageous to the host.[19] The important point is that homeostasis is flexible and may be either plastic or elastic as host and microbes respond to their niche and that short-term processes may have long-term biologic significance.

Microbes produce hundreds of metabolites and molecules creating a cascade of stimuli beginning with binding to host epithelial cells; sometimes employing secretion systems that translocate bacterial effector molecules into host cells; invasion and/or engulfment; engaging numerous extracellular or intracellular pattern recognition receptors (such as TLRs, NODs, etc.); and the initiation of chemokine and cytokine production. This process, as described by Matzinger,[125] signals "danger," and the chemokines recruit and activate innate cells to phagocytose the offending microbe, amplify the host response, and induce appropriate effector mechanisms leading to the initiation of antigen-specific acquired immune response. This is illustrated by organisms such as *Salmonella* spp. that stimulate APCs to produce proinflammatory cytokines including IL-6, IL-12, or IL-23 that drive the differentiation of Th1 or Th17 cells.[126,127] Although these cells contribute to antimicrobial functions, host responses must be regulated carefully to avoid immune-mediated damage and to maintain the physiologic functions that are required to sustain life.

The conundrum of distinguishing pathogens from commensals emerges, as the latter can also express ligands for PRRs yet do not impart a strong danger signal.[71] There are several possible explanations for this paradox. It may be related to the burden of a particular microbial species; the age at which infection occurs or its duration; the structure of its PAMPs that may render them less proinflammatory; or possibly the absence of secretion systems, invasion mechanisms, or other virulence factors. The issue of duration may be best illustrated by organisms associated with gastric or colorectal cancer. In these cases the persistent gnawing of a low-grade oxidative stress associated with these infections chips away at the DNA until DNA damage leads to a key mutation. For example, mitochondrial DNA mutations have been associated with a loss of cytochrome-*c* oxidase activity that is directly correlated with age.[128] Time is essential for random mutations to accumulate in key genetic regions that impact cell growth.

At the other extreme, one may encounter pathogens that display an array of molecular patterns that allows them to send a different, perhaps "fresh" or more intense, set of signals that stimulates mucosal responses immediately. Furthermore, gene expression in the host can modulate transcription in microbes[129] resulting in changes in the metabolic profile and local immune responses that impact virulence and resonate throughout the body.[130–133]

Contrasting the inflammatory responses leading to a relative sterile immunity are infections that induce a degree of tolerance. For example, dendritic cells exposed to *H. pylori* favor the ability of these APCs to induce Treg cells[134,135] and contribute to persistent infection. Indeed, a pathogen such as *H. pylori* can coexist more or less peacefully for the host's entire life.[46,47] The molecular basis to explain this effect of *H. pylori* remains unclear but could include the modest inflammatory effect of its lipopolysaccharide[136] or any number of metabolic factors that remain to be defined. Adding to the complexity of this biology, *H. pylori* may even confer some advantages to health in the digestive tract[137] or other sites, as discussed later. Importantly, "sterile immunity" may be less advantageous to the host than a more mutually beneficial relationship.

Adaptive Immunity to Mucosal Stimulation

The transition from innate to adaptive immunity is based on the interaction among Th cells, APCs, and the molecular environment that drives Th cell differentiation. As illustrated in Fig. 7.3, the cytokine milieu induced following antigenic stimulation shapes the differentiation of Th cells. The combination of microbial factors, the conditioned responses of APCs that have adapted to their respective niche, and local cytokines (e.g., from ILCs or other T cells) all regulate Th cell differentiation. This is illustrated with nematode infections that induce thymic stromal lymphopoietin and IL-4 leading to the differentiation of naïve Th cells into Th2 cells.[138–140]

Th17 cells provide another important Th cell response in mucosal tissues. IL-17 production was first implicated in mucosal infections in studies of the pathogenesis of *H. pylori* in human gastric tissue.[141] Subsequently, Th17 cells, through the production of IL-17 and IL-22, have been shown to play an important role in immunity to mucosal pathogens[75] including *Citrobacter rodentium,*[142] *Klebsiella pneumoniae,*[143] *H. pylori,*[144] and *Candida albicans*.[81]

Most inflammatory responses are mixed, as cytokines capable of favoring different Th cell subsets are induced simultaneously. The plasticity of Th cells and the complexity of Th cell differentiation are attributable to the fact that multiple cytokines targeting a Th cell induce the same, complementary, or competitive signaling pathways. Whereas some responses can be uniform such as Th2 responses induced by nematodes[145] or Th1 responses induced by *Mycobacterium tuberculosis,*[146] other microbes induce mixed Th1/Th17 responses, and there are even reports of Th1 responses in Treg cells.[147] Moreover, IL-10–producing Treg cells expressing retinoic acid–related orphan receptor gamma (RORγt) are induced in the gut following infection.[121]

The Th cell–derived cytokines expand and differentiate $CD8^+$ cytotoxic T lymphocytes as well as B cells. In addition, these cytokines feedback on innate cells to increase the expression of cytokines produced by epithelial cells, ILCs, or APCs. For example, the production of interferon-γ, IL-2, and IL-15 is particularly effective at increasing cytotoxic activity mediated by NK cells, invariant NK-T cells, and cytotoxic T lymphocytes that would be effective at mediating immunity against intracellular pathogens including viruses and some invasive bacteria. For example, these responses would be induced most efficiently by Th1 cells in an environment in which the pathogen, or vaccine antigen, induced IL-1 and IL-12.

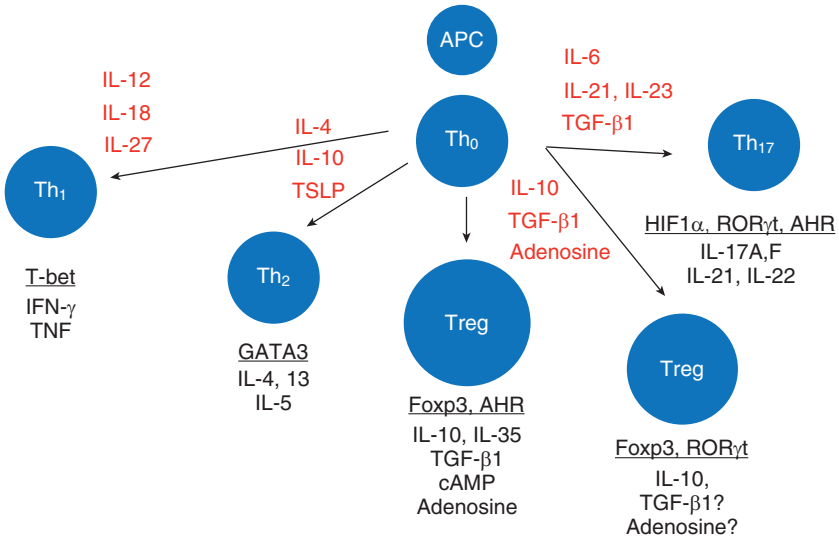

FIG. 7.3 Impact of T helper cell plasticity on immunologic function. Selection of Th cell subsets is driven by positive signals (in *red*) that favor the induction of specific transcription factors *(underlined)* and the genes they regulate. In addition, negative feedback provided by some of these cytokines favors the emergence of certain subsets. For example, production of IL-4 inhibits Th1 cells, whereas IFN-γ inhibits Th2 and Th17 cells. Based on the bias in cytokine production, these Th cell subsets mediate different functional activities. Th1 cells enhance cell-mediated immunity, which is well suited to protect against intracellular functions. Th2 cells enhance IgE and mast cell responses that are important for immunity against helminths. They also contribute to IgA production. Th17 responses are particularly important in antibacterial immunity and IgA regulation, whereas Treg cells not only favor IgA but also inhibit other responses and contribute to tolerance. Th17 cells induced by infection can also acquire Foxp3 and function as IL-10–producing Treg cells. However, when not appropriately regulated, these subsets contribute to disease, as evidenced by the association of Th1 and Th17 cells with gastritis and inflammatory bowel disease and the well-established role for Th2 cells in allergy. *AHR,* Aryl hydrocarbon receptor; *APC,* antigen-presenting cell; *cAMP,* cyclic adenosine monophosphate; *GATA3,* transcription factor binding to DNA sequence guanine-adenine-thymine-adenine; *HIF1α,* hypoxia inducible factor-1α; *IFN-γ,* interferon-γ; *IL,* interleukin; *RORγt,* retinoic acid–related orphan receptor gamma; *T-bet,* T-box expressed in T cells; *TGF-β1,* transforming growth factor-β1; *Th,* T helper; *TNF,* tumor necrosis factor; *Treg,* regulatory T; *TSLP,* thymic stromal lymphopoietin.

Mucosal B cells are noted for having undergone isotype switch from IgM-bearing cells to IgA-producing cells. Whereas IgA is usually the predominant antibody in mucosal tissues, other isotypes can be induced including various isotypes of IgG and IgE. IgG tends to be the predominant antibody isotype in the lower airway and reproductive tract. The process of isotype switch is mediated by different cytokines including IL-4, which favors IgE[148] and IgG1, as well as TGF-β1, B-cell–activating factor of the TNF family (BAFF), and A-proliferation–inducing ligand (APRIL), which direct switch to IgA.[149–151] One of the richest sources of TGF-β1 is the Treg cell subset.[123] Treg cells induce more IgA-producing cells than Th2 cells in addition to inhibiting proinflammatory cytokine responses. Treg cells can also differentiate into follicular Th cells in mouse Peyer patches for supporting IgA B-cell responses.[152] These multiple functions for current or former Treg cells illustrate the delicate balance that is required in mucosal immune homeostasis. Inhibition of host responses by Treg cells can favor persistent infection. Whereas some microbes induce host responses that eventually eliminate and prevent future infection, Treg cells may allow IgA antibodies that are sufficient to protect the host, while simultaneously limiting tissue damage that could emerge from excessive amounts of inflammation that may be induced by pathobionts.[123] Following the isotype switch to IgA, other Th cell–derived cytokines (e.g., IL-5, IL-6, IL-10) can expand the pool of IgA-producing cells[153] and coordinate an effective host response that remains under the control of Treg cells.

The production of IgE in the airway and digestive tract occurs in the context of nematode infections or allergies. Environmental triggers including microbial PAMPs can stimulate the production of cytokines that favor Th2 differentiation and IgE production.[69] Furthermore, rhinoviruses have also been implicated as a trigger that exacerbates atopy including food allergy as well as wheezing.[154] Thus careful regulation of mucosal immune responses is important for maintaining immunologic homeostasis to infections.

The innate-like mucosal-associated invariant T cells are a more recently described population of cells.[155] These cells reside in the mucosa and are unique in that they express T-cell receptors (usually Vα7.2) but with diversity limited to the recognition of nonpeptide antigens expressed by some microbes but not others. These antigens include modified vitamin B_2 metabolites derived from microbes and presented by an MHC class I–like molecule.[156] These MHC molecules are an evolutionarily conserved MHC-related protein 1 (MR1). It has been described that other metabolites create neoantigens by modifying the riboflavin metabolite. Some of these mucosal-associated invariant T cells have been shown to produce TNF-α in response to *Mycobacterium tuberculosis*–infected cells.[156] Although much remains to be studied, their significant presence in human tissues and blood compared with mice and their role in some bacterial and fungal infections, but not viral infections, suggest many advances will be reported in the near future.

Immunologic Homeostasis in Mucosal Tissues

Immune responses in mucosal tissues including the gut, airway, urogenital tract, and eye are regulated by $CD4^+$ Th cells, regulatory NK-T cells, $CD8^+$ T cells, and B cells. In response to benign antigens, which on their own do not deliver a loud danger signal, immune cells with a regulatory phenotype maintain immunologic homeostasis. Lineages with this function are represented unevenly in different tissues, but together they create a regulatory network to maintain equilibrium in mucosal immune reactivity.

Th cells are an important part of the adaptive immune system that contributes to the balance of immunity and immunologic restraint required for healthy mucosal tissues. A number of functionally distinct $CD4^+$ Th cell subsets have been elucidated including Treg cells, which mediate antiinflammatory activity. These cells are a topic of intense study because of their ability to control inflammatory responses and prevent autoimmune reactions.

Treg cells, or various Th cell subsets with regulatory function, inhibit immune and inflammatory responses through the production of IL-10 or TGF-β1 or both. In addition to these cytokines, interactions between surface cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) on Treg cells and receptor molecules on target cells limit immune responsiveness. Other data suggest that contact between Treg cells and effector T cells

stimulates the production of the antiinflammatory mediator IL-35.[157] A more intimate mechanism of intercellular contact was described by Bopp and coworkers,[158] who reported that the suppressive action of murine Treg cells depended on the transfer of cyclic AMP from Treg cells to responder T cells. Whereas pharmacologic manipulation may increase the levels of cyclic AMP available to mediate Treg cell function, there are biologically relevant stimuli that accomplish this. One example is adenosine.

There is now substantial evidence that adenosine is an important mediator of Treg cell function. For example, engagement of the A_{2A} adenosine receptor not only induces Treg cells[159] but is also required for optimal Treg cell function.[160,161] Subsequently, investigators reported that Treg cells had the ability to synthesize adenosine through the action of two ectoenzymes, CD39 (ectonucleoside triphosphate diphosphohydrolase-1 [NTPDase-1]) and CD73 (ecto-5′-nucleotidase). Many Treg cells in mucosal tissues express CD39 and CD73, which catalyze the transformation of ATP to adenosine.[162] ATP enhances dendritic cell maturation[163] and increases Th17 responses in the intestinal lamina propria.[114] As most Treg cells express CD39 and CD73, they not only generate adenosine that inhibits many responses including Th17 cells[164] but also degrade ATP and in so doing remove this proinflammatory stimulus from the immunologic synapse. As described earlier, ATP metabolism provides an intersection between the host and microbes that generate and use these products for their respective advantage.

The induction of Treg cells to infections has also been examined in the airway.[165,166] For example, infection with *Bordetella pertussis* stimulates the accumulation of Treg cells that can attenuate injury caused by immune and inflammatory cells induced by infection.[167] Mills et al. expanded this model to show that parenteral administration of an immunomodulatory antigen from *B. pertussis* protects against colitis induced by the adoptive transfer of $CD45RB^{high}$ Th cells.[168] This experiment shows that induction of one functional subset of Th cells by a specific microbial antigen can create a regulatory network that interferes with inflammation mediated by Th cells, even in other mucosal tissues. This notion is supported by the observations that Th2 cell responses induced by nematode infections attenuate disease mediated by Th1 or Th17 cells or both in the stomach[169] or intestine.[170,171] These reports provide evidence that immune deviation induced by mucosal infection deflects a potentially adverse host response to a more tolerable condition. These studies also demonstrate that new knowledge gleaned from research in one tissue or with one organism may have relevant applications in the management of other diseases in unrelated tissues.

When Mucosal Responses Go Wrong

Although mucosal immunity is intended to maintain immunologic homeostasis and health through protective responses, the host pays a price when inappropriate responses are stimulated by infection. The burst of cytokines, reactive oxygen or nitrogen species, and the release of enzymes or vasoactive mediators allow the infiltrating immune and inflammatory cells to contribute to disrupted tissue architecture and loss of function. In addition to tissue damage, these responses can cause pathologic amounts of catharsis, pain, or motility disorders including bronchoconstriction. Furthermore, the oxidative stress induced by chronic inflammation also increases the risk of various cancers.[172,173]

In some infections the onset of immune-mediated damage can begin with the acquisition of an infection that is sufficiently robust to break tolerance. For example, viral infections that induce prodigious amounts of IL-2 can break tolerance in autoreactive T cells leading to diabetes.[174] In the context of mucosal immunity, infection with one microbe not only activates host responses to this challenge but also simultaneously activates other T cells.[175] One well-known example of this is Guillain-Barré syndrome that is triggered by a mucosal infection with *Campylobacter jejuni*.[176,177] The microbe may express molecules that resemble the host (molecular mimicry). Thus if other significant immunologic events are occurring concurrently, the triggering infection may disrupt the generation or maintenance of tolerance contributing to allergies or immune-mediated diseases such as the induction of Guillain-Barré syndrome by *C. jejuni*.[177]

There are several signature changes in mucosal immunity in the context of chronic inflammation or immune-mediated disease. There is a predictable shift from the normal IgA responses to the accumulation of IgM and IgG as well as activated complement. These responses are observed in chronically inflamed tissues including the oral cavity with periodontal disease, the small intestine affected by celiac disease,[178] the stomach during infection with *H. pylori,*[179] and the intestines in response to inflammatory bowel disease (IBD).[180] Although many of the host responses are aimed at controlling these inflammatory conditions (e.g., through an increase in Treg cells and inhibitory cytokine production such as IL-10), the antigenic drive fueled by leaky epithelial cells and aberrant host responses prevents resolution of the inflammation. In addition, the recruitment of cells from the blood that lack the hyporesponsive mucosal phenotype[181] or disruption of proper differentiation toward this more anergic phenotype[182] or both lead to heightened responses not normally encountered in mucosal tissues. The contribution of the host response to the pathogenesis of immune-mediated disease triggered by an infection may be the price one pays for enhanced antimicrobial immunity.

Another concept that has emerged is related to the altered equilibrium that is associated with persistent or recurring inflammatory disease. Immune-mediated diseases can have a profound effect on microbial composition and vice versa. This has led to the concept of homeostatic scarring,[183] a process in which the equilibrium is perturbed and the simple treatment of a disease such as IBD with antiinflammatories may improve the inflammation but does little to restore homeostasis. Although it is not yet clear if the creation of a dysbiosis portends disease or if unrelated triggers lead to a proinflammatory microbiota, it increasingly appears that successful prevention or treatment of many diseases may require manipulation of the microbiota and some monitoring of mucosal immunity.

