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REVIEWS

TOLL-LIKE RECEPTOR SIGNALLING

Shizuo Akira and Kiyoshi Takeda‡*

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

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

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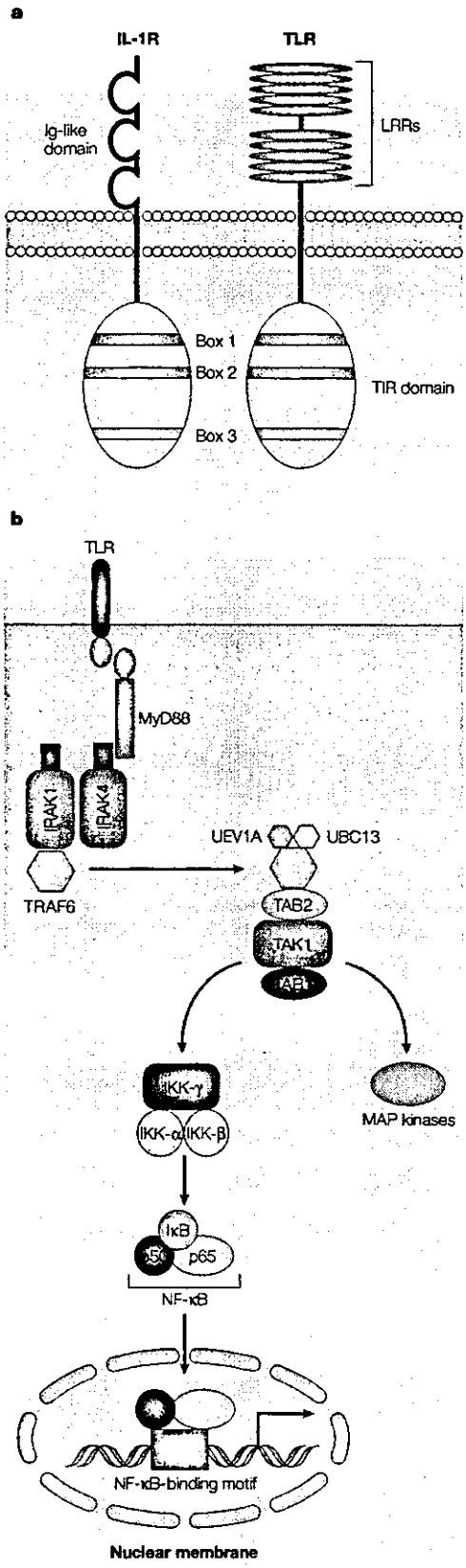
TLR/IL-1R superfamily: structure and function

The discovery of the TLR family began with the identification of Toll, a receptor that is expressed by insects and was found to be essential for establishing dorsoventral polarity during embryogenesis[5]. Subsequent studies revealed that Toll also has an essential role in the insect innate immune response against fungal infection[6]. Homologues of Toll were identified through database searches, and so far, 11 members of the TLR family have been identified in mammals. The TLRs are type I integral membrane glycoproteins, and on the basis of considerable homology in the cytoplasmic region, they are members of a larger superfamily that includes the interleukin-1 receptors (IL-1Rs). By contrast, the extracellular region of the TLRs and IL-1Rs differs markedly: the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs, whereas the extracellular region of IL-1Rs contains three immunoglobulin-like domains (FIG. 1a).

Toll/IL-1R domain. TLRs and IL-1Rs have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain[7]. Within the TIR domain, the regions of homology comprise three conserved boxes, which are crucial for



signalling (FIG. 1a). Amino-acid sequence conservation among the TIR domains is generally 20–30%, and these domains vary in size. The crystal structures of the TIR domains of human TLR1 and TLR2 have been obtained and analysed; they contain a central five-stranded parallel β-sheet, which is surrounded by five α-helices on each side[8]. These two secondary structural elements are connected by loops: for example, the BB loop connects the strand β-B and the helix α-B. The conserved boxes 1 and 2 and the BB loop are adjacent and display most of their side chains for interaction with adaptor molecules.

C3H/HeJ mice have a defect in their ability to respond to lipopolysaccharide (LPS) because of a missense mutation in the *Tlr4* gene[9], which alters the sequence located at the tip of the BB loop, farthest from the rest of the TIR domain. This indicates that the mutation abrogates LPS signalling not because it disrupts the TIR domain structure itself, but rather because it disrupts a direct point of contact with another molecule or molecules, specifically with other TIR-domain-containing molecules.

Leucine-rich repeats. The extracellular domain of TLRs contains 19–25 tandem copies of the LRR motif. Each repeat consists of 24–29 amino acids and contains the leucine-rich sequence XLXXLXLXX, and another conserved sequence XØXXØX$_4$FXXLX (REF. 10), where X denotes any amino acid and Ø a

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

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.

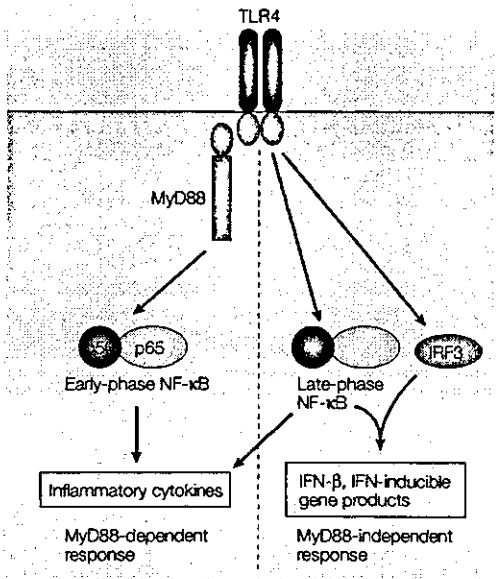


Figure 2 | **TLR4 signalling: MyD88-dependent and -independent pathways.** Stimulation of Toll-like receptor 4 (TLR4) facilitates the activation of two pathways: the MyD88 (myeloid differentiation primary-response protein 88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves 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 (IRF3) 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).

