

Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4

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SUMMARY

An understanding of lipopolysaccharide (LPS) signal transduction is a key goal in the effort to provide a molecular basis for the lethal effect of LPS during septic shock and point the way to novel therapies. Rapid progress in this field during the last 6 years has resulted in the discovery of not only the receptor for LPS – Toll-like receptor 4 (TLR4) – but also in a better appreciation of the complexity of the signalling pathways activated by LPS. Soon after the discovery of TLR4, the formation of a receptor complex in response to LPS, consisting of dimerized TLR4 and MD-2, was described. Intracellular events following the formation of this receptor complex depend on different sets of adapters. An early response, which is dependent on MyD88 and MyD88-like adapter (Mal), leads to the activation of nuclear factor- κ B (NF- κ B). A later response to LPS makes use of TIR-domain-containing adapter-inducing interferon- β (TRIF) and TRIF-related adapter molecule (TRAM), and leads to the late activation of NF- κ B and IRF3, and to the induction of cytokines, chemokines, and other transcription factors. As LPS signal transduction is an area of intense research and rapid progress, this review is intended to sum up our present understanding of the events following LPS binding to TLR4, and we also attempt to create a model of the signalling pathways activated by LPS.

Keywords CD14; LPS; Mal; MyD88; TLR4

INTRODUCTION

Lipopolysaccharides (LPS) are major components of the outer membrane of Gram-negative bacteria, which makes them prime targets for recognition by the immune system. LPS produced by bacteria such as *Staphylococcus aureus* are toxic to humans and responsible for the dangerously lowered blood pressure that occurs with septic shock. The host defence response to LPS includes expression of a variety of pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and interferon- β (IFN- β), and also other pro-inflammatory proteins such as inducible NO synthase (iNOS). The lack of effective therapeutic approaches for septic shock gives rise to the death of many people world-wide. Therefore, research into the molecular basis of LPS signal transduction is an area of great interest.

LPS-BINDING PROTEIN AND CD14

Probably the first host protein involved in LPS recognition is LPS-binding protein (LBP).¹ LBP is an acute-phase protein, produced in the liver, which circulates in the bloodstream where it recognizes and forms a high-affinity complex with the lipid A moiety of LPS, as free molecules, fragments, or still bound to the outer membrane of intact bacteria. The primary sequence of LBP shows sequence identity with another binding protein found in granulocytes – bactericidal/permeability-increasing protein – and also with a cholesterol ester transport protein of the plasma.¹ The role of LBP appears to be that of aiding LPS to dock at the LPS receptor complex by initially binding LPS and then forming a ternary complex with CD14, thus enabling LPS to be transferred to the LPS receptor complex composed of Toll-like receptor-4 (TLR4) and MD-2.^{2,3} CD14 is found in two forms. The first is soluble CD14 (sCD14), which occurs in plasma where it helps to convey LPS signalling in cells lacking membrane-bound CD14, e.g. endothelial and epithelial cells. The second, more extensively studied form of CD14, is membrane bound (mCD14), attached to the surface of myeloid cells via a glycosylphosphatidylinositol tail,^{4–7} enabling CD14 to be membrane

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proximal despite lacking a transmembrane domain. CD14 was initially thought to be the long sought after receptor for LPS, as antibodies to CD14 abrogated the binding of LPS/LBP.⁷ Because CD14 anchors without a transmembrane domain it was always unlikely that CD14 alone could convey a signal in response to LPS. Subsequent studies have since revealed that the actual receptor for LPS is TLR4.⁸ Since this discovery, studies on CD14 have focused on the role of CD14 in TLR4 signalling, which appears to be that of binding LPS and subsequently presenting it to MD-2 and TLR4. Furthermore, CD14 has been shown to be important in TLR2 signalling, whereby CD14 acts as a membrane receptor for bacterial products other than LPS (such as peptidoglycan and lipoarabinomannan), presenting these to TLR2.⁹

The regions of CD14 that are essential for binding of LPS, transfer to MD-2/TLR4 and subsequent optimal signalling by TLR4, have been mapped by Muroi *et al.*⁹ and include amino acids 35–44, 144–153, 235–243 and 270–275, although none of these regions is likely to participate in the direct association of CD14 with TLR4. Although Viriyakosol & Kirkland¹⁰ found that the C-terminal portion of human CD14 beyond amino acid 152 was not required by CD14 in its role as a signalling molecule for LPS, this has been disputed by Muroi *et al.*⁹ who claim that the region of amino acids 273–275 of mouse CD14 is essential for TLR4-mediated activation of the transcription factor nuclear factor- κ B (NF- κ B). This discrepancy may be caused by a difference between human and mouse forms of CD14 in terms of requirement of the C-terminal region for CD14 to act as a signalling molecule in TLR4-mediated LPS signalling. Despite all the evidence for CD14 in LPS signalling, studies in CD14 knockout mice demonstrate a significant response of these mice to LPS, suggesting that a CD14-independent loading of LPS onto MD-2/TLR4 exists.^{11,12}

