(92,93). Embryonic fibroblast cells obtained from TBK1-deficient mice showed impaired activation of IRF-3 and expression of IFN-β and IFN-inducible genes in response to TLR3 and TLR4 ligands (94–96). In contrast, embryonic fibroblast cells from IKKi/IKKε-deficient mice were not defective in their response to TLR3 and TLR4 ligands (95). However, TLR3-mediated activation of IRF-3 and expression of IFN-β and IFN-inducible genes were almost completely abolished in embryonic fibroblast cells lacking both TBK1 and IKKi/IKKε. Thus, TBK1 and IKKi/IKKε are critical regulators of IRF-3 activation in the MyD88-independent pathway.

The mechanisms by which the TRIF-dependent pathway leads to activation of NF-κB and IRF-3 are now under investigation. The TIR domain of TRIF is located in the middle portion of this molecule, flanked by the N-terminal and C-terminal portions. Both N-terminal and C-terminal portions of TRIF mediate activation of the NF-κB-dependent promoter, whereas only the N-terminal portion is involved in IFN-β promoter activation (85). Accordingly, the N-terminal portion of TRIF was shown to associate with IKKi/IKKε and TBK1, which mediate IRF-3-dependent IFN-β induction (93,97). The N-terminal portion of TRIF was also shown to associate with TRAF6 (97,98). Since TRAF6 is critically involved in TLR-mediated NF-κB activation (99), TRAF6 may regulate NF-κB activation derived from the N-terminal portion of TRIF. The C-terminal portion of TRIF was shown to associate with RIP1 (100). Embryonic fibroblast cells from RIP1-deficient mice showed impaired NF-κB activation in response to the TLR3 ligand. Thus, RIP1 is shown to be responsible for NF-κB activation that originates from the C-terminal portion of TRIF.

**Negative regulation of TLR signaling**

Stimulation of TLRs by microbial components triggers the induction of inflammatory cytokines such as TNF-α, IL-6 and IL-12. When all these cytokines are produced in excess, they induce serious systemic disorders with a high mortality rate in the host. It is therefore not surprising that organisms have evolved mechanisms for modulating their TLR-mediated responses.

Exposure to microbial components such as LPS results in a severely reduced response to a subsequent challenge by LPS. This phenomenon was first described over 50 years ago and is now called endotoxin (or LPS) tolerance, but the precise mechanisms remain unclear (101). The mechanisms are now being analyzed in the context of TLR signaling, and several models are proposed. LPS stimulation of macrophages results in reduced surface expression of the LPS receptor complex composed of TLR4 and MD-2, a co-factor that facilitates LPS binding (102,103). TLR2, TLR7 and TLR4 ligands induce reduced expression of IRAK-1 (104–106). Several other mechanisms are also shown to be involved in LPS tolerance (107).

In addition, molecules that negatively regulate TLR signaling have been identified. IRAK-M, a member of the IRAK family of serine/threonine kinases, is induced by TLR stimulation in monocyte/macrophages, and lacks kinase activity (108). IRAK-M-deficient mice show increased production of inflammatory cytokines in response to TLR ligands and defective induction of LPS tolerance (109). Inhibitory activity of IRAK-M seems to be elicited by IRAK-M prevention of IRAK-1/IRAK-4 dissociation from MyD88, thereby preventing formation of the IRAK-1–TRAF6 complex.

An alternatively spliced variant of MyD88 that lacks the intermediary domain of MyD88 (MyD88s) is induced in monocytes upon LPS stimulation. Overexpression of MyD88s results in impaired LPS-induced NF-κB activation through inhibition of IRAK-4-mediated IRAK-1 phosphorylation (110).

SOCS1 is a member of the SOCS family of proteins that are induced by cytokines and that negatively regulate cytokine signaling pathways (111). In addition to cytokines, TLR ligands such as LPS and CpG DNA induced expression of SOCS1 in macrophages (112,113). SOCS1-deficient mice were hypersensitive to LPS-induced endotoxin shock and showed defective induction of LPS tolerance (114,115). Ectopic expression of SOCS1 resulted in impaired LPS-induced NF-κB activation in macrophages. These findings indicate that SOCS1 directly down-modulates TLR signaling pathways, although the precise mechanism by which SOCS1 inhibits TLR signaling remains unclear.

Membrane-bound proteins harboring the TIR domain, such as SIGIRR (single immunoglobulin IL-1 receptor-related molecule) and T1/ST2, have also been shown to be involved in negative regulation of TLR signaling. In both SIGIRR- and T1/ST2-deficient mice, the LPS-induced inflammatory response was enhanced (116,117).

Ubiquitination-mediated degradation of TLRs is also proposed as a mechanism to inhibit activation of the signaling pathway. A RING finger protein, Triad3A, is shown to act as an E3 ubiquitin ligase and enhance ubiquitination and proteolytic degradation of TLR4 and TLR9 (118). Thus, several molecules are postulated to modulate TLR signaling pathways (Fig. 5). Combination of these negative regulators may finely coordinate the TLR signaling pathway to limit exaggerated innate responses causing harmful disorders.

**Involvement of TLRs and immune disorders**

Several lines of evidence indicate that TLRs are implicated in inflammatory and immune disorders. For example, constitutive activation of innate immune cells caused by defective IL-10 signaling results in development of chronic enterocolitis (119). Introduction of TLR4 deficiency into these mutant mice results in improvement of intestinal inflammation, indicating that TLR-mediated microbial recognition in the intestine triggers development of chronic enterocolitis (120). The MyD88-dependent pathway is seemingly involved in allograft rejection (121). Development of atherosclerosis observed in apolipoprotein E-deficient mice is rescued by introduction of MyD88 deficiency, indicating that the TLR-mediated pathway is responsible for the development of atherosclerosis (122,123). Involvement of the TLR9–MyD88-dependent pathway in the induction of auto-antibodies in SLE and rheumatoid arthritis was also demonstrated, as described above. In addition to these immune-related disorders, TLR recognition of commensal bacteria has been shown to play a crucial role in the maintenance of intestinal epithelial homeostasis (124). Thus, TLR-mediated pathways are probably involved in many aspects of immune responses, even in the absence of infection.

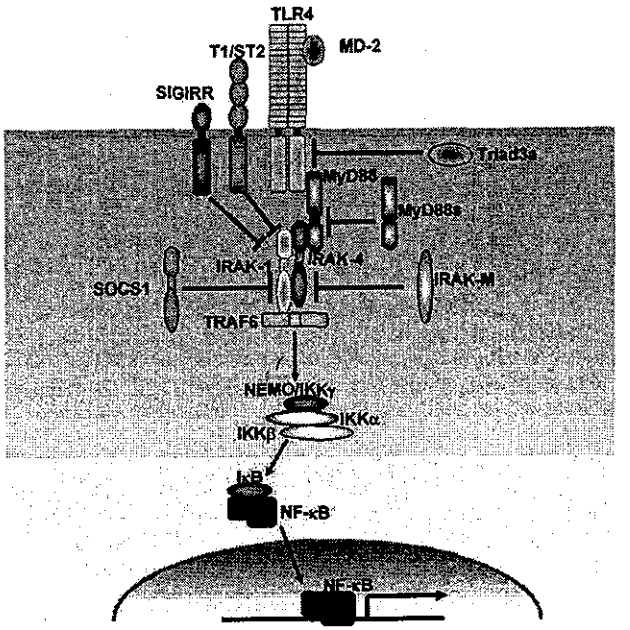


**Fig. 5.** Negative regulation of TLR signaling pathways. TLR signaling pathways are negatively regulated by several molecules. IRAK-M inhibits dissociation of IRAK-1/IRAK-4 complex from the receptor. MyD88s blocks association of IRAK-4 with MyD88. SOCS1 is likely to associate with IRAK-1 and inhibits its activity. TRIAD3A induces ubiquitination-mediated degradation of TLR4 and TLR9. TIR domain-containing receptors SIGIRR and T1/ST2 are also shown to negatively modulate TLR signaling.