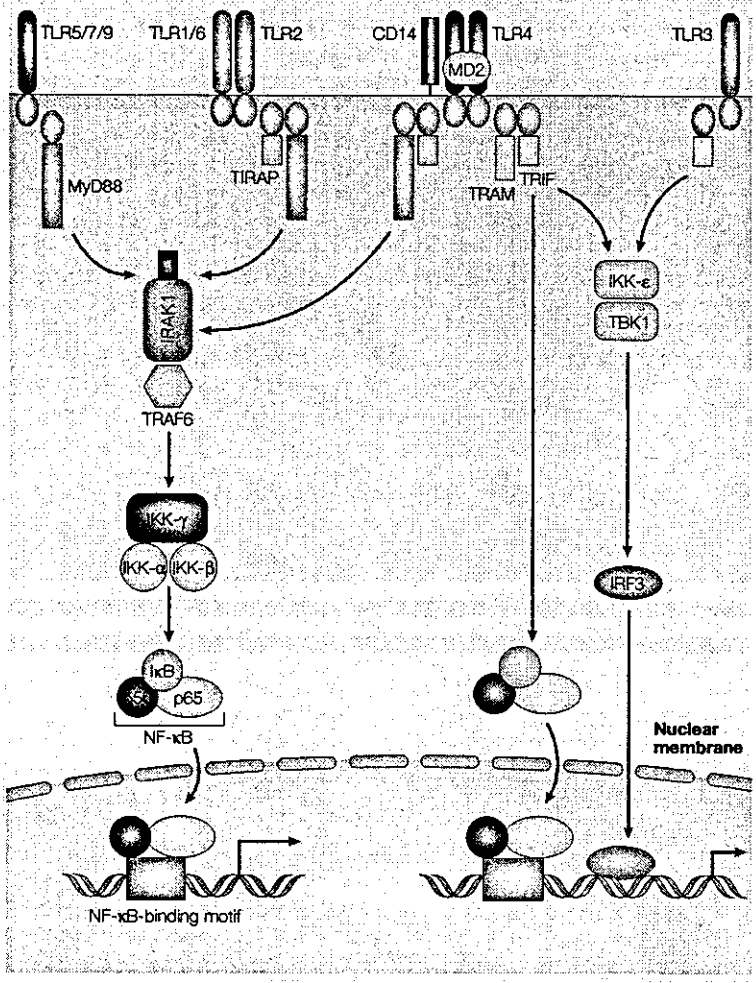


Figure 3 | **Involvement of TIR-domain-containing adaptors in TLR-signalling pathways.** The Toll/interleukin-1 (IL-1)-receptor (TIR)-domain-containing adaptor molecule MyD88 (myeloid differentiation primary-response protein 88) mediates the Toll-like receptor (TLR)-signalling pathway that activates 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[50]. 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].

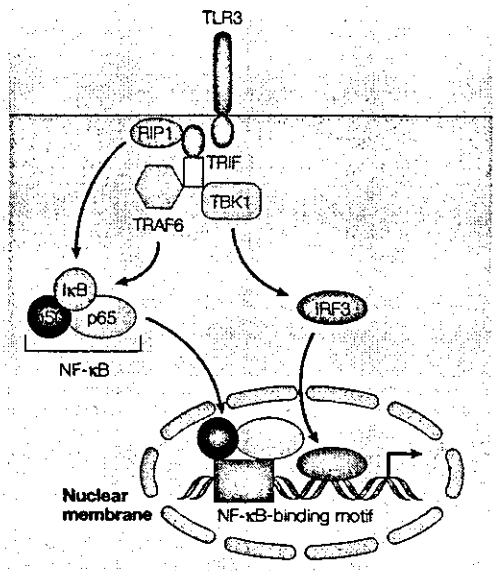


Figure 4 | **TRIF-dependent induction of IFN-β.** The amino-terminal region of TRIF (Toll/interleukin-1-receptor (TIR)-domain-containing adaptor protein inducing interferon (IFN)-β) interacts with both TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) and TBK1 (TRAF-family-member-associated 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.

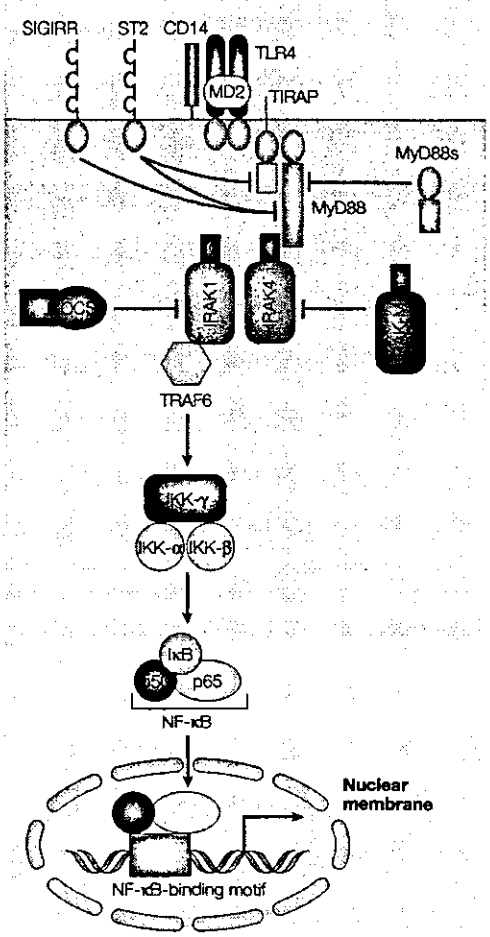


Figure 5 | **Negative regulation of TLR signalling.** Toll-like receptor (TLR)-signalling pathways are negatively regulated by several molecules that are induced by the stimulation of TLRs. IRAK-M (interleukin-1-receptor (IL-1R)-associated kinase M) inhibits the dissociation of the IRAK1–IRAK4 complex 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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Competing interests statement

The authors declare that they have no competing financial interests.

Online links

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Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ

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Toll-like receptors (TLRs) recognize microbial components and trigger the inflammatory and immune responses against pathogens. IκBζ (also known as MAIL and INAP) is an ankyrin-repeat-containing nuclear protein that is highly homologous to the IκB family member Bcl-3 (refs 1–6). Transcription of IκBζ is rapidly induced by stimulation with TLR ligands and interleukin-1 (IL-1). Here we show that IκBζ is indispensable for the expression of a subset of genes activated in TLR/IL-1R signalling pathways. IκBζ-deficient cells show severe impairment of IL-6 production in response to a variety of TLR ligands as well as IL-1, but not in response to tumour-necrosis factor-α. Endogenous IκBζ specifically associates with the p50 subunit of NF-κB, and is recruited to the NF-κB binding site of the IL-6 promoter on stimulation. Moreover, NF-κB1/p50-deficient mice show responses to TLR/IL-1R ligands similar to those of IκBζ-deficient mice. Endotoxin-induced expression of other genes such as *Il12b* and *Csf2* is also abrogated in IκBζ-deficient macrophages. Given that the lipopolysaccharide-induced transcription of IκBζ occurs earlier than transcription of these genes, some TLR/IL-1R-mediated responses may be regulated in a gene expression process of at least two steps that requires inducible IκBζ.

