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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).

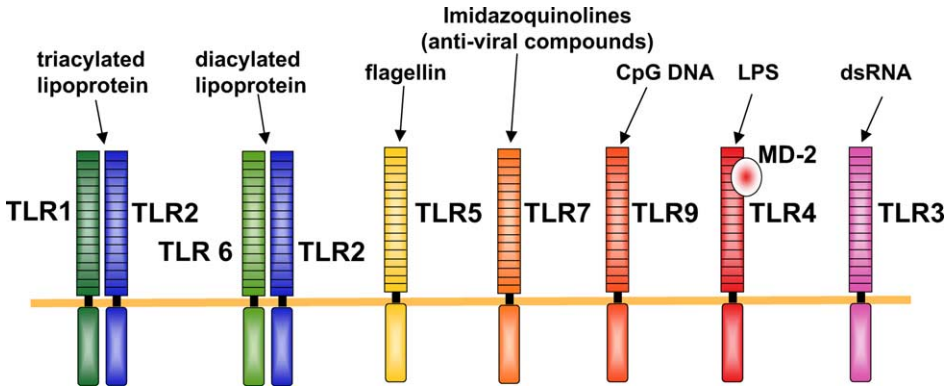

Fig. 1. TLRs and their ligands. TLR1–TLR7 and TLR9 have been characterized to recognize microbial components. TLR2 is essential for the recognition of microbial lipopeptides. TLR1 and TLR6 associate with TLR2, and discriminate subtle differences between triacyl- and diacyl lipopeptides, respectively.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

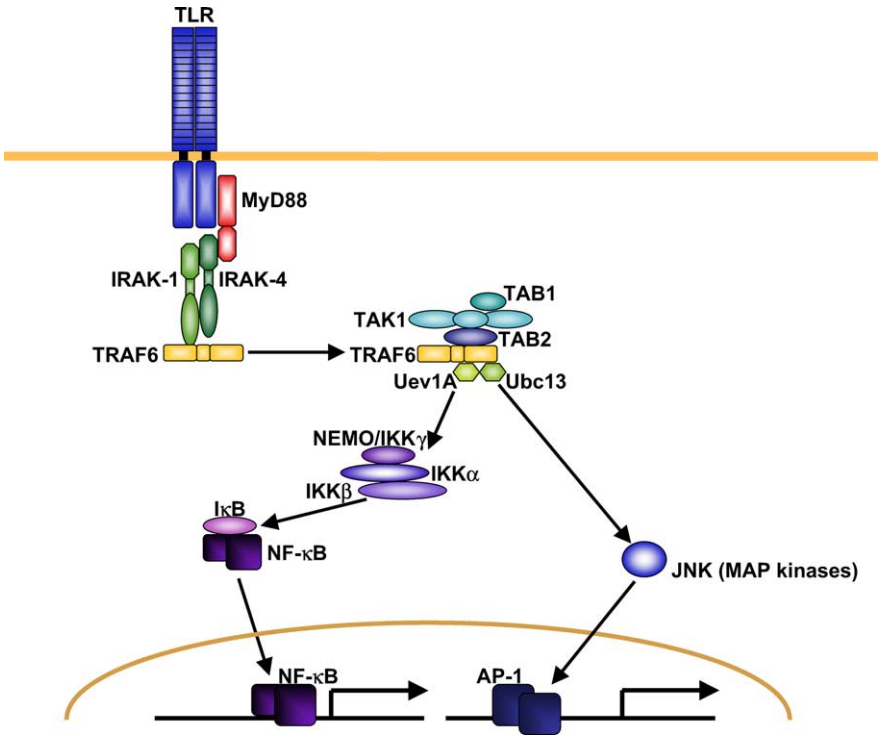

Fig. 2. TLR-mediated MyD88-dependent signaling pathway. MyD88 binds to the cytoplasmic portion of TLRs through interaction between individual TIR domains. Upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor, which induces association of IRAK-1 and MyD88 via the death domains. IRAK-4 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

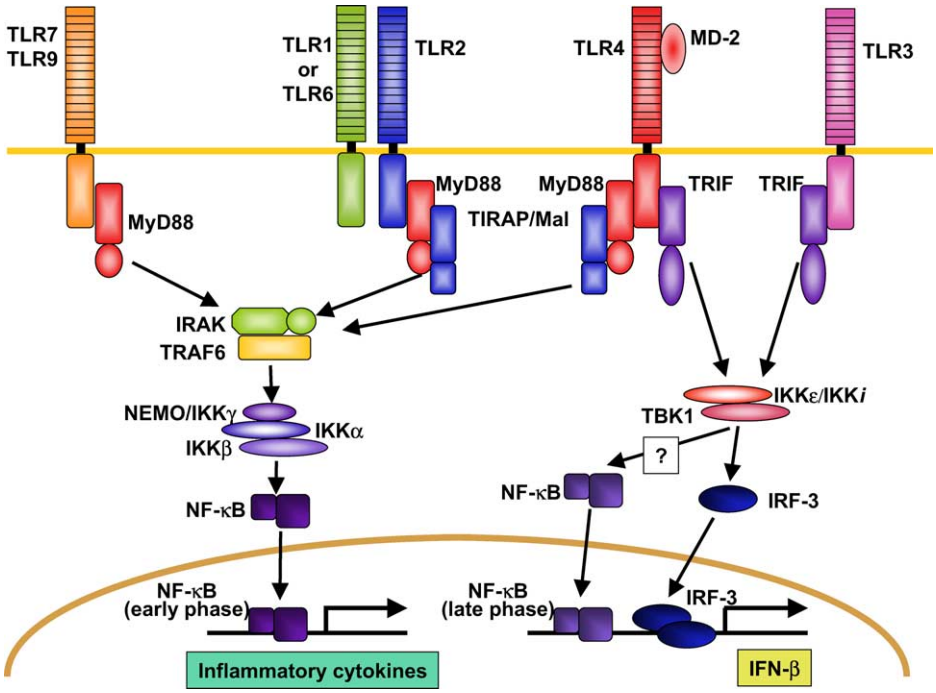

Fig. 3. TIR domain-containing adaptors and TLR signaling. MyD88 is an essential TIR domain-containing adaptor for the induction of inflammatory cytokines via all the TLRs. TIRAP/Mal is a second TIR domain-containing adaptor that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. In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

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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