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# Review

# Toll-like receptor signalling and the clinical benefits that lie within

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**Abstract.** TLRs are of crucial importance to the innate immune system by recognising molecules that are broadly shared by pathogens but distinguishable from host molecules. The innate immune system works to defend the body from microbial infection by initiating inflammation, the extreme form of which is sepsis. The discovery that endogenous ligands, as well as microbial components, are recognised by TLRs, raise the possibility of these receptors and their associated adaper molecules, as potential targets for the development of agonists and antogonists for the treatment of various pathological diseases, and their manipulation as potential adjuvants in vaccine development. By elucidating the mechanisms of TLR signalling pathways involving adapter molecules like MyD88, Mal, TRIF and TRAM combined with the identification of single nucleotide polymorphisms (SNPs) within these receptors and the unique genes that are expressed upon recognition, will assit in the development of therapeutics to alleviate the consequences of microbial-mediated inflammation, which include inflammatory disorders and septic shock.

## 1. Introduction

Since the discovery of Toll-like receptors (TLRs), rapid progress has been made as to the understanding of the molecular and biochemical mechanisms of innate immunity. The innate immune system defends the body from microbial infection by initiating inflammation and orchestrating the acquired immune response. Uncontrolled innate response can lead to chronic inflammation, the extreme form of which is sepsis and autoimmune disease. The prototypic inducer of inflammation is Lipopolysaccharide (LPS), the major cell wall component of Gram-negative bacteria. The signalling receptor for LPS is Toll-like Receptor-4 (TLR4), which upon ligand-induced receptor dimerisation, initiates a signal transduction pathway involving one or more adapter molecules leading to activation of the prototypic inflammatory transcription factor, nuclear factor-κB (NF-κB) and interferon regulatory factors (IRF).

TLRs play a pivotal role in recognition of molecular patterns displayed by micro-organisms that subsequently lead to an immune response. Developments in the TLR field have focused on four main areas: identification of additional TLR ligands including putative endogenous ligands, further elucidation of components of individual TLR signalling pathways, and *in vivo* studies of the involvement of TLRs in the resistance to infections.

The purpose of this review is to discuss TLR4 signal transduction with an emphasis on the adaptor molecules and their role in mediating downstream events that induce an inflammatory response and the activation of transcription factors such as NF-κB, IRF-3 and activating protein-1 (AP-1). A greater understanding of the signalling pathways regulating the pro-inflammatory response to microbial infection is of crucial importance to developing new therapeutics to treat septic shock and chronic inflammation in human medicine.

## 2. Toll-like receptors

Toll-like receptors are a family of innate immune receptors whose critical role involves the recognition of invading pathogens. They are evolutionarily conserved, their homologs found in mammals, plants and insects. They were first described for their involvement in innate immunity in *Drosophila melanogaster*, where fruitflies with a mutant Toll receptor demonstrated high susceptibility to fungal infection [1]. This study led to the finding that Toll receptor is responsible for detecting fungal invasion and triggering a host defence [2]. Subsequently, mammalian orthologues of Toll receptors were identified and their role in innate immunity elucidated [3, 4].

TLRs are broadly distributed on the cells of the immune system such as macrophages, dendritic cells (DC), neutrophils, B cells, as well as mucosal epithelial and endothelial cells [5–9]. The family of mammalian TLRs are type I transmembrane receptors characterised by an ectodomain com-

