

## Review

# Toll-like receptor downstream signaling

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## Abstract

The family of Toll-like receptors (TLRs) senses conserved structures found in a broad range of pathogens, causing innate immune responses that include the production of inflammatory cytokines, chemokines and interferons. The signal transduction is initiated from the Toll/interleukin-1 receptor (TIR) domain of TLRs after pathogen recognition. Almost all TLRs use a TIR-containing adapter MyD88 to activate a common signaling pathway that results in the activation of NF- $\kappa$ B to express cytokine genes relevant to inflammation. Recently, three further TIR-containing adapters have been identified and shown to selectively interact with several TLRs. In particular, activation of the TRIF-dependent pathway confers antiviral responses by inducing anti-viral genes including that encoding interferon- $\beta$ . Taken together, these results indicate that the interaction between individual TLRs and the different combinations of adapters directs appropriate responses against distinct pathogens.

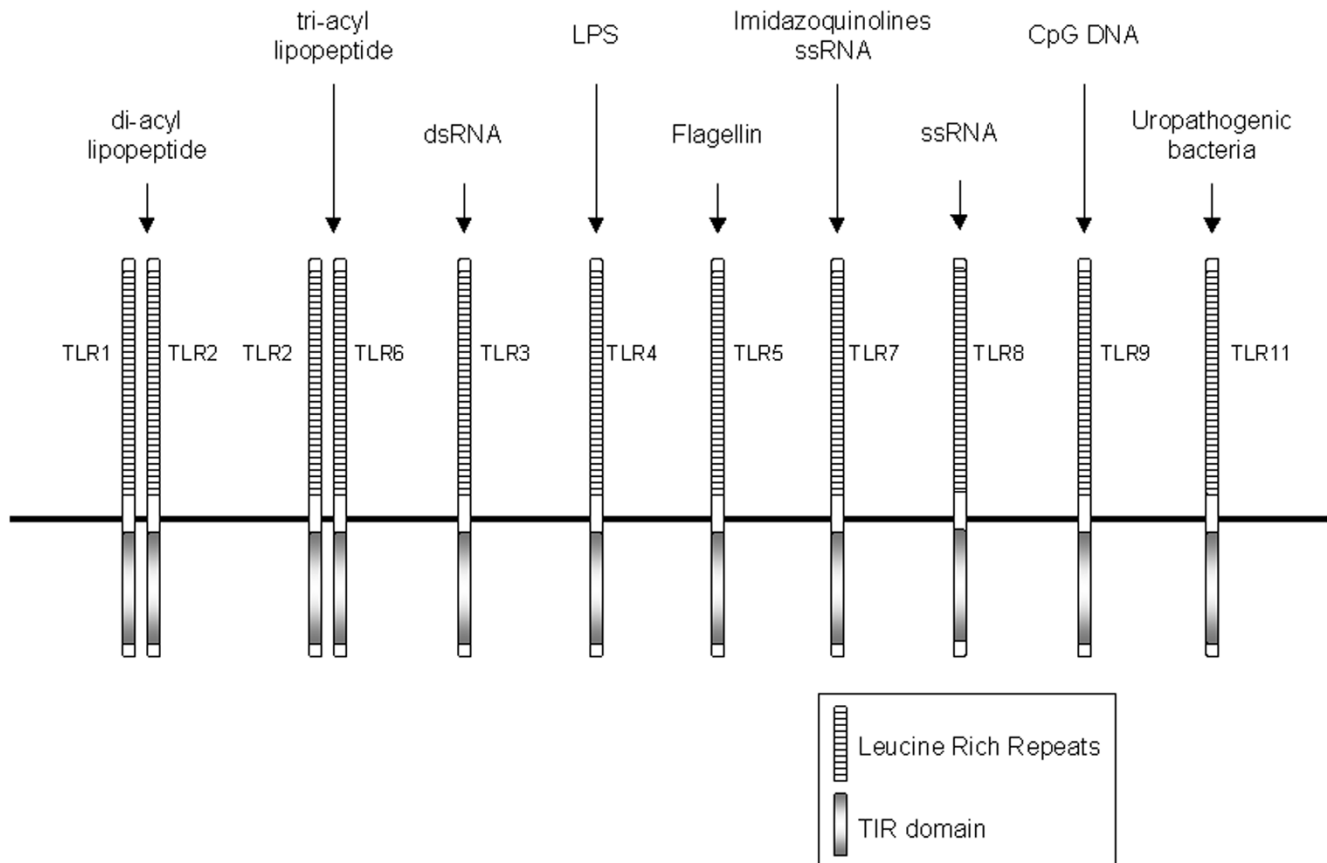
**Keywords:** inflammatory cytokines, innate immunity, interferons, TIR-domain containing adapter, TLR

