

7. Future prospects

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

Acknowledgements

We thank M. Hashimoto for excellent secretarial assistance. This work was supported by grants from the Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology, and the Japan Research Foundation for Clinical Pharmacology.

References

- [1] Hashimoto C, Hudson KL, Anderson KV. The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 1988;52:269–79.
- [2] Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA. The dorsoventral regulatory gene cassette *spatzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* 1996;86:973–83.
- [3] Medzhitov R, Preston-Hurtburt P, Janeway Jr CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394–7.
- [4] Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutation in *Tlr4* gene. *Science* 1998;282:2085–8.
- [5] Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci USA* 1998;95:588–93.
- [6] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J Immunol* 1999;162:3749–52.
- [7] Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, et al. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 1999;401:811–5.
- [8] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675–80.
- [9] Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999;11:115–22.
- [10] Takeuchi O, Takeda K, Hoshino K, Adachi O, Ogawa T, Akira S. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int Immunol* 2000;12:113–7.
- [11] Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, et al. Cutting edge: preferentially the *R*-stereoisomer of the Mycoplasma lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. *J Immunol* 2000;164:554–7.
- [12] Hacker H, Vabulas RM, Takeuchi O, Hoshino K, Akira S, Wagner H. Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF)6. *J Exp Med* 2000;192:595–600.
- [13] Schnare M, Holt AC, Takeda K, Akira S, Medzhitov R. Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr Biol* 2000;10:1139–42.
- [14] Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo S, Hoshino K, et al. Small antiviral compounds activate immune cells via TLR7/MyD88-dependent signalling pathway. *Nat Immunol* 2002;3:196–200.
- [15] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor-5. *Nature* 2001;410:1099–103.
- [16] Janssens S, Burns K, Tschopp J, Beyaert R. Regulation of interleukin-1 and lipopolysaccharide-induced NF- κ B activation by alternative splicing of MyD88. *Curr Biol* 2002;12:467–71.
- [17] Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J. Inhibition of IL-1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *J Exp Med* 2003;197:263–8.
- [18] Cao Z, Henzel WJ, Gao X. Irak: a kinase associated with the interleukin-1 receptor. *Science* 1996;271:1128–31.
- [19] Janssens S, Beyaert R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol Cell* 2003;11:293–302.
- [20] Swantek JL, Tsen MF, Cobb MH, Thomas JA. IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. *J Immunol* 2000;164:4301–6.
- [21] Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, et al. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* 2002;416:750–6.
- [22] Li S, Strelow A, Fontana EJ, Wesche H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci USA* 2002;99:5567–72.
- [23] Kobayashi K, Hernandez LD, Galan JE, Janeway Jr CA, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 2002;110:191–202.
- [24] Arch RH, Gedrich RW, Thompson CB. Tumor necrosis factor receptor-associated factors (TRAFs)—a family of adapter proteins that regulates life and death. *Genes Dev* 1998;12:2821–30.
- [25] Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, et al. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 1999;13:1015–24.
- [26] Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, et al. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* 1999;4:353–62.
- [27] Deng L, Wang C, Spencer E, Yang L, Braun A, You J, et al. Activation of the I κ B kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 2000;103:351–61.
- [28] Wang C, Deng L, Hong M, Akkaraju GR, Inoue J-I, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 2001;412:346–51.
- [29] Burns K, Clatworthy J, Martin L, Martinon F, Plumpton C, Maschera B, et al. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat Cell Biol* 2000;2:346–51.
- [30] Bulut Y, Faure E, Thomas L, Equils O, Arditi M. Cooperation of Toll-like receptor 2 and 6 for cellular activation by soluble tuberculosis factor and *Borrelia burgdorferi* outer surface protein A lipoprotein: role of Toll-interacting protein and IL-1 receptor signaling molecules in Toll-like receptor 2 signaling. *J Immunol* 2002;167:987–94.
- [31] Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. *J Biol Chem* 2002;277:7059–65.

