Control of *Mycobacterium tuberculosis* through mammalian Toll-like receptors

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An efficient immune response against the intracellular pathogen *Mycobacterium tuberculosis* is critically dependent on rapid detection of the invader by the innate immune response and the activation of the adaptive immune response. Toll-like receptors (TLRs) contribute to innate immunity by the detection of *Mycobacteria*-associated molecular patterns and mediating the secretion of antibacterial effector molecules. TLRs influence the adaptive immune response by upregulation of immunomodulatory molecules supporting the development of a Th1-biased T cell response. In this manner, activation of TLRs contributes to defense against microbial infection.

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Abbreviations

DC	dendritic cell
iNOS	inducible NO synthase
LAM	lipoarabinomannan
NFκB	nuclear factor κB
PIM	phosphatidylinositolmannan
STF	soluble tuberculosis factor
TLR	Toll-like receptor

Introduction

In *Drosophila*, activation of Toll leads to the induction of a variety of antimicrobial peptides, including metchnikowan, defensins, cecropins and drosomycin [1].

The mammalian Toll-like receptor (TLR) family is structurally conserved and homologous to the *Drosophila* Toll system [2]. Microbial ligands and bacterial lipoproteins have been shown to activate mammalian TLRs, facilitating the transcription of genes — including those for cytokines and costimulatory molecules — that regulate the adaptive immune response [2]. Ten members of the TLR family have been reported to date [3]. Most human tissues express at least one TLR, and the complete panel of TLR mRNA is expressed in the spleen and the peripheral blood [4•]. The greatest variety of TLR mRNAs is found in professional phagocytes, suggesting a key role of TLRs in innate immunity.

TLRs are type I membrane proteins containing an extracellular domain with leucine-rich repeats and a cytoplasmic portion with homology to the IL-1 receptor (IL-1R) family [2]; the cytoplasmic portion interacts with MyD88, IL-1R-associated protein kinase and TNFR-activated factor 6 [5].

Mycobacterium tuberculosis, the causative agent of tuberculosis, is an intracellular bacterium that is capable of surviving and persisting within host mononuclear cells. *Mycobacteria* have evolved sophisticated evasion strategies to escape from destruction by resting macrophages. The host response against tubercule bacilli is dominated by the fine-tuned interaction of innate and adaptive immune responses. One of the most intriguing questions in tuberculosis concerns the cross-talk between the pathogen, its host cells and T cells. TLRs are believed to represent key molecules in the orchestration of this interaction. The following review is aimed at discussing current knowledge about the interaction between TLRs and *M. tuberculosis*, with special emphasis on the biological implications.

