TOLL-LIKE RECEPTORS

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Abstract The innate immune system in drosophila and mammals senses the invasion of microorganisms using the family of Toll receptors, stimulation of which initiates a range of host defense mechanisms. In drosophila antimicrobial responses rely on two signaling pathways: the Toll pathway and the IMD pathway. In mammals there are at least 10 members of the Toll-like receptor (TLR) family that recognize specific components conserved among microorganisms. Activation of the TLRs leads not only to the induction of inflammatory responses but also to the development of antigen-specific adaptive immunity. The TLR-induced inflammatory response is dependent on a common signaling pathway that is mediated by the adaptor molecule MyD88. However, there is evidence for additional pathways that mediate TLR ligand-specific biological responses.

INTRODUCTION

The immune system detects and eliminates invading pathogenic microorganisms by discriminating between self and non-self. In mammals the immune system can be divided into two branches: “innate immunity” and “adaptive immunity.” Adaptive immunity detects non-self through recognition of peptide antigens using antigen receptors expressed on the surface of B and T cells. In order to respond to a wide range of potential antigens, B and T cells rearrange their immunoglobulin and T cell receptor genes to generate over $10^{11}$ different species of antigen receptors. Engagement of antigen receptors by the cognate antigen triggers clonal expansion of the lymphocyte and further production of antigen-specific antibodies. This highly sophisticated system is observed only in vertebrates and is a potent defense against microbial infection. In contrast, the innate immune system, which was first described over a century ago, is phylogenetically conserved and is present in almost all multicellular organisms (1). Whereas the system of adaptive immunity
has been the subject of considerable study in the past century, innate immunity has been less well appreciated. Therefore, the mechanism by which innate immunity recognizes non-self remained unknown until quite recently. However, the recent identification of Toll-like receptors in mammals has made immunologists aware that innate immunity plays an important role in the detection of invading pathogens. Recent evidence shows that Toll-like receptors recognize specific patterns of microbial components, especially those from pathogens, and regulates the activation of both innate and adaptive immunity. In this review we focus on recent progress regarding the functions of Toll-like receptors and their signaling pathways.

TOLL IN DROSOPHILA

The Toll Pathway

The involvement of the Toll receptors in innate immunity was first described in drosophila. Drosophila Toll was originally identified as a transmembrane receptor required for the establishment of dorso-ventral polarity in the developing embryo (2). Stimulation of Toll by the secreted Spätzle factor, a ligand of Toll, activates the cytoplasmic serine/threonine kinase Pelle via the adaptor protein Tube. Activation of Pelle promotes degradation of the ankyrin-repeat protein Cactus, which associates with the Rel-type transcription factor Dorsal in the cytoplasm. Once Cactus is degraded in response to the Toll-mediated signal, Dorsal is free to translocate to the nucleus, where it regulates transcription of specific target genes (3). The signaling pathway of drosophila Toll shows remarkable similarity to the mammalian IL-1 pathway, which leads to activation of NF-κB, a transcription factor responsible for many aspects of inflammatory and immune responses. Indeed, the cytoplasmic domains of drosophila Toll and the mammalian IL-1 receptor are highly conserved and are referred to as the Toll/IL-1 receptor (TIR) domain. Based on this similarity, it was proposed that the Toll-mediated pathway might be involved in regulating immune responses (3). This was clearly demonstrated in a study of mutant flies lacking individual components of the Toll-mediated pathway, i.e., Toll, Spätzle, Tube, or Pelle (4). Each mutant fly was highly sensitive to fungal infection owing to a lack of expression of the antifungal peptide Drosomycin. In drosophila two additional Rel-type transcription factors, Dorsal-type immune factor, and Relish, have been identified in addition to Dorsal. Dorsal-type immune factor is mainly involved in the induction of antifungal peptide genes in adult flies, whereas Dorsal is involved in dorso-ventral patterning in the embryo (5, 6). Relish regulates the induction of peptides active against Gram-negative bacteria (7). A recent study indicated that the Toll pathway is required for resistance to Gram-positive bacterial infections in addition to fungal infections (8). Indeed, infection with either Gram-positive bacteria or fungi induced Toll-dependent expression of the antifungal peptide Drosomycin.

Tube is an adaptor that functions downstream of Toll and upstream of Pelle. Although Tube and Pelle have been shown to interact via conserved death domains
(9, 10), no direct interaction has been demonstrated between Tube and Toll. However, the protein DmMyD88 appears to function as an adaptor linking Toll and Pelle; the TIR domain of DmMyD88 associates with the TIR domain of Toll, and the death domain of DmMyD88 associates with the death domain of Pelle (11, 12). DmMyD88 mutant flies are highly sensitive to fungal infection, suggesting that DmMyD88 is an essential component of the Toll pathway in drosophila. However, the functional relation between Tube and DmMyD88 remains unclear.

The protein Spätzle is secreted as a precursor form that is cleaved to its active form by a serine protease in response to immune challenge. The cleaved Spätzle then activates Toll. Mutant flies with a loss-of-function mutation in the gene encoding the serine protease inhibitor Spn43Ac exhibit constitutive expression of cleaved Spätzle and, consequently, constitutive expression of Drosomycin (13). These data demonstrate that Toll is indirectly activated by Spätzle, rather than directly by microbial components. The precise mechanism by which Toll is activated in response to microbial infection is not well understood, but a recent genetic study has provided some possible clues. An ethyl-methyl-sulfonate–induced mutation of the semmelweis (sem) gene was shown to cause impaired expression of Drosomycin in response to infection by Gram-positive bacteria but not fungi (14). The gene responsible for the mutation was analyzed and found to encode PGRP-SA, the peptidoglycan recognition protein. PGRP-SA recognizes peptidoglycans that are abundant in Gram-positive bacterial cell walls (15). Thus, infection by Gram-positive bacteria is detected by PGRP-SA, which in turn activates the Toll-mediated pathway. A factor that is involved in the detection of fungal infection and activation of the Toll-mediated pathway has recently been identified. Ethyl-methyl-sulfonate mutagenesis of drosophila produced a mutant with impaired activation of the Toll pathway in response to fungal infection but not to Gram-positive bacterial infection (16). This mutation was localized to the Persephone gene, which encodes a serine protease but possesses no obvious microbial pattern recognition domain (Figure 1). Most likely, there is a molecule upstream of Persephone that detects fungal infection. Identification of this molecule may provide new insights into the mechanisms of microbial recognition in drosophila.

IMD Pathway

The immune response against Gram-negative bacteria is mediated by a distinct pathway first identified by a mutation in the immune deficiency (imd) gene of drosophila (17). Imd mutant flies are highly susceptible to infection by Gram-negative bacteria but not to fungi, whereas Toll mutants are highly susceptible to fungi but not to Gram-negative bacteria. The imd gene encodes an adaptor protein containing a death domain with similarity to the mammalian receptor interacting protein (18). Genetic studies have identified several molecules involved in the IMD pathway that are involved in the response against Gram-negative bacteria. These include DmIKKβ, DmIKKγ, dTAK1, and Relish (7, 19–22). Fruitflies with mutations in these genes are defective in expression of the antibacterial peptide
Diptericin and highly susceptible to Gram-negative bacterial infection. No receptor
involved in the IMD pathway has been identified. Mutant flies lacking 18-wheeler,
a member of the Toll family, are susceptible to Gram-negative bacterial infection,
but expression of Diptericin is normal in these mutants (23, 24). Moreover, there
are nine Toll family members in drosophila, but none of them has been shown
to induce expression of Diptericin (25). These results indicate the existence of a
non-Toll-related receptor that initiates signaling in the IMD pathway. Indeed, a
member of the PGRP family, PGRP-LC, has been implicated in the activation of
the IMD pathway, because induction of Diptericin in response to Gram-negative
bacterial infection was shown to be defective in PGRP-LC mutant flies (26–28).
Unlike PGRP-SA, PGRP-LC is a transmembrane protein (15). Although it remains
unclear whether the intracellular portion of PGRP-LC possesses a domain required
for activation of signaling cascades, it is possible that this protein may act as a
receptor linking the recognition of Gram-negative bacteria to the activation of the
IMD pathway (Figure 1). PGRPs in drosophila consist of a large family containing
12 members (15) and thus could be involved in sensing a variety of different
microbes in drosophila.

One component of the IMD pathway, Relish, is activated by a cleavage into
two domains: the DNA-binding Rel homology domain and the inhibitory ankyrin
repeat domain. Although the mechanism by which Relish is cleaved is unclear,
it has been suggested that Dredd, a homologue of mammalian caspase-8, may be
somehow involved. Dredd mutant flies are defective in cleavage of Relish, and
are very susceptible to infection by Gram-negative bacteria (29–31). Dredd asso-
ciates with drosophila Fas associating death domain (dFADD), a homologue of
mammalian FADD (12, 32). In mammals the FADD/caspase8-dependent pathway
is activated as a result of signaling from the type I TNF receptor (TNF-R1) and
mediates induction of apoptosis. Overexpression of IMD, a homologue of mam-
malian receptor interacting protein that associates with TNF-R1, induces apoptosis
in drosophila, whereas imd mutant flies are resistant to UV-induced apoptosis (18).
Thus, the IMD pathway is presumably involved in the induction of apoptosis as
well as the response against Gram-negative bacteria. This also indicates that IMD
may act upstream of dFADD-Dredd, similar to receptor interacting protein’s act-
ing upstream of FADD-caspase-8 in the mammalian TNF-R1-mediated signal
pathway.