IκBζ is thought to be induced in response to IL-1 or lipopolysaccharide (LPS) (TLR4 ligand)[4–6]. In addition to IL-1 and LPS, IκBζ messenger RNA was strongly upregulated on stimulation with peptidoglycan (PGN) (TLR2 ligand), bacterial lipoprotein (BLP) (TLR1/TLR2), flagellin (TLR5), MALP-2 (TLR6/TLR2), R-848 (TLR7) and CpG DNA (TLR9), but not with tumour-necrosis factor-α (TNF-α) (Fig. 1a). In contrast, other IκB family members such as IκBα and Bcl-3 were induced in response to TNF-α as well as the TLR ligands and IL-1. Thus, IκBζ is induced in the TLR/IL-1R signalling pathway but not the TNF signalling pathway. Furthermore, IL-1- or LPS-induced expression of IκBζ was completely abolished in *MyD88*$^{-/-}$ embryonic fibroblasts (MEFs; Fig. 1b), showing that IκBζ is inducible in the MyD88-dependent part of the TLR/IL-1R signalling pathway[7–15].

To elucidate the physiological role of IκBζ in the TLR/IL-1R response, we generated *IκBζ*$^{-/-}$ mice by targeted gene disruption (see Supplementary Discussion 1 and Supplementary Fig. 1a–d). *IκBζ*$^{-/-}$ splenocytes showed defective proliferation in response to LPS but not to anti-CD40, IL-4 and anti-IgM (Supplementary Fig. 1e, f), suggesting that the TLR4 response is impaired in *IκBζ*$^{-/-}$ cells. Moreover, although *IκBζ*$^{-/-}$ mice grew normally after birth, some of them started to develop atopic dermatitis-like skin lesions with acanthosis and lichenoid changes at the age of 4–5 weeks (Supplementary Fig. 2a, b). All *IκBζ*$^{-/-}$ mice developed the disease by the age of 10 weeks. Histological analysis of 5-week-old *IκBζ*$^{-/-}$ mice showed pathological changes in the conjunctiva, including a heavy lymphocyte infiltration into the submucosa and loss of goblet cells in the conjunctival epithelium (Supplementary Fig. 2c–f).

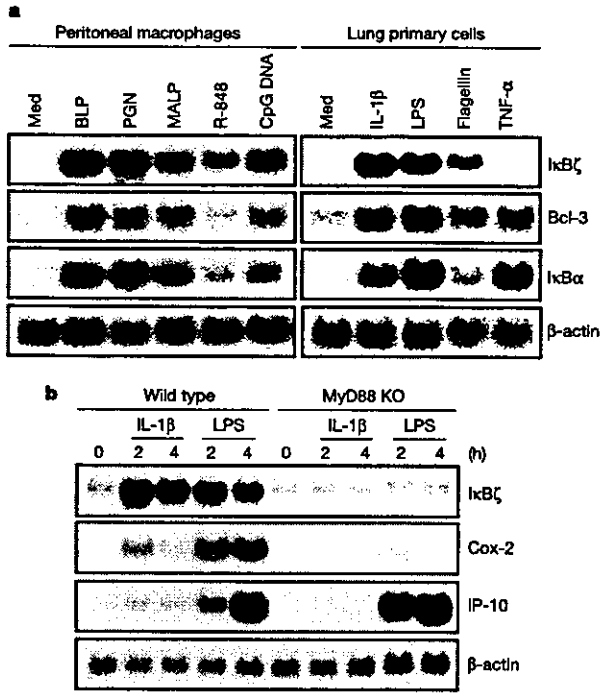

Figure 1 Specific induction of IκBζ on stimulation by TLR/IL-1R ligands. **a**, The indicated cells were stimulated with 100 ng ml^{-1} BLP, 10 μg ml^{-1} PGN, 30 ng ml^{-1} MALP-2, 100 nM R-848, 3 μM CpG DNA, 10 ng ml^{-1} IL-1β, 10 μg ml^{-1} LPS, 100 ng ml^{-1} flagellin and 10 ng ml^{-1} TNF-α for 2 h. Total RNA (10 μg) was extracted and subjected to northern blot analysis for expression of IκBζ, Bcl-3, IκBα and β-actin. Med, medium. **b**, *MyD88*$^{+/+}$ (wild type) and *MyD88*$^{-/-}$ (KO) MEFs were stimulated with 10 ng ml^{-1} IL-1β and 10 μg ml^{-1} LPS for the indicated periods. Total RNA (10 μg) was extracted and subjected to northern blot analysis for expression of IκBζ, Cox-2, IP-10 and β-actin.

We analysed the LPS-induced production of inflammatory mediators in macrophages. $I\kappa B\zeta^{+/+}$ macrophages produced TNF-α, IL-6 and nitric oxide (NO) in response to LPS (Fig. 2a). Although LPS-induced production of TNF-α and NO was normal, production of IL-6 was severely impaired in $I\kappa B\zeta^{-/-}$ macrophages. In addition, production of IL-6 via stimulation by various TLR ligands was also profoundly inhibited in $I\kappa B\zeta^{-/-}$ cells (Fig. 2b–d). Moreover, $I\kappa B\zeta^{-/-}$ cells exhibited defective IL-1-induced IL-6 production; however, IL-1-induced activation of NF-κB and mitogen-activated protein kinases was not impaired in these cells, indicating that there is no defect in the intracellular signalling pathways (Supplementary Fig. 3a, b). On the other hand, TNF-α-induced IL-6 production was not impaired in $I\kappa B\zeta^{-/-}$ cells (Fig. 2e). The impaired production of IL-6 in response to LPS correlated well with the reduced induction of IL-6 mRNA in $I\kappa B\zeta^{-/-}$ cells (Fig. 2f).

