Table 1 | **Toll-like receptors and their ligands**

Receptor	Ligand	Origin of ligand	References
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria	112
	Soluble factors	*Neisseria meningitidis*	113
TLR2	Lipoprotein/lipopeptides	Various pathogens	114
	Peptidoglycan	Gram-positive bacteria	115,116
	Lipoteichoic acid	Gram-positive bacteria	116
	Lipoarabinomannan	Mycobacteria	117
	Phenol-soluble modulin	*Staphylococcus epidermidis*	118
	Glycoinositolphospholipids	*Trypanosoma cruzi*	119
	Glycolipids	*Treponema maltophilum*	120
	Porins	*Neisseria*	121
	Atypical lipopolysaccharide	*Leptospira interrogans*	122
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*	123
	Zymosan	Fungi	124
	Heat-shock protein 70*	Host	125
TLR3	Double-stranded RNA	Viruses	52
TLR4	Lipopolysaccharide	Gram-negative bacteria	9
	Taxol	Plants	126
	Fusion protein	Respiratory syncytial virus	127
	Envelope protein	Mouse mammary-tumour virus	128
	Heat-shock protein 60*	*Chlamydia pneumoniae*	129,130
	Heat-shock protein 70*	Host	131
	Type III repeat extra domain A of fibronectin*	Host	132
	Oligosaccharides of hyaluronic acid*	Host	133
	Polysaccharide fragments of heparan sulphate*	Host	134
	Fibrinogen*	Host	135
TLR5	Flagellin	Bacteria	136
TLR6	Diacyl lipopeptides	*Mycoplasma*	137
	Lipoteichoic acid	Gram-positive bacteria	116
	Zymosan	Fungi	138
TLR7	Imidazoquinoline	Synthetic compounds	139
	Loxoribine	Synthetic compounds	12
	Bropirimine	Synthetic compounds	12
	Single-stranded RNA	Viruses	140,141
TLR8	Imidazoquinoline	Synthetic compounds	142
	Single-stranded RNA	Viruses	140
TLR9	CpG-containing DNA	Bacteria and viruses	143
TLR10	N.D.	N.D.	–
TLR11	N.D.	Uropathogenic bacteria	144

*It is possible that these ligand preparations, particularly those of endogenous origin, were contaminated with lipopolysaccharide and/or other potent microbial components, so more-precise analysis is required to conclude that TLRs recognize these endogenous ligands. N.D., not determined; TLR, Toll-like receptor.

hydrophobic amino acid. The repeats comprise a β-strand and an α-helix connected by loops. The LRR domains of TLRs form a horseshoe structure, and it is thought that the concave surface of the LRR domains is involved directly in the recognition of various pathogens. The main ligands recognized by different TLRs are summarized in TABLE 1. Remarkably, despite the conservation among LRR domains, different TLRs can recognize several structurally unrelated ligands[1,3,4]. The subcellular localization of different TLRs correlates to some extent with the molecular patterns of their ligands. TLR1, TLR2 and TLR4 are located on the cell surface and are recruited to phagosomes after activation by their respective ligands. By contrast, TLR3, TLR7 and TLR9, all of which are involved in the recognition of nucleic-acid-like structures, are not expressed on the cell surface[11–13]. For example, TLR9 has recently been shown to be expressed in the endoplasmic reticulum, and it is recruited to endosomal/lysosomal compartments after stimulation with CpG-containing DNA[14].

TLR/IL-1R-superfamily signalling cascade

After ligand binding, TLRs/IL-1Rs dimerize and undergo the conformational change required for the recruitment of downstream signalling molecules. These include the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6)[15,16].

MyD88. *MyD88* was isolated originally as a gene that is induced rapidly during the IL-6-stimulated differentiation of M1 myeloleukaemic cells into macrophages[17]. The encoded protein has an amino (N)-terminal death domain (DD), which is separated from its carboxy (C)-terminal TIR domain by a short linker sequence. *MyD88* was subsequently cloned as an adaptor molecule that functions to recruit IRAK to the IL-1R complex following stimulation with IL-1 (REFS 18–20). The association between MyD88 and IRAK is mediated

through a DD–DD interaction. MyD88 forms homodimers through DD–DD and TIR-domain–TIR-domain interactions and exists as a dimer when recruited to the receptor complex[21]. Therefore, MyD88 functions as an adaptor linking TLRs/IL-1Rs with downstream signalling molecules that have DDs.

IRAK family. Four IRAKs — IRAK1, IRAK2, IRAK4 and IRAK-M — showing distinct gene-expression patterns have been identified in mammals[22]. IRAKs contain an N-terminal DD and a central serine/threonine-kinase domain. IRAK1 and IRAK4 have intrinsic kinase activity, whereas IRAK2 and IRAK-M have no detectable kinase activity. The kinase activity of IRAK1 increases strongly following TLR/IL-1R stimulation, and its kinase domain is essential for signalling through nuclear factor-κB (NF-κB). However, kinase activity itself is not essential for signalling, because in IRAK1-deficient cells, the overexpression of a kinase-defective mutant of IRAK1 can strongly induce NF-κB activation[23].

By contrast, the overexpression of IRAK4 does not result in robust NF-κB activation, yet the expression of a kinase-inactive mutant of IRAK4 inhibits IL-1-mediated NF-κB activation. It has also been shown that IRAK1 is a direct substrate of IRAK4 but not vice versa[24]. In IRAK1-deficient mice, cytokine production in response to IL-1 and LPS was diminished but not abolished[25–27], whereas IRAK4-deficient mice showed virtually no response to IL-1, LPS or other bacterial components, demonstrating that IRAK4 has an important role in IL-1R/TLR signalling[28]. Recently, patients with an inherited IRAK4 deficiency have been identified[29]. These patients failed to respond to IL-1, to IL-18 or to stimulation of each of five TLRs (TLR2, TLR3, TLR4, TLR5 and TLR9). Together, these results show that IRAK4 and its kinase activity are required for TLR signalling and that IRAK4 functions upstream of IRAK1.

TRAF6. TRAFs constitute a family of evolutionarily conserved adaptor proteins[30]. So far, six members of the TRAF family have been identified in mammals, and they are characterized by the presence of an N-terminal coiled-coil domain (known as TRAF-N) and a conserved C-terminal domain (known as TRAF-C). The N-terminal portion of most TRAF proteins contains a RING (really interesting new gene)-finger/zinc-finger region, which is essential for downstream signalling events, whereas the TRAF-C domain mediates self-association and interactions with upstream receptors and signalling proteins. TRAF6 functions as a signalling mediator for both the TNF-receptor superfamily and the TLR/IL-1R superfamily, interacting directly with members of the TNF-receptor superfamily (CD40 and TNF-related activation-induced cytokine receptor, TRANCER) or being coupled indirectly to TLR/IL-1R superfamily members through its association with IRAKs. The consensus sequence for the TRAF6-binding domain has been identified as P-X-E-X-X-(D/E/F/W/Y)[31]. This motif is found in CD40, TRANCER, and the IRAKs; three of these TRAF6-binding motifs are found in IRAK1, two in IRAK2 and one in IRAK-M.

TAK1 and TABs. The activation by TRAF6 of the transcription factors NF-κB and activator protein 1 (AP1) involves TAK1 and two adaptor proteins TAB1 and TAB2. TAK1 is a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family[32], which has been shown to be essential for both IL-1/LPS- and TNF-induced NF-κB activation[33]. Two TAK1-binding proteins, TAB1 and TAB2, have been identified[34,35]. When co-expressed ectopically, TAB1 enhances the kinase activity of TAK1, indicating that TAB1 functions as an activator of TAK1[34]. By contrast, TAB2 functions as an adaptor, linking TAK1 to TRAF6 and thereby facilitating TAK1 activation[35]. However, embryonic fibroblasts obtained from TAB2-deficient mice show no impairment in either IL-1/LPS- or TNF-induced activation of NF-κB[36]. Furthermore, a new TAB2-like molecule, TAB3, has been identified recently, and similar to TAB2, it has been shown to associate with TAK1 and activate NF-κB[37]. Co-transfection of SMALL INTERFERING RNAS (siRNAs) directed against both TAB2 and TAB3 inhibited both IL-1- and TNF-induced activation of TAK1 and NF-κB, indicating that TAB2 and TAB3 function redundantly as mediators of TAK1 activation.

It has been shown that UBIQUITYLATION has an important role in TAK1 activation and that TRAF6 functions as an E3 ubiquitin ligase[38]. TRAF6 can interact through its RING-finger domain with ubiquitin-conjugating enzyme 13 (UBC13), and this UBC13–TRAF6 complex triggers TAK1 activation through the assembly of a lysine63-linked polyubiquitin chain[39].

NF-κB. The NF-κB family of transcription factors is composed of five members — p65 (REL-A), REL-B, cytoplasmic (c) REL, p50 and p52 — which function as homo- and heterodimers. NF-κB dimers are usually sequestered in the cytoplasm in an inactive form by molecules of the inhibitor of NF-κB (IκB) family. Activation of NF-κB involves the phosphorylation and proteolysis of the IκB proteins and the concomitant release and nuclear translocation of the NF-κB factors. This acute activation process is mediated by the IκB kinase (IKK) complex, which comprises two catalytic subunits — IKK-α and IKK-β (also known as IKK1 and IKK2) — and a regulatory subunit, IKK-γ (also known as NF-κB essential modulator, NEMO)[40]. After activation by upstream signals, IKK phosphorylates the IκBs, leading to their polyubiquitylation and proteasome-mediated degradation.

Recently, an alternative pathway of NF-κB activation has been suggested, in which NF-κB-inducing kinase (NIK) activates IKK-α, which then phosphorylates the NF-κB2 precursor protein p100 (REFS 41,42). After phosphorylation, p100 is recognized by an SCF (S-phase kinase-associated protein 1–Cullin1–F box)-family E3 ubiquitin ligase, β-transducin repeat-containing protein (β-TRCP), which catalyses the polyubiquitylation of p100 and thereby triggers its processing by the proteasome to the transcriptionally active p52 form. This proteolytic event is tightly regulated by sequences located in the C-terminal region of p100 (REF. 43).

SMALL INTERFERING RNAS (siRNAs). Synthetic double-stranded RNA molecules of 19–23 nucleotides, which are used to knockdown (silence the expression of) a specific gene. This is known as RNA interference (RNAi) and is mediated by the sequence-specific degradation of mRNA.

UBIQUITYLATION
The attachment of the small protein ubiquitin to lysine residues present in other proteins. This tags these proteins for rapid cellular degradation.