Recognition of mycobacterial antigens by TLRs

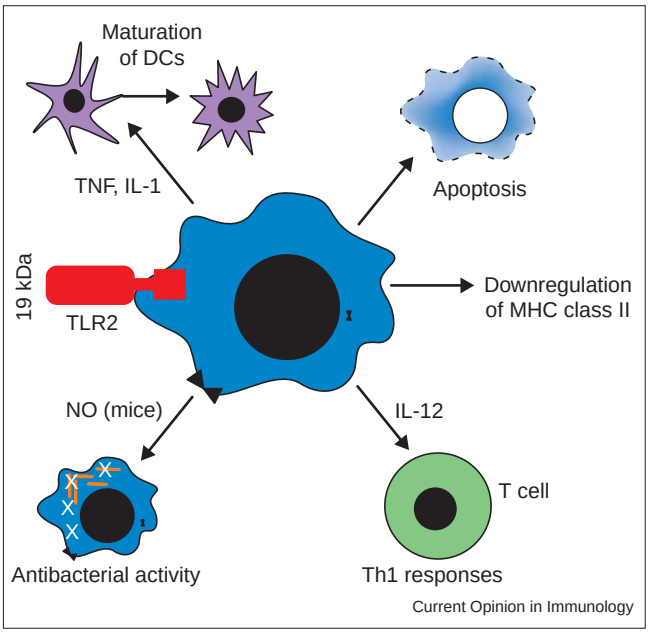
The 19 kDa lipoprotein

Bacterial lipoproteins belong to a group of molecules categorized as pathogen-associated molecular patterns that contain molecular motifs that are present in microorganisms, but are absent from mammalian hosts. Recognition of pathogen-associated molecular patterns, including mycobacterial lipoproteins, modulates the functions of both innate and acquired immunity (Figure 1). The 19 kDa lipoprotein, which is a potent inducer of T cell responses, is a secreted antigen of *M. tuberculosis* [6]. Recent studies have demonstrated that the 19 kDa lipoprotein activates murine and human macrophages to secrete TNF and NO via interaction with TLR2, but not TLR4 [7]. Similarly, lipoproteins derived from *Borrelia burgdorferi* [8], *Treponema pallidum* [7], *Mycoplasma fermentans* [9] or synthetic lipopeptides activate cells via TLR2 [10]. It needs to be emphasized that definitive evidence for a physical interaction between lipoproteins and TLRs is lacking. It is intriguing, however, that the lipid portion is required for activation of TLR2 [7,10]. The recognition of 19 kDa lipoprotein by TLR2 is not limited to macrophages — the preferred host cell of *M. tuberculosis* — but also occurs in dermal endothelial cells [11]. This suggests that endothelial cells participate in TLR2-dependent monitoring for microbial invaders, thereby enabling rapid detection of antigen at the site of entry.

Lipoarabinomannan

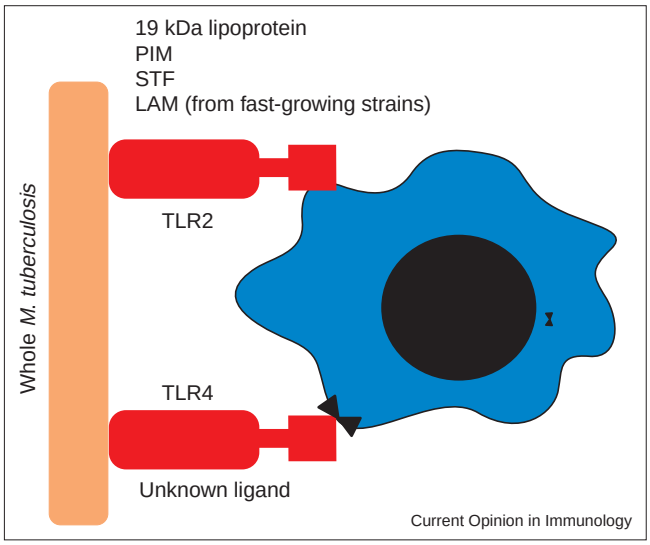
Lipoarabinomannan (LAM) is a glycolipid that dominates the mycobacterial cell wall and is a key molecule in eliciting cytokine secretion by macrophages [12]. Earlier studies

Figure 1



Model of TLR2-mediated effects in tuberculosis. Mycobacterial products such as the 19 kDa lipoprotein have various pro- or anti-bacterial effects on macrophages (blue); these include induction of host cell apoptosis, downregulation of MHC class II and enhanced activity against mycobacteria (orange), for example via NO in mice. Macrophages also produce cytokines that activate other cell types in the adaptive immune response such as DCs and T cells.

Figure 2



Mycobacterial TLR-ligands. Whereas all of the purified ligands examined so far activate macrophages via TLR2 (possibly in combination with other TLRs), none that acts via TLR4 has been characterized. Whole *M. tuberculosis* interact with TLR2, TLR4 and other TLRs to various extents, depending on mycobacterial strain and study system.

had indicated that macrophage activation by LAM is initiated by binding to the cell surface molecule CD14. Since CD14 lacks an intracellular signaling domain, the downstream events leading to the biological response remained elusive. The recent discovery that TLRs are required for LAM-mediated cellular activation has resolved the issue. LAM derived from ubiquitous, non-pathogenic mycobacteria (AraLAM) induced increased IL-2 receptor expression in Chinese hamster ovary (CHO) cells genetically engineered to express CD14 and TLR2. In striking contrast, the biochemically distinct LAM purified from virulent *M. tuberculosis* (ManLAM) failed to activate TLR2- or TLR4-transfected cells [13]. The capability of pathogenic bacteria to circumvent TLR-mediated cellular activation may support their intracellular survival and persistence. In summary, activation of macrophages via LAM is achieved by the combined action of the adaptor molecule CD14 and the signal transducing moleculeTLR2.

