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INNATE IMMUNE RECOGNITION

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■ **Abstract** The innate immune system is a universal and ancient form of host defense against infection. Innate immune recognition relies on a limited number of germline-encoded receptors. These receptors evolved to recognize conserved products of microbial metabolism produced by microbial pathogens, but not by the host. Recognition of these molecular structures allows the immune system to distinguish infectious nonself from noninfectious self. Toll-like receptors play a major role in pathogen recognition and initiation of inflammatory and immune responses. Stimulation of Toll-like receptors by microbial products leads to the activation of signaling pathways that result in the induction of antimicrobial genes and inflammatory cytokines. In addition, stimulation of Toll-like receptors triggers dendritic cell maturation and results in the induction of costimulatory molecules and increased antigen-presenting capacity. Thus, microbial recognition by Toll-like receptors helps to direct adaptive immune responses to antigens derived from microbial pathogens.

INTRODUCTION

Innate immunity covers many areas of host defense against pathogenic microbes, including the recognition of pathogen-associated molecular patterns (PAMPs) (1). In vertebrates, which are the only phylum that can mount an adaptive immune response, there are also mechanisms to inhibit the activation of innate immunity. An example is the inhibition of killing by natural killer (NK) cells, which are known to receive an inhibitory stimulus from MHC class I molecules. We concentrate in this review on the mechanisms of recognition that are truly innate, such that the genes are encoded in the germline DNA and do not require the gene rearrangement essential to adaptive immune recognition (Table 1).

Innate immunity is an evolutionarily ancient part of the host defense mechanisms: The same molecular modules are found in plants and animals, meaning that it arose before the split into these two kingdoms (2). Adaptive immunity is a relative newcomer on the evolutionary landscape. Because the mechanism of generating receptors in the adaptive immune system involves great variability and rearrangement of receptor gene segments, the adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection,

TABLE 1 Innate and adaptive immunity

Property	Innate immune system	Adaptive immune system
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns (LPS, LTA, mannans, glycans)	Details of molecular structure (proteins, peptides, carbohydrates)
Self-Nonself discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Cytokines (IL-1β, IL-6) Chemokines (IL-8)	Clonal expansion or anergy IL-2 Effector cytokines: (IL-4, IFNγ)

and pathogen-specific adaptor proteins. However, the adaptive immune response is also responsible for allergy, autoimmunity, and the rejection of tissue grafts.

Innate immunity also lies behind most inflammatory responses; these are triggered in the first instance by macrophages, polymorphonuclear leukocytes, and mast cells through their innate immune receptors. What adaptive immunity adds to the underlying innate immune system is specific recognition of proteins, carbohydrates, lipids, nucleic acids, and pathogens, using the same activated, but not antigen-specific, effector cells generated by innate immune recognition. So the two systems are also linked in the use of the same effector cells (1). However, the real question is, how are they linked in the generation of an adaptive immune response? Unfortunately, defects in innate immunity though very rare are almost always lethal. They are rarely observed in a physician's office, unlike defects in adaptive immunity, and only appeared once the wonder drug penicillin became available to treat infections. Therefore, we have relatively few patients surviving the lack of one or the other of their innate immune mechanisms, and thus we have relatively little data on the role of the innate immune system from such patients.

In this article, we focus on how the innate immune system plays a role in discrimination between what we like to call infectious non-self and its obverse, which we refer to as noninfectious self. That is, we believe that the major decision to respond or not respond to a particular ligand is in the main decided by the genome-encoded innate immune system receptors. The positive and negative selection of developing lymphocytes plays a secondary but important role in this decision. We also should not forget recent evidence that a third type of cell could participate in the discrimination of self and nonself. These are called by various authors suppressor T cells (Ts) or regulatory T cells (Treg). But we argue that the main decision to respond or not to respond to a particular antigen is made by

innate immune recognition receptors when they encounter pathogen-associated molecular patterns (PAMPs), such as LPS or bacterial CpG DNA.

The innate immune system is made of many cells, such as those white blood cells that are not B lymphocytes or T lymphocytes of the adaptive immune system. It also can be considered to be a property of the skin and the epithelia that line our internal organs such as the gut and lungs. These tissues are normally populated with what are called commensal microbes, although when one gets a cut or, more seriously, a perforating ulcer, these commensals become pathogens. This is also seen in individuals who receive antibiotics, the main effect of which is to kill most or all microorganisms, with the result that there is frequently overgrowth of pathogenic microbes. This could in part be due to the loss of *Escherichia coli*, which produce potent antimicrobial peptides called colicins; in the absence of colicins, other, more dangerous pathogens may grow out and colonize the gut.