### Phylogenetic divergence in the role of Toll

It is well established that mammalian TLRs recognize specific molecular patterns found in microbial components, possibly through a close physical interaction. The Toll receptor in *Drosophila melanogaster* plays an essential role in the host defense against infection by fungi and Gram-positive bacteria. In *Drosophila*, fungal and Gram-positive bacterial infection triggers activation of the Toll-mediated pathway. Toll signaling induces the activation of the Pelle serine/threonine kinase via the adaptor DmMyD88 and degradation of the ankyrin-repeat protein Cactus, and causes activation of the Rel-type transcription factors Dorsal and DIF. Thus, the *Drosophila* Toll signaling pathway is very similar to that of mammalian TLR, especially the participation of the adaptor MyD88, the serine/threonine kinase IRAK, the ankyrin-repeat protein IκB and the Rel-type transcription factor NF-κB (125,126). However, there are some functional differences between the mammalian TLR system and the *Drosophila* Toll system (Fig. 6). First, unlike the mammalian TLR-mediated pathway, the *Drosophila* Toll pathway does not seem to utilize homologs of the mammalian IKKβ and IKKγ/NEMO proteins. These molecules have been shown to be involved in the IMD pathway that senses Gram-negative bacterial infection (125,126). In addition, *Drosophila* Toll is activated by an endogenous ligand, Spätzle (127). Spätzle is initially produced as a pro-Spätzle, and is cleaved into the active signaling form by an as yet unidentified serine protease in response to invasion by fungi or Gram-positive bacteria. This indicates that Toll is not directly involved in the pattern recognition of micro-organisms. In the case of fungal infections, a serine protease that is encoded by the *Persephone* gene has been shown to activate Toll (128). The *Persephone* gene product possesses no obvious pattern recognition motif. Thus, the molecule that is responsible for the recognition of fungi remains unclear. In the case of Gram-positive bacterial infections, Gram-negative binding protein (GNBP) and PGRP-SA, a member of the peptidoglycan recognition protein (PGRP) family, play an essential role in the activation of Toll. This is demonstrated by the finding that mutant flies lacking GNBP or PGRP-SA are defective in the activation of the Toll-mediated pathway in response to Gram-positive bacterial infection (129,130). Subsequently, PGRP-SA has been shown to be responsible for the recognition of Gram-positive lysine-type peptidoglycan, demonstrating that PGRP-SA is a pattern recognition molecule in *Drosophila* (131). In addition, another member of the PGRP family, PGRP-LC, has been shown to play an essential role in the host defense against Gram-negative bacterial infection through the recognition of Gram-negative *diaminopimelic* acid-type peptidoglycan (132–134). In sharp contrast, mammalian PGRP-S has been shown to play only a minor role in the recognition of pathogens (135). Thus, although the signaling molecules associated with *Drosophila* Toll and mammalian TLR are shared, the actual pattern recognition of pathogens is mediated by quite different mechanisms. In particular, it is of note that only one Toll is actually involved in the *Drosophila* immune response (126). The complete lack of immune functions in other members (18-Wheeler/Toll-2 to Toll-9) of the *Drosophila* Toll family strongly suggests that the ancestral function of the Toll family is not to mediate immune response. In contrast, all members of the mammalian TLR family are specialized for functioning in immune responses, indicating that they have arisen from a common ancestor possessing immune recognition function. Thus, the systems by which pathogens are recognized in vertebrates and insects seem to have evolved separately.

### Future prospects

We now know that innate immunity plays an important role in the initiation of an immune response that follows the activation of antigen-specific acquired immunity. Although signaling pathways via TLRs are now being unveiled, there still remain several unanswered questions. For example, activation of TLR7, TLR8 and TLR9 leads to induction of IFN-α/β in a MyD88-dependent manner in PDC. It is possible that there might be a unique pathway downstream of MyD88 that specifies the signaling cascade of these TLRs. The mechanism for regulation of TLR-mediated gene induction is also of interest. A TLR-inducible nuclear factor, IκBζ, has been shown to regulate a subset of TLR-inducible genes (136). In this model, IκBζ, which is immediately induced by TLR stimulation, mediates induction of a certain group of TLR-inducible genes such as IL-6, IL-12p40 and GM-CSF in macrophages. It is of interest to analyze whether this two-step TLR-mediated gene induction model can be applied to other subsets of genes that are induced by TLRs. A complete understanding of the mechanisms of innate immunity will be helpful for the future

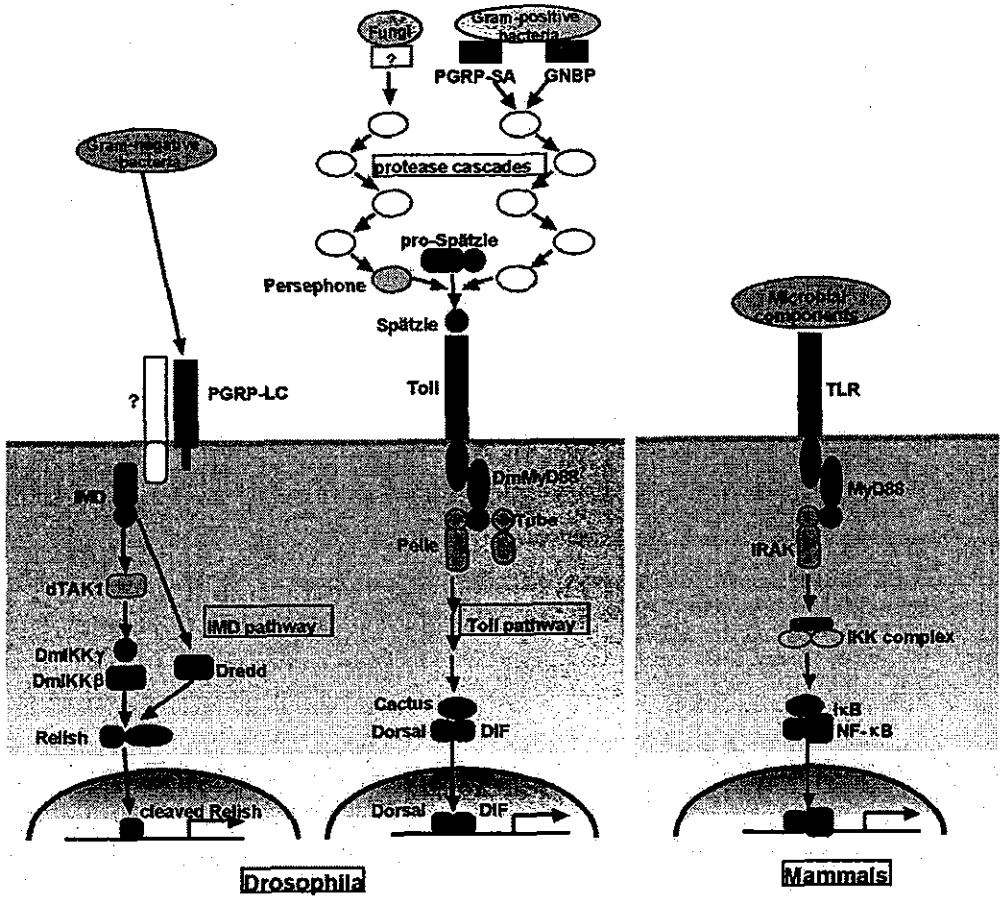


**Fig. 6.** Toll pathway in *Drosophila* and TLR pathway in mammals. In *Drosophila*, fungal and Gram-positive bacterial infections are sensed by pattern recognition proteins. GNBP1 and PGRP-SA are responsible for the recognition of Gram-positive bacteria. Recognition of micro-organisms is followed by activation of proteolytic cascades, leading to the cleavage of Spätzle. Spätzle activates Toll, which leads to degradation of Cactus and nuclear translocation of the Rel-type transcription factor DIF. In the case of Gram-negative bacterial infection, the IMD pathway is activated in *Drosophila*. In mammals, microbial infections are sensed by TLRs, which leads to activation of the Rel-type transcription factor NF-κB.

development of innovative therapies for manipulation of infectious diseases, cancer and allergies.


## Acknowledgements

We thank M. Hashimoto and M. Kurata for secretarial assistance. This work was supported by grants from the Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology, the Uehara Memorial Foundation and the Naito Foundation.


## Abbreviations

| | |
|---|---|
| CARD | caspase-recruitment domain |
| dsRNA | double-stranded RNA |
| HSP | heat shock proteins |
| iE-DAP | γ-D-glutamyl-meso diaminopimelic acid |
| IKK | IκB kinase |
| LRRs | leucine-rich repeats |
| Mal | MyD88-adaptor-like |
| MCMV | mouse cytomegalovirus |
| MDP | MurNAc-L-Ala-D-isoGln |
| NOD | nucleotide-binding oligomerization domain |
| PDC | plasmacytoid dendritic cells |
| PGN | peptidoglycan |
| PGRP | peptidoglycan recognition protein |
| PKR | dsRNA-dependent protein kinase |
| RNAi | RNA interference |
| SIGIRR | single immunoglobulin IL-1 receptor-related molecule |
| ssRNA | single-stranded RNA |
| TICAM | TIR domain-containing adaptor molecule |
| TIR | Toll/IL-1 receptor |
| TIRAP | TIR domain-containing adaptor protein |
| TLRs | Toll-like receptors |
| TRIF | TIR domain-containing adaptor inducing IFN-β |

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Molecular Immunology 40 (2004) 861–868
Molecular Immunology
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# TIR domain-containing adaptors define the specificity of TLR signaling

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**Abstract**

The concept that Toll-like receptors (TLRs) recognize specific molecular patterns in various pathogens has been established. In signal transduction via TLRs, MyD88, which harbors a Toll/IL-1 receptor (TIR)-domain and a death domain, has been shown to link between TLRs and MyD88-dependent downstream events leading to proinflammatory cytokine production and splenocyte proliferation. However, recent studies using MyD88-deficient mice have revealed that some TLRs possess a MyD88-independent pathway, which is represented by interferon (IFN)-β production induced by LPS stimulation. This indicates that additional signaling molecules other than MyD88 exist in the TLR signaling pathway. Indeed, two additional TIR domain-containing adaptors, TIRAP/Mal and TRIF, have recently been identified. Both define the specific biological responses of each TLR.