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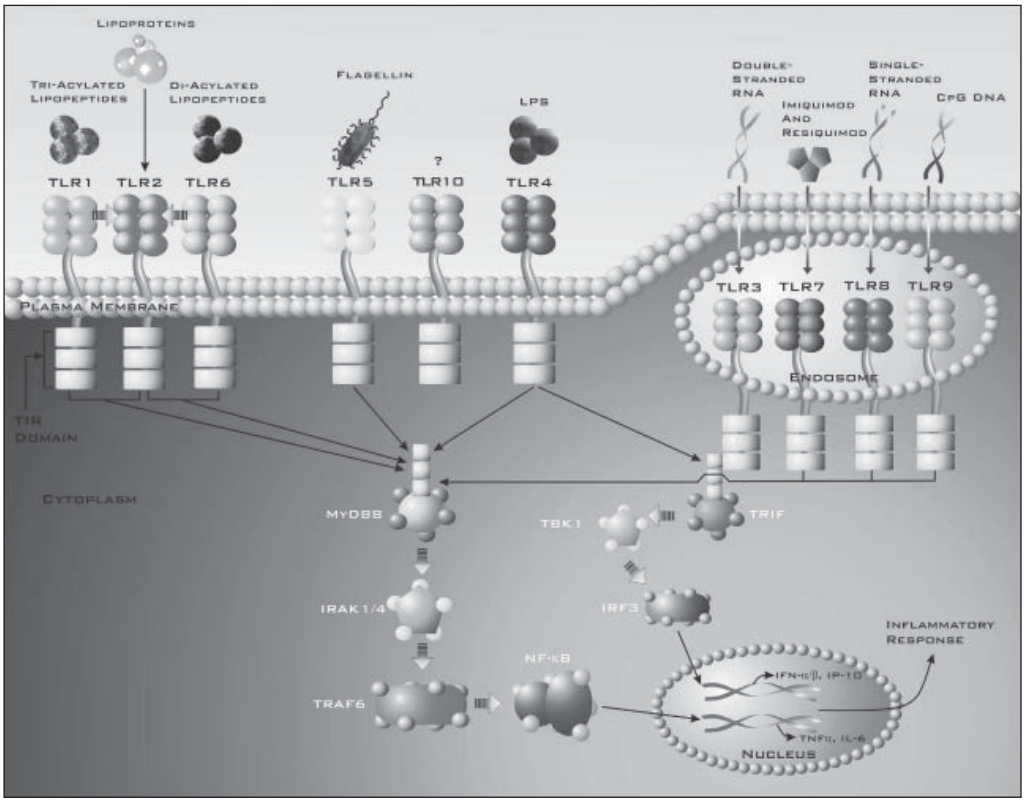


**Fig. 1.1.** TLR family discriminates between specific pathogen associated molecular patterns. At present, there are only 10 TLR family members that have been identified in humans, with TLR11–13 identified in mouse only. The binding of a microbial molecule to its TLR, transmits a signal to the cell's nucleus through a series of downstream events, ultimately leading to an immune response. All TLRs, except for TLR3, are thought to signal through a MyD88-IRAK-TRAF6 pathway to induce NF-κB and MAP kinases.

posed of multiple copies of leucine-rich motifs and a Toll/interleukin-1 receptor (TIR) motif in the cytoplasmic domain. The TIR domain, found in other members of the interleukin (IL)-1 receptor family, mediates homophilic and heterophilic interactions between TLRs and TIR-containing adaptors.

### *2.1. Ligand specificity in Toll-like receptor signalling*

In mammals, the TLR superfamily of receptors consists of 13 family members [10–15] (Fig. 1.1).

When bacteria enter hosts, they are recognised by pathogen recognition receptors, which elicit signal transduction events leading to both innate and adaptive immunity. The defence against invading micro-organisms is triggered by the ability of TLRs to recognise structurally conserved pathogen-associated molecular patterns (PAMP's) of microbial origin [3]. Such specific microbial products include LPS, bacterial lipoproteins, peptidoglycan, bacterial DNA and viral nucleic acids. Activation of these conserved motifs initiates an inflammatory cascade that attempts to clear the offending pathogen and set in motion a specific immune response.

The recognition of a microbial molecule to its respective TLR, transmits a signal to the cell's nucleus initiating the expression of genes coding for the synthesis of regulatory molecules such as cytokines, chemokines, adhesion molecules etc.. The cytokines, in turn, bind to cytokine receptors on other defence cells. Cytokines such as IL-1, tumour necrosis factor (TNF)-α and interferons, trigger innate immune defences and provide an immediate response against the invading micro-organism. However, excessive activation of these inflammatory cytokines leads to septic shock or sepsis, a leading cause of death in patients with bacterial infections [16]. TLRs also participate in adaptive immunity by triggering various secondary signals needed for humoral and cell-mediated immunity. TLRs have also been implicated in the detection of several endogenous or 'self' proteins associated with cellular damage, such as fibronectin [17], fibrinogen [18], heat shock proteins by TLR4 [19–21]; necrotic cells by TLR2 [22–25]; and chromatin-IgG complexes by TLR9 [26]. This has led to the suggestion that TLRs might also act as 'danger' receptors, rather than solely involved in microbial recognition as the aforementioned proteins can also be autoantigens. For the purpose of this review however, we will concentrate on the role of TLRs in microbial detection.

Perhaps the most widely studied event of TLR signalling is the downstream events mediated by the recognition and response to LPS with TLR4, in conjunction with MD-2, CD-14 and LBP (Fig. 1.2). LPS is an integral component of the outer membranes of Gram-negative bacteria and is regarded as the major prototypic activator of innate immunity [27, 28]. Its structure is a complex glycolipid head comprised of a hydrophilic polysaccharide portion and a hydrophobic domain termed Lipid A, responsible for LPS biological activity. LPS has a profound effect on the mammalian immune system and results in the production of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1, IL-8 and IRF-3-dependent IFN-β.