When full-length or a deletion mutant form of IκBζ was transfected into MEFs, full-length IκBζ, but not the deletion mutant, rescued the defective production of IL-6 on stimulation with IL-1 in $I\kappa B\zeta^{-/-}$ cells (Fig. 2g), suggesting that expression of IκBζ is required for TLR/IL-1-mediated production of IL-6. As the genes for IκBζ and IL-6 are inducible in response to TLR ligands and IL-1 (refs 4–6, 16, 17), we compared the time course of mRNA expression in macrophages. On stimulation with LPS, induction of IκBζ expression was observed at 30 min and reached maximal levels after 120 min. On the other hand, induction of IL-6 mRNA occurred at later time points compared with IκBζ or TNF-α (Fig. 2h). Taken together, these results indicate that the TLR/IL-1R-mediated expression of the IL-6 gene (*Il6*) requires the preceding induction of IκBζ. Given that IκBζ is also an inducible protein in TLR/IL-1R-mediated signalling pathways, the TLR/IL-1R-mediated production of pro-inflammatory IL-6 may be controlled in at least a two-step fashion.

Our data and those of a previous study[5] indicate the positive role of IκBζ in the TLR/IL-1R-mediated expression of IL-6. To test

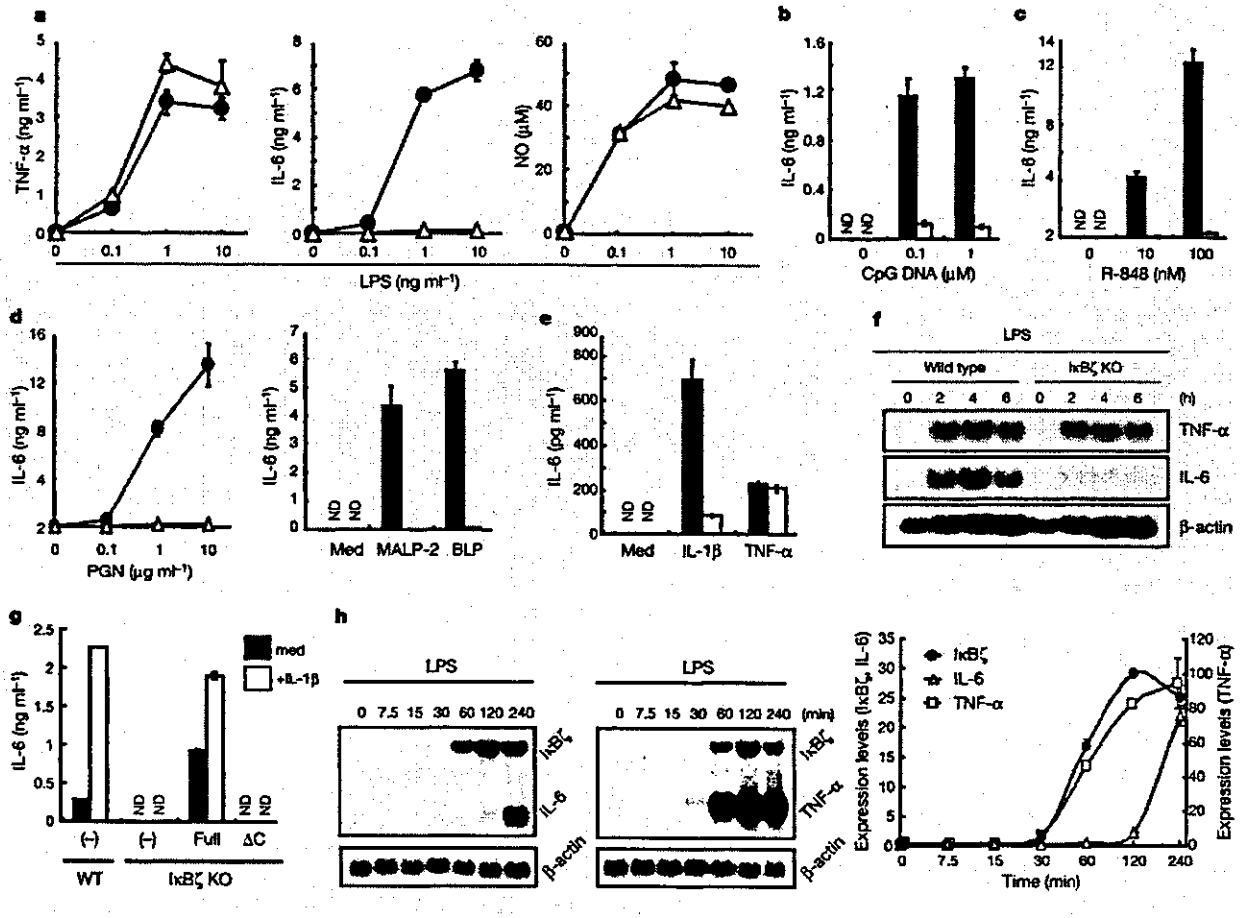

Figure 2 Immune responses in $I\kappa B\zeta^{-/-}$ cells and kinetics of IκBζ induction. **a–d**, $I\kappa B\zeta^{+/+}$ (filled symbols/columns) and $I\kappa B\zeta^{-/-}$ (open symbols/columns) peritoneal macrophages were cultured with 10 ng ml^{-1} LPS, 100 ng ml^{-1} BLP, 30 ng ml^{-1} MALP-2, and the indicated concentrations of PGN, R-848 and CpG DNA in the presence of 30 ng ml^{-1} IFN-γ for 24 h. Values are means ± s.d. of triplicate experiments. ND, not detected. **e**, $I\kappa B\zeta^{+/+}$ (filled columns) and $I\kappa B\zeta^{-/-}$ (open columns) MEFs were stimulated with 10 ng ml^{-1} IL-1β and 10 ng ml^{-1} TNF-α. Values are means ± s.d. of triplicate experiments. **f**, Peritoneal macrophages were stimulated with 10 ng ml^{-1} LPS for the indicated periods. Total RNA (5 μg) was extracted and subjected to northern blot analysis for expression of IL-6, TNF-α and β-actin. **g**, Rescue of IL-1 responsiveness in $I\kappa B\zeta^{-/-}$ MEFs by retroviral transfection with full-length (Full), but not deletion mutant (ΔC), IκBζ. Indicated values are means ± s.d. of triplicate experiments. **h**, Double RNA products indicative of IκBζ, IL-6 and TNF-α mRNA transcripts after LPS simulation of wild-type peritoneal macrophages resolved by electrophoresis. Two independent experiments with independently derived wild-type cells were quantified by PhosphoImager (left and middle panels), and mRNA abundance (right panel) is shown for the indicated genes in arbitrary units (left axis, IκBζ and IL-6; right axis, TNF-α) relative to β-actin. Indicated values are means ± s.d. of duplicate experiments.

