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TIR domain-containing adaptors define the specificity of TLR signaling

Masahiro Yamamoto^a, Kiyoshi Takeda^{a,b}, Shizuo Akira^{a,b,*}

^a Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

^b ERATO, Japan Science and Technology Corporation, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

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.

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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- κ B, 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-1 β , 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- κ B and MAPK through almost all of the TLRs is abolished in MyD88-deficient cells,

* Corresponding author. Tel.: +81-6-6879-8303; fax: +81-6-6879-8305.

E-mail address: sakira@biken.osaka-u.ac.jp (S. Akira).

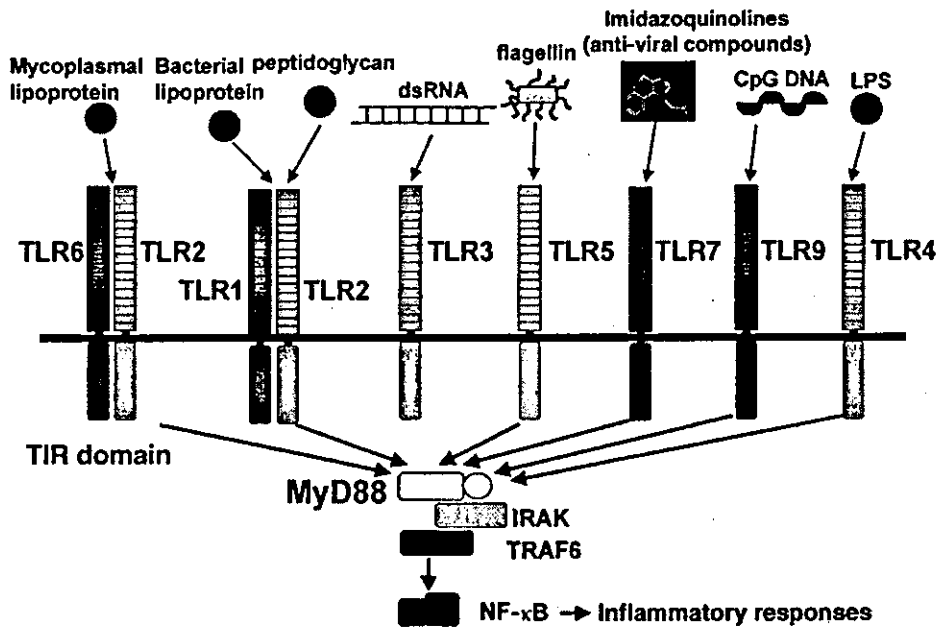


Fig. 1. TLRs recognize molecular pattern associated with bacterial pathogens. triacylated lipoprotein for TLR1; peptidoglycan for TLR2; double-stranded RNA for TLR3; lipopolysaccharide (LPS) for TLR4; flagellin for TLR5; diacylated lipoprotein for TLR6; imidazoquinoline and its derivative R-848, for 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

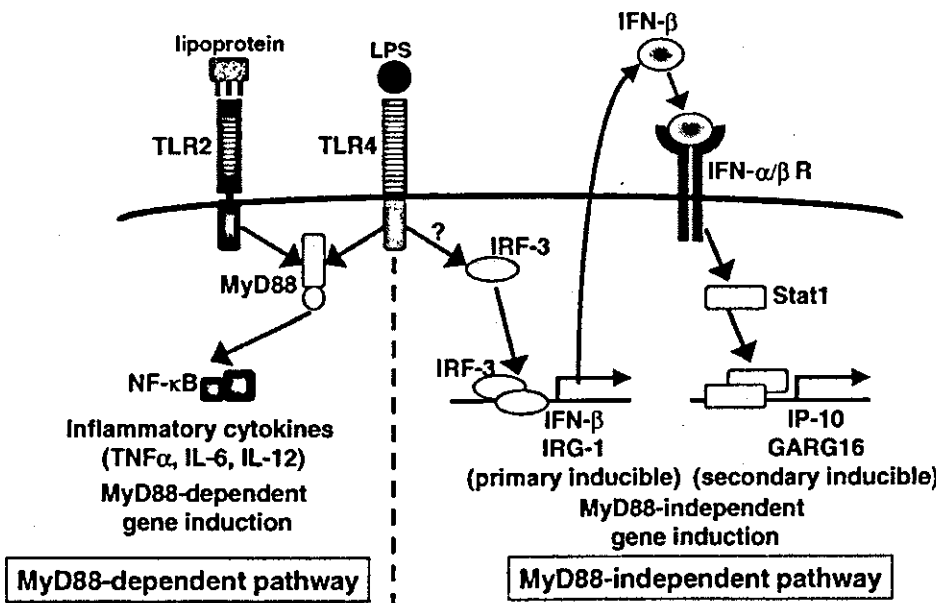


Fig. 2. TLR signaling pathways are separated into two groups. A MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK, and a MyD88-independent pathway associated with the induction of IFN-β and IFN-inducible genes, and maturation of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