*Keywords:* TLRs; MyD88; TIRAP/Mal; TRIF; MyD88-independent pathway

## 1. Introduction

Toll-like receptors (TLRs) are germ-line-encoded receptors and play an important role in innate immunity from insects to mammals. TLRs recognize pathogen-associated molecular patterns (PAMPs) such as microbial components. To date, 10 TLRs (TLR1-TLR10) have been reportedly encoded in the human genome, and at least one ligand has been identified for each TLR, except for TLR8 and TLR10: triacylated lipoprotein for TLR1; peptidoglycan, the major gram-positive bacterial cell wall component, for TLR2; double-stranded RNA, which is considered to be generated in the lifecycle of RNA viruses, for TLR3; lipopolysaccharide (LPS), a gram-negative cell wall component, for TLR4; flagellin, a component of bacterial flagella, for TLR5; diacylated lipoprotein for TLR6; imidazoquinoline, an anti-viral drug, and its derivative R-848, for TLR7; and bacterial unmethylated CpG DNA for TLR9. Stimulation of TLRs by these ligands activates a signaling cascade, which culminates in proinflammatory cytokine production and subsequent immune responses. The signaling cascade downstream of TLR is generated from its Toll/interleukin-1 receptor (TIR)-domain (Takeda et al., 2003; Janeway and Medzhitov, 2002; Dunne and O'Neill, 2003; Imler and Hoffmann, 2001). Here, we describe an overview of the mechanisms of intracellular TLR signaling, with special emphasis on the events proximal to TLRs.

## 2. MyD88-dependent pathway

MyD88, a common adaptor protein also harboring a TIR domain, is associated with the TIR domain of TLRs, and leads to the activation of IRAK-1/4 and TRAF-6, culminating in the activation of NF-κB, a transcription factor crucial for the expression of pro-inflammatory cytokines and various mediators (Suzuki et al., 2002a,b; Janssens and Beyaert, 2003; O'Neill, 2002a; Ghosh and Karin, 2002). Significant roles of MyD88 in TLR/interleukin-1 receptor signaling were demonstrated by analysis of MyD88-deficient mice. Macrophages from MyD88-deficient mice are completely defective in the production of inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IL-12 p40 in response to bacterial components. MyD88-deficient splenocytes are incapable of proliferation in response to stimulation with LPS, CpG DNA, or IL-1 (Poltorak et al., 1998; Hoshino et al., 1999; Hacker et al., 2000; Schnare et al., 2000; Adachi et al., 1998) (Fig. 1).

## 3. MyD88-independent pathway

Although the activation of NF-κB and MAPK through almost all of the TLRs is abolished in MyD88-deficient cells,

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0161-5890/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2003.10.006

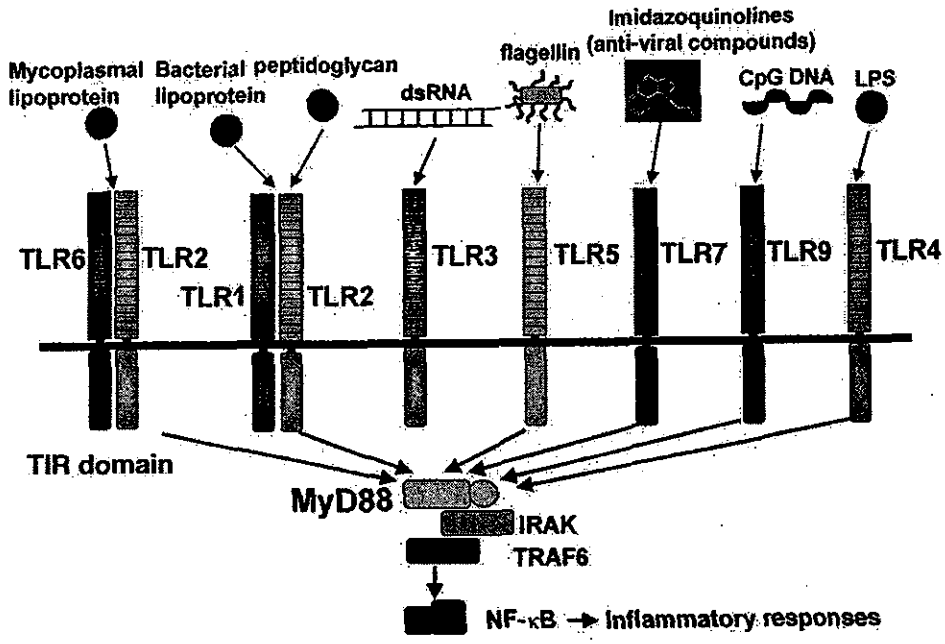


Fig. 1. TLRs recognize molecular pattern associated with bacterial pathogens. triacylated lipoprotein for TLR1; peptidoglycan for TLR2; double-stranded RNA for TLR3; lipopolysaccharide (LPS) for TLR4; flagellin for TLR5; diacylated lipoprotein for TLR6; imidazoquinoline and its derivative R-848, for TLR7; and bacterial unmethylated CpG DNA for TLR9. MyD88 associates with the TIR domain of TLRs and transduces signals to induce immune responses.

the TLR4 ligand LPS stimulation still activates NF-κB and MAPK in MyD88-deficient cells, but with delayed kinetics compared with those of wild-type cells (Kawai et al., 1999). LPS-stimulated MyD88-deficient cells remain intact in their capacity to induce interferon (IFN)-inducible genes, such as IP-10, GARG-16, or IRG-1, and augment surface activation markers, including CD40, CD80, or CD86, in dendritic cells (Kaisho et al., 2001; Kawai et al., 2001; Doyle et al., 2002). These observations indicate that the LPS-induced TLR4 signaling cascade is separated into two groups: a MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK, and a MyD88-independent pathway associated with the induction of IFN-inducible

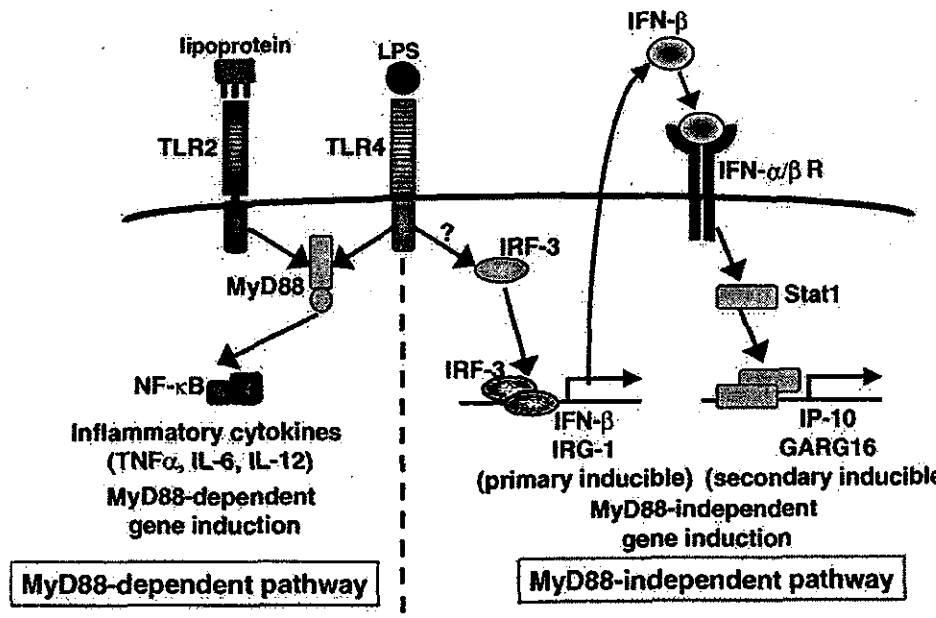


Fig. 2. TLR signaling pathways are separated into two groups. A MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK, and a MyD88-independent pathway associated with the induction of IFN-β and IFN-inducible genes, and maturation of dendritic cells with slow activation of NF-κB and MAPK.

genes and maturation of dendritic cells with slow activation of NF-κB and MAPK. TLR3 has also been shown to utilize the MyD88-independent pathway, since poly(I:C)-induced NF-κB activation and maturation of dendritic cells were observed normally in MyD88-deficient mice (Alexopoulou et al., 2001) (Fig. 2).