## 3. TIR domain-containing adapter molecules in TLR signalling

The ability of a TLR to tailor an inflammatory response, specific for individual ligands, has recently focused attention

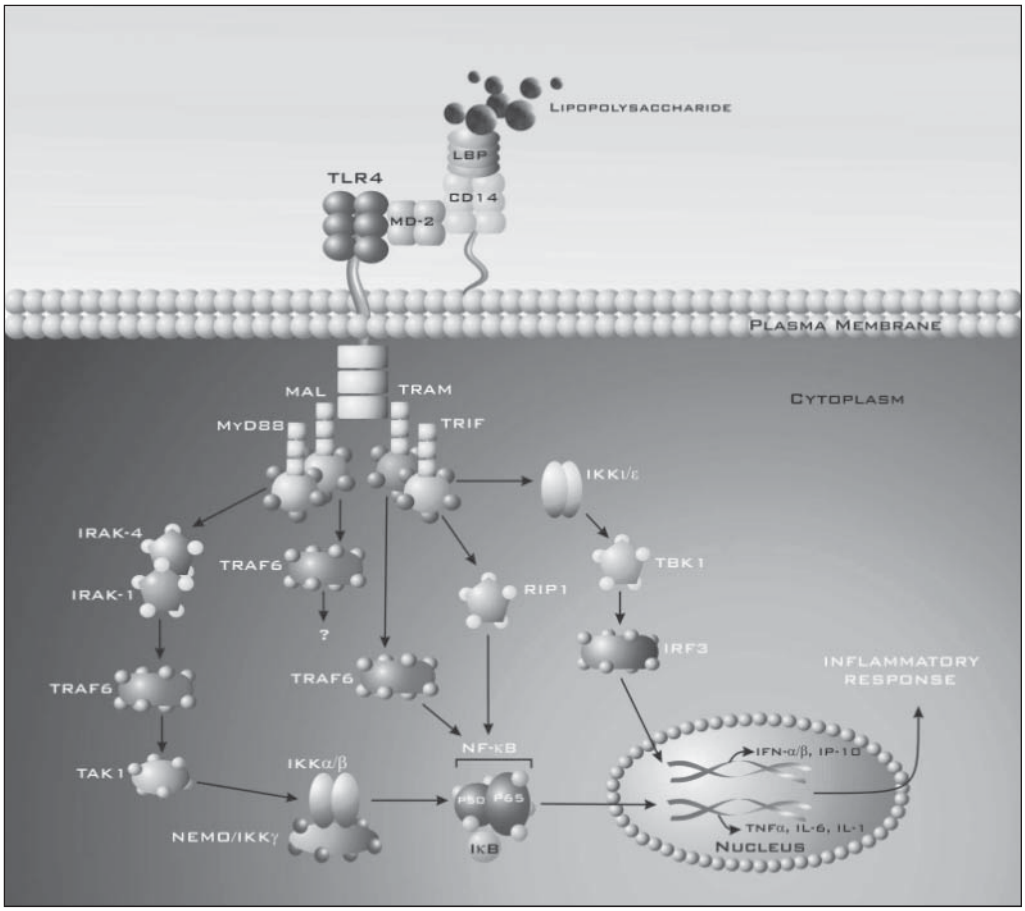


**Fig. 1.2.** TLR4 recognition and the involvement of its adapters in signalling.
LPS is opsonised by lipid binding protein (LBP), and the complex is recognised by the opsonic receptor, CD14, on the macrophage surface. CD14 associates with the cell surface by means of a glycolipid linkage rendering it incapable of generating a transmembrane signal. MD-2 confers LPS response by binding to the extracellular region of TLR4 and is an important requisite in its signalling. TIR containing adapter molecule MyD88, associates with the cytoplasmic TIR domain of TLRs, which upon stimulation mediates the association with a serine-threonine kinase, IRAK and the subsequent activation of TRAF6. TRAF6 activates TAK1 mediating the phosphorylation of the IKK complex consisting of IKKα/β and NEMO/IKKγ, subsequently phosphorylating IκB, which degrades and translocates NF-κB into the nucleus where it activates the transcription of proinflammatory genes. Mal acts as a second adapter that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4 signalling. TRIF mediates the MyD88-independent pathway via IKKε/IKKι and TBK1activating IRF-3 and subsequent IFN-β induction, in addition TRIF activates NF-κB via RIP1 and TRAF6. TRAM is essential for mediation of the TLR4 MyD88-independent pathway and TRIF-dependent pathway.

onto the cytosolic subfamily of adapter molecules that orchestrate and fractionate these downstream signalling events. There are currently five cytosolic TIR-containing proteins (MyD88, Mal, TRIF, TRAM, SARM) that are thought to play a crucial role in specificity of individual TLR-mediated signalling pathways, where most TLR members differentially utilise many of these signalling components.