Mucosal Host Response Defines Pathogenicity

Much has been written about the role of microbial factors in disease pathogenesis[19]; however, it is also important to consider the contribution of local mucosal immune responses. Some of the most compelling evidence for immune-mediated damage following infection comes from genetic studies. First, with the emergence of genetically engineered animal models, it became clear that disruption of scores of genes that regulate immunity was sufficient to cause inflammation in the gastrointestinal tract, usually manifesting as colitis.[184–187] Of significance, disease is markedly attenuated or prevented by reducing the microbial burden with broad-spectrum antibiotics or raising animals in gnotobiotic conditions. These findings demonstrated that the normal, resident microbiota, often referred to as *commensals*, were sufficient to trigger disease. Second, more recent genome-wide studies of patient populations have shown that polymorphisms in genes encoding proteins that regulate host responses are associated with gastrointestinal diseases including gastric cancer associated with *H. pylori* infection,[188-191] Crohn disease, and ulcerative colitis.[192] These data have supported the concept that chronic, mucosal diseases such as IBD or gastric cancer are due to the inappropriate regulation of mucosal immune responses to microbial antigens in genetically susceptible hosts.[193]

Other reports implicate the host response in more acute, infectious diarrhea (Table 7.1). For example, challenging mice lacking stem cell factor with cholera toxin[194] or *Salmonella*[195] does not induce disease. Similarly, inhibiting the recruitment of leukocytes in chemokine receptor–deficient mice attenuates disease caused by *C. difficile* toxin.[196] By impeding the accumulation or activation of mast cells or neutrophils, these approaches remove a major source of mediators that stimulate epithelial cell secretion and diarrhea.

An aberrant host response does not provide a complete explanation for the pathogenesis of complex diseases, but immune responses contribute through their effects on colonization, translocation, tissue damage, and dissemination of infections. However, the interaction between the microbiota and the host is not always detrimental.

Good Bugs, Bad Bugs, and the Hygiene Hypothesis

The evolution of mucosal immunity was driven in part by the benefit of microbial-derived nutrients to the host and the need to keep microbial

TABLE 7.1 Role of Mucosal Immune Reponses in Microbial Pathogenesis

PATHOGEN	EVIDENCE FOR IMMUNOPATHOGENESIS
Vibrio cholerae	Absence of disease SCF-deficient mice[194]
Bacillus anthracis	Attenuation of disease in mice depleted of macrophages[251]
Bordetella pertussis	Clearance prevented by IL-10–producing Treg cells[167]
Clostridioides difficile (formerly *Clostridium difficile*)	Inhibiting leukocyte recruitment in chemokine-deficient mice prevents disease[196]
Salmonella spp.	Absence of disease in SCF-deficient mice[195]
Helicobacter pylori and gastric cancer	Class II MHC/HLA acts as receptor, and its expression is increased by cytokines[38] Tissue damage and epithelial cell apoptosis attributed to host response[252–257] Treg cells and antiinflammatory mediators control gastritis and contribute to persistence[258–263] Immunogenetic studies in humans implicate polymorphisms in genes that regulate host response (e.g., IL-1β, TNF-α, IL-10)[188,190]
Helicobacter hepaticus	Exacerbates disease in IL-10–deficient mice[264,265] Disease prevention associated with induction of IL-10–producing Treg cells[265]
Inflammatory bowel disease	Immunogenetic studies in humans implicating polymorphisms in genes that regulate host response to infections (e.g., NOD2, IL-17, TNF-α, IL-10)[192]

HLA, Human leukocyte antigen; *IL,* interleukin; *MHC,* major histocompatibility complex; *NOD2,* nucleotide oligomerization domain 2; *SCF,* stem cell factor; *TNF-α,* tumor necrosis factor-α; *Treg,* T regulatory.

communities under control. Increasingly the host response is recognized for its ability to shape microbial communities rather than sterilize them.[25,197–199] The notion that tissues should be sterile has never reflected reality, and attempts to scrub the mucosa to some artificial state of cleanliness are increasingly viewed as a contributor to many diseases. This notion has evolved from the hygiene hypothesis, which can be defined as the theory that children who are not exposed to robust microbial challenges have altered immune homeostasis and an increased risk for several immune-mediated diseases. Advocates of the hygiene hypothesis suggest that the symbiosis of a complex microbiota with the host response tends to establish a protective equilibrium. The cumulative effects of microbial virulence factors or metabolites, diet, environmental factors, and host genetics and epigenetics are all thought to impact mucosal immunologic homeostasis.

One clinical example that is often cited in support of the hygiene hypothesis is the continued increase in IBD in Western Europe and North America[200] and more recently in Asia.[201] Although many factors are associated with this trend, one hypothesis is that these Th1/Th17 predominant diseases occur less in countries that have a higher burden of infections, including nematodes. Indeed, helminth infection can attenuate IBD in humans[171] and animal models.[170] Other reports describe an antiinflammatory effect of nematodes on *H. pylori*–induced gastritis,[169] whereas probiotic bacteria[202–206] or fungal infections with *C. albicans* protect against colitis in animal models[207] by increasing IL-10 and decreasing IL-17A and IL-17F as well as TNF-α. In the case of *C. albicans,* the protection has been traced to its ability to induce indoleamine-2,3-dioxygenase production by dendritic cells that in turn affects the differentiation of Th cells.[208] Several other studies support the notion that PRRs and signaling through MyD88 are required to maintain mucosal homeostasis.[209,210]

Although diseases such as asthma and IBD occur less frequently in countries lacking the degree of hygiene found in Europe and North America, one cannot ignore the huge morbidity and mortality from mucosal infections in these populations that affect nutrition, growth, and cognitive development.[211] Ideally a limited microbial community that is more complex but lacks the most deleterious pathogens or virulence factors could achieve the potential benefit inferred by the hygiene hypothesis. *H. pylori* is one species that is widespread in countries cited as having lower rates of asthma or IBD. Epidemiologic studies suggest that *H. pylori* confers protection against esophageal cancers[137,212] or other infections including tuberculosis.[213] Müller et al. performed a series of studies in a murine model of asthma showing that neonatal infection with *H. pylori* induces Treg cells that inhibit gastritis and favor persistence but also attenuate airway disease.[134,135] Whereas one would have to weigh the risk of gastric cancer with the benefits of carrying *H. pylori,* it is possible that sufficiently attenuated strains will be generated that may be proven beneficial when included in a probiotic mix.

Additional evidence for the beneficial role of a complex microbiota comes from the use of fecal transplants as a strategy for preventing or attenuating diarrhea caused by *C. difficile*.[214] Furthermore, a natural shift in microbial communities has been implicated in recovery from cholera.[215] The current artificial approaches for "rePOOPulation"[216] lack the optimal specificity, assured freedom from pathogens, and the control of dose that are preferred in therapeutic approaches in medicine. Nonetheless, as the beneficial and deleterious microbial communities or metabolites/virulence factors continue to be identified, it may be possible to modify a host's microbiota for the benefit of the host's health through the use of prebiotics, probiotics, antibiotics, or even vaccines.

Mucosal Immunization

Given that many infections of significance to humans enter by a mucosal route, designing safe and effective mucosal vaccines remains a high priority to protect against infectious diseases including tuberculosis, HIV, various diarrheas, and influenza. Intact organisms, encountered naturally or as vaccine vectors, remain the most effective immunogens, as they entail multiple "danger" signals, some of which are not well understood. To make vaccines safe, scientists have opted for the reductionist approach; however, these approaches often lack the ligands that define tropism to a preferred inductive site and the requisite array of proinflammatory signals that determine if an antigen is ignored or acted on.

Effective mucosal immunization begins by providing the immunogen to a mucosal inductive site.[217] Traditionally, this has included administration by intranasal, sublingual, oral, and intravaginal routes, all of which have the goal of targeting mucosal tissues directly. In humans, one of the more successful mucosal vaccines is the oral polio vaccine. This is a live-attenuated virus vaccine and thus brings the advantage of replication and packaging that enhances the immunogenicity. Killed or subunit vaccines, particularly those delivered orally, are challenged by their relative lack of immunogenicity, as proteins are considered "food" more often than a danger signal. Other strategies to augment the immunogenicity have included the use of bacterial toxins as an adjuvant. Cholera toxin is profoundly immunogenic, although it carries significant side effects when administered orally. Mutated toxins, engineered as a fusion protein with the antigen of interest and possibly targeted to delivery by inhalation, could circumvent many unwanted side effects and yield a promising strategy in the future.[218] More recent efforts of fusion science in mucosal immunology, plant biology, and drug delivery engineering have provided an opportunity to advance strategies to develop effective antigen delivery vehicles targeting mucosal surfaces for the initiation of potent antigen-specific SIgA and serum IgG responses.[219,220] For example, a rice-based oral vaccine system has been shown to be effective for the induction of antigen-specific protective immunity against intestinal toxin produced by *Vibrio cholerae* and enterotoxigenic *E. coli*.[221,222] For nasal vaccination, the cationic form of pullulan-based nanogel is also effective for the induction of protective immunity against *Clostridium botulinum* type A neurotoxin and *Streptococcus pneumoniae*.[223]

Although the systemic route of immunization rarely induces protective mucosal immunity, exceptions exist; an example is the human papillomavirus vaccine.[224] Live virus vaccines, live vectors, or virus-like particles can be effective, as they are assumed to retain the tropism for mucosal tissues where they can replicate or be processed by APCs. This approach is believed to have the advantage of bringing a collection of signals that enhance immunogenicity and stimulate a response in an otherwise hyporesponsive environment—what Janeway eloquently

referred to as the "immunologist's dirty little secret."[225] It is theoretically possible that the strategic use of adjuvants or biologic response modifiers with systemic immunization may induce a phenotype that enables effector cells to populate mucosal tissues. For example, the role of retinoic acid in imparting the ability of APCs or lymphocytes to home to mucosal tissues could provide one strategy.[117] At the present time, no such adjuvants or biologic response modifiers that intentionally target mucosal trafficking are used routinely for this purpose in humans.

Considering the epidemiology of some transmissible diseases, there may be a strategic advantage to control infections through mucosal immunization of food-producing animals rather than attempt widespread immunization of humans. As a case in point, one would need a fraction of the vaccine preparations to control enterohemorrhagic *E. coli* (O157) by immunizing cattle rather than humans. By targeting the virulence factor Tir in these *E. coli,* infection in cattle is decreased,[226,227] and meat is thus rendered safer for human consumption without the need to extensively test another vaccine for use in humans.

Although some successes have been achieved in mucosal immunization, many challenges remain, including the pressing need for a vaccine against HIV and herpes simplex virus (HSV). When considering strategies for the successful induction of immunity, it is important to consider the known biology so that the immunogen gets delivered to the appropriate inductive site to stimulate a protective response in the tissue of interest. For HIV and HSV, the infection is spread naturally through mucosal routes of sexual contact including the rectum or female reproductive tract. However, very little is known, especially in humans, about which effector cell responses should be induced in these sites and how they can be induced. Based on other mucosal diseases and the vaccines that have been produced, one can predict that systemic immunization will be most effective at preventing infection if it can induce the required responses in these sites. Current efforts for preventing HIV and HSV infection at mucosal sites rely on a degree of blind luck to achieve immunity in the rectum or vagina given how little is known about the induction, regulation, effector mechanisms, and trafficking of immunologic cells in these sites. As evidenced by the parenterally administered polio vaccine, it is possible that systemic immunity may be sufficient to limit tissue injury and disease from a pathogen acquired through mucosal routes.

At the other end of the spectrum, mucosal immunization may be exploited as a strategy to decrease deleterious host responses through the induction of tolerance—through either immune deviation or active suppression. For example, if autoimmune diseases are driven by Th1/Th17 responses, the induction of Th2 cells may change the local homeostasis by dampening the Th1/Th17 cells. As described earlier (see "Good Bugs, Bad Bugs, and the Hygiene Hypothesis"), infection with nematodes has been shown to decrease gastritis[169] and colitis[170,171] in murine models as well as in humans with IBD.[228] Current efforts are focused on identifying specific molecules produced by the nematodes that could be incorporated into a mucosal delivery system. Other attempts to enhance tolerance to transplanted tissue or attenuate allergies or autoimmune diseases by the oral or inhaled delivery of the offending antigen have been contemplated, but limited success has been achieved in humans to date.

The field of mucosal immunology in disease prevention was advanced based on the importance of antibody production in breast milk. Ingestion of colostrum and breast milk is of great benefit to infants due to the presence of antibodies, glycans, and cytokines that protect the infant and condition the maturation of the infant's immune system.[229] Furthermore, the nutritional medium provided by breast milk (including oligosaccharides) creates a prebiotic effect that shapes microbial communities in the offspring.[230] Maternal antibodies are extremely relevant, as they reflect the microbial environment of the mother and hence that into which the infant is born. Consequently there is a great deal of interest in how specific antibody can be enriched by natural or artificial immunization. This has been pursued both in human medicine to decrease influenza and HIV transmission and in veterinary medicine to decrease zoonotic infections. The current successes in limiting *Coxiella burnetii* infection in ruminants has enhanced herd health by decreasing the pathogen burden and protected people working closely with these animals.[231] However, strategic immunization of mothers to protect infants from neonatal diarrheal or respiratory diseases has yet to be fully realized.

ROLE OF MUCOSAL IMMUNITY: BEYOND THE MUCOSA

In the gut, the microbial burden contributes directly to diarrhea, epithelial cell turnover,[232] and cancer[191,233,234] as well as to malnutrition, stunted growth, and impaired cognitive development.[211,235] However, this discussion would not be complete without pointing out the profound scope of bodily functions that are affected by interactions between the host and its microbial burden in mucosal tissues. Although it has been challenging to demonstrate that systemic antigen exposure modulates mucosal immunity, there is overwhelming evidence that mucosal exposure to antigens has profound effects on systemic responsiveness (e.g., through oral tolerance).[6,236,237] Another illustration is the emerging evidence that the host-microbial interactions in the gut modify diseases in systemic sites including hepatitis,[238] obesity,[238,239] diabetes,[240] and autoimmune diseases such as autoimmune encephalitis[241] and even memory[242] or behavior.[59,243] This ability to regulate disease or complex functions in multiple tissues adds a much broader relevance to the host-microbial interactions that constitute mucosal immunity.

One specific example of how bacteria in a mucosal site regulate host responses elsewhere was identified by the observation that mice from different vendors had sharp variations in the percentage of Th17 cells in the intestinal mucosa.[244] Subsequently, segmented filamentous bacteria (SFB) were identified as being sufficient to cause an expansion of Th17 cells.[21] Whereas mice can tolerate this expansion without obvious effects, on further provocation, the colonization with SFB and associated increase in Th17 cells exacerbated experimental autoimmune encephalitis.[241] These experiments yet again illustrate the outreach of the interactions between mucosal immune responses and the microbiota and the ambiguity in describing bacteria such as SFB as commensals when they may be better thought of as amphibionts.[19]

The current evidence does not provide a cogent explanation for these profound effects of the host-microbial interactions. It is safe to assume that to mediate systemic effects, molecules produced by mucosal microorganisms have to be "sensed" and tethered to changes in cell signaling, differentiation, and function that account for their effects—likely through immunophysiologic effects as much as pure immunologic responses. Increasingly, scientists are viewing the problem from a network perspective.[245] In a network, one can consider the entire microbiome—not merely from a taxonomic perspective but from metabolic and antigenic views.[246] Furthermore, the interaction of metabolites with other microbes as well as the host can be examined using a network analysis.[247] Similar rigor is required to understand the host genome, epigenome, transcriptome, and metabolome as well as environmental factors (including diet) and their collective impact on host responses and health.[248–250] These enormous data sets will require computational models that create a map of the biologic landscape that is defined by key intersections within the interactome.[245] F studies will subsequently be needed to validate the impact of these factors so that more strategic approaches can be used to improve health.

CONCLUSIONS

Understanding how diseases are caused or prevented by infection and the host response is in its infancy. The emerging evidence suggests that mucosal and systemic immune responses adapt throughout life in response to an array of dynamic changes in dietary, microbial, and other influences. Knowing the changes in metabolites that microbiota impart; how these are sensed, that is, in utero, in the neonatal period, and throughout life; and their effect on transcriptomes and function will enhance our understanding of the mechanisms by which microbes modulate disease. These complex interactions will require many advances in systems biology and computational modeling so that strategies to enhance immunity with vaccines or by improving health by manipulating microbial communities will be more effective. With more attention to the endless possibilities, technically difficult approaches will be overcome, and manipulation of the host-microbial interactions in the mucosa will become an important target for interventions that prevent or treat many diseases.

Key References

The complete reference list is available online at Expert Consult.

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8 Granulocytic Phagocytes

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Vertebrate host defense against microbes represents the integration of the innate and acquired immune systems, which together respond to a diverse array of infectious threats.[1,2] Innate (natural) immunity provides the host with the capacity to respond immediately to an infectious challenge, regardless of previous exposure to the specific invading agent, by using response elements encoded in germline genes. Elements of the innate system include phagocytic cells, polymorphonuclear leukocytes, mononuclear phagocytes, and circulating soluble proteins, including components of the complement system (see Chapters 4 and 9). This sensitive system for the recognition of structural elements that are inherently and uniquely microbial has functional analogues in the immune systems of a wide variety of multicellular organisms, including plants and insects. As such, innate immune elements comprise an evolutionarily ancient system that provides a rapid and sensitive surveillance mechanism to protect the host when challenged with any invading microorganism. However, on par with the importance of rapid recognition of and response to microbial threats is the need to resolve the inflammatory response and restore homeostasis. Granulocytes, the most numerous leukocytes in the peripheral circulation of humans, include neutrophils, eosinophils, and basophils. They represent the predominant cell type in the acute innate immune response and figure more broadly in the integration of innate and adaptive immunity.[2,3] Structurally, these cells share with one another a multilobed nucleus and the presence of numerous membrane-bound, characteristically staining cytoplasmic granules, but functionally they differ significantly.