During analysis of the molecular basis for MyD88-independent events, some groups hypothesized that a molecule(s) responsible for the MyD88-independent pathway downstream of TLR4 may harbor the TIR domain like MyD88, and successfully identified another TIR domain-containing molecule named TIRAP (for TIR domain-containing adaptor protein)/Mal (for MyD88-adaptor like) through database searches. Human TIRAP encodes a 232 amino acid cytoplasmic protein harboring the TIR domain in the C-terminal portion (Horng et al., 2001; Fitzgerald et al., 2001). Ectopic overexpression of TIRAP activated the NF-κB-dependent promoter as strongly as MyD88, and the P125H mutant of TIRAP, which acted in a dominant negative manner, specifically blocked TLR4-dependent NF-κB activation but not IL-1R- or TLR9-dependent activation. Furthermore, epitope-tagged TIRAP interacted directly with MyD88 and the TIR domain of TLR4 but not TLR9, suggesting that, unlike MyD88 which participates in all of the TLR and IL-1 receptor signaling pathways, TIRAP is an adaptor molecule specific for TLR4 signaling. Furthermore, cell membrane-permeable TIRAP peptide inhibited not only pro-inflammatory cytokine production, such as IL-12 or IL-6, but also maturation of dendritic cells in terms of the expression of co-stimulatory molecules and proliferation in response to LPS stimuli, indicating that TIRAP takes part in the MyD88-independent pathway downstream of TLR4 signaling in vitro (Toshchakov et al., 2002).

## 4. The second adaptor TIRAP: its physiological role

To examine the physiological in vivo role of TIRAP in the TLR signaling pathway, TIRAP knockout mice were generated by targeted gene disruption (Yamamoto et al., 2002a; Horng et al., 2002). Since TIRAP was identified as a molecule for the TLR4-specific signaling pathway, the growth rate of splenocytes was first compared between wild-type and TIRAP-deficient mice. Wild-type splenocytes proliferated in a dose-dependent manner in response to LPS, whereas TIRAP-deficient splenocytes were completely unresponsive to LPS stimulation. On the other hand, both wild-type and TIRAP-deficient cells proliferated to the same extent in response to CpG DNA, indicating that the TLR4-MyD88-dependent, but not TLR9-MyD88-dependent, B cell response was impaired in the TIRAP-deficient mice. Regarding cytokine production, LPS stimulation of wild-type macrophages resulted in the production of TNF-α, IL-6 and IL-12 p40 in a dose-dependent manner, whereas the production of these cytokines was completely abolished in TIRAP-deficient cells. Furthermore, all of the wild-type mice died within 96 h after LPS-induced shock. In contrast, TIRAP-deficient mice were completely resistant to the septic shock induced by LPS injection. In addition, the IL-6 level in sera from injected TIRAP-deficient mice was significantly lower than that of wild-type mice. These results indicated that TIRAP is a molecule involved in, at least, TLR4-mediated MyD88-dependent events.

The responses to other TLR ligands such as double-stranded RNA (for TLR3), flagellin (for TLR5), R-848 (for TLR7), and CpG DNA (for TLR9) were indistinguishable between wild-type and TIRAP-deficient macrophages. IL-1R and IL-18R also utilize MyD88 to signal in the cytoplasm. The responses to IL-1 and IL-18 were not impaired in TIRAP-deficient cells. As for the response to TLR2 ligands, including peptidoglycan and MALP-2, proinflammatory cytokines were produced from wild-type cells in a dose-dependent fashion, whereas TIRAP-deficient cells showed severely impaired production in response to the stimulation. Thus, MyD88-dependent events through TLR2 as well as those of TLR4 were affected by TIRAP deficiency, demonstrating that TIRAP play an important role in the MyD88-dependent immune responses shared by TLR2 and TLR4.

Although LPS-induced activation of NF-κB was normally observed at 20 or 60 min after stimulation, even in TIRAP-deficient macrophages, early NF-κB activation at 10 min was not observed in TIRAP-deficient cells. The delayed NF-κB activation in TIRAP-deficient cells in response to LPS was reminiscent of that of MyD88-deficient cells, which also showed LPS-stimulated NF-κB activation with delayed kinetics. Activation of MAPK was also delayed in LPS-stimulated TIRAP-deficient cells. Stimulation with MALP-2, a TLR2 ligand, showed NF-κB activation and MAPK phosphorylation in wild-type cells, but these were profoundly inhibited in TIRAP-deficient cells. Finally, the response to R-848, a TLR7 ligand, was normal in TIRAP-deficient cells. Taken together, TIRAP deficiency also specifically affected the activation of the intracellular signaling cascade for both TLR2 and TLR4, but not for other TLRs.

Several previous in vitro studies demonstrated that TIRAP was involved not only in MyD88-dependent events but also in MyD88-independent events, including maturation of dendritic cells and IRF3 activation (Toshchakov et al., 2002; Shinobu et al., 2002). It was investigated whether or not MyD88-independent as well as MyD88-dependent events were impaired in TIRAP-deficient cells. LPS stimulation led to the induction of IRGs, including IP-10, GARG-16, and IRG-1, in both wild-type and TIRAP-deficient cells in a similar manner. TIRAP-deficient dendritic cells showed increased expression of activation markers, such as CD40, CD80 or CD86, as in the case for wild-type dendritic cells. IRF3 is a key transcription factor for LPS-mediated expression of IFN-β and IRGs (Sato et al., 2000; Taniguchi et al., 2001). Activated IRF3 forms a homodimer after LPS stimulation and translocates into the nucleus to switch on the induction of IFN-β and other IRGs. In TIRAP-deficient

cells, dimeric forms of IRF3 were detected normally in response to LPS. Thus, LPS-induced IRF3 activation was not affected by the absence of TIRAP. However, the possibility that TIRAP deficiency might be compensated by MyD88 could not be ruled out. To exclude this, mice lacking both TIRAP and MyD88 were generated. Macrophages from TIRAP and MyD88 doubly deficient mice expressed the same levels of IRGs as those of wild-type cells. Dendritic cells from the doubly deficient mice also responded to LPS comparably to those from wild-type mice in terms of the up-regulation of co-stimulatory molecules. Taken together, these results clearly demonstrate that TIRAP deficiency was not compensated by MyD88. Therefore, TIRAP is not a molecule responsible for the MyD88-independent pathway.

## 5. The third adaptor TRIF

The analysis of TIRAP-deficient mice suggested that other molecules were responsible for the MyD88-independent pathway downstream of TLR3 or TLR4, and prompted us to search for other TIR domain-containing molecules. Database search screening resulted in the identification of some novel proteins harboring the TIR domain with unknown functions. One of these was named TRIF for TIR domain-containing adaptor inducing interferon-β (Yamamoto et al., 2002b; O'Neill, 2002b; Imler and Hoffmann, 2003; Barton and Medzhitov, 2003). Human TRIF consisted of 712 amino acids. TRIF was much larger than the known TIR domain-containing adaptor molecules such as MyD88 or TIRAP, whose sizes are 296 and 232 amino acids, respectively. TRIF possessed the TIR domain in the C-terminal side of the protein. The TIR domain of TRIF was not very similar to the other TIR domains registered in the database such as those of MyD88, TIRAP, and TLRs. The conserved proline residue among TLRs that is essential for the activation of TLR-mediated signal transduction was observed in the TIR domain of TRIF. Northern blotting analysis revealed that TRIF was expressed widely in all human tissues, among which the liver showed especially strong expression.

As reported previously, overexpression of MyD88 or TIRAP in 293 cells strongly induced NF-κB activation as detected by a luciferase-mediated reporter assay. Overexpression of TRIF activated NF-κB, albeit at a low level compared with MyD88- or TIRAP-induced activation. A reporter assay that monitors the activation of the IFN-β promoter was also performed in order to analyze the activation of the MyD88-independent pathway. Neither MyD88 nor TIRAP overexpression activated the IFN-β promoter, whereas strong activation was observed in the case of TRIF introduction. These results showed that TRIF is unique as a potent activator of the IFN-β promoter.

Truncated forms of TRIF were generated to determine the internal domain crucial for the activation of NF-κB and the IFN-β promoter: ΔN lacked the N-terminus except for the TIR domain, ΔC lacked the C-terminus but possessed the TIR domain, and ΔNΔC lacked both the N-terminus and C-terminus and was only composed of the TIR domain. When transfected together with an NF-κB-dependent reporter plasmid, both ΔN and ΔC induced activation of the NF-κB-dependent promoter, albeit at reduced levels compared with full-length TRIF. ΔNΔC did not activate the NF-κB-responsive luciferase reporter. On the other hand, ΔC, but not ΔN or ΔNΔC, induced activation of the IFN-β promoter to the similar level as the full-length TRIF. Taken together, while both the N-terminus and the C-terminus possessing the TIR domain potentially activate NF-κB, only the N-terminal portion of TRIF is involved in activation of the IFN-β promoter, indicating that distinct domains of TRIF are differentially responsible for the activation of NF-κB and the IFN-β promoter.