### *3.1. Role of the first adapter molecule MyD88*

The most widely utilised adapter molecule in TLRs is myeloid differentiation factor 88 (MyD88). MyD88 (296 amino acid protein), was originally identified as a novel myeloid differentiation primary response gene in M1 monoleukemic cell lines that show IL-6-mediated myeloid cell differentiation [29]. Subsequently, it was shown to be involved in IL-1 and IL-18 signalling pathways. [30]. It is regarded as a common adapter protein that signals by all members of the TLR and IL-1 receptor type I (IL-1R) superfamily. TLRs and receptors of the IL-1 family interact with MyD88 via their respective TIR domains to activate the Rel transcription factor NF-κB. These interactions are mediated by homophilic associations involving two well-defined structural domains of MyD88: the carboxy-terminal TIR domain interacts with the cognate domains in the cytoplasmic tails of the TLRs (residues 155–196), and the amino-terminal death domain mediates interaction with the corresponding domain of IL-1R-associated protein kinase (IRAK), a member of a family of serine-threonine kinases [31–33].

IRAK's are important mediators in the signal transduction of the TIR family as they may act to potentiate the downstream signalling that involves both IRAK-1 and IRAK-4. Initially, IRAK-1 undergoes autophosphorylation, dissociating from the receptor complex and interacting with tumour necrosis factor receptor-associated factor 6 (TRAF6) [34]. The kinase activity of IRAK-1 is not required to induce downstream signals, and while IRAK-4 is able to phosphorylate IRAK-1, the reverse is not the case. Furthermore, in IRAK-1-deficient mice, responses to LPS were diminished but not abrogated, whereas IRAK-4-deficient mice display virtually no response to a range of bacterial components, highlighting the critical role of IRAK-4 in TLR signalling [32, 35].

The abrogated response to TLR ligands by MyD88-deficient mice (MyD88$^{-/-}$), provided an invaluable tool for analysing the critical role of MyD88 in TLR signalling [30]. MyD88$^{-/-}$ mice were resistant to LPS-induced shock compared to that of wild type [36]. Interestingly, while LPS stimulated MyD88$^{-/-}$ macrophages failed to produce inflam-

matory cytokines, TLR4-mediated activation of NF-κB was still observed, albeit with delayed kinetics compared to that of wild-type cells. Importantly, the LPS-mediated functions, such as the production of TNF-α, IL-1, IL-6 and macrophage inflammatory protein-1α/β (MIP-1 α/β) were abolished, signifying MyD88's critical role in the production of a pro-inflammatory response [36].

Further studies found that LPS induced the expression of several type I interferon (IFN)-inducible genes, such as IP-10 and GARG16, in MyD88$^{-/-}$ mice [37]. This supported and explained our earlier work which demonstrated the importance of type-1 IFNs in the LPS response [38, 39]. LPS was found to directly induce the expression of IFN-β via activation of the transcription factor IRF-3, mediated by the MyD88-independent pathway. This was further supported by the lack of LPS-induced IFNβ and IFN-inducible genes in IRF-3 deficient mice [40]. In addition to TLR4 signalling, TLR3 signalling also activates IRF-3 and induces IFN-β in a MyD88-independent manner.

The discovery of a MyD88-independent pathway led to speculation that another adapter molecule, containing a TIR domain, participated in TLR4-mediated downstream signalling [36].

### *3.2. Mal/TIRAP*

MyD88 adapter-like (Mal) or TIR domain-containing adapter protein (TIRAP) was simultaneously described as the second TIR-containing adapter protein capable of mediating NF-κB activation and responsible for differential signalling by TLR4 [41, 42]. Mal was identified as a 235 amino acid protein, homologous to MyD88, containing a TIR domain at the carboxy-terminal (residues 74–235). Overexpression of Mal activated NF-κB, MAPK, c-jun N-terminal kinase (JNK), and p38 in human embryonic kidney cells (HEK293) defining its association with TLR4, but not with other receptors of the TLR family [41, 42]. *In vitro* studies suggested Mal interacted with TLR4 specifically, perhaps acting as an adapter in the MyD88-independent pathway. Although Mal has similarities to MyD88, they differ in the sense that the N-terminal portion of Mal is 75 amino acids shorter and it lacks a death domain [42].