TOLL-LIKE RECEPTORS IN MAMMALS

A year after the discovery of the role of the drosophila Toll in the host defense
against fungal infection, a mammalian homologue of the drosophila Toll was
identified (33). Subsequently, a family of proteins structurally related to drosophila
Toll was identified, collectively referred to as the Toll-like receptors (TLRs). The
TLR family is known to consist of 10 members (TLR1-TLR10), and no doubt more
will be found in the future (33–38). The chromosomal location of each human TLR
gene has been determined. \( TLR1 \) and \( TLR6 \) map very close to 4p14 (34, 35); \( TLR2 \) and \( TLR3 \) map to 4q32 and 4q35, respectively; \( TLR4 \) and \( TLR5 \) map to 9q32-33 and 1q33.3, respectively (34). \( TLR7 \) and \( TLR8 \) are located in tandem in Xp22, whereas \( TLR9 \) maps to 3p21.3 (36, 38).

TLR family members are characterized structurally by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a TIR domain in their intracellular domain. A comparison of the amino acid sequences of the human TLRs reveals that members of the TLR family can be divided into five subfamilies: the TLR3, TLR4, TLR5, TLR2 and TLR9 subfamilies (Figure 2). The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10; the TLR9 subfamily is composed of TLR7, TLR8, and TLR9. In the TLR2 subfamily TLR1 and TLR6 are highly similar proteins and exhibit 69.3% identity in overall amino acid sequence, but the TIR domains of both receptors are highly conserved, with over 90% identity (35). Because \( TLR1 \) and \( TLR6 \) have similar genomic structures, consisting of one exon, and are located in tandem in the same chromosome, they may be the products of an evolutionary duplication. Division of TLRs into five subfamilies is also based on genomic structure. The \( TLR2 \) gene has two exons, but all of the coding sequences are contained within, exon 2. In contrast, the TLR9 subfamily members including TLR7, TLR8, and TLR9 are encoded by two exons (36, 38). The genes for TLR3 and TLR8 show 42.3% identity and 72.7% similarity in their amino acid sequences, have similar genomic structures, and are located close to each other on the X chromosome (36, 38). The \( TLR4 \) and \( TLR5 \) genes have four and five exons,

![Figure 2](image_url)  
**Figure 2** Phylogenetic tree of human Toll-like receptors (TLRs). The phylogenetic tree was derived from an alignment of the amino acid sequences for the human TLR members using the neighbor-joining method.
respectively. TLR3 has a unique structure among the TLRs in that it has five exons and the protein is encoded by exons 2 through 5. This is in contrast to all of the other TLRs, which are encoded by only one or two exons.

**ROLES OF TLRs IN RECOGNITION OF MICROBIAL COMPONENTS**

Ectopic overexpression of TLR4, the first mammalian TLR identified, was shown to cause induction of the genes for several inflammatory cytokines and costimulatory molecules (33). Therefore, it was anticipated that the TLRs might be involved in immune responses, especially in the activation of innate immunity. In 1998 TLR4 was shown to be involved in the recognition of lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacteria. Subsequently, other members of the TLR family have been shown to be essential for the recognition of a range of microbial components (Table 1). The structural similarity of TLRs seems to reflect their common function in the recognition of microbial components.

**TLR4**

**TLR4 RECOGNIZES LIPOPOLYSACCHARIDE** Two mouse strains, C3H/HeJ and C57BL10/ScCr, have long been known to be hypo-responsive to LPS. Two independent groups searching for the genes responsible for this hyporesponsiveness identified mutations in Tlr4 (39, 40). The C3H/HeJ mouse strain has a point mutation in the intracellular region of the Tlr4 gene leading to the replacement of a highly conserved proline with histidine. This mutation results in the generation of a dominant negative allele, defects in TLR4-mediated signaling, and consequent suppression of the response to LPS (41). Another LPS hypo-responsive strain, C57BL10/ScCr, has a null mutation in the Tlr4 gene (39, 40). TLR4-deficient mice generated by gene targeting are also hypo-responsive to LPS, confirming that TLR4 is an essential receptor for the recognition of LPS (41).

Recognition of LPS requires other molecules in addition to TLR4. LPS binds to LPS-binding protein, present in the serum, and this LPS–LPS-binding protein complex is subsequently recognized by CD14, a glycosylphosphatidylinositol-anchored molecule preferentially expressed in monocytes/macrophages and neutrophils. LPS stimulation is followed by increased physical proximity between CD14 and TLR4, suggesting that CD14 and TLR4 may interact in LPS signaling (42, 43). MD-2 was identified as a molecule that associates with the extracellular portion of TLR4 and enhances LPS responsiveness (44, 45). Chinese hamster ovary cell lines that are hypo-responsive to LPS have mutations in the MD-2 gene (46). Generation of MD-2-deficient mice demonstrated its essential role in the response to LPS. Macrophages, dendritic cells, and B cells from MD-2-deficient mice display severely impaired responses to LPS. Furthermore, MD-2-deficient mice are resistant to LPS-induced endotoxin shock, similar to TLR4-deficient mice (47). MD-2 associates with TLR4 in the endoplasmic reticulum/cis Golgi and then the
TABLE 1  Toll-like receptors and their ligands

<table>
<thead>
<tr>
<th>TLR family</th>
<th>Ligands (origin)</th>
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| TLR1       | Tri-acyl lipopeptides (bacteria, mycobacteria)  
|            | Soluble factors (Neisseria meningitides) |
| TLR2       | Lipoprotein/lipopeptides (a variety of pathogens)  
|            | Peptidoglycan (Gram-positive bacteria)  
|            | Lipoteichoic acid (Gram-positive bacteria)  
|            | Lipoarabinomannan (mycobacteria)  
|            | A phenol-soluble modulin (Staphylococcus epidermidis)  
|            | Glycoconjugated phospholipids (Trypanosoma Cruzi)  
|            | Glycolipids (Treponema pallidum)  
|            | Porins (Neisseria)  
|            | Zymosan (fungi)  
|            | Atypical LPS (Leptospira interrogans)  
|            | Atypical LPS (Porphyromonas gingivalis)  
|            | HSP70 (host)  
| TLR3       | Double-stranded RNA (virus)  
| TLR4       | LPS (Gram-negative bacteria)  
|            | Taxol (plant)  
|            | Fusion protein (RSV)  
|            | Envelope proteins (MMTV)  
|            | HSP60 (Chlamydia pneumoniae)  
|            | HSP60 (host)  
|            | HSP70 (host)  
|            | Type III repeat extra domain A of fibronectin (host)  
|            | Oligosaccharides of hyaluronic acid (host)  
|            | Polysaccharide fragments of heparan sulfate (host)  
|            | Fibrinogen (host)  
| TLR5       | Flagellin (bacteria)  
| TLR6       | Di-acyl lipopeptides (mycoplasma)  
| TLR7       | Imidazoquinoline (synthetic compounds)  
|            | Loxoribine (synthetic compounds)  
|            | Bropirimine (synthetic compounds)  
| TLR8       | ?  
| TLR9       | CpG DNA (bacteria)  
| TLR10      | ?  

TLR4/MD-2 complex moves to the cell surface, where excess MD-2 is secreted (48). Whereas TLR4 normally resides on the cell surface in wild-type cells, it is found in the Golgi apparatus in cells deficient for MD-2, indicating that MD-2 is essential for the intracellular distribution of TLR4 (47). Another cell-surface protein, RP105, is also involved in the recognition of LPS. RP105 contains an LRR domain that is structurally related to those found in the extracellular portion of the TLRs...
and is preferentially expressed on the surface of B cells (49). B cells from RP105–deficient mice show a severely reduced response to LPS. RP105 functionally associates with TLR4 to recognize LPS (50). Thus, several components are implicated in the recognition of LPS, indicating that the functional LPS receptor forms a large complex.

OTHER TLR4 LIGANDS  In addition to LPS, TLR4 recognizes several other ligands. Taxol is a product of the Pacific yew (*Taxus brevifolia*) and exhibits potent antitumor activity in humans. The antimitotic action of Taxol is due to its ability to bind and stabilize microtubules, which prevents proper cell division during mitosis. Taxol possesses similar immunostimulatory activities to those of LPS in mice but not in humans. Mouse TLR4 and MD-2 mediate the LPS-mimetic activity of Taxol (51–53). TLR4 and CD14 recognize the fusion protein of respiratory syncytial virus (54, 55). Accordingly, C3H/HeJ and C57BL/10ScNcr mice, which are mutated for TLR4, exhibited a reduced inflammatory response against and impaired clearance of respiratory syncytial virus. Activation of B cells by murine retroviruses such as mouse mammary tumor virus is dependent on TLR4. The envelope proteins of mouse mammary tumor virus and Moloney murine leukemia virus were reported to co-immunoprecipitate with TLR4 (56). Thus, TLR4 is presumably involved in the recognition of a certain group of viruses.