Full-length TRIF-mediated activation of NF-κB or the IFN-β promoter was profoundly inhibited by cotransfection of ΔNΔC, suggesting that ΔNΔC acts as a dominant negative inhibitor like those of MyD88 and TIRAP. In 293 cells transfected with TLR2 or TLR7 expression vectors, MALP-2 or R-848 induced activation of the NF-κB-dependent luciferase reporter, respectively. Cotransfection of ΔNΔC significantly inhibited the TLR2- or TLR7-meditated NF-κB activation. LPS stimulation activated the NF-κB responsive reporter in cells transfected with both TLR4 and MD-2, but this activation was abolished by cotransfection of ΔNΔC. In addition, activation of NF-κB induced by overexpression of MyD88 and TIRAP was predominantly blocked by ΔNΔC cotransfection. 293 cells stably transfected with TLR3 showed poly(I:C) stimulation-dependent activation of NF-κB and the IFN-β promoter (Matsumoto et al., 2002). The dominant negative form of MyD88 or TIRAP did not block poly(I:C)-induced activation of either NF-κB or the IFN-β promoter. Most notably, TRIF ΔNΔC completely inhibited not only the activation of poly(I:C)-induced NF-κB but also that of the IFN-β promoter. These data suggest that TRIF, unlike other adaptor molecules such as MyD88 and TIRAP, may have a unique role in, at least, the TLR3-mediated MyD88-independent pathway.

Coimmunoprecipitation analysis revealed that TRIF associated with the TIR domain of TLR3, indicating that TRIF may recognize TLR3 through homophilic interaction between the TIR domains. An interaction between epitope-tagged TRIF and endogenous IRF3 was also detected.

In summary, first, TRIF overexpression culminated in activation of not only the NF-κB-responsive element but also the IFN-β promoter. Second, the dominant negative form of TRIF inhibited activation of the IFN-β promoter by TLR3 as well as that of NF-κB through various TLRs. Third, TRIF interacted with TLR3 through the TIR domain and endogenous IRF3. Thus, the in vitro studies described above demonstrate that TRIF may be a molecule involved in the MyD88-independent pathway, at least that mediated by TLR3 activation (Fig. 3).

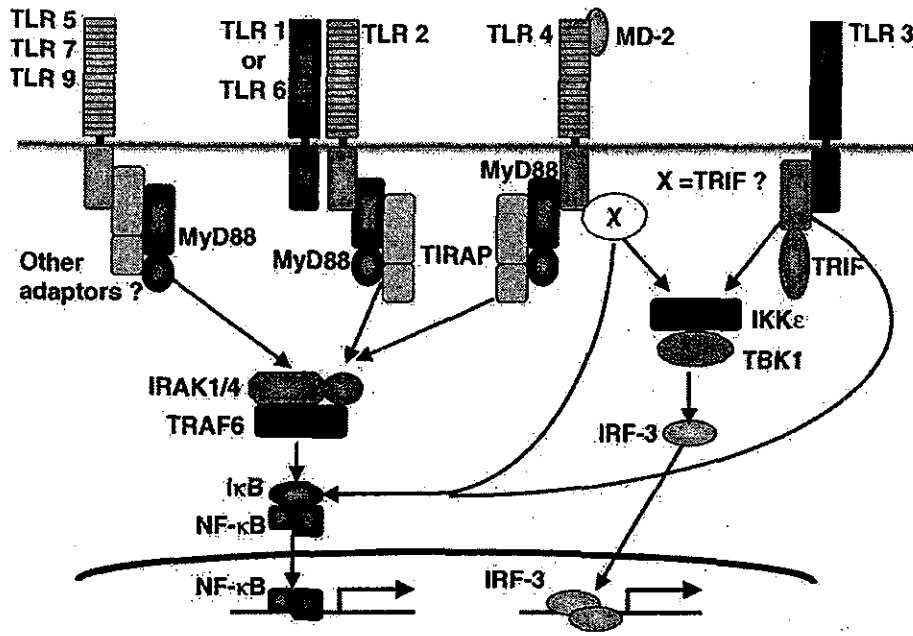


Fig. 3. TIRAP is an adaptor molecule shared with TLR2 and TLR4 signaling cascade. Other adaptors may also exist downstream of TLR5, TLR7, and TLR9 in the MyD88-dependent pathways like TIRAP in TLR2- and TLR4-mediated signaling pathway. TRIF may exist downstream of TLR3/TLR4, and upstream of IKKε and TBK1 to mediate the MyD88-independent pathways leading to production of IFN-β and IFN-inducible genes.

## 6. The biological significance of TIR domain-containing adaptors

Analysis of MyD88-deficient mice clearly showed that both the TLR2- and TLR9-signaling pathways are completely dependent on MyD88 (Takeuchi et al., 2000). However, the production of type I interferon from certain cell types is stimulated by activation of TLR9 signaling but not by that of TLR2. If MyD88 was the only molecule connecting TLRs and downstream signaling molecules such as IRAK or TRAF6, the difference between the TLR2- and TLR9-mediated responses would be hard to explain. Analysis of the physiological role of TIRAP using TIRAP-deficient mice could give clues to the answers to these currently incomprehensible questions. TIRAP acts in the MyD88-dependent pathway shared by TLR2 and TLR4, indicating that even MyD88-dependent pathways can be specified by dependency on TIRAP. It is of note to point out that the signaling pathways for TLR5 and TLR7 are MyD88-dependent, but not TIRAP-dependent. It is reasonable to say that the remaining TLR-mediated MyD88-dependent signaling pathway also possesses an adaptor molecule(s) like TIRAP. Recently, two independent groups reported the identification of such a candidate called TRAM, for TRIF-related adaptor molecule, or TIRP, for TIR domain-containing protein (O'Neill et al., 2003; Bin et al., 2003). One group showed that overexpression of TRAM led to the activation of both the NF-κB-responsive luciferase reporter and the IFN-β promoter. On the other hand, the other group showed that overexpression of TRAM/TIRP only stimulated NF-κB activation but not that of the IFN-β promoter. In addition, the latter group found that TRAM/TIRP associated with other TIR domain-containing adaptors, including TIRAP and TRIF, but not with MyD88. It is noteworthy that the dominant negative form of TRAM/TIRP specifically inhibited the activation of NF-κB via IL-1R. Since TIRAP is not involved in IL-1R signaling, specific inhibition of IL-1R signaling by the dominant negative form of TRAM/TIRP and binding of TRAM/TIRP to TIRAP seem to be incompatible. Thus, the true nature of the function of TRAM/TIRP remains controversial. Whether TRAM/TIRP is only involved in IL-1R signaling or with some other TLRs under physiological conditions will be determined in an animal model lacking this gene.

TRIF is an adaptor molecule identified as a possible candidate responsible for linking TLRs and type I IFN production. Signaling pathways of TLR-mediated type I IFN production are classified into two groups: one is MyD88-dependent and mainly observed in TLR7- and TLR9-mediated signaling, and the other is MyD88-independent and observed in TLR3- and TLR4-mediated signaling (Hemmi et al., 2000, 2002, 2003). Furthermore, it is unclear whether the TLR3-mediated MyD88-independent pathway is governed by the same mechanism as that for TLR4. For example, a report showed that the virus-induced phosphorylation site of IRF-3 was different from that of LPS (Servant et al., 2001). Given that dsRNA is derived from viral infection, this finding suggests that TLR3-mediated IRF-3 activation is regulated by a mechanism distinct from that of TLR4. In vitro studies concluded that TLR3-mediated activation of NF-κB and the IFN-β promoter was not dependent on MyD88 or TIRAP but on TRIF, indicating a role for TRIF in the TLR3-mediated

MyD88-independent pathway. Nevertheless, there is the possibility that TRIF is involved in other TLR-mediated pathways, since epitope-tagged TRIF interacted with TLR2 as well as TLR3 and dominant negative forms of TRIF had negative effects on various TLRs-mediated NF-κB activation (Yamamoto et al., 2002a,b; Oshiumi et al., 2003).

Very recently, two different groups have reported the generation of mice carrying mutation of the *Trif* gene. One group generated TRIF-deficient mice by conventional reverse genetics. The other used *N*-ethyl-*N*-nitrosourea (ENU) to produce germline mutant mice called Lps2-mice that carries a mutated form of the *Trif* gene. Both mutant mice showed defective responses in TLR3-mediated MyD88-independent IFN-β induction and signaling pathways such as IRF-3 and NF-κB activation. In addition, TLR4-mediated MyD88-independent responses were severely impaired in TRIF-mutated mice. These results indicate that TRIF plays an essential role in both TLR3- and TLR4-mediated MyD88-independent signaling pathways (Yamamoto et al., 2003; Hoebe et al., 2003).