In Mal-deficient mice (Mal$^{-/-}$) the response of TLR members TLR5, TLR7 and TLR9 to their respective ligands, as well as IL-1 and IL-18 were normal, however deficiency in the activation of NF-κB, MAPK and cytokine production was observed in response to LPS stimulation, though with delayed kinetics [43], similar to that observed in MyD88$^{-/-}$ mice [36, 44]. Mal$^{-/-}$ mice were described as having a higher resistance to the toxic effects of LPS, and Mal$^{-/-}$ macrophages resembled MyD88$^{-/-}$-derived macrophages, in that there was no LPS-induced expression of IL-6, TNF-α, or IL-8.

Surprisingly, Mal$^{-/-}$ mice also displayed impairment in TLR2-mediated responses, suggesting Mal as an important component of TLR2-mediated innate host defence [43]. However, it was apparent that LPS was still able to induce activation of IRF-3 leading to subsequent IFN-β production. Furthermore, a double knock-out, deficient in both MyD88 and Mal, was still responsive to LPS-induced activation of NF-κB, clearly demonstrating that the MyD88-independent pathway was still intact. Conclusively, Mal was therefore found to be essential for TLR4/TLR2-mediated MyD88-dependent signalling but was not the missing adapter responsible for the 'MyD88-independent' signalling pathway.

Recent studies have identified a novel feature of Mal, distinct from MyD88, in that it contains a putative TRAF6 binding motif (T6BM) [45]. Located in the TIR domain of Mal (amino acid residues 188–193) the structural determinant for TRAF6 interaction has been described as a Pro-X-Glu-X-X-(Aromatic/Acidic residue) motif [46]. Cell-permeable CD40 and TRANCE-R peptides containing the T6BM were used *in vitro* to inhibit TRAF6 signalling, which indicates their potential as therapeutic modulators. Mal associates with TRAF6 and mutation of the critical $P_0$ glutamic amino acid residue of the T6BM (termed MalE190A) inhibits Mal-induced MAP-kinase activation and transactivation of the p65 subunit of NF-κB and was able to inhibit TLR2- and TLR4-mediated activation of NF-κB [45]. We have also recently found that Mal is phosphorylated and targeted by SOCS-1 for polyubiquitination and subsequent degradation thereby rendering cells temporarily refractory to further stimulation [47].

Taken together, a greater mechanistic understanding of Mal and TRAF6 interaction, together with the pathways this mediates, will make a significant contribution to understanding innate immune responses to pathogen challenge, but also provide insights into possible therapeutics for use in controlling chronic inflammatory responses such as sepsis or septic shock.

### *3.3. TRIF/TICAM-1*

Further database searches identified a third TIR-containing putative adaptor molecule termed TIR domain-containing adapter inducing interferon-β (TRIF, also known as TICAM-1, 712 amino acids) [48, 49]. Overexpression of TRIF in HEK293 cells induced activation of NF-κB in response to TLR4 and TLR3, albeit at lower levels then MyD88 and Mal [48]. Furthermore, TRIF very strongly activated the IFN-β promoter, which was not the case in MyD88 and Mal. Similar to Mal, TRIF was found to recruit TRAF6 via its putative T6BM, a site essential for NF-κB, but not IRF-3 activation. Contradictory to these findings was a recent paper delineating the involvement of TRAF6 in TLR signalling, where TRAF6 is involved in MyD88-mediated NF-κB activation but not TRIF-mediated NF-κB activation [50].

In TRIF-deficient mice, activation of IRF-3 and IFN-β were found to be defective following TLR3 and TLR4-stimulation. In addition, TRIF-deficient mice demonstrated defective production of inflammatory cytokines in response to TLR4 activation [49].

Hoebe *et al.* identified a germline mutation in TRIF termed Lps2 using N-ethyl-N-nitrosourea (ENU), which in mice led to hyporesponsiveness to LPS [51]. Lps2 was found to abolish cytokine response to double-stranded RNA and LPS, in addition Lps2 mutant mice entirely failed to produce IFN-α/β in response to a viral infection.

A dominant negative form of the TRIF TIR domain was found to inhibit the activation of the IFN-β promoter by TLR3 as well as NF-κB by TLR2, TLR3, TLR4 and TLR7

[48]. The fact that TRIF preferentially activates the IFN-β promoter suggests the involvement of TRIF in the MyD88-independent pathway of TLR3 and TLR4 signalling yet its involvement with TRAF6 is still contentious.

### *3.4. TRAM/TIRP/TICAM-2*

TRIF-related adapter molecule (TRAM, also known as TIRP/TICAM-2, 235 amino acids) was identified as a small TIR domain-containing protein [52, 53]. Like TRIF, TRAM activates both IRF-3 and NF-κB [54], which coordinates the transcription of IFN-β and chemokines [52].