- [32] Yu KY, Kwon HJ, Norman DA, Vig E, Goebel MG, Harrington MA. Cutting edge: mouse Pellino-2 modulates IL-1 and lipopolysaccharide signaling. *J Immunol* 2002;169:4075–8.
- [33] Jiang Z, Johnson HJ, Nie H, Qin J, Bird TA, Li X. Pellino 1 is required for interleukin-1 (IL-1)-mediated signaling through its interaction with the IL-1 receptor-associated kinase 4 (IRAK4)-IRAK-tumor necrosis factor receptor-associated factor 6 (TRAF6) complex. *J Biol Chem* 2003;278:10952–6.
- [34] Kawai T, Takeuchi O, Fujita T, Inoue J, Muhradt PF, Sato S, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IRF-3 and the expression of a subset of LPS-inducible genes. *J Immunol* 2001;167:5887–94.
- [35] Doyle SE, Vaidya SA, O'Connell R, Dadgostar H, Dempsey PW, Wu T-T, et al. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 2002;17:251–63.
- [36] Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, et al. TLR4, but not TLR2, mediates IFN- β -induced STAT1 α / β -dependent gene expression in macrophages. *Nat Immunol* 2002;3:392–8.
- [37] Hoshino K, Kaisho T, Iwabe T, Takeuchi O, Akira S. Differential involvement of IFN- β in Toll-like receptor-stimulated dendritic cell activation. *Int Immunol* 2002;14:1225–31.
- [38] Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature* 2001;413:732–8.
- [39] Weaver BK, Kumar KP, Reich NC. Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAFI. *Mol Cell Biol* 1998;18:1359–68.
- [40] Yoneyama M, Suhara W, Fukuhara Y, Fukuda M, Nishida E, Fujita T. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* 1998;17:1087–95.
- [41] Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α / β gene induction. *Immunity* 2000;13:539–48.
- [42] Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003;300:1148–51.
- [43] Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, et al. IKK ϵ and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 2003;4:491–6.
- [44] Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, et al. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF- κ B-dependent gene transcription. *EMBO J* 2000;19:4976–85.
- [45] Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the Toll signaling pathway. *Nat Immunol* 2001;2:835–41.
- [46] Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies C, Mansell AS, Brady G, et al. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* 2001;413:78–83.
- [47] Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN- β promoter in the Toll-like receptor signaling. *J Immunol* 2002;169:6668–72.
- [48] Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon- β induction. *Nat Immunol* 2003;4:161–7.
- [49] Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* 2002;420:329–33.
- [50] Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role of TIRAP/Mal for activation of the signaling cascade shared by TLR2 and TLR4. *Nature* 2002;420:324–9.
- [51] Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 2003;301:640–3.
- [52] Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, et al. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 2003;424:743–8.
- [53] Bin LH, Xu LG, Shu HB. TIRP: a novel TIR domain-containing adapter protein involved in Toll/interleukin-1 receptor signaling. *J Biol Chem* 2003;278:24526–32.

REVIEWS

TOLL-LIKE RECEPTOR SIGNALLING

Shizuo Akira* and Kiyoshi Takeda†

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

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

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

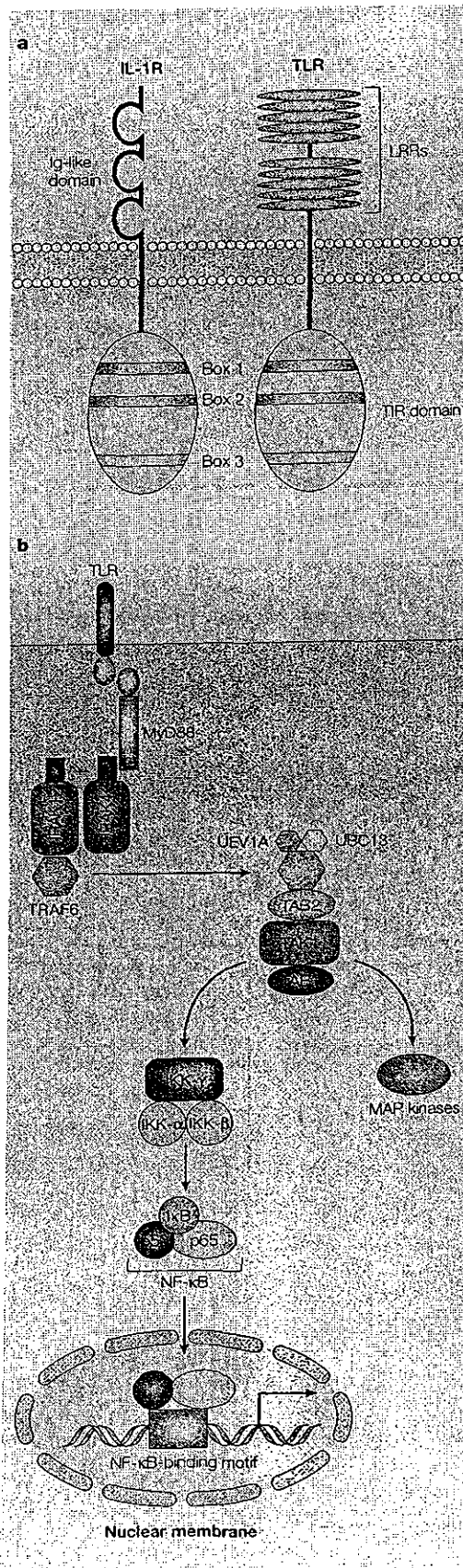
TLR/IL-1R superfamily: structure and function

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

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

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

†Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Correspondence to S.A. e-mail: sakira@biken.osaka-u.ac.jp doi:10.1038/nri1391



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 side⁸. These two secondary structural elements are connected by loops: for example, the BB loop connects the strand β -B and the helix α -B. The conserved boxes 1 and 2 and the BB loop are adjacent and display most of their side chains for interaction with adaptor molecules.