TLR4 seems to recognize some endogenous ligands as well. Heat shock proteins are highly conserved among organisms ranging from bacteria to mammals. A wide variety of stressful conditions such as heat shock, ultraviolet radiation, and viral and bacterial infection induce the increased synthesis of heat shock proteins. The primary functions of heat shock proteins are to chaperone nascent or aberrantly folded proteins. In addition, heat shock proteins activate macrophages and dendritic cells to secrete proinflammatory cytokines and to express costimulatory molecules. Thus, heat shock proteins may be representative of a type of endogenous “danger signal,” i.e., molecules or molecular structures that are released or produced by cells undergoing stress or abnormal cell death (necrosis). These signals are recognized by macrophages and dendritic cells and thereby initiate immune responses (57).

The ability of heat shock protein to activate the immune cells is best documented for the heat shock protein HSP60. The immuno-stimulatory activity of HSP60 is mediated by TLR4 (58, 59). For example, HSP60 has been implicated in inflammation accompanying atherosclerosis, development of which is associated with chronic infection by *Chlamydia pneumoniae*. HSP60 derived from *Chlamydia pneumoniae* (cHSP60) colocalizes with macrophages in the atheromatous lesion and induces an inflammatory response. Therefore, cHSP60 is thought to be one of the factors causing atherosclerosis in chronic Chlamydial infection. cHSP60 also activates vascular smooth muscle cells and macrophages through TLR4 (60, 61). Mice defective for TLR4 show defective production of inflammatory cytokines in response to HSP70 as well as HSP60 (62–64). Thus, TLR4 seems to be responsible for the inflammatory responses elicited by heat shock proteins. However,
both TLR2 and TLR4 are required for recognition of HSP70 (63, 64). CD91 (α-macroglobulin receptor) has been identified as a receptor for several heat shock proteins, including HSP70 (65). Furthermore, HSP60 binds to macrophages from TLR4-deficient C57BL/10ScCr mice, despite the fact that no HSP60-induced production of inflammatory cytokines is observed (66). These data suggest that TLR4 is not directly involved in the recognition of heat shock proteins.

Extracellular matrix components, including fibronectin, hyaluronic acid, and heparan sulfate, are produced in response to tissue injury and play important roles in tissue remodeling, such as containing the agent of injury, closing the wound, and completing the healing. The type III repeat extra domain A of fibronectin has immuno-stimulatory activities similar to those provoked by LPS. This response to extra domain A of fibronectin is mediated by TLR4 (67). In addition, low molecular weight oligosaccharides of hyaluronic acid have been reported to be potent activators of dendritic cells, and activation of dendritic cells by hyaluronic acid is mediated by TLR4 (68). Furthermore, polysaccharide fragments of heparan sulfate have been reported to induce maturation of dendritic cells via TLR4 (69).

Extravascular fibrin deposits are an early and persistent hallmark of inflammation accompanying injury, infection, and immune disorders. Fibrin is generated from plasma-derived fibrinogen, which escapes the vasculature in response to endothelial cell retraction at sites of inflammation. The capacity of fibrinogen to induce the production of chemokines from macrophages is elicited through recognition by TLR4 (70). Thus, TLR4 is presumably involved in several aspects of the inflammatory response by recognizing endogenous ligands produced during inflammation, even in the absence of infection. However, it should be noted that all of these endogenous TLR4 ligands activate immune cells only at very high concentrations, which is in sharp contrast to the low concentrations required for lipopolysaccharide (LPS). Therefore, there remains the possibility that these endogenous ligands might be contaminated with a true TLR4 ligand such as LPS.

One intriguing question is whether TLR4 recognizes its ligands directly or not. Some groups have proposed that recognition of LPS by TLR4 involves direct binding, while others have suggested that LPS binds to MD-2, and this complex somehow stimulates TLR4 (71–74). Species-specific recognition of different ligands provides one kind of genetic evidence for direct interaction. For example, mouse but not human cells recognize Taxol, and this species-specific recognition is conferred by MD-2 (53). Another group showed that human but not murine TLR4 recognizes the highly acylated LPS from Pseudomonas aeruginosa (75).

TLR4-INDEPENDENT RECOGNITION OF LIPOPOLYSACCHARIDE  TLR4 has now been established as an essential component in the recognition of LPS. However, several reports have indicated that LPS can also be recognized independently of TLR4. A study using affinity chromatography, peptide mass fingerprinting, and fluorescence resonance energy transfer identified four molecules on the cell surface that bind LPS. These are HSP70, HSP90, chemokine receptor 4 (CXCR4), and growth differentiation factor 5 (76).
LPS is rapidly delivered into the cytoplasm after binding to the cell surface. This intracellular movement appears to be necessary for certain cellular responses, since agents that block vesicular transport such as wortmannin or cytochalasin D block the integrin-mediated adhesion of neutrophils in response to LPS (77). This suggests that LPS may be recognized in the cytoplasm as well as on the cell surface. A candidate molecule that confers the intracellular recognition of LPS is Nod1. Nod1 was originally identified as a molecule that is structurally related to the apoptosis regulator, Apaf-1, which contains the caspase-recruitment domain and the nucleotide-binding oligomerization domain. Nod1 possesses an N-terminal caspase-recruitment domain linked to a nucleotide-binding domain and a C-terminal LRR domain. Unlike Apaf-1, Nod1 induces activation of NF-κB (78). Nod1 mediates activation of NF-κB in response to LPS and cell-invasive *Shigella flexneri*, indicating that Nod1 is a cytoplasmic receptor for LPS (79, 80). These findings suggest that the Nod family of proteins is involved in inflammatory responses, possibly through the recognition of LPS in the cytoplasm. Nod2, a molecule in the same family as Nod1 and Apaf-1, also confers LPS-induced activation of NF-κB. Furthermore, frameshift and missense mutations in *NOD2* are associated with susceptibility to Crohn’s disease (81, 82). However, the mutations found in these patients are restricted to the LRR domain, which presumably recognizes LPS, and the mutant NOD2 protein is defective in LPS-induced NF-κB activation (81, 82). Therefore, it remains unclear exactly how mutation of *NOD2* may be associated with susceptibility to Crohn’s disease.

**TLR2, TLR1, and TLR6**

**TLR2 recognizes a variety of microbial components** TLR2 recognizes components from a variety of microorganisms. These include lipoproteins from pathogens such as Gram-negative bacteria, Mycoplasma and spirochetes (83–87), peptidoglycan and lipoteichoic acid from Gram-positive bacteria (88–91), lipolamin from mycobacteria (90–93), glycoinositolphospholipids from *Trypanosoma Cruzi* (94), a phenol-soluble modulin from *Staphylococcus epidermidis* (95), zymosan from fungi (96), glycolipids from *Treponema maltophilum* (97), and porins that constitute the outer membrane of *Neisseria* (98). Analysis of TLR2-deficient mice showed that TLR2 is critical to the recognition of peptidoglycan and lipoproteins (99, 100). Accordingly, TLR2-deficient mice showed higher susceptibility to infection by the Gram-positive bacteria *S. aureus* than wild-type mice (101). Another TLR2-deficient mouse strain showed defective clearance of spirochetes after infection by *Borrelia burgdorferi* and unresponsiveness to *B. burgdorferi* lipoproteins (102). Furthermore, TLR2 recognizes several atypical types of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*, in contrast to TLR4, which recognizes LPSs from enterobacteria such as *Escherichia coli* and *Salmonella* spp. (103, 104). The properties of the atypical LPSs recognized by TLR2 differ structurally and functionally from the enterobacteria LPS recognized by TLR4. In particular, the two types of LPS differ structurally in the number of
acyl chains in the lipid A component (105). TLR2 and TLR4 may differentially recognize these structural variations in LPS. However, as with many of these studies, it remains possible that very small amounts of contaminating TLR2 ligand in the LPS preparations might obscure some of these results.

