Review

Bench-to-bedside review: Toll-like receptors and their role in septic shock

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Abstract

The Toll-like receptors (TLRs) are essential transmembrane signaling receptors of the innate immune system that alert the host to the presence of a microbial invader. The recent discovery of the TLRs has rapidly expanded our knowledge of molecular events that initiate host–pathogen interactions. These functional attributes of the cellular receptors provide insights into the nature of pattern recognition receptors that activate the human antimicrobial defense systems. The fundamental significance of the TLRs in the generation of systemic inflammation and the pathogenesis of septic shock is reviewed. The potential clinical implications of therapeutic modulation of these recently characterized receptors of innate immunity are also discussed.

Keywords CpG motifs, sepsis, septic shock, Toll-like receptors, Toll receptors

The recent recognition of the TLRs as the principal inducers of the innate immune system is among the most important and fundamental discoveries in microbial pathogenesis in the past decade. This discovery fills an essential gap in our understanding of the molecular events that follow microbial infection and the initial host defense to invasive pathogens. The TLRs are the critical pattern recognition molecules that alert the host to presence of a microbial pathogen [1,2]. The initial engagement of TLRs to specific and highly conserved microbial constituents largely determines the ultimate fate of each host–pathogen interaction. Once an organism breaches the integument or mucous membrane barriers, the innate immune system must recognize this potential threat and orchestrate an appropriate response to surround and eradicate the pathogen.

The remarkable ability of the human immune system to rapidly respond to a myriad of potential microbial invaders remains essential for survival. This fact is amply demonstrated in patients with a variety of inherited or acquired immune deficiencies. The mechanisms that underlie this antimicrobial defense mechanism have fascinated researchers for over a century. This vigorous early warning and microbial clearance mechanism must have been of fundamental evolutionary importance to our early hominid ancestors. The adoption of a predatory lifestyle and the loss of the thick coating of fur that separates *Homo sapiens* from our primate relatives must have produced heavy selection pressure, favoring a highly primed innate immune system because frequent injuries to our thin integument would inevitably occur.

Humans retain one of the most active host immune responses to microbial antigens known in the entire animal kingdom. The price paid for this defense system is a heightened sensitivity to endotoxin that makes humans more susceptible to lipopolysaccharide (LPS)-induced shock than almost all other mammalian species [3]. The essential role that the TLRs play in this early defense system, and the impact of the signaling system of the innate immune response in human septic shock are the focus of this review.

18W = 18-Wheeler; DIF = Dorsal-like immunity factor; DN = dominant-negative; ECSIT = evolutionarily conserved signaling intermediate in Toll pathways; HSP = heat shock protein; IL = interleukin; IL-1R = IL-1 receptor; IL-1R1 = type 1 IL-1 receptor; IRAK = IL-1 receptor-associated kinase; LBP = lipopolysaccharide-binding protein; LPS = lipopolysaccharide; MHC = major histocompatibility complex; MyD88 = myeloid differentiation factor; NF = nuclear factor; PRR = pattern recognition receptor; TIR = Toll/IL-1 receptor; TLR = Toll-like receptor; TNF = tumor necrosis factor.

Table 1

Microbial products implicated in the pathogenesis of sepsis

Mediator products	Host receptor
Lipopolysaccharide	LBP-CD14-TLR4-MD2
Peptidoglycan	CD14-TLR2, CD14-TLR2/6, CD14-TLR2/1, CD14-TLR2/?
Lipopeptides, lipoteichoic acid	CD14-TLR2, CD14-TLR2/6, CD14-TLR2/1, CD14-TLR2/?
Microbial DNA (unmethylated CpG motifs)	TLR9
Bacterial flagella	TLR5
Double-stranded viral RNA	TLR3

LBP, lipopolysaccharide-binding protein; TLR, Toll-like receptor.

The microbial mediators implicated in the pathogenesis of bacterial sepsis

While bacterial endotoxin expressed in Gram-negative bacteria is generally viewed as the principal mediator of Gram-negative septic shock, recent evidence indicates that LPS works in concert with a variety of other microbial mediators that contribute to systemic inflammation [4,5]. The current listing of those microbial mediators implicated in the pathogenesis of septic shock and the principal receptors that recognize them is presented in Table 1.

These mediators have several structural or functional attributes that are essential for microbial survival and pathogenesis. Importantly, these microbial elements are unique to prokaryotes or lower eukaryotes and thus are logical targets for recognition of these microbial invaders by the innate immune system. These mediators work in combination with each other and probably synergize to induce systemic inflammation and contribute to the pathogenesis of septic shock [3,4].

While LPS is the best studied and probably the most important microbial mediator of sepsis, it is sufficient but not necessary to induce shock. The most direct evidence that LPS is not essential for the induction of an inflammatory signal to host phagocytic cells is found with the LPS-deficient *Neisseria meningitidis* strain [6]. Deletion of lipid A synthetic genes in this strain of *N. meningitidis* results in a viable bacterium that lacks LPS in its outer membrane. Lipid A deletion in enteric Gram-negative bacteria such as *Escherichia coli* is a lethal mutation. The LPS-deficient strain of *N. meningitidis* is still capable of inducing inflammatory reactions (although at lower levels) via the TLR2 receptors found on macrophages. This indicates that Gram-negative bacteria have other cell wall components that induce inflammation even in the absence of bacterial LPS [6].

Host response to microbial mediators through the innate immune system

The innate immune system (macrophages, neutrophils, natural killer cells and the alternative complement pathway) has evolved as an early, rapid response system to microbial invasion. The actions against the invading pathogens are either direct (e.g. phagocytosis and killing) or indirect through the release of cytokines or other stimulatory molecules, which trigger the adaptive immune system by activating B cells and T cells.

Janeway [4] and Poltorak *et al.* [7] proposed a central concept in the understanding of the innate immune system: the identification of infectious agents by means of conserved structural features through pattern recognition receptors (PRRs). The components expressed by microbial agents that trigger the immune response are termed pathogen-associated molecular patterns. The discovery of the TLRs of the innate immune system provides the PRRs that detect these pathogen-associated molecular patterns.

