Toll-like receptors: function and roles in lung disease

Subhendu Basu and Matthew J. Fenton
Mucosal Biology Research Center, Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201

CHARACTERISTIC STRUCTURAL FEATURES

Mammalian Toll-like receptor (TLR) proteins derive their name from the Drosophila Toll protein, with which they share sequence similarity. Toll was originally shown to be critical for dorsal-ventral patterning in fly embryos (64). Generation of adult flies expressing mutant Toll revealed that this transmembrane receptor also served as a critical component of host immunity against fungal infection (44). Toll is a member of a family of related proteins in the fly. This family includes the proteins 18-wheeler, Toll-3 through Toll-9, MstProx, STSdm2245, Tollo, and Tehao (20, 29, 46, 50). Toll and 18-wheeler have been shown to be important for host defense against pathogens, most likely because they direct the production of several antimicrobial peptides (e.g., defensins). Importantly, the cytoplasmic domain of Toll was found to be similar to the cytoplasmic domain of the mammalian IL-1 receptor, suggesting that orthologous receptors might be encoded in the mammalian genome. The ensuing hunt for bona fide human orthologs of Drosophila Toll led to the discovery of “hToll” by Medzhitov et al. in 1997 (48). These investigators expressed a constitutively active mutant of hToll in THP-1 monocytic leukemia cells and demonstrated that this expression resulted in activation of the transcription factor NF-kB as well as expression of several immune-response genes. After these initial studies, additional mammalian TLR proteins were identified, and hToll was subsequently renamed TLR4.

TLR proteins are a family of type I transmembrane receptors characterized by an NH2-terminal extracellular leucine-rich repeat domain (LRR) and a COOH-terminal intracellular tail containing a conserved region called the Toll/IL-1 receptor (TIR) homology domain. The extracellular domain contains a varying number of LRR, which are presumably involved in ligand binding, although these domains may also be necessary for TLR dimerization. The extracellular domain of TLR4 is highly polymorphic compared with the proximal cytoplasmic domain of the protein (63). In addition, the extracellular domain of TLR4 contains an 82-amino acid region that is highly variable and contributes to species-specific differences in recognition of the prototypic TLR4 ligand LPS (27). The intracellular TIR domain region spans over 150 amino acids and contains three highly conserved regions (52). The TIR domain mediates protein-protein interactions between TLR proteins and their downstream signal transduction components. The TIR domain is the defining motif of the TLR/IL-1 superfamily, and it is likely to be one of the earliest signaling domains to have evolved (40). A TIR-like motif is also present in the intracellular domain of several plant receptors known to confer disease resistance (52). Although the IL-1 receptor and TLR proteins possess very different extracellular domains, their TIR domains allow both receptor types to activate similar transduction pathways.

TLR PROTEINS AND THE IMMUNE RESPONSE

Ten distinct mammalian TLR proteins have been identified to date (reviewed in Ref. 3). Agonists have been identified for some (TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9), but not all, of these TLR proteins (Fig. 1). TLR2 agonists include a variety of bacterial cell wall components, such as peptidoglycan (67) as well as lipoarabinomannan, which is a major cell wall-associated glycolipid derived from Mycobacterium tuberculosis. TLR3 recognizes poly(I-C) and double-stranded viral RNA (4), whereas TLR4 agonists include gram-negative bacterial LPS, respiratory syncytial virus protein F, and the plant product Taxol (13, 39, 42). Bacterial flagellin has been identified as a TLR5 agonist (30), and last, unmethylated CpG-containing DNA has been identified as a TLR9 agonist (33). Several mammalian TLR agonists have been identified. These include β-defensin 2 (8), heat shock protein 60 (75), and fibronectin fragments containing the alternatively spliced type.
III repeat extra domain A that is generated in response to tissue injury (51).