TLR2 COOPERATES WITH TLR1 AND TLR6  One aspect of TLR2 ligand recognition involves cooperation with other TLR family members, in particular TLR6 and TLR1, which confer discrimination among different microbial components. The role of TLR6 was analyzed by introducing a dominant negative form into the RAW264.7 macrophage cell line. Peptidoglycan and secreted modulin from S. aureus are TLR2 ligands that induce TNF-α production in RAW264.7 cells, but these responses are suppressed by expression of dominant negative TLR6 (95, 106). TLR2 and TLR6 co-immunoprecipitate, suggesting that they physically interact in the cell (106). Analysis of TLR6-deficient mice further demonstrated that TLR6 functionally cooperates with TLR2 to recognize microbial lipopeptides (107). For example, bacterial lipopeptides have a NH2-terminal cysteine residue that is triacylated, in contrast to mycoplasmal macrophage-activating lipopeptides 2 (MALP-2) which are only diacylated. Macrophages from TLR6-deficient mice did not show any inflammatory response to MALP-2, whereas these cells responded normally to bacterial lipopeptides. Macrophages from TLR2-deficient mice showed no response to either type of lipopeptide. Reconstitution experiments in TLR2/TLR6 doubly deficient embryonic fibroblasts demonstrated that both TLR2 and TLR6 are required for the response to MALP-2. Thus, TLR6 functionally associates with TLR2 to confer specific recognition of the subtle differences between triacyl and diacyl lipopeptides.

TLR1 has also been reported to functionally associate with TLR2. Cotransfection of TLR1 and TLR2 into HeLa cells confers responsiveness to soluble factors released from Neisseria meningitidis (108). Analysis of TLR1-deficient mice has demonstrated the importance of TLR1 in the recognition of triacyl lipopeptides (109). Macrophages from TLR1-deficient mice showed impaired production of inflammatory cytokines in response to several kinds of triacyl lipopeptides and lipoproteins from mycobacteria. When a range of triacyl lipopeptides with different lengths of fatty acid chains at their N-terminal cysteines was tested on cells from TLR1-deficient mice, the response to lipopeptides with an N-palmitoyl-S-dilauryl cysteine residue was found to be the most impaired. Although this impairment was not complete, this study suggests that TLR1 is responsible for recognizing subtle differences among the lipid moieties of lipopeptides. TLR1 is highly homologous to TLR6. Therefore, TLR6 may in some cases compensate for a deficiency in TLR1, or other TLRs, in the recognition of triacyl lipopeptides. Involvement of TLR1 in the recognition of the outer surface lipoprotein of B. burgdorferi was also shown (110).

Thus, TLR2 has been shown to functionally associate with several TLRs, at least TLR1 and TLR6, and it recognizes a wide variety of microbial components. It is unknown whether dimerization of TLR2 with other TLRs occurs constitutively
or if it is induced in response to ligand stimulation. It is also unknown whether TLR2 forms a large complex containing TLR1, TLR6, and other TLRs.

**TLR5**

Chinese hamster ovary cells expressing human TLR5 are responsive to the culture supernatants of *Listeria monocytogenes*. Purification of the culture supernatants containing TLR5-stimulating activity led to the identification of flagellin as the active component (111). Flagellin is the primary protein component of flagellar, a highly complex structure that extends out from the outer membrane of Gram-negative bacteria. Flagella serve as the propellers that move the bacteria through their aqueous environment. They also aid in the attachment of the bacteria to the host cells, assisting in bacterial invasion and thereby contributing to the virulence of pathogenic bacteria. The flagellin genes from a variety of Gram-negative bacteria share highly conserved regions at their amino- and carboxy-termini, and these regions are responsible for the immunostimulatory activity of flagellin (112).

Flagellin elicits a potent immune response not only in mammals but also in plants. The flagellin-induced immune response in plants is dependent on a MAP kinase signaling cascade (113). A screen for flagellin-insensitive arabidopsis mutants led to the isolation of a single genetic locus, *FLS2*. This gene encodes a transmembrane receptor–like kinase with a leucine-rich repeat (LRR) domain, which shows a structure similar to the extracellular portion of the mammalian TLR family (114). Thus, flagellin represents an evolutionarily conserved pathogenic molecular pattern that is recognized by conserved host receptors containing an LRR domain. In addition to the *FLS2* gene product, plants have several other gene products that are responsible for resistance to pathogens. Many of these products also possess an LRR domain, and some also possess a Toll/IL-1 receptor (TIR) domain, indicating that the LRR and TIR domains are important for the host defense against pathogens in many multicellular organisms (115).

**TLR3**

Double-stranded (ds) RNA is produced by many viruses during their replicative cycle, either as an essential intermediate in RNA synthesis or as a byproduct generated by symmetrical transcription of DNA virus genomes. dsRNA is a potent inducer of type I interferons (IFN-α and -β), which exert various physiological effects including antiviral and immuno-stimulatory activities. dsRNA also induces transcription of some IFN-inducible genes and promotes maturation of dendritic cells. Some synthetic dsRNAs, such as polyinosinic-polycytidylic acid [poly(I:C)], have similar activity to that of dsRNA. Some of the immunostimulatory activity of dsRNA is believed to be elicited by activation of dsRNA-dependent protein kinase (PKR). Embryonic fibroblasts from PKR-deficient mice showed impaired responses to dsRNA and poly(I:C), although some responses remained (116, 117). PKR-deficient mice were further shown to be susceptible to respiratory infection by vesicular stomatitis virus, but the responses to infections by other routes such
as intravenous and intraperitoneal routes were apparently normal. This indicates that additional molecules other than PKR might be responsible for the recognition of dsRNA and viruses (118, 119).

Expression of human TLR3 in the dsRNA-nonresponsive cell line 293 conferred enhanced activation of NF-κB in response to dsRNA and poly(I:C). Furthermore, TLR3-deficient mice showed impaired responses to dsRNA and poly(I:C), indicating that TLR3 is a receptor for dsRNA (120). However, further studies will be required to clarify the mechanisms by which PKR is linked with TLR3 in the dsRNA-mediated pathway. In addition, the more fundamental question of whether TLR3 is actually involved in the recognition of viruses remains an intriguing but unanswered one. The principal cell in human and mouse blood that produces type I interferon in response to viral challenge is plasmacytoid dendritic cell (121–123); however, TLR3 is not expressed in this cell type (124). Therefore, other receptors in addition to TLR3 might be responsible for the recognition of viral infection leading to production of IFN-α/β.

TLR3 has unique structural features among the TLRs. For example, TLR3 lacks the proline residue that is conserved among other TLRs. This proline is mutated in the Tlr4 gene of C3H/HeJ mice and is responsible for the LPS-hyposensitive phenotype of this strain. The genomic organization of TLR3 is also different from the other TLRs. TLR3 is also unique in that it is preferentially expressed in mature dendritic cells (125). Therefore, TLR3 may have a unique function in addition to the recognition of dsRNA.

**TLR9 and TLR7**

**TLR9 IS ESSENTIAL FOR RECOGNITION OF CPG DNA** Bacterial DNA is a potent activator of immune cells. The critical involvement of TLR9 in the recognition of bacterial DNA was demonstrated using TLR9-deficient mice (126). The immunostimulatory activity of bacterial DNA is attributed to the presence of unmethylated CpG motifs, which are relatively infrequent in the vertebrate genome and when they occur are typically methylated on their cytosine residues and lack any immunostimulatory activity. Thus, CpG DNA is another prototypic molecular pattern by which the immune system recognizes pathogens. Synthetic oligodeoxynucleotides containing unmethylated CpG motifs also activate immune cells. Administration of CpG DNA is sufficient to protect against infections by intracellular pathogens such as *Leishmania major* and *Listeria monocytogenes* in mice (127–129). Furthermore, CpG DNA activates dendritic cells to produce the Th1-polarizing cytokine IL-12, leading to the development of Th1-like immune responses. Therefore, CpG DNA has promising therapeutic value as an adjuvant and antiinfectious agent (130, 131).

Human and mouse immune cells are optimally activated by slightly different CpG motifs (132). This specificity can be explained by species differences among TLR9s. When mouse or human TLR9 was expressed in the CpG DNA-unresponsive cell line 293, these cells gained the ability to respond to the optimal mouse or human CpG sequence, respectively (133). These findings also indicate
that TLR9 directly recognizes CpG DNA. Thus, the identification of optimal CpG motifs or other synthetic agonists of TLR9 from humans and other diverse animals may lead to the establishment of effective adjuvants for each species.

Several studies have reported that CpG DNA is recognized in the endosome following nonspecific uptake into the cells (130, 131). This suggests that recognition of CpG DNA by TLR9 occurs in the endosome. Indeed, CpG DNA-induced activation of signaling cascades such as c-Jun N-terminal kinase (JNK) and NF-κB is delayed compared with LPS-induced activation in normal macrophages (126). Recently, a monoclonal antibody against TLR9 has been established, and staining with this antibody indicated the intracellular localization of endogenous TLR9 in a mouse macrophage cell line (134). This is in sharp contrast to TLR1, TLR2, and TLR4, which are expressed on the cell surface (44, 75, 135, 136). TLR2 is recruited to the phagosomes after stimulation with zymosan (96, 106). Thus, internalization of TLR ligands may be required for full activation of immune cells by TLRs, or signaling pathways via TLR9 may have some distinct characteristics from other TLRs.