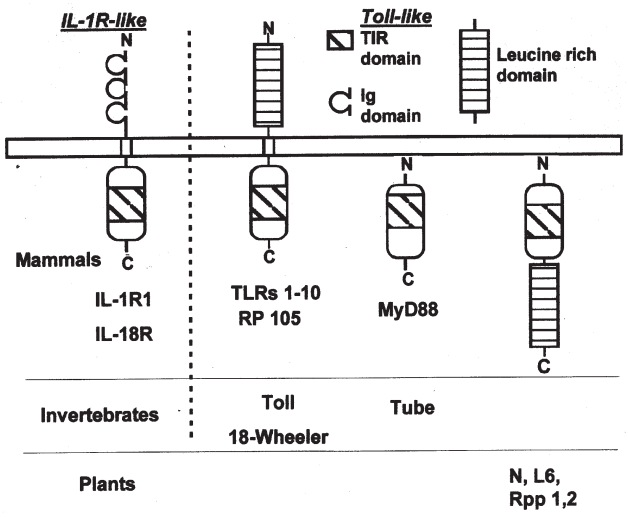
Comparative molecular biology has succeeded in unlocking the mystery of the cellular receptors to endotoxin and a large number of other microbial elements (Table 1). This evolutionarily conserved receptor system has allowed multicellular organisms (including plants as well as invertebrates and vertebrates) to rapidly recognize the presence of microbial invaders. Microbial pathogens represent an immediate threat to survival, and this requires a vigorous and coordinated immune response in defense of the viability of the host [5]. The following section summarizes the major findings that defined the TLR family as the central transmembrane receptor of the innate immune system.

IL-1 receptor/TLR superfamily

The IL-1 receptor (IL-1R)/TLR superfamily members (Fig. 1) are found in many plants and in vertebrate and invertebrate animal species. Those members with known function share the feature of being involved in host responses to injury and infection [8]. Most notably, in humans these include not only the receptor and accessory protein for IL-1, but also the IL-18 receptor and its accessory protein, and the long-sought signaling receptor for LPS, TLR4 [1,8].

Many proteins from diverse systems show homology to the cytoplasmic domain of the type 1 interleukin-1 receptor

Figure 1



The homologies and interconnections between the Toll-like receptors (TLRs) and the type 1 interleukin-1 receptor (IL-1R1) complex. AcP, accessory protein; Ig, immunoglobulin; MyD88, myeloid differentiation factor; R, receptor; TIR, Toll/IL-1 receptor.

(IL-1R1). This expanding IL-1R1-like family includes murine and human proteins, *Drosophila* (fruit fly) proteins, and a plant (tobacco) protein [7,8]. In *Drosophila*, Toll is involved in the rapid and transient transcriptional induction of several genes encoding potent antimicrobial peptides on septic injury [9]. A noteworthy plant member of the IL-1R1 family is the tobacco N gene [10]. The N gene encodes a protein with an amino-terminal domain that has significant homology to Toll and the cytoplasmic domain of the IL-1R1. Introduction of the N gene into tobacco mosaic virus-sensitive strains of tobacco confers resistance to tobacco mosaic virus via the ability to mount a hypersensitive response to the virus at the site of infection within the plant. This system is fundamentally similar to the acute inflammatory response pathway stimulated by injury and mediated by IL-1 in mammals [1].

A growing number of mammalian homologs of IL-1R1 have recently been identified that contain highly conserved regions in their cytosolic domains. Homologous regions were also found in a receptor-like protein of the *Drosophila* fly called Toll. This has resulted in the defining of the IL-1R/TLR superfamily, the conserved region being termed the Toll/IL-1R (TIR) domain [9].

Drosophila Toll members

Toll was originally described in *Drosophila* as a type I transmembrane receptor that controls dorsal–ventral polarity during embryogenesis [11]. Nüesslein-Volhard and Wieschaus discovered the first Toll mutants in fruit fly embryos. Wieschaus noted that the Toll mutant embryos failed to hatch and developed no ventral or lateral cell types. When Nüesslein-Volhard saw the particular embryos lacking the entire mesoderm and nervous system, she exclaimed 'Toll!' (German for jazzy or cool). The new gene was thus given its name.

Four members of the Toll family have been identified in *Drosophila* thus far: Toll, 18-Wheeler (18W), Mst and STSDm2245. Toll and 18W share the greatest similarity to each other and also to the cytoplasmic tail of the mammalian IL-1R1. The *Drosophila* 18W is required for morphogenesis and has many similarities to Toll. The extracellular regions of Toll and 18W contain multiple leucine-rich repeats and carboxyl-terminal cysteine-rich domains [12].

Drosophila Toll signaling pathway

Current understanding of the Toll pathway in *Drosophila* immune response came from studies of promoters of genes induced in response to infection. Insects respond to infection with antimicrobial peptides produced by the fat body and hemocytes. All of the antimicrobial peptide genes include NF-κB or Rel proteins in their upstream regions. The first insect protein discovered to regulate transcription through NF-κB sites was Dorsal. Subsequent genetic studies further identified Spätzle, Toll, Pelle, Tube and Cactus as necessary partners to induce Drosomycin, an antifungal *Drosophila* peptide, in response to fungal infection (Fig. 1) [9].

It has been demonstrated by genetic complementation tests that Spätzle is the Toll ligand. Spätzle is endogenously secreted as an inactive precursor molecule. Protease Easter creates an active form through proteolysis into the biologically active carboxyl-terminal polypeptide fragment [13]. Binding of Spätzle to Toll activates the receptor by ligand-dependent receptor dimerization. This is of interest because there might be an endogenous ligand for the mammalian homologs of Toll. Activated Toll recruits the adapter protein known as Tube and a protein kinase referred to as Pelle to the intracellular part of the Toll protein [9]. No true homolog to Tube has yet been defined in mammals, but the myeloid differentiation factor MyD88 appears to have a similar function.