NEUTROPHILS

Development

Neutrophils arise from pluripotent hematopoietic stem cells (HSCs) in the bone marrow through an orderly succession of phenotypically distinct cell types.[4,5] From the HSCs arise multipotential progenitors, a cell population with the capacity to differentiate into all hematopoietic lines but unable to multiply. The multipotential progenitors spawn common myeloid progenitors that serve as the source of precursors for the individual hematopoietic cell lines, including granulocyte/macrophage progenitors. The complex procession from HSC to granulocyte/macrophage progenitor and then to neutrophils is coordinated by specific transcription factors, including PU.1, CCAAT/enhancer-binding proteins (α, β, and ε), growth factor independent-1, and interferon regulatory factor 8.[5] Timely expression of such factors coordinates transcription of stage-specific genes that are responsible for the phenotypic and functional features that define myeloid intermediates along the differentiation pathway. In part, soluble proteins, such as interleukin-17 (IL-17), IL-23, and granulocyte colony-stimulating factor (G-CSF), modulate the relative levels of transcriptional factors within myeloid cells and thus influence the fate of the cell.[6] CSFs also alter the survival and direct the maturation and proliferation of myeloid cells. Each factor is named for the colony produced under its influence: GM-CSF, for granulocytes and macrophages; G-CSF, for granulocytes; M-CSF, for monocytes and macrophages; and multi-CSF (or IL-3), for a variety of colonies, including neutrophils, macrophages, eosinophils, megakaryocytes, and erythroid cells.

[a]All material in this chapter is in the public domain, with the exception of any borrowed figures or tables.

Emergency Granulopoiesis

In addition to the granulopoiesis essential to maintain steady-state levels of circulating neutrophils, the hematopoietic system has the capacity to mobilize additional functioning neutrophils in response to the increased demand imposed by infection.[7,8] Infection induces augmented production of cytokines, including G-CSF, GM-CSF, and IL-3, and these circulating proteins drive "emergency" granulopoiesis. IL-17, a cytokine produced by Th17 cells,[9] drives G-CSF production and promotes emergency granulopoiesis, as deduced from experimental models of chronic inflammation, but it does not contribute to homeostatic neutrophil production. G-CSF stimulates the production of granulocyte precursor cells, the proliferation of cells in the granulocyte lineage, and the survival of granulocyte precursors and neutrophils. Furthermore, G-CSF accelerates passage of granulocyte precursors through the bone marrow, thereby providing an immediate supply of young neutrophils into the circulation. Thus the control of granulocyte production can be modulated not only to maintain homeostatic levels of neutrophils as aged cells are cleared from the circulation but also to respond to increased demands created by infectious or other challenges.

Mitotic and Postmitotic Cells

The cell populations during steady-state granulocyte development in the bone marrow can be divided into three pools: a stem cell pool, a mitotic pool, and a postmitotic pool.[10] The stem cell pool includes undifferentiated HSCs, whereas the mitotic pool encompasses cells that proliferate and mature sequentially from myeloblasts into promyelocytes and myelocytes. Maturation is associated with the appearance of the cytoplasmic granules characteristic of neutrophils, eosinophils, and basophils.[11] The postmitotic phase of development includes metamyelocytes, band (or immature) neutrophils, and mature neutrophils, all cells held in reserve and ready for release.

Coincident with the appearance of morphologic changes, cells acquire the specific surface markers and functional properties of more mature cells.[12] For example, Fc receptors appear as the cells develop into promyelocytes, competence for phagocytosis arises in the early myelocyte stage, and complement receptors surface in the late myelocyte and metamyelocyte stages. Oxygen-dependent microbicidal activity appears in the early metamyelocyte stage, and cells in the late metamyelocyte–band stage demonstrate increased adhesiveness, cell motility, and chemotactic responses.[12] In addition, coordinated expression of genes encoding the granule proteins is synchronized with early stages of myeloid development, and normal granulocytic differentiation is intimately linked with expression of proteins localized in the specific granules.[12]

Morphologic and Structural Characteristics

The earliest histochemical studies of neutrophils classified the membrane-bound intracellular granules by their staining characteristics. Two populations of granules were distinguished based on staining with azure A: the positively staining azurophilic granules and the unstained specific granules. Sophisticated analyses of the composition of isolated neutrophil organelles have refined significantly our appreciation of the complexity and heterogeneity of neutrophil granules.[13,14] Such studies have provided novel insights into the biologic roles of the various proteins in the

matrix of the granule and have revealed functionally important proteins within the membranes of particular granule subsets.

Azurophilic Granules

At a first approximation, neutrophil granules can be categorized based on peroxidase staining. The peroxidase-positive granules are also known as *primary granules,* as they arise first in granulopoiesis, and as *azurophilic granules,* based on histochemical staining. Azurophilic granules contain myeloperoxidase (MPO)[15]; a variety of proteolytic enzymes, including the four serine proteases, cathepsin G, proteinase-3,[16] elastase,[17] and NSP4 (neutrophil serine protease 4)[18]; and the antimicrobial defensins[19] and bactericidal permeability-increasing protein (BPI) (Table 8.1).[20]

TABLE 8.1 Contents of Neutrophil Granules and Secretory Vesicles

GRANULE	MEMBRANE	MATRIX
Azurophilic (primary granule)	CD63,CD68, presenilin	MPO, elastase, cathepsin G, proteinase 3, defensins, BPI, lysozyme, sialidase, azurocidin, β-glucuronidase
Specific (secondary granule)	CD11b/CD18, CD66, CD67, gp91*phox*/p22*phox,* TNF receptor, SNAP-23, VAMP-2, stomatin	Collagenase, gelatinase, urokinase plasminogen activator, hCAP-18, NGAL, vitamin B_{12}–binding protein, lysozyme, lactoferrin, haptoglobin, pentraxin 3, prodefensin, SLPI, orosomucoid, heparanase, β_2-microglobulin, CRISP3
Gelatinase (tertiary granule)	CD11b/CD18, CD67, gp91*phox*/p22*phox,* MMP25, TNF receptor, SNAP-23, VAMP-2, Nramp1	Gelatinase, arginase 1, lysozyme, β_2-microglobulin, CRISP3
Secretory vesicles	CD11b/CD18, CD67, gp91*phox*/p22*phox,* MMP25, CD35, CD16, C1q receptor, CD14, fMLF receptor, SNAP-23, VAMP-2, Nramp1, alkaline phosphatase, DAF, CD10, CD13, CFTR	Plasma proteins

BPI, Bactericidal permeability-increasing protein; *CD,* cluster of differentiation; *CFTR,* cystic fibrosis transmembrane conductance regulator; *CRISP3,* cysteine-rich secretory protein 3; *DAF,* decay accelerating factor; *fMLF,* *N*-formyl-methionyl-leucyl-phenylalanine; *hCAP-18,* human cathelicidin protein-18; *MMP25,* matrix metalloproteinase 25; *MPO,* myeloperoxidase; *NGAL,* neutrophil gelatinase–associated lipocalin; *Nramp1,* natural resistance-associated macrophage protein; *SNAP-23,* synaptosomal-associated protein-23; *SLPI,* secretory leukocyte protease inhibitor; *TNF,* tumor necrosis factor; *VAMP-2,* vesicle-associated membrane protein-2.
From Borregaard N. Neutrophils, from marrow to microbes. Immunity. *2010;33:657–670; and Borregaard N, Sorensen OE, Theilgaard-Mönch K. Neutrophil granules: a library of innate immunity proteins.* Trends Immunol. *2007;28:340–345.*

Because of the acid hydrolase activity of the azurophilic granule contents, this compartment had been considered lysosomal in nature. However, azurophilic granules lack lysosome-associated membrane protein,[21] an identifying marker for lysosomes. Moreover, proteins such as MPO[22] and the defensins[23] segregate into the azurophilic granule independently of the mannose-6-phosphate receptor, a targeting system characteristic of lysosomal proteins. Taken together, these observations suggest that the azurophilic granule may be a specialized organelle that is distinctly different from conventional primary lysosomes.

Peroxidase-Negative Granules

The peroxidase-negative granules include *specific granules, gelatinase granules,* and *secretory vesicles.*[24] The contents of the specific and gelatinase granules overlap to a significant extent (see Table 8.1)[25] but differ from the contents of azurophilic granules and secretory vesicles. More striking, however, is the distribution of functionally important plasma membrane proteins in the membranes of peroxidase-negative granules.[26] These membranes contain flavocytochrome b_{558},[27,28] an essential component of reduced nicotinamide adenine dinucleotide phosphate (NADPH)–dependent oxidase (discussed later); receptors for chemotactic peptides[29]; extracellular matrix proteins[30]; cytokines[31]; opsonins[32]; and adhesion proteins.[33,34] The secretory vesicles are especially enriched for plasma membrane proteins[35] and can be rapidly recruited to fuse with the plasma membrane, amplifying the potential of the neutrophil to respond to stimulation. They therefore represent an intracellular reservoir of functionally important membrane proteins that can be quickly recruited to the cell surface during neutrophil activation. The existence of such compartments is ideally suited to the role of neutrophils as the major circulating cell in the innate immune system; a reservoir of readily accessible functional proteins allows a rapid response without the delays that would be incurred by requirements for new protein synthesis. The functional consequences of this compartmentalization of proteins in the matrix and in the membrane of granules are discussed later.

Nucleus and Actin Cytoskeleton

During granulocyte maturation, the nucleus becomes segmented (Fig. 8.1), and cytoskeletal elements, including microfilaments and microtubules, appear in the cytoplasm. A meshwork of microfilaments makes up the clear cortical veil that surrounds the cell and forms the lamellipodium of an advancing cell (see Fig. 8.1). These structures are polymers of actin, a protein representing 5% to 10% of the total cellular protein. Actin and its associated proteins constitute the contractile machinery necessary for cellular locomotion[36] and phagocytosis.[37] Actin monomers (G-actin), in the presence of actin-binding protein, polymerize to form cross-linked actin filaments (F-actin). Regulation of the length of the filaments and the degree of cross-linking provides the physicochemical dynamics of actin flux between the gel and sol states. Actin filaments are associated with the cytoskeleton or with the plasma membrane via membrane skeletal proteins.[38] Stimulation of the cell with chemotactic factors causes an abrupt increase in the amount of actin associated with the cytoskeleton[39] and a shift in microfilament organization from a parallel strand to a cross-hatched meshwork most evident at the leading edge of the directionally polarized cell.[40] Microtubules appear to be necessary

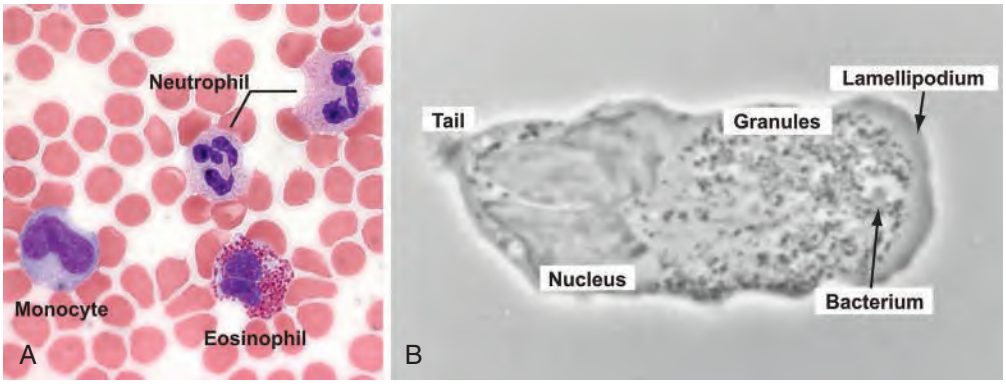

FIG. 8.1 Human neutrophils. Photomicrographs of phagocytes in whole blood (A) and a neutrophil as viewed by phase-contrast microscopy (B).

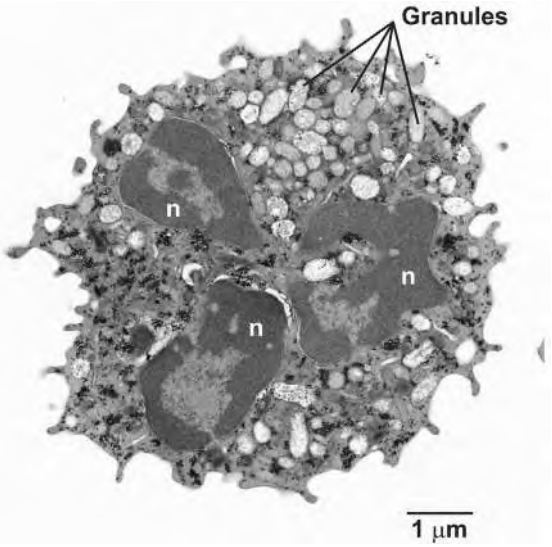

FIG. 8.2 Electron micrograph of a human neutrophil. Note the granules (as indicated) and glycogen particles *(small dark particles),* but few other prominent organelles. *n,* Nucleus (multilobed).

for the initial orientation of the cell in a chemotactic gradient; for the spatial organization of structures within the cell during locomotion; and for vesicle transport, degranulation, and the regulation of cell surface microviscosity during phagocytosis.

Organelles in the Cytoplasm

Mature neutrophils (Fig. 8.2; see Fig. 8.1) are characterized by a paucity of ribosomal material and mitochondria, in keeping with the relatively low levels of synthetic processes in these cells. However, studies over the past 3 decades have radically changed the image of neutrophils as biosynthetically inactive cells and have identified an array of proteins actively synthesized by neutrophils, both at rest and after stimulation. Among the proteins synthesized by neutrophils are major histocompatibility complex class I molecules,[41] complement receptors,[42] CXC chemokines, CC chemokines, proinflammatory and antiinflammatory cytokines, immunoregulatory molecules, colony-stimulating factors, tumor necrosis factor (TNF) superfamily members, and proteins important in angiogenesis and fibrogenesis.[3] As presented later, the transcriptional profile of recruited neutrophils includes genes directed toward wound healing.[43] Glycogen granules fill the cytoplasm and serve as a source of energy to support neutrophil activity.

Surface Receptors

As immune effector cells, neutrophils are equipped with surface receptors that sense extracellular signals and can recognize ligands to support the wide array of agonist-dependent activities within its functional repertoire. Surface receptors for immunoglobulin[44] and complement fragments[45] contribute to opsonin-dependent phagocytosis. Specific receptors on the plasma membrane initiate cell movement by recognizing interacting molecules on endothelial cells or extracellular matrix proteins, bacterially derived formylated proteins, chemotactic complement fragments C5a and C3a, platelet-activating factor, IL-8 and related chemokines, and leukotriene B_4 (LTB_4).[46–48,49,50] These receptors are homogeneously distributed over the surface of the resting cell but undergo an asymmetrical clustering at the front of the cell when it polarizes in response to a chemotactic stimulus. The distribution of receptors with different ligand specificities can be independently regulated, even though stimulation via these receptors may evoke similar functional responses.[51] Moreover, the various neutrophil functional activities exhibit differential requirements for receptor occupancy. For example, maximal degranulation requires brief receptor occupancy, whereas sustained oxidative responses depend on continuous ligand binding to the receptor.[52] Neutrophils also possess membrane receptors that signal to evade ("don't eat") or promote ("eat me") their own uptake by macrophages, a process known as efferocytosis (see later).[53]

Homeostasis of the Circulating Neutrophil Population

To maintain a stable number of circulating neutrophils, the production of new and functional cells must be balanced by clearance of cells that are aged and spent. The daily production of mature polymorphonuclear leukocytes in a healthy adult is remarkable, with approximately 10^9 cells/kg body weight entering the circulation from the bone marrow.[54] During acute infection or other inflammatory stresses, neutrophils are mobilized from the granulocyte marrow reserve, which is estimated to be $\approx 18.6 \times 10^9$ cells/kg (6.9×10^9 cells/kg are mature neutrophils).[54] Even in the presence of persistent stimulation, this reserve can be depleted only if there is nutritional deficiency or another disorder (e.g., ethanol abuse) that compromises mechanisms for augmenting delivery to meet demands. Augmented stem cell input, increased mitoses during the mitotic stage of development, use of a store of cells whose maturation had been inhibited (so-called hiatal cells), and shortening of the maturation time within the marrow all may occur.[10] Multiplication and differentiation of stem cells are stimulated by the CSF produced by peripheral blood monocytes, tissue macrophages, and stimulated lymphocytes.[54,55]

Distribution of Neutrophils

The total blood granulocyte pool ($\approx 7 \times 10^8$ cells/kg body weight) includes two compartments of similar size: intravascular circulating cells and marginating cells. The distribution of the marginated pool varies with the size and flow of the capillary bed in an individual organ. Liver, spleen, and bone marrow are included in this pool, but unsettled is the physiologic contribution of the pulmonary circulation.[10,56] Whereas experimental data based on intravascular transit time suggest that the lung is the predominant site of marginated neutrophils, studies using radionuclide imaging demonstrate relatively little neutrophil margination in normal human lungs. Overall, the contribution of the pulmonary circulation to the marginated pool of normal neutrophils in healthy humans remains unsettled.

A dynamic equilibrium exists between neutrophils in marginated and circulating compartments as cells marginate via transient endothelial interactions and then resume rapid flow, reflecting the balance between intercellular adherence and shear forces.[57–59] The intravascular half-life of circulating neutrophils is normally 6 to 8 hours, whereas their persistence in extravascular sites ranges from a few hours to several days. A report using in vivo labeling with ^2H_2O suggested that normal human neutrophils have a life span in circulation of 5.4 days,[60] more than 10-fold greater than previously thought. However, that conclusion has been challenged,[61,62] and alternative interpretations of the same data yield estimates that agree with the long-standing accepted value of 6 to 8 hours. Turnover is accelerated and half-life in circulation is shortened during infection, as neutrophils are recruited to sites of inflammation, where they ingest microbes and undergo accelerated apoptosis (see later). In fact, the redistribution of circulating neutrophils to sites of infection explains, in part, the failure of neutrophil blood counts to rise after granulocyte transfusions. Whereas granulocyte transfusions may promote resolution of acute infection, decrease the time to resolution of fever, and shorten the duration of antibiotic therapy, they do not typically increase peripheral white blood cell counts.[63,64]

Granulocytosis, a common feature of acute inflammation, is a consequence of certain physiologic and pharmacologic stimuli that typically redistribute neutrophils among the various granulocyte pools as well as increase cell production. For example, the acute administration of corticosteroids or endotoxin, perhaps mimicking pathophysiologic events that occur in severe infection, promotes granulocyte release from the marrow reserve. Sustained steroid administration produces granulocytosis primarily by decreasing neutrophil adherence and shifting cells from the marginating to the circulating pool. Similarly, exercise, stress, epinephrine, hypoxia, aspirin, and alcohol cause granulocytosis by mobilizing marginating cells.