There are many aspects to innate immunity that fall outside the purview of this article, but they are nevertheless important components of host defense. Among these are antimicrobial peptides produced by polymorphonuclear leukocytes in most vertebrate species and by the fat body in the fruit fly *Drosophila melanogaster*. The complement pathway can also be triggered by the mannose-binding lectin, an acute phase protein. That makes the control of these responses important, and we discuss them later in the article.

Among the cells that bear innate immune or germline-encoded recognition receptors are macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, and the so-called NK cells. These cells can become activated during an inflammatory response, which is virtually always a sign of infection with a pathogenic microbe. Such cells rapidly differentiate into short-lived effector cells whose main role is to get rid of the infection; in this they mainly succeed without recourse to adaptive immunity. However, in certain cases, the innate immune system is unable to deal with the infection, and so activation of an adaptive immune response becomes necessary. In these cases, the innate immune system can instruct the adaptive immune system about the nature of the pathogenic challenge. It does so through the expression of costimulatory molecules, such as CD80 and CD86, on the surface of specialized antigen-presenting cells, the most important of which are the dendritic cells that guard against infection in virtually all tissues (1, 3, 4). Tissue dendritic cells also play a featured role in the initiation of tissue graft rejection.

The genes for CD80 and CD86 are regulated by a transcription factor called NFκB, which can lead to rapid induction of their expression on the cell surface of dendritic cells and other antigen-presenting cells. The mechanism of NF-κB regulation is discussed in detail in this article, and it also has been reviewed extensively in this series (5).

The T and B lymphocytes of the adaptive immune system have receptors that need to be assembled from gene segments. This allows great variability in adaptive immune recognition, but it cannot discern the nature of the pathogen infecting the body (6). Adaptive immunity also allows the unfortunate effects of autoimmune

disease, allergy, and allograft rejection. These latter are all a consequence of immune responses to nonpathogen antigens. They also appear to be a result of the random nature of receptor gene segment rearrangement. Nevertheless, in animals or plants with only an innate immune system at their disposal, there is no sign of any of these processes, despite numerous attempts to demonstrate such effects. Instead, what appears to be graft rejection is more readily explained by invoking cells like NK cells, which recognize the absence of self rather than the presence of nonself. Whether this is at the root of the mechanism is outside the scope of this article. However, invertebrates and plants lack the essential genes to make an adaptive immune response, and they also lack the associated tissue architecture to induce such a response. Therefore, the problems generated by having an adaptive immune system, as well as the benefits of having one, are found only in the descendents of the teleost fish, including ourselves.

In recent times, the origin of the adaptive immune response has been uncovered. It turns out that the two recombinase-activating genes are encoded in a short stretch of DNA, in opposite orientations and lacking exons. This suggested an origin in a retroposon, as did the presence of the recognition signal sequences that lie 3′ of all V gene segments and 5′ of all J gene segments (7). This hypothesis was tested in vitro and shown to be true (8, 9). Other processes expand diversity tremendously, such as the generation of D gene segments in the first chain to rearrange, the nucleotide-adding enzyme TdT that inserts nucleotides in the junctions of V-D-J junctions, and somatic hypermutation.

All of these processes are found exclusively in vertebrates and not in plants and invertebrates. Therefore, these organisms are wonderful tools for studying innate immunity, and the fact that the earth's surface is covered with more species of invertebrates and plants than vertebrates speaks to the success of innate immune systems. This gives testimony to the necessity of having both an adaptive and an innate immune system; the vertebrates just added an antigen-specific mechanism for recognizing specific pathogens to a pre-existing system of non-antigen-specific innate immunity.

Some of the advantages of having an adaptive immune system are the ability to remember or adapt to an infectious agent, but this memory is confined to an individual. Apart from the trans-placental transfer of antibody from mother to fetus, there is little carryover of this memory from one generation to the next. This may be good because many have speculated that adaptive immunity toward pathogenic microbes is an initiating signal for autoimmunity (10).

One can think in very simple terms about the virtues of a nonclonal system of host defense. First, it serves to make adaptive immunity more useful, in part by delaying the need for an adaptive immune response by the three to five days that it takes to generate the clonal expansion and differentiation to effector lymphocytes. Second, it serves to alert the clonal, adaptive immune system that it is under attack by a pathogenic microorganism. The nonclonal system also cannot mediate all the bad effects of adaptive immunity because it involves rapid activation of effector cells that, if they were directed against self tissues, would be lethal to the host and

thus expunged by evolutionary processes (6). The virtue of having both innate and adaptive systems of recognition is that the interplay of these two distinct systems allows the discrimination of an infectious attack on the host from noninfectious self.

