Toll-like Receptors and Innate Immunity

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Toll-like receptors (TLRs) are the best-characterized membrane-bound receptors in innate immune cells, including macrophages and dendritic cells. Upon recognition of specific ligands originating from pathogen- and modified self-derived molecules, TLRs trigger intracellular signaling cascades that involve various adaptor proteins and enzymes, resulting in the generation of proinflammatory and antimicrobial responses through the activation of transcription factors such as nuclear factor-κB. TLR-dependent signaling pathways are tightly regulated during innate immune responses by a variety of negative regulators. This review focuses on the newly described regulation of TLR-dependent signaling pathways, and emphasizes the roles of TLRs in innate immunity. Efforts to modulate these regulatory pathways and signaling molecules may result in the development of new therapeutic strategies through TLR-based therapy.

Key Words: Toll-like receptor, Nuclear factor-κB, Innate immunity

INTRODUCTION

The mammalian innate immune system acts as a sentinel by facilitating the efficient recognition of infectious microbes and providing protective mechanisms that eradicate microbial infections. In this context, Toll-like receptors (TLRs) are the best-characterized innate receptors, can be rapidly activated, and consist of functional modules that provide crucial host defense during microbial infection (1). Different TLRs sense the unique molecular signatures of microbes and trigger the innate immune system (2). The sensing of distinct microbial components by TLRs triggers intracellular signal transduction pathways, which induce the expression of proinflammatory mediators and cytokines through the activation of transcription factors, such as nuclear factor (NF)-κB (3). Several TLR domain-containing adaptors, such as Myeloid differentiation primary response gene 88 (MyD88), toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP)/MyD88 adaptor like (Mal), TIR-domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM), play a role in the regulation of TLR-mediated signaling pathways (3). The activated NF-κB pathway contributes to both antiapoptotic and proinflammatory functions (4). Indeed, dysregulation of the NF-κB pathway is responsible for sustained inflammatory responses and may promote to malignancy. Thus, NF-κB activation must be tightly controlled by post-translational modifications including phosphorylation and ubiquitination (4).

The innate receptors activated by stimulation with different infectious stimuli also involve other signaling pathways, such as the mitogen-activated protein kinases...
(MAPK) and phosphoinositide 3-kinase (PI3K) pathways, through which they play key roles in integration of the innate and inflammatory immune responses (5). The ability of the TLR-induced NF-κB pathway to cross-talk with other signaling molecules is a key element in shaping the overall pattern of host responses, such as antimicrobial killing mechanisms, production of cytokines and chemokines, maturation of antigen presenting cells, and recruitment of the adaptive immune response (5–7). Research into TLR-dependent intracellular signal transduction pathways has highlighted roles for the NF-κB and MAPK pathways in determining the outcomes of various infectious and inflammatory conditions.

In addition, a variety of regulatory factors that control TLR activation have been reported to be involved in the negative feedback of TLR-dependent signaling (7). This coordinated activation of immune signaling pathways is required for the optimal and effective induction of host defense, eradication of invading pathogens, and maintaining cellular homeostasis. This review focuses on recent advances in our understanding of the role and regulatory mechanisms of TLR-induced signaling as it relates to innate and inflammatory responses. This issue not only has crucial implications for understanding host innate mechanisms, but also for controlling harmful inflammatory conditions.

1. Overview of TLR family

Toll was the first protein in the fruit fly Drosophila melanogaster to be described as a key receptor for dorso-ventral polarity in the developing fly embryo, and is required for host defense against fungal infections (8). The subsequent identification of mammalian TLRs has provided key insights into microbial pathogenesis and human protective immunology. The TLR family of innate receptors plays a critical role in recognition and effector functions during infection. To achieve this, different TLRs sense distinct conserved molecular patterns of various microorganisms, thus providing the innate immune system with a degree of specificity against different pathogens (1). To date, 10 functional TLRs have been identified in humans and 12 in mice. The mammalian TLR1-9 are conserved; however, mouse TLR10 is not functional, and TLR11-13 have been lost from the human genome (9). The TLR family can be divided into extracellular and intracellular receptors: TLR1, 2, 4, 5, 6, and 10 are on the cell surface, whereas TLR3, 7, 8, and 9 are present in intracellular endosomal/lysosomal compartments and the endoplasmic reticulum (ER) (10).