genes and maturation of dendritic cells with slow activation of NF- κ B and MAPK. TLR3 has also been shown to utilize the MyD88-independent pathway, since poly(I:C)-induced NF- κ B 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- κ B-dependent promoter as strongly as MyD88, and the P125H mutant of TIRAP, which acted in a dominant negative manner, specifically blocked TLR4-dependent NF- κ B 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- κ B was normally observed at 20 or 60 min after stimulation, even in TIRAP-deficient macrophages, early NF- κ B activation at 10 min was not observed in TIRAP-deficient cells. The delayed NF- κ B activation in TIRAP-deficient cells in response to LPS was reminiscent of that of MyD88-deficient cells, which also showed LPS-stimulated NF- κ B 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- κ B 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- κ B activation as detected by a luciferase-mediated reporter assay. Overexpression of TRIF activated NF- κ B, 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- κ B and

the IFN- β promoter: Δ N lacked the N-terminus except for the TIR domain, Δ C lacked the C-terminus but possessed the TIR domain, and Δ N Δ C lacked both the N-terminus and C-terminus and was only composed of the TIR domain. When transfected together with an NF- κ B-dependent reporter plasmid, both Δ N and Δ C induced activation of the NF- κ B-dependent promoter, albeit at reduced levels compared with full-length TRIF. Δ N Δ C did not activate the NF- κ B-responsive luciferase reporter. On the other hand, Δ C, but not Δ N or Δ N Δ C, 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- κ B, 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- κ B and the IFN- β promoter.

Full-length TRIF-mediated activation of NF- κ B or the IFN- β promoter was profoundly inhibited by cotransfection of Δ N Δ C, suggesting that Δ N Δ C 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- κ B-dependent luciferase reporter, respectively. Cotransfection of Δ N Δ C significantly inhibited the TLR2- or TLR7-mediated NF- κ B activation. LPS stimulation activated the NF- κ B responsive reporter in cells transfected with both TLR4 and MD-2, but this activation was abolished by cotransfection of Δ N Δ C. In addition, activation of NF- κ B induced by overexpression of MyD88 and TIRAP was predominantly blocked by Δ N Δ C cotransfection. 293 cells stably transfected with TLR3 showed poly(I:C) stimulation-dependent activation of NF- κ B 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- κ B or the IFN- β promoter. Most notably, TRIF Δ N Δ C completely inhibited not only the activation of poly(I:C)-induced NF- κ B 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- κ B-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- κ B 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).