The virtue of having clonally distributed receptors is also obvious, in that they allow the recognition of particular features of pathogens. Such receptors also remember having seen pathogens before, and so they mount a stronger and more specific response on re-encounter with the same microbe. Those organisms, all of which are vertebrates, that have both innate and adaptive immune systems gain a tremendous benefit in longevity through stimulation of having both systems. This may be of particular importance in vaccination, which was initiated a bit over 200 years ago by Edward Jenner. Recently, it was reported that the vaccinia virus introduced by Jenner had genetic elements that could inhibit an intracellular domain found in innate immune recognition receptors (11). This finding, just made, says that the vaccinia can grow for a while in a nonvaccinated individual because it can shut off the innate immune response. This property is undoubtedly of importance to the virus in infecting cows (and milkmaids), but it can also allow vaccinia to be a very potent vaccine that leads to resistance to small pox of variola, with which it cross-reacts.

Thus, the virtues of having an innate immune system of pathogen recognition lie not only in the delaying tactics of inflammation upon infection, but also in the activation of the adaptive immune system only when the body is under attack by a specific pathogen. This system works to allow long life in most vertebrates by controlling the expression of the cell surface costimulatory molecules, and by inducing secretion of appropriate cytokines and chemokines that direct the lymphocytes of the adaptive immune system to their appropriate locations. Together these function to give optimal host defense.

PATTERN RECOGNITION RECEPTORS

The innate immune system uses a variety of pattern recognition receptors that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (12). The principal functions of pattern recognition receptors include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis.

Mannan-binding lectin (MBL), C-reactive protein (CRP), and serum amyloid protein (SAP) are secreted pattern recognition molecules produced by the liver during the acute phase response at the early stages of infection (13–15). CRP and SAP are members of the pentraxin family, and both can function as opsonins upon binding to phosphorylcholine on bacterial surfaces (13, 14). CRP and SAP can also bind to C1q and thus activate the classical complement pathway (16). MBL is a member of the collectin family, which also includes pulmonary surfactant proteins A and D (17, 18). The collectins are characterized by the presence of a collagenous

region and a C-type lectin (CTL) domain; typically they form oligomeric receptors (17, 18). MBL binds specifically to terminal mannose residues, which are abundant on the surface of many microorganisms, and associates with MBL-associated serine proteases (MASP). MASP1 and MASP2 are activated by MBL and initiate the lectin pathway of complement by cleaving C2 and C4 proteins (15).

Several cell surface receptors expressed on macrophages function as pattern recognition receptors that mediate phagocytosis of microorganisms. Macrophage mannose receptor (MMR) is a member of the C-type lectin family and is closely related to DEC205, a receptor expressed on dendritic cells. MMR interacts with a variety of gram-positive and gram-negative bacteria and fungal pathogens (15). The main function of the MMR is thought to be phagocytosis of microbial pathogens, and their delivery into the lysosomal compartment where they are destroyed by lysosomal enzymes (15). The function and ligand specificity of DEC205 has not yet been characterized, but its similarity to MMR and its expression on dendritic cells suggest that it may also function as a phagocytic receptor.

Macrophage scavenger receptor (MSR) is another phagocytic pattern recognition receptor expressed on macrophages. MSR belongs to the scavenger receptor type A (SR-A) family and has an unusually broad specificity to a variety of polyanionic ligands, including double-stranded RNA (dsRNA), LPS, and LTA (19). MSR protects against endotoxic shock by scavenging LPS and has a role in host defense, as demonstrated by increased susceptibility of MSR-deficient mice to *Listeria monocytogenes*, herpes simplex virus, and malaria infections (20, 21). In addition to recognition of microbial PAMPs, MSR also plays a role in lipid homeostasis by binding and endocytosing acetylated low-density lipoproteins (22). Another SR-A family member, MARCO, is a macrophage receptor that binds to bacterial cell walls and LPS, and it also mediates phagocytosis of bacterial pathogens (23).

INTRACELLULAR RECOGNITION SYSTEMS

Viruses and some bacterial pathogens can gain access to the intracellular compartments, such as the cytosol. Several pattern recognition receptors are expressed in the cytosol where they detect these intracellular pathogens and induce responses that block their replication. The protein kinase PKR is activated upon binding to dsRNA, which is produced during viral infection (24). Activated PKR phosphorylates and inactivates the translation initiation factor eIF2α, which results in a block of viral and cellular protein synthesis (24). In addition, PKR activates NF-κB and MAP kinase signaling pathways, which leads to the induction of the antiviral type-I IFN genes (25). PKR also inhibits viral spread by inducing apoptosis in infected cells (25).