MD-2

The secreted glycoprotein, MD-2, acts as an extracellular adaptor protein in the activation of TLR4 by LPS and is essential for LPS signalling to occur, as a mutant form (C95Y) completely abolishes LPS responses and also because wild-type MD-2 was able to restore LPS responsiveness in TLR4-expressing cells lacking MD-2.^{13,14} MD-2 seems to play a role in ligand recognition by TLR4. Visintin *et al.*¹⁵ demonstrated that LPS does indeed bind to MD-2, which in turn associates with TLR4 via the extracellular leucine-rich repeats of TLR4, thus inducing TLR4 aggregation and signal transduction. Knockout studies in mice have demonstrated that MD-2 is indispensable for LPS responses, because mice lacking MD-2 are unresponsive to LPS.^{16,17} Akashi *et al.*¹⁸ have demonstrated, using immunoprecipitation studies, the direct binding of lipid A to the MD-2/TLR4 complex ($K_d \approx 3$ nM) on the surface of cells expressing CD14. Furthermore, the same group showed that although CD14 is not necessary for the interaction of LPS with soluble MD-2, mCD14 was in fact needed for LPS to associate with the MD-2/TLR4

complex.¹⁸ In this case, a direct interaction of CD14 with the signalling complex could not be demonstrated, thus reinforcing that the main role for CD14 is that of loading LPS onto the MD-2/TLR4 complex, and that the direct binding of CD14 to the receptor complex is of minimal importance for signalling.

E5531 is a potent LPS antagonist that was initially developed for therapeutic intervention in endotoxic shock by neutralizing endotoxins *in vivo*. E5531 was first thought to antagonize LPS signalling by interfering with LPS binding to membrane CD14; however, further studies have now established that the mechanism for the antagonistic effect of E5531 comes from a direct inhibition on the MD-2/TLR4 complex. E5531 also inhibits CD14-independent ligands, but does not inhibit other CD14-dependent microbial products,¹⁸ suggesting that the antagonistic effects of E5531 are independent of CD14.

Originally it was suggested, by Latz *et al.*, that upon binding LPS, TLR4/MD-2/CD14, together with LPS, become internalized and traffick to the Golgi apparatus. The group suggested that if this trafficking did not occur, LPS would fail to activate the cell.¹⁹ However, it has subsequently been suggested that TLR4 resides in the plasma membrane and LPS initiates aggregation of TLR4, which is not dependent on LPS trafficking to the Golgi.²⁰

TLR4

The growing evidence for TLR4 as the signalling receptor for LPS is compelling. TLR4 was, in fact, the first TLR to be described, originally being named human Toll (hToll).²¹ Preliminary investigations suggested the involvement of hToll/TLR4 in innate immunity and, specifically, a key role in this immune response for the intracellular Toll/IL-1R (TIR) domain of this receptor. A fusion protein of TLR4 with CD2 gave rise to a constitutively active version of TLR4, which resulted in the expression of B7.1 (the costimulatory ligand to CD28) and inflammatory cytokines. TLR4 has now been established as the receptor for LPS; however, in addition to this, TLR4 also recognizes lipoteichoic acid (LTA), fibronectin, the fusion protein of respiratory syncytial virus (RSV) and taxol, a plant diterpene structurally unrelated to LPS but exhibiting LPS-mimetic effects on murine cells.^{8,17,22–24} The evidence that TLR4 was the receptor for LPS came about when C3H/HeJ mice, which are insensitive to LPS, were shown to have a single point mutation in the TIR domain of TLR4. Furthermore, another LPS-insensitive mouse strain, C57BL/10ScCr, was shown to have a null mutation in the TLR4 gene.⁸

To date, 13 TLRs have been described in mice and 10 in humans. The key features of these type 1 receptors are the extracellular leucine-rich repeats, a single transmembrane region, and the intracellular TIR domain (which is the domain of the TLRs that share sequence similarity with *Drosophila* Toll.²⁵ The TIR domain of TLRs is shared by the interleukin-1 receptor (IL-1R) family and by the IL-1R-related family of signalling adapter proteins. Together, these three groups of receptors/adapters (with

the defining TIR domain) make up the IL-1R/TLR superfamily (reviewed in ref. 26). Small differences in the extra- and intracellular regions of TLRs give rise to distinct responses to specific microbial products.