## 7. Other recent advances in understanding the MyD88-independent pathway

Recently, two groups reported that IKK-I/IKK-ε and TBK1/T2K act as molecules for mediating IRF3 phosphorylation (Fitzgerald et al., 2003; Sharma et al., 2003). IKK-I/IKK-ε and TBK1/T2K were primarily cloned as molecules possessing sequence homology with preexisting IKK such as IKKα and IKKβ, and therefore possibly having a role in NF-κB activation (Shimada et al., 1999; Peters et al., 2000; Pomerantz and Baltimore, 1999; Bonnard et al., 2000). Surprisingly, overexpression of both molecules led to strong activation of the IFN-β and RANTES promoters as well as NF-κB (Fitzgerald et al., 2003; Sharma et al., 2003). In addition, activation of both the IFN-β and RANTES promoters by overexpression of TRIF was inhibited by dominant negative forms of either IKK-I/IKK-ε or TBK1/T2K, suggesting roles for both IKK-I/IKK-ε and TBK1/T2K as molecules downstream of TRIF. TRIF bound to IKK-I/IKK-ε and TBK1/T2K through regions other than the TIR domain (Fitzgerald et al., 2003). Regarding IRF3 activation by TRIF, since the N-terminus, but not the C-terminus, of TRIF could stimulate IRF3 activation, some regions in the N-terminus of TRIF may be required for linkage between TRIF and IKK-I/IKK-ε or TBK1/T2K or both. The question of whether both molecules are really downstream of TRIF will also be resolved using TRIF-deficient cells in the future.

Elucidating the molecular mechanisms of TLR4-mediated MyD88-independent events at the molecular level will be important for the treatment of LPS-induced septic shock, which occasionally causes devastating results in the medical field. The recent discovery of central roles for type I interferons in LPS-induced endotoxin shock using IFN-β-deficient mice and Tyk2-deficient mice may provide a certain aspect of the biological role of the TLR4-mediated MyD88-independent pathway (Karaghiosoff et al., 2003). Tyk2-deficient mice were resistant to high doses of LPS-induced septic shock even though systemic inflammatory cytokines were secreted normally. Instead, LPS-mediated production of type I interferons, such as IFN-β and IFN-α4, was perturbed in Tyk2-deficient mice. Considering that IFN-β-deficient mice were also resistant to high doses of LPS, type I interferon may be indispensable for LPS-mediated septic shock. Thus, although type I interferons have been primarily interpreted as players responsible for host anti-viral defense, they also participate in a biological event which specifically occurs in the case of anti-bacterial defense.

## 8. Concluding remarks

Previously, TLR signaling has been considered to consist of MyD88-dependent and MyD88-independent pathways such that MyD88 and molecules for the MyD88-independent pathway were the only molecules which existed between TLR and downstream of the signal transducers. However, recent findings regarding the specific role of TIRAP in the MyD88-dependent pathway in TLR2 or TLR4 signaling change the hypothesis to a new paradigm that adaptor molecules harboring a TIR domain fractionate TLR signal transduction to provide the signaling specificities. Furthermore, the recent discovery of another TIR domain-containing adaptor, TRIF, opens the door to understanding the remaining molecular mechanisms of the MyD88-independent pathway. Future studies will clarify some of the remaining puzzles concerning TLR signaling specificities that are just beginning to be resolved.

## Acknowledgements

We thank the rest of Dr Akira's laboratory members for helpful discussions, M. Hashimoto for secretarial assistance, and N. Okita and N. Iwami for technical assistance. This work was supported by grants from Special Coordination Funds, the Ministry of Education, Culture, Sports, Science and Technology, Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists, The Uehara Memorial Foundation. The Naito Foundation, and the Junior Research Association from RIKEN.

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# TLR signaling pathways

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**Abstract**

Toll-like receptors (TLRs) have been established to play an essential role in the activation of innate immunity by recognizing specific patterns of microbial components. TLR signaling pathways arise from intracytoplasmic TIR domains, which are conserved among all TLRs. Recent accumulating evidence has demonstrated that TIR domain-containing adaptors, such as MyD88, TIRAP, and TRIF, modulate TLR signaling pathways. MyD88 is essential for the induction of inflammatory cytokines triggered by all TLRs. TIRAP is specifically involved in the MyD88-dependent pathway via TLR2 and TLR4, whereas TRIF is implicated in the TLR3- and TLR4-mediated MyD88-independent pathway. Thus, TIR domain-containing adaptors provide specificity of TLR signaling.


*Keywords:* TLR; Innate immunity; Signal transduction; TIR domain

## 1. Introduction

Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorso-ventral pattern in developing embryos [1]. In 1996, Hoffmann and colleagues demonstrated that Toll-mutant flies were highly susceptible to fungal infection [2]. This study made us aware that the immune system, particularly the innate immune system, has a skilful means of detecting invasion by microorganisms. Subsequently, mammalian homologues of Toll receptor were identified one after another, and designated as Toll-like receptors (TLRs). Functional analysis of mammalian TLRs has revealed that they recognize specific patterns of microbial components that are conserved among pathogens, but are not found in mammals. In signaling pathways via TLRs, a common adaptor, MyD88, was first characterized as an essential component for the activation of innate immunity by all the TLRs. However, accumulating evidence indicates that individual TLRs exhibit specific responses. Furthermore, they have their own signaling molecules to manifest these specific responses. In this review, we will focus on the recent advances in our understanding of the mechanism of TLR-mediated signaling pathways.

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## 2. Toll-like receptors

A mammalian homologue of *Drosophila* Toll receptor (now termed TLR4) was shown to induce the expression of genes involved in inflammatory responses [3]. In addition, a mutation in the *Tlr4* gene was identified in mouse strains that were hyporesponsive to lipopolysaccharide [4]. Since then, Toll receptors in mammals have been a major focus in the immunology field. First, several proteins that are structurally similar to TLR4 were identified and named TLRs [5]. The TLR family now consists of 10 members (TLR1–TLR10). The cytoplasmic portion of TLRs shows high similarity to that of the interleukin (IL)-1 receptor family, and is now called the Toll/IL-1 receptor (TIR) domain. Despite of this similarity, the extracellular portions of both types of receptors are structurally unrelated. The IL-1 receptors possess an Ig-like domain, whereas TLRs bear leucine-rich repeats (LRRs) in the extracellular domain. Genetic approaches have mainly been conducted to analyze the physiological function of TLRs, and have revealed essential roles for TLRs in the recognition of pathogens. Each TLR has been shown to recognize specific components of pathogens, thus demonstrating that the mammalian immune system detects invasion by pathogens via the recognition of microbial components by TLRs (Fig. 1).

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doi:10.1016/j.smim.2003.10.003

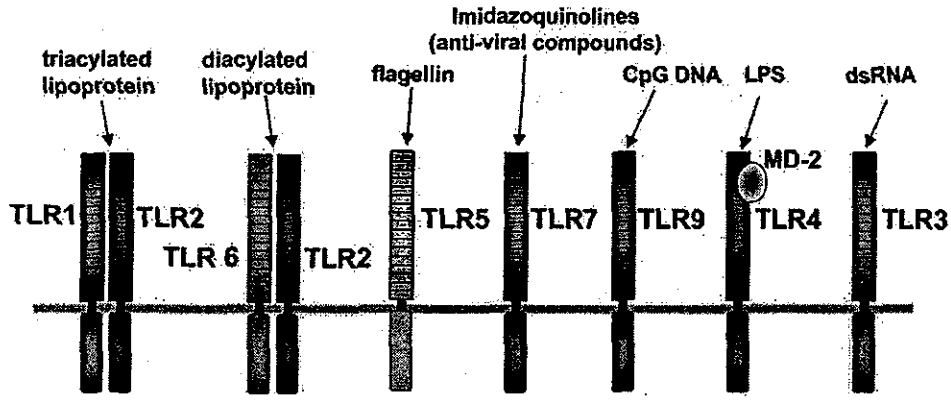


Fig. 1. TLRs and their ligands. TLR1–TLR7 and TLR9 have been characterized to recognize microbial components. TLR2 is essential for the recognition of microbial lipopeptides. TLR1 and TLR6 associate with TLR2, and discriminate subtle differences between triacyl- and diacyl lipopeptides, respectively. TLR4 recognizes LPS. TLR9 is the CpG DNA receptor, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 is a receptor for flagellin. Thus, the TLR family discriminates between specific patterns of microbial components.

## 3. Signaling pathways via TLRs

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. A crucial role for the TIR domain was first revealed in the C3H/HeJ mouse strain, which had a point mutation that resulted in an amino acid change of the cytoplasmic proline residue at position 712 to histidine [4,6]. This proline residue in the TIR domain is conserved among all TLRs, except for TLR3, and its substitution to histidine caused a dominant negative effect on TLR-mediated signaling [6,7]. In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, was first characterized to play a crucial role. In addition, recent accumulating evidence indicates that TLR signaling pathways consist, at least, of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR3- and TLR4 signaling pathways [8].

## 4. MyD88-dependent pathway

MyD88 possesses the TIR domain in the C-terminal portion, and a death domain in the N-terminal portion. MyD88 associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of the death domains of both molecules. IRAK is activated by phosphorylation and then associates with TRAF6, leading to the activation of two distinct signaling pathways, and finally to the activation of JNK and NF-κB (Fig. 2).

### *4.1. MyD88*

MyD88 knockout mice showed no responses to the TLR4 ligand LPS in terms of macrophage production of inflammatory mediators, B cell proliferation, or endotoxin shock [9]. The cellular responses to the TLR2 ligands peptidoglycan and lipoproteins were abolished in MyD88 knockout mice [10,11]. Furthermore, cells from MyD88 knockout mice showed no responses to the TLR9 ligand CpG DNA and the TLR7 ligand imidazoquinoline [12–14]. Finally, MyD88 knockout mice did not produce any IL-6 in response to the TLR5 ligand flagellin [15]. These findings demonstrated that the TIR domain-containing adaptor MyD88 is essential for the inflammatory responses mediated by all the TLR family members.