In the setting of acute inflammation, spent and apoptotic neutrophils are ingested by macrophages by the regulated process of efferocytosis.[65] Neutrophils that are activated during their brief tour in circulation become senescent, a proapoptotic state characterized by increased surface expression of CXCR4 and decreased ability to perform antimicrobial

and proinflammatory functions.[66] Resident macrophages in liver, spleen, and likely bone marrow[56] routinely ingest and thereby clear senescent neutrophils from circulation in a manner that is immunologically silent—that is, without release of proinflammatory cytokines.

Inflammatory Response

Inflammation represents a remarkably integrated cascade of events involving both cellular and soluble factors that are precisely orchestrated spatially and temporally. As such, it is best conceptualized as a complex network of signals that modulate the responses of different cells and circulating molecules that, in turn, interact and are subject to a variety of regulatory checkpoints operating by local, systemic, and neural mechanisms.[3,67,68] Within the context of host response to invading microbes, the innate immune system sits poised to respond rapidly to perceived threats and in a stepwise fashion to recognize, contain, kill, and destroy potential pathogens. Because all successful biologic systems represent a balance among competing forces, the return of the host to homeostasis after an acute inflammatory response requires execution of a properly timed and appropriately proportioned antiinflammatory cascade. Thus the acute response of neutrophils requires a sensitive afferent limb to allow systemic recognition of a local threat at very low levels as well as an effector arm targeted against the noxious source to contain, kill, and degrade potential pathogens.[67] The coordinated response needs to occur before antiinflammatory events supervene to trigger neutrophil apoptosis and removal en route to resolution of the inflammatory reaction.[69] In addition, the fact that the noninflammatory homeostatic state is actively maintained by modulation of proinflammatory surveillance systems, rather than simply the absence of inflammatory stimuli, adds another layer of complexity and feedback signaling to an already intricate system.[68–70]

Circulating neutrophils are functionally heterogeneous, with most (80%), but not all, cells having the capacity to form immunoglobulin G (IgG) rosettes.[71] Because release from the bone marrow is not synchronized, this heterogeneity probably reflects in part maturational differences within a single cell line. More sophisticated analyses suggest that subsets of circulating neutrophils have distinct and important functional phenotypes.[72–78] In contrast to the heterogeneity of circulating neutrophils, those in tissue are relatively homogeneous, and more than 96% are capable of IgG rosette formation.[71] They contain fewer lysosomal granules and more glycogen than do their circulating counterparts because anaerobic glycolysis provides the energy for cell movement through the tissues.[79] The phenotypic differences between circulating and tissue neutrophils could reflect determinants required for neutrophils to immigrate into tissue, influences of transmigration per se, the impact of trophic elements in the tissue compartment, or other factors. In any case, the tissue neutrophil exhibits a phenotype different from that of the circulating, unstimulated neutrophil. For example, exudative neutrophils synthesize significantly more IL-8 and activate genes that collectively contribute to wound healing.[43,80] Furthermore, neutrophils exposed to concentrations of mediators that are too low to stimulate directly nevertheless prepare the cell for an enhanced response to a second, unrelated stimulus in a phenomenon known as *priming*.[81,82]

Priming

A broad array of proinflammatory mediators, including chemotactic factors, bacterial molecules, chemokines, cytokines, and certain lipids, can prime neutrophils, as can transmigration across the endothelium and migration into tissue. The primed state exists with respect to each of the major aspects of neutrophil function, persists for an extended period (longer than 20 minutes under experimental conditions in vitro) in relation to the response elicited by direct stimulation of the cell, and is reversible. It is not known whether priming agents share the same mechanism of action or if all the essential molecular events causing priming have been elucidated. Consistent with the diverse phenotypic features of primed neutrophils, partial assembly of the NADPH oxidase by phosphorylation and translocation of p47*phox,* secretory vesicle exocytosis and partial mobilization of specific granules to the plasma membrane resulting in increased surface expression of flavocytochrome b_{558} (see later), reorganization of the plasma membrane and distribution of receptors and signaling molecules into lipid rafts, modulation of intracellular signaling intermediates, and transcription of several gene families have been implicated in contributing to the more responsive state of the cell.[83–89] Most neutrophil priming agents also delay neutrophil apoptosis and thus prolong functional capacity, a phenomenon consistent with the potential for an enhanced proinflammatory response.[90]

Step 1: Neutrophil Recruitment

To combat invading microorganisms, neutrophils must emigrate from the circulation and into the extravascular tissue space, a process that reflects both the responses of neutrophils to the shear stress in the circulation and the summation of coordinated interactions of cells, specific receptors, and soluble mediators.[48,91,92] In fact, neutrophils in tissue, interacting with elements in the extracellular matrix, differ functionally from circulating neutrophils.[93–95] The process of neutrophil transmigration out of the circulation involves at least four discrete steps: rolling adhesion, integrin activation, firm adhesion, and transmigration (Fig. 8.3).[91] These

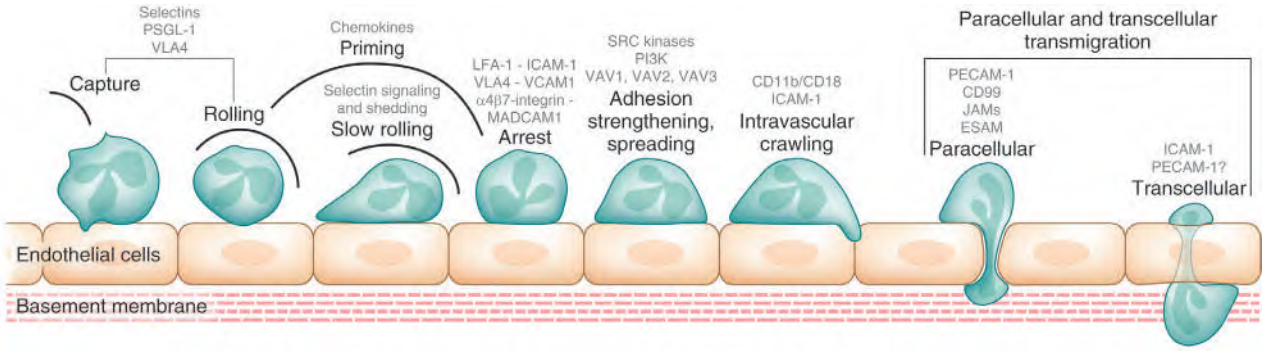

FIG. 8.3 Steps in the emigration of neutrophils from the vascular space. Neutrophils are depicted entering the marginating pool in a postcapillary venule, and the effector molecules participating in each event are indicated in gray text. Initial capture is mediated by interactions between neutrophil/leukocyte (L)-selectin and endothelial cell (E, P)-selectins and their respective carbohydrate ligands on the opposing cell surface (e.g., P-selectin glycoprotein ligand-1 *[PSGL-1]*), whereas integrins (e.g., very late antigen 4 *[VLA4]*) are responsible for firm adherence. Selectin-mediated signaling slows the rolling or tumbling neutrophils, partially countering the shear forces resulting from blood flow. Chemokines diffusing into the bloodstream from sites of microbial invasion in the tissues bind to specific receptors and activate the neutrophil, which is then arrested by integrin-dependent interactions. The activated neutrophil flattens against the endothelium and crawls along the luminal surface of the vessel wall. Platelet–endothelial cell adhesion molecule-1 *(PECAM-1)*, junctional adhesion molecules *(JAMs)*, and endothelial cell–selective adhesion molecule *(ESAM)* localized to interendothelial cell junctions interact with PECAM-1 and CD99 on the neutrophil surface to permit neutrophil migration between and through endothelial cells. Once in the tissues, the polarized neutrophils move up the mediator concentration gradient to reach the site of microbial invasion. See text for further details. *ICAM-1,* Intercellular adhesion molecule; *LFA-1,* leukocyte function–associated antigen; *MADCAM1,* mucosal addressin cell adhesion molecule 1; *PI(3)K,* phosphatidylinositol-3-phosphate kinase; *SRC,* sarcoma; *VAV1,* vav1 guanine nucleotide exchange factor; *VCAM1,* vascular cell adhesion molecule 1.

events are mediated in turn by four classes of adhesion proteins: selectins, integrins, immunoglobulin-like proteins, and mucin-like selectin ligands. In addition to neutrophils and endothelial cells, platelets figure prominently in the initiation of the inflammatory response, colocalizing with neutrophils and participating in P-selectin–dependent leukocyte binding.[92] The cooperation of several cell types and their secreted products culminates in events necessary to recruit circulating neutrophils to the site of inflammation, and the activation of autocrine and paracrine feedback loops modulates the extent of the host response.[91,92]

Selectin-Mediated Adhesion and Rolling

Through a process of repetitive ligand-receptor binding and release, marginating granulocytes become reversibly adherent to endothelial cells in the postcapillary venules and, under the influence of the physiologic shear forces of blood flow,[91,92] tumble or roll slowly along the vessel wall (see Fig. 8.3). The molecules mediating rolling adhesion are called *selectins,* to indicate that the amino-terminal lectin domain mediates their selective function and cellular expression. Homologous to C-type lectins, selectins require calcium for expression of binding activity. Individual members of the selectin protein family are named for the cell type on which they were originally identified (E, endothelia; L, lymphocytes; P, platelets). Selectins interact with sialylated Lewis X and A glycoproteins on the surface of interacting cells as well as sulfated and phosphorylated polysaccharides, such as heparin and mannose-6-phosphate. Individual selectins exhibit different but overlapping binding specificities, the bases for which largely remain to be determined.[96]

The presence of unique selectins on endothelial cells and neutrophils means that rolling adhesion can be modulated bidirectionally. For example, L-selectin is constitutively expressed on neutrophils and is shed after cell activation. In contrast, little or no E-selectin is present on resting endothelial cells, either in vitro or in vivo, but stimulation triggers inducible and transient E-selectin expression, which peaks within 4 hours after stimulation and dissipates over 24 hours.

β_2-Integrin–Mediated Cell-Cell Interaction

When neutrophils tumbling along the venule wall encounter inflammatory mediators and stimulated endothelial cells, adhesive interactions between the two cell types rapidly shift to a high-affinity state, reflecting activation of β_2-integrins.[91,92] The β_2-integrins, or leukocyte integrins, are members of a large family of heterodimeric molecules that mediate cell-cell and cell-matrix interactions.[91] The integrin protein family is subdivided on the basis of eight different β chains, any one of which can associate with multiple α chains to form a unique $\alpha\beta$ pair. Both the α and the β chains are transmembrane molecules with short cytoplasmic tails and large extracellular globular heads that interact to form the ligand-binding site. The three integrins on neutrophils express a common 95-kDa β_2 chain, CD18, but distinct α chains.[97] These molecular complexes are also referred to as leukocyte function–associated antigen-1 (LFA-1; $\alpha_L\beta_2$, CD11a/CD18), Mo-1 or Mac-1 ($\alpha_M\beta_2$, CD11b/CD18), and p150, 95 ($\alpha_X\beta_2$, CD11c/CD18).[91,97] CD11b/CD18 and CD11c/CD18 also function as receptors (CR3 and CR4, respectively) for the opsonic C3 fragments iC3b and C3d. Endothelial counterreceptors for the β_2-integrins include intercellular adhesion molecule-1 (ICAM-1) and ICAM-2. LFA-1 binds to both ICAM-1 and ICAM-2, whereas CD11b/CD18 and CD11c/CD18 bind only to ICAM-1 but at different sites from that for LFA-1. An additional LFA-1 counterreceptor, ICAM-3, is not present on endothelium but is expressed on all hematopoietic cells, where it may be involved in leukocyte-leukocyte interactions.[98,99] In addition to the β_2-integrins, neutrophils possess on their surface the leukocyte response integrin,[100] which, together with integrin-associated protein, modulates cellular responses, particularly responses induced by extracellular matrix proteins.[101–103] Although the precise details of the interactions of these various proteins are unknown, their importance is inferred from the observation that mice deficient in integrin-associated protein are unable to mount an inflammatory response after intraperitoneal challenge.[104]

To be functional, integrins require calcium, a specific membrane environment, and appropriate stimuli such as chemoattractant peptides, chemokines, or cytokines.[97] These stimuli appear to modulate integrin binding affinity by inducing conformational changes in the receptor, which, in turn, alter the interaction of the intracytoplasmic tails with each other and with the cytoskeleton.[97] Integrin activation results in increased surface number and avidity of β_2-integrins as well as clustering of receptors and cytoskeletal rearrangement. This conformational change–mediated process has been named "inside-out signaling."[91,97] By comparison, "outside-in signaling" refers to events that occur after ligand-induced integrin clustering and the change in receptor avidity (e.g., firm cell adherence and events leading to degranulation and neutrophil superoxide production).[91,97] Neutrophils change from spherical granulocytes with relatively little surface area involved in cell-cell contact to flattened, adherent cells with a broad surface area for cellular interactions (Fig. 8.4). Blood flow shear forces are no longer able to propel them forward along the vessel wall. This firm adhesion, the third step in transmigration, is mediated through interactions between β_2-integrins on neutrophils and ICAM-1 and ICAM-2 on endothelial cells.[91] Recent studies of neutrophil extravasation, using mice deficient in individual members of the β_2-integrin family, suggest that CD11a/CD18 may participate in neutrophil adhesion, whereas CD11b/CD18 supports crawling of neutrophils along the cells lining the vessel lumen.[105]

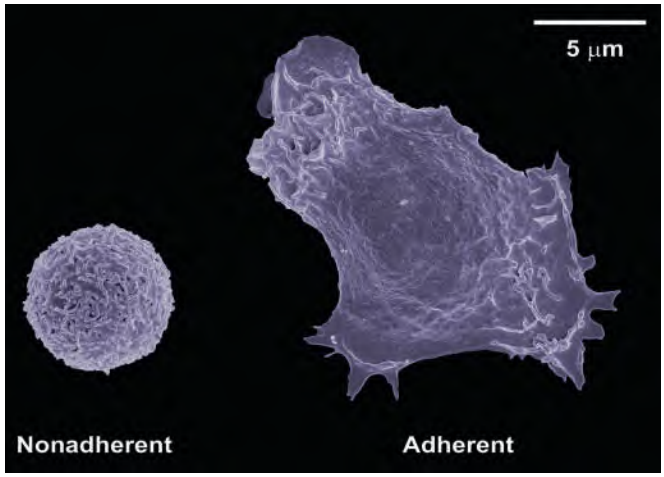

FIG. 8.4 Scanning electron micrographs of nonadherent and adherent neutrophils.

Tissue Migration

Transmigration, the final step in neutrophil immigration into tissue (see Fig. 8.3), involves multiple leukocyte and endothelial cell molecules, including platelet-endothelial cell adhesion molecule-1 (CD31), junctional adhesion molecules (e.g., JAM-A, JAM-B, JAM-C), endothelial cell selective adhesion molecule, ICAM-1 and ICAM-2, integrin-associated protein (CD47), CD11b/CD18, and CD99 (on neutrophils).[91,106] CD31 and CD47 are localized at the intercellular junctions between endothelial cells, whereas CD99 is expressed on neutrophils. JAMs are expressed on multiple cell types, including endothelial cells and neutrophils, and facilitate leukocyte adhesion to endothelial cells during transmigration.[107] Models reflecting current understanding dictate that transmigration typically occurs between endothelial cells, perhaps by neutrophil-dependent transient disorganization of adherens junctions at cell-cell interfaces.[91] However, there is accumulating evidence that neutrophil extravasation can occur by transcellular pathways.[91,108] Transcellular leukocyte movement involves formation of actin and vimentin-stabilized channels through which leukocytes migrate.[91]

Transmigration is rapid, occurring in less than 2 minutes after leukocyte-endothelial contact is established, and remarkably efficient in that neutrophils translocate without compromising the integrity of the endothelial monolayer. As neutrophils migrate through tissue, additional factors contribute to their ameboid movement. Complex signaling integrates actin-dependent cytoskeletal rearrangements, activity of small-molecular-weight guanosine triphosphate (GTP)ases, localized generation of specific phosphoinositides, and lipid mediators into complex feedback loops that promote responsive changes in cell shape coordinated in time and space.[92,109–115]

Although this paradigm applies to neutrophil extravasation from the vascular lumen into tissue, transmigration of neutrophils across epithelial barriers from tissue into visceral lumina, which is necessary in infections involving the gastrointestinal, genitourinary, and respiratory tracts, deviates in several ways from this theme.[116,117] Major insights into the mechanisms of neutrophil interaction with polarized epithelial cells have been derived from studies with human cell lines such as T84 and HT29.[118–120] Neutrophils can bind to either surface of these cells, and transmigration can occur in either direction. Evidence suggests that the mechanism for transmigration in each direction is distinct because immunomodulators such as lipoxin A_4[121] and interferon-γ (IFN-γ)[122] stimulate movement toward the basolateral surface but inhibit luminal migration. The interactions between epithelial cells and migrating neutrophils are remarkably coordinated, as evidenced by reciprocal secretion of adenosine and IL-6 by monolayers of T84 cells.[123] Furthermore, transmigration across intestinal epithelium triggers neutrophil release of elastase, which then disrupts the apical junctions in a very localized fashion, perhaps contributing to the loss of epithelial cell integrity during colitis.[124] Eventual characterization of regulated neutrophil movement across epithelium and the interactions between epithelial cells and migrating neutrophils will provide important insights into the pathophysiology of infectious diseases at such epithelial surfaces.

Production of Inflammatory Mediators

Neutrophil migration through tissue is likewise the consequence of a carefully regulated process involving the sequential release and compartmentalization of a wide variety of inflammatory mediators.[48,92] Early (0 to 5 hours) neutrophil influx into an area of induced injury appears predominantly to reflect the effects of IFN-γ, C5a, and LTB_4. IL-8 and IL-6 appear in a second wave of mediator activity (at 5 to 24 hours), and IL-1α, GM-CSF, and TNF-α appear in a third wave of activity (8 to 24 hours). Meanwhile, concentrations of IL-1, IL-2, and IL-4 remain unchanged. C5a, LTB_4, and IL-8 are potent neutrophil chemoattractants, as are hydroxyeicosatetraenoic acids and microbial oligopeptides analogous to *N*-formyl-methionyl-leucyl-phenylalanine (fMLF).