Adding to the complexity of LPS signalling, LPS derived from different strains of bacteria appears to be recognized by different receptor clusters, giving rise to different cellular responses.^{27,28} One study²⁹ suggests that the shape of the LPS molecule is critical for the response of a cell to LPS and demonstrates that only LPS with a conically shaped lipid A portion (such as that made by *Escherichia coli*) will act as a ligand for TLR4. Cylindrically shaped lipid A portions of LPS (such as those from several non-enterobacteria, e.g. *Rhodobacter sphaeroides* and *R. capsulatus*), on the other hand, as well as precursors and analogues of toxic lipid A [such as lipid IVa and pentaacyl LPS (pLA)] from *E. coli* LPS will bind and activate TLR2 and may even act as antagonists to TLR4. For example, LPS analogues, such as lipid IVa, seem to act as LPS antagonists in human cells but show agonistic properties in mouse cells. Triantafyllou *et al.*³⁰ have shown distinctive responses to LPS compared to the LPS analogues pLA or 406 with different combinational associations of receptors (pLA and 406 also recruiting CD55, heat shock proteins 70 and 90, and GDF5 to the MD-2/TLR4 complex). The group also demonstrated that less recruitment of TLR4/MD-2 within lipid rafts leads to activation of the mitogen-activated protein kinase (MAPK) cascades without activating NF- κ B. It therefore seems clear that the structure of LPS is essential for the formation of specific TLR receptor clusters in response to diverse bacterial products.

In addition to causing MD-2/TLR4 to homodimerize, LPS also causes several other combinations of signalling proteins to become associated with the receptor complex. As described above, the specific combinations and make-up of these receptor clusters can, to a large extent, be determined by the form of LPS bound to MD-2/TLR4. Proteins found to be associated with LPS/MD-2/TLR4 include heat shock proteins 90 and 70. Furthermore, CD55, as well as CD11/CD18, have been implicated as components of the TLR signalling complex, although, to date, the importance of these signalling proteins remains somewhat uncertain.^{30–33}

WHAT HAPPENS NEXT? ADAPTORS IN TLR4 SIGNALLING

The most extensively studied signalling pathways activated by TLR4 in response to LPS all make use of adaptor proteins in order to operate. These adaptors include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also known as TIRAP), TIR-containing adaptor molecule (TRIF, also known as TICAM-1), and TRIF-related adaptor molecule (TRAM, also known as TICAM-2). From the evidence that is emerging, the TLR4-mediated response to LPS can be divided into two categories: an early MyD88-dependent response; and a delayed MyD88-independent response.

MyD88-dependent signalling

MyD88 is made up of a C-terminal TIR domain which is separated via a short linker from an N-terminal death domain (DD).³⁴ This DD is related to a motif of ≈ 90 amino acids that was originally defined as the region of similarity between the cytoplasmic tails of the FAS and the TNF receptors required for their induction of apoptosis. The DD mediates protein–protein interactions through other DD sequences, a mechanism utilized by many signalling complexes to induce responses such as cytotoxicity, activation of MAPK and activation of transcription factors such as NF- κ B. MyD88 was initially shown to associate with the Type I IL-1R (IL-1RI) through its TIR domain, which subsequently also was shown to occur for TLR4.³⁵ Studies using MyD88^{−/−} mice revealed both MyD88-dependent and MyD88-independent pathways of TLR4 signalling. Activation of NF- κ B and MAPK still occurred in these mice, although in a delayed manner compared to the activation occurring in wild-type mice. In addition, the induction of dendritic cell maturation, the activation of the transcription factor IFN-regulated factor-3 (IRF-3) and the induction of IFN- β in response to LPS were all unaffected in MyD88-deficient mice.³⁶ Despite all of these signalling pathways being intact, albeit delayed, MyD88-deficient mice exhibit resistance to the lethal effects of LPS. Furthermore, MyD88-deficient splenocytes are incapable of proliferation in response to stimulation with LPS.^{8,17,37–39} Taken together, these results point to a role for MyD88 in an early response to LPS.