Cells of the innate immune system are not only important because of their antimicrobial and cytokine responses to pathogens, but they are also critical to host defense by virtue of their presentation of antigens to cells involved in acquired/adaptive immunity. Therefore, it is not surprising that much attention has recently been focused on the roles of TLR proteins in the maturation and cellular activation of dendritic cells (DCs), the antigen-presenting cell type considered most relevant to the development of acquired immunity. DC maturation is characterized by the production of proinflammatory cytokines (IL-12 and TNF-α), upregulation of costimulatory molecules (CD40, CD80, CD86, and B7RP-1/inducible costimulator ligand), and changes in the expression levels of chemokine receptors (CCR2, CCR5, and CCR7). These mature DCs have enhanced antigen-presenting capacity and migrate from the peripheral tissues to draining lymph nodes where they activate the adaptive immune system. Purified agonists for TLR2 (49), TLR4, and TLR9 (33) have all been shown to stimulate the maturation and antigen-presenting capacity of DCs in vitro. However, it has been suggested that the priming of the adaptive immune system by mature DCs varies based on the type of TLR that is engaged (3, 57). It has also been reported that stimulation of DCs via TLR2 vs. TLR4 stimulates DCs to produce different cytokines and to direct the development of distinct types [e.g., Th helper type 1 (Th1) vs. Th2] of adaptive immune responses (56).

In recent years, a subset of T lymphocytes that are CD4+CD25+ have been identified that prevent a number of immune-mediated diseases, including autoimmune disorders and inflammatory bowel disease (47). These regulatory T cells (Tregs) play an active role in modulating the initiation of adaptive immune responses by preventing the activation of autoreactive T cells. However, these Tregs are themselves regulated by TLR-dependent DC responses. A recent study showed that TLR agonists, such as LPS or CpG DNA, could overcome the inhibitory effect of the Treg cells. The upregulation of costimulatory molecules on the DCs that lacked TLR4 or myeloid differentiation factor 88 (MyD88) was unable to overcome the inhibitory effect of Treg cells and was absolutely dependent on the production of IL-6 by the DCs (55). A more recent study shows that Treg cells express TLR4, 5, 7, and 8, that TLR agonists such as LPS can exert a direct effect on these cells through TLR engagement, and that this effect was independent of antigen-presenting cells. The exposure of these Treg cells to LPS enhances their survival and their suppressor activity (11). Therefore, stimulation of TLRs seems to have opposing effects on the innate and adaptive immune response. TLR engagement leads to the maturation of DCs and activation of the adaptive immune response by releasing T effector cells from suppression by the Treg cells. This release may be mediated by IL-6 secretion from innate immune cells. In contrast, TLR engagement appears to have a direct effect on the Treg cells, resulting in enhancement of their suppressive activity. These different effects may be triggered at different concentrations of TLR agonist, with TLR-mediated suppression being favored at high concentrations of agonist (60). Therefore, it seems that TLR proteins play direct roles in the innate and adaptive immune responses.

**TLR SIGNAL TRANSDUCTION**

The sequence similarities between Toll and IL-1 receptors suggested that these receptors share a common signal transduction pathway that begins at their conserved intracellular TIR domain. Subsequent investigation revealed that different TLR proteins do utilize a similar signaling cascade that ultimately culminates in the activation of NF-κB, activator protein-1, phosphatidylinositol 3-kinase, and mitogen-activated protein (MAP) kinases. In mammals, engagement of TLR proteins and the IL-1 receptor leads to the sequential activation of the adapter protein MyD88, the IL-1 receptor-associated kinases, TNF receptor-associated factor-6, and eventually, the IkB kinase complex (17) (Fig. 2). Most TLR-dependent responses in innate immune cells (e.g., proinflammatory cytokine production) were found to be MyD88 dependent. However, the maturation of DCs in vitro in response to TLR3 and TLR4 engagement was not abolished in MyD88 knockout mice (4, 38), demonstrating the existence of a MyD88-independent signaling pathway. Subsequently, Toshchakov and colleagues (70) reported that macrophages from MyD88-deficient mice could also be stimulated with LPS to secrete IFN-β. In contrast, the response of these mice to CpG DNA via TLR9 was found to be completely MyD88 dependent (38). These data indicate that at least one other adapter molecule was involved in TLR4 signaling. A second adapter protein was subsequently identified by two independent groups and given the names TIR domain-containing adapter protein (TIRAP) or MyD88 adapter like (Mal) (23, 35). TIRAP/Mal was originally shown to bind to TLR4, but not TLR9 (32), and subsequent studies showed that TIRAP/Mal was necessary for signaling via both TLR2 and TLR4 (73).