TLR signaling is initiated by stimulation of ligands and activated by intracellular adaptor proteins (Fig. 1). Of the plasma membrane-associated TLRs, TLR4 is perhaps the best-investigated, and is the receptor for the Gram-negative bacterial lipopolysaccharide (LPS). Bacterial LPS is composed of three distinct regions: a hydrophobic lipid A (endotoxin), a non-repeating core oligosaccharide, and a distal polysaccharide (or O side chain) (11). Through the potent immunostimulatory activity of LPS, TLR4/LPS signaling has been implicated in a variety of diseases, such as septic shock (12). TLR2, as a heterodimer with either TLR1 or TLR6, can recognize lipoproteins from certain non-entrobacteria and the lipoarabinomannan of mycobacteria (13). In addition, TLR2 is functionally associated with dectin-1, a lectin family receptor for the fungal cell wall component β-glucan (14). TLR5 and TLR11 are able to recognize protein moieties from bacteria and parasites. Bacterial flagellin, a component of the bacterial flagellum, can be recognized by TLR5 and induces proinflammatory responses and host defenses at epithelial and mucosal surfaces (15). TLR11 recognizes a profiling-like protein of Toxoplasma gondii and is required for interleukin (IL)-12 production and resistance to infection with protozoan pathogens (16).

Additionally, TLRs located in intracellular organelles, such as endosomal/lysosomal compartments and the endoplasmic reticulum, can recognize viral and/or synthetic nucleic acid ligands. TLR3 can recognize viral double-stranded RNA (dsRNA), which is generated by RNA viruses during infection (17). TLR7 recognizes the imidazoquinoline family antiviral compounds imiquimod (also known as Aldara, R-837 or S-26308) and resiquimod (also known as R-848 or S-28463) (18). Additionally, TLR7 and 8 recognize single-stranded RNA from RNA viruses (19, 20). TLR9 recognizes non-methylated 2′-deoxyribo (cytidine-phosphate-
Figure 1. Toll-like receptor signaling pathways. Toll-like receptor (TLR) signaling is activated by stimulation of TLR ligands. TLR stimulation recruits MyD88 adaptor protein to the all TLRs except for TLR3. MyD88 interacts with a complex of the IRAKs and TRAF6 to activate the TAK1, which subsequently induces translocation of NF-κB and AP-1 to the nucleus through degradation of IκB proteins and activation of MAPKs, respectively. It leads to the expression of genes encoding the pro-inflammatory cytokines. Mal is also recruited to the TLR2/1, TLR2/6 and TLR4 to activate the MyD88-dependent pathway. TRIF protein is recruited to TLR3 and TLR4, which induces the interaction with a complex of TRAF3, TBK1 and IκB to activate phosphorylation of IRF3. Activated IRF3 is dimerized and translocated into the nucleus, which induces protein expression of type I IFNs. TRIF also interacts with a TRAF6-RIP1 complex to activate NF-κB. TRAM is responsible for activation of TRIF-dependent pathway in TLR4, but not TLR3 signaling. Stimulation with ligands for TLR7, TLR8 and TLR9 forms a signaling complex consisting of MyD88, IRAK4, TRAF6, TRAF3, and IRAK1. TRAF6 and TRAF3 are responsible for activation of NF-κB (for proinflammatory cytokines) and IRF7 (for type I IFNs), respectively.
guanosine) (CpG) DNA motifs derived from host or pathogen genomes (21).

Various endogenous molecules originating from stressed or damaged cells have also been associated with the initiation of TLR responses (10). Although this review cannot cover all of the endogenous molecules that function as TLR ligands, they are comprised of a variety of proteins, nucleic acids, and glycosaminoglycans, including heat shock proteins that stimulate innate and inflammatory immune responses. Controlling the interactions between endogenous ligands and TLRs may provide new therapeutic strategies for various human inflammatory and neurologic diseases (22).