Functional results from overexpression of TRIF with TRAM in HEK293 cells suggested that TRAM facilitates activation of TRIF, placing TRAM upstream of TRIF in the IFN-β induction pathway [53]. Studies by Fitzgerald *et al.* have determined by using immunoprecipitation studies that apart from TRAMs interaction with TRIF, it is also associated with IRF-3, IRF-7, and the putative IRF-3, -7 kinases, IKKε, and TBK1 [52].

In response to the TLR4 ligand LPS, TRAM-deficient mice demonstrated defects in cytokine production. Furthermore, TLR4- but not TLR3-mediated MyD88-independent IFN-β production, IFN-inducible genes and activation of signalling pathways were completely abolished [54], identifying TRAM as an essential adapter specifically mediating the MyD88-independent pathway of TLR4 signalling.

### *3.5. SARM*

A fifth adapter molecule termed sterile α and HEAT–Armadillo motifs (SARM, 690 amino acids) was described as containing a C-terminus TIR domain [55]. In addition, the protein contains two sterile α motif (SAM) domains and an Armadillo repeat motif (ARM). The SAM domain is known to mediate protein-protein interactions whereas the Armadillo repeat mediates the interaction of β-catenin with its ligands as well as forming structural complexes with other proteins. C-elegans contains 3 putative TIR-containing proteins, two of which are closely related to SARM, suggesting SARM may be involved in TLR signalling. However its role as either a positive or negative signalling component in TLR signalling is still to be fully elucidated [56].

## 4. Therapeutic approach to targeting TLRs and their adapter molecules

There is a growing body of evidence that drugs targeting TLRs and their signalling mediators can provide new opportunities for the design of therapeutics to treat such human inflammatory diseases as sepsis syndrome, asthma, rheumatoid arthritis, atherosclerosis, systemic lupus erythematosus (SLE) and inflammatory-bowel disease [57, 58]. However, the fight against chronic inflammatory diseases and the search for therapeutics has proven to be a challenge due to the significantly varied response to infection between individuals and the genetic factors contributing to this variation, among many other factors [59].

The discovery that endogenous ligands, as well as microbial components, are recognised by TLRs, raise the possibility of these receptors as potential targets for the development of agonists and antagonists for the treatment of various pathological inflammatory diseases of non-infectious etiology, in addition to their manipulation as potential adjuvants in vaccine development. The development of innate immune ligands as adjuvants in vaccination studies, produced from either recombinant strains or synthesised as chemical analogues, has proven to be an effective means in counteracting the harmful pro-inflammatory response associated with systemic microbial infections. At present, almost all standard vaccines contain innate immune ligands as adjuvants, one such ligand in particular is monophosphorylated lipid A, which is derived from *Salmonella minnesota* [60]. A multitude of synthetic lipid A analogs, such as monosaccharide, acyclic and disaccharide compounds are also in development for use as vaccine adjuvants, most of which act as agonists and antagonists to TLR4 having clinical benefits as standalone immunomodulators [61–63].

The prospect of blocking TLR signalling for the treatment of the symptoms of infections or inflammatory disease has been one of the main focuses in TLR study [64].

Asthma is one of the most common chronic inflammatory diseases, which despite its well characterised cellular mechanism and therapeutic drugs, is increasing in incidence in patients worldwide. As the main cause of exacerbations in asthma are viral and bacterial infections, strategies for blocking and activating TLR docking sites for viruses and bacteria may be an effective means for treating the disease. The use of TLRs for treatment of allergic diseases has already shown promising results as shown by Broide and colleagues who demonstrated from allergen-induced mice a reduced airway hyperresponsiveness and airway (eosinophilic) inflammation from administration of unmethylated CpG DNA [65].

Recently, a paper has described a possible therapeutic option for sepsis patients involving neutralisation of TLR4-activating ligands. The paper outlines the use of designer molecules TLR4-IgGFc (T4Fc) and soluble TLR4, fused to the MD-2 complex by a flexible linker, significantly inhibited LPS activity *in vitro* [66].

Currently, the ability to reduce signal transduction through TLRs in chronic inflammation has focused primarily on the thought of specific small-molecular-mass inhibitors of adapter molecules involved in the signalling pathway that may inhibit such interactions as Mal, TAK-1 or IRAK-1 with TRAF6 or MyD88 recruitment [57]. Targeting TLR adapter molecules specifically by interfering with the homophilic and domain-domain interactions that regulate the selective recruitment to a given receptor, might provide an attractive opportunity for therapeutic intervention in chronic inflammatory responses [67]. As protein-protein interactions are central to most biological processes, the underlying mechanism represents a large and important class of targets for human therapeutics, although developing small molecules that modulate protein-protein interaction is difficult, important progress has attracted much attention [68].