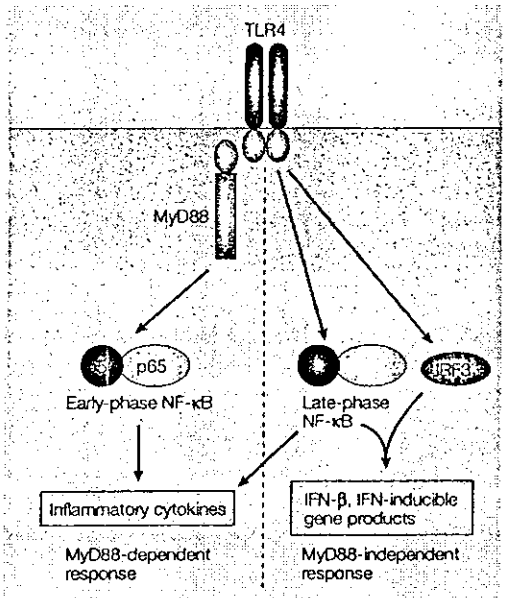

Figure 2 | **TLR4 signalling: MyD88-dependent and -independent pathways.** Stimulation of Toll-like receptor 4 (TLR4) facilitates the activation of two pathways: the MyD88 (myeloid differentiation primary-response protein 88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early phase of nuclear factor-κB (NF-κB) activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF)3 and involves the late phase of NF-κB activation, both of which lead to the production of IFN-β and the expression of IFN-inducible genes.

Despite these findings, the nuclear translocation of NF-κB alone might not be sufficient for activation of NF-κB-dependent transcription. Some NF-κB proteins, particularly p65, are post-translationally regulated, and various kinases — including cyclic AMP (cAMP)-dependent protein kinase (PKA), casein kinase II, protein kinase C-ζ (PKC-ζ) and IKK itself — have been implicated in this process[40].

Fitting the TLR/IL-1R signalling pathway together. After TLR/IL-1R stimulation, MyD88 is recruited to the cytoplasmic TIR domain, where it facilitates the association of IRAK4 with the receptor complex through a homophilic DD interaction. The binding of MyD88 to IRAK4 facilitates IRAK4-mediated phosphorylation of a crucial residue or residues in the kinase-activation loop of IRAK1, which induces the kinase activity of IRAK1. Activated IRAK1 then autophosphorylates residues in its N-terminus, and this hyperphosphorylation of IRAK1 enables TRAF6 to bind to this complex. The IRAK1–TRAF6 complex then disengages from the receptor and interacts at the plasma membrane with another preformed complex consisting of TAK1, TAB1, and TAB2 or TAB3. This interaction induces phosphorylation of TAB2/TAB3 and TAK1, which then translocate together with TRAF6 and TAB1 to the cytoplasm. TAK1 is subsequently activated in the cytoplasm, leading to the activation of IKKs, which then phosphorylate the IκBs. This phosphorylation leads to the degradation of IκB and consequently the release of NF-κB. Activation of TAK1 also results in the activation of MAPKs, including JUN N-terminal kinase (JNK) (FIG. 1b).

MyD88-dependent and -independent pathways

MyD88-deficient mice do not produce TNF or IL-6 when exposed to IL-1 or microbial components that are recognized by TLR2, TLR4, TLR5, TLR7 or TLR9 (REFS 15,44). So, MyD88 is essential for responses against a broad range of microbial components. However, closer study of MyD88-deficient cells has revealed the existence of MyD88-dependent and -independent pathways, both of which mediate signalling in response to LPS[45] (FIG. 2). For example, the activation of NF-κB in response to mycoplasmal lipopeptide, a TLR2 ligand, is completely abolished in MyD88-deficient macrophages, whereas NF-κB activation still occurs in response to LPS, a TLR4 ligand, although with delayed kinetics. MAPK activation is also delayed in LPS-stimulated MyD88-deficient macrophages.

The MyD88-independent pathway was further characterized by determining the genes expressed in MyD88-deficient macrophages following exposure to LPS[46]. A number of genes known to be interferon (IFN)-inducible genes were identified, such as glucocorticoid-attentuated response gene 16 (*GARG16*), immunoresponsive gene 1 (*IRG1*) and the gene encoding CXC-chemokine ligand 10 (*CXCL10*; the product of which is also known as IFN-γ-inducible 10 kDa protein, *IP10*). As expected, genes encoding inflammatory cytokines, such as TNF, IL-6 and IL-1β, were not expressed. Induction of the IFN-inducible genes was completely abolished in TLR4-deficient macrophages, demonstrating that CXCL10, GARG-16 and IRG1 are produced in a TLR4-dependent but MyD88-independent manner. By contrast, stimulation with TLR2 ligands was found not to upregulate the expression of IFN-inducible genes, which is consistent with the idea that TLR2 does not use this MyD88-independent pathway. Further studies that used mice deficient in the IFN-α/β receptor subsequently showed that the production of CXCL10 in response to LPS is mainly a secondary consequence of IFN-β production[47,48].

In addition to inducing the expression of IFN-inducible genes, the MyD88-independent pathway leads to the LPS-mediated maturation of dendritic cells (DCs)[49]. When cultured with LPS, MyD88-deficient bone-marrow-derived DCs upregulate the cell-surface expression of co-stimulatory molecules, such as CD40, CD80 and CD86, and induce the proliferation of T cells. By contrast, TLR4-deficient DCs fail to mature in response to LPS, indicating that DC maturation proceeds in a MyD88-independent manner[49]. However, because stimulation of wild-type DCs, with either TLR2 ligands (through the MyD88-dependent pathway) or TLR4 ligands (through the MyD88-dependent and -independent pathways), was observed to increase the cell-surface expression of co-stimulatory molecules, then either the MyD88-dependent or -independent

pathway is sufficient for the induction of co-stimulatory molecules. For TLR4, the MyD88-independent production of co-stimulatory molecules is mainly secondary to the production of IFN-β[47]. By contrast, the TLR-mediated expression of genes that encode inflammatory cytokines is controlled by the MyD88-dependent pathway, although both the MyD88-dependent and -independent pathways are involved in TLR4-mediated production of inflammatory cytokines[50,51]. Therefore, the expression of the genes that encode inflammatory cytokines and co-stimulatory molecules is differentially regulated during TLR signalling.

Although MyD88 has been reported to be involved in TLR3 signalling[52], TLR3 seems to transduce its signals mainly through the MyD88-independent pathway, because stimulation with the TLR3 ligand polyinosinic–polycytidylic acid (poly I:C) does not result in impaired production of inflammatory cytokines and co-stimulatory molecules in MyD88-deficient mice (S.A. and K.T., unpublished observations).

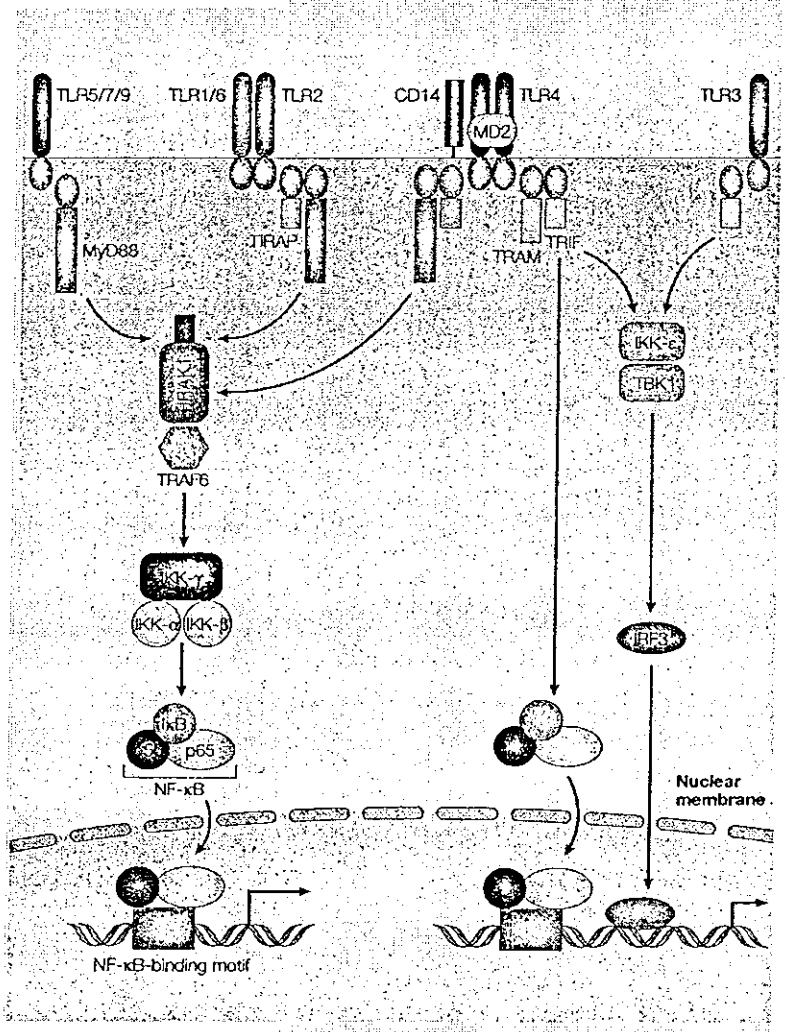

Figure 3 | **Involvement of TIR-domain-containing adaptors in TLR-signalling pathways.** The Toll/interleukin-1 (IL-1)-receptor (TIR)-domain-containing adaptor molecule MyD88 (myeloid differentiation primary-response protein 88) mediates the Toll-like receptor (TLR)-signaling pathway that activates IRAKs (IL-1-receptor-associated kinases) and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6), and leads to the activation of the IKK complex (inhibitor of nuclear factor-κB (IκB)- kinase complex), which consists of IKK-α, IKK-β and IKK-γ (also known as IKK1, IKK2 and nuclear factor-κB (NF-κB) essential modulator, NEMO, respectively). This pathway is used by TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 and releases NF-κB from its inhibitor so that it translocates to the nucleus and induces expression of inflammatory cytokines. TIRAP (TIR-domain-containing adaptor protein), a second TIR-domain-containing adaptor protein, is involved in the MyD88-dependent signalling pathway through TLR2 and TLR4. By contrast, TLR3- and TLR4-mediated activation of interferon (IFN)-regulatory factor 3 (IRF3) and the induction of IFN-β are observed in a MyD88-independent manner. A third TIR-domain-containing adaptor, TRIF (TIR-domain-containing adaptor protein inducing IFN-β), is essential for the MyD88-independent pathway. The non-typical IKKs IKK-ε and TBK1 (TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1) mediate activation of IRF3 downstream of TRIF. A fourth TIR-domain-containing adaptor, TRAM (TRIF-related adaptor molecule), is specific to the TLR4-mediated, MyD88-independent/TRIF-dependent pathway.

Adaptor family

The discovery of the MyD88-independent pathway led researchers to characterize the signalling pathways of the various TLRs, the activation of which leads to different patterns of gene expression. As a result, the molecular mechanisms underlying such differences can now be explained, at least in part, by the existence of several adaptors, which are used by different TLRs. These adaptors, which all have TIR domains, include the following (in order of identification): MyD88; TIRAP (TIR-domain-containing adaptor protein; also known as MyD88-adaptor-like protein, MAL); TRIF (TIR-domain-containing adaptor protein inducing IFN-β; also known as TIR-domain-containing molecule 1, TICAM1); and TRAM (TRIF-related adaptor molecule; also known as TIR-domain-containing molecule 2, TICAM2) (FIG. 3).