The mammalian IL-1R-associated kinase (IRAK) appears to be a homolog to *Drosophila* Pelle, which interacts with another protein, the *Drosophila* homolog of mammalian TRAF6 (dTRAF6). The close relationship within Toll-related signaling also becomes evident from the fact that MyD88, IRAK and TRAF6 participate not only in Toll or TLR signaling, but also in IL-1 and IL-18 signaling, leading to the activation of NF-κB [16].

The next downstream step in the signaling cascade is a direct association of the *Drosophila* homolog of TRAF6 with the *Drosophila* homolog to evolutionarily conserved signaling intermediate in Toll pathways (ECSIT). Transfection of dECSIT in insect cells leads to production of diptericin, attacin and defensin (antimicrobial peptides) [15].

The final members of the signal transduction family in *Drosophila* are Dorsal and Dorsal-like immunity factor (DIF). These are released from Cactus, which is a cytoplasmic anchoring protein and the *Drosophila* homolog to I-κB; Dorsal and DIF are the *Drosophila* NF-κB homologs [9].

After release from Cactus, Dorsal and DIF translocate into the nucleus and induce gene transcription. There is also evidence for a *Drosophila* homolog to the mammalian I-κB kinase *Drosophila* LPS-activated kinase, which phosphorylates Cactus. In transfection models, *Drosophila* LPS-activated kinase-deficient cell lines fail to respond to LPS stimulation [12].

The role of the Toll receptor in the adult fly is the induction of immune response in fungal infection. Toll-deficient flies fail to express Drosomycin. In infection models, Toll-mutant flies died from overwhelming growth of *Aspergillus fumigatus* [16].

The immune response of Toll-deficient flies is not altered against bacteria, suggesting a different receptor and a different pathway. Williams *et al.* demonstrated that 18W-deficient flies have an increased mortality from *E. coli* infections [17]. This might be due to a reduced expression of the antibacterial *Drosophila* protein attacin.

Another clue to the pathogen specificity is that antifungal and antibacterial signaling is also divergent further downstream: 18W-deficient flies in bacterial infection have a reduced translocation of DIF into the nucleus, whereas Dorsal translocation is unaffected [18]. Toll, however, does not require DIF for intracellular signaling. This suggests that there might be a selective immune response to particular microbial pathogens.

Homologies between *Drosophila* Toll and the TLR family

All members of the Toll family are membrane proteins that cross the membrane once and share similar extracellular domains [14]. Typical examples are 18–31 leucine-rich repeats. The extracellular domain of human TLR4 contains 22 copies of the leucine-rich repeats. The extracellular domains are very divergent among the different members: TLR2 and TLR4 share only 24% of identical sequences. This makes it probable that they bind different ligands.

The TLRs of different species are very different: mouse TLR4 and human TLR4 are only 53% identical. Genetic studies of leucine-rich repeat structures among different individuals also revealed that polymorphisms are responsible for a different reaction on microbial challenge. The intracellular part contains a cytoplasmic domain of approximately 200 amino acids that is evolutionarily conserved. This highly conserved region is known as the TIR domain [14].

Mammalian Toll homologs

The mammalian homologs of *Drosophila* Toll are termed TLR proteins and have been most intensively studied [14,17]. To date, 10 human TLRs (TLR1–TLR9) have been described and their structures have been published [19–22]. TLR1–TLR6 have been characterized by their distinctive expression patterns with mRNA detection assays.

TLR1 is expressed ubiquitously and at rather high levels. TLR2 has a particular strong expression pattern in peripheral blood mononuclear cells, but is also expressed in lymphoid tissue [23]. TLR3 mRNA is expressed in the lung, muscle, the heart, the brain and intestinal cells. Alternative splicing variants have been reported from the pancreas and the placenta. Among peripheral blood cells, TLR3 is selectively expressed in specific subsets of dendritic cells [24]. TLR4 is expressed in lymphocytes, the spleen and the heart. TLR5 mRNA is detectable in peripheral blood monocytes, leukocytes, the ovary and the prostate. TLR6 expression was located in the spleen, the thymus, the ovary and the lung [21].

The fact that TLR mRNA is also expressed in cells of epithelial surfaces like intestinal epithelial cells [25] suggests that they act as sentinels for invading microbes. Among these human TLRs, the microbial ligands for TLR2, TLR4, TLR9 and, most recently, for TLR5, and perhaps TLR3, have been identified (see following sections).

Understanding the regulation of TLR expression will provide further insight into their tissue distribution and their role in combating infection. There has been an initial report of the TLR4 promoter region. The upstream region shares similarities with many myeloid-specific genes: it contains purine-rich sequences, recognized through transcription factors of the Ets family. Through supershift analysis, the Ets member PU.1 was shown to have affinity to the promoter region [26]. In another report, IL-4 was shown to downregulate TLR expression. The T helper 2-type adaptive immune response may thus inhibit TLR activation [27].

LPS tolerance is a phenomenon where pre-exposure to LPS induces a reduced sensitivity to a subsequent challenge of LPS [28]. *In vivo*, this phenomenon is related to a decreased febrile response and to a diminished response to subsequent severe infection. LPS tolerance, also termed LPS refractoriness, might be related to differential regulation of the TLR family [28,29]. There are contradictory reports about the regulation of TLR mRNA and surface TLR protein on stimulation with its ligands. Nomura *et al.* [28] reported that LPS stimulation leads to a decrease in TLR4 mRNA levels and surface expression of the TLR4–MD2 complex in mouse macrophages. The LPS pretreated monocytes also secreted less proinflammatory cytokines. Other reports show an increased amount of intracellular TLR mRNA on stimulation with LPS [29,30].

These contradictory findings may be related to differences in experimental conditions used in each laboratory. The potential impact of endotoxin tolerance (or reprogramming) in human

sepsis is of critical importance. The differential expression of TLRs in patients over the course of different stages of early and late severe sepsis is a priority in current sepsis research.

TLR signaling and homology with the IL-1R

As already mentioned, the intracellular part of members of the TLR family has a high degree of homology to the intracellular domain of the IL-1R. This remarkable level of homology suggests similar intracellular signaling pathways (see Fig. 2) [19,31].