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

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

Figure 1 | TLR structure and signaling. 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 ubiquitination of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor- κ B (I κ B)-kinase complex), which consists of IKK- α , IKK- β and IKK- γ (also known as IKK1, IKK2 and nuclear factor- κ B (NF- κ B) essential modulator, NEMO, respectively). The IKK complex then phosphorylates I κ B, which leads to its ubiquitination and subsequent degradation. This allows NF- κ B 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	<i>Neisseria meningitidis</i>	113
TLR2	Lipoprotein/lipopeptides	Various pathogens	114
	Peptidoglycan	Gram-positive bacteria	115, 116
	Lipoteichoic acid	Gram-positive bacteria	116
	Lipoarabinomannan	Mycobacteria	117
	Phenol-soluble modulim	<i>Staphylococcus epidermidis</i>	118
	Glycosylated phospholipids	<i>Trypanosoma cruzi</i>	119
	Glycolipids	<i>Treponema maltophilum</i>	120
	Porins	<i>Neisseria</i>	121
	Atypical lipopolysaccharide	<i>Leptospira interrogans</i>	122
	Atypical lipopolysaccharide	<i>Porphyromonas gingivatis</i>	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*	<i>Chlamydia pneumoniae</i>	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 sulfate*	Host	134
	Fibrinogen*	Host	135
TLR5	Flagellin	Bacteria	136
TLR6	Diacyl lipopeptides	<i>Mycoplasma</i>	137
	Lipoteichoic acid	Gram-positive bacteria	116
	Zymosan	Fungi	138
TLR7	Imidazoquinoline	Synthetic compounds	139
	Loxoribine	Synthetic compounds	12
	Bropiramine	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^{1,44}. 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 surface¹¹⁻¹³. 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 DNA¹⁴.

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 macrophages¹⁷. 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 complex²¹. 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 mammals²². 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- κ B (NF- κ B). 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- κ B activation²³.

By contrast, the overexpression of IRAK4 does not result in robust NF- κ B activation, yet the expression of a kinase-inactive mutant of IRAK4 inhibits IL-1-mediated NF- κ B activation. It has also been shown that IRAK1 is a direct substrate of IRAK4 but not vice versa²⁴. 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 signalling²⁵. Recently, patients with an inherited IRAK4 deficiency have been identified²⁶. 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 proteins²⁹. 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, TRIGGER) 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, TRIGGER, 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- κ B 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) family³², which has been shown to be essential for both IL-1/LPS- and TNF-induced NF- κ B activation³³. 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 TAK1³⁴. By contrast, TAB2 functions as an adaptor, linking TAK1 to TRAF6 and thereby facilitating TAK1 activation³⁵. However, embryonic fibroblasts obtained from TAB2-deficient mice show no impairment in either IL-1/LPS- or TNF-induced activation of NF- κ B³⁶. 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- κ B³⁷. 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- κ B, 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 ligase³⁸. 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 chain³⁹.

NF- κ B. The NF- κ B 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- κ B dimers are usually sequestered in the cytoplasm in an inactive form by molecules of the inhibitor of NF- κ B (I κ B) family. Activation of NF- κ B involves the phosphorylation and proteolysis of the I κ B proteins and the concomitant release and nuclear translocation of the NF- κ B factors. This acute activation process is mediated by the I κ B 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- κ B essential modulator, NEMO)⁴⁰. After activation by upstream signals, IKK phosphorylates the I κ Bs, leading to their polyubiquitylation and proteasome-mediated degradation.

Recently, an alternative pathway of NF- κ B activation has been suggested, in which NF- κ B-inducing kinase (NIK) activates IKK- α , which then phosphorylates the NF- κ B2 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.

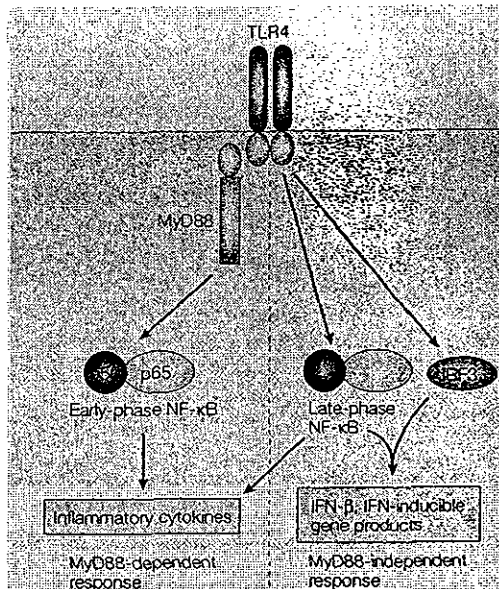


Figure 2 | TLR4 signalling: MyD88-dependent and -independent pathways. Stimulation of Toll-like receptor 4 (TLR4) facilitates the activation of two pathways: the MyD88 (myeloid differentiation primary-response protein 88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early phase of nuclear factor- κ B (NF- κ B) 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- κ B 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- κ B alone might not be sufficient for activation of NF- κ B-dependent transcription. Some NF- κ B 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 process⁴⁰.

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 I κ Bs.

This phosphorylation leads to the degradation of I κ B and consequently the release of NF- κ B. 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 LPS⁴⁵ (FIG. 2). For example, the activation of NF- κ B in response to mycoplasmal lipopeptide, a TLR2 ligand, is completely abolished in MyD88-deficient macrophages, whereas NF- κ B 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 LPS⁴⁶. A number of genes known to be interferon (IFN)-inducible genes were identified, such as glucocorticoid-attenuated 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-1 β , 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 manner⁴⁹. 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 signalling⁵², 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).

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- κ B 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- κ B-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.