TIRAP. Identification of the MyD88-independent pathway of TLR4 signalling led to the discovery of the second TIR-domain-containing adaptor, which is known as TIRAP[53,54]. Unlike MyD88, TIRAP does not have a DD and was initially thought to mediate the MyD88-independent pathway of TLR4 signalling. However, the physiological role of TIRAP was revealed by generating knockout mice: the production of inflammatory cytokines in response to LPS was found to be defective in TIRAP-deficient mice, but the expression of IFN-inducible genes and the delayed activation of NF-κB was still observed[54,55]. This phenotype was similar to that of MyD88-deficient mice, and it indicated that TIRAP is essential for the TLR4-mediated, MyD88-dependent signalling pathway but not the MyD88-independent pathway. The possibility that MyD88 and TIRAP might function redundantly in the MyD88-independent pathway was excluded by generating mice deficient in both MyD88 and TIRAP. Enforced overexpression of MyD88 in TIRAP-deficient embryonic fibroblasts, but not vice versa, activates the NF-κB-dependent promoter, indicating that TIRAP probably acts upstream of MyD88 (S.A. and K.T., unpublished observations). Interestingly, TIRAP-deficient mice also show impaired cytokine production in response to TLR2 ligands, despite having normal responses to TLR3, TLR7 and TLR9 ligands[55,56]. Therefore, TIRAP is essential for MyD88-dependent signalling through TLR2 and TLR4.

TRIF. Because analyses of TIRAP-deficient mice indicated that TIR-domain-containing molecules might mediate the specificity of different TLR signalling pathways, further database searches for such proteins were conducted, leading to the identification of a third TIR-domain-containing adaptor, TRIF[57]. The same molecule was also identified as a TLR3-binding molecule in a yeast two-hybrid screen, but in this report, it was called TICAM1[58]. The enforced expression of TRIF, but not of MyD88 or TIRAP, led to activation of the IFN-β promoter in HEK293 (human embryonic kidney 293) cells, whereas a dominant-negative form of TRIF inhibited TLR3-dependent activation of the IFN-β promoter. These *in vitro* studies indicate that TRIF functions in the MyD88-independent pathway to induce IFN-β. The physiological role of TRIF was subsequently revealed through the targeted deletion of *Trif* in mice. In response to TLR3 and TLR4 ligands, these TRIF-deficient mice showed both impaired activation of IFN-regulatory factor 3 (IRF3) and decreased expression of IFN-inducible genes[59]. Consistent with this, analysis of LPS-hyporesponsive mice, which were generated by random germline mutagenesis, also led to the identification of *Trif* as a gene responsible for TLR3- and TLR4-mediated responses[51]. Therefore, studies that used two independently generated strains of *Trif*-mutant mice demonstrated that TRIF is essential for the TLR3- and TLR4-mediated activation of the MyD88-independent pathway, which subsequently leads to the production of IFN-β.

In addition, TRIF-deficient mice showed defective production of inflammatory cytokines in response to TLR4 ligands but not to other TLR ligands. However, TLR4-mediated activation of the MyD88-dependent pathway was not impaired, as determined by phosphorylation of IRAK1 and early-phase activation of NF-κB[50,51]. These findings indicate that TLR4 requires both MyD88-dependent and MyD88-independent/TRIF-dependent signals to induce the expression of inflammatory cytokines. By contrast, activation of the MyD88-dependent pathway alone is sufficient to induce inflammatory-cytokine production in response to the ligation of TLR2, TLR5, TLR7 or TLR9 — none of which activate the MyD88-independent/TRIF-dependent pathway. It remains unclear why TLR4-mediated signalling pathways use both MyD88-dependent and MyD88-independent pathways to induce the expression of inflammatory cytokines. However, these findings might indicate that, as well as NF-κB, an unidentified molecule or molecules activated by the MyD88-independent/TRIF-dependent pathway is required for inflammatory-cytokine induction.

TRAM. A fourth TIR-domain-containing adaptor, TRAM, was recently identified through sequence homology in database searches[59–62]. *In vitro* studies indicated that TRAM associates with TRIF and TLR4 but not with TLR3 (REFS 61,62), and the inhibition of TRAM expression by siRNA demonstrated its important role in the TLR4- but not TLR3-mediated induction of IFN-β and IFN-inducible genes[61,62]. Analysis of TRAM-deficient mice further established that TRAM has an essential role in the MyD88-independent cascade of TLR4-induced signals. In response to TLR4 ligands, TRAM-deficient mice, similar to TRIF-deficient mice, showed impaired activation of IRF3 and reduced expression of IFN-inducible genes. However, unlike TRIF-deficient mice, TRAM-deficient mice showed a normal response to TLR3 stimulation[60]. So, TRAM is involved specifically in the activation of the MyD88-independent/TRIF-dependent signalling pathway through TLR4. In addition, similar to TRIF-deficient mice, TRAM-deficient mice are defective in their production of inflammatory cytokines in response to LPS, despite the fact that the activation of IRAK1 and early-phase NF-κB is normal[60]. This indicates that TRAM and TRIF are involved in the TLR4-mediated induction of inflammatory cytokines, although the precise mechanisms remain unknown.

Differential use of adaptors in TLR signalling. The characterization of TIR-domain-containing adaptors has established the essential roles of these adaptors in TLR signalling (FIG. 3). MyD88 is essential for all TLR-mediated production of inflammatory cytokines. However, stimulation of TLR3 or TLR4 results in induction of type I IFNs (IFN-α/β) in a MyD88-independent manner. This MyD88-independent response is entirely dependent on TRIF. In addition, TIRAP is involved specifically in TLR2- and TLR4-mediated activation of the MyD88-dependent pathway, and TRAM is a specific adaptor in the TLR4-mediated, MyD88-independent/TRIF-dependent pathway. Therefore, TIRAP and TRAM provide the specificity for different TLR-signalling pathways. Because the cytoplasmic portion of TLR4 binds directly to TRAM but not TRIF, as shown by *in vitro* experiments, TLR4 might acquire the ability to induce type I IFNs by associating with TRAM, which bridges TLR4 and TRIF[62]. Interestingly, all of these adaptors are involved in the TLR4-signalling pathway; however, it remains unknown why only TLR4 requires all of the TIR-domain-containing adaptors to induce gene expression. But this use of various adaptors, and the synergistic activation of both the MyD88-dependent and MyD88-independent/TRIF-dependent pathways, might explain why the TLR4 ligand LPS is such a strong immunostimulator, sufficient to induce ENDOTOXIC SHOCK. In contrast to the induction of type I IFNs through TLR3 and TLR4, TLR7 and TLR9 mediate the production of type I IFNs through a MyD88-dependent signalling cascade. However, it remains unclear which molecule or molecules provides specificity in these signalling pathways. There is one further TIR-domain-containing molecule, which is known as SARM (sterile α- and armadillo-motif-containing protein)[63]. An orthologue of mammalian SARM, the *Caenorhabditis elegans* TIR-domain-containing protein (TIR1), has recently been shown to mediate the expression of genes that encode antimicrobial peptides. However, this response is independent of the *C. elegans* TLR[64]. Nonetheless, elucidation of the role of mammalian SARM might improve our understanding of TLR signalling.

ENDOTOXIC SHOCK
A serious systemic disorder that leads to multiple organ failure and death. It is caused by an excessive release of lipopolysaccharide (also known as endotoxin) during Gram-negative bacterial infection.

MyD88-independent/TRIF-dependent pathway

IRF3 activation. Previous studies have shown that activation of the gene encoding IFN-β and the IFN-inducible genes requires IRF3 (REF. 65). The IRFs are a family of transcription factors that are involved both in the induction of type I IFNs and in the response to IFNs[66]. So far, of the nine known members of the IRF family, IRF3, IRF5 and IRF7 have been shown to function as direct transducers of virus-mediated signalling and have a crucial role in the expression of type I IFNs[67]. IRF3 is expressed constitutively by various cells, and in response to viral infection, its C-terminal regulatory domain is activated by phosphorylation, which allows the formation of IRF3 dimers. After dimerization, IRF3 translocates rapidly to the nucleus, where despite lacking intrinsic transcriptional activity, it activates transcription of the type I IFN genes by recruiting the co-activators p300 and CBP (cAMP-responsive-element-binding protein (CREB)-binding protein). IRF3 mediates the initial induction of type I IFNs during viral infection, and these secreted type I IFNs activate the expression of IFN-inducible genes, such as *CXCL10* and *IRG1*, through the JAK (Janus activated kinase)–STAT (signal transducer and activator of transcription) signalling pathway[68].

In contrast to IRF3, mRNA that encodes IRF7 is produced in most cell types in response to IFN and viral infection. During viral infection, the activation of the constitutively expressed IRF3 molecules and the consequent production of type I IFNs leads to the induction and activation of IRF7 through the JAK–STAT signalling pathway. Subsequently, both IRF3 and IRF7 are involved in the production of delayed-type IFNs (IFN-α/β), thereby amplifying the expression of IFNs[69].

Stimulation with LPS also activates IRF3, and because LPS can induce the expression of both IFN-β and IFN-inducible genes in a MyD88-independent manner, this indicates that IRF3 activation does not require MyD88. In fact, IRF3 activation, as shown by dimer formation and nuclear translocation, can be observed in MyD88-deficient cells[47].

The activation of IRF3 by signalling through TLR3 is more rapid and potent than that triggered by TLR4 signalling, and this correlates with increased production of IFN-β (S.A. and K.T., unpublished observations). Whereas activating TLR3 with poly I:C results in the C-terminal phosphorylation of IRF3, as detected using a phosphospecific antibody, stimulation with LPS does not induce detectable C-terminal phosphorylation[70]. However, we think that TLR3- and TLR4-mediated IRF3 activation probably differ quantitatively, rather than qualitatively, because it is more plausible that the phosphospecific antibody is not sensitive enough to detect IRF3 phosphorylation following TLR4 activation.

Two IKK-related proteins — IKK-ε (also known as inducible IKK, IKKi) and TBK1 (TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1; also known as NF-κB-activating kinase, NAK) — have recently been identified as the kinases that phosphorylate IRF3 in response to viral infection and stimulation of TLR3 (REFS 71,72). Overexpression of IKK-ε or TBK1 activates the promoter of the *IFN-β* gene and of IFN-inducible genes and induces the phosphorylation and nuclear localization of IRF3. In addition, siRNA targeting of IKK-ε and TBK1, but not of IKK-β, was observed to considerably reduce the TRIF-dependent activation of a reporter gene containing an IRF DNA-binding motif and to decrease the viral induction of *IFN-α4* and *IFN-β* reporter genes[71,72]. Furthermore, analysis of TBK1-deficient embryonic fibroblasts confirmed the essential role of TBK1 in IRF3-dependent gene expression mediated by TLR3 and TLR4 signalling[73].