Already there have been many reports of small-molecule inhibitors of protein-protein interactions such as the novel inhibitor of inflammatory mediator production, [alkyl 6-(N-substituted sulfamoyl)cyclohex-1-ene-1-carboxylate,

**Table 1.1.** Single nucleotide polymorphisms in genes associated with TLR signalling.

| Gene | Polymorphism | Associated Disease/Condition |
|---|---|---|
| TLR4 | Asp299Gly | Possible susceptibility to late-onset Alzheimer's disease [92]; association with Legionnaires disease susceptibility [93]; bacterial vaginosis [94]; acute coronary events [95]; acute myocardial infarction & stroke [96]; significantly correlated with severity of asthma [97]; associated with decreased susceptibility to rheumatoid arthritis [83]; lower rate of acute allograft rejection after transplantation [98]; reduced risk for carotid artery atherosclerosis [82]; susceptibility to Helicobacter pylori infection and gastric lymphoma [99]. |
| | Thr399Ile | Blunted response to inhaled LPS in humans [84]; significant increase in ulcerative colitis [100]. |
| | Asp299Gly/ Thr399Ile | Interruption of TLR4-mediated LPS signalling and hyporesponsiveness [84]; Meningococcal disease [101]; respiratory syncytial virus infection [102]; possibly predispose people to develop septic shock with gram-negative micro-organisms [103]; severe inflammatory response syndrome [81]; increased risk of bacterial infection (Gram-negative) [104]; premature birth [105]; myocardial infarction [106]; reduced prevalence of diabetic neuropathy in type 2 diabetes [107]; allograft rejection [98]; associated chronic periodontal disease [108]. |
| TLR2 | Thr16933Ala/ Arg753Gln | Higher prevalence of Gram-positive bacterial infections [109]. Significantly less responsive to bacterial peptides derived from B. *burgdorferi* and T. *pallidum* [85]; strongly associated with acute rheumatic fever in children [110]; risk of developing tuberculosis [86]. |
| | Arg677Trp | Enhanced susceptibility to leprosy [111] and tuberculosis [112], abolished activation of NF-κB by *Mycobacterium leprae* and *Mycobacterium tuberculosis* [113] |
| TLR3 | Cys2593Thr/ Cys2642Ala/ Ala2690Gly | A significant association with type 1 diabetes in South Africans of the major allele for Cys2593Thr and minor alleles for Cys2642Ala and Ala2690Gly [114] |
| TLR6 | Ser249Pro | Pathogenesis of childhood asthma [115] |
| TLR9 | Thr1237Cys/ Thr1486Cys | New potential binding sites for transcription factors [116] |
| CD14 | Cys159Thr | Higher prevalence of Gram-negative bacterial infections [109]; associated with biliary atresia and idiopathic neonatal cholestasis [117]. |
| LBP | Cys89Gly/ Pro436Leu | Associated with an increased risk for the development of sepsis [118] |
| TNF-α | Gly308Ala | TNFalpha -308 allele could be an additional genetic risk factor for coeliac disease in trisomy 21 [119] |

TAK-242], which was discovered through chemical library screening as a new therapeutic agent for sepsis [69]. Li and co-workers published the involvement of TAK-242 to selectively inhibit TLR4 mediated cytokine production through the suppression of LPS-induced TNFα, IL-6 and IL-12 production by selectively inhibiting upstream TLR4 intracellular signalling. The suppression of these cytokines through possible MD-2 or TRAM inhibition, suggests TAK-242 as a novel small molecule TLR4 signalling inhibitor that could be a promising therapeutic agent for inflammatory diseases, whose pathogenesis involves TLR4 [70]. It would not be unexpected that small compounds able to inhibit TIR-TIR interactions will be developed soon. Also recently, a peptide derived from vaccinia virus immunoregulatory protein A52R, a protein shown to inhibit NF-κB activation through inhibiting TIR signalling of TRAF6 and IRAK2 [71], was found to significantly inhibit *in vitro* cytokine secretion in response to TLR activation [72]. Treatment of this cell permeable peptide was also found to dramatically reduce middle ear inflammation in BALB/c mice injected with heat-inactivated *Streptococcus pneumoniae*.

Other proposals that would reduce inflammatory cytokine expression include soluble TLRs that specifically bind and neutralise the microbial ligands, for example soluble TLR4 acting a feedback mechanism to inhibit excessive LPS responses [73], or soluble TLR2 for the treatment of sepsis caused by staphylococcal exotoxins. An additional alternative is the use of small molecules or antibodies capable of blocking specific protein-ligand complexes to the receptors, for example an LPS antagonist that interferes with MD-2 recruitment to LPS in TLR4 signalling [74]. However, the pharmaceutical industry is still polarised in the most appropriate method of drug development [68, 75].