Phosphatidylinositolmannan

Soluble tuberculosis factor (STF) is a protein-free component of a short-term-culture filtrate of *M. tuberculosis*. Similar to 19 kDa lipoprotein and LAM, STF interacts with TLR2, but not TLR4. Biochemical studies revealed phosphatidylinositolmannan (PIM), a biosynthetic precursor of LAM, as the critical component of STF for the activation of nuclear factor κB (NFκB), activator protein-1 and mitogen-activated protein kinases in murine macrophages [14•]. Interestingly, dominant-negative constructs of TLR2 or TLR6 inhibit the responses induced by STF, suggesting a functional interaction between TLR2 and TLR6 in the cellular response to appropriate ligands [15•]. Similar observations have been made using non-mycobacterial lipoproteins [16]. The combined ligation of TLRs may increase the diversity of responses to microbial antigens and enhance the spectrum of pathogen-associated molecular patterns that can be detected by the innate immune system.

Whole *M. tuberculosis*

Whole *M. tuberculosis* will be the initial challenge for the host cell after infection. Therefore, one of the most intriguing questions is whether the tubercle bacilli stimulates macrophages via TLRs. Viable or dead *M. tuberculosis* bacilli activated murine macrophages that were transfected with TLR2 or TLR4 [17]. Even though the interaction between *M. tuberculosis* and TLRs was partially mediated by cell associated molecules, it was independent of LAM [17]. At the same time an independent study confirmed these findings using an alternative experimental approach; murine macrophages expressing a dominant negative construct for TLR2 secreted reduced levels of TNF after stimulation with avirulent or virulent mycobacteria [18]. However, as opposed to macrophages expressing a dominant negative form of the adaptor molecule MyD88, inhibition was incomplete, suggesting the involvement of other TLRs. As opposed to LPS-induced macrophage activation, the overexpression of MD-2, a protein that is associated with TLR4, did not augment TNF production induced by viable *Mycobacteria* [19•]. Furthermore, the

production of NO, a potent antibacterial effector molecule, was not impaired in macrophages derived from mice deficient in TLR2 and TLR4 [19•]. As observed for LAM, the TLR response to whole bacilli varies with the mycobacterial species under investigation: *M. avium*, a fast-growing *Mycobacterium*, activates cells specifically via TLR2, but not TLR4 [20].

In summary, these results suggest that purified mycobacterial antigens preferentially interact with TLR2, possibly in combination with additional TLRs (Figure 2). However, infection with whole bacilli evokes a more complex activation pattern involving at least TLR2 and TLR4 and leads to differential activation of antibacterial effector pathways. The mycobacterial ligand that interacts with TLR4 remains to be determined.

TLR-mediated modulation of the adaptive immune response

Since mycobacterial TLR-ligands are secreted by the pathogen, they could concentrate in the extracellular environment and act on uninfected bystander cells. This hypothesis is consistent with previous studies showing that LAM can be exported from *M. tuberculosis*-infected cells [21]. It is tempting to speculate that glycolipids (e.g. LAM), lipoproteins (e.g. 19 kDa) or other bacterial products (e.g. PIM) secreted by pathogens are capable of governing the development of an acquired immune response. The following section will discuss the complex biological effects elicited by the induction of TLR signaling with focus on the impact of infection with *M. tuberculosis*.

Regulation of TLRs by *Mycobacteria*

Infection of murine macrophages with *M. avium* resulted in differential expression of TLRs: TLR2 mRNA and protein were upregulated, whereas TLR4 mRNA decreased [22]. Induction of TLR2 mRNA was dependent on protein kinase C and independent of TNF receptor I and NFκB [22], but required two NFκB elements and two stimulating factor 1 elements in the TLR2 promotor [23]. Since upregulation of TLR2 was mimicked by TNF, IL-1α or GM-CSF (but not IFN-γ), these cytokines might mediate this response *in vivo* [22]. The ability of bacterial pathogens to modulate the expression of TLRs indicates that this system is tightly regulated in order to support a balanced activation of the immune system.

Maturation of dendritic cells

Dendritic cells (DCs) have a key role in linking innate and adaptive immunity. In accordance with this function, DCs are equipped with a complete set of TLRs, including TLR3 that is absent from other cell types [24]. Maturation of DCs, which is a prerequisite for an efficient activation of T cells, is induced by infection with numerous pathogens, including *M. tuberculosis* [25]. The maturation *in vivo* is likely to be driven by mycobacterial pathogen-associated molecular patterns such as the 19 kDa lipoprotein [26•] or cell wall components [27], and is mediated by TLR2 or TLR4. Therefore, lipopeptides can promote DC maturation, thereby providing a mechanism by which bacterial products can participate in the initiation of an immune response.