## Introduction

In *Drosophila*, Toll was initially identified as an essential protein for the determination of dorsoventral polarity during early embryogenesis. Subsequently, it was shown that flies with a mutant *Toll* gene are highly susceptible to fungal infection because of a defective production of specific anti-fungi peptides upon infection, demonstrating that Toll is a receptor that detects fungi invasion to trigger immune responses [1]. In 1997, a human gene similar to the *Toll* gene was identified through the bioinformatic approach [2]. Initial study demonstrated that this gene product can promote the expression of genes encoding inflammatory cytokines, suggesting that Toll in mammals also has a function in innate immune responses. A further five genes homologous to the *Toll* gene were subsequently identified, and these genes were referred to as the Toll-like receptor (TLR) family. So far, 11 members of the TLR family (TLR1–TLR11) have been identified. In both *Drosophila* and humans, Toll/TLRs consists of two

major domains characterized by leucine-rich repeats (LRR domain) and Toll/interleukin-1 receptor (TIR domain) domain (Fig. 1). The first evidence of TLRs in the recognition of pathogens was reported from studies with mice carrying a point-mutated or disrupted *Tlr4* gene [3,4]. These mice are unresponsive to bacterial lipopolysaccharide (LPS), an integral component of the outer membranes of Gram-negative bacteria that can cause endotoxin shock. Subsequently, the generation of knockout mice for each TLR gene has revealed respective pathogens that can be recognized by each TLR. TLR2 is involved in the responses to a variety of bacterial components that include peptidoglycan, lipoproteins/lipopeptides, glycosyl-phosphatidylinositol anchors from *Trypanosoma cruzi*, and zymosan [5–7]. However, recognition of these TLR2 ligands requires another TLR family member. The mycoplasmal diacylated lipopeptide MALP-2 is recognized by a heterodimer of TLR2 and TLR6, whereas the bacterial triacylated lipopeptide

AP-1 = activator protein-1; DCs = dendritic cells; dsRNA = double-stranded RNA; GARG16 = glucocorticoid-attenuated response gene 16; IFN = interferon; IKK = I $\kappa$ B kinase; IL-1R = interleukin-1 receptor; IP = interferon-inducible protein; IRAK = interleukin-1 receptor-associated kinase; IRF = interferon regulatory factor; IRG-1 = immune response gene 1; LPS = lipopolysaccharide; LRR = leucine-rich repeats; MAL = MyD88-like adaptor; MALP-2 = macrophage-activating lipopeptide-2; MAP = mitogen-activated protein; MyD88 = myeloid differentiation factor 88; NF- $\kappa$ B = nuclear factor kappa B; RHIM = RIP homotypic interaction motif; RIP = receptor-interacting protein; TIR = Toll/interleukin-1 receptor; TLR = Toll-like receptor; TRAF = TNF receptor associated factor; TRAM = TRIF-related adaptor molecule; TRIF = TIR-domain-containing adaptor-inducing interferon beta.

**Figure 1**

Structure and ligands for Toll-like receptors (TLRs). dsRNA, double-stranded RNA; LPS, lipopolysaccharide; TIR, Toll/interleukin-1 receptor.