Tissue migration of neutrophils also reflects remarkable temporal regulation, well illustrated by the shift in the biosynthesis of lipid mediators during inflammation.[70] Proinflammatory molecules, such as leukotrienes and prostaglandins, are generated endogenously at inflammatory sites and stimulate neutrophil degranulation and chemotaxis. The arachidonate released by activated neutrophils is converted to LTB_4 by neutrophil 5'-lipoxygenase, whereas the arachidonate in the exudate likewise becomes a substrate for the 15'-lipoxygenase expressed by tissue macrophages recruited to the site. The latter reaction generates lipoxin A_4, which inhibits neutrophil activation in a receptor-dependent fashion and blocks inflammation. In addition, lipid-derived mediators known as resolvins and protectins, which are produced from eicosapentaenoic acid and docosahexaenoic acid, have strong antiinflammatory properties and participate in the resolution of acute inflammation (see later).[70] Taken together, the shift from the production of proinflammatory leukotrienes and prostaglandins early to antiinflammatory lipoxins, resolvins, and protectins late provides a mechanism for the sequential promotion of exudate formation, followed by resolution mediated by transcellular metabolism of lipid mediators generated in situ.[70]

Role of Chemokines in Neutrophil Recruitment

Among the soluble mediators that can recruit leukocytes, the chemokines represent a diverse and biologically important class of proteins. Chemokines are a family of structurally related, pluripotent proteins that trigger leukocyte activation, including adherence, chemotaxis, degranulation, and priming of the neutrophil oxidase; participate in angiogenesis; and figure prominently in the host response to infection.[3,48] Chemokines are classified into two major families, CXC and CC, distinguished by the presence or absence of an amino acid between the first two cysteines in the protein. Chemokines interact with specific receptors on target cells, although there is significant promiscuity and redundancy in the chemokine system, with some members exhibiting very limited ligand-receptor interaction and others binding to more than one receptor. All chemokines targeted for neutrophils are in the CXC family, including IL-8, which binds to the G protein–coupled receptors CXCR1 and CXCR2. Secreted by leukocytes, platelets, fibroblasts, epithelial cells, and activated endothelium, IL-8 triggers the full range of cellular responses in neutrophils, promoting cell migration, degranulation, priming of NADPH oxidase activity, and cell survival in tissue.

Chemotactic stimuli bind to high-affinity receptors on the leukocyte surface. Receptors for IL-8, fMLF, and C5a are members of a large family of proteins characterized by an external ligand-binding domain, seven membrane-spanning segments, and cytoplasmic regions that couple to G proteins.[125] In the presence of chemoattractant gradients across the cell as small as 0.1% to 1.0% (e.g., as the attractant diffuses from a focus of infection), ligand-linked receptors distribute asymmetrically and trigger the directed movement (chemotaxis) and net accumulation of neutrophils at sites of increasing concentrations of attractant. Chemoattractant signals effect cell movement by promoting changes in intracellular calcium, the polymerization state of actin, and a number of actin-binding and regulatory proteins of the cytoskeleton[126] as well as receptor-dependent interactions between leukocyte adherence molecules and the extracellular matrix.[48,92] Just as mystifying as the myriad signal transduction pathways that promote neutrophil movement is the complexity of how the migrating neutrophil deciphers and prioritizes competing signals that drive chemotaxis. For example, within the inflammatory site, the advancing neutrophil encounters host chemoattractants, such as IL-8 and LTB_4; bacterially derived factors, such as formylated peptides; and C5a, generated by the microbial activation of the complement cascade. An intracellular signaling hierarchy exists that favors neutrophil responses to targets derived from bacteria in preference to host chemokines.[127] The binding of chemoattractants to their receptors also primes neutrophils for the microbicidal response, which includes degranulation and the respiratory burst, although these responses generally require higher concentrations of the stimulus than chemotaxis does. For this reason, activation of these distal events is likely delayed until the cell reaches the infected tissue site.

Step 2: Phagocytosis

Phagocytosis is the intracellular uptake of particles greater than 0.5 μm by a mechanism independent of clathrin but dependent on the polymerization of actin.[128,129] After attachment to the cell surface (Fig. 8.5), the phagocytic particle is internalized, with subsequent phagosome maturation (described later) and eventual fusion with the intracellular granules to form a mature phagosome (analogous to a phagolysosome in macrophages) (Fig. 8.6; see Table 8.1).[130,131] Neutrophils may ingest some microorganisms in the absence of opsonins, as occurs with recognition of β-glucan on fungi by dectin-1 on the neutrophil surface.[132] However, most microorganisms must be opsonized for binding and ingestion by neutrophils to occur efficiently.

Opsonins

Specific IgG, complement, and mannose-binding lectin are the major opsonic factors promoting recognition and ingestion of most microorganisms by neutrophils, although mannose-binding lectin figures

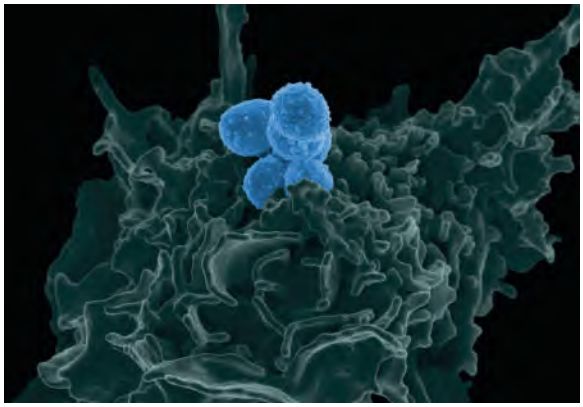

FIG. 8.5 Scanning electron micrograph of *Staphylococcus aureus* bound to the surface of a human neutrophil.

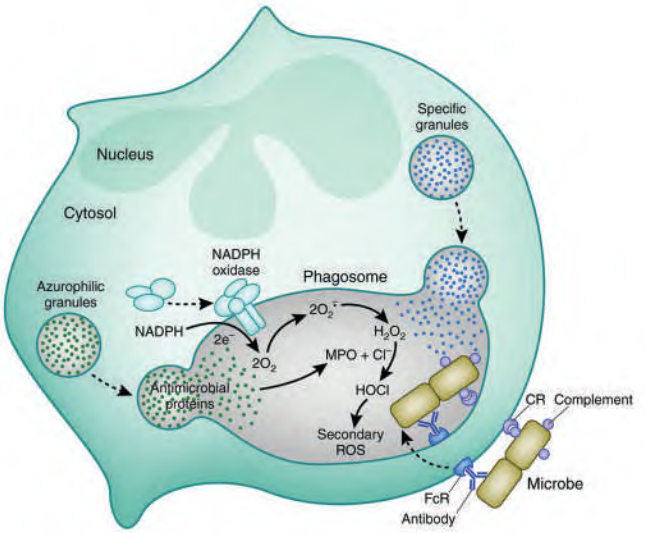

FIG. 8.6 Neutrophil phagocytosis and activation of microbicidal processes. See text for details. *CR,* Complement receptor; *FcR,* Fc receptor; *H_2O_2,* hydrogen peroxide; *HOCl,* hypochlorous acid; *MPO,* myeloperoxidase; *NADPH,* reduced nicotinamide adenine dinucleotide phosphate; *ROS,* reactive oxygen species.

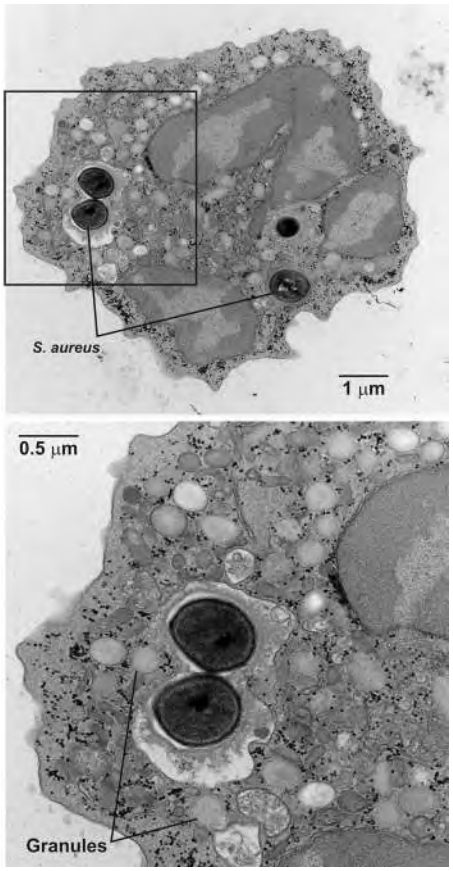

FIG. 8.7 Transmission electron micrograph of a neutrophil that has ingested *Staphylococcus aureus*. Bacteria are in phagocytic vacuoles formed by invagination of the external cell membrane. Degranulation into a phagocytic vacuole can be seen in the lower panel, which is a higher magnification image of the same neutrophil *(boxed area in top panel).*

predominantly in recognition of fungi rather than bacteria.[133] Antibody promotes phagocytic uptake by neutralizing antiphagocytic molecules on the bacterial surface, such as capsular polysaccharide; efficiently activating the classical pathway of complement, thereby promoting deposition of opsonic fragments of C3 on the bacterial surface; and mediating the interactions of organisms with the Fc receptor on the neutrophil membrane.[129] Activation of complement via either the classical or the alternative pathway leads to C3b and iC3b deposition on the microbial surface (see Chapter 9), and C1q deposition enhances Fc receptor–dependent ingestion.

Phagocytic Receptors

Distinct receptors for IgG (FcγRI through FcγRIII), but not other immunoglobulins, and for C3b (CR1) and iC3b (CR3) are present in the neutrophil membrane.[134,135] In addition to its iC3b-binding site, CR3 also bears a carbohydrate recognition domain that may bind to glycoproteins on the microbial surface. Data suggest that Fcγ receptors mediate phagocytosis via calcium-dependent pathways, whereas CR1 and CR3 use calcium-independent pathways.[136] FcγRII and FcγRIII are low-to-moderate affinity receptors that are constitutively expressed, whereas the high-affinity FcγRI is present only after cell stimulation (e.g., by IFN-γ).[137] Intracellular pools of receptors exist in the membranes of specific granules and secretory vesicles[14,130] and thereby provide a reservoir of functionally important membrane proteins that can be mobilized to the surface after exposure of the cell to a variety of inflammatory mediators. Such a structural organization affords neutrophils a mechanism to rapidly increase their capacity to recognize and respond to targets. In general, phagocytosis is most efficient when organisms are opsonized with both IgG and C3, thereby allowing cooperative interaction of the two types of receptors.

Ingestion

The sequential interaction between opsonic ligands distributed over the particle surface and their receptors on the phagocyte membrane culminates in ingestion of a surface-bound target (Fig. 8.7; see Fig. 8.6).[129,130] The sequential interaction of these opsonic ligands with their receptors in the phagocytic membrane initiates electrophysiologic changes in transmembrane potential, polymerization of actin microfilaments in the cytoplasm underlying the site of particle attachment, lipid signaling, and membrane remodeling, all of which result in circumferential flow of the cell membrane about the opsonized particle to create a phagosome (see Figs. 8.6 and 8.7).[129,138–140,141] The phagocytic capacity of neutrophils is remarkable: they can ingest more than nine yeast particles, each measuring 2 μm × 3 μm, despite having a diameter of only about 10 μm. They accomplish this impressive feat without generating new membrane or redistributing membrane from granules or endoplasmic reticulum, presumably by unfolding the membrane wrinkles that dominate the neutrophil surface (see Fig. 8.5).[142] The recruitment of membrane from the endoplasmic reticulum into nascent phagosomes, a potential mechanism in macrophages that has elicited significant controversy,[143–148] likely does not apply to neutrophils because of their relative paucity of endoplasmic reticulum.

Step 3: Fate of the Ingested Microbe

In addition to restricting access of ingested microbes to nutrient sources, internalization of organisms within phagosomes provides the host with an isolated compartment that can be made toxic both by the delivery of preformed cytotoxic agents and by the generation of reactive species de novo in the phagosome. Because of the small size of this specialized compartment (≈1.2 fL), extremely high concentrations of neutrophil-generated toxins are achieved, often in the micromolar range. Phagosome maturation, the development of phagosomes replete with a complete array of microbicidal agents, is a stepwise process whereby its contents and membrane composition are modified by sequential association with endosomal components.[37] Once internalization has begun, actin polymerization in the cytoplasm immediately adjacent to the nascent phagosome, actin-binding proteins are recruited to the periphagosomal space, and the phospholipid composition of the local membrane undergoes remodeling.[140,143,149–151] In an orderly fashion, cytosolic proteins associate with and disassociate from the phagosome sequentially, with eventual fusion of the phagosome with the neutrophil granules and generation of a mature phagosome. In

the mature phagosome, optimal microbicidal activity represents the coordinated generation of oxygen-derived species by activation of the NADPH-dependent oxidase and release of granule components.[152] Despite their shared lineage and overlapping functions, neutrophils and macrophages differ in many important ways, and the change in intraphagosomal pH that accompanies phagocytosis is a notable example. The intraphagosomal pH must be modified for optimal activation of some of the granule contents. To that end, activation recruits to the phagosomal membrane Na^+/H^+ exchangers, Na^+K^+-adenosine triphosphate (ATP) ases, and vacuolar-type proton ATPases from secretory vesicles and from primary and tertiary granules.[153,154] In contrast to the profound drop in phagosomal pH to 5.0 or less seen in macrophages, the phagosome in neutrophils is transiently alkaline (pH ≈ 7.8) and then drops to just below neutrality.[37,155–157,158,159] The consumption of protons by dismutation of superoxide anion ($O_2^{\bullet}$) to hydrogen peroxide (H_2O_2), the slowing of granule fusion, concurrent recruitment of vacuolar-type proton ATPases as degranulation proceeds, and altered phagosomal permeability to protons as reactive oxygen species are generated[157] all contribute to the blunted acidification of the neutrophil phagosome.[37]

Respiratory Burst

The oxidative or respiratory burst is mediated by a multicomponent enzyme complex in the plasma membrane and phagosomal membrane of stimulated neutrophils; oxidase activity is not present in resting, nonstimulated neutrophils.[160,161] An identical NADPH oxidase system exists in eosinophils, monocytes, and macrophages, with a hierarchy of relative activity of eosinophils > neutrophils > monocytes > macrophages. The phagocyte NADPH oxidase is a flavin adenine dinucleotide (FAD)–dependent electron transferase that shuttles electrons across the membrane, from cytosolic NADPH to molecular oxygen, to generate $O_2^{\bullet}$, the one-electron reduction product of oxygen, as the immediate product:

$$2O_2 + NADPH \rightarrow 2O_2^{\bullet} + H^+ + NADP^+ \qquad \text{(equation 1)}$$

The K_m for oxygen is low, ≈10 μM, thus supporting phagocyte oxidase activity at low oxygen tensions present in infected or damaged tissues.[162] The speed and capacity of electron transfer by the NADPH oxidase are remarkable; for example, more than 10^{10} electrons translocate within 5 minutes in response to fMLF.[163] Uncompensated, this magnitude of electron redistribution would depolarize the plasma or phagosomal membrane at greater than 200 mV within milliseconds and thereby terminate oxidase activity. However, the action of a voltage-gated proton channel compensates for more than 95% of the negative charge created by the translocation of electrons. Encoded by the gene *Hvn1,* the highly selective proton channel promotes the electrogenic activity of the phagocyte NADPH oxidase as well as calcium influx into and acid extrusion from cytoplasm, thereby protecting vulnerable systems from a low pH. Phagocytes from mice in which *Hvn1* is deleted exhibit reduced NADPH oxidase activity, defective antimicrobial action, and excess cytoplasmic acidification.[158]

Reactive Oxygen Species

Most of the superoxide formed readily undergoes dismutation to H_2O_2 and oxygen:

$$2O_2^{\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(equation 2)}$$

In the neutrophil phagosome, superoxide is converted quantitatively to H_2O_2.[164] This reaction occurs spontaneously with rapid kinetics at acidic pH, where a significant portion of the superoxide exists in its protonated form, the perhydroxy radical ($HO_2^{\bullet}$), because of the high rate constant for the reaction between $O_2^{\bullet}$ and the perhydroxy radical. MPO can act as a superoxide dismutase (SOD) by reacting with $O_2^{\bullet}$ to form compound III as an intermediate.[165] Although dismutation can be catalyzed by SOD, SOD is a cytoplasmic enzyme and absent from phagosomes. Because 1 mole of oxygen is regenerated for each mole of H_2O_2 formed, there is a net 1:1 stoichiometry between oxygen consumption and H_2O_2 formation, but a 2:1 relationship between $O_2^{\bullet}$ and H_2O_2 (Eqs. 1 and 2). Although the final product of the NADPH oxidase is H_2O_2, up to 72% of oxygen consumed by stimulated normal neutrophils can be recovered as hypochlorous acid (HOCl, or bleach), a consequence of the reactions of H_2O_2, MPO, and chloride (see later).[166] Although murine phagocytes generate nitric oxide (NO) that contributes to killing of microbes and tumors,[167] human neutrophils appear much less capable of generating reactive nitrogen species.[168,169] There is no nitration of fluorescein, a susceptible target, nor evidence for interactions between NO and $O_2^{\bullet}$ in phagosomes of human neutrophils.[170] Although it is possible that investigators have not applied the optimal in vitro conditions to support NO production by human neutrophils, the absence of agonist-dependent NO production may be another of the many differences between human and murine phagocytes.[171–173] To protect cytoplasmic contents from oxidative damage mediated by the reactive products of the NADPH oxidase that inadvertently leak from phagosomes, cytoplasmic SOD consumes $O_2^{\bullet}$, whereas both catalase and glutathione peroxidase catabolize H_2O_2, the latter in a fashion dependent on reduced glutathione. Taurine, present at a very high concentration (19 mM)[174] in neutrophils, may provide a sink for escaped HOCl, although it is also plausible that the product, taurine monochloramine, may modify susceptible targets in the cytoplasm. NADPH provides the reducing equivalents for the glutathione reductase–catalyzed regeneration of glutathione from glutathione disulfide. Consequently, NADPH levels must be maintained to support both the superoxide-forming oxidase and the glutathione cycle. The reduced pyridine nucleotide is regenerated by the activity of the hexose monophosphate shunt, which is enhanced 15-fold to 30-fold during phagocytosis.

