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 the (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.

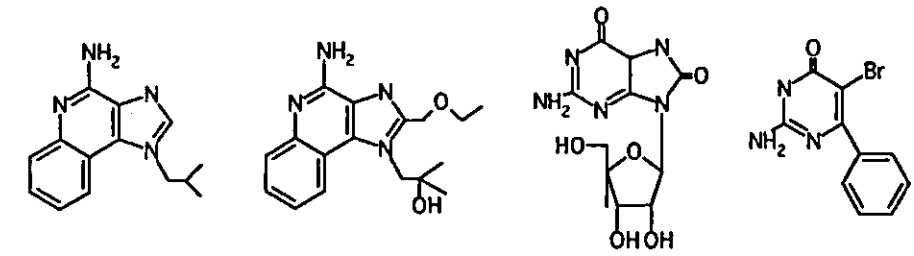

Figure 3 Structures of synthetic compounds that activate TLR7. Analysis of TLR7-deficient mice revealed that TLR7 recognizes several synthetic compounds, which are structurally related to nucleic acids. These include imidazoquinoline (Imiquimod and R-848), loxoribine, and bropirimine.

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. infulenzae* 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 $\gamma\delta$T 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 show 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 phosphatidyl-inositol-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-1RAcP. Interaction with MyD88, which is also recruited to the signaling complex, then triggers IRAK autophosphorylation, 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 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/p52, 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 E_2 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 *Porphylomonas 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 disregulation 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 $CD11c^{dull}B220^{+}Gr\text{-}1^{+}$ 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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