PAM3CSK4 is recognized by a heterodimer of TLR2 and TLR1 [8,9]. Flagellin, a 55 kDa monomer obtained from bacterial flagellum, the polymeric rod-like appendage extending from the outer membrane of Gram-negative bacteria, is also a potent pro-inflammatory inducer, which is recognized by TLR5 [10]. TLR3 recognizes double-stranded (ds) RNA that is generated in the lifecycle of RNA viruses during infection [11]. TLR7 recognizes the pharmaceutical compounds imiquimod (also known as Aldara, R-837 or S-26308) and resiquimod (also known as R-848 or S-28463) [12]. These compounds of the imidazoquinoline family are known to have potent antiviral and antitumor activities. TLR7 and its close relative TLR8 also recognize the single-stranded RNA present in numerous viruses [13,14]. TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs commonly present in bacterial and viral genomes that have immunostimulatory activities [15]. It has recently been shown that TLR11, which is abundant in the kidney and bladder, senses uropathogenic bacteria [16] (Fig. 1).

### Signal transduction pathway of interleukin-1 receptor (IL-1R) and TLRs

TLRs signal through a pathway conserved in the IL-1R family [17]. The TIR domain, which is present in all TLRs and IL-1R family members, is responsible for initiating a signaling cascade through homophilic or heterophilic interactions with TIR-domain-containing adapters. Almost all TLRs and IL-1Rs recruit a TIR-domain-containing adapter protein MyD88. Upon association with TLRs or IL-1Rs, MyD88 in turn recruits members of IL-1R-associated kinase (IRAK) family through interactions between the death domains. Once phosphorylated, IRAK1 and IRAK4 dissociate from the receptor complex and then associate with TRAF6, a member of the TRAF family. In contrast, IRAK-M, which lacks the kinase activity, has been shown to prevent dissociation of IRAK1 and IRAK4 from receptor complex, thereby negatively regulating the TLR signaling [18,19]. Activated TRAF6 can form a complex with the ubiquitin-conjugating enzymes Ubc13 and Uev1A; TRAF6 then acts as the ubiquitin E3 ligase to activate the kinase

TAK1, a member of the mitogen-activated protein (MAP) kinase kinase kinase family [20,21]. TAK1 is thought to activate two divergent pathways that lead to the transcription factors NF- $\kappa$ B and AP-1 through kinase cascades involving the canonical I $\kappa$ B kinase complex (IKK- $\alpha$ , IKK- $\beta$ , and IKK- $\gamma$ ) and MAP kinases (ERK, JNK, p38), respectively, to induce the expression of target genes. However, there is no evidence showing that TAK1 is really involved in these pathways *in vivo*.

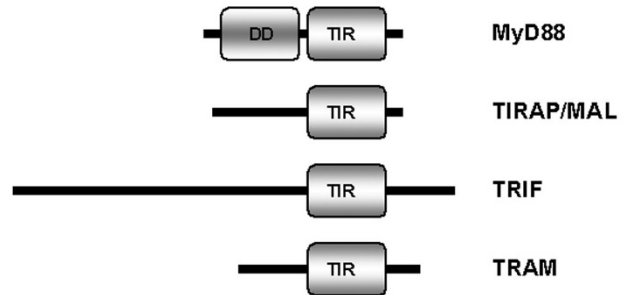
## TIR-domain containing adapters

### MyD88

MyD88 was originally identified as one of the myeloid differentiation primary response genes rapidly induced by IL-6 in M1 myeloleukemic cells. MyD88 contains two domains characterized by a death domain and a TIR domain, but lacks a putative transmembrane region, suggesting that MyD88 might function as an adapter protein in cytoplasm (Fig. 2). Consequently, MyD88 was shown to be recruited to the IL-1R after IL-1 ligation and to associate with IRAK1, resulting in the activation of transcription factors NF- $\kappa$ B and AP-1 [22–24]. Studies on MyD88-deficient mice clearly demonstrated that MyD88 is an essential component in the responses to IL-1 and the IL-1-related cytokine IL-18 [25]. All of the responses to IL-1 and IL-18 and the activation of NF- $\kappa$ B and MAP kinases were completely defective in cells from MyD88-deficient mice. Subsequent investigation confirmed that MyD88 is also used in TLR signaling. In MyD88-deficient mice, the production of inflammatory cytokines such as tumor necrosis factor- $\alpha$ , IL-1 $\beta$  and IL-6, the proliferation of B cells, and the induction of endotoxin shock in response to LPS (TLR4 ligand) are also completely abolished, demonstrating that MyD88 is indispensable for the responses to LPS *in vivo* [26]. In addition, cells from MyD88-deficient mice are totally unresponsive to peptidoglycan, lipoprotein, CpG DNA, and imidazoquinolines in terms of cytokine production [12,27,28] (Fig. 3).