A second adaptor protein, Mal, was subsequently described⁴⁰ as a novel adaptor in TLR4 signalling. Hopes were raised at the time that it might be the adaptor involved in the MyD88-independent pathway. However, subsequent studies using Mal knockout mice proved instead that Mal was an essential adaptor that works together with MyD88.^{41–43} In a similar manner to MyD88, Mal-deficient mice displayed a resistance to the toxic effects of LPS and did not produce TNF, IL-6 or IL-12p40 in response to LPS. Furthermore, Mal-deficient mice showed a delayed activation of NF- κ B and MAPK in response to LPS, which strengthens the theory that Mal is essential for, and acts together with, MyD88. Supporting this, Mal has been shown to form homodimers and also to heterodimerize with MyD88. Interestingly, Mal has been shown to be differentially involved in signalling by different TLRs and is thought to provide some of the specificity in the downstream events of the different receptors. As shown simultaneously by Yamamoto *et al.*⁴⁴ and Horng *et al.*,⁴² Mal-deficient mice respond normally to ligands to TLR5, TLR7 and TLR9, as well as to IL-1 and IL-18, suggesting that these receptors signal independently from Mal. However, these mice exhibit impaired responses, not just to LPS but also to the ligands for TLR2 (in combination with TLR1 and TLR6).⁴²

A current working model for the early MyD88-mediated signalling pathway in response to LPS is starting to emerge (Fig. 1). LPS binds, with the help of CD14, to MD-2 and TLR4. This induces homodimerization of TLR4, which in