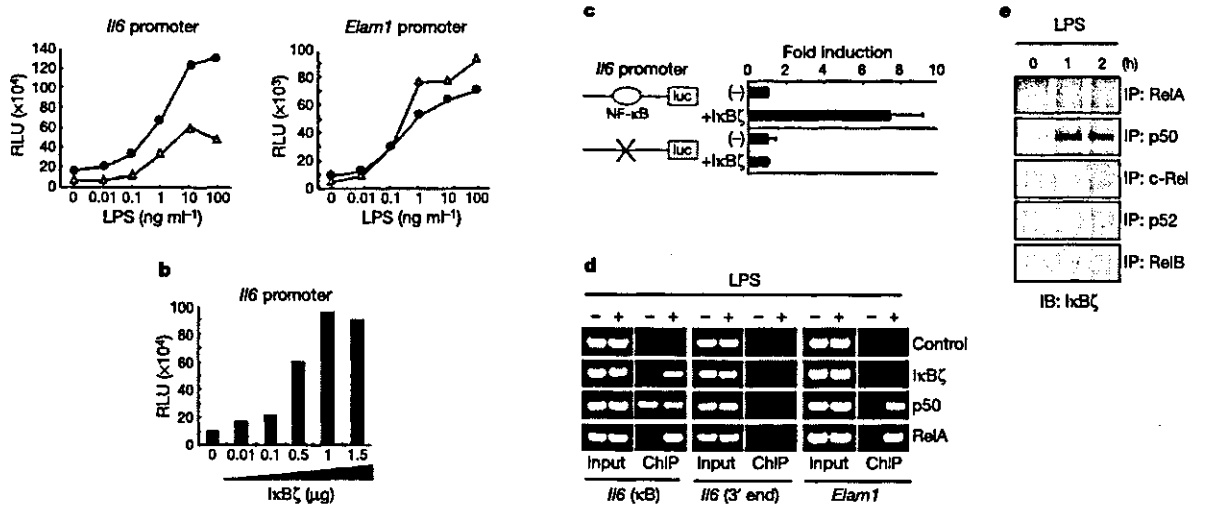

Figure 3 *In vitro* analysis of IκBζ on the *Il6* promoter. **a**, RAW 264.7 cells were transiently transfected with luciferase reporter constructs of either the murine *Il6* promoter or the *Elam1* promoter together with either control (open symbols) or the IκBζ expression plasmid (filled symbols). Luciferase activities are expressed as fold-increase values over the background shown by lysates prepared from untransfected cells. Data are representative of three separate experiments. Cells were stimulated with the indicated concentrations of LPS. RLU, relative luciferase units. **b**, Untreated RAW 264.7 cells were transiently transfected with the IκBζ expression vector together with constant amounts of the *Il6* reporter plasmid. Data are representative of three separate experiments. **c**, P19 cells were transiently transfected with either wild-type or mutant *Il6* promoter reporter constructs together with either control or the IκBζ expression plasmid. Luciferase activities were normalized in each case by dividing the fold-increase values of IκBζ-expressed cells over the background values of lysates with that of mock-expressed cells. Values are means ± s.d. of triplicate experiments. **d**, Chromatin from untreated (−) or LPS-treated (+) (1 μg ml^{-1} for 3 h) RAW 264.7 cells was used for ChIP assays with the indicated antibodies. Precipitated DNA for the *Il6* κB site (left), the 3′ region of the *Il6* gene (centre), or the *Elam1* promoter (right) was assayed by PCR (ChIP). Data are representative of two independent experiments. **e**, Unstimulated or LPS-stimulated (10 ng ml^{-1}) peritoneal macrophages were immunoprecipitated with the indicated antibodies. The immunoprecipitated (IP) lysates were subsequently immunoblotted (IB) with anti-IκBζ.