NF-κB activation. MyD88-deficient mice still activate NF-κB in response to stimulation with LPS, although the kinetics are delayed compared with wild-type mice. However, the activation of NF-κB in response to LPS is completely abrogated in mice lacking both MyD88 and TRIF, demonstrating that TRIF is essential for NF-κB activation through the MyD88-independent pathway[50]. Transcriptional activation of the *IFN-β* gene requires activation of both NF-κB and IRF3, and in contrast to inflammatory cytokine production — which requires both early- and late-phase NF-κB activation, mediated by MyD88 and TRIF, respectively — production of IFN-β can be induced by TRIF-mediated late-phase NF-κB activation alone (FIG. 4).

In vitro analyses showed that both the N-terminal and C-terminal regions of TRIF can independently activate an NF-κB-responsive promoter, whereas only the N-terminal region is involved in the activation of the IFN-β promoter[57]. The mechanism of NF-κB activation through the N-terminal region of TRIF was further studied in a yeast two-hybrid screen, which led to the finding that TRAF6 physically interacts with TRIF[74]. The TRAF-C domain of TRAF6 is reported to bind to a consensus motif — P-X-E-X-X-(D/E/F/W/Y) — as discussed earlier[31]. Interestingly, both human and mouse TRIF contain three TRAF6-binding motifs in the N-terminal region. Although mutating each of the three TRAF6-binding motifs (TRIF3A mutant) abolished the association of TRIF with TRAF6, activation of NF-κB by this TRIF3A mutant was only partially reduced. The activation that still occurs is probably a result of the C-terminal region of TRIF, which activates NF-κB independently of the N-terminal region: indeed, a version of the TRIF3A mutant that lacks the C-terminal region was found to lose its ability to activate NF-κB. In addition, it was shown that TBK1, which activates IRF3 and thereby induces IFN-β, associates with the N-terminal region of TRIF[72,74]. Therefore, the N-terminal region of TRIF directly associates with TRAF6 and TBK1, leading to activation of NF-κB and the *IFN-β* gene, respectively (FIG. 4). Furthermore, TRIF uses at least two pathways for NF-κB activation. The first involves its N-terminal region and is mediated by TRAF6, and the second involves its C-terminal region.

A recent study indicates that TRIF-dependent NF-κB activation is dependent on receptor-interacting protein 1 (RIP1), which associates with the C-terminus of TRIF[75].

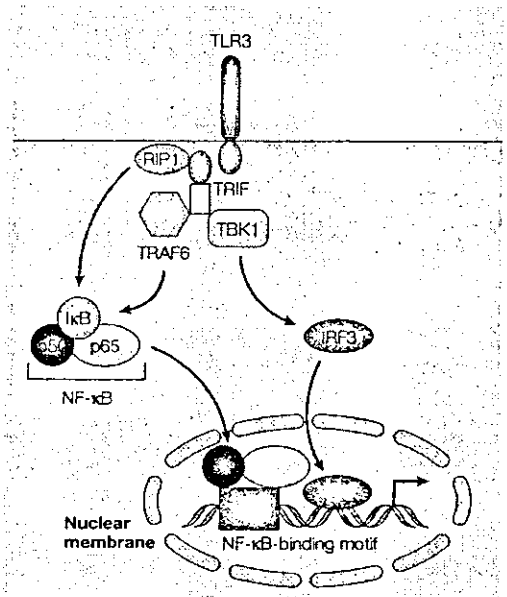

Figure 4 | **TRIF-dependent induction of IFN-β.** The amino-terminal region of TRIF (Toll/interleukin-1-receptor (TIR)-domain-containing adaptor protein inducing interferon (IFN)-β) interacts with both TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) and TBK1 (TRAF-family-member-associated nuclear factor-κB (NF-κB) activator (TANK)-binding kinase 1). TRIF-dependent activation of TBK1 leads to the phosphorylation of IRF3 (IFN-regulatory factor 3), and TRAF6 mediates NF-κB activation. RIP1 (receptor-interacting protein 1) mediates the NF-κB activation that is induced through the carboxy-terminal region of TRIF. Activation of both NF-κB and IRF3 contributes to the activation of the *IFN-β* gene. IκB, inhibitor of NF-κB; TLR, Toll-like receptor.

This region of TRIF contains a RIP homotypic-interaction motif, which is required for association with RIP1. A dominant-negative form of RIP1 inhibits TRIF-mediated NF-κB activation, and embryonic fibroblasts from RIP1-deficient mice showed impaired TLR3-mediated NF-κB activation. So, RIP1 probably mediates NF-κB activation through the C-terminal region of TRIF.

Other molecules involved in TLR signalling

After ligand binding, TLRs activate various intracellular signalling molecules in addition to those discussed earlier. These include Toll-interacting protein (TOLLIP), the pellinos, phosphatidylinositol 3-kinase (PI3K), AKT (also known as protein kinase B, PKB), evolutionarily conserved signalling intermediate in Toll pathways (ECSIT), the SRC-family tyrosine kinases and MAPKKKs. These molecules are potentially involved in TLR-signalling pathways and are discussed briefly here.

TOLLIP. Originally, TOLLIP was cloned as a protein that interacts with the IL-1R accessory protein[76]. Subsequently, it has been shown to associate directly with the cytoplasmic TIR domain of IL-1Rs, TLR2 and TLR4, following the stimulation of these receptors, and to inhibit TLR-mediated cellular responses by suppressing the phosphorylation and kinase activity of IRAK1 (REF. 77). In resting cells, TOLLIP forms a complex with members of the IRAK family, thereby preventing NF-κB activation by blocking the phosphorylation of IRAK1. After receptor activation, TOLLIP–IRAK1 complexes are recruited to the receptor, which results in the rapid autophosphorylation of IRAK1 and its dissociation from the receptor. At the same time, IRAK1 phosphorylates TOLLIP, which might then lead to the dissociation of TOLLIP from IRAK1 and its rapid ubiquitylation and degradation. TOLLIP is therefore thought to function mainly to maintain immune cells in a quiescent state and to facilitate the termination of TLR/IL-1R-induced cell signalling during inflammation and infection.

Pellino. Pellino was first identified in *Drosophila* as a protein that binds to Pelle, the *Drosophila* homologue of the IRAKs[78]. Three mammalian homologues of Pellino have since been identified — pellino-1, pellino-2 and pellino-3 — and these show a high degree of evolutionary conservation[79–81]: human pellino-2 is 60% identical to *Drosophila* Pellino. Mammalian pellino-1 and -2 interact with IRAK1 and have been shown to be required for NF-κB activation in the TLR/IL-1R-signalling pathways[79,80]. Under steady-state conditions, IRAK1 and pellino-2 remain separate, but following TLR/IL-1R stimulation, they form a complex[79]. Because of their ability to interact with IRAKs and their lack of any domain capable of enzymatic activity, it is likely that the pellinos function as scaffolding proteins that facilitate the release of phosphorylated IRAK from the receptor.

PI3K. PI3Ks are activated during TLR/IL-1R signalling, as a result of the direct interaction of the PI3K p85 regulatory subunit with the receptor[82]. This interaction involves the SRC homology 2 (SH2) domain of the p85 subunit and a domain in the receptor containing the motif Tyr-Xaa-Xaa-Met. The subsequent association of the p110 catalytic subunit of PI3Ks results in complete activation, leading to the phosphorylation and activation of its downstream target, AKT.

Interestingly, the PI3K-binding motif Tyr-Xaa-Xaa-Met, where Xaa denotes any amino acid, is present only in a subset of TLRs: TLR1, TLR2 and TLR6, but not TLR3, TLR4 or TLR5 (REF. 83). However, a putative PI3K-binding site (Tyr257-Lys258-Ala259-Met260) is present in the C-terminus of MyD88, and LPS stimulation has been shown to result in the tyrosine phosphorylation of MyD88 and the formation of a PI3K–MyD88 complex[84]. MyD88 also interacts directly with AKT, and a dominant-negative mutant of AKT causes a defect in MyD88-dependent NF-κB transcriptional activity. However, the binding of NF-κB to DNA is not affected by inhibiting AKT, indicating that AKT might be involved in the phosphorylation of the p65 transactivation domain. A dominant-negative mutant of MyD88 was shown to block the kinase activity of AKT generated in response to LPS and IL-1, and a dominant-negative mutant of p85 inhibited the NF-κB activity elicited by LPS and IL-1 but not that elicited by TNF[85]. These findings indicate that PI3K is a positive mediator of the signalling induced by LPS and IL-1 that

leads to NF-κB activation. However, recent studies using mice that lack the p85 regulatory subunit showed an increased production of IL-12 by DCs, possibly because of enhanced activation of the p38 MAPK, indicating that PI3K might have a negative role in TLR signalling in DCs[86].

ECSIT. ECSIT has no homology with any known protein and was cloned as a TRAF6-interacting protein by yeast two-hybrid screening[87]. ECSIT interacts with the conserved TRAF domain of TRAF6. A *Drosophila* homologue of ECSIT has been identified, and the interaction between TRAF6 and ECSIT is also conserved in *Drosophila*. ECSIT also interacts with MEKK1 (MAPK/ERK (extracellular signal-regulated kinase) kinase kinase 1), which can phosphorylate and activate the IKK complex. Expression of a dominant-negative mutant of ECSIT blocks signalling through TLR4, indicating that ECSIT might transduce TLR signals by bridging TRAF6 and the IKK complex. Furthermore, the inhibition of ECSIT expression, using siRNA in a macrophage cell line, resulted in impaired LPS-induced, but not TNF-induced, NF-κB activation[88]. The physiological function of ECSIT was studied by generating ECSIT-deficient mice, which were found to die on about embryonic day 7.5 (REF. 88). Further characterization showed that ECSIT is an obligatory intermediate in bone morphogenetic protein (BMP) signalling, and therefore ECSIT is an essential component in both the TLR- and BMP-signalling pathways.