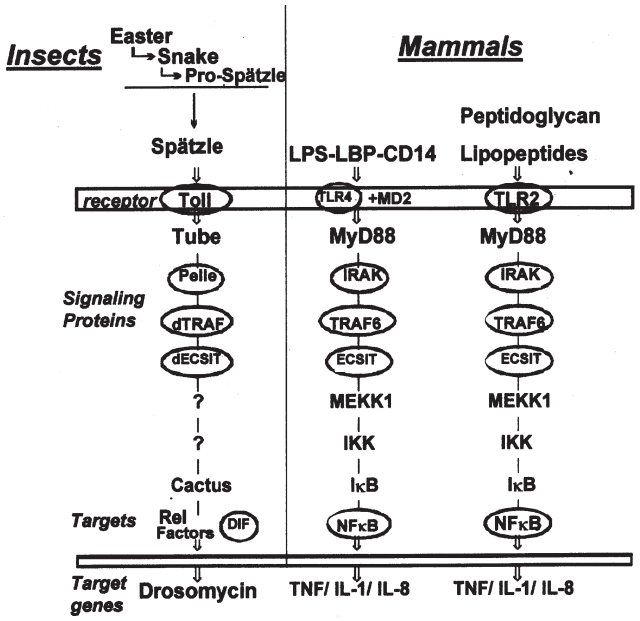
Postreceptor signaling of the IL-1R is well understood. Binding of the ligand causes receptor dimerization and, with involvement of IL-1RAP (an accessory protein), the intracellular part of the receptor forms a complex of MyD88 (an adapter protein) and IRAK (a kinase). IRAK then phosphorylates TRAF6, which leads to NF-κB-inducing kinase and I-κB kinase activation. The I-κB kinase phosphorylates I-κB, which then dissociates from NF-κB [14]. NF-κB translocates to the nucleus and initiates gene transcription. When human homologs to the *Drosophila* Toll were discovered, there was great interest regarding their role in innate immunity. Medzhitov *et al.* reported NF-κB activation as well as cytokine release through TLR4 in transfected monocytic cell lines, which were able to express TLR4 constitutively at a high level [32].

TLR2 and TLR4 have so far been the most intensively studied members of mammalian homologs to *Drosophila* Toll. Currently recognized ligands for TLR4 are presented in Table 2, and the known ligands for TLR2 are presented in Table 3. Both TLR2 and TLR4 require the adapter protein MyD88 for signaling, and immunoprecipitation studies showed direct interaction of MyD88 and IRAK [32]. MyD88 was originally isolated and characterized as a myeloid differentiation primary response gene. MyD88 itself consists of a carboxyl-terminal TIR domain, making it a member of the TLR family. IRAK has been shown both to interact with both MyD88 and TRAF6 [20].

Further evidence for the role of TRAF6 and NF-κB-inducing kinase in TLR4 signaling came from the study of TRAF6 and NF-κB-inducing kinase dominant-negative (DN) variants, which could not activate NF-κB [33]. Another very interesting observation came from this study; activation of the c-Jun N-terminal kinase activation through TLR4 is prevented by the MyD88-DN variant, but not by the TRAF6-DN variant. This indicates an alternative signaling pathway that diverges at the MyD88 level.

Two novel members of the IL-1R/TLR intracellular complex have recently been reported: Tollip, and the TIR domain-containing adapter protein TIRAP. Tollip is present in a complex with IRAK and, during activation of the IL-1R/TLR complex, Tollip associates with IL-1RAcP. IRAK autophosphorylation is then triggered by MyD88, which leads to rapid dissociation of IRAK from Tollip and the receptor complex [34].

Figure 2



Comparisons between the signaling pathways in the insect and mammalian IL-1/Toll-like receptor (TLR) pathways. (d), *Drosophilia* homolog; ECSIT, evolutionarily conserved signaling intermediate of Toll; HSP, heat shock protein; IKB, inhibitory subunit κB; IKK, I-κB kinase; IRAK, IL-1 receptor-associated kinase; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MEKK, mitogen-activated protein extracellular activated receptor kinase kinase; MyD88, myeloid differentiation factor; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor receptor-associated factor 6.

Horng *et al.* defined TIRAP, another adapter that mediates MyD88-independent signaling in response to TLR4 stimulation in MyD88-deficient mice [35]. It appears that the TIRAP-dependent, MyD88-independent signaling pathway is particularly important in the expression of MHC class II molecules and accessory molecules (B.7 antigens) on antigen-presenting cells and T-cell activation (see Fig. 3).

The protein ECSIT is specific for TLR/IL-1 signaling, and is involved in the proteolytic cleavage and activation of the mitogen-activated protein kinase MEKK-1 after TRAF6 activation [15]. ECSIT-DN mice fail to activate NF-κB through MEK-1 and MEKK-1.

Recent observations by Miyake and colleagues demonstrated the necessity of another cell surface molecule for TLR4 signal transduction [36]. The protein MD-2 has no intracellular domain, but on co-expression with TLR4 enhances LPS sensitivity in transfection models. MD-2 cotransfection with TLR2 had no effect on LPS response. Recent data support a direct binding of MD-2 to LPS. This effect was independent of CD14 or LPS-

Table 2

Currently recognized ligands for Toll-like receptor 4

Microorganism	Microbial product
Gram-negative bacteria	Lipopolysaccharide, lipid A
Gram-positive bacteria	Lipoteichoic acid
Mycobacteria	Live *Mycobacteria tuberculosis*
Spirochetes	*Treponema brennaborense* glycolipids
Virus	Respiratory syncytial virus protein F
Other ligands	Heat shock protein 60, Taxol

binding protein (LBP) and suggests a specific and unique role for MD-2 in LPS recognition that contributes to modulation of the proinflammatory response of effector cells [37].