In addition to inflammatory responses mediated by the MyD88-dependent pathway, a subset of TLRs can also induce appropriate responses depending on the types of pathogens. In TLR4-deficient macrophages, all of the responses to LPS tested are completely abolished, and the LPS-induced activation of NF- $\kappa$ B and MAP kinases fails totally, indicating that TLR4 is an essential signaling receptor for LPS [4,26]. In MyD88-deficient cells, the production of inflammatory cytokines and the activation of IRAK1 in response to LPS are also diminished. However, NF- $\kappa$ B and MAP kinases are unexpectedly activated after stimulation with LPS in MyD88-deficient cells, but the activation was delayed in comparison with that in wild-type cells [26]. In contrast, the activation of NF- $\kappa$ B and AP-1 in response to other stimuli such as ligands of TLR2, TLR5, TLR7, and TLR9 was completely defective in MyD88-deficient cells. These aspects strongly suggest that one or

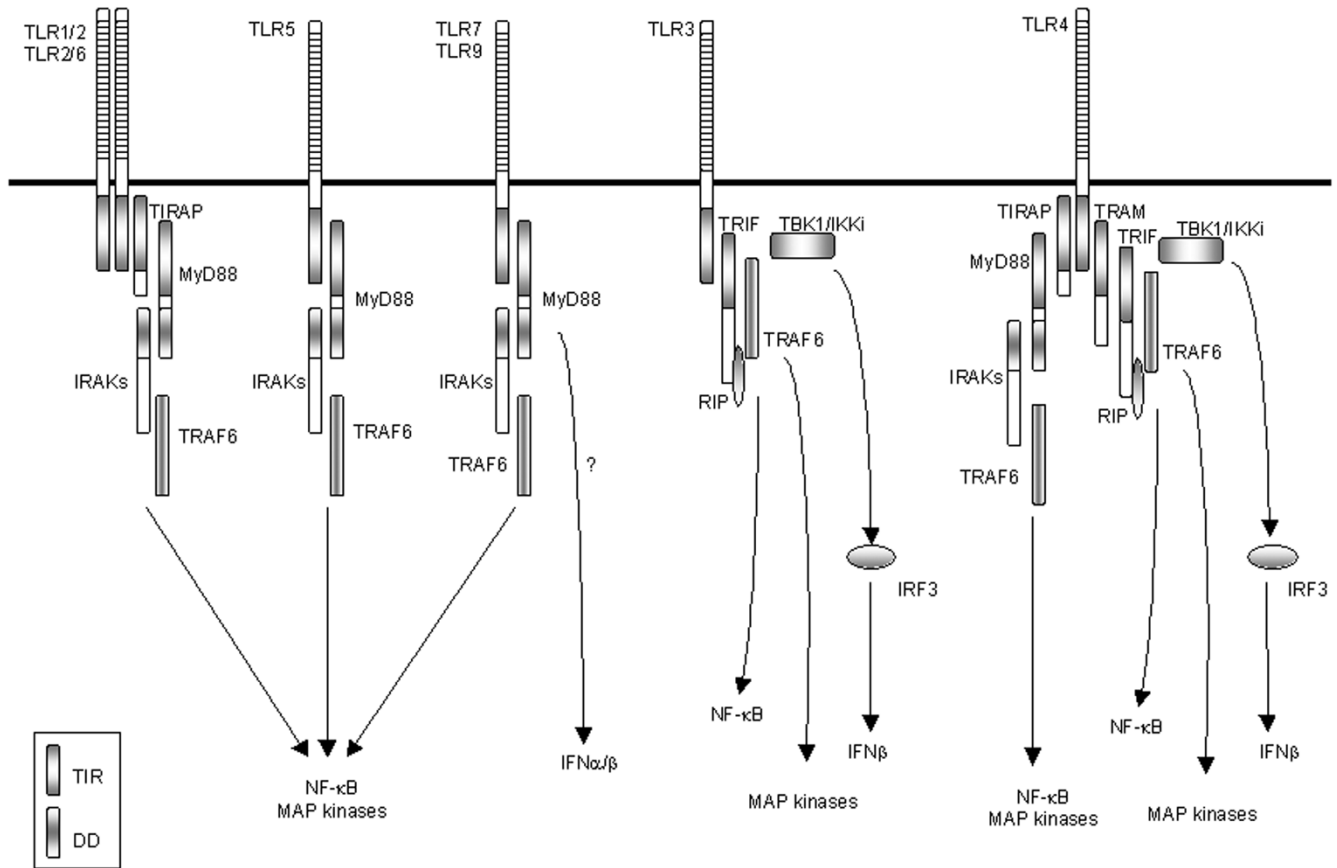
**Figure 2**



Structure of Toll/interleukin-1 receptor (TIR)-containing adapter proteins. DD, death domain.

more alternative pathways leading to the expression of certain genes associated with the delayed activation of NF- $\kappa$ B and AP-1 probably exist in TLR4 signaling. In this regard, genes induced by LPS in a MyD88-independent manner were sought by subtractive screening, which revealed that several genes were considerably induced after stimulation with LPS in MyD88-deficient cells [29]. These include interferon-inducible protein (IP)-10, a member of the CXC chemokines, glucocorticoid-attenuated response gene 16 (GARG16) and immune-responsive gene 1 (IRG-1), all of which are so-called IFN-inducible genes. Northern blot analyses showed that induction of these genes by LPS is comparable between wild-type and MyD88-deficient macrophages, but is completely abolished in TLR4-deficient cells. Thus, there is a MyD88-independent pathway that mediates the induction of IFN-inducible genes in TLR4 signaling [29].