An alternatively spliced variant of MyD88, MyD88s, which lacks the intermediate domain, has been shown to be induced by LPS stimulation and to inhibit LPS-induced NF-κB activation through inhibition of IRAK activity [16,17]. Thus, MyD88s may negatively regulate the inflammatory responses triggered by LPS.

### *4.2. IRAK*

IRAK was originally identified as a serine/threonine kinase associated with the IL-1 receptor, which also harbors the TIR domain [18]. Four members of the IRAK family have been identified so far: IRAK-1, IRAK-2, IRAK-M, and IRAK-4. IRAK proteins consist of an N-terminal death domain, which is responsible for interaction with MyD88, and a central kinase domain. IRAK-1 and IRAK-4 harbor a critical aspartate residue in the kinase domain, but this residue is not conserved in IRAK-2 or IRAK-M, which causes them to be catalytically inactive [19]. The importance of the IRAK family members in TLR-mediated signaling pathways was first demonstrated in IRAK-1 knockout mice, which showed defective LPS-induced responses [20]. IRAK-1 knockout mice showed defective LPS responses, however, this impairment was only partial. In contrast, IRAK-4 knockout mice showed almost complete impairment in the response to microbial components that stimulate TLR2, TLR3, TLR4, and TLR9 [21]. A biochemical study revealed that IRAK-4 acts upstream of, and phosphorylates, IRAK-1 upon stimulation [22]. Thus, IRAK-4 is a central mediator of TLR signal-

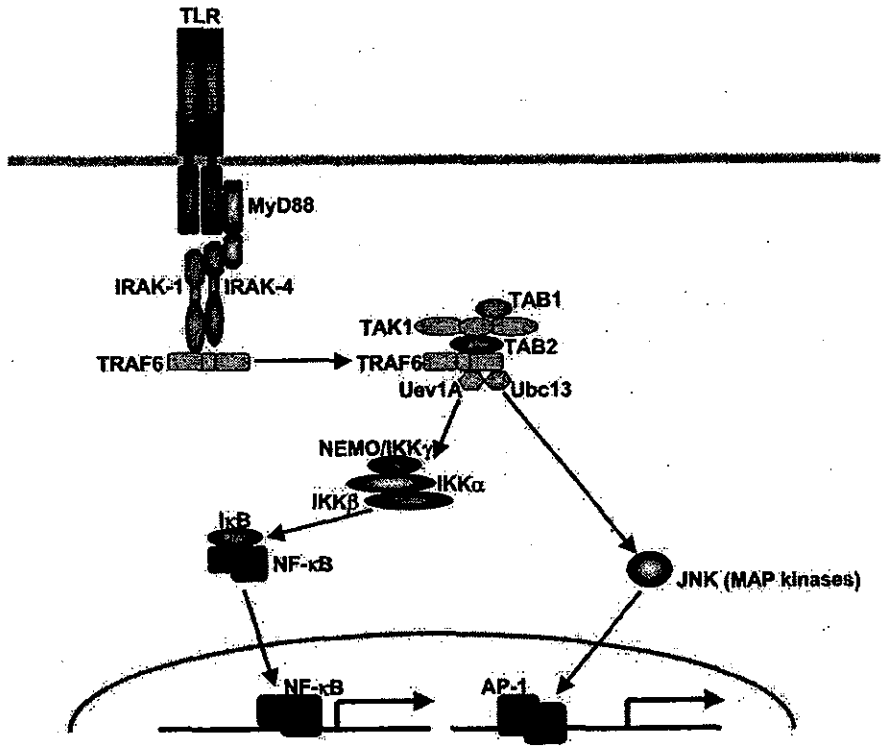


Fig. 2. TLR-mediated MyD88-dependent signaling pathway. MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. Upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor, which induces association of IRAK-1 and MyD88 via the death domains. IRAK-4 then phosphorylates IRAK-1. Phosphorylated IRAK-1, together with TRAF6, dissociates from the receptor and then TRAF6 interacts with TAK1, TAB1, and TAB2. The complex of TRAF6, TAK1, TAB1, and TAB2 further forms a larger complex with Ubc13 and Uev1A, which induces the activation of TAK1. Activated TAK1 phosphorylates the IKK complex, consisting of IKKα, IKKβ, and NEMO/IKKγ, and MAP kinases, such as JNK, and thereby induces the activation of the transcription factors NF-κB and AP-1, respectively.

ing by activating IRAK-1. In sharp contrast to mice lacking IRAK-1 and IRAK-4, IRAK-M knockout mice showed increased production of inflammatory cytokines in response to the TLR ligands and exaggerated inflammatory response to bacterial infection, demonstrating that IRAK-M plays a negative inhibitory role in the TLR signaling pathway [23].

### 4.3. TRAF6 and downstream molecules

TRAF6 is a member of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family that mediates cytokine signaling pathways [24]. TRAF proteins consist of two C-terminal TRAF domains (TRAF-N and TRAF-C), which are responsible for interaction with TRAF proteins and other signaling molecules, N-terminal RING finger, and zinc finger domains. Among the TRAF family members, TRAF6 has been shown to be involved in the TLR signaling pathway in addition to signaling pathways via the OPGL receptor and CD40 [25,26]. Upon stimulation of TLRs, TRAF6 is recruited to the receptor complex, and activated by IRAK-1 that binds to the TRAF domain of TRAF6. Then, the IRAK-1/TRAF6 complex dissociates from the receptor and associates with TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2, at the membrane portion. IRAK-1 stays in the membrane and is degraded, whereas the complex of TRAF6, TAK1, TAB1, and TAB2 moves into the cytoplasm, where it forms a large complex with other proteins, such as the E2 ligases Ubc13 and Uev1A [27]. The Ubc13 and Uev1A complex has been shown to catalyze the synthesis of a Lys 63-linked polyubiquitin chain of TRAF6 and thereby induce TRAF6-mediated activation of TAK1 and finally of NF-κB [28].

### 4.4. Other molecules

In addition to the molecules described above, several other molecules have been implicated in the TLR-mediated signaling pathway. Toll-interacting protein (Tollip) was first identified in an analysis of IL-1 signaling [29]. Tollip is present in a complex with IRAK-1. Upon stimulation with IL-1, the Tollip-IRAK-1 complex is recruited to the IL-1 receptor complex. IRAK-1 is then phosphorylated, which leads to the rapid dissociation of IRAK-1 from Tollip, thereby inducing activation of TRAF6. Subsequently, Tollip has been shown to negatively regulate the TLR-mediated signaling pathway [30,31]. Overexpression of Tollip inhibited activation of NF-κB in response to IL-1, the TLR2 and TLR4 ligands. However, it remains unclear how Tollip is physiologically involved in TLR signaling.

Pellino was originally identified in *Drosophila* as a molecule that associates with Pelle, a *Drosophila* homologue of IRAK. In mammals, two Pellino homologues,

Pellino-1 and Pellino-2, have been identified. Both Pellino-1 and Pellino-2 have been shown to interact with IRAK-1 in response to IL-1 stimulation [32,33]. Ectopic expression of the Pellino-2 antisense construct inhibited IL-1- or LPS-induced activation of the NF-κB-dependent promoter, indicating that Pellino-2 is involved in the IL-1 and TLR4 signaling pathways. Thus, several molecules that may modulate TLR signaling have been identified.

## 5. MyD88-independent pathway

As described above, MyD88 knockout mice did not show any production of inflammatory cytokines, such as TNF-α and IL-12, in response to any of the TLR ligands. Furthermore, activation of NF-κB and JNK in response to the TLR2, TLR7, and TLR9 ligands was not observed in MyD88 knockout mice. However, in the case of TLR4 stimulation, LPS-induced activation of NF-κB and JNK was observed with delayed kinetics, even in MyD88 knockout cells, although these cells did not produce any inflammatory cytokines in response to LPS [9]. In an attempt to assess the role of LPS-induced signal activation in a MyD88-independent manner, a subtraction analysis was performed using mRNA extracted from non-stimulated and LPS-stimulated MyD88 knockout macrophages [34]. This analysis revealed that IFN-inducible genes, such as IP-10 and GARG16, were induced in response to LPS in MyD88 knockout cells. Subsequent studies clearly demonstrated that there is a MyD88-independent pathway as well as a MyD88-dependent pathway in TLR signaling. In the MyD88-independent pathway, LPS stimulation leads to activation of the transcription factor IRF-3, and thereby induces IFN-β. IFN-β, in turn, activates Stat1, leading to the induction of several IFN-inducible genes [35–37].