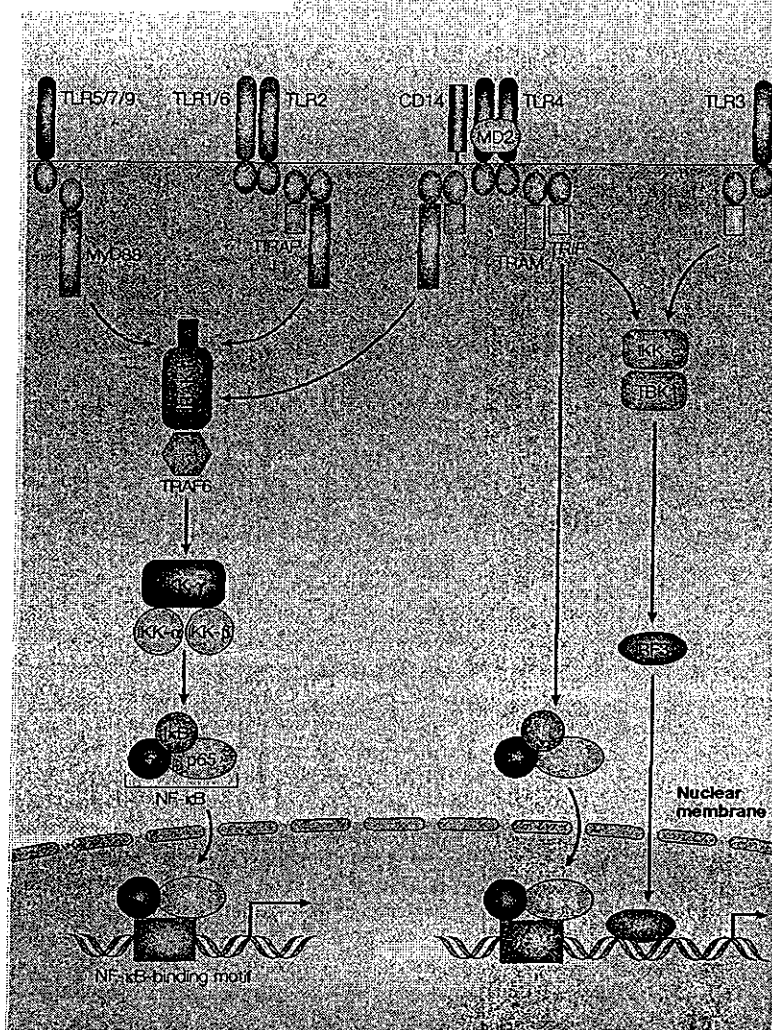


Figure 3 | Involvement of TIR-domain-containing adaptors in TLR-signalling pathways. The Toll/interleukin-1 (IL-1)-receptor (TIR)-domain-containing adaptor molecule MyD88 (myeloid differentiation primary-response protein 88) mediates the Toll-like receptor (TLR)-signalling pathway that activates IRAKs (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- κ B (I κ B)-kinase complex), which consists of IKK- α , IKK- β and IKK- γ (also known as IKK1, IKK2 and nuclear factor- κ B (NF- κ B) essential modulator, NEMO, respectively). This pathway is used by TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 and releases NF- κ B 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- κ B 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.

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, TRIF⁵⁷. The same molecule was also identified as a TLR3-binding molecule in a yeast two-hybrid screen, but in this report, it was called TICAM1⁵⁸. 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 genes⁵⁹. 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 responses⁶⁰. 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- κ B^{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- κ B, 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 stimulation⁶⁰. 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- κ B is normal⁶⁰. 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 TRIF⁶². 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 endotoxemic 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* TLR⁶⁴. 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 IFNs⁶⁶. 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 IFNs⁶⁷. 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 pathway⁶⁸.

In contrast to IRF3, miRNA 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 IFNs⁶⁹.

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 cells⁶⁷.

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 phosphorylation⁷⁰. 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- κ B activator (TANK)-binding kinase 1; also known as NF- κ B-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- α 4* 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 signalling⁷³.

NF- κ B activation. MyD88-deficient mice still activate NF- κ B in response to stimulation with LPS, although the kinetics are delayed compared with wild-type mice. However, the activation of NF- κ B in response to LPS is completely abrogated in mice lacking both MyD88 and TRIF, demonstrating that TRIF is essential for NF- κ B activation through the MyD88-independent pathway⁵⁹. Transcriptional activation of the *IFN- β* gene requires activation of both NF- κ B and IRF3, and in contrast to inflammatory cytokine production — which requires both early- and late-phase NF- κ B activation, mediated by MyD88 and TRIF, respectively — production of IFN- β can be induced by TRIF-mediated late-phase NF- κ B activation alone (FIG. 4).

In vitro analyses showed that both the N-terminal and C-terminal regions of TRIF can independently activate an NF- κ B-responsive promoter, whereas only the N-terminal region is involved in the activation of the IFN- β promoter⁷⁴. The mechanism of NF- κ B 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 TRIF⁷⁴. 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 earlier²¹. 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- κ B 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- κ B 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- κ B. 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- κ B and the *IFN- β* gene, respectively (FIG. 4). Furthermore, TRIF uses at least two pathways for NF- κ B 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- κ B activation is dependent on receptor-interacting protein 1 (RIP1), which associates with the C-terminus of TRIF⁷⁵.