The induction of some IFN-inducible genes is thought to be regulated in part by the transcription factor IRF3, a member of the interferon regulatory factor (IRF) family. IRF3 is normally present in cytoplasm. Upon stimulation, IRF3 is phosphorylated at multiple serine residues to form a homodimer, moving to the nucleus, where it regulates the expression of IFN- $\beta$  or other target genes [30]. IRF3-deficient mice are defective in the LPS-mediated induction of IFN- $\beta$  and IFN-inducible genes but are intact in the production of inflammatory cytokines [31]. Furthermore, the induction of some of IFN-inducible genes is regulated in part secondarily to LPS-inducible IFN- $\beta$ , which binds to the IFN- $\alpha/\beta$  receptor and activates the classical JAK-STAT pathway [32,33]. These results suggest that IRF3 is responsible for the MyD88-independent induction of IFN-inducible genes. Indeed, IRF3 is normally activated in response to LPS in MyD88-deficient cells [29,34]. Thus, TLR4 is capable of inducing two different pathways, namely the MyD88-dependent pathway responsible for the induction of a core set of inflammatory cytokines and the MyD88-independent pathway leading to the activation

**Figure 3**

Schematic representation of Toll-like receptor (TLR) signaling pathways. All TLRs except for TLR3 are thought to share the MyD88-dependent pathway that activates NF- $\kappa$ B and mitogen-activated protein (MAP) kinases, leading to the induction of inflammatory cytokine genes. Interleukin-1 receptor-associated kinases (IRAKs) and TRAF6 are located downstream of MyD88. TIRAP is involved in the MyD88-dependent pathway downstream of TLR2 and TLR4. TRIF is utilized in the TLR3-mediated and TLR4-mediated activation of interferon regulatory factor (IRF)3 and the subsequent induction of IRF3-dependent gene expression such as interferon- $\beta$  (IFN- $\beta$ ). TRAM is specifically involved in the activation of IRF3 in TLR4 signaling. The complex of TBK1/I $\kappa$ B kinase-i (IKK-i) is responsible for the activation of IRF3 downstream of TRIF in TLR3 and TLR4 signaling. TRAF6 is also involved in the TRIF-dependent activation of NF- $\kappa$ B and MAP kinases. Receptor-interacting protein (RIP) mediates TRIF-dependent NF- $\kappa$ B activation. DD, death domain.

of IRF3 and IFN- $\beta$  production (Fig. 3). In addition, stimulation with LPS also augments the surface expression of co-stimulatory molecules such as CD40, CD80 and CD86 to induce the maturation of dendritic cells (DCs) in MyD88-deficient mice, indicating that the maturation of DCs also proceeds without the MyD88-dependent pathway [35].