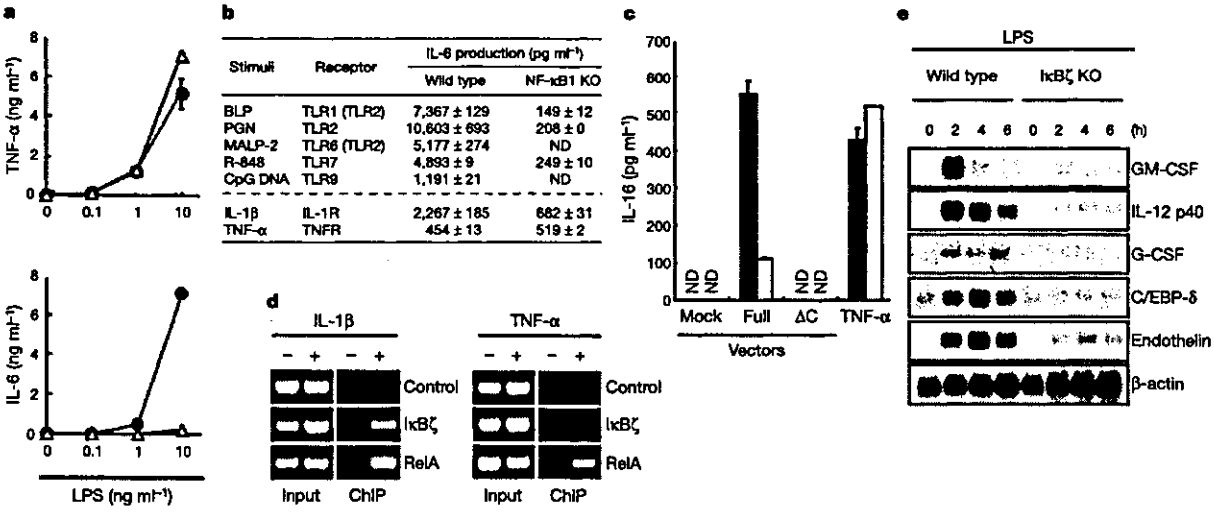

Figure 4 The TLR/IL-1R responses in NF-κB1/p50-deficient cells and microarray analysis of *IκBζ*$^{-/-}$ cells. **a**, *NF-κB1*$^{+/+}$ (filled symbols) and *NF-κB1*$^{-/-}$ (open symbols) peritoneal macrophages were cultured with 10 ng ml^{-1} LPS in the presence of 30 ng ml^{-1} IFN-γ for 24 h. Values are means ± s.d. of triplicate experiments. **b**, *NF-κB1*$^{+/+}$ and *NF-κB1*$^{-/-}$ peritoneal macrophages and MEFs were cultured with 100 ng ml^{-1} BLP, 10 μg ml^{-1} PGN, 30 ng ml^{-1} MALP-2, 100 nM R-848, 3 μM CpG DNA, 10 ng ml^{-1} IL-1β or 10 ng ml^{-1} TNF-α in the presence of 30 ng ml^{-1} IFN-γ for 24 h. IL-1β- and TNF-α-induced IL-6 production were analysed by MEFs. Values are means ± s.d. of triplicate experiments. **c**, *NF-κB1*$^{+/+}$ (filled columns) and *NF-κB1*$^{-/-}$ (open columns) MEFs were retrovirally transfected with either the full-length (Full) or the deletion mutant (ΔC) of IκBζ. Furthermore, the same lines of untransfected cells were stimulated with 10 ng ml^{-1} TNF-α. Values are means ± s.d. of duplicate experiments. **d**, Chromatin from untreated (−), IL-1β-treated (left panel (+); 10 ng ml^{-1} for 3 h) or TNF-α-treated (right panel (+); 10 ng ml^{-1} for 3 h) wild-type MEFs were used for ChIP assays with the indicated antibodies. Precipitated DNA for the input (left) or the *Il6* κB site (right) was assayed by PCR. **e**, *IκBζ*$^{+/+}$ and *IκBζ*$^{-/-}$ peritoneal macrophages were stimulated with 10 ng ml^{-1} LPS for the indicated periods. Total RNA (5 μg) was extracted and subjected to northern blot analysis for expression of the indicated probes. GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

whether IκBζ promotes the ligand-induced activation of the *Il6* promoter, we introduced reporter plasmids containing the promoter of either *Il6* or *Elam1* (also known as *Sele*) into RAW 264.7 cells together with control or IκBζ expression vectors. LPS stimulation activated both the *Il6* and *Elam1* promoters in a dose-dependent manner. Under conditions of IκBζ overexpression, we found that the promoter activities of *Il6*, but not *Elam1*, were further enhanced (Fig. 3a). Ectopic expression of IκBζ alone also upregulated the activity of the *Il6* promoter in unstimulated cells (Fig. 3b). As an NF-κB binding site in the *Il6* promoter has been shown to have an important function in its activation, we transfected P19 cells with either a wild-type reporter or a mutant reporter in which the NF-κB binding site was disrupted[18]. IκBζ overexpression activated the wild-type reporter, but not the mutant NF-κB reporter (Fig. 3c). To probe directly the specific involvement of IκBζ in the κB site of the *Il6* promoter, we performed a chromatin immunoprecipitation assay (ChIP) to investigate the proteins bound to the region. In unstimulated cells, the NF-κB p50 subunit was readily detected in the *Il6* κB site as described previously[19]. On stimulation with LPS, RelA as well as p50 bound to the κB sites of the *Il6* and the *Elam1* promoter, but not the 3′ end of the *Il6* gene. In contrast, we found that IκBζ only bound to the *Il6* κB site, but not the other sites tested, in LPS-stimulated cells (Fig. 3d), demonstrating a specificity of IκBζ for the κB site in the *Il6* promoter. Finally, we addressed association of IκBζ with NF-κB family members. Immunoprecipitation analysis showed that IκBζ proteins interacted with p50, but not with RelA, RelB, c-Rel or the p52 subunit (Fig. 3e). These findings indicate that the positive effects by IκBζ may be exerted through association with the p50 subunit.

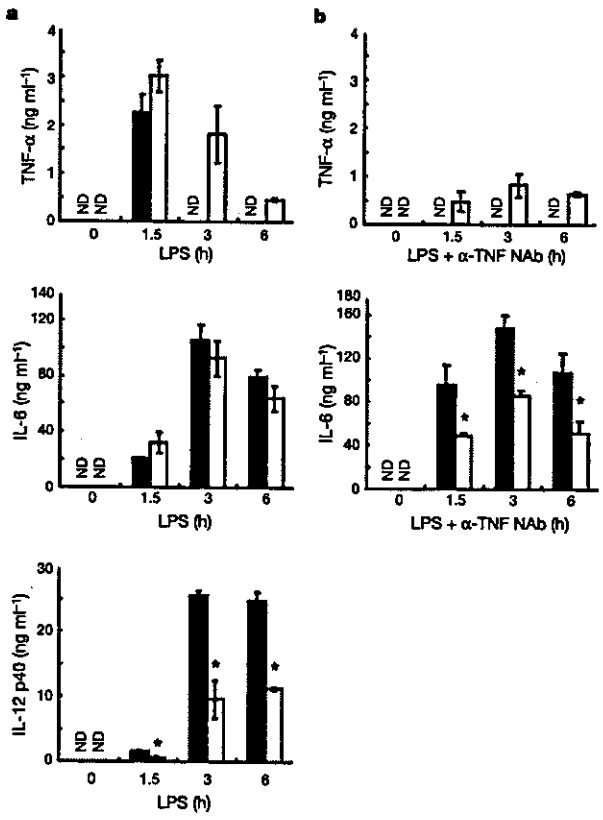

Figure 5 *In vivo* cytokine production in $I\kappa B\zeta^{-/-}$ mice. **a**, **b**, Age-matched $I\kappa B\zeta^{-/-}$ (open columns; $n = 3$ in **a** and 2 in **b**) and $I\kappa B\zeta^{+/+}$ (filled columns; $n = 3$ in **a** and **b**) mice were intraperitoneally injected with 1.5 mg LPS only (**a**), or with 10 μg anti-TNF NAb 1 h before the LPS injection (**b**). Sera were collected at the indicated times. Values are means ± s.d. of sera samples at the indicated times. Asterisk, statistical significance ($P < 0.05$) in a two-tailed Student's *t*-test comparing $I\kappa B\zeta^{+/+}$ and $I\kappa B\zeta^{-/-}$ mice.