TLR7 RECOGNITION OF SYNTHETIC AGONISTS  TLR7 and TLR8 are highly homologous to TLR9, as mentioned above. Although the natural ligands of TLR7 and TLR8 remain unclear, the TLR9 subfamily including TLR7, TLR8, and TLR9 may participate in the discrimination of nucleic acid-like structures in microorganisms. This parallels the situation in the TLR2 subfamily, which discriminates between differences in lipoproteins. One such example was demonstrated in TLR7-deficient mice. Several synthetic imidazoquinolines have demonstrated potent antiviral and antitumor properties, owing to their ability to induce inflammatory cytokines, especially IFN-α. One of these imidazoquinoline compounds, Imiquimod, has been approved for the treatment of genital warts caused by infection of human papillomavirus. Recently, it was shown that TLR7-deficient mice do not respond to synthetic imidazoquinolines (137). These compounds have structures similar to nucleic acids, and TLR7 may sense viral infection by recognizing a similar, as yet undetermined viral component or product, or a host compound induced in response to virus. In addition, our unpublished data (S Akira) indicate that two other immunomodulators, loxoribine and bropirimine, also activate immune cells through TLR7 (Figure 3). Loxoribine (7-allyl-8-oxoguanosine) enhances natural killer (NK) cell activity and induces production of cytokines including IFNs; it is anticipated to be useful for the clinical treatment of cancer (138). Bropirimine (2-amin-5-bromo-6-phenyl-4(3)-pyrimidinone) is an orally active immunomodulator that induces production of cytokines including IFN-α and is in clinical use against renal cell carcinoma (139). Thus, the TLR family recognizes not only microbial components but also clinically useful synthetic compounds, suggesting that a screen for TLR-activating agents will be useful for clinical applications. We anticipate that new therapies utilizing the TLR-mediated innate immune activation will be developed to treat several disorders such as infection, cancer, and allergy.
EXPRESSION OF TLRs

Distribution of TLRs

The expression of TLR family members has been elucidated in several studies. Monocytes/macrophages express mRNA for most TLRs except TLR3 (125). Expression of TLRs in dendritic cells differs among their subsets (124, 140). In humans blood dendritic cells contain two subsets, myeloid dendritic cell (MDC) and plasmacytoid dendritic cell (PDC) (141–143). MDCs express TLR1, 2, 4, 5, and 8, and PDCs exclusively express TLR7 and TLR9, although there are some reports that TLR7 is also expressed in MDC (124, 140, 144, 145). Immature dendritic cells mature in response to microbial components (146–149), and the expression of different TLRs shows distinct patterns during maturation. Expression of TLR1, 2, 4, and 5 is observed in immature dendritic cells but decreases as the dendritic cells mature (136). TLR3 is expressed only in mature dendritic cells (125). Thus, TLRs are differentially expressed in different subsets and maturation stages of dendritic cells. Another study has examined expression of all the human TLR mRNAs in a range of tissues (150). This study indicated that most tissues express at least one TLR, and that phagocytes in particular show abundant expression of all known TLRs, although several TLRs are preferentially expressed in B cells. Further study will be required to clarify the tissue distribution of each TLR.

Mast cells have been preserved throughout evolution and have the capacity to phagocytose pathogens, process antigens, and produce inflammatory cytokines, indicating their potential role in the innate immune response against infectious organisms as well as in allergic diseases (151). Mast cells express TLR2, 4, 6, and 8 but not TLR5 (152, 153). Furthermore, mast cells from TLR4-mutated mice
showed defective production of inflammatory cytokines in response to LPS. When mice lacking mast cells were reconstituted with TLR4-mutated mast cells, it was observed that recruitment of neutrophils in the peritoneal cavity after enterobacteria infection was impaired (152). Intradermal injection of peptidoglycan (PGN) caused TLR2-mediated activation of mast cells in skin, which may be involved in the inflammatory lesions of atopic dermatitis (154). Thus, TLRs are expressed in mast cells and may play a role in their innate immune responses.

In addition to innate immune cells, TLRs are expressed in several other types of cells that contribute to inflammatory responses. The mucosal surfaces of the respiratory and intestinal tract are covered by a single layer of epithelial cells, forming a protective barrier against pathogens. In the intestine the apical surfaces of epithelial cells are continually exposed to bacteria, but this does not result in exaggerated inflammation. These epithelial cells elicit inflammatory responses only against pathogenic bacteria that invade into the basolateral compartment from the apical side. For example, exposing the basolateral, but not apical, surface of model intestinal epithelia to the TLR5 ligand, flagellin, induces an inflammatory response. Furthermore, TLR5 is expressed exclusively on the basolateral surface of the intestinal epithelial cells (155). TLR4 is expressed at relatively low levels in intestinal epithelial cells, which may explain why lipopolysaccharide (LPS) does not elicit a strong inflammatory response in the intestine (156, 157). In contrast, intestinal epithelium from patients with inflammatory bowel diseases showed augmented expression of TLR4 (158). This is consistent with the idea that inflammatory bowel diseases may result from exaggerated inflammatory responses to intestinal bacterial flora. Thus, TLR expression is finely regulated in epithelial cells, perhaps explaining why pathogenic Gram-negative bacteria, but not commensal bacteria, induce inflammatory responses in the intestine.

An epithelial cell line from the small intestine shows a peculiar type of LPS response: In response to LPS it does not produce inflammatory cytokines, but instead produces the chemokine MIP-1. TLR4 is not expressed on the cell surface of small intestine epithelial cells, but resides in the Golgi apparatus and is colocalized with LPS (159). LPS is internalized and delivered to the Golgi apparatus, thereby enabling LPS-induced cell activation (160). Therefore, the expression of TLR4 in the Golgi apparatus would be important for LPS-induced induction of chemokines by LPS in the small intestinal epithelia. Renal epithelial cells are important barriers to Gram-negative pyelonephritis. Expression of TLR2 and TLR4 in renal epithelial cells is induced by IFN-γ and TNF-α and contributes to the detection of bacterial invasion in the lumen of tubules and induction of the inflammatory response (161). TLR4-deficient mice are defective in the production of inflammatory cytokines after intrapulmonary administration of *Haemophilus influenzae*. This finding indicates that TLR4 plays an important role in sensing *H. influenzae* infection in the pulmonary epithelia (162). TLR4 is also expressed on corneal epithelial cells and contributes to the inflammatory responses leading to river blindness following invasion of parasitic filarial nematodes (163). Microvascular endothelial cells are the first lines of defense against invading microorganisms. Human dermal
endothelial cells express TLR4, indicating a possible role in detection of pathogens by endothelial cells (164).

Regulation of TLR Expression

Expression of TLRs is modulated by a variety of factors such as microbial invasion, microbial components, and cytokines. Infection by Mycobacterium avium induces augmented TLR2 mRNA expression and decreased TLR4 mRNA expression in macrophages (165) and leads to increased TLR2 promoter activity accompanied by chromatin remodeling (166, 167). Nontypeable H. influenzae activates NF-κB through TLR2 and induces expression of TLR2 in epithelial cells in an autocrine manner (168, 169). Infection of mice with E. coli induces expression of TLR2 mRNA in γδT cells, which is thought to represent a more primitive, early line of cellular defense, preprogrammed to recognize a limited set of antigens (170). Viral infection also induces expression of the TLR1, TLR2, TLR3, and TLR7 mRNAs in macrophages. Increased TLR expression is suppressed by treatment with anti-IFN-α/β antibody, indicating that IFN-α/β mediates virus-induced activation of innate immunity via modulation of TLR expression (171). LPS enhances expression of TLR2 in macrophages and adipocytes (172, 173). In contrast, LPS stimulation of mouse macrophages causes a reduction in surface expression of the TLR4/MD-2 complex, and this may be one mechanism underlying the phenomenon of LPS tolerance (74, 174).

Several cytokines regulate expression of the TLRs. Colony-stimulating factor 1 is induced in vivo after infection or challenge with LPS and can prime macrophages to respond to further LPS stimulation with enhanced inflammatory cytokine production. Colony-stimulating factor 1 can downregulate TLR9 expression in macrophages and strongly suppresses CpG DNA-induced production of inflammatory cytokines (175). Macrophage migration inhibitory factor (MIF) is an important cytokine that mediates inflammation and sepsis (176). MIF-deficient mice are defective in their responses to LPS. Recently, this defect was shown to be the result of decreased expression of TLR4. Introduction of antisense MIF mRNA into normal cells resulted in reduced TLR4 promoter activity and a reduced LPS response, indicating that MIF regulates TLR4 expression (177). IFN-γ, which primes phagocytes to respond to LPS, enhances surface expression of TLR4 in human monocytes and macrophages (178). Expression of the Tlr2 gene in macrophages is induced by LPS and inflammatory cytokines such as IL-2, IL-15, IL-1β, IFN-γ, and TNF-α (172). IL-15, a cytokine that promotes extrathymic development and survival of T cells, especially CD8+ T cells and NK cells, induces expression of the Tlr2 gene in T cell lines through the activation of Stat5 (179). T1/ST2, a member of the IL-1 receptor (IL-1R) family, is expressed by fibroblasts, mast cells, and Th2 cells, but not Th1 cells, and exists in both membrane-bound and soluble forms. Blocking ligand activation of T1/ST2 causes downregulation of TLR4. For example, incubation of macrophage cultures with the soluble form of T1/ST2 downregulates TLR4 mRNA expression, and administration of
anti-T1/ST2 antibody to mice reduces the mortality of LPS-induced endotoxin shock (180).