TLR3 and 4 are unique among TLR proteins in that they not only signal through the MyD88-dependent pathway, but also through an MyD88-independent pathway that activates the transcription factors interferon regulatory factor-3 and -7. This in turn leads to the production of IFN-β, inducible nitric
oxide synthase, and the chemokines RANTES and interferon inducible protein-10. It was later shown that this pathway was mediated by the TIR domain-containing, adapter-inducing IFN-β protein (TRIF) (72, 74), also known as TIR-containing adapter molecule-1 or TICAM1 (53). Two groups have recently shown that TRIF-deficient (72) or TRIF-mutant mice (34) were defective in their responsiveness to TLR3 and TLR4 agonists, whereas mice that lacked both TRIF and MyD88 were completely unresponsive to LPS stimulation via TLR4. It has been proposed that TRIF may also be involved in double-stranded, RNA-induced activation of NF-κB and MAP kinases via TLR3 (37). TRIF can physically associate with TLR3, but not with TLR2, via its TIR domain (74). Current data indicate that TRIF is necessary to mediate MyD88-independent activation of IFN-β expression by Escherichia coli LPS (via TLR4) and poly(I-C) (via TLR3).

More recently, another adapter protein TRIF-related adapter molecule (TRAM) has been identified (24). TRAM can bind to TLR4, TRIF, and TIRAP, but it does not interact with TLR3. The selective ability of TRAM to bind to TLR4 may explain the existence of a residual response to LPS that was still observed in macrophages from TRIF knockout mice (34). Hoebe and colleagues (34) postulated that a distinct adapter protein, termed “adapter X,” could mediate these TRIF-independent responses to LPS. Whether TRAM is the hypothetical adapter X remains to be determined. The capacities of TRAM and TRIF to activate the transcription factors IRF-3 and IRF-7 are likely to explain why engagement of TLR3 and TLR4, but not TLR2, leads to the expression of IFN-β. Recent studies have identified IkB kinase (IKK)-related kinase (IKKe) and TRAF-associated NF-κB activator-binding kinase 1 (TBK1) as the upstream signaling molecules involved in the activation of IRF-3 and -7 (22, 62). Overexpression of these kinases led to the activation of IRF-3 and -7 as well as the IFN-β promoter. Viral infection, or the binding of double-stranded RNA to TLR3, results in the coordinate activation of IRF-3 and NF-κB. It is certainly possible that engagement of TLR3 and TLR4 recruits TRIF/TICAM, leading to the activation of IKKe and TBK1 and ultimately the activation of IRF-3 and IRF-7. This signaling pathway could explain the MyD88-independent nature of IFN-β expression induced following engagement of TLR3 and TLR4.

In an effort to identify potentially critical residues within the TLR4 TIR domain, alanine-scanning mutagenesis of the TLR4 TIR domain was performed. These experiments identified two exposed surfaces in the TLR4 TIR domain (the BB and DD loops) that seemed critical for the activation of proinflammatory (IL-12 p40) and anti-inflammatory (IL-10) promoters (59). To date, the binding sites on TLR4 for MyD88, TIRAP/Mal, TRIF, and TRAM have not been identified.

TLR PROTEINS IN THE LUNG

TLRs have been implicated in a number of lung-associated immune responses and pathologies. In situ hybridization has revealed that human alveolar epithelial cells type II and alveolar macrophages express both TLR2 mRNA and protein (18). TLR2 also plays an important role in mediating inflammatory responses to gram-positive and mycobacterial products such as peptidoglycan, lipoteichoic acid, lipoproteins, and lipoarabinomannan. In developing mice, studies of TLR mRNA expression in the lung and other tissues show that there is a severalfold increase in TLR2 and 4 mRNA levels from the fetal age of 14–15 days to term. This trend continues after birth, with the adult lung expressing two- to fivefold higher levels of TLR2 and 4 mRNA compared with the newborn. In contrast, the levels of TLR2 and 4 mRNA in the liver are 1–2 orders of magnitude higher than the lung. TLR expression in the liver is comparable between the fetal, newborn, and adult stages (28).