NADPH Oxidase (Respiratory Burst Oxidase)

Dormant in resting neutrophils, the respiratory burst oxidase assembles and becomes active concomitant with cell stimulation, as during phagocytosis. The lag period between stimulus exposure and expression of NADPH oxidase activity varies from several seconds to a few minutes, depending on the agonist, and reflects the time required for assembly of the multiple components of the oxidase at the cytoplasmic face of the plasma membrane or phagosomal membrane. On the basis of studies both in intact neutrophils and from in vitro cell-free systems, the components of the NADPH oxidase include integral membrane proteins as well as soluble cytosolic proteins (Fig. 8.8).

Four proteins have been demonstrated to be essential in all settings for a functional phagocyte NADPH oxidase, with a fifth required for stable assembly on phagosomes.[160,161,175] Within the plasma membrane and membranes of specific granules and secretory vesicles is flavocytochrome b_{558}, so designated because of a characteristic 558-nm peak in its redox difference spectrum.[176–178] It is a heterodimer composed of large and small subunits, gp91*phox* and p22*phox* (where *phox* stands for *ph*agocyte *ox*idase), respectively, which are firmly but noncovalently associated with each other. In addition, the low-molecular-weight protein Rap1A frequently copurifies with flavocytochrome b_{558}, although the functional significance is unknown.[179] Flavocytochrome b_{558} contains two different types of redox centers, a FAD-binding domain and two inequivalent heme prosthetic groups,[180–182] and is the catalytic subunit of the oxidase, operating as an electron transferase. NADPH, the source of electrons driving the system, binds to a cytoplasmic domain in gp91*phox* and is oxidized by the transfer of two electrons to FAD, followed by two single-electron reductions of the heme groups ($Fe^{3+} \rightarrow Fe^{2+}$). The very low midpoint potential of the reduced hemes allows them to react directly with molecular oxygen, reoxidizing the iron moieties and forming two molecules of superoxide, $O_2^{\bullet}$. Because flavocytochrome b_{558} spans the membrane, electrons from the oxidation of cytoplasmic NADPH are shuttled to molecular oxygen outside the cell or inside the phagosome (in both cases, sites separated from cytoplasm by a membrane). Although flavocytochrome b_{558} operates as an electron transferase and mediates the catalytic functions of the respiratory burst oxidase, elements in two protein complexes that reside independently in the cytoplasm of resting neutrophils are required for enzyme activation and activity in vivo. One complex includes p47*phox,* p67*phox,* and p40*phox.*[183–192] p47*phox* possesses a very cationic carboxyl-terminal domain containing several serine residues that serve as substrates for agonist-dependent phosphorylation by various kinases, including protein kinase C, p21-activated kinase, and mitogen-activated protein kinases—extracellular signal-regulated kinase 1/2 and AKT, the individual kinases involved dictated by the specific agonist.[193] In the stimulated neutrophil,

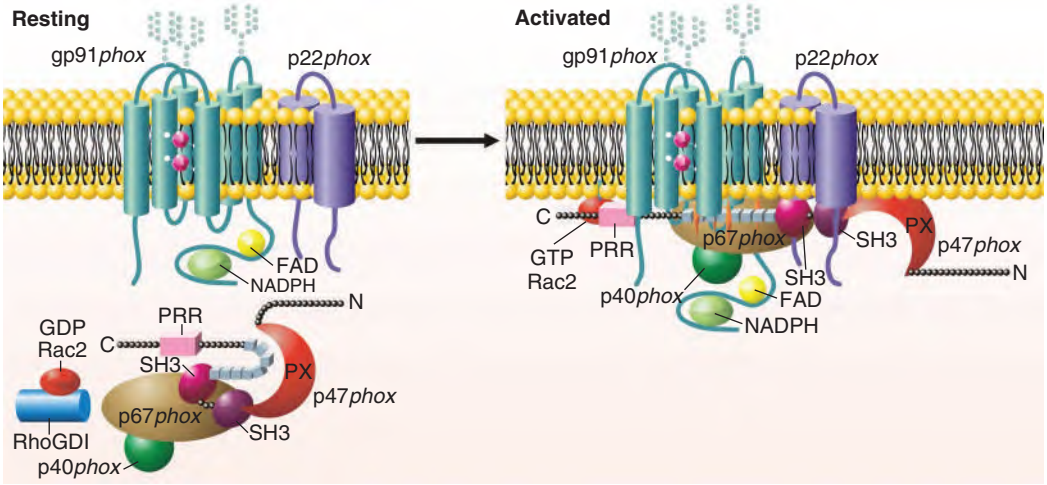

FIG. 8.8 Model of the agonist-dependent assembly of the neutrophil respiratory burst oxidase. Control of the activity of the reduced nicotinamide adenine dinucleotide phosphate *(NADPH)* oxidase reflects the spatial segregation of essential elements into two compartments of the cell. In resting neutrophils, flavocytochrome b_{558}, composed of gp91*phox* and p22*phox,* rests in the plasma membrane and membranes of secretory vesicles and specific granules. In contrast, inactive Rac2 bound to (Rho) guanosine diphosphate dissociation inhibitor *(RhoGDI)* as well as a complex of p47*phox,* p67*phox,* and p40*phox* resides in the cytoplasm. The latter complex depends on intermolecular interactions mediated by Src homology 3 domains *(SH3)* and proline-rich regions *(PRR)*. When the neutrophil is stimulated, several signal transduction events occur, including the phosphorylation of RhoGDI, which releases Rac2, with subsequent conversion from the guanosine diphosphate *(GDP)*–bound to the guanosine triphosphate *(GTP)*–bound state and association with the membrane. Concurrently, there is phosphorylation of multiple sites in the autoinhibitory region of p47*phox* (chain of small, light blue boxes). The latter event triggers a conformational change in p47*phox* that exposes otherwise cryptic sites on p47*phox* and p67*phox,* which subsequently support assembly of the oxidase at the membrane. *FAD,* Flavin adenine dinucleotide.

the partially phosphorylated protein translocates to the plasma membrane, where additional sites are phosphorylated.[194–198] As a consequence of conformational changes triggered by its phosphorylation, domains of p47*phox* that mediate interactions with specific phospholipids or cognate protein domains are exposed, thereby enabling association with flavocytochrome b_{558} at the plasma or phagosomal membrane to form the functional oxidase.[193,199–206]

Translocation of Cytosolic Components

Docking of cytoplasmic oxidase components at the plasma or phagosomal membrane depends on both protein-protein and protein-lipid interactions,[207] and phospholipid remodeling of neutrophil plasma membrane during phagocytosis serves as a critical step in oxidase organization.[208,209] Specific sites of gp91*phox* and p22*phox* have been implicated as mediating interactions with the cytosolic components.[175,210–217] p47*phox,* which lacks inherent enzymatic activity, serves as a docking platform or adaptor protein that organizes the functional oxidase at target membranes. Neutrophil stimulation also results in membrane translocation of p67*phox,* probably as a result of its association with p47*phox*; p67*phox* fails to translocate in the absence of p47*phox,* although p47*phox* is competent for translocation by itself.[197] p67*phox* possesses a domain that regulates NADPH reduction of FAD[218] and thus serves as an essential cofactor for the phagocyte oxidase. Enzymatic activity of p67*phox* requires its binding of Rac, a GTPase of the Rho protein family, to a domain in its N-terminus.[219] Both p47*phox* and p67*phox* contain two copies of a 50–amino acid domain related to a region of the Src oncoprotein, Src homology region 3 (SH3).[185,187] Proline-rich motifs similar to those that bind to SH3 domains in other proteins[220] are present in p47*phox,* p67*phox,* and p22*phox*. In general, SH3 domains are thought to mediate binding of proteins, expressing them to cytoskeletal and membrane elements. Both SH3 and proline-rich segments of the oxidase proteins are involved in the protein-protein interactions required for oxidase activation and function.[221,222] The net effect of phosphorylation and translocation of cytosolic proteins is the assembly on the membrane of the active oxidase complex, composed of the flavocytochrome b_{558} subunits, p47*phox,* p67*phox,* Rac2, p40*phox,* and perhaps other components.[179,223–228]

p40*phox*

p40*phox,* the third component of the complex with p47*phox* and p67*phox,* exhibits homology to p47*phox,*[190,229,230] including the presence of a PX or phox homology domain that associates with phosphoinositides in target membranes.[231–234,235,236] The PX domain of p40*phox* binds to phosphatidylinositol-3-phosphate (PI(3)P) that is generated on the cytoplasmic leaflet of phagosomal membranes by the action of class III PI(3)P kinases.[236–241] Defective oxidase activity in and oxidant-dependent killing by neutrophils from mice lacking p40*phox* suggested that p40*phox* played a specialized role in directing assembly of a functional oxidase.[233] However, the identification of a patient who lacks normal p40*phox* protein and whose neutrophils fail to generate oxidants in phagosomes or kill ingested *Staphylococcus aureus* normally demonstrated that class III PI(3)P kinase–mediated PI(3)P generation is essential for p40*phox* binding to phagosomes, a step critical to sustained oxidant generation and optimal microbicidal action in that compartment.[242] Disruption of the PI(3)P binding region by a single mutation (R105Q) in the PX domain of p40*phox* undermines stable oxidase assembly on phagosomes, although neutrophils have nearly normal oxidant production at the cell surface. Of note, the patient did not display signs and symptoms typical of chronic granulomatous disease (see Chapter 12) but rather presented with refractory inflammatory bowel disease. Genome-wide association studies have linked Crohn disease with *NCF4,* the gene encoding p40*phox,*[243,244] so elucidation of this association promises to provide new insights into links between innate immunity and inflammatory bowel disease.

Rac2

In addition to the ternary complex of p47*phox*-p67*phox*-p40*phox,* the low-molecular-weight GTP-binding protein Rac2 exists in its guanosine diphosphate–bound state coupled with the guanosine diphosphate dissociation inhibitor RhoGDI in the cytoplasm. Activation-dependent phosphorylation of RhoGDI results in a change in its conformation and the release of Rac2 from the complex, permitting Rac2GTP to bind to the target membrane and interact there with the flavocytochrome b_{558} and p67*phox*.[245–251] During neutrophil stimulation, Rac translocates to the plasma membrane, independent of the redistribution of p47*phox* and p67*phox*.[245–247]

In contrast to the major advances made in elucidating the biochemistry and cell biology of oxidase activation, relatively little is known about how activity of the NADPH oxidase is terminated. The cytoplasmic NADPH concentration is one limiting factor in the activity of the phagocyte oxidase,[252] and indirect evidence suggests that reactive products

of the oxidase, particularly those formed via the catalytic action of MPO, inactivate the enzyme,[253] but the precise mechanisms by which oxidase activity is regulated and terminated are not known.

Nonphagocyte Oxidases: The NOX Protein Family

The NADPH oxidase was thought to be an oxidant-generating system uniquely expressed by phagocytes and dedicated exclusively to antimicrobial action until the Lambeth group[254] cloned a homologue of gp91*phox* from a library of human colon complementary DNA; with that discovery, the NADPH oxidase (NOX) protein family was born.[161,238–251,252,253,254,255,256,257,258] The NOX family includes NOX1 through NOX2 (gp91*phox* is also known as NOX2) and two dual oxidases (DUOX1 and DUOX2), which are widely expressed throughout the plant and animal kingdoms.[256,259] Not only do most cells express a NOX protein, but many also possess multiple isoforms, each in a different subcellular compartment. Functions served by NOX proteins are as diverse as their tissue distribution is wide. In the context of host defense against infection, epithelial cells in the respiratory and gastrointestinal tracts express DUOX proteins, whose oxidant production mediates antimicrobial action, both directly against microbes and indirectly by disrupting microbe-driven signaling.[260–262] Furthermore, NOX1-dependent oxidants have been linked to epithelial cell repair of mucosal injury that accompanies inflammation.[263] The extent to which NOX-generated oxidant systems in epithelial cells contributes to mucosal immunity in humans awaits further delineation.

Degranulation

In parallel with activation of the respiratory burst oxidase, stimulated neutrophils release their granule contents either to the extracellular space or into the nascent phagosome (see Figs. 8.6 and 8.7).[264,265] Stimulated exocytosis, or degranulation, by neutrophils is a remarkable process, given that there are at least four distinct classes of membrane-bound vesicles (i.e., azurophilic granules, specific granules, gelatinase granules, and secretory vesicles) that are released and two potential target destinations for fusion events (i.e., plasma and phagosomal membrane). There is a hierarchy among these compartments with respect to the order in which each releases its contents after exposure to an agonist (i.e., secretory vesicles, tertiary granules, secondary granules, primary granules) and to the calcium requirements for exocytosis.[266,267]

Rearrangement of the actin-based cytoskeleton is a prerequisite for the release of all granule subtypes, with different processes occurring in specific sites in the cell for each granule type.[268] In the case of primary granules, there is a decrease in actin polymerization at the membrane, likely reflecting reorganization of the subplasmalemmal membrane cytoskeleton[38,269] and access to target membrane for granule membrane fusion.[270] Concurrently, actin polymerization occurs in the cytoplasm and promotes primary granule release.[270] The actin reorganization that accompanies primary granule release is regulated by Rac2, the same GTPase that is essential for activity of the NADPH oxidase (see earlier discussion).[270,271,272,273] Human neutrophils possess other low-molecular-weight GTPases, including members of the Rab family,[274] and Rab27a has been implicated in contributing to degranulation.[275,276] There is evidence that the Rab effector protein JFC/Slp1 and Munc 13-4 participate in regulation of exocytosis.[276,277]

As part of the cytoskeletal rearrangement that accompanies degranulation,[278,279] several actin-binding proteins are redistributed, including myristoylated alanine-rich C-kinase substrates (MARCKS).[280] A MARCKS-related peptide inhibits MPO release from stimulated neutrophils, suggesting that the N-terminal region of MARCKS contributes to a critical step in granule release.[281] Fusogenic proteins on the granules, including annexins,[282] synaptosomal-associated protein (SNAP)-23, syntaxin-4 and syntaxin-6, vesicle-associated membrane protein (VAMP)-1, VAMP -2, and VAMP-7, and likely other molecules, mediate association at the target membranes with specific receptors, including various target SNAP receptors (t-SNAREs).[283] Lipid modification probably contributes critically to fusion of the granule membrane with the plasma or phagosomal membrane, and such remodeling depends on the redistribution and activation of phospholipase D.[284,285] In addition, the activity of signaling proteins such as MEG2 requires specific membrane phosphoinositides generated locally.[286]

As discussed previously, the granules of neutrophils differ not only in the luminal contents but also in the proteins inserted in their membranes. Surface expressions of CD63, CD66, and CD35 are monitored experimentally as specific markers of plasma membrane fusion with primary granules, secondary granules, and secretory vesicles.[14] Primary, or azurophilic, granules fuse predominantly with the phagosome, thereby delivering microbicidal and hydrolytic proteins at high concentrations close to the ingested organisms. Specific granules and secretory vesicles fuse preferentially with the plasma membrane, releasing their contents extracellularly and bringing to the cell surface a variety of functionally important membrane proteins, including integrins, flavocytochrome b_{558}, and receptors for chemotactic agents and opsonins (see Table 8.1). Taken together, the process of degranulation affords concurrent recruitment of critical membrane proteins to the cell surface or phagosome and discharge of proteins that directly or indirectly contribute to the death and destruction of ingested microbes.