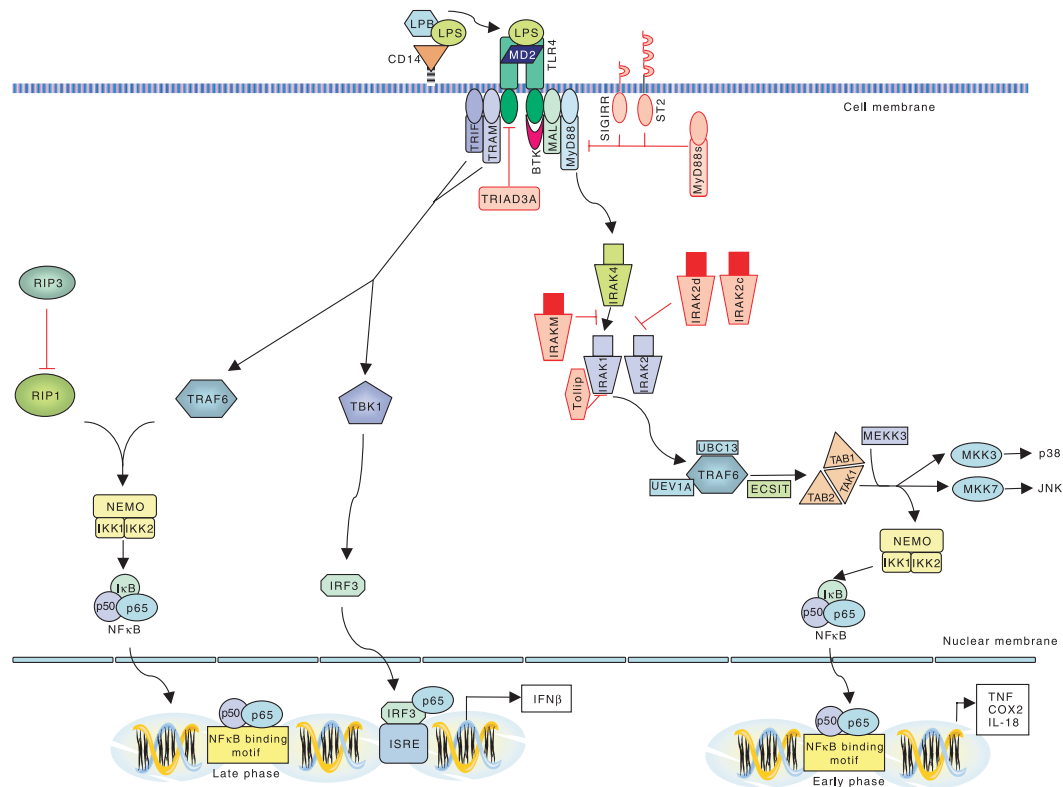


Figure 1. Simplified model of lipopolysaccharide (LPS) signalling (the abbreviations used are defined at the end of the figure legend). Circulating LBP recognizes LPS in the plasma and brings it to CD14. This aids the loading of LPS onto the LPS receptor complex, which is composed of dimerized TLR4 receptors and two molecules of the extracellular adapter MD-2. Subsequent signals activated by TLR4 can be subdivided into those dependent on MyD88 (and Mal), which occur early (represented by the events illustrated on the right hand side of the diagram), and those independent of MyD88, which occur later and use the adapters TRIF and TRAM (depicted on the left). LPS signalling leads to the early activation of NF- κ B, IRF3 and MAPK kinase pathways, which is mediated by the adapters MyD88 and Mal. After the subsequent activation and phosphorylation of IRAK, TRAF6 becomes activated, which gives rise to the expression of numerous pro-inflammatory genes. As a later response to LPS, TLR4 gives rise to the activation of TRAF6 and TBK1, an event mediated by the adapters TRIF and TRAM. Details of these interactions are given in the text. ST2, SIGIRR, MyD88s, IRAK-M, Tollip, IRAK2c and IRAK2d are negative regulators and are shown in red. Abbreviations: IFN, interferon; IKK, I κ B kinase; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon response factor; ISRE, interferon-sensitive response element; LPS, lipopolysaccharide; LBP, LPS-binding protein; Mal, MyD88 adapter like; MAPK, mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase; MyD88, myeloid differentiation marker; NF- κ B, nuclear factor- κ B; RIP, receptor interacting protein; SIGIRR, single immunoglobulin interleukin-1 receptor-related molecule; TBK, TANK-binding kinase; TLR, Toll-like receptor; TRAF6, tumour necrosis factor receptor-associated factor; TRIF, TIR-containing adapter molecule; TRAM, TRIF-related adapter molecule; Tollip, TAB, TAK-1 binding protein; TAK, transforming growth factor- β -activated kinase. Uev1a/Ubc13 are TRAF6 ubiquitin ligases.⁹⁸

turn recruits MyD88 and Mal to the receptor complex. Several studies have been carried out in regard to the amino acids essential for signalling by TLR4 and the interactions between TLR4 and MyD88/Mal. The single, most dramatic effect of a single point mutation is a proline to histidine substitution at amino acid 712 in the TIR domain of TLR4 (also called the *lps^d* mutation).^{8,45} This substitution totally abolishes the ability of the receptor to signal. The crystal structure of the corresponding mutation in TLR2 (Pro681His) has been described and reveals that this mutation resides in a large conserved surface patch where the Pro681His mutation has no effect on the structure of the TIR domain. However, the mutation has major disruptive

effects on signalling, and it has been postulated that the inhibitory effect of the *lps^d* mutation may be caused by the interference in the interaction of the TIR domain of TLR4 with MyD88. However, Dunne *et al.*⁴⁶ demonstrated (using models of the human TIR domains of TLR4, Mal and MyD88 in docking studies) that MyD88 and Mal bind to different regions in TLR2 and TLR4, and that Mal and MyD88 are predicted to interact at a third, non-overlapping site, suggesting a heterotetrameric complex formation. GST pull-downs, as well as co-immunoprecipitation studies, confirmed the prediction from the docking studies, that the *lps^d* mutation does not affect the ability of the TIR domain to bind either MyD88 or Mal. The corresponding

proline → histidine mutations of the BB loops of Mal and MyD88 demonstrated that an association with TLR4 can still take place. This again shows that this specific mutation is of no consequence for the interaction to occur. It is therefore possible that the role of the proline in TLR4 is in homodimerization of the receptors.

Further substitution mutant analysis of a chimeric fusion of CD4/TLR4 by Ronni *et al.*⁴⁷ revealed two important surface patches on the human TLR4 TIR domain, one confirming the importance of the BB loop and another within the DD loop. The study also shows that the C-terminal α -helix is essential for structural integrity, which supports previous findings where a 30 amino acid deletion of the C-terminal of IL-1R caused a null phenotype, and a 42 amino acid deletion of mouse IL-1R disrupted function.^{48,49}

Another ligand utilizing MyD88 is IL-1. The events following the binding of IL-1 to IL-1R binding have been well documented (recently reviewed in ref. 26). After forming a complex with IL-1R/IL-1RAcP, MyD88 associates with IL-1R-associated kinase (IRAK)-1 and IRAK-2. This appears to be true also for the TLR4/MyD88/Mal complex. The IRAK family of proteins have, in recent years, grown to include additional members other than IRAK-1 and IRAK-2.⁵⁰ These include IRAK-M, which was first described as being expressed mainly in cells of monomyeloid origin (thereby the name IRAK-M). In a similar manner to IRAK-2, and in contrast to IRAK-1, IRAK-M showed only minimal autophosphorylation activity.^{51,52} Furthermore, IRAK-M was shown to form heterocomplexes with IRAK-1 together with IRAK-2, and has been postulated to influence the responsiveness of a cell to LPS.