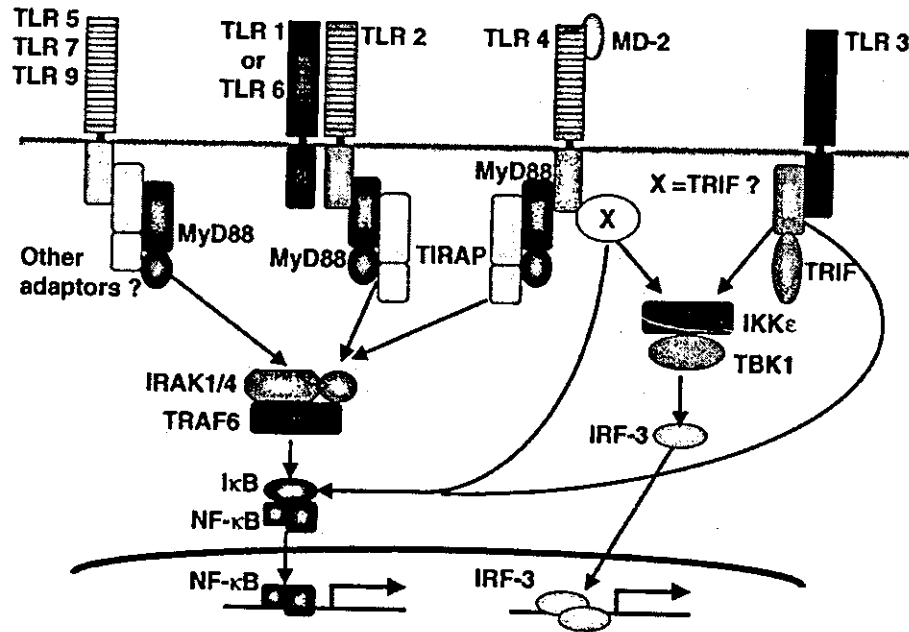


Fig. 3. TIRAP is an adaptor molecule shared with TLR2 and TLR4 signaling cascade. Other adaptors may also exist downstream of TLR5, TLR7, and TLR9 in the MyD88-dependent pathways like TIRAP in TLR2- and TLR4-mediated signaling pathway. TRIF may exist downstream of TLR3/TLR4, and upstream of IKK ϵ and 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- κ B-responsive luciferase reporter and the IFN- β promoter. On the other hand, the other group showed that overexpression of TRAM/TIRP only stimulated NF- κ B 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- κ B 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- κ B 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- κ B 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- κ B 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 IKK α and IKK β , and therefore possibly having a role in NF- κ B 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- κ B (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- α 4, 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.

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TLR signaling pathways

Kiyoshi Takeda, Shizuo Akira*

Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, and ERATO,
Japan Science and Technology Corporation, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

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.

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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.

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).

* Corresponding author. Tel.: +81-6-6879-8303; fax: +81-6-6879-8305.
E-mail address: sakira@biken.osaka-u.ac.jp (S. Akira).

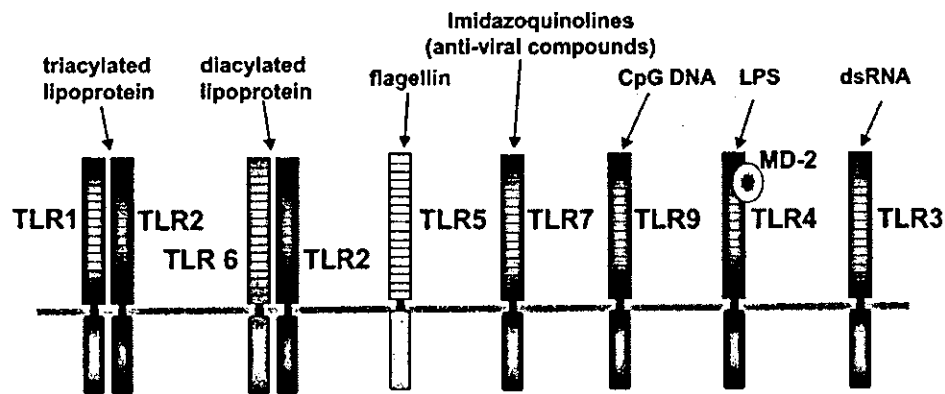


Fig. 1. TLRs and their ligands. TLR1–TLR7 and TLR9 have been characterized to recognize microbial components. TLR2 is essential for the recognition of microbial lipopeptides. TLR1 and TLR6 associate with TLR2, and discriminate subtle differences between triacyl- and diacyl lipopeptides, respectively. 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- κ B (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- κ B 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-