TLR2 signaling shares many similarities with IL-1R/TLR4 signaling. TLR2-dependent NF-κB activation requires the TIR domain, which is the epitope for the TIR–MyD88–IRAK complex that induces TRAF6 [23]. Nonetheless, there are severe unique features involved in TLR2 signaling. TLR2 forms heterodimeric structures with other TLR members such as TLR1 and TLR6. A recent report demonstrated that the p85 regulatory subunit of phosphatidylinositol-3′-kinase can directly associate with the intracellular domain of TLR2 [38]. The Rho-type GTPase Rac1 also appears to be associated with TLR2-mediated signaling. This alternative signaling pathway activates a number of phosphorylated lipids and results in the generation of the intracellular protein kinase Akt. This pathway directly activates NF-κB, independent of the phosphorylation and degradation of I-κB [38] (see Fig. 4).

TLR4 as the LPS receptor

Another exciting development in the history of TLR research has been the report that TLR4 is utilized by LPS and therefore is the long-sought LPS receptor [7,39].

Probably the most powerful microbial stimulant of innate immune responses is LPS. LPS has been known to induce signals very similar to IL-1, and also to bind to CD14 on macrophages. The best-characterized interaction is between LPS and LBP. LPS is first bound by LBP, a plasma lipid transfer protein that moves LPS monomers from aggregates or bacterial membranes to a binding site on surface receptor CD14. Alternatively, the LBP–LPS complex can be recognized by a soluble version of CD14 that subsequently activates non-myeloid cells [40].

CD14 is a known PRR on the surface of monocytes/macrophages. It has been clear for many years that CD14 has a major role for the effects of LPS on macrophages, monocytes, and neutrophils, and that CD14 increases the sensitivity of macrophages to LPS [46]. But the precise role of CD14 in LPS signaling remains unclear. There has to be a molecule with the ability to identify the binding partner and to discriminate LPS from host lipids and to transduce signals across the membrane. Neither LBP nor CD14 have been shown to have the binding specificity to discriminate LPS from host lipids. The interest in TLR biology increased greatly as evidence accumulated that these proteins participate in intracellular signaling initiated by LPS and Gram-negative bacteria.

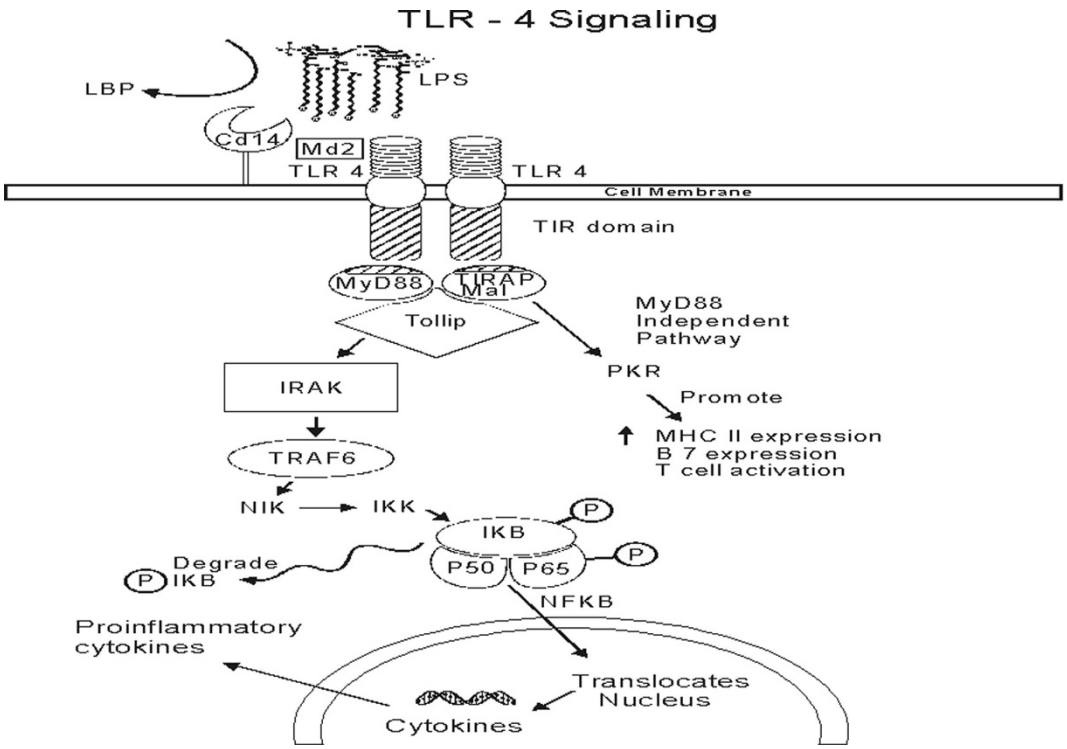
Medzhitov *et al*. showed in 1997 that a constitutively active mutant of human TLR4 induces the expression of the NF-κB-controlled cytokines IL-1, IL-6 and IL-8. This implicated the role of TLR4 in innate immunity [19]. The precise nature of the transmembrane receptor was complicated by the fact that alternative evidence developed in parallel by other investigators implicated TLR2 as the LPS receptor. Researchers demonstrated in 1998 that overexpression of TLR2 in mammalian cells renders cells responsive to LPS in a CD14-dependent manner. TLR2 was stably transfected in human kidney cell lines and did activate NF-κB in the presence of CD14 and LBP, and after LPS stimulation [23].

Table 3

Currently recognized ligands for Toll-like receptor 2

Organism	Product
Gram-negative bacteria	Lipopolysaccharide (LPS) from various sources; possibly contaminated with peptides (*Salmonella, Shigella* spp., *Escherichia coli*). Purified LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* LPS
Gram-positive bacteria	*Listeria monocytogenes*, *Bacillus*, *Streptococcus* spp., *Staphylococcus aureus*-lipoteichoic acid, peptidoglycan, lipopeptides
Mycobacteria	Heat-killed *Mycobacteria tuberculosis, Mycobacteria avium* lipopeptides, lipoarabinomannan, mannosylated phosphatidylinositol
Spirochetes	*Borrelia burgdorferi*, *Treponema pallidum*, *Treponema maltophilum* (lipopeptides, glycolipids, outer surface protein A)
Mycoplasma	*Mycoplasma fermentans* (R-MALP, lipopeptides)
Yeast	Zymosan

Figure 3



The intracellular signaling pathways of the Toll-like receptor 4 (TLR4) complex. IKB, inhibitory subunit κB; IKK, I-κB kinase; IRAK, IL-1 receptor-associated kinase; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; Mal, myeloid differentiation factor MyD88-like; MyD88, myeloid differentiation factor; NF-κB, nuclear factor-κB; NIK, nuclear factor-κB inducing kinase; PKR, RNA binding protein kinase; TIR, Toll IL-1 receptor; TIRAP, Toll IL-1 receptor adapter protein; Tollip, Toll interactive protein; TRAF6, tumor necrosis factor receptor-associated factor 6.