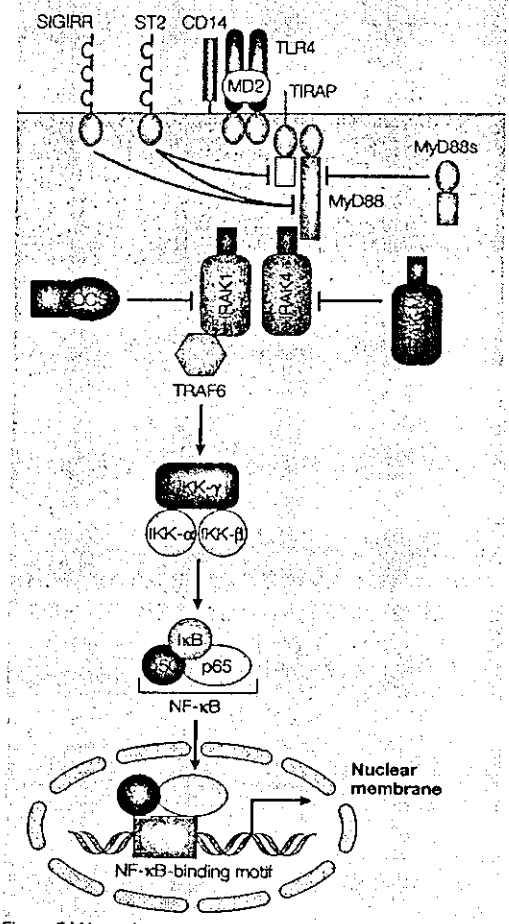

Figure 5 | **Negative regulation of TLR signalling.** Toll-like receptor (TLR)-signalling pathways are negatively regulated by several molecules that are induced by the stimulation of TLRs. IRAK-M (interleukin-1-receptor (IL-1R)-associated kinase M) inhibits the dissociation of the IRAK1–IRAK4 complex from the receptor. SOCS1 (suppressor of cytokine signalling 1) probably associates with IRAK1 and inhibits its activity. MyD88s (myeloid differentiation primary-response protein 88 short) blocks the association of IRAK4 with MyD88. The TIR (Toll/IL-1R)-domain-containing receptors SIGIRR (single immunoglobulin IL-1R-related molecule) and ST2 have also been shown to negatively modulate TLR signalling. IκB, inhibitor of NF-κB; IKK, IκB kinase; NF-κB, nuclear factor-κB; TIRAP, TIR-domain-containing adaptor protein; TRAF6, tumour-necrosis-factor-receptor-associated factor 6.

SRC family of tyrosine kinases. Bruton's tyrosine kinase (BTK) is a member of the SRC-related TEC-family of protein tyrosine kinases and has an essential role in B-cell development and B-cell receptor (BCR)-mediated signalling. Macrophages from X-linked-immunodeficient mice that lack BTK show reduced responses to LPS, and BTK has also been found to associate with the TIR domain of TLR4,TLR6, TLR8 and TLR9 (REF. 89). It has also been shown to associate with MyD88, TIRAP and IRAK1, and to be tyrosine phosphorylated in response to LPS, whereas a dominant-negative form of BTK inhibits LPS-induced activation of NF-κB, indicating that BTK is involved in the TLR-mediated signalling pathway. During BCR-mediated signalling, BTK interacts with and is activated by the SRC family of tyrosine kinases, such as FYN, LYN and haematopoietic-cell kinase, HCK; however, SRC-family kinases have only a minor role in LPS signalling[90]. Therefore, the involvement of BTK in TLR signalling needs to be further investigated.

MAPKKK. Members of the MAPKKK family — such as TAK1, MEKK1, MEKK2, MEKK3, TPL2 (tumour-progression locus 2; also known as cancer Osaka thyroid, COT) and NIK — are implicated in IKK–NF-κB and MAPK activation. Among these members, MEKK3 has been shown to be involved in signalling through TLR4 but not through TLR9 (REF. 91): in response to stimulation with a TLR4 ligand but not a TLR9 ligand, embryonic fibroblasts from MEKK3-deficient mice were shown to have impaired IL-6 production and defective activation of NF-κB, JNK and the p38 MAPK. Stimulation of TLR4 also induced association of MEKK3 with TRAF6. So, MEKK3 is involved in the TLR4-mediated signalling pathway. Another member of the MKKK family, TPL2 has been shown to be involved in the TLR4-mediated activation of ERK[92]. In response to TLR4 ligand, TPL2-deficient mice showed impaired TNF production and defective activation of ERK. Taken together, it is clear that several MAPKKKs mediate TLR-signalling pathways.

LPS TOLERANCE
A transient state of hyporesponsiveness to subsequent stimulation with lipopolysaccharide (LPS), which is induced by the administration of Toll-like receptor ligands *in vivo* and *in vitro*.

Negative regulation of TLR signalling

The inflammatory cytokines produced as a result of TLR signalling, when released in excess, induce serious systemic disorders that are associated with a high mortality rate — such as endotoxic shock, which can be induced by the TLR4 ligand LPS. It is therefore not surprising that organisms have evolved mechanisms for modulating their TLR-mediated responses (FIG. 5). The molecules thought to negatively regulate TLR signalling are discussed briefly here; these include IRAK-M, SOCS1 (suppressor of cytokine signalling 1), MyD88 short (MyD88s), SIGIRR (single immunoglobulin IL-1R-related molecule) and ST2.

Unlike the other IRAKS, which are ubiquitously expressed, the expression of IRAK-M is restricted to monocytes and macrophages and increases following stimulation with TLR ligands. IRAK-M also lacks kinase activity[92]. In response to TLR ligands, IRAK-M-deficient mice show increased production of inflammatory cytokines and defective induction of LPS TOLERANCE[93]. Biochemical analysis has revealed that IRAK-M prevents the dissociation of the IRAK1–IRAK4 complex from MyD88, thereby preventing the formation of the IRAK1–TRAF6 complex. These findings indicate that IRAK-M negatively regulates TLR-signalling pathways.

SOCS1 is a member of the SOCS family of proteins, which are induced by cytokines and negatively regulate cytokine-signalling pathways[94]. LPS and CpG-containing DNA have been shown to induce the expression of SOCS1 in macrophages[95,96], and SOCS1-deficient mice have been shown to be hypersensitive to LPS-induced endotoxic shock (that is, to show increased production of inflammatory cytokines)[97,98]. Furthermore, LPS tolerance was not induced in SOCS1-deficient mice and the ectopic introduction of SOCS1 into macrophages inhibited LPS-induced NF-κB activation. These findings indicate that SOCS1 directly downmodulates TLR-signalling pathways. Although SOCS1 has been shown to associate with IRAK1 (REF. 98), the precise mechanism by which SOCS1 inhibits TLR signalling remains unclear.

MyD88s, an alternatively spliced variant of MyD88 that lacks the intermediary domain, is induced in monocytes following stimulation with LPS. Unlike MyD88, MyD88s does not bind IRAK4, and overexpression of MyD88s does not induce IRAK1 phosphorylation. Therefore, MyD88s inhibits LPS-induced NF-κB activation because of its inability to bind to IRAK4 and promote IRAK1 phosphorylation[99].

In addition to these cytoplasmic molecules, the negative effects of which are induced by TLR signalling, membrane-bound molecules that contain a TIR domain — such as SIGIRR and ST2 — have recently been shown to be involved in the negative regulation of TLR signalling. SIGIRR-deficient mice were found to be highly sensitive to LPS-induced endotoxic shock[100]. Following TLR stimulation, SIGIRR has also been shown to interact transiently with TLR4, IRAK1 and TRAF6, thereby negatively regulating TLR-signalling pathways. Similarly, ST2-deficient mice showed increased production of inflammatory cytokines in response to LPS; moreover, they also showed defective induction of LPS tolerance[101]. Overexpression of ST2 was found to inhibit NF-κB activation, because ST2 associated with, and probably sequestered, MyD88 and TIRAP. Therefore, TIR-domain-containing orphan receptors, such as SIGIRR and ST2, are implicated in the negative regulation of TLR signalling.

Conclusions and future prospects

The molecular mechanisms by which the TLRs activate innate immunity are being elucidated by analysing mice that lack either individual TLRs or other molecules involved in TLR signalling. TIR-domain-containing adaptors, such as MyD88, TIRAP, TRIF and TRAM, have been found to have crucial roles in TLR-signalling pathways, because they provide specificity to the response generated by signalling through each TLR. However, several questions remain to be answered. For example, TLR7 and TLR9, but not TLR2, induce type I IFNs in a MyD88-dependent manner, indicating that TLR7 and TLR9 have a unique signalling pathway. It is possible that an additional TIR-domain-containing adaptor, SARM[63], is involved in this TLR7/TLR9-mediated pathway. Alternatively, some TLR signalling might be regulated by molecules that do not contain a TIR domain, and elucidation of this unique TLR7/TLR9 pathway should improve our understanding of the mechanisms of the TLR-mediated activation of innate immunity. In addition, we are now able to study mice that are deficient in both MyD88 and TRIF, which therefore lack all of the TLR-signalling pathways that have been characterized so far. We now need to intensively analyse the role of TLR signalling in host defence against various infectious microorganisms. For example, MyD88-deficient mice have been shown to be sensitive to Gram-negative bacterial and Gram-positive bacterial infections[102], yet they still generate immune responses against intracellular bacteria

Box 1 | TLR-independent recognition of microorganisms

The intracellular recognition of certain pathogens seems to involve a Toll-like receptor (TLR)-independent system. The most well-characterized is the nucleotide-binding oligomerization domain (NOD) family of proteins, which includes NOD1 and NOD2 — proteins that recognize the core structures of bacterial peptidoglycan in the cytoplasm[107]. In addition, TLR-independent mechanisms have been shown to be involved in the recognition of viral products. For example, TLR3-deficient mice retain responsiveness to double-stranded (ds) RNA, indicating that dsRNA is recognized by both TLR3-dependent and -independent mechanisms[50,52,108]. Furthermore, the introduction of dsRNA into the cytoplasm of dendritic cells leads to the induction of type I interferons (IFNs) (IFN-α/β) through a mechanism that is partly dependent on PKR (IFN-inducible dsRNA-dependent protein kinase) but independent of TLR3 (REF. 109). PKR was originally proposed to mediate the cellular recognition of and response to dsRNA; however, PKR-deficient mice do not show considerable impairment in their response to dsRNA or viral infection[110–111]. It is still unclear whether PKR is involved in the TLR3-independent response to dsRNA, and it is possible that another molecule or molecules mediates this recognition. The generation of mice lacking both TLR3 and PKR will further elucidate the role of PKR in the recognition of dsRNA.

(*Listeria monocytogenes* and mycobacteria)[103–106] and viruses. Consistent with this, TLR-independent mechanisms for the recognition of intracellular bacteria and viruses have been proposed (BOX 1). Analysis of mice that lack both MyD88 and TRIF should reveal the extent to which MyD88-dependent and TRIF-dependent/MyD88-independent pathways of TLR signalling contribute to host defence. Mutant mice that lack components of the TLR-signalling pathways should provide powerful models for the *in vivo* analysis of immune responses, host defence against infectious diseases and anticancer responses.

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The authors declare that they have no competing financial interests.

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TOLL-LIKE RECEPTORS: LIGANDS AND SIGNALING

Kiyoshi Takeda and Shizuo Akira

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Innate immunity has recently been revealed to have a skillful system that detects microbial invasion by virtue of Toll-like receptors (TLRs). TLRs comprise a large family consisting of at least 10 members. Genetic studies have established that each TLR recognizes specific components of pathogens. The signaling pathway via TLRs originates from the conserved cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain. The TIR domain-containing adaptor myeloid differentiation marker 88 (MyD88) is common to TLR-mediated signaling, which leads to the production of inflammatory cytokines. However, individual TLRs seem to have their own signaling cascades. In this chapter we focus on recent advances in our understanding of the function of TLRs, particularly with regard to their ligands and signaling.