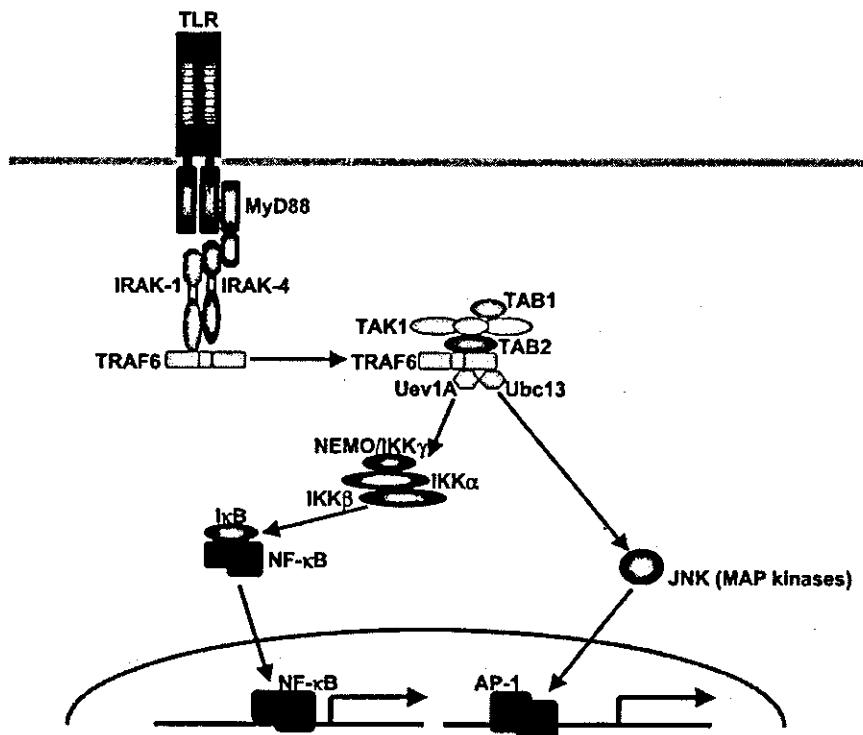


Fig. 2. TLR-mediated MyD88-dependent signaling pathway. MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. Upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor, which induces association of IRAK-1 and MyD88 via the death domains. IRAK-4 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 IKK α , IKK β , and NEMO/IKK γ , and MAP kinases, such as JNK, and thereby induces the activation of the transcription factors NF- κ B 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- κ B [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- κ B 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- κ B-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- κ B 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- κ B 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- κ B 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 I κ B kinases (IKKs) [42]. IKKs are composed of IKK α and IKK β , both of which phosphorylate Ser32 and Ser36 of I κ B α , thereby inducing NF- κ B activation. In addition, there are two noncanonical IKKs, TANK-binding kinase 1 (TBK1) and IKK ϵ /IKK ι , which have distinct kinase activities compared with the canonical IKK α and IKK β . They analyzed whether these four IKKs could phosphorylate IRF-3 using an *in vitro* kinase assay, and found that TBK1 and IKK ϵ /IKK ι induced IRF-3 phosphorylation. RNAi-mediated ablation of TBK1 and IKK ϵ /IKK ι resulted in inhibition of virus-induced phos-

phorylation of IRF-3. Maniatis and colleagues also found that overexpression of TBK1 and IKK ϵ /IKK ι led to activation of IRF-3 and induction of IFN- β [43]. They also showed that reduced expression of TBK1 and IKK ϵ /IKK ι by RNAi led to impaired induction of IFN- β in response to virus and dsRNA. Thus, TBK1 and IKK ϵ /IKK ι 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 IKK ϵ /IKK ι 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- κ B 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

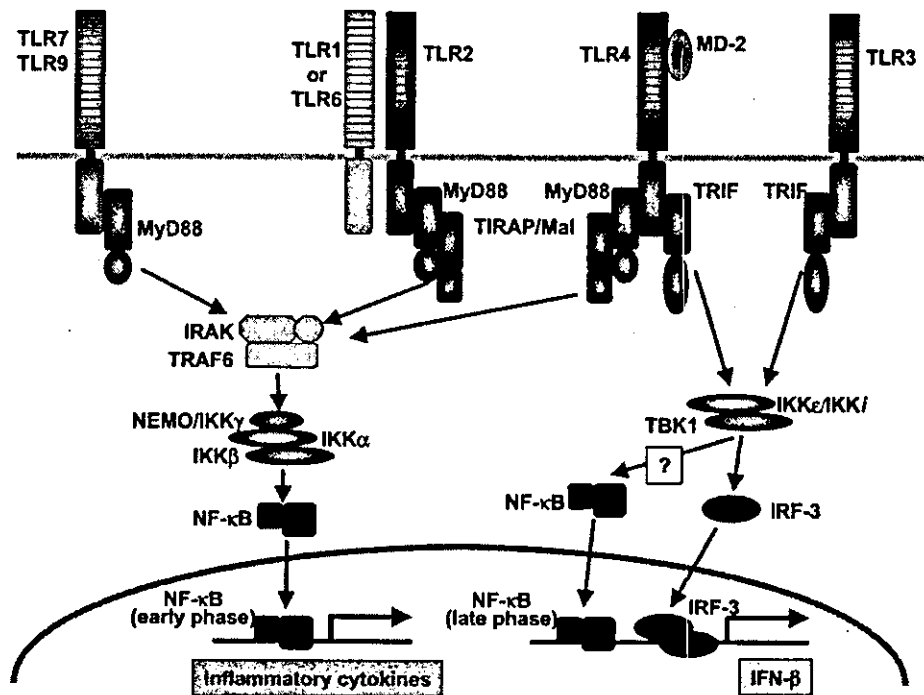


Fig. 3. TIR domain-containing adaptors and TLR signaling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via all the TLRs. TIRAP/Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In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