In addition to the TLR4 ligand, the TLR3 ligand dsRNA has been shown to induce activation of NF-κB in MyD88 knockout cells [38]. Virus and viral-derived dsRNA are potent activators of IRF-3, which leads to the initial phase of IFN-β induction [39–41]. Thus, the TLR3 ligand dsRNA also activates the MyD88-independent signaling pathway, in which IRF-3 plays a key role. Recently, two independent groups identified kinases responsible for the activation of IRF-3. Hiscott and colleagues tried to identify molecules that interact with IRF-3 by two-hybrid screening, and found that IRF-3 was associated with IκB kinases (IKKs) [42]. IKKs are composed of IKKα and IKKβ, both of which phosphorylate Ser32 and Ser36 of IκBα, thereby inducing NF-κB activation. In addition, there are two noncanonical IKKs, TANK-binding kinase 1 (TBK1) and IKKε/IKK*i*, which have distinct kinase activities compared with the canonical IKKα and IKKβ. They analyzed whether these four IKKs could phosphorylate IRF-3 using an in vitro kinase assay, and found that TBK1 and IKKε/IKK*i* induced IRF-3 phosphorylation. RNAi-mediated ablation of TBK1 and IKKε/IKK*i* resulted in inhibition of virus-induced phosphorylation of IRF-3. Maniatis and colleagues also found that overexpression of TBK1 and IKKε/IKK*i* led to activation of IRF-3 and induction of IFN-β [43]. They also showed that reduced expression of TBK1 and IKKε/IKK*i* by RNAi led to impaired induction of IFN-β in response to virus and dsRNA. Thus, TBK1 and IKKε/IKK*i* have been shown to be critical regulators of IRF-3 activation, leading to the induction of IFN-β in response to the TLR3 ligand. At present, it remains unclear whether these noncanonical IKKs are involved in TLR4-mediated IRF-3 activation. Although TBK1 knockout mice have been characterized, involvement of TBK1 in the MyD88-independent pathway has not been analyzed in these mice [44]. Studies with TBK1 and IKKε/IKK*i* knockout mice will clarify the involvement of these IKKs in the MyD88-independent pathway.

## 6. TIR domain-containing adaptors

During analysis of the MyD88-independent pathway, two TIR domain-containing adaptors, TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) and TIR domain-containing adaptor inducing IFN-β (TRIF)/TIR domain-containing adaptor molecule (TICAM-1), were identified [45–48]. Analysis of these two adaptors indicated that TIR domain-containing adaptors regulate the TLR-mediated signaling pathways by providing specificity for individual TLR signaling cascades (Fig. 3).

### *6.1. TIRAP/Mal*

Database search analyses led to the identification of a second TIR domain-containing molecule, which was named TIRAP or Mal [45,46]. TIRAP/Mal harbors the TIR domain in the C-terminus. Initial *in vitro* studies indicated that TIRAP/Mal specifically interacts with TLR4, and is involved in the TLR4-mediated MyD88-independent signaling pathway. However, generation of TIRAP/Mal knockout mice revealed an unexpected role of TIRAP/Mal in TLR signaling [49,50]. Similarly to MyD88 knockout macrophages, TIRAP/Mal knockout macrophages showed impaired inflammatory cytokine production and delayed activation of JNK and NF-κB in response to the TLR4 ligand. However, TLR4 ligand-induced activation of IRF-3 and expression of IFN-inducible genes was normally observed in TIRAP/Mal knockout macrophages. Even in mice lacking both MyD88 and TIRAP/Mal, the TLR4 ligand-induced expression of IFN-inducible genes was not impaired. Thus, TIRAP/Mal is critically involved in the MyD88-dependent pathway, but not in the MyD88-independent pathway, via TLR4. TIRAP/Mal knockout mice showed normal responses to the TLR3, TLR5, TLR7, and TLR9 ligands, but were defective in TLR2 ligand-induced inflammatory cytokine production. Taken together, these studies clearly established that TIRAP/Mal is essential for the

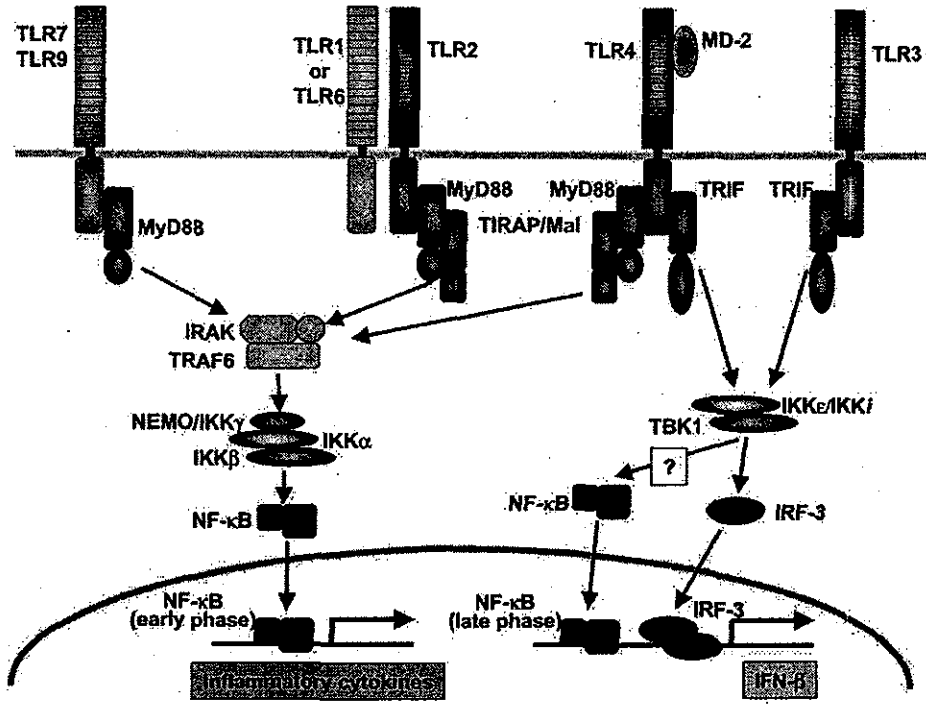


Fig. 3. TIR domain-containing adaptors and TLR signaling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via all the TLRs. TIRAP/Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In the TLR4- and TLR3-mediated signaling pathways, a MyD88-independent pathway exists that leads to activation of IRF-3 via TBK1 and IKKε/IKKi. The TIR domain-containing adaptor TRIF mediates this MyD88-independent pathway.

MyD88-dependent signaling pathway via TLR2 and TLR4, but not for MyD88-independent signaling.

### 6.2. TRIF

A third TIR domain-containing adaptor, TRIF/TICAM-1 was identified by a database search and as a TLR3-associated molecule by two-hybrid screening [47,48]. Unlike MyD88 and TIRAP/Mal, TRIF is a large protein consisting of 712 amino acids in humans. Overexpression of TRIF as well as MyD88 and TIRAP caused activation of the NF-κB-dependent promoter in 293 cells. Furthermore, overexpression of TRIF, but not MyD88 or TIRAP, induced activation of the IFN-β promoter. Dominant negative TRIF inhibited the TLR3 ligand-induced activation of the IFN-β promoter, and RNAi-mediated knockdown of TRIF caused impairment in the TLR3 ligand-induced IFN-β expression. Thus, these in vitro studies indicated that TRIF is involved in the TLR3-mediated MyD88-independent pathway.

Most recently, TRIF knockout mice have been generated. In TRIF knockout mice, TLR3-mediated expression of IFN-β and IFN-inducible genes was impaired [51]. Furthermore, TRIF knockout mice displayed defective expression of IFN-inducible genes in response to the TLR4 ligand. A study of random germline mutagenesis in mice, using the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU), also revealed that TRIF-mutant mice were defective in the TLR3- and TLR4-mediated responses [52]. Thus, TRIF has been demonstrated to be essential for the TLR3- and TLR4-mediated MyD88-independent pathway. These studies clearly established that TIR domain-containing adaptors provide specificity for individual TLR-mediated signaling pathways. In addition to the impaired MyD88-independent pathway, TRIF knockout mice displayed defective TLR4-mediated inflammatory cytokine production, although activation of the MyD88-dependent pathway, such as IRAK-1 phosphorylation and early phase of NF-κB activation, was not impaired. Therefore, the TLR4 signaling pathway is likely to require activation of both the MyD88-dependent and -independent pathways to induce inflammatory cytokines.

### 6.3. Other TIR domain-containing adaptors

In addition to MyD88, TIRAP, and TRIF, a fourth TIR domain-containing adaptor, TIRP, has recently been identified [53]. Human TIRP protein consists of 235 amino acids, and the TIR domain was located in the middle portion of the protein. Although TIRP has been shown to be involved in the IL-1 receptor-mediated signaling pathway, it remains unclear whether TIRP mediates the TLR signaling pathway. In addition, there is another TIR domain-containing adaptor, SARM. This molecule is a large protein consisting of about 700 amino acids, and the TIR domain is located in the C-terminal portion. At present, we do not know whether this molecule is involved in the TLR-mediated signaling pathway. Generation of knockout mice of all of these adaptors will provide definite evidence of their roles in TLR signaling.