One further IRAK protein has been identified, denoted IRAK-4.⁵³ IRAK-4 is the closest human homologue to *Drosophila* Pelle, the fly equivalent to IRAK, and has been shown to interact with both IRAK-1 and TNF-receptor associated factor (TRAF)-6 in response to IL-1. IRAK-4 differs from the other members of the IRAK family as it is dependent on its kinase activity in order to activate NF- κ B. Furthermore, IRAK-4 is thought to act upstream of IRAK-1, as IRAK-4 can phosphorylate IRAK-1 and because overexpression of dominant-negative IRAK-4 can block IL-1-induced activation of IRAK-1. Co-immunoprecipitation studies have shown how only kinase-inactive IRAK-4 was able to interact with MyD88, and also that the phenotype of IRAK-4 knockout mice⁵⁴ display virtually no TIR signalling response. This can be compared to mice lacking IRAK-1, in which TIR signalling, to some degree, remains intact. The mice lacking IRAK-4 were also shown to be completely resistant to LPS-induced septic shock and showed virtually no cytokine response when injected intraperitoneally with a lethal dose of LPS.⁵⁴ LPS treatment of purified B lymphocytes normally induces these cells to enter the cell cycle; however, IRAK-4^{-/-} B cells were completely defective in this response. In addition, bone marrow-derived macrophages and murine embryonic fibroblasts (MEFs) derived from the same mice were both unresponsive to LPS stimulation, as determined by measuring the levels of IL-6, IL-1 and TNF. Therefore, IRAK-4

appears to be a principal mediator of LPS signalling, on both MyD88-dependent and -independent pathways. Another recent study⁵⁵ further emphasizes the importance of IRAK4, showing that it is required for recruitment of IRAK to the IL-1R complex. However, this group disputes the importance of the kinase activity of IRAK4 in IL-1 signalling.

Downstream events in the activation of the MyD88-dependent pathway by LPS, leading to the activation of NF- κ B and the MAPK pathways, are shared with the well-studied IL-1 pathway. A simplified model of the activation of NF- κ B starts by association of IRAK-1 and IRAK-4 with the receptor complex (as described above). Auto-phosphorylation of IRAK-1 (with Tollip acting as a negative regulator, see below) occurs in two substeps, giving rise to hyperphosphorylated IRAK-1, which causes dissociation from the receptor complex and association of IRAK with TRAF6.⁵⁶ TRAF6 then becomes activated and associated with TAB-2, which activates the MAPK kinase TAK1 (transforming growth factor- β -activated kinase), which is constitutively associated with its adapter protein, TAB1.^{57–59} At this point, TAK-1 acts as a common activator of NF- κ B as well as of the p38 and c-jun N-terminal kinase (JNK) MAPK pathways. The activation of NF- κ B starts by the assembly of a high-molecular-weight protein complex known as the signalosome. This complex is made up of inhibitory-binding protein κ B kinase (IKK) α and IKK β , together with a scaffolding protein named IKK γ (also known as NEMO). Subsequent phosphorylation of a set of inhibitory-binding proteins κ B (I κ B) results in their ubiquitination and degradation, releasing NF- κ B which can then translocate into the nucleus.

Apart from activating NF- κ B, p38 and JNK MAPK, the early MyD88-dependent response to LPS also induces an early activation of IRF3 and induction of IFN- β (see below).

MyD88-independent signalling

Mal and MyD88 had now been shown to both be responsible for conveying the early response to LPS. This meant that the adapter(s) responsible for the delayed MyD88-independent pathway were still missing. The next adapter to be identified was given the name of TRIF,⁴⁴ (or TICAM-1⁶⁰). TRIF was shown to activate NF- κ B when over-expressed, although not as potently as the activation of NF- κ B by MyD88 and Mal. On the other hand, TRIF was a more potent inducer of IFN- β than MyD88 or Mal. Important evidence for the role of TRIF in TLR4 signalling came from Yamamoto *et al.*,⁶¹ who used TRIF-deficient mice to demonstrate the involvement of TRIF in the MyD88-independent pathway in response to LPS. This was shown by measuring the activation of NF- κ B in response to LPS in TRIF^{-/-} mice, which was almost normal, although when the cells were deficient in TRIF as well as MyD88, the NF- κ B response to LPS was totally abolished.⁶¹ Furthermore, TRIF was shown to play a critical role in the activation of IRF3 and induction of IFN- β by LPS. Most importantly, TRIF was shown to be the sole adapter used by TLR3.