Step 4: Resolution of the Inflammatory Response

Briefly, resolution of inflammation occurs in a remarkably complicated yet orderly manner: locally produced CXC chemokines that elicit neutrophil migration switch to CC chemokines that enlist nonneutrophil leukocytes to migrate[287–289]; recruited neutrophils spontaneously, or in response to agonists (e.g., late after phagocytosis), undergo cell death; and macrophages and dendritic cells, both resident and recruited, ingest the apoptotic neutrophils in a nonphlogistic (noninflammatory) fashion and contribute to wound healing and reestablishment of the normal state. Ingestion of apoptotic neutrophils by macrophages or dendritic cells or both, known as efferocytosis, and subsequent removal from the inflammatory site are important for resolution of the acute inflammatory response.[53]

Apoptosis

Neutrophils have a relatively short half-life, approximately 6.5 hours, in the circulation,[290,291] and they live an additional 1 to 2 days after migrating into tissue, with senescent cells undergoing spontaneous apoptosis before being cleared by tissue macrophages.[53,292–295] Apoptotic neutrophils have markedly depressed functional capacity,[296–299] thereby preparing them for removal by tissue macrophages.[294] Collectively, the loss of proinflammatory potential in apoptotic neutrophils, their physical removal by macrophages, and the noninflammatory phenotype of macrophages that ingest apoptotic neutrophils[300] result in cell turnover in the inflammatory site that provokes little tissue damage. Nonsenescent neutrophils can become apoptotic in response to a variety of soluble agents[90,297,301–303] and after phagocytosis of bacteria.[304,305] The mechanisms underlying apoptosis in human neutrophils are complex; differ as a function of the agonist and cytokine context; and are, in some cases, modulated by pathogens.[306,307]

Neutrophils express members of the Bcl-2 family of apoptotic proteins, notably the proapoptotic proteins Bax, Bid, Bak, and Bad, but not the antiapoptotic Bcl-2.[307–309] Neutrophil apoptosis is caspase mediated, reflecting the involvement of mitochondria and complex crosstalk among several signaling pathways, including the caspases, reactive oxygen species, and mitogen-activated protein kinases.[307,310–312] Several molecules on the neutrophil surface have been implicated as participants in receptor-mediated uptake of apoptotic neutrophils by tissue macrophages, including phosphatidylserine exposed from the inner leaflet of the plasma membrane, CD47, CD31, calreticulin, proteinase 3, altered membrane carbohydrates, and oxidized membrane phospholipids.[53] Likewise, a variety of macrophage receptors have been proposed to mediate uptake, including a phosphatidylserine receptor, scavenger receptors, CD14, CD44, and the coordinated activity of CD36 and the integrin $\alpha_v\beta_3$.[53]

Proinflammatory Molecules Alter Apoptosis

Within the context of the acute inflammatory response, cytokines released at the site delay apoptosis, thereby extending the life span of neutrophils and permitting their participation in host defense.[90] However, the regulation of neutrophil apoptosis by cytokines such as TNF-α is complex; for instance, low concentrations of TNF-α delay neutrophil apoptosis, whereas high concentrations promote it.[303,313] Cytokines modulate proapoptotic and antiapoptotic pathways in a variety of ways, and

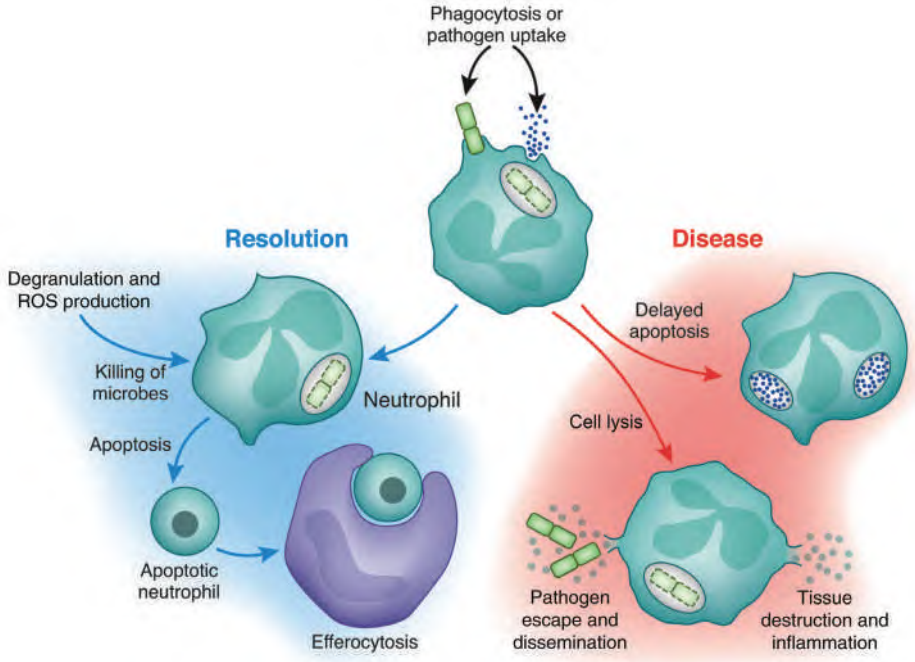

FIG. 8.9 Potential outcomes of the interaction of microbes with neutrophils. See text for details. *ROS,* Reactive oxygen species.

phagocytosis by neutrophils typically accelerates apoptosis—a phenomenon also known as phagocytosis-induced cell death, with an associated downregulation of their proinflammatory capacity (Fig. 8.9).[90,298,306,314,315] More recent studies also provide strong evidence that levels of neutrophils in circulation follow circadian cycles, and neutrophil clearance generates signals that modulate hematopoiesis.

Thus, the stimulated neutrophil at the peak of its response to an invading microbe initiates pathways leading to decreased proinflammatory activity and to apoptosis, en route to resolution of the acute phase of the inflammatory response.[299,306,316–322] Evidence supporting this interpretation comes from a consideration of the local consequences when accelerated apoptosis is not triggered by neutrophil stimulation. For example, neutrophils from individuals with chronic granulomatous disease (see "Neutrophils and Microbial Evasion of Host Defense")[316,323] exhibit delayed phagocytosis-induced apoptosis and produce less prostaglandin D_2, an antiinflammatory mediator, two deficiencies that may contribute to the chronic inflammation and granuloma formation that are hallmarks of this disease.

Microbial Pathogens Influence Neutrophil Fate

As a mode of cell death, apoptosis of neutrophils spares the host local tissue damage that might arise from necrosis and the attendant release of cytotoxic cellular contents. This aspect of neutrophil biology has been the focus of intense research over the past 15 years, and understanding of the process has progressed rapidly. Indeed, more recent studies on the cell biology of death and its evolving definitions based on morphologic, enzymatic, functional, and immunologic criteria[324] promise to provide important new insights into the overall economy of neutrophil homeostasis in the absence and presence of infection and other proinflammatory stimuli.

Inasmuch as neutrophil apoptosis is critical for the resolution of the inflammatory response, it is perhaps not surprising that some pathogens can alter this process to survive and thereby cause disease (see Fig. 8.9).[304,305] For example, *Anaplasma phagocytophilum,* the causative agent of human granulocytic anaplasmosis,[325] is internalized by receptor-mediated endocytosis rather than phagocytosis[326,327] and inhibits neutrophil apoptosis.[328] *A. phagocytophilum* is one of the few microbes that can replicate within neutrophils, and the ability of this pathogen to delay neutrophil apoptosis is important for its intracellular replication and ultimately for the organism to cause human infection.

Neutrophils as "Trojan Horses"

Apoptotic neutrophils may also serve as "Trojan horses," providing a vehicle for viable parasites, such as *Leishmania major,* to gain entry into recruited macrophages.[329] How widespread this phenomenon is and the extent to which it promotes infections are not known. *S. aureus* may use a variation of the Trojan horse phenomenon, in which neutrophils themselves serve as a vehicle to disseminate the pathogen.[330–332] Although *S. aureus* is ingested readily by neutrophils, some strains have significant capacity to survive after phagocytosis and eventually cause neutrophil lysis (see Fig. 8.9).[333–336] Lysis of *S. aureus*–containing neutrophils liberates previously ingested bacteria (which may then disseminate) and releases cytotoxic contents that can potentially cause undesired host tissue damage. The mechanism for neutrophil lysis after phagocytosis of *S. aureus* remains incompletely characterized, but neutrophils have some morphologic characteristics of apoptosis before lysis.[337]

Bioactive Lipid Mediators

Studies within the past 10 years have identified another family of biologic agents that contribute to the active resolution of the inflammatory response. Bioactive lipids, such as resolvins, protectins, and maresins derived from leukocytes at an inflammatory site, dampen the inflammatory reaction and promote its resolution.[69,70,338] Seen collectively, endogenous factors derived from cells recruited to the site of inflammation orchestrate the orderly recruitment of different types of inflammatory cells and their subsequent clearance. Under optimal circumstances, these endogenously generated lipids direct a controlled response that eliminates the infectious threat with minimal collateral injury and promptly restores the preinfectious healthy state.

Microbicidal Mechanisms

Intraphagosomal Killing of Microbes

Postphagocytic events deliver the products of degranulation and the respiratory burst to the phagocytic vacuole, a membrane-bound compartment in which an ingested microbe is exposed to high concentrations of toxic substances (see Fig. 8.6). Components of the antimicrobial response of neutrophils are often classified by their dependence, or lack thereof, on the products of the respiratory burst. Elegant studies defining the actions of these cytotoxic agents generally focus on their behavior in isolation or with only a subset of the phagosomal contents that would otherwise be present in vivo. However, intraphagosomal

events are much more dynamic and complex.[152,165,339,340] Reactive species present in the phagosome at any point in time act simultaneously on microbial targets and, in many cases, interact with each other as well. Oxidant-mediated modifications of surface structures on ingested bacteria may alter the susceptibility of bacterial targets to granule proteins, or granule protein interactions may change access or susceptibility of sites to oxidant attack. Individual granule proteins can synergize with each other or oxidants or both to exact damage not possible with any single agent. Some direct interactions among neutrophil products will benefit the host, as when the granule protein MPO catalyzes generation of HOCl by using NADPH oxidase–derived H_2O_2 as substrate.[166] Other interactions may be counterproductive, as when the MPO-generated HOCl oxidatively inactivates elastase[341] or cathepsin G.[342] Furthermore, antimicrobial collaborations in the phagosome are not limited to agents derived from neutrophils but include soluble factors internalized from the extracellular environs during phagocytosis. For example, group IIA phospholipase A_2, an acute-phase plasma protein with antimicrobial activity against gram-positive bacteria,[343–345] synergizes, at concentrations that alone are too low to be active, with products of the NADPH oxidase to exhibit potent antimicrobial and phospholipase activity within neutrophil phagosomes.[345] Adding to the complexity of intraphagosomal antimicrobial events is the inherent variability among ingested organisms. The microbial surface structure and composition vary among different organisms and within individual species differ with respect to growth phase. Consequently, surface components that are the immediate substrates for antimicrobial toxins will vary widely among and within species. Furthermore, phagocytosed microbes are not passive prey. The transcriptional responses of ingested organisms to the toxic phagosomal environment initiate microbial stress responses, structural modifications of the microbial surface, and release of secreted products, some of which serve as competing targets for host toxic agents. Overall, ingested organisms respond rapidly to intraphagosomal stress and thereby alter the array of potential targets for neutrophil attack. Given the combined number and complexity of host and microbial variables, it should not be surprising that no single neutrophil product, biochemical modification, or specific microbial target has been identified as being uniformly essential for the death and degradation of all types of organisms sequestered within the phagosome. The same collaborative and integrated approach that provides a flexible and generally effective host defense system also keeps understanding of the underlying mechanisms at bay. Nonetheless, with these challenges and limitations in mind, studies using a reductionist approach have provided substantial insights into the complexities of antimicrobial action within phagosomes.

Oxygen-Dependent Killing of Microbes

Optimal antimicrobial action against most microorganisms depends on the MPO-H_2O_2-chloride system.[166] First described by Klebanoff,[346,347] the system has three essential components uniquely present in neutrophils; in the presence of H_2O_2 (generated by the respiratory burst), MPO (released from primary granules) oxidizes chloride to generate the potent microbicide HOCl. MPO is unique among peroxidases in its capacity to oxidize chloride anion at physiologic pH (reviewed in Nauseef[348]). The resultant chemistry demands a steady flux of chloride ion into the phagosome. The relatively high chloride concentration in neutrophil cytoplasm provides the source for chloride, redistributed to phagosomes through membrane transporters, including cystic fibrosis transmembrane conductance regulator, to support HOCl generation and efficient antimicrobial action.[349–353] Although H_2O_2 itself has bactericidal properties, its microbicidal potency is enhanced approximately 50-fold in the presence of MPO. In the presence of suitable substrates, HOCl can generate chloramines, chlorine gas, chlorinated sterols, tyrosyl radicals, and nitric oxide–derived reactants that can extend the spectrum and duration of antimicrobial action.[166,339,354–356] However, reactivity of products of the MPO system lacks selectivity, and chlorinated bacterial and host proteins can be recovered from the phagosome of human neutrophils after microbial challenge.[357–359] However, the killing of ingested microbes correlates with the extent of chlorotyrosine generation on bacteria,[358] thereby linking the MPO-dependent modifications to antimicrobial action. The precise events responsible for the microbicidal activity of the MPO system are incompletely characterized[339]; candidate target sites in bacteria include components of the electron transport chain, iron-sulfur centers, penicillin-binding proteins, and sites in bacterial membranes that are necessary for initiation of chromosomal replication[360–363] as well as the generalized stress secondary to HOCl-mediated oxidative protein unfolding.[364] Oxidation of particular microbial targets may lead to the release of free iron, which can then participate in the formation of the highly reactive hydroxyl radical ($OH^{\bullet}$) and augmentation of the oxidant attack.[365] Studies have implicated the MPO-H_2O_2-halide system in tissue injury, in oxidation of lipids, and possibly in atherogenesis, extending the biologic importance of this system beyond its microbicidal activity.[366–369]

Metabolites of oxygen for which a role in neutrophil bactericidal activity has been suggested include H_2O_2, $O_2^{\bullet-}$, singlet oxygen, and hydroxyl radical. The fact that catalase, which degrades H_2O_2 into O_2 and H_2O, protects some bacteria from the bactericidal effects of neutrophils[370] supports a direct germicidal effect of H_2O_2. However, the permeability of the phagosomal membrane to H_2O_2 makes it unlikely that sufficient H_2O_2 accumulates within the lumen to support bacterial killing.[340] Superoxide by itself is thought to play little role in the killing of microorganisms but under appropriate conditions can react with other products of oxygen metabolism to generate hydroxyl radical and singlet oxygen. Evidence suggests that $O_2^{\bullet-}$ in the phagosome reacts directly with MPO in a catalytic cycle in which superoxide acts as a reductant and culminates in dismutation of superoxide to H_2O_2.[371] The bactericidal effect of these reactive oxidants may result from the initiation of a chain of oxidizing events in the bacterial cell wall.[339,372] Damage proceeds from outside to the inside of the target bacteria, with dysfunction or inactivation of critical functions localized to the bacterial inner membrane.[339] For example, the death of *Escherichia coli* parallels the oxidation of methionine residues in the inner membrane and cytoplasm but not in the outer membrane or periplasm of bacteria fed to human neutrophils.[373]

The hydroxyl radical is a potent bactericidal agent that can be formed by the direct reaction of superoxide with H_2O_2, a reaction that occurs slowly on its own but is catalyzed by the ferric ion.[374] Sensitive analytic systems indicate that activated neutrophils produce hydroxyl radicals by two different mechanisms, one dependent on the catalytic activity of MPO[375] and the other requiring transition metals in the Haber-Weiss reaction.[376] The relative contribution of each mechanism to the overall production of hydroxyl radicals in vivo depends on the availability of exogenous transition metals, usually iron. In the presence of supplemental iron, hydroxyl radical generation occurs through the Haber-Weiss reaction. However, lactoferrin and transferrin can interfere with this reaction by binding iron in a noncatalytic form. Therefore, under physiologic conditions, it appears that the small amounts of hydroxyl radical generated by stimulated neutrophils are derived from the MPO-dependent pathway.

Oxygen-Independent Killing of Microbes

Antimicrobial systems that operate in the absence of exogenous oxidants contribute greatly to overall innate immunity and neutrophil-dependent host defense,[377–379] as clearly demonstrated by the ability of neutrophils to kill certain organisms under anaerobic conditions, where the NADPH oxidase would not be functional. Agents contributing to oxygen-independent microbicidal activity include defensins, BPI, lactoferrin, lysozyme, peptidoglycan recognition proteins (PGRP), neutrophil gelatinase–associated lipocalin (NGAL), cathelicidins, and defensins. In many cases, these are highly charged cationic proteins that bind to negatively charged prokaryotic cell envelopes, thereby compromising the capacity of microbes to perform chemiosmotic work and maintain viability. BPI is a 59-kDa protein located in the primary granules of neutrophils. Its antimicrobial activity resides in a 25-kDa amino-terminal fragment.[380–384] In addition, BPI binds to lipopolysaccharide[385] and blocks the release of TNF-α elicited by bacteria.[382] Lactoferrin is an iron-binding protein found in secretions bathing mucosal membranes and in the specific granules of neutrophils.[386] Its bacteriostatic properties reflect an ability to deprive bacteria of the iron required for their growth, an effect eliminated by saturation of its iron-binding sites.[386] Lactoferrin plays a role in the alteration of the physicochemical properties of the neutrophil membrane that occurs during degranulation,[387] in the

modulation of hydroxyl radical production, in the regulation of granulopoiesis,[388] and in the modulation of complement function.[389]

Lysozyme, found mainly in the specific granules but also present in the primary granules, hydrolyzes the glycoside bond between *N*-acetylmuramic acid and *N*-acetylglucosamine, components of the peptidoglycan in bacterial cell walls. Although the bactericidal properties of lysozyme reflect this activity, peptide substitutions on the *N*-acetylmuramic acid residue in most bacteria make this bond inaccessible to lysozyme, thereby limiting its bacteriolytic properties. However, in the context of the complex environment of an inflammatory reaction, a bacterial cell wall already damaged by complement or granule proteins may allow access of lysozyme to its site of action.

Human PGRP-S binds avidly to the many forms of peptidoglycan on the surface of bacteria as dissimilar as *S. aureus* and *E. coli*, where it damages microbes by interfering with peptidoglycan biosynthesis and inducing conformational changes that prevent cross-linking of cell wall peptides.[390] Furthermore, PGRP-S acts synergistically with lysozyme to promote the lysis of *E. coli*.[391]

During investigation of gelatinase in specific granules, Kjeldsen and coworkers[392] discovered an associated protein (NGAL) that indirectly contributes to antimicrobial action by binding to catecholate-based bacterial siderophores, thereby interfering with iron acquisition by microbes. The contribution of NGAL to overall murine host defense against infection is illustrated by the failure of NGAL knockout mice to survive intraperitoneal challenge with *E. coli* that produced enterochelin, a catecholate-based siderophore.[393]

Specific granules also house precursor forms of a family of antimicrobial peptides collectively termed *cathelicidins* because of their sequence homology at their N-terminus with cathelin, an inhibitor of cathepsin.[394–396] The antimicrobial activity of cathelicidins resides in the cationic C-terminal portion of the protein, which is released from the holoprotein precursor during neutrophil degranulation. The spectrum of organisms susceptible to cathelicidins is broad, and the toxicity to target organisms is a consequence of disruption of the bacterial membrane. Among mammals there is significant variation in the number of cathelicidins expressed in individual species. Only one, human cathelicidin protein-18, which is processed to the antimicrobial peptide LL-37, has been identified in humans.[397] Of note, defensins, human cathelicidin protein-18, and NGAL are three antimicrobial proteins that are expressed in both keratinocytes and neutrophils, raising provocative questions about the synergistic interactions between recruited neutrophils and resident keratinocytes in contributing to tissue repair and wound healing at the site of cutaneous infection.[398,399]

Defensins are potent antimicrobial peptides in the primary granules of neutrophils,[377,400,401] along with prodefensins in specific granules,[402] and in epithelial cells of the gut and the genitourinary tract.[403,404] Defensins and closely related proteins are widely distributed in nature (e.g., the hemolymph of insects[405]) and probably represent an ancient approach to host defense. The precursor forms of the α-defensins present in azurophilic granules undergo proteolytic processing by the action of elastase and proteinase 3, two proteins in the same granule compartment, to yield active antimicrobial peptides.[406] In general, defensins are small molecules (3 to 4 kDa) rich in arginine and containing a characteristic disulfide motif. Elegant studies have defined many of the physical properties of purified defensins[407–409] and provide insight into their mechanism of action, which involves insertion into microbial membranes, resulting in the formation of pores that allow efflux of cytoplasmic components. The spectrum of organisms against which defensins are active is extremely broad, including gram-positive and gram-negative bacteria, fungi, and enveloped viruses.