TLRs DETECT MICROBIAL INVASION

Host defense is believed to be triggered by the detection of microbial invasion into the host. However, the receptors that detect pathogens remained unclear for a long time. Genetic studies in *Drosophila* indicated that Toll was a receptor that detected pathogens (Lemaitre et al., 1996). One year later, a mammalian Toll receptor (now termed TLR4) was shown to induce the expression of genes involved in inflammatory responses (Medzhitov et al., 1997). Subsequent studies revealed that there were several Toll receptors in mammals, and they were designated TLRs. TLRs bear leucine-rich repeats (LRRs) in the extracellular portion and the TIR domain in the cytoplasmic portion. The TIR domain of TLRs shows high similarity with the cytoplasmic region of the IL-1 receptor family and further similarity with several cytoplasmic adaptors, including MyD88 and the TIR adaptor protein (TIRAP). TLRs in mammals have been shown to recognize microbial components that are not present in mammals but are conserved between pathogens, and thereby to detect the invasion of microorganisms such as bacteria, fungi, protozoa, and viruses. So far, the roles of eight members of the TLR family have been established (Fig. 1).

TLRs in Bacterial Recognition

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria and a potent activator of innate immune cells, including macrophages and dendritic cells (DCs). Therefore, the identifica-

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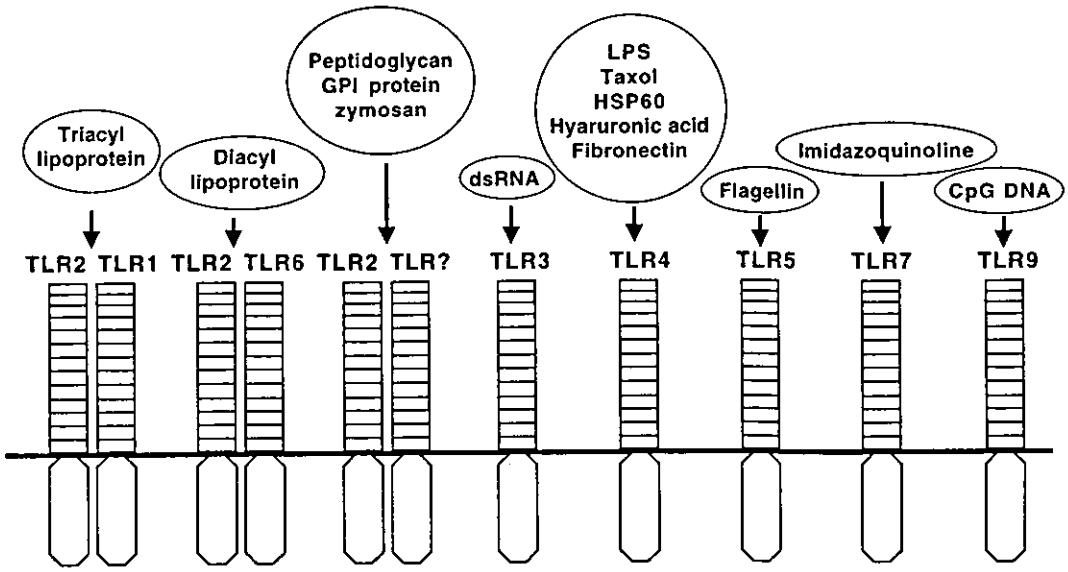

FIGURE 1 TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 recognizes flagellin. Thus, the TLR family members recognize specific patterns of bacterial components.

tion of a LPS signaling receptor has long been anticipated. It is well known that two mouse strains, C3H/HeJ and C57BL/10ScCr, are hyporesponsive to LPS and sensitive to gram-negative bacterial infection. In 1998, Beutler and colleagues identified the gene responsible for the hyporesponsiveness to LPS and found mutations in *Tlr4* in these strains (Poltorak et al., 1998). Another group also found mutations in the *Tlr4* gene in these strains (Qureshi et al., 1999). The C3H/HeJ mouse strain has a point mutation in the cytoplasmic region of the *Tlr4* gene that results in an amino acid change from proline to histidine. This mutation has been shown to result in defective TLR4-mediated signaling and to have a dominant negative effect on LPS responses (Hoshino et al., 1999). The other LPS-hyporesponsive strain, C57BL/10ScCr, has a null mutation in the *Tlr4* gene (Poltorak et al., 1998; Qureshi et al., 1999). The generation of TLR4 knockout mice further revealed the essential role of TLR4 in LPS recognition (Hoshino et al., 1999).

TLR2 has been shown to recognize peptidoglycan, which is abundantly present in the cell walls of gram-positive bacteria (Schwandner et al., 1999; Takeuchi et al., 1999; Yoshimura et al., 1999). In addition, TLR2 recognizes lipoteichoic acids from gram-positive bacteria (Schwandner et al., 1999; Lehner et al., 2001). Accordingly, TLR2-deficient mice are highly sensitive to infection by the gram-positive bacterium *Staphylococcus aureus* (Takeuchi et al., 2000a). TLR2 is involved in the recognition of several additional bacterial components, such as lipoproteins and lipopeptides from a variety of bacteria (Takeuchi et al., 2000b), lipoarabinomannan from mycobacteria (Underhill et al., 1999a; Means et al., 1999a, 1999b), a phenol-soluble modulin from *Staphylococcus epidermidis* (Hajjar et al., 2001), glycolipids from *Treponema maltophilum* spirochetes (Opitz et al., 2001), and porins present

in the outer membrane of *Neisseria* spp. (Massari et al., 2002). The mechanism by which TLR2 recognizes a variety of bacterial components is now partly explained by the fact that TLR2 associates with other TLRs, particularly TLR1 and TLR6. TLR2 ligands, such as peptidoglycan and secreted modulin from *S. epidermidis,* induced tumor necrosis factor alpha (TNF-α) production in RAW264.7 cells, which was inhibited by the expression of the dominant negative form of TLR6 (Hajjar et al., 2001; Ozinsky et al., 2000). Macrophages from TLR2-deficient mice show no inflammatory response to all the kinds of lipoproteins and lipopeptides analyzed to date. Macrophages from TLR6-deficient mice did not show any TNF-α production in response to diacyl lipopeptides from *Mycoplasma* spp., but showed a normal response to triacyl lipopeptides (Takeuchi et al., 2001). In contrast, TLR1-deficient mice were impaired in TNF-α production induced by triacyl lipopeptides, but not that induced by diacyl lipopeptides (Takeuchi et al., 2002). Thus, TLR1 and TLR6 cooperate functionally with TLR2 and participate in the discrimination of subtle structural differences among lipopeptides. TLR1 is also involved in the recognition of lipoprotein from *Mycobacterium* spp. and *Borrelia burgdorferi* (Takeuchi et al., 2002; Alexopoulou et al., 2002).

In addition to TLR2 and TLR4, several TLRs are involved in the recognition of bacterial components such as flagellin and CpG DNA. Flagellin is a protein component of the flagellum, which extends out from the outer membrane of gram-negative bacteria. Flagellin has been shown to activate immune cells via TLR5 (Hayashi et al., 2001). CpG DNA is characteristic of the genomic DNA of bacteria, in which unmethylated CpG motifs are present in the expected frequency. In the mammalian genome, CpG motifs are suppressed in frequency and are highly methylated, which causes no immunostimulatory activity. Generation of TLR9-deficient mice revealed its essential role in the recognition of CpG DNA (Hemmi et al., 2000).

TLRs in Fungal and Protozoan Recognition

TLRs recognize components of not only bacteria but also fungi and protozoa. Zymosan is a crude mixture of glucans, mannan, proteins, chitin, and glycolipids extracted from the cell walls of fungi, which activates immune cells. Zymosan has been shown to be recognized by TLR2 (Underhill et al., 1999b). The immunostimulating activity of zymosan is seemingly attributed to the presence of β glucan (Kataoka et al., 2002). Infection with the protozoan parasite *Trypanosoma cruzi* causes Chagas' disease in humans. Glycosylphosphatidylinositol (GPI) anchors that are present in the membrane of *T. cruzi* have been shown to activate the innate immune cells via TLR2 (Campos et al., 2001; Ropert et al., 2002).

TLRs in Viral Recognition

Accumulating evidence indicates that TLRs are involved in the recognition of viral invasion. TLR4 and CD14 have been shown to recognize the fusion protein of respiratory syncytial virus (Kurt-Jones et al., 2000). TLR4-mutated C3H/HeJ and C57BL/10ScCr mice were impaired in the inflammatory response to respiratory syncytial virus infection and accordingly impaired in virus clearance (Haynes et al., 2001). Mouse mammary tumor virus has been shown to activate B cells through association of that virus's envelope glycoprotein and TLR4 (Rassa et al., 2002).

Double-stranded RNA (dsRNA) is produced by many viruses during their replicative cycle and is representative of the viral components that activate immune cells mainly by inducing type I interferons (alpha/beta interferons [IFN-α/β]) and some of the IFN-inducible genes. Synthetic dsRNA, such as poly(I:C), has activity similar to that of dsRNA. TLR3-deficient mice were impaired in the response to dsRNA and poly(I:C) (Alexopoulou et al., 2001). In addition, expression of human TLR3 in the dsRNA-nonresponsive cell line 293 enabled the cells to activate NF-κB and the

IFN-β promoter in response to dsRNA and poly(I:C) (Alexopoulou et al., 2001; Matsumoto et al., 2002). These findings indicate that both TLR3 and TLR4 are involved in viral recognition.

Synthetic compounds, imidazoquinolines, exhibit potent antiviral and antitumor properties by inducing inflammatory cytokines, especially IFN-α. One of the imidazoquinoline compounds, Imiquimod, has been approved for the treatment of genital warts caused by infection with human papillomavirus. TLR7-deficient mice did not show any response to the imidazoquinolines (Hemmi et al., 2002). Therefore, TLR7 may also be involved in viral recognition. Identification of a natural ligand for TLR7 will reveal the precise role of TLRs in viral recognition.

TLRs in the Recognition of Endogenous Ligands

As described above, TLRs play a critical role in the detection of microbial invasion by recognizing specific components of pathogens. However, several reports indicate that some TLRs, particularly TLR4, are involved in the recognition of endogenous ligands regardless of infection. Heat shock proteins (HSPs) are highly conserved between bacteria and mammals. Several stressful conditions such as heat shock, radiation, and infection induce the synthesis of HSPs, which act to chaperone nascent or aberrantly folded proteins. HSPs, especially HSP60 and HSP70, activate innate immune cells such as macrophages and DCs. The immunostimulatory activity of HSP60 has been shown to be induced by TLR4 (Ohashi et al., 2000; Vabulas et al., 2001). TLR4 mutant mice were impaired in the production of inflammatory cytokines in response to HSP70 as well as HSP60 (Dybdahl et al., 2002; Vabulas et al., 2002; Asea et al., 2002). Thus, TLR4 seems to be responsible for the inflammatory responses elicited by HSPs. In addition to TLR4, TLR2 has also been shown to be required for the recognition of HSP70 (Vabulas et al., 2002; Asea et al., 2002).