TRIF had now been established as a key adapter in the MyD88-independent signalling pathway by TLR4, but because the studies had shown poor direct interaction between TLR4 and TRIF, questions still remained as to what further adapter molecule was involved in this pathway. A candidate adapter for bridging TRIF with TLR4 was identified independently by two different groups.^{62,63} (This adapter was given the names TRAM⁶² and TICAM-2.⁶³) TRAM was shown to activate IRF-3, IRF-7 and NF- κ B, independently of MyD88. TRAM was also shown to be uniquely required by TLR4 in order to signal. This was in contrast to TRIF, which was also required by TLR3.⁶² TRAM structurally resembles Mal and appears to have no signalling role other than acting as a bridge between TLR4 and TRIF. This was suggested because TRAM, on its own, displays little or no signalling activity⁶³ when measuring the activation of NF- κ B and IFN- β promoter activity. Further evidence of the TLR4 specificity of TRAM came when it was shown that TRAM could not interact with TLR2, TLR5, TLR6, TLR7, TLR8 or TLR9, and only weakly or not at all with TLR3. Furthermore, TRAM was also shown to co-immunoprecipitate with IRF3,⁶² and overexpression of TRAM, together with TRIF, caused IRF3 to translocate to the nucleus. Oshiumi *et al.*⁶³ have demonstrated an inhibitory effect of dominant-negative TRIF on the activation of NF- κ B and the induction of IFN- β by TRAM, in contrast to the dominant-negative mutants of MyD88 and Mal, which had no effect on the same responses. TRAM has also been shown to act upstream of TRIF, as TRAM cannot restore a functional induction of IFN- β when overexpressed in TRIF-deficient cells. Studies on mice lacking functional TRAM conclusively showed that TRAM was essential for the MyD88-independent signalling pathway in response to LPS, as they were defective in their response to LPS in a manner similar to that of the TRIF^{-/-} mice.⁶⁴ In these studies, the MyD88-dependent pathway appeared to function normally, whereas the MyD88-independent response to LPS was completely eliminated.

The MyD88-independent pathway is responsible for the later activation of NF- κ B and IRF3. The model for delayed MyD88-independent activation of NF- κ B involves TRIF binding to TRAF6 via several N-terminal TRAF6-binding domains on TRIF.⁶⁵ Subsequent events leading to the activation of NF- κ B may be presumed to follow the model described above, involving the activation of components of the signalosome, followed by the ubiquitination and degradation of I κ B, leading to translocation of NF- κ B to the nucleus. However, recent work has shown that activation of IRF3 appears to be somewhat more complex. IKK ϵ , as well as TANK-binding protein (TBK)-1, act as IRF3 kinases, and TBK-1 and IKK ϵ ⁶⁶ have been shown to bind to TRIF, which has led to the proposed model of a complex of TRIF, TBK-1, IKK ϵ and IRF3, leading to phosphorylation and activation of IRF3. IRF3 can then bind to the interferon-sensitive response element (ISRE), inducing a subset of genes including IFN- β .⁶⁶ TBK-1 has been shown to be particularly important, as TBK-1-deficient cells display no IRF3 activation by LPS and polyI:C. Wietek *et al.*⁶⁷ have recently implicated a role for the NF- κ B subunit p65 in the

activation of IRF3 by LPS. p65 is required for the activation of the ISRE and was also shown to occur in the ISRE-binding complex together with IRF3. Intriguingly, this requirement appears to be specific for the TLR4 signalling pathway and confirms the results from other studies showing a co-operation between the NF- κ B and IRF3 pathways.^{68–70}

OTHER IMPORTANT SIGNALLING COMPONENTS: SARM, TOLLIP, PKR AND BTK

To date, little is known about the role of the fifth TIR-containing adapter, SARM, in TLR signalling. As the name suggests, the protein, apart from a TIR domain, also contains a unique combination of the sterile alpha (SAM) and the HEAT/Armadillo motifs, and is a protein expressed to a high degree in liver and kidney tissue⁷¹ where it shares a promoter region with vitronectin, causing the two genes to be co-expressed. Further studies should provide us with more information on the specific function of this adapter-like protein.

Tollip is another potential adapter protein that may be important in TLR4 signalling.⁷² This protein was shown to reside in a complex with IRAK, and was recruited to the IL-1R complex together with IRAK in response to IL-1. Association with MyD88 then triggers autophosphorylation of IRAK, following which Tollip dissociates from IRAK. Studies have subsequently shown Tollip to block activation of NF- κ B in response to TLR4, suggesting a negative regulatory role for Tollip in TLR4 signalling.

Another contender for the MyD88-independent branch of signalling pathways induced by LPS is dsRNA-dependent protein kinase (PKR), which has been shown to associate with Mal.⁴³ Cells lacking PKR display a severely defective activation of NF- κ B in response to LPS, suggesting a role for this protein in TLR4 signalling. However, further investigations are needed in order to fully establish the role of PKR in LPS signalling.