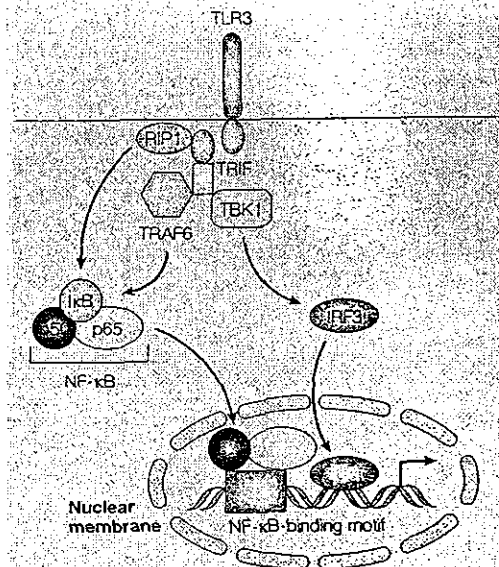


Figure 4 | TRIF-dependent induction of IFN- β . The amino-terminal region of TRIF (Toll/interleukin-1-receptor (TIR)-domain-containing adaptor protein inducing interferon (IFN)- β) interacts with both TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) and TBK1 (TRAF-family-member-associated nuclear factor- κ B (NF- κ B) activator (TANK)-binding kinase 1). TRIF-dependent activation of TBK1 leads to the phosphorylation of IRF3 (IFN-regulatory factor 3), and TRAF6 mediates NF- κ B activation. RIP1 (receptor-interacting protein 1) mediates the NF- κ B activation that is induced through the carboxy-terminal region of TRIF. Activation of both NF- κ B and IRF3 contributes to the activation of the IFN- β gene. I κ B, inhibitor of NF- κ B; 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- κ B activation, and embryonic fibroblasts from RIP1-deficient mice showed impaired TLR3-mediated NF- κ B activation. So, RIP1 probably mediates NF- κ B 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 MAPKKs. 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 protein⁷⁶. 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- κ B 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 IRAKs⁷⁸. Three mammalian homologues of Pellino have since been identified — pellino-1, pellino-2 and pellino-3 — and these show a high degree of evolutionary conservation⁷⁹⁻⁸¹: 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- κ B 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 complex⁷⁹. 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 receptor⁸². 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 complex⁸⁴. MyD88 also interacts directly with AKT, and a dominant-negative mutant of AKT causes a defect in MyD88-dependent NF- κ B transcriptional activity. However, the binding of NF- κ B 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- κ B activity elicited by LPS and IL-1 but not that elicited by TNF⁸⁵. These findings indicate that PI3K is a positive mediator of the signalling induced by LPS and IL-1 that

leads to NF- κ B 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 DCs⁸⁶.

ECSIT. ECSIT has no homology with any known protein and was cloned as a TRAF6-interacting protein by yeast two-hybrid screening⁸⁷. ECSIT interacts with the conserved TRAF domain of TRAF6. A *Drosophila* homologue of ECSIT has been identified,

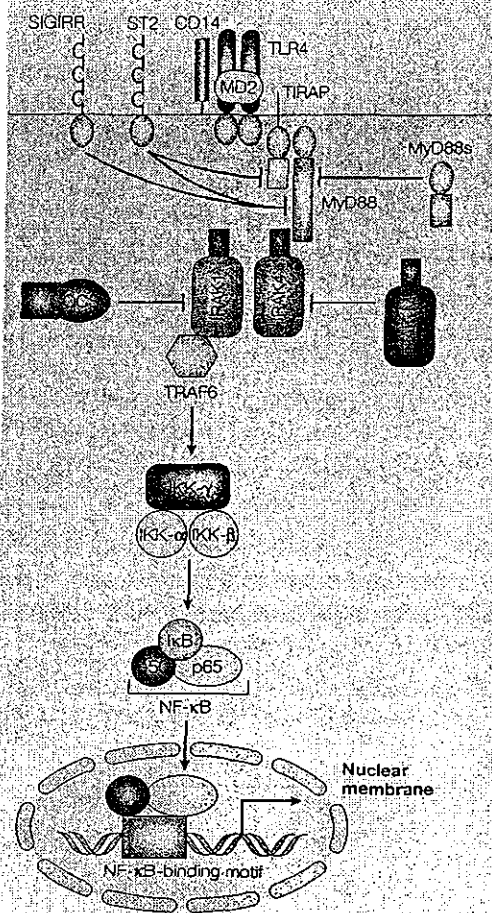


Figure 5 | Negative regulation of TLR signalling. Toll-like receptor (TLR)-signalling pathways are negatively regulated by several molecules that are induced by the stimulation of TLRs. IRAK-M (interleukin-1-receptor (IL-1R)-associated kinase M) inhibits the dissociation of the IRAK1-IRAK4 complex from 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. I κ B, inhibitor of NF- κ B; IKK, I κ B kinase; NF- κ B, nuclear factor- κ B; TIRAP, TIR-domain-containing adaptor protein; TRAF6, tumour-necrosis-factor-receptor-associated factor 6.

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- κ B activation⁸⁸. 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.