TLR-MEDIATED SIGNALING PATHWAYS

The pathways that transduce TLR signals in mammals have both similar and dissimilar characteristics from those in drosophila. In drosophila the Toll- and IMD-pathways are essential for antifungal and anti-Gram negative bacterial responses, respectively. In mammals the host defense against microorganisms mainly relies on pathways that originate from the common TIR domain of TLRs. The TLR family signaling pathway is highly homologous to that of the IL-1R family. Both TLR and IL-1R interact with an adaptor protein MyD88, which has a TIR domain in its C-terminal portion but a death domain in its N-terminal portion instead of the transmembrane domain found in TLRs. MyD88 associates with both the TLRs and the IL-1R via interaction between the respective TIR domains. Upon stimulation, MyD88 recruits a death domain–containing serine/threonine kinase, the IL-1R-associated kinase (IRAK). IRAK is activated by phosphorylation and then associates with TRAF6, leading to activation of two distinct signaling pathways, JNK and NF-κB (181–185).

MyD88-Dependent Signaling Pathway

Studies of MyD88-deficient mice revealed that this protein plays a critical role in the response to IL-1 and LPS (186, 187). Macrophages from MyD88-deficient mice do not produce any inflammatory cytokines in response to peptidoglycan, lipoproteins, CpG DNA, dsRNA, or the imidazoquinolines (100, 120, 137, 188–190). MyD88-deficient mice are also unable to produce any detectable level of IL-6 in response to flagellin (111). These results demonstrate that MyD88 is critical to the production of inflammatory cytokines induced by the TLR family. Indeed, no activation of NF-κB or JNK was observed in MyD88-deficient macrophages in response to peptidoglycan, lipoprotein, CpG DNA, or the imidazoquinolines. Accordingly, MyD88-deficient mice were found to be highly susceptible to infection by S. aureus (101). Similarly, TRAF6-deficient mice exhibit impaired responses to both IL-1 and LPS, indicating that TRAF6 is a critical component of both the IL-1R- and TLR4-mediated signaling pathways at a level downstream of MyD88 (191, 192). The IRAK family is comprised of four members that contain a conserved death domain and kinase domain: IRAK-1, IRAK-2, IRAK-M, and IRAK-4 (193). IRAK-1-deficient mice were found to have partial defects in their responses to IL-1 and LPS (194–196). In contrast, IRAK-4-deficient mice show almost no inflammatory responses to either IL-1 or LPS (197). Among the IRAK homologs, IRAK-4 is most structurally related to its drosophila counterpart, Pelle (197). These findings indicate that IRAK-4 is an essential component in IL-1- and TLR4-dependent signaling pathways.

The MyD88-dependent pathway signals via MyD88, IRAK, and TRAF6 and leads to NF-κB activation. The activity of NF-κB is regulated by association with
IκB, which sequesters NF-κB in the cytoplasm until phosphorylated on serine residues by the IκB kinase (IKK) complex. This phosphorylation leads to the dissociation and nuclear translocation of NF-κB. The IKK complex contains two catalytic subunits, IKKα and IKKβ, as well as a scaffold protein, IKKγ. LPS stimulation enhances the activity of IKK in a human monocytic cell line (198, 199). Although IKKα is dispensable for IL-1- and LPS-induced NF-κB activation, cells from mice deficient in IKKβ or IKKγ show impaired NF-κB activation and IL-6 production in response to IL-1 and LPS (117, 200). This shows that these IKK components are critical to the TLR-mediated signaling pathway.

In drosophila dTAK1 acts upstream of dIKKβ and dIKKγ. Studies using dTAK1-mutant flies showed that dTAK1 plays an essential role in Gram-negative bacteria-induced activation of Relish, an NF-κB-like transcription factor in the IMD pathway (21). In vitro over-expression studies showed that both IL-1 and LPS activate mammalian TAK1, which in turn activates NF-κB (201–203). However, the physiological role of TAK1 remains to be elucidated. Recent studies have suggested a unique mechanism by which TRAF6 is linked to the IKK complex. A mammalian protein complex that activates IKK was purified and analyzed and found to be composed of two subunits: TAK1 and a ubiquitin conjugating enzyme complex composed of Ubc13 and Uev1A. TRAF6 functions together with Ubc13/Uev1A to catalyze the Lys 63 (K63)–linked polyubiquitination of TRAF6 itself (204). TAK1 is consequently activated via its association with the ubiquitinated TRAF6. Once activated, TAK1 mediates phosphorylation of the IKK complex (205). Ubiquitination is thought to be a step that directs modified target proteins to the proteasome, where they are degraded. However, ubiquitination of TRAF6 mediates activation of NF-κB through a process that does not require protein degradation.

A candidate molecule that links TRAF6 and NF-κB was identified in a screen of TRAF6-interacting molecules. This molecule is designated ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) (206) and it interacts with TRAF6 and MEKK1, a MAP kinase kinase kinase family member that mediates the activation of NF-κB. However, the biological function of ECSIT remains to be elucidated.

An additional molecule that mediates TLR-induced signaling has been reported. Receptor interacting protein-2 (RIP2) contains a C-terminal caspase-recruitment domain and was originally identified as a serine/threonine kinase that associates with the TRAFs and with TNF receptor family members such as the type I TNF receptor and CD40 to induce NF-κB activation and apoptosis (207, 208). Mice deficient in RIP2 exhibit partial impairment in their response to LPS, peptidoglycan, and dsRNA (209, 210). Furthermore, RIP2 associates with TLR2, indicating that RIP2 is somehow involved in TLR signaling pathways.

**MyD88-Independent Signaling Pathway**

**LPS-INDUCED RESPONSE IN THE ABSENCE OF MYD88** MyD88 is essential for the production of inflammatory cytokines in response to a variety of microbial
components. However, LPS is still able to induce activation of NF-κB and JNK in MyD88-deficient macrophages, but with delayed kinetics (187). This indicates that although MyD88 is important for LPS-induced production of inflammatory cytokines, there exists an MyD88-independent component in the LPS signaling pathway. Evidence is accumulating that MyD88-independent activation of the LPS-TLR4 signaling pathway is of biological importance. Dendritic cells from MyD88-deficient, but not from TLR4-deficient mice showed enhanced expression of costimulatory molecules and increased T cell allo-stimulatory activity in response to LPS. This indicates that LPS-induced maturation of dendritic cells depends on the MyD88-independent pathway (211, 212). LPS stimulation induces caspase-1-dependent cleavage of the IL-18 precursor into its mature form in Kupffer cells from MyD88-deficient mice (213). Analysis of LPS-induced genes in MyD88-deficient macrophage showed that a number of IFN-regulated genes are upregulated, such as those encoding IP-10 and GARG16 (214). Thus, several LPS-induced responses occur in MyD88-deficient mice. In addition to LPS, dsRNA induced activation of NF-κB in MyD88-deficient mice, although no dsRNA-induced production of inflammatory cytokines was observed (120). It is not known whether activation of MyD88-independent signaling induced by dsRNA and LPS are equivalent or not.

MOLECULES INVOLVED IN THE MYD88-INDEPENDENT PATHWAY  In the course of analyzing the MyD88-independent activation of LPS signaling, a novel adaptor molecule named TIR domain–containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) was identified (215, 216). Similar to MyD88, TIRAP/Mal possesses a C-terminal TIR domain but lacks an N-terminal death domain. It specifically associates with TLR4 through interaction between their respective TIR domains. The dominant-negative form of TIRAP/Mal inhibited TLR4-mediated activation but not TLR9-mediated activation of NF-κB. Furthermore, LPS-induced maturation was abolished in both wild-type and MyD88-deficient dendritic cells treated with a cell-permeable TIRAP peptide that blocks TIRAP-mediated signaling. These in vitro findings indicate that TIRAP/Mal is a possible adaptor molecule involved in LPS-induced, MyD88-independent signaling (Figure 4). The generation and analysis of TIR domain–containing adaptor protein/MyD88-adaptor-like (TIRAP/Mal)–deficient mice will clarify its physiological role in TLR4-mediated signaling.

LPS stimulation of MyD88-deficient macrophages also activates IRF-3 (214). LPS-induced activation of IRF-3 causes expression of several IFN-inducible genes (216). Activation of IRF-3 was observed when cells were stimulated with ligand for TLR4 but not TLR2 (214, 217). Viral infection and dsRNA, which also activate the MyD88-independent pathway, are also known to activate IRF-3, thereby inducing the IFN-α/β- and IFN-regulated genes (218–220). Therefore, IRF-3 may play an important role in the MyD88-independent pathway.