TLR proteins are also important in clearing *M. tuberculosis* infections in the mouse. TLR4 has been shown to enhance the control of chronic *M. tuberculosis* infection and improve survival in one study (1). Another study reported that CD14, TLR2, and TLR4 are not important in controlling low-dose [100 colony-forming units (CFU)/mouse] *M. tuberculosis* infections (58). In the latter study, these investigators reported that only in high-dose (2,000 CFU/mouse) infection models does TLR2 play a role in clearing *M. tuberculosis* (58). The reason for these differing conclusions has not been determined to date. It has been shown that TLR6 is expressed in the murine lung (68), but this receptor does not appear to play a role in *M. tuberculosis* infection (65). Immunohistochemical studies of lung granulomas obtained from nine patients with active tuberculosis showed that TLR1–5 and TLR9 were expressed in all the granulomas (21). Only five out of nine granulomas were
also positive for IL-4 expression. A negative association between TLR2 and IL-4 expression was observed, further indicating differential TLR protein expression and the host response to *M. tuberculosis* (21). TLR2-deficient mice that were infected intraperitoneally with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) had tenfold higher bacterial loads in the lung, and macrophages from these mice made low levels of proinflammatory cytokines compared with TLR4-deficient or wild-type mice (32). This was most likely due to a defect in the adaptive immune response of the TLR2-deficient mice since these mice showed impaired T cell proliferation in vitro, whereas the macrophages were able to suppress intracellular bacterial growth of BCG in an interferon-γ-dependent manner.

TLR proteins play an important role in clearing respiratory syncytial virus (RSV), which infects the lower respiratory tract and is a major respiratory pathogen in humans. The RSV coat protein F initiates NF-κB binding activity and induces the expression of a number of genes in human and murine monocytes and macrophages. Depletion of alveolar macrophages abrogated the early NF-κB activation after RSV infection, and a similar effect was seen in TLR4-deficient mice (26). This indicates that alveolar macrophages are the major cell type responding to RSV infection via TLR4. TLR4-deficient (C57BL/10ScN) mice infected with RSV had impaired natural killer T cell trafficking, lower expression of IL-12, IL-6, and IL-1β, and delayed clearing of the virus compared with mice that had TLR4 (31, 42).

TLR proteins have been shown to bind soluble innate immune proteins such as lung surfactant protein A (SP-A) (25, 54). Studies using macrophages from TLR4-mutant C3H/HeJ mice have shown that TLR4 is necessary for SP-A-induced activation of NF-κB and the upregulation of TNF-α and IL-10 expression (25). Also, studies in human tracheobronchial epithelial cells have shown that stimulation with *E. coli* LPS, a TLR4 agonist, leads to the CD14-dependent upregulation of human β-defensin 2 mRNA expression (6), as well as the upregulation of IL-8 production. TLR4 has also been shown to play a role in the acute lung injury model of hemorrhage followed by septic challenge, by facilitating the influx of neutrophils into the lung. This contrasts with TLR4-deficient mice that had fewer neutrophilic infiltrates in the lung. However, the local cytokine expression profile in these mice was found to be comparable (5).

In a chronic fungal asthma model induced by *Aspergillus fumigatus*, IL-18 production was shown to be upregulated and to prevent the development of fungal disease. Depletion of IL-18 exacerbates the disease, correlating with a decrease in TLR2 expression and retention of the fungus (9). This finding implies that TLR2 may play a role in the amelioration of *A. fumigatus*-mediated asthma and correlates with the antifungal role of TLR proteins in *Drosophila* (44). Alveolar macrophages from TLR4-deficient mice expressed normal levels of TNF-α on stimulation with *Pneumocystis carinii*, whereas alveolar macrophages from MyD88-deficient mice did not, thereby indicating that the response to *P. carinii* is mediated by an MyD88-dependent and TLR4-independent pathway that leads to the activation of NF-κB (43).