2. TLR signaling pathways: activation of NF-κB and MAPK

TLRs consist of three major domains: (1) a leucine rich extracellular domain; (2) a transmembrane domain; and (3) a cytoplasmic TIR domain. TLR signaling is initiated by the interaction of an agonistic ligand with the extracellular domain that harbors a leucine rich repeat (LRR), which is composed of 19–25 tandem copies of the LRR motif (23). Ligand recognition by TLRs results in the structural rearrangement of the extracellular domains or changes the conformation of a pre-existing dimer; such changes facilitate the close apposition of the cytoplasmic domain and recruitment of signaling adaptors (10, 24).

Specific adaptor molecules such as MyD88, Mal, TRIF, and TRAM play a crucial role in the activation of the TLR signaling cascade. These adaptor proteins commonly contain TIR domains and mediate TIR-TIR interactions among receptors and adaptor molecules during TLR signaling (25). Upon ligand activation of all TLRs, except for TLR3, the adaptor MyD88 associates with the TIR domain, and recruits IL-1 receptor-associated kinase (IRAK) family members to the TLR. IRAK is then activated through phosphorylation and associates with the downstream adaptor tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). The resulting IRAK-1/TRAF6 complex dissociates from the TLR and then associates with TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins, such as TAB1 and TAB2. During this signaling, the Lys 63-linked polyubiquitination of TRAF6 is crucial for the induction of TRAF6-mediated activation of TAK1, and finally of the NF-κB pathway (26). This activated TAK1 complex then activates the IkB kinase (IKK) complex, consisting of IKKα, IKKβ, and IKKγ/NF-κB essential modulator (NEMO), which catalyzes IkB phosphorylation. IkB is then destroyed by the proteosome pathway, allowing for NF-κB translocation into the nucleus. This results in the production of inflammatory cytokines and mediators (27).

Additionally, TLR3 uses TRIF to activate interferon-regulated factor 3 (IRF3) through a MyD88-independent and TRIF-dependent pathway. TLR4 is the only TLR that uses MyD88 and TRIF-dependent pathways. The TRIF pathway has been reported to induce interferon (IFN)-β production through the activation of IRF3 (28, 29). TRIF associates with TRAF6 and TANK-binding kinase (TBK)-1 in an independent manner (30). A novel inhibitory role for MyD88 in TLR3-TRIF signaling was also reported wherein MyD88 prevents activation of the TRIF pathway upon TLR3 stimulation through inhibition of c-Jun N-terminal kinase (JNK) phosphorylation (31).

Further, other adaptors including Mal, TRAM, and sterile α and HEAT/armadillo (ARM) motif protein (SARM) have been demonstrated to play an essential role in the regulation of TLR-dependent signaling pathways as bridging adapters (32). Mal recruits MyD88 to TLR2 and TLR4, whereas TRAM recruits TRIF to TLR4 (32). Although Mal is required for TLR2 and 4 signaling, it also inhibits TLR3 signaling to the JNK pathway and IL-6 induction (33).

There are four groups of mammalian MAPKs: extracellular signal-regulated kinase 1/2 (ERK1/2), p38 proteins (p38α/β/γ/δ), JNKs and ERK5 (34). The MAPK pathways regulate numerous cellular events, including cellular proliferation, survival, and inflammatory responses (34). Regarding TLR activation, TAK1 is a member of the MAP kinase kinase kinase (MAPK3K) family and phosphorylates MKK3 and MKK6, which subsequently activates the MAPK pathways JNK and p38 MAPK (27). All three MAPKs (ERK1/2, p38, and JNK) are activated by various TLR agonistic ligands including LPS, peptidoglycan, polyI:C, and unmethylated CpG DNA (35). Many of the roles of
TLRs cell proliferation and/or apoptosis might be associated with the signaling components of the MAPK cascades and their crosstalk with PI3K and other signaling molecules (5).