Similar to TLR4-mediated signaling, each TLR seems to have its own signaling pathway in addition to the common MyD88-dependent pathway. In
TLR2-mediated signaling, stimulation with heat-killed *S. aureus* results in the recruitment of active RacI and phosphatidylinositol-3 to the cytoplasmic portion of TLR2. This in turn causes activation of Akt, which is followed by the activation of the p65 subunit of NF-κB in a process that is independent of IκBα degradation (221). Stimulation of dendritic cells with TLR2 and TLR4 agonists induces mRNA expression for distinct types of cytokines and chemokines (222). The existence of individual pathways for each TLR may explain the distinct biological responses elicited by different TLR agonists.

In addition to MyD88 and TIRAP/Mal, another adaptor molecule named Toll-interacting protein (Tollip) has been identified (223). Tollip was first identified in the context of IL-1 signaling and was shown to be present in a complex with IRAK. Upon stimulation with IL-1, the Tollip-IRAK complex is recruited to the IL-1R complex through the association of Tollip with IL-1RαC. Interaction with MyD88, which is also recruited to the signaling complex, then triggers IRAK auto-phosphorylation, which in turn leads to the rapid dissociation of IRAK from Tollip. A subsequent study showed that Tollip negatively regulates the TLR-mediated signaling pathway (224, 225). Overexpression of Tollip blocked activation of NF-κB in response to IL-1, TLR2, and TLR4 agonists. However, it remains unclear what physiological roles Tollip plays in TLR signaling.

**Transcription Factors Activated in the TLR-Mediated Signaling Pathway**

NF-κB is a transcription factor that was originally identified as a nuclear factor necessary for the transcription of immunoglobulin light chain in B cells. Subsequently, NF-κB was shown to be expressed in a variety of cell types. The NF-κB family of transcription factors is evolutionarily conserved. In *drosophila*, three members have been identified, as mentioned earlier: Dorsal, Dorsal-type immune factor, and Relish. In mammals five family members have been identified: RelB, c-Rel, p65 (RelA), p100/p105, and p105/p50 (226). Each member of the NF-κB family plays an important role in LPS-mediated responses. For example, B cells from mice deficient in p50, RelA, c-Rel, or RelB displayed an impaired growth response to LPS (226). Mice lacking individual NF-κB subunits were very susceptible to microbial infections (227–229). The critical involvement of NF-κB in the development and function of dendritic cells has also been shown. RelB-deficient mice showed defective development of a dendritic cells subset (230–232). In mice doubly deficient for p50 and p65, the development of dendritic cells was also impaired. In contrast, the development of dendritic cells was normal, but IL-12 production was severely impaired in mice doubly deficient for p50 and c-Rel (233). This indicates that the function and development of dendritic cells is finely regulated by distinct NF-κB subunits. Necrotic cells, but not apoptotic cells, induce inflammatory responses (57). Necrotic cells induce TLR2-dependent activation of NF-κB, and embryonic fibroblast cells from mice deficient in the p65 subunit of NF-κB are defective in the necrotic cell–induced expression of chemokines (234).
**AP-1** The AP-1 (activating protein-1) family of transcription factors consists of homodimers and heterodimers of the Jun and Fos family, which bind to the 12-O-tetradecanoylphorbol-13-acetate response element (235). Jun proteins form not only homo- and hetero-dimers within the AP-1 family but also form heterodimers with members of the CREB/ATF family of transcription factors, such as ATF, and therefore are able to bind to the cAMP response element (CRE). The activity of AP-1 is upregulated through phosphorylation by the MAP kinases JNK and ERK (236). LPS and peptidoglycan enhance the transcriptional activity of AP-1 and the CREB/ATF family of transcription factors (237–239). In addition, viral infection and dsRNA activate AP-1 through induction of JNK (117).

**NF-IL6** NF-IL6 is a member of the C/EBP family of transcription factors, which contain basic and leucine zipper domains (240). NF-IL6 was originally identified as a nuclear factor that specifically binds to an IL-1 responsive element in the IL-6 gene promoter (241). NF-IL6 was subsequently shown to be activated by phosphorylation in response to inflammatory stimuli and to play an important role in macrophage responses (242). Indeed, macrophages from NF-IL6-deficient mice display defective killing activity against *Listeria monocytogenes* (243). NF-IL6 is critical for LPS-induced gene expression in macrophages. Macrophages from NF-IL6-deficient mice show defective expression of LPS-inducible genes such as Cox-2, a C-type lectin Mincle, and membrane-bound glutathione-dependent prostaglandin E2 synthase (244–246). Thus, NF-IL6 is a nuclear target in the TLR-mediated signaling pathway.

**IRF** The IRF family of transcription factors is composed of nine members that are critical regulators of innate immune responses (247). Among these, IRF-3 is presumably involved in the MyD88-independent signaling pathway, as described above. The expression of IRF-1 is markedly induced by viral infection. Macrophages from IRF-1-deficient mice show defective induction of IL-12 and iNOS in response to LPS (248, 249). IRF-7 is also induced by viral infection and critically involved in the biphasic system of IFNα/β gene induction in conjunction with IRF-3 (220). IRF-8/ICSBP is critical for induction of the IL-12 gene. As a result, IRF-8/ICSBP-deficient mice are highly susceptible to infection with *Toxoplasma gondii* and *Leishmania major* owing to defective Th1 responses (250, 251).

**OTHER TRANSCRIPTION FACTORS**

LPS stimulation induces activation of the STAT family of transcription factors (252). Bacterial infection or LPS stimulation of macrophages leads to the rapid phosphorylation of a serine residue in Stat1 (253). In macrophages from Stat1-deficient mice, LPS-induced expression of IFN-regulated genes such as IP-10, IRF-1, and iNOS was reduced. These findings indicate that Stat1 may be involved...
in the response to LPS (254). The STAT family of transcription factors has been established as critical molecules in cytokine signaling pathways (255). Indeed, other studies showed that LPS stimulation of macrophages induced expression of IFN-β through activation of the MyD88-independent pathway, and IFN-β in turn induced IFN-regulated gene expression through activation of Stat1 (256, 257). Therefore, Stat1 seems to be indirectly involved in the LPS-induced expression of IFN-regulated genes.

The Sp1 transcription factor is also involved in LPS-induced gene expression and plays a prominent role in the induction of IL-10 gene expression in both human and mouse macrophages (258, 259).

MODULATION OF IMMUNE RESPONSES BY TLRs

Regulation of Adaptive Immunity by TLRs

Recognition of microbial components by TLRs triggers activation of not only innate immunity but also adaptive immunity. The signals for activation of adaptive immunity are largely provided by dendritic cells. Immature dendritic cells residing in the periphery have a high capacity for endocytosis, which facilitates antigen uptake. They are activated by various microbial components to undergo maturation and express many of the TLRs, such as TLR1, 2, 4, and 5 (136). Furthermore, maturation of dendritic cells by a variety of microbial components is elicited through TLRs; this includes LPS, CpG DNA, peptidoglycan, lipoprotein, and the cell wall skeleton of Mycobacteria (126, 147–149, 212). TLR-mediated recognition of microbial components by dendritic cells induces the expression of costimulatory molecules such as CD80/CD86 and production of inflammatory cytokines such as IL-12 (260). Once matured, dendritic cells lose their capacity for endocytosis and migrate into the draining lymph nodes. Here they present microorganism-derived peptide antigens expressed on the cell surface with MHC class II antigen to naive T cells, thereby initiating an antigen-specific adaptive immune response (261, 262). The involvement of TLRs in the regulation of the adaptive immune response was demonstrated in vivo using MyD88-deficient mice. MyD88-deficient mice immunized with Ag mixed with complete Freund’s adjuvant (CFA) exhibited defective production of both IFN-γ from CD4+ T cells and Ag-specific IgG2a (263, 264). Furthermore, the Th1 immune response provoked by a protozoan parasite was abolished in MyD88-deficient mice (265). Thus, the Th1 immune response is regulated by the MyD88-dependent signaling pathway.

It has been proposed that distinct types of dendritic cell subsets differentially induce Th1 and Th2 responses (141–143). However, the functions of these dendritic cell subsets are rather flexible, and their ability to steer a particular type of Th cell development can depend on the microbial microenvironment (162, 266). Activation of TLR4 or TLR9 in dendritic cells induces production of IL-12, thereby skewing Th cell differentiation toward the Th1 type. LPSs from E. coli (TLR4
ligand) and *Porphyromonas gingivalis* (a putative TLR2 ligand) induce Th1-type and Th2-type responses, respectively, in vivo (267). This differential outcome was attributed to the ability of *E. coli* LPS but not *P. gingivalis* LPS to induce production of IL-12 from CD8+ dendritic cells. Thus, TLR signaling in dendritic cells is critically involved in determining the Th1/Th2 balance. MyD88-deficient mice exhibit a skewed Th2 response against Ag administered along with CFA or Th1-inducing microbial stimuli (263–265). The skewed Th2 response in MyD88-deficient mice does not seem to be caused by a default pathway active in the absence of IL-12 production, because IL-12-deficient mice do not show a Th2 response (265). Furthermore, TLR4 signaling stimulates wild-type and MyD88-deficient dendritic cells to support Th1 and Th2 cell differentiation, respectively (264). Although this finding indicates that activation of the MyD88-independent pathway downstream of TLR4 leads to differentiation of dendritic cells into Th2-supporting dendritic cells, there is little evidence to show that TLRs are involved in the helminth-induced Th2 response (268). It remains unclear whether the Th2 response is TLR-independent or not.