The MyD88-independent pathway is also used in TLR3 signaling. dsRNA (TLR3 ligand) also activates IRF3 and induces the expression of IFN-inducible genes, and these inductions are normal in MyD88-deficient cells [34]. Furthermore, the induction of IFN-inducible genes and the activation of IRF3 are not observed after stimulation of macrophages with TLR2 ligands, showing the specific use of the MyD88-independent pathway in TLR3 and TLR4 signaling [29].

#### TIRAP (MAL)

The finding that a MyD88-independent pathway exists in TLR4 signaling suggests that another protein, presumably containing the TIR domain, acts downstream of TLR4 to activate IRF3 independently of MyD88. In this model, a second TIR-domain-containing adapter TIRAP (also known as MAL) was identified by means of a database search, on the basis of the similarity to the TIR domain (Fig. 2) [36,37]. Expression of dominant-negative TIRAP, which encodes only the TIR domain, blocked NF- $\kappa$ B activation by TLR4 but not that by TLR9, and cell-permeable peptide-mediated inhibition of TIRAP function also decreased the induction of IP-10 by TLR4. Thus, TIRAP was expected to participate specifically in the TLR4-mediated MyD88-independent pathway. However, TIRAP-deficient mice were unexpectedly impaired in the activation of NF- $\kappa$ B and MAP kinases and in the

production of inflammatory cytokines induced by TLR1, TLR2, TLR6, and TLR4 ligands [38,39]. In contrast, the TLR4-mediated maturation of DCs and the activation of IRF3 were intact in TIRAP-deficient mice. Taken together, these results indicate that TIRAP is unlikely to mediate the MyD88-independent pathway; rather, it participates in the MyD88-dependent pathway downstream of TLR2 and TLR4 (Fig. 3).

#### **TRIF (TICAM1)**

A third TIR-domain containing the adapter TRIF (also known as TICAM1) was independently identified by the database search and as an interacting partner with TLR3 by yeast two-hybrid screening (Fig. 2) [40,41]. Initial studies indicated that the overexpression of TRIF strongly activates the IFN- $\beta$ -dependent promoter, in contrast to the lack of MyD88-mediated or TIRAP-mediated activation of the IFN- $\beta$ -dependent promoter. TRIF, as well as MyD88 and TIRAP, also activates NF- $\kappa$ B after overexpression. Moreover, the expression of dominant-negative TRIF or the siRNA-mediated reduction of TRIF expression blocked TLR3-dependent activation of IRF3. These findings suggested that TRIF is involved in the MyD88-independent pathway. Subsequent generations of TRIF-deficient mice clearly revealed that they were severely impaired in the induction of IFN- $\beta$  and IFN-inducible genes mediated by TLR3 and TLR4 ligands [42]. Furthermore, activation of IRF3 was not observed in TRIF-deficient cells. Analyses of mutant mice designated *lps2*, which were generated by *N*-ethyl-*N*-nitrosourea-induced random mutations, have also revealed that TRIF is an *lps2* gene product that is essential for responses mediated by TLR3 and TLR4 [43]. Thus, TRIF is a critical protein that mediates the MyD88-independent pathway in TLR3 and TLR4 signaling (Fig. 3). Furthermore, it is noteworthy that the production of inflammatory cytokines induced by LPS was severely impaired in both TRIF-deficient mice and MyD88-deficient mice. However, LPS-induced activation of IRAK1 and early-phase activation of NF- $\kappa$ B and MAP kinases was normally found in TRIF-deficient cells; this is in contrast to MyD88-deficient cells, which show impaired activation of IRAK1 and late-phase activation of NF- $\kappa$ B and MAP kinases [29,42]. These findings strongly suggest that both the MyD88-dependent and MyD88-independent pathways are required for the induction of genes for inflammatory cytokines, whereas activation of the MyD88-independent (TRIF-dependent) pathway is sufficient to induce IRF3-dependent IFN- $\beta$  and IFN-inducible genes. In contrast, activation of the MyD88-dependent pathway is sufficient for the induction of inflammatory cytokines in the case of TLR2, TLR5, TLR7, TLR9, and IL-1R. Thus, only TLR4 uses both the MyD88-dependent and MyD88-independent pathways for the induction of inflammatory cytokines, although it remains unclear why both pathways are necessary.