3. The roles of TLR signaling in innate immunity: animal studies

The roles of TLRs in innate immunity have been characterized in mice deficient in individual TLRs. TLR4 and 2 are sequentially involved in the innate immune responses to the Gram-negative bacterial pathogen Salmonella (36). TLR2-TLR4 double-deficient mice were more susceptible than TLR4-deficient mice, although MyD88-deficient mice were the most susceptible, when challenged with Salmonella typhimurium (36). Other studies have shown that MyD88-deficient mice have an increased susceptibility to, and decreased cytokine responses upon acute infection with, Trypanosoma cruzi; however, TLR2-deficient mice had no major defect in parasite control (37). In a mouse model of Clostridium difficile infection, MyD88-deficient mice had severe and often fatal intestinal disease (38). Moreover, TLR5 ligation using flagellin enhances host resistance to C. difficile infection in vivo (39).

Earlier studies reported that TLR2- and MyD88-deficient mice exhibit an increased susceptibility and bacterial burden in the kidneys and blood after systemic infection with Staphylococcus aureus (S. aureus) (40). MyD88-deficient macrophages did not produce cytokines in response to S. aureus, although TLR2-deficient macrophages produced detectable cytokine levels. Both TLR2 and IL-1 are required for host protection from systemic and cutaneous S. aureus infection (41). Generally, the phenotype of MyD88-deficient mice is more severe than that of TLR2-deficient mice (41). In nasal, cutaneous, and corneal infection models, TLR2 deficiency is associated with higher bacterial loads and an increased disease severity (41). However, TLR9-deficient mice did not show an impaired response to S. aureus corneal infection (41). MyD88- and IL-1R-deficient mice were more susceptible to S. aureus infection than TLR2-deficient mice (42), suggesting that other family members contribute to IL-1R/TLR signaling.

TLR5 has a dual role in host defense against microbial infection in terms of infection route and infectious dose (43). TLR5-deficient mice exhibited increased susceptibility to urinary tract infection with Escherichia coli, and increased inflammation in the bladder and kidneys (44). TLR5 contributes to protection after systemic infection with S. typhimurium and intranasal infection with Pseudomonas aeruginosa, although this can be masked by TLR4 and other flagellin-sensing pathways (45). In contrast, a deleterious role for TLR5, which is mainly expressed on intestinal CD11c+ lamina propria cells, was reported in mice orally infected with S. typhimurium (46). In this study, susceptibility and survival were dependent on the transport of pathogens from the intestinal tract to the mesenchymal lymph nodes, and TLR5-deficient mice showed improved protection against S. typhimurium (46).

Recent studies by Arpaia, et al. (47) suggested a role for TLR signaling in the induction of signals for bacterial expression of virulence genes. Mice deficient in both TLR2 and TLR4 are highly susceptible to oral infection with S. typhimurium, as well as depressed innate responses. However, TLR2-TLR4-TLR9 triple-knockout mice were less susceptible to infection than TLR2-TLR4 double-knockout mice, although they showed a marked reduction in cytokine production (47). Interestingly, induction of Salmonella pathogenicity island 2 (SPI-2) genes that encode proteins for survival within the Salmonella-containing vacuole (SCV) was absent in cells from TLR2-TLR4-TLR9 triple-knockout mice, thereby inhibiting intracellular replication (47).