Extracellular matrix components, including fibronectin, hyaluronic acid, and heparan sulfate, are produced when tissue is injured and play important roles in wound healing. The type III repeat extra domain A of fibronectin has been shown to activate immune cells through recognition by TLR4 (Okamura et al., 2001). Low-molecular-weight oligosaccharides of hyaluronic acid have been shown to be potent activators of DCs, which are mediated by TLR4 (Termeer et al., 2002). Polysaccharide fragments of heparan sulfate have been reported to induce the maturation of DCs via TLR4 (Johnson et al., 2002). Inflammatory responses to injury, immune disorders, and infection often accompany extravascular deposits of fibrin, which is generated from plasma-derived fibrinogen. Fibrinogen has also been shown to induce the production of chemokines from macrophages through recognition by TLR4 (Smiley et al., 2001). Thus, TLR4 is presumably involved in several inflammatory responses by recognizing endogenous ligands even in the absence of infection. However, all of the endogenous TLR4 ligands activate immune cells only when stimulated at very high concentrations. In addition, the ability of HSP70 to activate macrophages has recently been shown to be attributable to contaminating LPS in the HSP70 preparation (Gao and Tsan, 2003). LPS is the most powerful immunostimulator among microbial components, and the contamination will result in TLR4-dependent immune activation. Therefore, more careful experiments are required before we can conclude that TLR4 recognizes these endogenous ligands.

Molecules that Cooperate with TLRs

Although TLRs have been established to recognize specific patterns of microbial components, several additional molecules associate with some TLRs, particularly TLR4, to detect LPS. These include the LPS-binding protein (LBP), CD14, RP105, and MD-1 and -2 (Fig. 2).

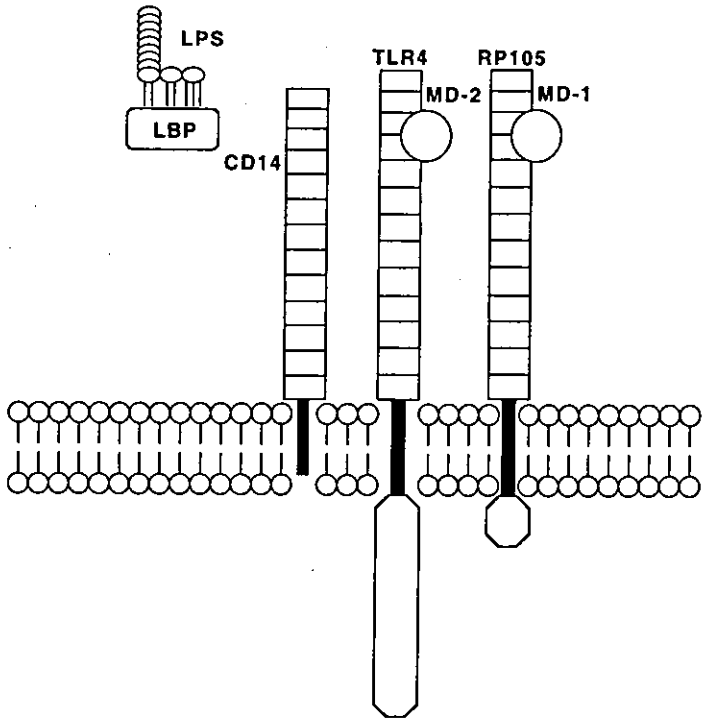

FIGURE 2 The LPS receptor complex. The LPS receptor comprises several components. TLR4 is an essential receptor component for the signal transduction via the LPS receptor complex. MD-2 associates with the extracellular portion of TLR4 and is involved in the LPS recognition. LBP is a soluble molecule that binds to the lipid A portion of LPS. The LPS–LBP complex binds to CD14 and then this complex associates with TLR4. In B cells, additional components, RP105 and MD-1, are involved in the LPS recognition.

LBP and CD14. LBP, which was identified as a plasma protein that binds to the lipid A moiety of LPS, is a member of a family of lipid-binding proteins that act as lipid transport proteins in some cases (Tobias et al., 1986; Schumann et al., 1990). The generation of LBP-deficient mice has revealed a nonredundant role for LBP in the response to LPS (Jack et al., 1997; Wurfel et al., 1997).

The formation of LPS and LBP complexes triggers the association of this complex with another LPS-binding molecule, CD14. CD14 is a GPI-anchored protein, which is preferentially expressed on the surface of mature myeloid cells. Soluble forms of CD14 are also produced through escape from the GPI anchoring and proteolytic cleavage of the membrane-bound CD14. The importance of CD14 in the response to LPS has been demonstrated in CD14-deficient mice, which showed a reduced response to LPS (Haziot et al., 1996; Moore et al., 2000). Thus, LPS first binds LBP, and then this complex is transferred to CD14. CD14 has no cytoplasmic region that would be required for cellular activation. Therefore, the LPS–LBP–CD14 complex requires an additional receptor that transduces the signal from the membrane into the cytoplasm, and it is TLR4 that is responsible for this signal transduction via the LPS–LBP–CD14 complex. Indeed, physical association between CD14 and TLR4 in response to LPS stimulation has been demonstrated (Jiang et al., 2000; Da Shilva Correia et al., 2001).

RP105 and MD-1. RP105 bears an extracellular LRR domain that is structurally similar to those found in TLRs. However, unlike TLRs, RP105 has only a short cytoplasmic tail and is preferentially expressed on B cells (Miyake et al., 1995). RP105-deficient mice showed a severely impaired response to LPS in B cells, indicating that RP105 is an essential component in the recognition of LPS

in B cells (Ogata et al., 2000). Miyake and colleagues (1998) also identified MD-1 as a molecule that associates with the extracellular portion of RP105. Similarly to RP105-deficient mice, MD-1-deficient mice showed impairment in LPS-induced B-cell proliferation, antibody production, and CD86 upregulation (Nagai et al., 2002). Furthermore, surface expression of RP105 was abolished in MD-1-deficient B cells, indicating that MD-1 is essential for the responsiveness to LPS and surface expression of RP105 in B cells. It remains unclear whether RP105/MD-1 is involved in the LPS recognition in other cells that express RP105/MD-1, such as DCs and macrophages.

MD-2. Miyake and colleagues further identified MD-2, which is structurally related to MD-1 (Shimazu et al., 1999). Expression of both MD-2 and TLR4, but not TLR4 alone, conferred LPS-induced NF-κB activation in LPS-nonresponsive Ba/F3 cells, indicating that MD-2 associates functionally with TLR4. Physical association of MD-2 and TLR4 on mouse peritoneal macrophages was also shown using a monoclonal antibody against the TLR4/MD-2 complex (Akashi et al., 2000; Nomura et al., 2000). The importance of MD-2 in the LPS responsiveness was further demonstrated in genetic studies. Chinese hamster ovary cell lines that showed an impaired response to LPS have been shown to be mutated in the *MD-2* gene (Schromm et al., 2001). MD-2-deficient mice displayed severely impaired responses to LPS, and the phenotype was very similar to that of TLR4-deficient mice (Nagai et al., 2002). Analysis of the MD-2-deficient mice further demonstrated that the surface expression of TLR4 was abolished in these mice, indicating that MD-2 is required for the surface expression of TLR4 (Nagai et al., 2002).

Thus, several molecules which associate functionally with TLR4 have been identified. However, no molecules have been reported to associate with the other TLRs. It is very intriguing how TLRs, which have the conserved LRR domains in the extracellular portion, recognize quite distinct types of microbial components such as the lipid moiety, peptides, and nucleic acids. In this regard, we can hypothesize that, although molecules that associate with individual TLRs have not yet been reported, they might exist and directly recognize the microbial components. Indeed, there is a report that indicates that MD-2 directly regulates the species-specific recognition of the lipid moiety by TLR4 (Akashi et al., 2001).

SIGNALING PATHWAYS VIA TLRs

The signaling pathways via TLRs originate from the TIR domain. MyD88 harboring the TIR domain in the carboxy-terminal portion associates with the TIR domain of TLRs. Upon stimulation by TLR ligands, MyD88 recruits a family of IL-1 receptor-associated kinases (IRAKs) to TLRs. IRAKs then activate tumor receptor-associated factor 6 (TRAF6), thereby inducing activation of mitogen-activated protein (MAP) kinases and NF-κB (Fig. 3). Important roles for each molecule have been elucidated through the generation of gene-targeted mice.

MyD88 is a Common Adaptor in TLR Signaling

MyD88-deficient mice showed impaired responses to the IL-1 family of cytokines, whose receptors have the cytoplasmic TIR domain (Adachi et al., 1998). Subsequent studies of MyD88-deficient mice demonstrated that these mice did not produce any inflammatory cytokines in response to LPS, peptidoglycan, lipoproteins, CpG DNA, flagellin, dsRNA, or the imidazoquinolines (Kawai et al., 1999; Takeuchi et al., 2000b, 2000c; Hayashi et al., 2001; Alexopoulou et al., 2001; Hemmi et al., 2002; Hacker et al., 2000; Schnare et al., 2000). These findings indicate that MyD88 is an essential adaptor in the signaling pathways activated via all the TLR family members that lead to the production of inflammatory cytokines. Indeed, macrophages from MyD88-deficient mice showed no activation of NF-κB or JNK in response to

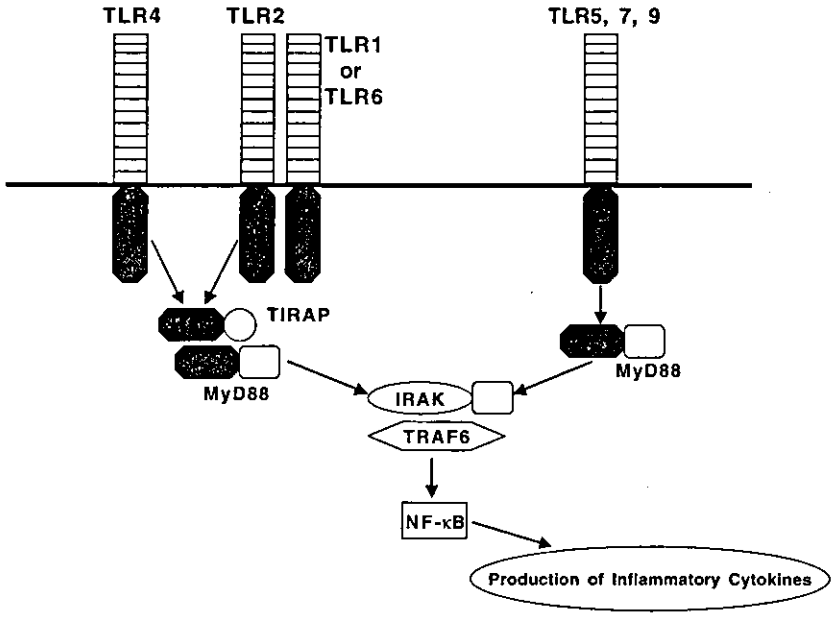

FIGURE 3 MyD88-dependent signaling pathway. A TIR domain-containing adaptor molecule, MyD88, associates with the cytoplasmic TIR domain of TLRs and recruits IRAKs to the receptor upon receptor activation. IRAKs then activate TRAF6, leading to the activation of MAP kinases and NF-κB. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4.

peptidoglycan, lipoprotein, CpG DNA, or the imidazoquinolines.