Additional cationic proteins isolated from neutrophil primary granules[379] demonstrate preferential activity against specific bacterial species. These proteins include p15s,[410] azurocidin,[411] and indolicidin.[412] Understanding of the principles of antimicrobial activity of these proteins is incomplete at this time, but their mechanisms of action include both enzymatic and nonenzymatic components.

Extracellular Microbicidal Activity

Intraphagosomal killing of microorganisms is the primary method used by neutrophils to eliminate invading bacteria and fungi. However, studies indicate that nuclear DNA released from a small subset of neutrophils forms weblike or netlike structures known as neutrophil extracellular traps (NETs) that ensnare bacteria and fungi.[92,413,414–421] NETs are composed of decondensed nuclear DNA, histones, and azurophilic granule proteins such as MPO and elastase.[418] Although early studies indicated NETs immobilize and kill bacteria, more recent work suggests that NETs lack significant microbicidal activity.[422,423] Rather, these later data indicate that NETs may trap microbes and thereby prevent dissemination. Although the biologic relevance of NETs is the subject of controversy, three separate mechanisms for the formation of NETs have been proposed.

NETs were first proposed to form by a novel cytolytic cell death process eventually named *NETosis*.[419] During NETosis, nuclear and granule membranes lose integrity, and DNA mixes with the cytoplasm and granule proteins just before cell lysis. Subsequently, neutrophil elastase degrades histones, and the cell ruptures and releases decondensed DNA to form a NET decorated with antimicrobial proteins.[414,419] This process depends on reactive oxygen species produced by the NADPH oxidase.[414,419] Whether this process is truly distinct from traditional necrosis or cytolysis remains a topic of debate. It is also important to note that formation of NETs has been linked to host tissue damage and associated with specific human diseases,[415,424–426] findings consistent with processes that occur after typical neutrophil necrosis or lysis.

As an alternative to NETosis, reports have shown that a subset of live neutrophils form NETs by ejecting the nucleus, and the anuclear neutrophils remain functional and viable.[416,427] NETs formed by this mechanism appear functionally similar to NETs formed by the cytolytic process; they capture bacteria and prevent their dissemination. These intriguing findings were demonstrated in mouse bacterial infection models using live imaging techniques.[415,416] A third mechanism for formation of extracellular traps involves rapid "catapult-like" release of mitochondrial DNA from neutrophils[417] or eosinophils.[428] This mechanism of extracellular trap formation does not involve death of the phagocyte nor does it alter the life span of these cells.[417]

Although each proposed mechanism is unique and there is controversy related to extracellular traps formed by cytolysis or living cells, a feature common to the three mechanisms is the capture of microbes, which in animal models prevents microbial dissemination.

Neutrophils and Microbial Evasion of Host Defense

Ongoing investigation in microbial pathogenesis reflects the scientific advances in molecular microbiology and eukaryotic cell biology. In many ways, results from studies that apply these newer analytic approaches challenge our conventional understanding of microbial pathogenesis and the biologic meaning of colonization, commensalism, infection, and disease.[429] The application of gene microarrays to the study of host-microbe interactions has revealed the remarkably dynamic and interactive nature of both the invading microorganism and the responsive host cell. This area of research is rapidly evolving and encompasses infections with a variety of microbial species. The topic is too extensive to cover here in any detail. Instead, we provide a few selected examples of bacterial evasion of neutrophil function and refer the reader to more comprehensive reviews on specific topics.

Microbial Responses to Neutrophils

Phagocytosis elicits transcription of an array of neutrophil genes[299,430–433] and bacterial genes, including genes representing complex transcriptional responses that allow the organism to evade the attack by neutrophil-generated cytotoxins.[317,334,433–439] Such analyses demonstrate that the ingested microbe responds to its immediate environment in the phagosome rapidly and specifically. For example, among the many genes expressed by *E. coli* 7 minutes after being ingested by normal neutrophils are those regulated by *OxyR*, an oxygen-sensing transcription factor.[437] However, the same strain of *E. coli* does not express *OxyR*-regulated genes when ingested by chronic granulomatous disease neutrophils, which lack the capacity to generate reactive oxygen species to create oxidant stress within the phagosome.

Within the complex context of the interactive cell biology between host and microbe, it is clear that pathogens have evolved molecular

strategies for neutralizing one or more of the discrete steps in normal host defense.[335,438,440–442] To that end, the invading microorganism may exploit specific aspects of normal mammalian cell biology, including adhesive properties, signal transduction pathways, cytoskeletal rearrangements, and vacuolar trafficking.[440] In some cases, these properties are manifest only when microbes are in the appropriate host, demonstrating the exquisitely precise manner in which the invading microorganism has adapted to the context of the mammalian host.[443,444] *S. aureus* is perhaps an ideal example organism in this regard because it produces molecules that have the potential to inhibit virtually each step of the neutrophil response to invading microbes, including recruitment, phagocytosis, and bactericidal activity.[335,445]

Microbial Targeting of Neutrophil Surface Molecules

In some cases, bacteria adhere to surface proteins on target phagocytes, as with certain *Neisseria* species and CD66 on the neutrophil surface.[446] In other situations, bacterial proteins secreted into target cells may modify the host cell response, as is the case with the Yop proteins of *Yersinia*.[438] Once secreted into the host cell, the various members of the Yop family of proteins impair phagocytosis, induce apoptosis, paralyze cellular actin, and, in the case of the tyrosine phosphatase YopH, block Fc receptor–mediated activation of the respiratory burst oxidase.[447,448] In other settings, multiple mammalian cell types are targeted. For example, a glycoprotein of Ebola virus in its secreted form engages the neutrophil Fc receptor and inhibits cell activation and in its transmembrane form interacts with endothelial cells. In this way, the virus simultaneously inhibits the neutrophil-dependent early inflammatory response and induces endothelial cell damage, the clinical hallmark of Ebola virus infection.[449]

Survival of Microbes After Ingestion by Neutrophils

Certain microbes have evolved means to subvert or avoid host defenses and survive even after ingestion by neutrophils. For example, *S. aureus* produces many molecules that inhibit the function of neutrophil antimicrobial peptides and moderate the effects of reactive oxygen species.[335] During interaction with neutrophils, *Streptococcus pyogenes* upregulates a two-component signal transduction system that controls cell envelope biosynthesis and production of molecules that moderate the effects of reactive oxygen species.[436] In addition, M and M-like proteins of this pathogen promote survival after phagocytosis.[450] The activity or function of these molecules likely explains the ability of ingested *S. aureus* or *S. pyogenes* to survive long enough after phagocytosis to cause neutrophil lysis (see Fig. 8.9). Whereas microbes such as *S. aureus* survive because of high resistance to neutrophil microbicides, *A. phagocytophilum* and *Francisella tularensis* inhibit neutrophil superoxide generation and thereby avoid exposure to key microbicides.[451,452] Even with the limited understanding available, it is clear that there is as broad a range of microbial tactics for pathogenesis as there are host cell targets, and their elucidation will provide insights into the biology of both the host and the pathogen.

EOSINOPHILS

Eosinophils are bone marrow–derived, tissue-based granulocytes located subjacent to the skin and mucosal lining of the respiratory and gastrointestinal tracts (Fig. 8.10).[453,454] In these locations, they play a role in host defense against helminthic infections and figure prominently in the pathology of hypersensitivity diseases, such as asthma and certain dermatologic and gastrointestinal disorders.[453,454] In many ways, eosinophils have a functional repertoire that resembles that of neutrophils,[453,454] but there are several significant differences in the activities of these two types of granulocytes.[455] Recent studies have expanded recognition of the central role of eosinophils in modulation of inflammatory responses in selected situations.[453,454] The granules of eosinophils harbor not only cytotoxic contents but also pleiotropic inflammatory agents, including cytokines, chemokines, lipid mediators, and neuroactive substances.[453,454] Consequently, the biology of eosinophils extends beyond their contribution to host defense against helminths, and the elucidation of the scope of the role of eosinophils as immune cells promises to provide exciting insights into mechanisms for the integration of innate and adaptive immune systems.

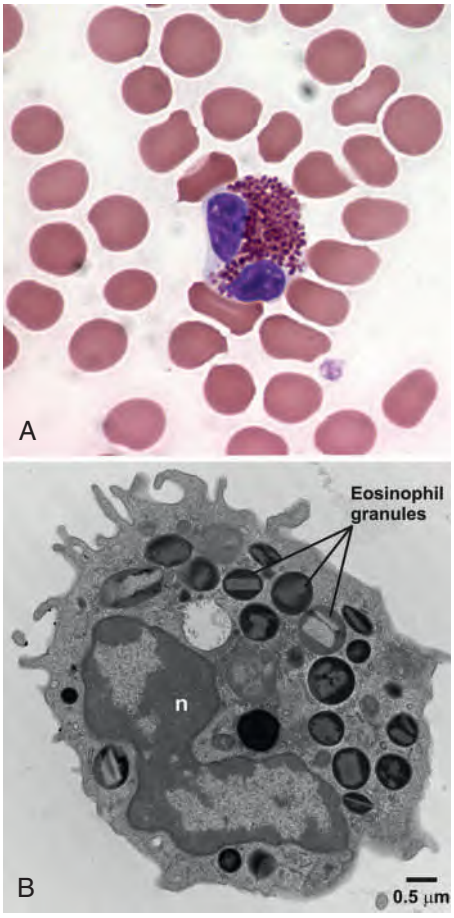

FIG. 8.10 Human eosinophils. Bright-field (A) and transmission electron (B) microscopy images of human eosinophils. Note the prominent granules with crystalloid cores. *n,* Nucleus.

Development and Characteristics

Eosinophils develop from bone marrow stem cells in 5 to 6 days.[456] Eosinophilopoiesis in humans appears to be uniquely dependent on IL-5, with IL-3 and GM-CSF also contributing to a lesser degree. IL-5 concentrations correlate with the appearance and magnitude of eosinophilia in vivo.[453,454] Eosinophil maturation is accompanied by the appearance of electron-dense primary granules, which appear during the promyelocyte stage of development, and large cytoplasmic granules known as crystalloid, specific, or secondary granules.[453,454,457–460] Primary granules contain Charcot-Leyden crystal protein (galactin 10) and lipid bodies.[453,460] Crystalloid granules are distinguished by their large size, an electron-dense crystalloid core containing major basic protein (MBP), and an electron-lucent matrix containing eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase (see Fig. 8.10).[454,457–459] Of note, these crystalloid granules also contain a wide range of chemokines (macrophage inhibitory protein-1α/CCL3, RANTES [regulated on activation, normal T cell expressed and secreted]/CCL5, eotaxin-1/CCL11, growth-regulated oncogene α/CXCL1, epithelial neutrophil-activating peptide-78/CXCL5), cytokines (e.g., IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, GM-CSF, IFN-γ, TNF-α, transforming growth factor-α, and transforming growth factor-β), enzymes (e.g., arylsulfatase B and collagenase), and growth factors (e.g., vascular endothelial growth factor and stem cell factor).[453,454,457,458,460]

Eosinophil Surface Receptors

Mature eosinophils can express an extensive repertoire of surface receptors—some constitutively expressed and others induced by stimuli—and thus have high immunomodulatory potential.[453] For

example, eosinophils express receptors for the Fc portion of IgA, IgD, IgM, IgG (FcγRII), and IgE (FcεRI and FcεRII), although the presence of IgE receptors on eosinophils is a subject of controversy.[460] Receptors for complement (CR1, CR3, CR4, ClqR, C3a, C5a), cytokines (e.g., receptors for IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IL-23, IL-27, IL-31, IL-33, GM-CSF, IFN-γ, and TNF-α), platelet-activating factor, prostaglandins, and LTB_4 have also been reported to be present on eosinophils.[453,454] Eosinophils also express on their surface two members of the CD2 subfamily of receptors, CD48 and CD244. Engagement of CD48 elicits degranulation. Several inhibitory receptors on the eosinophil surface suppress their activation when engaged and thereby contribute to the immunomodulatory activities of eosinophils.

Eosinophil Granules

Eosinophils release their granules via several distinct mechanisms.[457,458] Eosinophils can undergo traditional granule exocytosis, a process similar to neutrophil degranulation, during which the entire contents of granules are released into the extracellular environment. This process may involve compound exocytosis, a variation of exocytosis whereby multiple granules fuse within the cell cytoplasm (thus forming a large, single granule) before exocytosis.[457,458] Eosinophil granule products can also be released by piecemeal degranulation, which is the primary mechanism for release of granule contents. During piecemeal degranulation, secretory vesicles or eosinophil sombrero vesicles transport selected molecules from crystalloid granules to the cell surface for secretion.[457,458] Release of granule contents can also occur by cytolysis,[457,458] and there is evidence that cell-free granules can be triggered to release selected molecules by specific agonists.[457,459,461]

Recruitment From Circulation

Circulating eosinophils can be separated into two populations based on buoyant density. Most eosinophils in healthy individuals are characterized as dense or normodense. Hypodense eosinophils are cells that have been activated. They express a greater number of functionally competent receptors, exhibit a higher resting level of oxidative metabolism, and predominate in the blood and tissues of persons with eosinophilia.

The intravascular half-life of eosinophils is approximately 2 hours. Eosinophil migration involves different adhesion molecules than neutrophil transmigration.[453,454,460,462,463] In addition to the β_2-integrins also expressed by neutrophils, eosinophils express β_1 and β_7 integrins[464] and display a form of P-selectin glycoprotein ligand-1 that binds more avidly to endothelial P-selectin than the form expressed by neutrophils.[460,465–467] That eosinophil transmigration is normal in leukocyte adhesion deficiency-1 provides evidence that β_2-integrins are not required for this process. Association with endothelium in the process of eosinophil trafficking appears to be mediated by IL-5, IL-4, IL-13, and chemokines and the coordinated expression and activation of integrins and their cognate binding partners.[453,468]

Role in Host Defense

Substantial evidence supports a role for eosinophils in immunity to helminthic parasites, as demonstrated by the greater worm burden and tissue damage in animals treated with antieosinophil serum and by the finding that the transfer of passive immunity requires the presence of these cells. This conclusion is buttressed by the demonstration of eosinophils on and around degenerating parasites in vivo and by the ability of eosinophils to kill these organisms in vitro.[460,469,470] It is noteworthy that many of the studies of the role of eosinophils in host defense against parasitic helminths have been performed in mouse infection models, and there are significant functional and morphologic differences between human and mouse eosinophils.[460]

Killing of parasites is related to exocytosis of eosinophil granule contents onto the parasite surface while it is in close apposition to the eosinophils.[453,460,471,472] The eosinophil peroxidase–H_2O_2–halide oxidation system plays a minor role in anthelmintic activity,[473] whereas the cationic granule proteins are responsible for the bulk of this activity. On a molar basis, eosinophil cationic protein exerts a more potent anthelmintic effect than MBP does, but the greater quantity of the latter in the eosinophil makes its contribution more significant. The anthelmintic effects of these proteins are also specific for different stages in the life cycle of the parasite. In addition to contributing to host defense against infections with helminths, eosinophils respond to RNA viruses, including respiratory syncytial virus,[460,474] perhaps through the ribonuclease activity of eosinophil cationic protein and eosinophil-derived neurotoxin.

Moderation of Type I Hypersensitivity Reactions

Recognition that eosinophil granules contain a number of substances capable of inactivating the chemical mediators of anaphylaxis has led to the suggestion that eosinophils may moderate the severity of type I hypersensitivity reactions.[453,475] In this scenario, stimulation of basophils and mast cells by the interaction of surface IgE with specific antigen results in the release of substances important in type I hypersensitivity reactions. These include vasoactive amines, slow-reacting substances of anaphylaxis (leukotrienes C, D, and E), platelet-activating factor, and eosinophil chemotactic factor of anaphylaxis (ECF-A). Histamine and ECF-A attract eosinophils to the site of antigen reaction with basophils and mast cells. ECF-A can also stimulate eosinophil degranulation, as can immune complexes that are phagocytosed by eosinophils. Histaminase secreted by the eosinophil may inactivate local histamine, and a substance present in eosinophils may inhibit further histamine secretion by basophils. Arylsulfatase and phospholipase present in the smaller eosinophil granules are capable of inactivating leukotrienes C, D, and E and platelet-activating factor. Therefore eosinophils may moderate immediate hypersensitivity reactions by inhibiting the release of mediators of the type I reaction as well as by destroying mediators that have already been released.[473]

Eosinophil-Mediated Tissue Injury

The association of eosinophilia of several weeks' duration with the development of endocardial lesions and the isolation of an eosinophil-derived neurotoxin capable of reproducing the neurologic picture observed in patients with cerebrospinal fluid eosinophilia strongly support roles for eosinophils in the pathogenesis of tissue injury in certain disorders, the most prominent of which is asthma. Substantial evidence indicates that eosinophil MBP is an important mediator of asthmatic tissue injury. For example, increased quantities of MBP are detectable in the bronchial washings from patients with asthma but not from bronchial washings from patients with other pulmonary disorders. Nanomolar concentrations of MBP, but not of other cationic proteins, cause exfoliation of epithelial cells, impaired ciliary function, net chloride secretion, and bronchial hyperreactivity. Immunofluorescent staining of bronchial epithelium in autopsy specimens from patients who died as a result of asthma revealed extensive deposition of MBP in the peribronchial areas and overlying regions of bronchial epithelial denudation. These findings were not observed in autopsy material obtained from patients who died of nonasthmatic pulmonary diseases. The importance of epithelial denudation lies in the resultant enhanced responsiveness of the underlying bronchial smooth muscle to contractile agonists, including acetylcholine and histamine, as well as to leukotriene C_4 produced by eosinophils.[476]

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The complete reference list is available online at Expert Consult.

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