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- κ B, 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 signalling⁹⁰. 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- κ B 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- κ B, 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 MCKK family, TPL2 has been shown to be involved in the TLR4-mediated activation of ERK⁹². 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 activity²¹. In response to TLR ligands, IRAK-M-deficient mice show increased production of inflammatory cytokines and defective induction of LPS TOLERANCE²³. 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 pathways²⁴. LPS and CpG-containing DNA have been shown to induce the expression of SOCS1 in macrophages^{25,26}, and SOCS1-deficient mice have been shown to be hypersensitive to LPS-induced endotoxic shock (that is, to show increased production of inflammatory cytokines)^{27,28}. Furthermore, LPS tolerance was not induced in SOCS1-deficient mice and the ectopic introduction of SOCS1 into macrophages inhibited LPS-induced NF- κ B 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- κ B activation because of its inability to bind to IRAK4 and promote IRAK1 phosphorylation⁹⁹.

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 shock¹⁰⁰. 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 tolerance¹⁰¹. Overexpression of ST2 was found to inhibit NF- κ B activation, because ST2 associated with, and probably sequestered, MyD88 and TRAF6. 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, TRAP, 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, SARM⁶³, 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 infections¹⁰², 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 cytoplasm¹⁰⁷. 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.

1. Janeway, C. A. Jr & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
2. Beutler, B. & Rietschel, E. T. Innate immune sensing and its roots: the story of endotoxin. *Nature Rev. Immunol.* **3**, 169–176 (2003).
3. Medzhitov, R. Toll-like receptors and innate immunity. *Nature Rev. Immunol.* **1**, 135–145 (2001).
4. Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunol.* **2**, 675–680 (2001).
5. Hashimoto, C., Hudson, K. L. & Anderson, K. V. The *Toll* gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52**, 269–279 (1988).
6. Lemaître, B., Nicolas, E., Michaut, L., Reichhart, J.-M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983 (1996).
7. Slack, J. L. et al. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. *J. Biol. Chem.* **275**, 4670–4678 (2000).
8. Xu, Y. et al. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. *Nature* **408**, 111–115 (2000).
9. Pottorak, A. et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* **282**, 2085–2088 (1998).
10. Beil, J. K. et al. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* **24**, 528–533 (2003).
11. Ahmad-Nejad, P. et al. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol.* **32**, 1958–1968 (2002).
12. Heif, F. et al. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. *Eur. J. Immunol.* **33**, 2987–2997 (2003).
13. Matsumoto, M. et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J. Immunol.* **171**, 3154–3162 (2003).
14. Latz, E. et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature Immunol.* **5**, 190–198 (2004).
15. Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. *Annu. Rev. Immunol.* **21**, 335–376 (2003).
16. Dunne, A. & O'Neill, L. A. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci. STKE* [online], 2003, re3 (2003).
17. Lord, K. A., Hoffman-Liebermann, B. & Liebermann, D. A. Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL-6. *Oncogene* **5**, 1095–1097 (1990).
18. Muzio, M., Ni, J., Feng, P. & Dixit, V. M. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612–1615 (1997).
19. Wesche, H., Henzel, W. J., Shillington, W., Li, S. & Cao, Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**, 837–847 (1997).
20. Burns, K. et al. MyD88, an adaptor protein involved in interleukin-1 signaling. *J. Biol. Chem.* **273**, 12203–12209 (1998).
21. Dunne, A., Ejdebäck, M., Ludford, P. L., O'Neill, L. A. & Gay, N. J. Structural complementarity of Toll/interleukin-1 receptor domains in Toll-like receptors and the adaptors Mal and MyD88. *J. Biol. Chem.* **278**, 41443–41451 (2003).
22. Janssens, S. & Beyaert, R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol. Cell* **11**, 293–302 (2003).
23. Li, X. et al. Mutant cells that do not respond to interleukin-1 (IL-1) reveal a novel role for IL-1 receptor associated kinase. *Mol. Cell Biol.* **19**, 4643–4652 (1999).
24. Li, S., Strelow, A., Fontana, E. J. & Wesche, H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc. Natl Acad. Sci. USA* **99**, 5567–5572 (2002).
25. Kanakaraj, P. et al. Defective interleukin (IL)-18-mediated natural killer and T helper cell type 1 responses in IL-1 receptor-associated kinase (IRAK)-deficient mice. *J. Exp. Med.* **187**, 2073–2079 (1998).
26. Thomas, J. A. et al. Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase. *J. Immunol.* **163**, 978–984 (1999).
27. Swantek, J. L., Tsen, M. F., Cobb, M. H. & Thomas, J. A. IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. *J. Immunol.* **164**, 4301–4306 (2000).
28. Suzuki, N. et al. Severe impairment of interleukin-1 and Toll-like receptor signaling in mice lacking IRAK-4. *Nature* **416**, 750–756 (2002).
29. Picard, C. et al. Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science* **299**, 2076–2079 (2003).
30. Bradley, J. R. & Pober, J. S. Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* **20**, 6482–6491 (2001).
31. Ye, H. et al. Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* **418**, 443–447 (2002).
32. Yamaguchi, K. et al. Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. *Science* **270**, 2008–2011 (1995).