Another antiviral pathway activated by dsRNA is the 2′-5′-oligoadenylate synthase (OAS)/RNaseL pathway (26). OAS is activated upon binding to viral dsRNA and produces an unusual nucleotide second messenger - 2′-5′ oligoadenylate. These oligonucleotides then activate the RNaseL, which destroys both viral and cellular

RNAs. The antiviral effect of this pathway is therefore due to cleavage of viral RNA and induction of apoptosis in infected cells due to cleavage of cellular RNA and block of protein synthesis (26).

Another group of proteins likely involved in intracellular pattern recognition is the family of NOD proteins. NOD proteins contain an N-terminal CARD domain, a nucleotide binding domain (NBD), and a C-terminal leucine-rich repeat (LRR) region (27, 28). This domain arrangement is characteristic of the NB-ARC family, which, in addition to the NODs, includes the mammalian apoptosis regulator APAF-1 and a class of plant resistance genes (R genes). R genes in plants can detect microbial infection and induce the hypersensitivity response—a major effector response that blocks pathogen replication and spread (29). The CARD domains of NOD1 and NOD2 associate with a protein kinase, RIP2, which in turn activates NF-κB and MAP kinase signaling pathways (27, 28, 30). The full range of ligands recognized by NOD proteins is currently unknown, but both NOD1 and NOD2 are reported to activate NF-κB in response to LPS, presumably through binding to their LRR regions (30, 31). It is interesting that mutations in the *nod2* gene cause a predisposition to Crohn's disease—a chronic inflammatory disorder of the gut (32, 33).

DROSOPHILA TOLL

The first member of the Toll family, *Drosophila* Toll, was discovered as one of 12 maternal effect genes that function in a pathway required for dorso-ventral axis formation in fly embryos (34, 35). Other genes in this pathway encode a Toll ligand, Spatzle, an adapter protein, Tube, a protein kinase, Pelle, an NF-κB family transcription factor, Dorsal, and a Dorsal inhibitor, Cactus, which is a homologue of mammalian IκB. Spatzle is secreted as a precursor polypeptide and requires proteolytic cleavage by serine proteases for activation. This cleavage is controlled by a protease cascade that includes four serine proteases: gastrulation defective, easter, snake, and nudel (34, 36).

Soon after *Drosophila* Toll and the human IL-1R were identified, it became apparent that they had possible functional similarities. In addition to the presence of homologous cytoplasmic TIR domains, both receptors could induce NF-κB activation and could signal through homologous protein kinases—Pelle and IRAK (34, 36). The analysis of the promoter regions of the genes encoding antimicrobial peptides in *Drosophila* revealed consensus NF-κB binding sites (37). Since these peptides are rapidly induced in flies in response to infection, these observations suggested a possible involvement of the Toll pathway in *Drosophila* immunity. Indeed, analysis of *Drosophila* strains carrying loss-of-function mutations in the *Toll* gene demonstrated a striking defect in immune responses: These flies were highly susceptible to fungal infection but had normal responses to gram-negative bacterial infection (38).

The systemic immune response in *Drosophila* is mediated by a battery of antimicrobial peptides produced largely by the fat body, an insect organ analogous to

the mammalian liver. These peptides lyse microorganisms by forming pores in their cell walls. Functionally, the antimicrobial peptides fall into three classes depending on the pathogen specificity of their lytic activity (2). Thus, Drosomycin is a major antifungal peptide, whereas Diptericin is active against gram-negative bacteria, and Defensin works against gram-positive bacteria (2). Interestingly, infection of *Drosophila* with different classes of pathogens leads to preferential induction of the appropriate group of antimicrobial peptides (39). For example, fungal infection results in the induction of Drosomycin, but not Diptericin (39). Mutation of the *Toll* gene blocks the induction of Drosomycin in response to fungal infection but does not affect significantly the induction of Diptericin in response to gram-negative infection. Importantly, mutations in *Spatzle*, *Tube*, *Pelle*, and *Cactus* genes also specifically affect the resistance of *Drosophila* to fungal pathogens (38).