Inhalation of particulate matter may contribute to chronic inflammatory airway diseases such as asthma, especially if the particles are associated with endotoxins. Alveolar macrophages usually respond to particulate matter of average size 2.5–10 μm (7) and produce cytokines that lead to the airway-associated inflammatory responses. It is thought that the primary protective mechanism to asthma is mediated by a Th1 immune response since IFN-γ production inhibits IgE synthesis and eosinophilia (14, 36), whereas a Th2 bias exacerbates asthma. However, in humans, elevated levels of IFN-γ in the serum (16) and bronchoalveolar lavage (12) have been directly correlated as a contributing factor to the pathophysiology of severe asthma. Therefore, although Th2 cells play a role in the pathogenesis of asthma, it is naïve to conclude that IFN-γ-producing Th1 cells are protective, whereas Th2 cells are detrimental. Nevertheless, this is a useful paradigm.

TLR4 has also been shown to play a role in allergic asthma, perhaps by modulating the Th1 vs. Th2 responses (19). It was proposed that low doses of LPS signal through the MyD88-independent TLR4 pathway and skew the response to a Th2 phenotype that leads to asthma, whereas high doses of LPS would lead to a Th1 response that is protective. These results may also be explained by the finding that high doses of LPS activate the CD4⁺ CD25⁺ Tc cells that may prevent the activation of pathogenic T cell clones (11), whereas low doses of LPS activate Tc cells that in conjunction with IL-6 production release T cells from the inhibitory effect of the Tc cells (55), thereby leading to the activation of the pathogenic T cell clones. Lung-associated antigen presenting cells secrete IL-10 (2, 15) and IL-6 (15) upon exposure to allergen and LPS (61). The production of IL-6 suggests that the lung environment may be skewed toward a Th2 response, but the situation may be more complex as engagement of TLR4 or 9, in conjunction with IL-6 production, releases T cells from the inhibitory effect of CD4⁺ CD25⁺ Tc cells (55), which may lead to the activation of T cell clones that exacerbate the pathogenesis of asthma. However, this effect may be counterbalanced by the production of IL-10 by the pulmonary DCs that induce antigen-specific tolerance in the responding T cell population (2) and therefore may prevent airway reactivity to inhaled antigens.

Unmethylated CpG DNA is a TLR9 agonist that seems to have therapeutic potential in modulating eosinophilia and asthma (10, 41, 66, 71). The predominant effect of stimulation through TLR9 with CpG DNA is a skewing toward a Th1 immune response that prevents allergic inflammation. It has also been shown that direct conjugation of CpG to allergen reduces that dose of CpG DNA needed to elicit a Th1 response and the potency of the allergen (69). Therefore, modulation of the signals through TLR proteins may either ameliorate or exacerbate allergen-induced asthma, depending on the microenvironment of the lung. However, it is important to bear in mind that in the mouse model, effects mediated through TLR proteins may vary depending on the strain studied (45). Sequence analysis of TLR4 in 18 strains of inbred mice showed a large degree of heterogeneity in its sequence. The effect of inhaled LPS was studied in these strains, and there was a large variation in airway responses between the various strains. In general, airway responses to inhaled LPS did not usually correlate with TLR4 microheterogeneity, with the exception of neutrophil recruitment to the lung, which negatively correlated with the loss of TLR4 function.

**SUMMARY**

Many laboratories are actively investigating the roles of TLR proteins in pulmonary infectious disease, lung-specific
immune responses, and inflammation, as well as allergic asthma. It is likely that TLR proteins contribute in some manner to all of these complex responses, participating in ways that can be both beneficial and detrimental to the host. Key questions that remain to be determined are whether TLR proteins will prove to be important targets for the development of novel therapies and whether TLR polymorphisms will prove to be useful markers of disease in human populations. One remarkable feature of the TLR protein family is the diversity of chemical structures that can be recognized by the various receptors. In spite of this diversity, there is still dramatic specificity in the recognition of these agonists by their cognate TLR proteins. To date, there has been no report of overlapping TLR agonist activity, such as is observed among the chemokines and other cytokines. This specificity is likely to be the key to the development of TLR-specific agonists and antagonists that can be used to modulate TLR activity in vivo as part of therapeutic intervention. TLR agonists have already been used as adjuvants to increase vaccine efficacy and safety, and some TLR antagonists have been used to protect against septic shock in some mouse models. These early successes show promise for the use of TLR-based therapies in the future.

REFERENCES


Invited Review

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