Analysis of the in vivo antigen-specific responses in MyD88-deficient mice suggested that the immuno-stimulatory activity of adjuvants such as CFA is elicited through the TLRs (263). Indeed, CFA contains a complex mixture of mycobacterial components. In addition to CFA, several microbial components have potent immuno-stimulatory activity as adjuvants. CpG DNA, which is recognized by TLR9, is a potent adjuvant that elicits a skewed Th1 response (269, 270). The outer membrane proteins of *Neisseria*, porins, have potent immunogenicity and are used as adjuvants in various vaccine formulations. The Neisserial porins have been shown to be recognized by TLR2 (98). Similar to CFA, the cell-wall skeletal fraction from *Mycobacterium bovis* BCG strain (BCG-CWS) has potent immunogenicity and is used as an adjuvant for immunotherapy in cancer (271). Recognition of BCG-CWS is dependent on TLR2 and TLR4 (147). Thus, several pathogen-derived adjuvants are recognized by TLRs, which may explain the molecular mechanism of their adjuvanticity.

### Crosstalk Between Type I IFNs and TLRs

Activation of TLRs in dendritic cells leads to production of type I IFNs (IFN-α/β), which promote dendritic cell maturation and induce some Th1-type chemokine genes (257, 272). Type I IFNs induce production of antigen-specific immunoglobulins with all isotypes in a dendritic cell–dependent manner (273). CFA-induced immune responses are abolished in IFN-α/βR-deficient mice (273). Thus, type I IFNs are critical to the link between innate and adaptive immunity. Patients with systemic lupus erythematosus manifest elevated levels of serum IFN-α, which induces dendritic cell differentiation, indicating that deregulation of type I IFN production can lead to immunological disorders (274). Dendritic cell subsets, such as myeloid dendritic cells (MDC) and plasmacytoid dendritic cells (PDC), respond to different repertoires of pathogenic stimuli. In humans
MDCs produce IL-12 in response to a variety of stimuli including LPS, whereas PDCs preferentially produce IFN-α upon viral infection and in response to CpG DNA (121, 275). Different TLRs are expressed between MDC and PDC, as described above. However, the pattern of TLR expression alone does not determine how dendritic cell subsets differentially respond to pathogenic stimuli. It has recently been shown that the same TLR7 ligand induces production of IL-12 in MDC, but IFN-α in PDC, indicating that distinct patterns of response are determined not only by TLR expression but also by the dendritic cell lineage (145).

A murine counterpart of human PDCs has been identified as an IFN-α-producing cell population (MIPC) (122, 123). MIPCs are CD11c dull B220 + Gr-1 + and reside in the spleen or bone marrow. Similar to human PDCs, MIPCs express TLR7 and TLR9 and produce IL-12 in response to CpG DNA (122, 123, 276). MIPCs play crucial roles in the production of type I IFN and IL-12 during MCMV infection (277). It remains unknown how TLRs on MIPC are involved in antiviral immune responses.

Involvement of the TLRs in Microbial Killing

In addition to controlling the development of adaptive immunity, activation of TLRs appears to be directly involved in induction of antimicrobial activity. TLR2 activation leads to nitric oxide–dependent and –independent killing of intracellular Mycobacterium tuberculosis in mouse and human macrophages, respectively (278). In drosophila activation of the Toll and IMD pathways by microbial invasion leads to the synthesis of antimicrobial peptides. Expression of a single antimicrobial peptide is sufficient to rescue the susceptibility of Spätzle/IMD double mutant flies to microbial infection, indicating that antimicrobial peptides play an essential role in the host defense in drosophila (279). These antimicrobial peptides are evolutionarily ancient and conserved between humans and plants and have been shown to directly kill microbes (280). In mammals antimicrobial peptides such as β-defensins are produced in several kinds of epithelial cells residing in the gastrointestinal tracts, respiratory tracts, and skin (280). Paneth cells in the base of the crypts of gastrointestinal tracts secrete α-defensins in response to LPS or bacterial challenge (281). Thus, mammalian antimicrobial peptides are produced in response to microbial stimuli at the epithelial surface, the front line of defense between pathogen and host. Strong expression of TLR4 occurs in the crypts of the small intestine (159). LPS induces expression of mouse β-defensin-2, -3, and -6 (282). Stimulation of the human lung epithelial cell line A549 with lipoprotein led to TLR2-mediated induction of β-defensin-2 (283). These findings indicate that TLRs are likely to mediate the secretion of antimicrobial peptides, thereby regulating the direct killing of microbes at the epithelial surface. This potential involvement of TLRs in induction of mammalian antimicrobial peptides needs to be analyzed more precisely.

Macrophages infected with invasive bacteria undergo apoptosis (284). Although the implications of this phenomenon remain elusive, the induction of apoptosis...
may limit the spread of pathogens by localizing cell death at the site of pathogen invasion. Apoptosis of macrophages and endothelial cells is triggered by several microbial components such as LPS and lipoprotein. TLR2 confers lipoprotein-induced apoptosis of macrophages, indicating the possible involvement of TLRs in infection-induced cell death (83). TLR2-mediated apoptosis involves MyD88 and an apoptotic pathway involving FADD and caspase 8. MyD88 associates with FADD via their respective death domains (84). LPS-induced apoptosis in endothelial cells is mediated by MyD88, IRAK-1, and FADD (285, 286). Thus, TLRs are presumably involved in apoptosis induced by microbial components. MyD88 and IRAK-1, both of which possess death domains, may induce apoptosis via interaction with FADD and consequent activation of the FADD–caspase 8 apoptotic signaling pathway. In addition to the induction of apoptosis, the FADD-dependent pathway mediates the activation of NF-κB and induction of inflammatory gene expression, indicating the possible involvement of FADD in TLR-mediated pathways (287, 288). However, there is a report showing that FADD suppresses activation of NF-κB by LPS (289). Thus, more experiments are required to clarify the role of FADD in TLR signaling.

FUTURE PROSPECTS

Since the discovery of the TLRs a few years ago, much progress has been made in our understanding of the mechanisms of innate immune recognition. The innate immune system detects the invasion of microorganisms through the TLRs, which recognize microbial components and trigger inflammatory responses. The TLRs also play a role in instructing the adaptive immune response. However, many questions remain to be answered. There are some TLRs that have unknown microbial ligands. It remains unclear whether TLR recognizes microbial components by direct binding or by some indirect mechanism. It is also unclear where each TLR recognizes these components—on the cell surface or in some intracellular compartment such as the phagosome or endosome. Many questions also remain to be answered with regard to TLR signaling pathways: How does activation of individual TLRs lead to differential gene expression and biological responses, and what kinds of signaling cascades do individual TLRs activate in addition to the common MyD88-dependent pathway? Finally, the fact that activation of TLRs leads to the induction not only of innate immunity but also of adaptive immunity suggests that the TLRs could be involved in some immune disorders as well as infectious diseases. Indeed, several autoimmune diseases have been shown to be associated with infection and dysregulation of innate immune activation (290). Furthermore, autoreactive B cells specific for self-IgG have been shown to be activated by an IgG/chromatin immune complex that synergistically activates the antigen receptor—and MyD88-dependent signaling pathways (291). This strongly suggests that autoimmune disorders are induced by the cross talk between adaptive and innate immune signaling pathways. By elucidation of these issues, we should
be able to increase our understanding of the complex nature of both the innate and adaptive immune systems.

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Host defense responses in drosophila. In drosophila, the Toll and IMD pathways confer host defense against pathogen invasion. The Toll pathway regulates production of antimicrobial peptides against fungi and Gram-positive bacteria. PGRP-SA is essential for activation of the Toll pathway in response to Gram-negative bacteria. Persephone is involved in activation of the Toll pathway in response to fungi. PGRP-LC recognizes the invasion of Gram-negative bacteria and is required for activation of the IMD pathway, which is essential for anti-Gram negative bacterial responses.
Figure 4  Toll-like receptor (TLR) signaling pathway. TLRs recognize specific patterns of microbial components. MyD88 is an essential adaptor for all TLRs and is critical to the inflammatory response. In the case of the TLR4-mediated pathway, lipopolysaccharide (LPS)-induced activation of signaling molecules such as IRF-3, PKR, MAP kinase, and NF-kB has been reported, indicating the presence of the MyD88-independent pathway. TIRAP/Mal was identified as a component specifically involved in TLR4-mediated signaling.