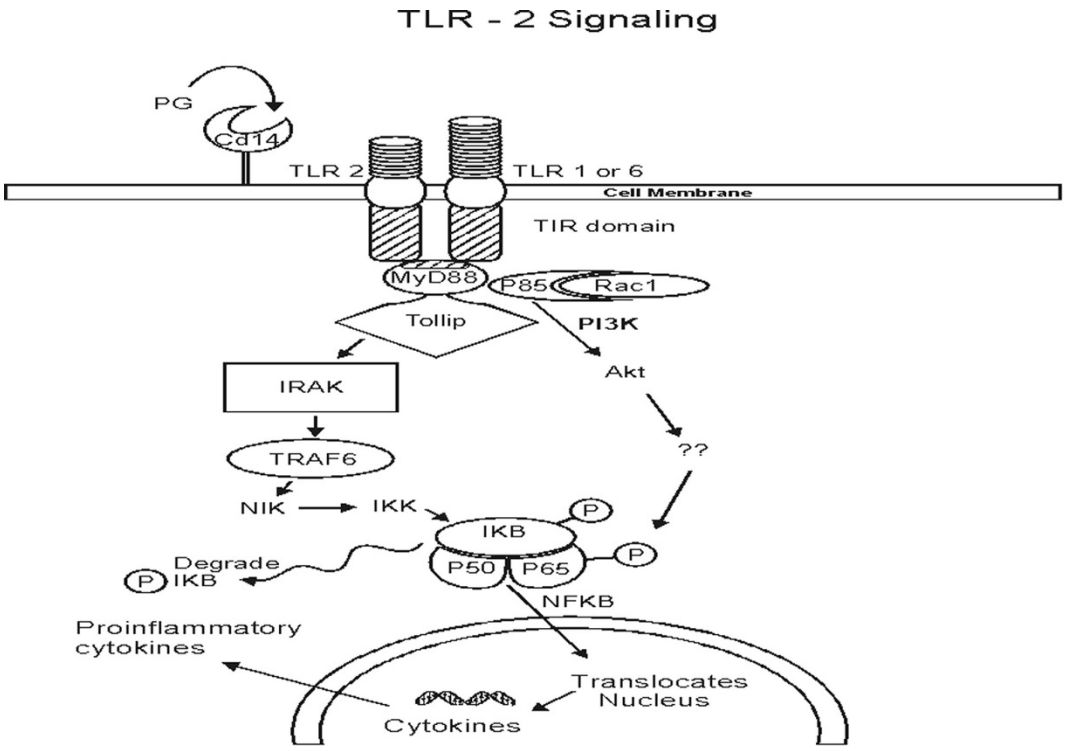
A second approach by Beutler and colleagues proved on a genetic level that TLR4 is involved in LPS signaling [7]. These proofs were carried out in genetic analysis in two strains of mice, C3H/HeJ and C57BL/10Scr, both known for their hyporesponsiveness to LPS and their increased mortality from Gram-negative sepsis. Over the past 20 years it has been shown that hyporesponsiveness to LPS maps to a single autosomal locus (lps^d), and impaired responses could be documented both in whole animals and in cells taken from these animals.

Through extensive genetic mapping work performed by Poltorak *et al*. and Qureshi *et al*., the lps^d allele was shown to map to the gene encoding TLR4. The *tlr*4 locus located to the target region in chromosome 4 [7,39].

A missense mutation in the *Tlr4* gene locus was demonstrated to be responsible for the altered LPS responsiveness in the C3H/HeJ strain. A point mutation converts a proline residue at position 712 to histidine (P712H), thus rendering the receptor inactive [7]. A further proof is that C57BL/10ScCr mice carry a large deletion mutation and do not express TLR4 mRNA. Additional evidence of the ability of TLR4 to bind to LPS is found in a recent report about a newly identified soluble form of TLR4 [41]. Soluble TLR4 can be spliced and shed following LPS stimulation and can prevent LPS signaling at the cell membrane of LPS-sensitive target cells.

After the initial observations with TLR2 as a physiologic LPS receptor [23], the relative role of TLR2 in LPS signaling has been re-examined. Hirschfeld *et al*. [42] reported that many LPS preparations in early experiments had bioactive contaminants that activate TLR2. Thorough purification of LPS eliminated signaling through TLR2. This paradigm has been challenged again by the report that purified LPS from *Prophyromonas gingivalis* and *Leptopira interrogans* only signals through TLR2, and not through TLR4 [43]. Therefore, the role of TLR2 in LPS signaling still remains ambiguous. TLR2 exerts a cellular response to LPS, but with a lower affinity than TLR4. TLR2 does not therefore appear to be essential for LPS signaling in native cells that also express TLR4. TLR2 could also be an alternative LPS receptor in TLR4-deficient cells [44]. Finally, TLR2 might be the genuine LPS receptor for LPS from non-enteric Gram-negative bacteria [43]. TLR2 has subsequently been demonstrated to be a broad spectrum PRR that interacts with a wide variety of other bacterial components besides LPS. TLR2 has thus far been involved in the recognition of multiple microbial products from Gram-

Figure 4



The intracellular signaling pathways of the Toll-like receptor 2 (TLR2) complex. Akt, intracellular protein kinase; IKB, inhibitory subunit κB; IKK, I-κB kinase; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation factor; NF-κB, nuclear factor-κB; NIK, nuclear factor-κB inducing kinase; PG, peptidoglycan; PI3K, phosphatidyl inositol-3′-kinase; Rac, Rho-type GTPase; TIR, Toll IL-1 receptor; Tollip, Toll interactive protein; TRAF6, tumor necrosis factor receptor-associated factor 6.

positive bacteria, mycobacteria, spirochetes, mycoplasma and yeast antigens [1,45] (Table 3).