TRAF6-deficient mice also exhibited impaired responses to both IL-1 and LPS, indicating that TRAF6 is also a critical component of both the IL-1 receptor- and the TLR4-mediated signaling pathways (Lomaga et al., 1999; Naito et al., 1999). The IRAK family has four members: IRAK-1, IRAK-2, IRAK-M, and IRAK-4 (Li et al., 2002). Physiological roles for these family members have been elucidated, except for IRAK-2. IRAK-1-deficient mice were partially impaired in their responses to IL-1 and LPS (Kanakaraj et al., 1998; Thomas et al., 1999; Swantek et al., 2000). IRAK-4-deficient mice showed almost no response to IL-1 and TLR ligands (Suzuki et al., 2002). IRAK-4 associates with IRAK-1 in response to IL-1 stimulation, and the introduction of the dominant negative form of IRAK-4 resulted in impaired IRAK-1 activation in response to IL-1. Thus, IRAK-4 presumably acts as a central mediator in the IL-1 receptor and TLR signaling, upstream of IRAK-1 (Li et al., 2002).

Additional molecules that are involved in the TLR signaling have been reported. Receptor interacting protein-2 (RIP2), harboring a carboxy-terminal CARD domain, was originally identified as a serine/threonine kinase that associates with TRAF family members and with TNF receptor family members, such as the type I TNF receptor and CD40, and induces NF-κB activation and apoptosis (McCarthy et al., 1998; Inohara et al., 1998). RIP2-deficient mice were partially impaired in their response to LPS, peptidoglycan, and dsRNA, indicating that RIP2 is involved in the TLR signaling pathways (Kobayashi et al., 2002; Chin et al., 2002). It remains to be elucidated how RIP2 is connected to the TLR signaling. Toll-interacting protein (Tollip) has

been identified as a molecule present in a complex with IRAK (Burns et al., 2000). Upon stimulation with IL-1, the Tollip–IRAK complex is recruited to the IL-1R complex by the association of Tollip with IL-1RAcP. IRAK is then activated by phosphorylation, which in turn leads to dissociation of IRAK from Tollip. Tollip is seemingly involved in the negative regulation of the TLR signaling pathway because overexpression of Tollip blocked activation of NF-κB in response to TLR2 and TLR4 ligands (Bulut et al., 2002; Zhang and Ghosh, 2002). However, the physiological roles of Tollip remain to be elucidated through the generation of gene-targeted mice.

Signaling Pathways That Are Independent of MyD88

MyD88 is essential for the production of inflammatory cytokines in response to all the TLR ligands, as described above. However, unlike the case for stimulation with other TLR ligands, LPS stimulation resulted in the activation of NF-κB and JNK in MyD88-deficient macrophages, although the kinetics were delayed compared to wild-type macrophages (Kawai et al., 1999). This finding indicates that the LPS-induced inflammatory cytokine production is completely mediated by the MyD88-dependent signaling pathway, but that a pathway exists that is independent of MyD88 in the LPS response (see Fig. 4). Indeed, some LPS responses were observed in MyD88-deficient mice. DCs from MyD88-deficient, but not from TLR4-deficient, mice matured in response to LPS (Kaisho et al., 2001). Kupffer cells from MyD88-deficient mice showed caspase-1-dependent cleavage of the IL-18 precursor into the mature form after LPS stimulation (Seki et al., 2001). MyD88-deficient macrophages showed LPS-induced expression of several genes, such as those

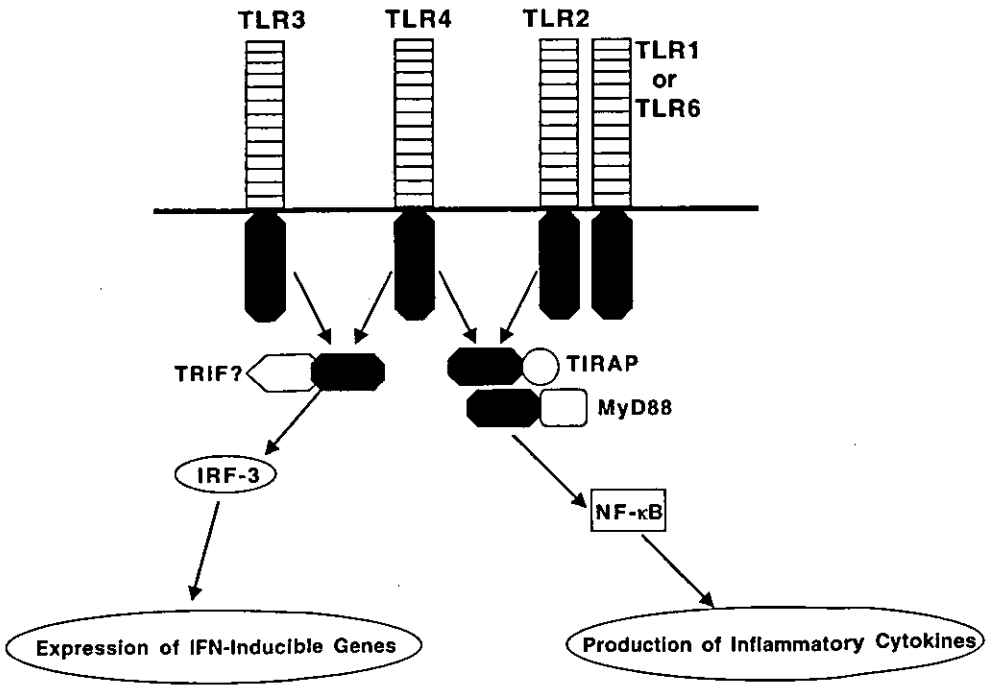

FIGURE 4 MyD88-independent signaling pathway. In the TLR3- and TLR4-mediated signaling pathways, LPS-induced activation of IRF-3 is observed in MyD88-deficient mice, indicating the presence of a MyD88-independent pathway. It remains unclear how IRF-3 is activated. Recently, a TIR domain-containing adaptor, TRIF, was found to associate with IRF-3 and TLR3, indicating a possible role for TRIF in the MyD88-independent pathway.

encoding IP-10 and GARG16, all of which are known as IFN-inducible genes (Kawai et al., 2001). In addition, MyD88-deficient macrophages showed NF-κB activation in response to dsRNA, as is the case in LPS stimulation (Alexopoulou et al., 2001). Thus, TLR4 and TLR3 utilize the MyD88-independent pathway, although it remains unclear whether the pathways activated by TLR4 and TLR3 are equivalent.

Stimulation with dsRNA or viral infection results in the activation of interferon regulatory factor 3 (IRF-3), a member of the IRF family of transcription factors, and thereby induces IFN-α/β and the IFN-inducible genes (Weaver et al., 1998; Yoneyama et al., 1998). IRF-3-deficient mice were impaired in the viral infection-induced expression of IFN-α/β, demonstrating the essential role of IRF-3 in virus-induced IFN-α/β expression (Sato et al., 2000). LPS has also been shown to activate IRF-3 (Kawai et al., 2001; Navarro and David, 1999). Thus, IRF-3 is activated in the TLR3 and TLR4 signaling pathways. Furthermore, LPS stimulation induced the activation of IRF-3 in MyD88-deficient mice, indicating that IRF-3 activation occurs in a MyD88-independent manner (Kawai et al., 2001). Subsequent studies demonstrated that LPS-induced activation of IRF-3 induced the MyD88-independent expression of IFN-β and then IFN-β induced Stat1-dependent expression of the IFN-inducible genes in macrophages and DCs (Toshchakov et al., 2002; Hoshino et al., 2002; Doyle et al., 2002). Thus, IRF-3 presumably plays an important role in the MyD88-independent pathway of TLR3 and TLR4 signaling. Analysis of the role of IRF-3 in the TLR signaling pathways would be of great interest.

IFN-α has been shown to be induced in response to the activation of TLR7 as well as TLR4 (Ito et al., 2002; Hemmi et al., 2002). However, unlike TLR4, TLR7-dependent induction of IFN-α was not observed in MyD88-deficient mice (Hemmi et al., 2002). In addition, a certain type of CpG DNA that activates TLR9 also induced IFN-α in plasmacytoid DCs (Krug et al., 2001). TLR7 and TLR9 are structurally related and presumably utilize a similar signaling cascade, thereby leading to similar biological outcomes. Therefore, we presume that an unknown signaling cascade that is dependent on MyD88 induces IFN-α in the TLR7- and TLR9-mediated signaling pathways.

TIR Domain-Containing Adaptors

In attempts to characterize the MyD88-independent signaling pathway, a second adaptor molecule containing the TIR domain was identified and designated TIRAP or MyD88-adaptor-like (Horng et al., 2001; Fitzgerald et al., 2001). The initial in vitro studies suggested that TIRAP specifically associates with TLR4 and acts as an adaptor in the MyD88-independent signaling pathway. However, TIRAP-deficient mice have recently been generated, and analysis of these mice has revealed an unexpected role for TIRAP in the TLR signaling (Yamamoto et al., 2002; Horng et al., 2002). TIRAP-deficient mice showed no production of inflammatory cytokines in response to the TLR4 ligand. However, TIRAP-deficient macrophages showed delayed activation of NF-κB and MAP kinases in response to LPS, as is the case in MyD88-deficient macrophages. Furthermore, TIRAP-deficient mice were not impaired in the LPS-induced expression of IFN-inducible genes and maturation of DCs. Even in TIRAP/ MyD88 double-deficient mice, these LPS responses were normal. These findings indicate that TIRAP is essential for the TLR4-mediated MyD88-dependent, but not for the MyD88-independent, signaling pathway. In addition, although TIRAP-deficient mice showed normal responses to the TLR3, TLR7, and TLR9 ligands, they were defective in their response to the TLR2 ligands. Thus, TIRAP has been demonstrated to be essential for the MyD88-dependent signaling pathway via TLR2 and TLR4, but not for the MyD88-independent signaling. These studies further indicate that the TIR domain-containing molecules provide the specificity for individual TLR-mediated signaling pathways.