Finally, Bruton's tyrosine kinase (Btk) has recently been implicated in TLR4 signalling.⁷³ Btk is a member of the Tec family of protein tyrosine kinases which is expressed in haematopoietic cells and involved in B-cell activation and survival.⁷⁴ It was first identified as the gene involved in human X-linked agammaglobulaemia, and mice expressing a mutated Btk display X-linked immunodeficiency.⁷⁵ Jeffries *et al.*⁷³ have provided evidence of a role for Btk in the activation of NF- κ B by TLR4. Apart from being able to bind directly to TLR4, co-immunoprecipitation studies have also shown an interaction between Btk and MyD88, Mal and IRAK-1, although not with TRAF6. Furthermore, stimulation of the monocytic cell line, THP-1, with LPS resulted in the activation of Btk. In addition, overexpression of a dominant-negative version of Btk had an inhibitory effect on the activation of NF- κ B by LPS. In another study, Horwood and colleagues⁷⁶ demonstrated that monocytes from subjects with XLA did not produce TNF in response to LPS. Taken together, these results point to a clear role for Btk in LPS signalling through TLR4, and further investigations will no doubt

Table 1. A list of proteins involved in lipopolysaccharide (LPS) responses (based on the phenotype of knockout mice)

		References
Mice deficient in the following proteins are resistant to the lethal effects of LPS		
Proteins involved in LPS recognition	MD-2, CD14, TLR4	8, 84, 85
Signalling proteins	MyD88, Mal, TRIF, TRAM, IRAK, IRAK4, PKC ϵ , TYK2	42, 44, 54, 61, 64, 86–89
Other receptors	IFNAR-1	90
Enzymes	iNOS, COX2	91, 92
Cytokines	TNF, IL-1, IFN- β	93–95
Mice deficient in the following proteins are hyper-responsive to LPS		
Receptors	ST2, SIGIRR	79, 96
Signalling proteins	IRAK-M	52
Other	SOCS1	97

COX2, cyclo-oxygenase 2; IFN, interferon; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; IRAK, IL-1 receptor-associated kinase; PKC, protein kinase C; SIGIRR, single immunoglobulin IL-1R-related molecule; SOCS1, suppressor of cytokine signalling-1; ST2; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRIF, TIR-containing adapter molecule; TRAM, TRIF-related adapter molecule; TYK, tyrosine kinase.

shed light on the exact mechanism and consequences of these interactions.

NEGATIVE REGULATION OF TLR4 SIGNALLING

In the past 2 years, a number of proteins which inactivate TLR4 signalling have been identified and described. ST2 and SIGIRR are cell-surface receptors with TIR domains which inhibit TLR4 by sequestering signalling proteins from the pathway.^{77–79} IRAK-M and two splice variants of IRAK2 (termed IRAK2c and IRAK2d) also block TLR4 signalling,⁸⁰ as does a splice variant of MyD88, termed MyD88s.^{81,82} Finally, Traid3 has been identified as a ubiquitin ligase that ubiquitinylates TLR4, leading to its down-regulation and degradation.⁸³ Several of these negative signals have been implicated in the process of LPS tolerance, whereby mice become resistant to the lethal effects of LPS if they are first primed with a non-lethal dose of LPS. The identification of these negative signals attests to the importance of controlling LPS responses. Septic shock may therefore occur as a result of altered negative signals as much as activating ones.

CONCLUSIONS

The number of proteins identified as being involved in TLR4 signalling has grown steadily over the past 3 years. That they are all important can be seen in the phenotype of mice which lack the proteins. Table 1 summarizes several of the proteins identified, to date, which have been knocked out and resulted in an altered phenotype. As stated above, some of the proteins are negative regulators and so mice deficient in these show a potentiated response. For completeness, the table includes downstream effectors of LPS action, as well as proteins directly on the TLR4 signalling pathway. What can be concluded, at this stage, is that the LPS response system is highly controlled and complex. It is somewhat surprising that the system appears to lack

redundancy and has many 'Achilles heels'. The removal (or mutation) of so many individual genes experimentally results in mice that are resistant to the effects of LPS. If the response to LPS is critical for the host to survive infection, one might expect it to be more robust and not so vulnerable as to be ablated with the removal of single proteins. In spite of all the evidence to date, it is clear that we still have major deficiencies in our understanding of the true nature of LPS signalling and how it might be controlled and regulated. The phenotypes tested in knockout mice are crude and it could well be that there are specificities and subtleties in TLR4 signalling which might be cell type or context-dependent. Furthermore, an understanding of the transition from host defence to death during LPS sensing is still far from clear, and how such a transition (or lack thereof) might relate to LPS tolerance is also unclear. In spite of these unresolved questions, the discovery of the set of proteins listed in Table 1 is a major triumph and a credit to the curiosity and commitment of teams of researchers. We have probably reached the end of the beginning in our understanding of how the host responds to LPS during infection.

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