7. Future prospects

Since the discovery of TLRs in mammals, rapid progress has been made on our understanding of the molecular mechanisms of innate immunity. Individual TLRs recognize their specific microbial components and activate signaling pathways. The TLR signaling pathways also have their own cascades for exhibiting their specific responses, which are characterized by several TIR domain-containing adaptors. Elucidation of the physiological roles of these adaptors will provide important clues for understanding how individual TLRs induce their specific innate immune responses.

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REVIEWS

TOLL-LIKE RECEPTOR SIGNALLING

Shizuo Akira* and Kiyoshi Takeda†

One of the mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms is through the Toll-like receptors (TLRs), which recognize specific molecular patterns that are present in microbial components. Stimulation of different TLRs induces distinct patterns of gene expression, which not only leads to the activation of innate immunity but also instructs the development of antigen-specific acquired immunity. Here, we review the rapid progress that has recently improved our understanding of the molecular mechanisms that mediate TLR signalling.

All living organisms are exposed constantly to microorganisms that are present in the environment and need to cope with invasion of these organisms into the body. The vertebrate immune response can be divided into innate and acquired immunity, with innate immunity being the first line of defence against pathogens. By contrast, acquired immune responses are slower processes, which are mediated by T and B cells, both of which express highly diverse antigen receptors that are generated through DNA rearrangement and are thereby able to respond to a wide range of potential antigens. This highly sophisticated system of antigen detection is found only in vertebrates and has been the subject of considerable research. Far less attention has been directed towards innate immunity, as it has been regarded as a relatively nonspecific system, with its main roles being to destroy pathogens and to present antigen to the cells involved in acquired immunity. However, recent studies have shown that the innate immune system has a greater degree of specificity than was previously thought and that it is highly developed in its ability to discriminate between self and foreign pathogens¹. This discrimination relies, to a great extent, on a family of evolutionarily conserved receptors, known as the Toll-like receptors (TLRs), which have a crucial role in early host defence against invading pathogens^{1,2}. Furthermore, accumulating evidence indicates that activation of the innate immune system is a prerequisite for the induction of acquired immunity, particularly for the induction of a T helper 1 (T_H1)-cell response^{3,4}. This marked shift in our thinking has changed our ideas about the

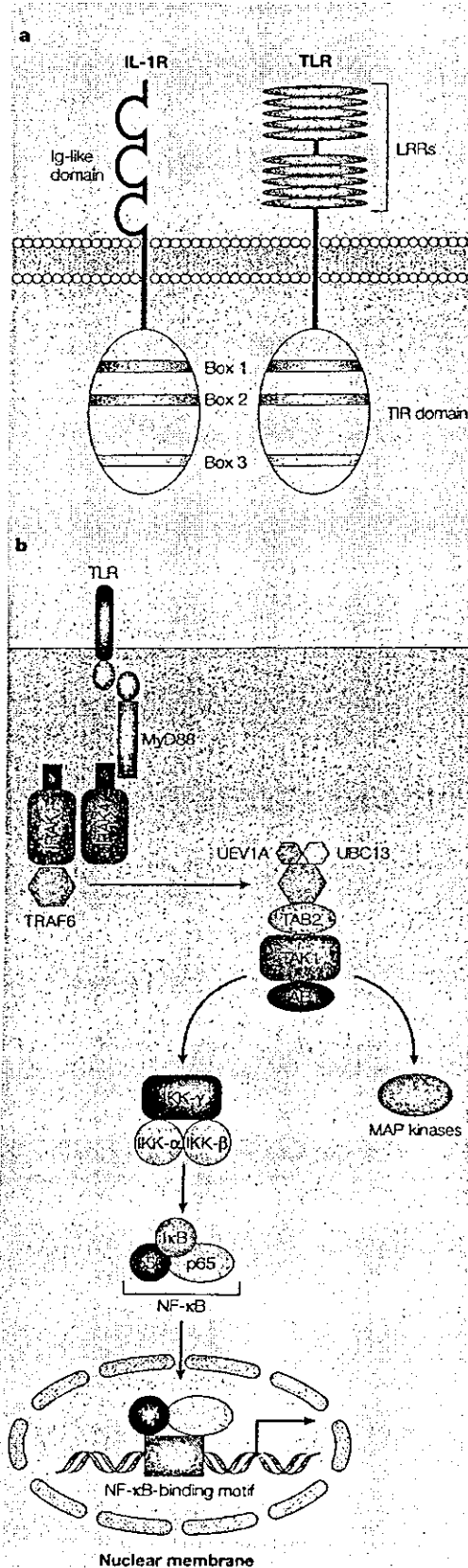
pathogenesis and treatment of cancers, and infectious, immune and allergic diseases. In the past few years, our knowledge of TLR signalling and the responses these receptors control has greatly increased. In this review, we discuss the TLRs, focusing on their signalling pathways.