Induction of apoptosis

Bacterial lipoproteins were found to induce apoptosis in a human monocytic cell line through TLR2 [10]. Thus, TLR2 is a molecular link between microbial products and host cell apoptosis. Although many bacterial pathogens induce apoptosis in host cells, the implications of this phenomenon remain elusive. Apoptosis could support the protective immune response by trapping pathogens in apoptotic bodies, which can be taken up by bystander cells thereby inducing adaptive immune responses. Furthermore, host cell apoptosis could allow for clearance of the pathogen without inflammation and subsequent induction of immunopathological tissue damage.

Downregulation of antigen-presenting-cell function

M. tuberculosis inhibits antigen processing by murine macrophages via a mechanism involving decreased synthesis of MHC class II molecules [28]. More recently, the 19 kDa lipoprotein has been identified as the causative mycobacterial component for this phenomenon [29•]. Inhibition of MHC class II processing by either *M. tuberculosis* bacilli or 19 kDa lipoprotein is dependent on TLR2 and independent of TLR4 [29•]. In addition, transfection of the fast-growing mycobacterial strain *M. smegatis* with the 19 kDa lipoprotein of *M. tuberculosis* reduces their capability to induce TNF and IL-12 release by human macrophages [30]. These observations may explain why vaccination with recombinant *M. vaccae* or *M. smegmatis* expressing the 19 kDa lipoprotein results in less protection than vaccination with non-recombinant strains [31].

Induction of a Th1 response

Infection of human macrophages with a variety of bacteria, including *M. tuberculosis*, results in the upregulation of several genes required to mount an efficient immune response against pathogens (e.g. transcription factors and signal transduction molecules) [32••]. The activation of these genes is similarly induced by bacterial components that are agonists for TLR2 or TLR4, suggesting that TLRs are responsible for triggering the adaptive immune response. Nevertheless, the expression of IL-12 and IL-15 — two proteins required for protection against tuberculosis — was upregulated less efficiently after exposure to *M. tuberculosis* as compared with the extracellular pathogens *Staphylococcus aureus* and *Escherichia coli*. This observation is consistent with a recent report showing that LAM purified from *M. tuberculosis* inhibits LPS-induced IL-12 production by human DCs [33].

Another study demonstrated by RNAse protection assay that activation of human, immature DCs via TLR2 (using peptidoglycan, zymosan or synthetic lipopeptides) or TLR4 (LPS) led to comparable activation of NFκB and mitogen-activated protein kinase [34•]. However,

cytokine- and chemokine-gene transcription revealed a significantly distinct pattern. TLR4 ligation supported the production of IL-12p70 and the chemokine IFN-γ-inducing protein (IP-10). TLR2 agonists failed to induce these mediators, but promoted the production of IL-23p19 and IL-8.

In contrast, the 19 kDa lipoprotein was identified as the major activator of IL-12 induction by mycobacterial lysates in human monocytes [7]. Also, stimulation of human monocyte-derived DCs by the 19 kDa lipoprotein elicits high levels of IL-12, but little IL-10, in a TLR2-dependent manner [35]. The reason for the discrepancy regarding IL-12-induction by TLR2 ligands might reflect the plasticity of TLR-mediated responses, depending on the fine specificity of the microbial ligand.

The emerging concept of TLRs as key molecules for shaping the quality of the immune response against microbes is further supported by experiments with MyD88-deficient mice, which do not respond to TLRs. These mice are incapable of developing antigen-specific Th1 responses after immunization with ovalbumin mixed with complete Freund's adjuvant (containing dead *Mycobacteria* as the active component) [36••]. This observation indicates that the well-known adjuvant activity of mycobacterial components is mediated by TLRs.

Induction of antibacterial activity

The production of reactive nitrogen intermediates is a major defense mechanism of mammalian hosts against infection with intracellular pathogens [37]. TLR signaling has been linked to the induction of inducible NO synthase (iNOS) mRNA and the production of NO [7]. The *M. tuberculosis* 19 kDa lipoprotein and the *Borrelia* OspA activate a reporter gene under control of the iNOS promoter in a transiently transfected murine macrophage cell line (RAW) [7]. In contrast, stimulation of RAW cells by the glycolipid PIM failed to induce iNOS mRNA and NO secretion [14•]. This discrepancy might be due to the different TLR2 agonists that were used. Alternatively, the employment of different readout systems may offer an explanation. It is noteworthy that microbes can also induce iNOS through mechanisms independent of MyD88, which suggests that receptors other than TLR are involved [19•].