Downstream events of TRIF-dependent activation of NF- $\kappa$ B are still unclear. It has recently been reported that TRIF associates directly with TRAF6 [44]. Three TRAF-binding motifs are present in the amino-terminal region of TRIF, and a mutagenesis study demonstrated that these motifs are necessary for association with TRAF6. Disruption of TRAF-binding motifs in TRIF resulted in reduced activation of NF- $\kappa$ B. Given that TRAF6 activates NF- $\kappa$ B but not the IFN- $\beta$  promoter, TRIF activates NF- $\kappa$ B by recruitment of TRAF6 via the TRAF-binding motifs [44]. In addition to amino-terminal TRAF-binding motifs, NF- $\kappa$ B is also activated from the carboxy-terminal domain of TRIF, which lacks the TIR domain [40]. It has recently been reported that the kinases receptor-interacting protein (RIP)-1 and RIP3 are recruited to TRIF through the RIP homotypic interaction motif (RHIM) found in the carboxy-terminal region of TRIF [45]. In cells deficient for the *Rip1* gene, TLR3-mediated activation of NF- $\kappa$ B was selectively impaired, whereas activation of JNK or IFN- $\beta$  promoter was intact, indicating that TLR3 uses RIP1 for activation of NF- $\kappa$ B downstream of TRIF; this was in contrast to other TLRs, which use IRAKs for the activation of NF- $\kappa$ B (Fig. 3).

#### **TRAM (TICAM2, TIRP)**

The fourth TIR-domain-containing adapter was independently identified in the database search and termed TRAM, TIRP, or TICAM2 (Fig. 2) [41,46,47]. It has recently been demonstrated that responses of TRAM-deficient mice to TLR2, TLR7, and TLR9 ligands are intact in terms of production of inflammatory cytokines [48]. However, the LPS (TLR4 ligand)-induced production of inflammatory cytokines was specifically impaired in TRAM-deficient mice. Moreover, the induction of IFN- $\beta$  and activation of IRF3 in response to LPS was severely impaired in TRAM-deficient mice, indicating that TRAM is also involved in the MyD88-independent pathway. These responses in TRAM-deficient mice are very similar to those found in TRIF-deficient mice, except for TLR3, to which TRAM-deficient mice are normally responsive. Thus, TRAM specifically mediates the MyD88-independent pathway in TLR4 signaling but not in TLR3 signaling (Fig. 3).

#### **Non-canonical IKKs**

The canonical IKKs (IKK- $\alpha$  and IKK- $\beta$ ) are required for the activation of NF- $\kappa$ B mediated by TLRs and IL-1R [17]. These IKKs phosphorylate NF- $\kappa$ B inhibitor I $\kappa$ Bs, leading to the ubiquitination and degradation of I $\kappa$ Bs by the proteasome pathway. NF- $\kappa$ B in turn translocates into the nucleus, where it induces the expression of target genes. Two kinases related to the canonical IKKs, TBK1 (also known as NAK and T2K) and IKK-i (also known as IKK- $\epsilon$ ), were identified [49,50]. These 'non-canonical' IKKs were also implicated in NF- $\kappa$ B activation, although the precise mechanism is unclear. It has recently been shown that forced expression of either TBK1 or IKK-i strongly enhances IFN- $\beta$ -promoter-dependent reporter gene