Dorsal, which is activated by the Toll pathway during dorso-ventral axis formation, does not appear to play a role in the systemic immune response in adult flies. Instead, another NF-κB family member—Dif (drosophila immunity factor)—is required for the induction of Drosomycin by Toll (40–42). Additionally, Spatzle is required for the activation of Toll by fungal pathogens; however, the serine protease cascade that generates active Spatzle during development is not involved in the immune response (38). Therefore, a different protease cascade must regulate its processing. This putative protease cascade is presumably triggered by a PRR(s) specific for fungal PAMPs, such as mannan. Further support for this hypothesis came from the analysis of *necrotic* mutants (43). *Necrotic* encodes a serine protease inhibitor of the serpin family. Mutations in this gene result in the spontaneous activation of the Toll pathway and constitutive induction of the Drosomycin gene (43). These results suggest that in Drosophila, the pattern recognition event occurs upstream of Toll and triggers a protease cascade, much as complement is activated by the lectin pathway in mammals. Interestingly, the Toll pathway can also be activated in response to gram-positive infection, suggesting that multiple pattern recognition molecules may function upstream of the protease cascade that controls cleavage of Spatzle (44).

The *Drosophila* response to gram-negative bacterial infection is controlled by a distinct pathway, which was defined by the mutation in the *imd* (immune deficient) gene (45). *Imd* mutants have a profound defect in resistance to gram-negative bacterial pathogens, while remaining essentially normal with regard to fungal and gram-positive infection (45). Genetic analyses led to the identification of four additional genes that function in the Imd pathway: Dredd, dIKK-γ, dIKK-β, and Relish (46–51). Mutations in any of these genes yield phenotypes very similar to the *Imd* mutants, that is, susceptibility to gram-negative bacterial infection due to impaired induction of antibacterial peptides such as Diptericin. Dredd is a *Drosophila* caspase previously implicated in the control of apoptosis during fly development (52). dIKK-γ and dIKK-β are *Drosophila* homologues of human IKK-γ (also known as NEMO) and IKK-β. In human cells, IKK-β and NEMO are essential regulators of NF-κB activation (53). Relish is a *Drosophila* homologue of the mammalian Rel/NF-κB family members, p100 and p105 (54).

One major question in *Drosophila* immunity that remains unresolved is the identity of the receptor that controls activation of the imd pathway in response to gram-negative bacterial infection. Since there are nine Toll-like receptors in *Drosophila*, it is possible that one of them may be responsible for the activation of the imd pathway (55). A mutation in 18-Wheeler, another Toll family member, affected expression of the antibacterial peptide, Attacin (56). However 18-Wheeler does not appear to function in the imd pathway (44). Moreover, none of the *Drosophila* Tolls could induce activation of the Diptericin promoter in *Drosophila* cell lines, and only Toll and Toll-5 were able to activate Drosomycin (55). Alternatively, a receptor unrelated to Toll may control the imd pathway and function as a sensor for gram-negative PAMPs such as LPS.

TOLL-LIKE RECEPTORS IN MAMMALIAN IMMUNITY

Ten TLRs have been described to date in humans and mice. They differ from each other in ligand specificities, expression patterns, and presumably in the target genes they can induce. No developmental function has been ascribed to mammalian TLRs so far. Several TLRs are involved in the recognition of a variety of PAMPs. The exact mechanism of recognition has not yet been determined for any of them and remains an important avenue of future research.

LIGAND RECOGNITION BY TLRS

TLR4

The first indication that mammalian TLRs may function as pattern recognition receptors came with the description of a human homologue of Drosophila Toll, now known as TLR4 (57). A constitutively active form of this receptor induced the expression of inflammatory cytokines and the costimulatory molecule B7 in the monocytic cell line THP-I (57). Subsequently, positional cloning analysis of the LPS-nonresponsive mouse strain, C3H/HeJ, showed that a point mutation in the TIR domain of TLR4 was responsible for the defect in LPS signal transduction (58, 59). Another mouse strain, B10.ScCR, did not respond to LPS and turned out to lack the genomic region that contains the entire *tlr4* gene (58, 59). Finally, mice with a targeted deletion of the *TLR4* gene were unresponsive to LPS (60). Together, these studies demonstrated the essential role for TLR4 in recognition of a major component of gram-negative bacteria.

TLR4, however, is not the sole receptor involved in LPS recognition. Transport of LPS molecules in the serum is mediated by LPS-binding protein (LBP) (61). At the plasma membrane, LBP is thought to transfer LPS monomers to CD14, a GPI-linked cell surface protein (61). Exactly how CD14 facilitates recognition of LPS by TLR4 is not clear, but its critical role is underscored by the LPS-hyporesponsive phenotype of CD14-deficient mice (62, 63). Finally, a small protein called MD-2 is also a component of the LPS-recognition complex (64). MD-2 lacks

a transmembrane anchor but is associated with the extracellular region of TLR4 (64). MD-2 is required for cellular responsiveness to LPS, as demonstrated by both transfection studies and an analysis of a CHO cell line with a mutated MD-2 gene (64, 65). Although the cell-surface events that confer LPS recognition have not been unambiguously determined, most of the available evidence indicates that a complex of TLR4/MD-2/CD14 directly binds LPS (66–68). How other members of the mammalian TLR family recognize their cognate ligands is still an enigma, and one that waxes in complexity as more and more ligands are identified for some individual members of the TLR family.