TLR/IL-1R superfamily: structure and function

The discovery of the TLR family began with the identification of Toll, a receptor that is expressed by insects and was found to be essential for establishing dorsoventral polarity during embryogenesis⁵. Subsequent studies revealed that Toll also has an essential role in the insect innate immune response against fungal infection⁶. Homologues of Toll were identified through database searches, and so far, 11 members of the TLR family have been identified in mammals. The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs). By contrast, the extracellular region of the TLRs and IL-1Rs differs markedly: the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains (FIG. 1a).

Toll/IL-1R domain. TLRs and IL-1Rs have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain⁷. Within the TIR domain, the regions of homology comprise three conserved boxes, which are crucial for

*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, and ERATO, Japan Science and Technology Agency, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.
†Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Correspondence to S.A.
e-mail: sakira@biken.osaka-u.ac.jp
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signalling (FIG. 1a). Amino-acid sequence conservation among the TIR domains is generally 20–30%, and these domains vary in size. The crystal structures of the TIR domains of human TLR1 and TLR2 have been obtained and analysed; they contain a central five-stranded parallel β -sheet, which is surrounded by five α -helices on each side⁶. These two secondary structural elements are connected by loops: for example, the BB loop connects the strand β -B and the helix α -B. The conserved boxes 1 and 2 and the BB loop are adjacent and display most of their side chains for interaction with adaptor molecules.

C3H/HeJ mice have a defect in their ability to respond to lipopolysaccharide (LPS) because of a missense mutation in the *Tlr4* gene⁹, which alters the sequence located at the tip of the BB loop, farthest from the rest of the TIR domain. This indicates that the mutation abrogates LPS signalling not because it disrupts the TIR domain structure itself, but rather because it disrupts a direct point of contact with another molecule or molecules, specifically with other TIR-domain-containing molecules.

Leucine-rich repeats. The extracellular domain of TLRs contains 19–25 tandem copies of the LRR motif. Each repeat consists of 24–29 amino acids and contains the leucine-rich sequence XLXXLX, and another conserved sequence XØXXØX,FXXLX (REF.10), where X denotes any amino acid and Ø a

Figure 1 | TLR structure and signalling. **a** | Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs) have a conserved cytoplasmic domain, that is known as the Toll/IL-1R (TIR) domain. The TIR domain is characterized by the presence of three highly homologous regions (known as boxes 1, 2 and 3). Despite the similarity of the cytoplasmic domains of these molecules, their extracellular regions differ markedly: TLRs have tandem repeats of leucine-rich regions (known as leucine rich repeats, LRR), whereas IL-1Rs have three immunoglobulin (Ig)-like domains. **b** | Stimulation of TLRs triggers the association of MyD88 (myeloid differentiation primary-response protein 88), which in turn recruits IRAK4 (IL-1R-associated kinase 4), thereby allowing the association of IRAK1. IRAK4 then induces the phosphorylation of IRAK1. TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) is also recruited to the receptor complex, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor- β -activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane (not shown), which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor- κ B (I κ B)-kinase complex), which consists of IKK- α , IKK- β and IKK- γ (also known as IKK1, IKK2 and nuclear factor- κ B (NF- κ B) essential modulator, NEMO, respectively). The IKK complex then phosphorylates I κ B, which leads to its ubiquitylation and subsequent degradation. This allows NF- κ B to translocate to the nucleus and induce the expression of its target genes.