33. Takaesu, G. et al. TAK1 is critical for IκB kinase-mediated activation of the NF-κB pathway. *J. Mol. Biol.* **326**, 105–115 (2003).
34. Shibuya, H. et al. TAB1: an activator of the TAK1 MAPKKK in TGF-β signal transduction. *Science* **272**, 1179–1182 (1996).
35. Takaesu, G. et al. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol. Cell* **5**, 649–658 (2000).
36. Sanjo, H. et al. TAB2 is essential for prevention of apoptosis in fetal liver but not for interleukin-1 signaling. *Mol. Cell Biol.* **23**, 1231–1238 (2003).
37. Ishitani, T. et al. Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. *EMBO J.* **22**, 6277–6288 (2003).
38. Deng, L. et al. Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**, 351–361 (2000).
39. Wang, C. et al. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **412**, 346–351 (2001).
40. Karin, M. & Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol.* **18**, 621–663 (2000).
41. Senftleben, U. et al. Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway. *Science* **293**, 1495–1499 (2001).
42. DeJardin, E. et al. The lymphotxin-β receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity* **17**, 525–535 (2002).
43. Amir, R. E., Haecker, H., Karin, M. & Ciechanover, A. Mechanism of processing of the NF-κB2 p100 precursor: identification of the specific polyubiquitin chain-anchoring lysine residue and analysis of the role of NEDD8-modification on the SCF (β-TrCP) ubiquitin ligase. *Oncogene* **23**, 2540–2547 (2004).
44. Adachi, O. et al. Targeted disruption of the MyD88 gene resulted in impaired IL-1- and IL-18-mediated function. *Immunity* **9**, 143–150 (1998).
45. Kawai, T., Adachi, O., Ogawa, T., Takeda, K. & Akira, S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**, 115–122 (1999).
46. Provides the first evidence for the existence of the MyD88-independent pathway.
47. Kawai, T. et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J. Immunol.* **167**, 5887–5894 (2001).
48. Hoshino, K., Kaisho, T., Iwabe, T., Takeuchi, O. & Akira, S. Differential involvement of IFN-β in Toll-like receptor-stimulated dendritic cell activation. *Int. Immunol.* **14**, 1225–1231 (2002).
49. Toshchakov, V. et al. TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. *Nature Immunol.* **3**, 392–398 (2002).
50. Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K. & Akira, S. Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* **166**, 5688–5694 (2001).
51. Yamamoto, M. et al. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* **301**, 640–643 (2003).
52. Hoebe, K. et al. Identification of *Lps2* as a key transducer of MyD88-independent TIR signaling. *Nature* **424**, 743–748 (2003).
53. References 50 and 51 demonstrate that TRIF is an essential adaptor in the TLR3- and TLR4-mediated MyD88-independent pathway.
54. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–738 (2001).
55. Hong, T., Barton, G. M. & Medzhitov, R. TRAP: an adaptor molecule in the Toll signaling pathway. *Nature Immunol.* **2**, 835–841 (2001).
56. Fitzgerald, K. A. et al. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**, 78–83 (2001).
57. Yamamoto, M. et al. Essential role of TRAP/Mal for activation of the signaling cascade shared by TLR2 and TLR4. *Nature* **420**, 324–329 (2002).
58. Hong, T., Barton, G. M., Flavell, R. A. & Medzhitov, R. The adaptor molecule TRAP provides signaling specificity for Toll-like receptors. *Nature* **420**, 329–333 (2002).
59. References 55 and 56 show that TRAP is essential for the TLR2- and TLR4-mediated MyD88-dependent pathway.
60. Yamamoto, M. et al. A novel TIR domain-containing adaptor that preferentially activates the interferon-β promoter. *J. Immunol.* **169**, 6668–6672 (2002).
61. Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. & Seya, T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nature Immunol.* **4**, 161–167 (2003).
62. Bin, L. H., Xu, L. G. & Shu, H. B. TRP, a novel Toll/interleukin-1 receptor (TIR) domain-containing adaptor protein involved in TIR signaling. *J. Biol. Chem.* **278**, 24526–24532 (2003).
63. Yamamoto, M. et al. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nature Immunol.* **4**, 1144–1150 (2003).
64. Fitzgerald, K. A. et al. LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the Toll adaptors TRAM and TRIF. *J. Exp. Med.* **198**, 1043–1055 (2003).
65. Oshiumi, H. et al. TIR-containing adapter molecule (TICAM)-2: a bridging adaptor recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-β. *J. Biol. Chem.* **278**, 49751–49762 (2003).
66. References 60 to 62 show the involvement of the TIR-domain-containing adaptor TRAM in the TLR4-mediated, MyD88-independent/TRIF-dependent pathway.
67. O'Neill, L. A., Fitzgerald, K. A. & Bowie, A. G. The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol.* **24**, 286–290 (2003).
68. Coullault, C. et al. TLR-independent control of innate immunity in *Caenorhabditis elegans* by the TIR domain adaptor protein TIR-1, an ortholog of human SARM. *Nature Immunol.* **5**, 488–494 (2004).
69. Yoneyama, M. et al. Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J.* **17**, 1087–1095 (1998).
70. Taniguchi, T., Ogasawara, K., Takaoka, A. & Tanaka, N. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* **19**, 623–655 (2001).
71. Barnes, B., Lubyova, B. & Pitha, P. M. On the role of IRF in host defense. *J. Interferon Cytokine Res.* **22**, 59–71 (2002).
72. Taniguchi, T. & Takaoka, A. The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr. Opin. Immunol.* **14**, 111–116 (2002).
73. Sato, M. et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity* **13**, 539–548 (2000).
74. Servant, M. J. et al. Identification of the minimal phosphoacceptor site required for *in vivo* activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J. Biol. Chem.* **278**, 9441–9447 (2003).