4. The roles of TLR signaling in innate immunity: clinical evidence

Microbial infection initiates TLR responses, and this interaction between TLRs and pathogen-associated molecular patterns (PAMPs) results in the induction of an array of antimicrobial immune responses. Various cytokines and chemokines, including TNF-α, cytokines of the IL-1 family (IL-1β, IL-18), IL-12, and IFN-γ, can be induced by the recognition of PAMPs by TLRs. The appropriate activation of these inflammatory cytokines and antimicrobial proteins is required for the induction of host defense against diverse microbial infection (48).
Evidence for the essential role of human TLRs in host defense was obtained in patients with germline mutations or variations in TLR and TLR signaling proteins. Human primary immunodeficiencies, such as anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) that carry either X-linked recessive hypomorphic mutations in NEMO or autosomal dominant hypermorphic mutations in \( \text{IKBA} \), have a cellular defect in NF-\( \kappa \)B activation (degradation of NF-\( \kappa \)B inhibitor \( \alpha \)) and exhibit diminished responses to TLR stimulation (49). Most patients with IRAK-4 deficiency have invasive pyogenic bacterial diseases and/or peripheral infections, particularly those caused by \( \text{Streptococcus pneumoniae} \) (50). Previous studies have shown that patients with defects in UNC-93B (51), TLR3 (52, 53), or TRAF3 adaptor molecule (54), suffer from herpes simplex encephalitis. TLR3 signaling is associated with mutation of UNC-93B (51), a protein present in the endoplasmic reticulum and known to interact with TLR3, 7, 8, or 9 (55). Fibroblasts with an autosomal dominant TLR3 deficiency infected with herpes simplex virus 1 exhibited impaired IFN-\( \beta \) and -\( \lambda \) production, suggesting that TLR3-mediated immunity is essential for protection against HSV-1 in the central nervous system during primary infection in childhood (53).

5. Negative regulators in TLR signaling

TLR signaling pathways are tightly controlled to prevent excessive and uncontrolled inflammatory responses that often lead to deleterious pathogenesis with an increased mortality rate. In TLR signaling, several negative regulators that function through the prevention of ligand-receptor binding, degradation of the target protein, and inhibition of recruitment or transcription of intermediates, have been identified. We will briefly discuss several key regulators of TLR signaling (Fig. 2).

Soluble receptors play a central role in the regulation of inflammation in various conditions. Earlier studies demonstrated that the soluble forms of TLRs (sTLRs), including TLR2 and TLR4, function as a feedback mechanism for the inhibition of excessive TLR activation. The soluble form of TLR4 (sTLR4) significantly abrogates LPS-mediated TNF production and NF-\( \kappa \)B activation via blockade of TLR4-MD2 interactions in mouse macrophages (56). The soluble form of TLR2 (sTLR2), which is naturally expressed in breast milk and plasma, is produced by post-translational

![Figure 2. Negative regulators of Toll-like receptor signaling.](image-url)
modification of the TLR2 protein (57). sTLR2 is not constitutively released in the resting state, but is upon cell activation, and inhibits IL-8 and TNF production by stimulation of bacterial lipopeptide (TLR2 ligand) (57). These findings suggest that soluble forms of TLR2 and TLR4 function as a critical first-line negative regulator in TLR signaling.

The transmembrane receptors ST2 and single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) are involved in the negative regulation of TLR signaling (58, 59). ST2 (also known as T1, Fit-1 or DER4) is an orphan receptor that has two main forms, ST2L and sST2 (60). ST2L belongs to the IL-1 receptor family, which comprises three extracellular immunoglobulin-like domains and an intracellular TIR domain (60). ST2L overexpression was shown to attenuate NF-κB activation induced by IL-1 and LPS, but not poly I:C. Macrophages from ST2L-deficient mice enhanced the production of proinflammatory cytokines in response to IL-1 receptor and TLR4, but not TLR3 (58). In addition, ST2L interacts with the essential TLR adaptors MyD88 and Mal, but not TRIF or IRAK, through proline 431 in box2 of the TIR domain (58). These data indicate that ST2L can inhibit IL-1 and TLR signaling through sequestration of the TLR proximal signaling components MyD88 and Mal. SIGIRR is also an orphan receptor of the IL-1 receptor family, and contains a single immunoglobulin domain and a conserved TIR domain (61). SIGIRR-deficient bone marrow-derived dendritic cells, but not macrophages, had higher proinflammatory cytokine and chemokine production in response to LPS and CpG ODN. SIGIRR-deficient mice are more susceptible to intestinal inflammation, but not to systemic inflammation, such as endotoxic shock (59). SIGRR can inhibit TLR responses through binding to TLR4, IRAK, and TRAF6 in a ligand-dependent pathway (62).