Other ligands in TLR signaling

The concept of innate immunity demands the presence of non-clonal receptors that recognize a variety of highly conserved bacterial structures. More evidence has been generated that the TLR family represents this key group of innate immune response receptors. TLR2 has also been recognized as a signal transducer for numerous bacterial products. Spirochetes lack LPS, but possess membrane lipoproteins in the cell wall. TLR2 antibody administered to human peripheral blood mononuclear cells prevented cytokine release after stimulation with lipoproteins and lipopeptides from *Mycoplasma fermentans*, *Borrelia burgdorferi* and *Treponema pallidum* [45]. Chinese hamster ovary cells (CHO-K1 cell lines) fail to express a functional TLR2 due to a frame-shift mutation in their TLR2 mRNA sequence. These cells were transfected with TLR2 and tested for many bacterial products. *B. burgdorferi* lipoprotein has also been shown to stimulate NF-κB through TLR2 in transfected Chinese hamster ovary cells and also in TLR-2-transfected 293 cells [1,45].

TLR4 signaling is primarily activated after lipid A/LPS challenge, but is also sensitive to lipoteichoic acid from Gram-positive bacteria and live *Mycobacteria tuberculosis* bacteria (Table 2). Purified glycolipids from *Treponema brennaborense*, a spirochete that causes a bovine infectious disease, have been associated with TLR4-dependent signaling [46].

Ohashi *et al*. reported the potential first endogenous ligand for the TLR4 [47]. Recent studies have shown that heat shock protein (HSP) is a danger signal to the innate immune system. Macrophages respond with proinflammatory cytokine response to stimulation with both autologous HSP60 and microbial HSP60/65. Macrophages from C3H/HeJ responded with nitric oxide formation and tumor necrosis factor (TNF) secretion, whereas they remained unresponsive to LPS [48]. A recent publication has linked TLR4 signaling and expression in injured myocardium. Since there was no infection present in this model, these findings open up the perspective that TLR4 may also be responding to non-infectious and endogenous ligands in inflammation [49].

Viral particles also act as a ligand for TLR4. Kurt-Jones *et al*. showed that the innate immune response to respiratory syncytial virus coat protein F is mediated by signaling through

TLR4 and CD14. Respiratory syncytial virus infection persisted longer in the lungs of TLR4-deficient mice compared with normal mice [49].

Toll-like receptor 9

The third member of the mammalian TLR family for which ligand specificity has been defined is TLR9. Hemmi *et al*. [50] first defined, in Japan, the TLR9 molecule as the receptor for bacterial DNA. The newly recognized TLR9 was identified by a blast search of the murine gene library with sequence homology to the TIR regions of other known TLRs. Hemmi *et al*. determined that abundant mRNA transcripts of the TLR9 gene were found in many tissues, indicating that this TLR was likely to be expressed and to have a physiological role. This was confirmed by the generation of a $TLR9^{-/-}$ knockout mouse strain. These animals were shown to be incapable of responding to unmethylated CpG motifs of synthetic oligonucleotides.

Bacterial DNA has been known as a potent immunostimulant for mammalian cells for several years from the work of Krieg [51] and Sparwasser *et al*. [52]. Bacterial DNA stimulates proinflammatory cytokines, nitric oxide and MHC class II expression by macrophage/monocyte cell lines, promotes B cell activation, and induces a T helper 1-type cytokine response by T cells [51–53]. The mechanisms that underlie the recognition of microbial DNA are now increasingly understood.

Unmethylated CpG motifs are found in microbial DNA, while these sequences are relatively rare in human DNA. When these sequences do occur in mammalian DNA they are usually modified by methylation. Human cells are capable of recognizing sequences of DNA that are common to bacterial genomes but are rare in human DNA. When these unmethylated CpG sequences are flanked by two purines on the 5′ side and two pyrimidines on the 3′ end of the immunostimulatory nucleic acid, sequences in bacterial DNA induce a strong proinflammatory signal for human immune effector cells [51]. The specific sequences that are optimally recognized by human cells are GT-C-p-G-TT, while murine cells recognize GA-C-p-G-TT [53].

The molecular mechanism responsible for the ability to discriminate bacterial DNA from human DNA remained obscure until recent discoveries with TLR9. TLR9-DN mutants had no TNF, IL-12, IL-6 and interferon-γ response on exposure to oligonucleotides containing CpG motifs found in microbial DNA [50]. Moreover, TLR9 knockout mice are refractory to lethal shock from synthetic oligonucleotides bearing unmethylated CpG motifs that rapidly induce refractory hypotension and lethality in wild-type mice.

The precise mechanism by which TLR9 can recognize the subtle differences between mammalian and prokaryotic DNA with such exquisite precision is currently unknown. It may be necessary to solve the three-dimensional crystal structure of the ectodomain of TLR9 to determine the recognition sequences of this DNA receptor molecule.

The intracellular signaling pathways responsible for CpG DNA immunostimulation share some similarities with other pattern recognition molecules such as TLR4. All TLRs identified thus far signal in concert with MyD88 [33], although accessory pathways are known to exist at least for TLR2 [38] and TLR4 [35]. Engagement of TLRs to their appropriate ligands is followed by activation of a specific sequence of protein tyrosine kinases, mitogen-activated protein kinases and extracellular-activated receptor kinase molecules. The end result is nuclear translocation of the signal transducer NF-κB. This transcriptional activator promotes the increase in transcription frequency of numerous proinflammatory genes. Specific members of the HSP family (HSP90) appear to be critical in orchestrating these intracellular signaling pathways [54].