The aforementioned results prompted us to study NF-κB1/p50-deficient cells. As previously reported, whereas LPS-induced TNF-α production in *NF-κB1*$^{-/-}$ macrophages is normal, *NF-κB1*$^{-/-}$ macrophages show defective LPS-induced production of IL-6 (refs 20, 21; see also Fig. 4a). Additionally, production of IL-6 in response to stimulation by IL-1 and other TLR ligands was severely impaired in *NF-κB1*$^{-/-}$ cells (Fig. 4b). Thus, the TLR/IL-1R-mediated responses in *NF-κB1*$^{-/-}$ mice are similar to those in $I\kappa B\zeta^{-/-}$ mice. We tested further whether IκBζ overexpression induced IL-6 production in *NF-κB1*$^{-/-}$ cells. Compared with *NF-κB1*$^{+/+}$ cells, IκBζ-mediated production of IL-6 was markedly reduced in *NF-κB1*$^{-/-}$ MEFs, indicating that NF-κB1 is critical for the effect of IκBζ (Fig. 4c). Both IL-1 and TNF-α activate similar sets of signalling molecules such as NF-κB and mitogen-activated protein kinases, and culminate in IL-6 expression. However, signalling mediated by IL-1/TLR ligands, but not TNF-α, specifically recruited IκBζ to the *Il6* promoter (Fig. 4d), presumably accounting for the responsiveness difference between TLR/IL-1R and TNFR. Next, we searched for other LPS-inducible genes regulated by IκBζ using microarray analysis. Dozens of LPS-inducible genes were significantly affected by the IκBζ deficiency (Supplementary Fig. 4a). Several of them were subsequently tested by northern blot analysis for confirmation of accuracy. Among them, LPS-induced expression of granulocyte-macrophage colony-stimulating factor (*Csf2*), IL-12 p40 (*Il12b*), granulocyte colony-stimulating factor (*Csf3*), C/EBP-δ (*Cebpd*) and endothelin 1 (*Edn1*) was compromised in $I\kappa B\zeta^{-/-}$ macrophages (Fig. 4e). Indeed, time course northern blot analysis showed that the kinetics of LPS induction of these genes were similar to that for IL-6 rather than TNF-α (Fig. 2h, Supplementary Fig. 4b, and data not shown), further supporting the model of a two-step regulation of these genes in TLR/IL-1R signalling pathways. Regarding the relationship between the κB sequences of the genes tested and IκBζ requirement, the promoters of IκBζ-regulated genes contained distinct κB sequences. Therefore, it may be difficult to determine whether an arbitrary LPS-inducible gene is IκBζ-dependent through simple sequence comparison of the κB sites (ref. 22; see also Supplementary discussion 2 and Supplementary Fig. 4c–e).

Finally, we examined *in vivo* cytokine production after LPS injection. Although LPS-induced IL-12 p40 production was impaired, IL-6 production was almost normal in $I\kappa B\zeta^{-/-}$ mice. Surprisingly, $I\kappa B\zeta^{-/-}$ mice exhibited more prolonged TNF-α production than $I\kappa B\zeta^{+/+}$ mice (Fig. 5a). As TNF-α is a major IL-6 producer under conditions of endotoxin-induced shock, we next attempted to negate biological activities of TNF-α by prior treatment with anti-TNF-α neutralizing antibodies (anti-TNF NAbs) (ref. 23). In anti-TNF NAb-treated $I\kappa B\zeta^{-/-}$ mice the serum concentration of LPS-induced IL-6 was significantly reduced compared with anti-TNF NAb-treated $I\kappa B\zeta^{+/+}$ mice (Fig. 5b), demonstrating that the prolonged TNF-α production might compensate for impaired IL-6 production in $I\kappa B\zeta^{-/-}$ mice. The prolonged TNF-α production might be secondary to the loss of IκBζ-regulated factors that negatively modulate TNF-α production. Alternatively, IκBζ might act directly as a negative regulator for TNF-α production in certain cells. Although the molecular mechanism of the prolonged TNF-α production remains unknown, such prolonged TNF-α production might lead to development of the skin lesion in aged $I\kappa B\zeta^{-/-}$ mice as demonstrated in TNF-α-mediated inflammatory diseases occurring in other mouse models[24,25].

We provide genetic evidence that IκBζ is essential for TLR/IL-1R-mediated IL-6 production. As IκBζ itself is an inducible protein, TLR/IL-1R-mediated IL-6 expression may be regulated in a two-

step mechanism. Moreover, microarray analysis showed that IκBζ might control LPS-inducible genes other than *Il6*. Further analysis that clarifies the molecular basis of IκBζ-dependent gene expression will provide new insight into the TLR/IL-1R-mediated MyD88-dependent immune responses. □

Methods

Generation of $I\kappa B\zeta^{-/-}$ mice

Genomic DNA containing the *IκBζ* gene was isolated, as described previously[26]. We constructed the targeting vector by replacing a 2.0-kilobase (kb) fragment encoding the central portion of IκBζ with a neo^R cassette that was transfected into embryonic stem cells (E14.1). G418 and gancyclovir doubly resistant colonies were screened by polymerase chain reaction (PCR) and Southern blotting. We micro-injected two independent homologous recombinants into C57BL/6 blastocysts and intercrossed heterozygous F_1 progenies to obtain $I\kappa B\zeta^{-/-}$ mice. Mice from these independent clones displayed identical phenotypes. $I\kappa B\zeta^{-/-}$ mice and their wild-type littermates at the age of 6–12 weeks were used for the current studies. All animal experiments were conducted with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Reagents and mice

LPS, PGN, MALP-2, flagellin, R-848 and CpG oligodeoxynucleotides were prepared as described previously[26]. Polyclonal antibody against IκBζ was obtained by immunizing rabbit with the C-terminal portion of the murine IκBζ protein. We used antibodies against phosphorylated ERK and JNK (Cell signalling), antibodies against ERK, JNK, p38, RelA, p50, c-Rel RelB, p52 (Santa Cruz), antibodies against RelA in ChIP assays (Biomol) and antibodies against murine TNF-α for neutralization (R&D). Recombinant TNF-α and IL-1β were obtained from Genzyme. NF-κB1/p50-deficient mice were as described previously[20].