Other intracellular proteins, such as MyD88 short protein (MyD88s), interleukin-1 receptor-associated kinase-M (IRAKM), and A20, have been shown to negatively regulate TLR signaling. MyD88s, an alternatively spliced form of MyD88, can inhibit IL-1- and LPS-, but not TNF-, induced NF-κB activation. MyD88s-MyD88 heterodimers are more often recruited to the IL-1R complex than MyD88 homodimers, and fail to activate IRAK phosphorylation, although they still bind IL-1R and IRAK (63). Among the IRAK family members, IRAKM, which lacks intrinsic kinase activity, is mainly expressed in peripheral blood leukocytes and its expression is increased by TLR stimulation (64). In one study, IRAKM-deficient macrophages markedly enhanced the production of inflammatory responses to bacterial infection and reduced tolerance in response to endotoxin (65). The mechanisms by which IRAKM regulates TLR signaling are involved in the dissociation of the IRAK1 and IRAK4 complex from MyD88, thereby preventing formation of the IRAK1-TRAF6 complex (65). A20 is one of the best-characterized negative regulators of TLR signaling. A20 was initially reported to be a TNF-induced novel zinc-finger protein that inhibits TNF-induced NF-κB activation (66, 67). Further research revealed that A20 is an inducible cysteine protease de-ubiquitinylating enzyme that removes ubiquitin moieties from TRAF6 to terminate TLR signaling in both the MyD88-dependent and MyD88-independent TLR-signaling pathways (68). A20 regulates the production of inflammatory cytokines in response to TLR2, 3, and 9 ligands, and modulates the development of endotoxin-induced lethal shock (68).

Our recent studies have added an orphan nuclear receptor, small heterodimer partner (SHP) (69), to the known negative regulators of TLR signaling. SHP contributes to the transcriptional regulation of various metabolic pathways, including cholesterol homeostasis, duodenal expression of secretin, and hepatic glucose homeostasis (70). SHP-deficient mice exhibited increased susceptibility to endotoxin- or zymosan-induced sepsis in vivo. SHP-deficient myeloid lineage cells secreted larger amounts of proinflammatory cytokines and chemokines in response to various TLR or non-TLR ligands, with the exception of Dectin-1, when compared to wild-type cells (71). Dual mechanisms were determined to be involved in SHP inhibition of TLR signaling (71, 72). In resting cells, SHP inhibits NF-κB-dependent signaling through interaction with NF-κB p65. In addition, after TLR stimulation, SHP attenuates K63-linked polyubiquitination of TRAF6 through interaction
with TRAF6 via its RING-domain (71). These findings demonstrate a novel negative role for SHP in the modulation of TLR-dependent signaling.

**Conclusion**

The roles of TLRs in innate immunity and inflammation have been well-characterized. Upon stimulation, TLRs initiate intracellular signaling cascades to activate proinflammatory and innate immune responses. Each TLR recognizes distinct PAMPs to produce unique outcomes. TLR signaling pathways are activated by several intracellular adaptors and kinases and are associated with the signaling components of MAPK pathways. NF-κB-mediated transcription is required for the induction of the proinflammatory cytokines and mediators that contribute to innate and adaptive immunity. The diverse signaling pathways that cross-talk with TLRs and NF-κB are being progressively unraveled. A number of animal and clinical studies have revealed that TLR signaling pathways play a key role in innate immunity and host defense against pathogenic microbes. Recent insights into the function of several molecules involved in the negative regulation of TLR signaling have extended our understanding of the inhibitory feedback mechanisms through which a variety of extracellular and intracellular decoys fine-tune the activation of innate immune responses. TLR signaling plays a role in the pathogenesis of numerous human diseases; thus, therapies targeting TLR signaling are being developed. Understanding the roles of TLRs and their regulators in animals and humans will facilitate the development of novel therapeutics for TLR-mediated diseases.

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