TOLL-LIKE RECEPTORS: LIGANDS AND SIGNALING

Kiyoshi Takeda and Shizuo Akira

13

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-

Kiyoshi Takeda and Shizuo Akira, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, and SORST of Japan Science and Technology Corporation, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.

The Innate Immune Response to Infection
Ed. by S. H. E. Kaufmann, R. Medzhitov, and S. Gordon
©2004 ASM Press, Washington, D.C.

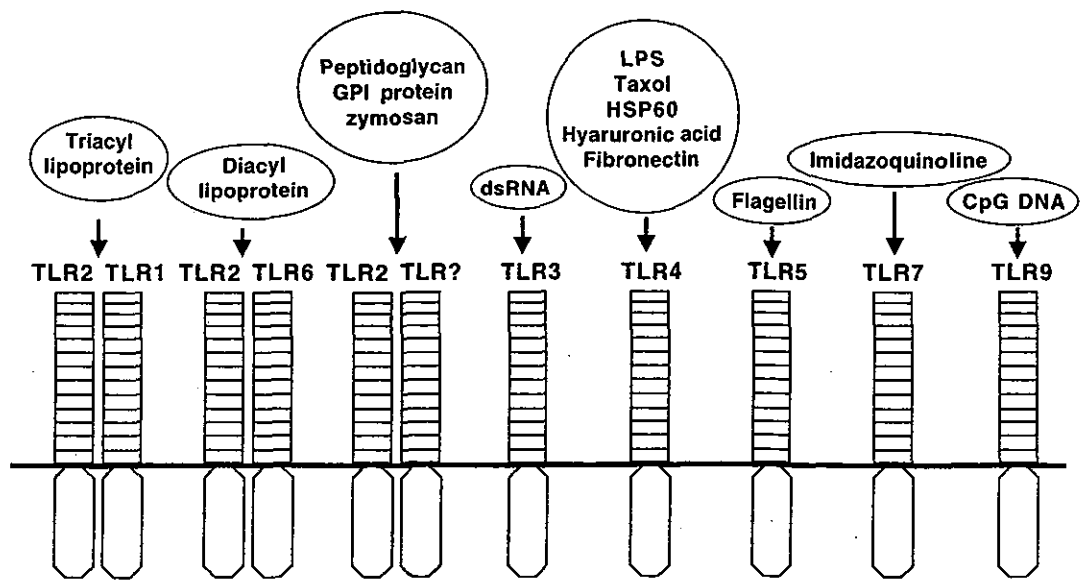


FIGURE 1 TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition, whereas 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 modulins 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- κ B 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).

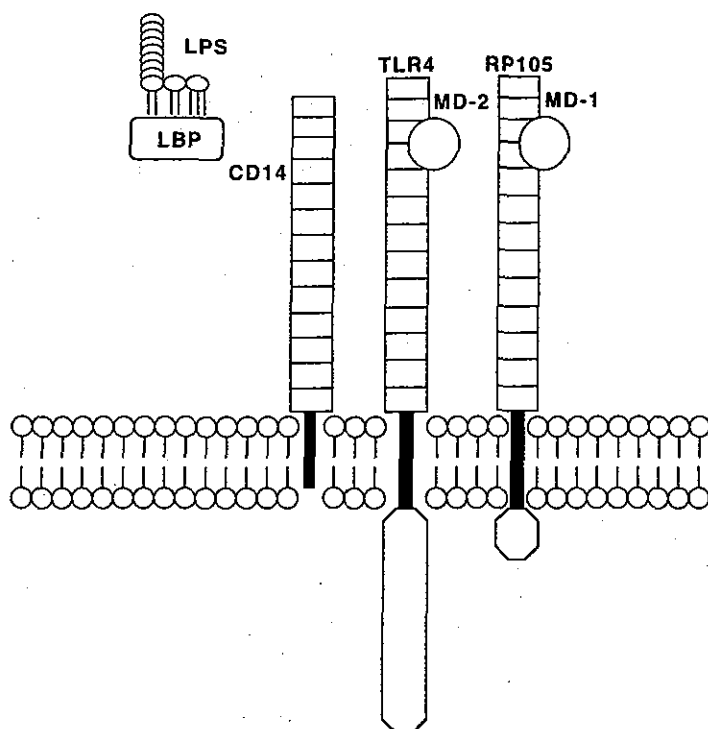


FIGURE 2 The LPS receptor complex. The LPS receptor comprises several components. TLR4 is an essential receptor component for the signal transduction via the LPS receptor complex. MD-2 associates with the extracellular portion of TLR4 and is involved in the LPS recognition. LBP is a soluble molecule 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