Measurement of pro-inflammatory cytokine and NO

Thioglycollate-elicited peritoneal macrophages or MEFs were cultured as described previously[26]. Concentrations of TNF-α (Genzyme), IL-6 (R&D) and IL-12 p40 (Genzyme) in the culture supernatant were measured by an enzyme-linked immunosorbent assay according to the manufacturer's instructions. Concentrations of NO were measured by the Griess method according to the manufacturers' instructions (DOJINDO). To measure *in vivo* cytokine concentrations, sera were taken from $I\kappa B\zeta^{+/+}$ or $I\kappa B\zeta^{-/-}$ mice. Anti-TNF NAbs were reconstituted in PBS to 20 μg ml^{-1}, and 10 μg (500 μl) of the reagent was intraperitoneally injected 1 h before LPS injection.

Electrophoretic mobility shift assay

This assay was performed as described previously[26].

Plasmids and retroviral transfection

The reporter plasmids consisted of the 5′ flanking region (−1240/+40) of the murine *Il6* gene, and were used in Fig. 3a, b. The reporter plasmids used in Fig. 3c were as described previously[18,27]. The full-length or the deletion mutant IκBζ, which lacks the C-terminal portion (ΔC; ref. 4), was cloned into pMRX retroviral vector, and the transfection was performed as described previously[28].

Luciferase reporter assay

Reporter plasmids were transiently co-transfected into RAW 264.7 and P19 cells with either the control or IκBζ expression vectors using SUPERFECT transfection reagent (Qiagen). Luciferase activities of total cell lysates were measured using the Dual-luciferase reporter assay system (Promega) as described previously[29].

Chromatin immunoprecipitation assay

The ChIP assay was performed essentially with a described protocol (Upstate Biotechnology). 2×10^6 RAW 264.7 cells or 5×10^5 MEFs were stimulated with LPS (1 μg ml^{-1} for 3 h), IL-1β (10 ng ml^{-1} for 3 h) or TNF-α (10 ng ml^{-1} for 3 h), respectively. Precipitated DNAs were analysed by quantitative PCR (35–40 cycles) using primers 5′-CGATGCTAAACGACGTCACATTGTGCA-3′ and 5′-CTCCAGAGCAGAAT GAGCTACAGACAT-3′ for the κB site in the *Il6* promoter, 5′-GCAGATGGACTTAGC TCGTCTCATTCA-3′ and 5′-CCACTCCTTCTGTGACTCCAGCTTATC-3′ for a 3′ gene segment in the *Il6* promoter and 5′-GATGCAGTTGAGAATTTCCTCTTAGCC-3′ and 5′-TGGAATAGTTGTTCTGGCGTTGGATCC-3′ for the κB site in the *Elam1* promoter.

Western blot analysis and immunoprecipitation

Western blot was performed as described previously[26].

Gene chip analysis

Miroarray analysis (Affimetrix) using $I\kappa B\zeta^{+/+}$ and $I\kappa B\zeta^{-/-}$ peritoneal macrophages was performed as described previously[30]. The colour image for gene expression was generated by GeneSpring6.0 (Silicon Genetics) software.

Histological analysis

Tissues were fixed in 10% phosphate-buffered formalin, and paraffin-embedded tissue sections were stained with haematoxylin and eosin or PAS staining using standard techniques.

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Review Article

Toll-like receptors in innate immunity

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Abstract

Functional characterization of Toll-like receptors (TLRs) has established that innate immunity is a skillful system that detects invasion of microbial pathogens. Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct development of antigen-specific acquired immunity. TLR signaling pathways are finely regulated by TIR domain-containing adaptors, such as MyD88, TIRAP/Mal, TRIF and TRAM. Differential utilization of these TIR domain-containing adaptors provides specificity of individual TLR-mediated signaling pathways. Several mechanisms have been elucidated that negatively control TLR signaling pathways, and thereby prevent overactivation of innate immunity leading to fatal immune disorders. The involvement of TLR-mediated pathways in autoimmune and inflammatory diseases has been proposed. Thus, TLR-mediated activation of innate immunity controls not only host defense against pathogens but also immune disorders.

Introduction

Host defense against invading microbial pathogens is elicited by the immune system, which consists of two components: innate immunity and acquired immunity. Both components of immunity recognize invading microorganisms as non-self, which triggers immune responses to eliminate them. To date, both components have been characterized independently, and the main research interest in the immunology field has been confined to acquired immunity. In acquired immunity, B and T lymphocytes utilize antigen receptors such as immunoglobulins and T cell receptors to recognize non-self. The mechanisms by which these antigen receptors recognize foreign antigens have been intensively analyzed, and the major mechanisms, such as diversity, clonality and memory, have been well characterized. However, these receptors are present only in vertebrates, and accordingly we do not fully understand the mechanism for non-self recognition in less evolved organisms. In addition, the innate immune system in mammals has not been well studied. As a result, although mammalian innate immune cells such as macrophages and dendritic cells are known to be activated by microbial components (non-self) such as lipopolysaccharide (LPS) from Gram-negative bacteria, a receptor responsible for the recognition remained unknown.

At the end of the 20th century, Toll was shown to be an essential receptor for host defense against fungal infection in *Drosophila*, which only has innate immunity (1). One year later, a mammalian homolog of the Toll receptor (now termed TLR4) was shown to induce expression of genes involved in inflammatory responses (2). In addition, a point mutation in the *Tlr4* gene has been identified in a mouse strain that is unresponsive to LPS (3). These studies have made innate immunity a very attractive subject of research, and in recent years there has been rapid progress in our understanding that the innate immune system possesses a skillful system that senses invasion of microbial pathogens by Toll-like receptors (TLRs). Furthermore, activation of innate immunity is a critical step to the development of antigen-specific acquired immunity. In this review, we will describe the mechanisms by which innate immunity is activated through TLRs.

Identification of the TLR family

After the characterization of the first mammalian TLR, TLR4, several proteins that are structurally related to TLR4 were identified and named Toll-like receptors (4). Mammalian TLRs comprise a large family consisting of at least 11 members. TLR1–9 are conserved between the human and mouse. However, although TLR10 is presumably functional in the human, the C-terminal half of the mouse *Tlr10* gene is substituted to an unrelated and non-productive sequence,

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