The induction of NO by mycobacterial lipopeptides led to the hypothesis that they would mediate antibacterial activity in phagocytes infected with *M. tuberculosis*. Indeed, TLR2 activation of infected macrophages by the 19 kDa lipoprotein resulted in killing of intracellular *M. tuberculosis* in both human and mouse macrophages [38•]. In mouse macrophages, killing was NO-dependent, but in human monocytes and alveolar macrophages this pathway was NO-independent. Thus, mammalian TLRs respond to microbial ligands and also have the ability to activate antimicrobial effector pathways. The presence of TLRs on cells of the monocyte/macrophage lineage in tuberculous lesions indicates that activation of TLR2 could contribute to host defense at the site of disease activity [38•].

Infections in TLR2- or TLR4-deficient mice

The first clues to the role of TLR4 *in vivo* were derived from observations in C3H/HeJ mice, which have a natural mutation in the *tlr4* genetic locus [39]. These animals are resistant to LPS-induced shock, but are highly susceptible to infection with *Salmonella typhimurium* [40]. These mice also show delayed clearance of intranasally applied *Haemophilus influenzae*. The decreased cytokine and chemokine release and the lack of neutrophils in the lung could be responsible for the increased susceptibility to the pulmonary challenge [41]. Finally, it was demonstrated that TLR4-deficient mice do not clear respiratory syncytial virus efficiently from the lung [42].

Studies investigating the course of infectious diseases in TLR2- or MyD88-deficient mice are still scarce as the tools have just recently become available. TLR2-deficient mice are highly susceptible to infection with *S. aureus* [43] and have a defect in the clearance of *B. burgdorferi* after intradermal infection [44].

The modulation of the course of tuberculosis by TLRs in mice is currently under investigation. One study suggests that the severity of disease in C3H/HeJ mice (i.e. with non-functional TLR4) infected intravenously with *M. tuberculosis* is not related to the *tlr4* genetic locus [45]. However, TLR4-deficient mice infected with a high-dose inoculum of attenuated *M. bovis* BCG (bacille Calmette–Guérin) cleared the infection more slowly than control mice (M Fenton, personal communication) did. Ongoing studies indicate that TLR2-deficient mice challenged with high-dose *M. bovis* BCG clear infection more slowly than control and TLR4-deficient mice (M Fenton, personal communication). The situation may be more complex in the low-dose model of aerosol infection with virulent *M. tuberculosis*. TLR2-deficiency did not result in significant differences in the inflammatory response or the bacterial burden in infected organs during the first 50 days (N Reiling, C Hölscher, S Ehlers, personal communication). Another study shows that after 3–4 months of infection, TLR2-deficient mice succumb to chronic infection (B Ryffel, personal communication). These preliminary observations suggest that the contribution of TLR2-signaling may be important at the chronic stage of infection when the majority of the bacterial burden has already been cleared.

TLR2-mutations in mycobacterial disease

The screening of the intracellular domain of TLR2 in the peripheral blood from leprosy patients demonstrated a band variant that was detected by single-stranded conformational polymorphism in 10 out of 45 subjects with severe disease [46•]. DNA sequencing detected an arginine→tryptophan substitution in one of the conserved regions of TLR2 intracellular signaling domain. The mutation was not present in healthy controls or patients with limited disease. This study indicates that polymorphisms in the human TLR2 gene may predict the ability to mount an efficient immune response to mycobacterial infection.

Conclusions

These results support the emerging paradigm that TLR4 is an important mediator of macrophage activation by LPS, whereas activation of macrophages by *Mycobacteria* is preferentially mediated by TLR2. In future, the repertoire of antigens that are known to mediate TLR signaling will continue to increase. The combinatorial recognition of pathogen-associated molecular patterns by more than one TLR opens a new dimension.

As TLR signaling appears to favor Th1-skewed immune responses, promotes the maturation of DCs and triggers antimycobacterial activity, this system provides an attractive target for preventive or therapeutic immune intervention. Ongoing studies in TLR-deficient mice will clarify the significance of the *in vitro* findings for infections *in vivo*. Particularly, the investigation of MyD88-deficient mice will uncover whether the TLR system is redundant or irreplaceable for protection against microbial pathogens.

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