Interestingly, B cells express on their cell surface a receptor called RP105 that is also involved in LPS recognition (69). RP105 is related to TLR4 in its extracellular domain, which likewise consists of leucine-rich repeats, but it lacks the intracellular TIR domain and instead has a short cytosolic tail that contains a tyrosine phosphorylation motif (69). RP105 is associated via its ectodomain with MD-1, a protein related to MD-2 that is required for RP-105 function (70, 71). In response to cross-linking or LPS stimulation, RP105 activates src kinases, including lyn (72). Since B cell responses to LPS are completely dependent on TLR4, the exact mechanism of LPS recognition is unclear, but it presumably involves cooperation between RP105 and TLR4 (72).

In addition to LPS, TLR4 has been implicated in the recognition of liptocheic acid (LTA), the heat shock protein hsp60, and the fusion protein of the respiratory syncytial virus (73–76). The physiological relevance of some of these putative TLR4 ligands remains to be demonstrated. However, it is clear that the original paradigm suggested by the example of *Drosophila* Tolls, in which different Tolls discriminate between classes of pathogens, is not applicable to mammalian TLRs.

TLR2

Of the mammalian TLRs, and perhaps of all PRRs, TLR2 recognizes the largest number of ligands. The list includes peptidoglycan (73, 77), bacterial lipoproteins (78–80), a phenol soluble factor from *Staphylococcus epidermidis* (81), LPS from *Prophyromonas gingivitis* (82) and *Leptospira interrogans* (which differs in structure from the LPS of gram-negative bacteria) (83), glycosylphosphotidylinositol lipid from *Trypanosoma cruzi* (84), and zymosan, a component of yeast cell walls (85). TLR2 does not recognize these PAMPs independently, but functions by forming heterodimers with either TLR1 or TLR6 (86, 87). A likely consequence of this cooperation is an increased repertoire of ligand specificities. Further studies are needed to determine whether heterodimerization is necessary for ligand recognition by any other TLR. No other TLR pairs have yet been identified, and some of the TLRs (such as TLR4 and TLR5) most likely function as homodimers (86).

TLR5

TLR5 recognizes flagellin, the protein subunits that make up bacterial flagella (88). Unlike most other PAMPs, flagellin is a protein and does not contain any obvious features to flag it as nonself or pathogen-associated. Nevertheless, flagellin

is extremely conserved at the N- and C-terminal ends that form its hydrophobic core and most likely is recognized by TLR5 in this region. It is probable that structural constraints have prevented mutations in these conserved regions and hence generation of escape mutants. Moreover, flagella, like other PAMPs, are essential for viability, and a mutation that compromises flagellin function would have deleterious consequences for the bacteria.

Flagellin from *S. adelaide* played an important role in early studies of immunity. It was a highly potent antigen that turned out to be thymus independent. It played a major role in establishing the clonal selection hypothesis of MacFarlene Burnet. The fact that this antigen was used intensively in Australia may account for most of the success of Australian immunology in the 1960s and 1970s; it drew numerous people to work at the Walter and Elisa Hall Institute.

TLR9

Unmethylated CpG DNA was long known for its immunostimulatory effects (89, 90), and we now know that TLR9 recognizes unmethylated CpG motifs present in bacterial DNA (91). The logic of this recognition is that most of the mammalian genome is methylated, while bacteria lack CpG methylation enzymes (90). One enigma concerning this PAMP is in its accessibility for recognition by TLR9; bacterial DNA, after all, should be neatly packaged in the bacteria and rarely, if ever, exposed for recognition at the bacterial cell surface. However, stimulation by CpG DNA can be inhibited by drugs that block its uptake, and therefore, TLR9 likely recognizes its ligand intracellularly, perhaps in endosomes or lysosomes, presumably following bacterial lysis (89, 92). It is worth noting here that although all the TLRs are assumed to reside at the cell surface, some of the TLRs (including TLR9) may in fact localize intracellularly. Furthermore, in some cases, cell-surface ligand binding may also be coupled to uptake, such that the TLR undergoes stimulus-dependent internalization, in the process delivering its cargo to an intracellular compartment. Thus, TLR2, for example, is recruited to macrophage phagosomes upon stimulation with zymosan (85).