One complication in designing a generic drug to combat any disease, including chronic inflammatory signalling, is the multiple genetic differences present within the human population. At present, such companies as Genome Therapeutics, and Gene Logic, have focused on personalised medicine – the tailoring of drugs to specific subpopulations of individuals that will most likely benefit from them [76, 77].

As each patient's genotype is different, an individuals response to TLR ligands varies greatly [78]. This variability may be due to single nucleotide polymorphisms (SNPs) in genes encoding TLRs and the molecules involved in the signalling pathway.

### *4.1. Polymorphisms of Toll-like receptors*

Since the completion of the Human Genome Project, advances in clinical applications involving genotyping technologies has led to the understanding of SNP being the most common form of variant in the human genome sequence [79]. SNPs are allelic variations, with a frequency of >1 % in comparison to "normal" allelic variants, that alter the amino acid sequence and affect promoter characteristics, which results in an altered susceptibility to infectious or inflammatory diseases [80]. Gene mutations and polymorphisms in TLRs have revealed the importance of TLRs in human defence against diseases. Research into specific polymorphisms in genes encoding TLRs, along with the downstream signalling molecules involved, plays an important role in identifying a link between TLR signalling and human disease. Although disease associations linked to SNPs in TLRs are in the early stage of experimental discovery, clinical insights have demonstrated a direct correlation to multiple immune and conditional diseases (Table 1.1).

It therefore raises a practical question at hand: can this understanding be exploited to a therapeutic advantage?

Studies of the TLR4 gene have shown that two cosegregating SNPs, gene-Asp299Gly and Thr399Ile, have been found to positively correlate with several infectious diseases such as increased incidence of systemic inflammatory response syndrome [81], reduced risk for carotid artery atherosclerosis [82] and decreased rheumatoid arthritis disease susceptibility [83] (Table 1.1). These genetic mutations in TLR4 are found within approximately 5–10 % of the total population and were first identified by Arbour and colleagues in 2000 from studies describing these SNP's association to a blunted response to inhaled LPS in humans and an increase susceptibility to Gram-negative bacterial infection [84].

Polymorphisms have also been identified in TLR2, where gene-Arg753Gln, was associated with a decreased response to bacterial peptides derived from *B. burgdorferi* and *T. pallidum* in septic shock patients [85]. In addition, this polymorphism may also predispose persons to staphylococcal infections [85] or tuberculosis [86]. A series of promoter polymorphisms present within CD14 at positions -1619, -1359, -1145, -809, and -159, have been implicated in bacterial ligands development of T-helper cell (Th)-2 responses [87]. Downstream TLR signalling molecules also report SNP's associated with human disease, these include: IRAK4, stop codons occurring at amino acid positions 287 and 293 resulting in increased Gram-positive infections [88, 89] and Caspase-12, which mediates the essential key proteolytic events in inflammatory cascades, stop codon TGA resulting in hyporesponsive to LPS and increased risk of sepsis [90]. Moreover, mutations present in the genes encoding transcription factor NF-κB essential modulator (NEMO) and IκBα give rise to X-linked anhidrotic extodermal dysplasia with immunodeficiencies [91]. In contrast, as more SNP's are discovered in signalling molecules such as the TIR-containing adapters, a greater understanding of their importance in disease progression and response will become apparent.

## 5. Concluding remarks

The innate immune system is the oldest mammalian defence against invading micro-organisms and provides the first line of defence against infection. Our comprehension of its complexity and mechanistic understanding of TLR signalling has increased greatly in the past few years.

The remarkable advancements made in defining the molecular mechanisms underlying TLR signalling have proven beneficial for their role as potential targets for therapeutic intervention. So far, such treatments strategies have included small molecular mass inhibitors as TAK-242 to suppress inflammatory cytokines, or lipid A as adjuvants in vaccine development. Moreover, the ability to target individual adapter molecules, that are responsible for mediating some of the specific responses to pathogens, provides the opportunity for the design of effector mimetics as novel antimicrobial drugs and therapeutic targets against chronic inflammation, which may be used in conjunction with genetic profiles of patients. Thus, elucidating the biological effects of innate immune SNPs in an individual's genotypic profile is crucial to determining how the individual functions towards susceptibility to infections and/or inflammatory diseases. This data may be beneficial in tailoring of specific drugs towards genetic polymorphisms associated with pro-inflammatory mediation or its downstream effects. Overall genetic and pharmacological studies on TLRs can hope to one day provide potential clinical therapies with the aim of decreasing infection and the treatment of human inflammatory diseases.

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