expression [51,52]. Furthermore, the kinase activities are required for IFN- $\beta$ -promoter activation, suggesting that TBK1/IKK-i activates IFN- $\beta$  promoter by phosphorylating one or more appropriate substrates. Indeed, TBK1/IKK-i is shown to be capable of phosphorylating the serine residues that are critical for activating IRF3 *in vitro* [51,52]. In cells deficient for the *Tbk1* gene, the dsRNA-mediated or LPS-mediated activation of IRF3 (dimer formation, nuclear localization) and induction of IRF3-dependent genes such as IFN- $\beta$ , GARG16 and IP-10 were considerably reduced, whereas the induction of inflammatory cytokines, which is regulated by the MyD88-dependent pathway and activation of NF- $\kappa$ B and MAP kinases, was not impaired [53]. Thus, TBK1 is specifically involved in the induction of IRF3-dependent genes mediated by TLR3 and TLR4 (Fig. 3). In contrast, IKK-i deficient cells normally respond to LPS and dsRNA to induce both IRF3-dependent and NF- $\kappa$ B-dependent gene expression. However, in TBK1/IKK-i doubly deficient cells the residual induction of IFN-inducible genes found in TBK1-deficient cells was completely abolished, indicating that IKK-i also participates in the pathway dependent on TLR3 and TLR4 [54]. It has recently been reported that TRIF also binds TBK1 through the amino-terminal region which shares the binding with TRAF6, and the association between TRIF and TBK1 seems to require the kinase activity of TBK1 [44]. Furthermore, expression of the kinase-negative mutant of TBK1 significantly blocked TRIF-dependent activation of the IFN- $\beta$  promoter, thus indicating that TBK1 and IKK-i act downstream of TRIF. TBK1 and IKK-i also bind TANK (also known as I-TRAF) and a newly identified TANK-related protein NAP1 [49,55,56]. Thus, it is speculated that TBK1 is recruited to the TRIF complex to become activated, then forms a signaling complex containing IKK-i, TANK, and perhaps NAP1, to phosphorylate IRF3.

### Conclusions and remarks

The discovery of a series of TIR-containing adapters revealed that there are differences in the signal transduction pathways of individual TLRs, which might induce different effector responses that are specific to each TLR, as well as redundant responses that are conserved in all TLRs. One of the effector's functions is to produce IFN- $\beta$ , which is mediated by a TRIF-dependent pathway in TLR3 and TLR4 signaling, thus implying roles of TLRs for the detection of virus infection and the induction of appropriate anti-viral responses. In addition, TLR7, TLR8, and TLR9 are also implicated in the recognition of viral products. Indeed, TLR7 and TLR9 ligands are known to have abilities to produce IFNs as well as inflammatory cytokines from a certain subset of DCs. Interestingly, responses to both TLR7 and TLR9 ligands are entirely dependent on MyD88 [57]. Thus, TLR7 and TLR9 seem to have one or more unique signaling pathways that produce IFNs. Given that TLR7 and TLR9 are structurally related,

these TLRs probably share the same signaling pathway. Although there is still a need to characterize a specific pathway that each TLR uses, understanding such pathways will be therapeutically useful in the control of inflammatory and anti-viral responses.

Roles of TLRs in the pathogenesis of rheumatoid arthritis have been investigated. It was demonstrated that peptidoglycan and bacterial DNA, which is recognized by TLR2 and TLR9, could be detected in the synovium of patients with rheumatoid arthritis [58]. Furthermore, synovial cells express low levels of TLR2 and TLR9, and TLR2 is upregulated by exposure to peptidoglycan [59,60]. In addition, TLRs have been shown to recognize endogenous ligands present in tissues and cells in the absence of bacterial infection. TLR2 activates cells through the recognition of heat shock protein 70 and necrotic cells, both of which are detected in synovial tissue of patients with rheumatoid arthritis [61]. These findings suggest that TLRs contribute to the pathogenesis of rheumatoid arthritis, and targeting the TLR signaling pathway will raise the possibility of a drug discovery to control inflammation induced in patients with rheumatoid arthritis.

### Competing interests

The author(s) declare that they have no competing interests.

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