TOLL SIGNALING PATHWAYS

Upon recognition of their cognate ligands, TLRs induce the expression of a variety of host defense genes. These include inflammatory cytokines and chemokines, antimicrobial peptides, costimulatory molecules, MHC molecules, and other effectors necessary to arm the host cell against the invading pathogen. TLRs accomplish this by activating an intracellular signaling pathway conserved from *Drosophila* to mammals. Furthermore, this pathway is remarkably similar to the one activated by the IL-1R (which also has a cytosolic TIR domain); indeed, identical molecules comprise the two signaling cascades (93), and until very recently we knew of no signaling components unique to one or the other pathway.

Upon ligation of TLR4 (and IL-1R), the adapter MyD88 is recruited to the receptor complex (94, 95). MyD88 has a C-terminal TIR domain that mediates

its homophilic interaction with the receptor and an N-terminal death domain that engages the death domain of its downstream target IRAK (96). IRAK may be recruited to the receptor via Tollip, an adapter that contains a C2 domain (97). Upon association with MyD88, IRAK, a serine threonine kinase, undergoes autophosphorylation. The RING-finger containing adapter TRAF6 is also part of this activated signaling complex (98). One study suggested that TRAF6 functions as an E3 ligase to ubiquitinate an as-yet-unidentified target that is necessary for TLR- and IL-1R-mediated IκB kinase β (IKK-β activation) (99). Activated IKK phosphorylates and targets for degradation the NF-κB inhibitor IκB, thereby freeing NF-κB to translocate into the nucleus and turn on transcription of target genes (5).

Although all TLRs signal through the conserved signaling cascade described above, the complexity of the TLR-induced cellular responses indicates that there must be additional regulatory mechanisms and signaling pathways downstream of TLRs. One example is provided by the existence of a Rac1-PI3K-AKT pathway activated by TLR2. This pathway leads ultimately to phosphorylation of NF-κB and is necessary for NF-κB transactivation activity (100). It will be important to test whether other TLRs also activate a similar pathway that enhances NF-κB transactivation potential. Because Rac1, PI3K, and AKT regulate diverse cellular functions in other pathways, this study also raises the interesting possibility of links connecting the TLR pathway to other signaling pathways.

Although analyses of knockout mice have confirmed that TLRs and IL-1Rs share in common many of the same signaling components, the members of the two families can induce distinct targets. Even different TLR members activate distinct albeit overlapping sets of target genes. Undoubtedly, there must be mechanisms that enable TLRs to achieve specificity in activation of cellular responses. The first indication of such a mechanism came with the analyses of MyD88-deficient mice (101–103). As expected, these mice were unable to activate NF-κB and MAP kinases, or to upregulate surface expression of MHC and costimulatory molecules in response to IL-1 and many TLR ligands, including peptidoglycan and unmethylated CpG motifs (80, 101–104). Surprisingly, however, the TLR4 ligand LPS could still activate NF-κB and MAP kinases (albeit with delayed kinetics) in the absence of MyD88 (101). Moreover, LPS-stimulated MyD88-deficient dendritic cells retain the ability to upregulate costimulatory and MHC molecules (102). Therefore, although MyD88 is required for all signaling events downstream of some TLRs, such as TLR2 and TLR9, MyD88 is clearly dispensable for some TLR4-induced signals. However, other inflammatory responses appear to be completely dependent on MyD88 regardless of the stimulus; MyD88-deficient mice do not produce the cytokine IL-12 in response to any of the tested PAMPs, including LPS and CpG DNA (102, 104).

These studies indicate that whereas signaling downstream of some TLRs (such as TLR9) and IL-1R is completely dependent on MyD88, TLR4 can activate two pathways, a MyD88-dependent pathway similar to that activated by other TLRs and IL-1R and a MyD88-independent pathway. Furthermore, the existence of a MyD88-independent pathway suggested that TLR4 may transduce some signals through a distinct adapter protein. This hypothesis was borne out by the

identification of the novel TIR domain-containing adapter protein, TIRAP (105). TIRAP contains a small N-terminal region of unknown function and a C-terminal TIR domain that mediates its interaction with TLR4. A dominant negative mutant of TIRAP specifically inhibits TLR4- but not IL-1R- or TLR9-induced NF-κB activation, indicating a specificity of TIRAP for the TLR4 pathway. TIRAP therefore represents the first identified molecule responsible for at least some of the known differences in signaling between TLR4 and other TLRs, as well as between TLRs and the IL-1Rs (105). Although many of the other components and targets of this TIRAP-regulated pathway remain to be identified, one downstream target of the TIRAP pathway is PKR, the interferon-regulated, dsRNA-activated protein kinase (105). Indeed, PKR is also a component of the MyD88-dependent pathway, as stimulation of wild-type (but not MyD88-deficient) macrophages with CpG results in PKR activation. Therefore, PKR, previously identified as a component of antiviral defense and stress responses, also functions in TLR signaling pathways (105).