Despite these similarities, it now appears that important differences exist in the cellular processes that follow ligand recognition by TLR4 and TLR9. The TLR4 signal is greatly enhanced by the simultaneous surface expression of the pattern recognition molecule CD14. The signaling induced by prokaryotic DNA is largely independent of CD14 expression. There is also a significant delay in cytokine generation following CpG DNA stimulation compared with LPS stimulation [58]. This delay may be accounted for by the necessity for endoctyosis of the CpG DNA–TLR9 complex before signaling begins. TLR4–MD2–LPS intracellular signaling occurs directly at the cell membrane surface.

Current evidence indicates that TLR9 is surface expressed on the membrane of immune effector cells, yet intracellular signaling may occur only after the TLR9–CpG sequence has been internalized. Oligonucleotides immobilized on solid surfaces fail to stimulate mammalian cells. Inhibitors of cellular uptake disrupt signaling by CpG DNA, indicating the need to internalize the prokaryotic DNA within the endosomal compartment before initiation of the specific signaling cascade. The essential adaptor molecule MyD88 colocalizes with tagged TLR9 at the endosomal compartment by confocal imaging [50].

LPS and CpG DNA synergistically activate immune effector cells [55]. Both microbial ligands promote cytokine gene expression at the transcriptional level. It has recently been shown that the combinations of these TLR ligands synergize at the post-transcriptional level by prolonging mRNA stability within the cytoplasm of macrophages. Undoubtedly, many other potential interactions may be found in the near future between multiple TLRs that are co-expressed on the cell surfaces of human cells.

Toll-like receptor 5

The major ligand for TLR5 has recently been discovered by Hayashi *et al*. [56]. Bacterial flagellin from either Gram-posi-

tive or Gram-negative bacteria has been found to induce mobilization of NF-κB and to promote expression of proinflammatory cytokines from mammalian mononuclear cells. Motility is an important virulence property of numerous bacterial pathogens including *Salmonella* spp., *Pseudomonas aeruginosa*, and *Listera monocytogenes.* Flagellin proteins are highly conserved among bacterial pathogens and considerable structural homology is essential to maintain the integrity of the locomotion system of bacterial organisms. Isolated and purified flagella protein itself, or flagellin proteins expressed on the cell surface of either Gram-positive or Gram-negative bacteria, stimulate monocyte/macrophage cells in a TLR5-specific, CD14-independent manner. The TLR5 receptor thus appears to be the principal means by which the innate immune system recognizes flagellated bacterial pathogens [56].

TLR protein heterodimers

While the precise biochemical nature of the 10 recognized human TLR proteins are being defined, there is great interest in discovering their organization and interactions in response to microbial infection. One hypothesis is that each TLR recognizes a distinct lipoprotein or glycolipid to elicit a specific response. In this manner, different pathogen-associated molecular patterns could activate different Toll family members, leading to activation of particular target genes. Results from *Drosophila* studies support this hypothesis. The 18W of the *Drosophila* system responds to bacterial stimuli and produces the antibacterial peptide gene attacin, while Toll itself induces the antifungal peptide drosomycin but no antibacterial peptides [8,9].

A similar mechanism could be true in humans or, more likely, the signaling mechanisms are more complex and interactive. The possibility of heterodimers with differing contributions of TLRs on their cell surface responding to different microbial mediators appears to be more probable. There has been concerted effort to answer the question of whether TLR heterodimers exist *in vivo* and induce different intracellular signals.

Underhill *et al*. constructed a mutation for TLR2 that is equivalent to the P712H mutation of TLR4 in C3H/HeJ mice. In TLR2-P681H, the proline at position 681 is replaced by a histidine, thus creating a DN mutation. This group also genetically engineered the TLR4-P712H mutation [57]. These mutant TLRs were transfected into RAW-TT10 murine macrophages. The cell line with the mutant TLR2 showed impaired TNF-α production when stimulated with *Staphylococcus aureus*, whereas there was normal reaction to LPS and *Salmonella minnesota*. Expression of the TLR4 mutant allowed a normal cytokine response to *S. aureus*, but strong inhibition of the TNF-α response to LPS and moderate inhibition of the response to *S. minnesota*.

There is no evidence to date that supports the importance of TLR2/TLR4 heterodimers in LPS signaling. It has recently been suggested that TLR6 may form heterodimers with TLR2 in response to bacterial peptidoglycan but not with lipopeptides where TLR2 homodimers are found [38]. Combinations of TLR signals could mediate the variable host response signals induced by a variety of different microbial pathogens and mediators.

Blocking TLR function as a potential therapeutic option?

The development of antagonists for TLR proteins may serve as a useful tool in counteracting the harmful proinflammatory response that complicates systemic microbial infections. There are at least three basic strategies for reducing signal transduction of TLRs with the specific goal of reducing the consequences of their biological effects.

First, the development of specific soluble TLR family members to bind and neutralize their respective microbial or mammalian ligands. Examples would be soluble TLR4 in Gram-negative sepsis [41], or soluble TLR2 for the treatment of toxic shock syndromes caused by staphylococcal exotoxins.

Second, the development of small molecules or antibody molecules that interfere with the extracellular domains of the TLRs. This approach could prevent interaction with distal intracellular signaling molecules before a natural ligand binds to TLR at the cell surface of effector cells. An example for this strategy would be an LPS antagonist molecule that binds to TLR4 but fails to activate an intracellular signal [1].

This strategy will be greatly facilitated once the ligand binding pockets of the TLRs are precisely characterized. The X-ray crystallographic structure analysis of the TLR protein with a specific ligand has so far not been successful. This structural information would allow for the design of novel therapeutic agonists and antagonists that could influence the outcome of a wide range of fatal infectious states.

Third, the development of small molecules that interfere with the intracellular domains of the TLRs. This approach could prevent interaction with distal intracellular signaling molecules after ligand binding to TLRs. An example for this strategy would be small molecules that might prevent recruitment of MyD88, a central intracellular member of the IL-1R/TLR family [32].

Concluding remarks

The human immune system is well endowed with potent detection and alarm systems to respond to the ever present threat of microbial pathogens. The recent discovery of the TLR family now permits a detailed evaluation of the molecular pathogenesis of sepsis. The availability of human and microbial functional genomics should allow us to more fully understand the complex interactions that exist between host and pathogen in septic patients in the future.

Competing interests

None declared.

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