Toll and Control of Adaptive Immunity

Dendritic cells are pivotally positioned at the interface of innate and adaptive immunity (4). Immature dendritic cells reside in the peripheral tissues, where they actively sample their environment by endocytosis and macropinocytosis. Upon encountering a pathogen, they undergo a developmental program called dendritic cell maturation, which includes induction of costimulatory activity, antigen processing, increased MHC molecule expression, and migration to the lymph node, where they can prime naïve antigen-specific T cells (4). In this way activation of the adaptive immune system occurs only upon pathogen recognition by dendritic cells. Pathogen recognition, of course, is mediated by TLRs on the surface of dendritic cells; not surprisingly, these cells express high levels of most members of the TLR family.

Analysis of MyD88-deficient mice demonstrated the critical role of TLRs in DC maturation and induction of adaptive immune responses. As mentioned above, stimulation of MyD88-deficient DCs with all tested PAMPs except LPS does not result in DC maturation (101, 106). Concomitant with the block in surface expression of costimulatory and MHC molecules, these DCs, not surprisingly, cannot prime antigen-specific naïve T cells in vitro (106). Furthermore, when MyD88-deficient mice are immunized with ovalbumin in complete Freund's adjuvant (CFA), no ovalbumin-specific T cell responses develop. In addition, these mice also fail to produce IFN-γ and ovalbumin-specific IgG2a antibodies (106). These defects are due at least in part to the inability of MyD88-deficient dendritic cells to make IL-12. Remarkably, however, B cells in MyD88-deficient mice make normal amounts of antigen-specific IgG1 and IgE, while T cells produce higher levels of IL-13 upon restimulation than their counterparts from wild-type mice (106). Clearly, then, these mice appear to have a selective defect for mounting Th1 but not Th2 responses, and TLRs seem to control induction of only Th1-type inflammation (106). Indeed, all the known PAMPs are derived from either prokaryotic, fungal, viral, or protozoan pathogens, which are conventionally targets of Th1 responses. TLRs are

essential for recognizing these categories of pathogens and for mounting adaptive immune responses, which are generally Th1-type responses that are appropriate for their elimination. Th2 responses, on the other hand, combat multicellular eukaryotic parasites, which probably are recognized not by TLRs but by a distinct set of pattern-recognition receptors. An interesting implication is that allergens, which also elicit Th2 responses, probably also activate the immune system through TLR-independent mechanisms.

Finally, the in vivo studies with the MyD88-deficient mice also demonstrate that adjuvants, an essential component of most vaccines, exert their immunostimulatory effects through activation of TLRs (106). The active ingredient of CFA, for example, is mycobacterial lysate, a TLR ligand (107, 108). Therefore, adjuvants function by stimulating TLRs expressed on dendritic cells, which in turn leads to dendritic cell maturation and the induction of antigen-specific adaptive immune responses (1, 106).

CONCLUSION

Innate immune recognition is very complex, as it has to be to protect the host against a highly diverse microbial world. But it seems to be in essence much simpler than the adaptive immune response, which operates by recognizing fine details of pathogenic microorganisms. In order to respond, naïve T cells need to recognize both the antigen bound to self-MHC ligands and a molecule of CD80 and/or CD86. These two proteins have to be expressed on the same antigen-presenting cell. The costimulatory molecules are induced by Toll-like receptors. There are ten TLRs in humans and mice, and they or their homologues are found in all multicellular organisms. Once a pathogen is recognized, the host antigen-presenting cell expressed on its surface costimulatory molecules and in the cytosol proinflammatory cytokines and chemokines. These molecules, together, can both attract naïve T cells through the secretions of chemokines and activate naïve T cells to respond to specific antigens of the pathogen. These antigens are displayed on the same cell surface as the induced costimulatory molecules, providing both an antigen-specific stimulus and the required costimulatory molecules to activate naïve, antigen-specific T cells. Once T cells are activated, the adaptive immune response takes over, and the pathogen is engulfed by a phagocyte and destroyed.

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