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“Toll-like receptor 2 in host defense against *Mycobacterium tuberculosis*: To be or not to be—that is the question”

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Abstract

Toll-like receptor (TLR) 2 is expressed on immune cells and respiratory epithelial cells lining the lung. TLR2 is not critical for protection during acute *Mycobacterium tuberculosis* (Mtb) infection but it has a significant multi-faceted role in containing chronic infection. This review highlights the contribution of TLR2 to host protection, immune evasion by Mtb and immune regulation during chronic Mtb infection. The TLR2-triggered pro-inflammatory cytokines initiate protective mechanisms and limit Mtb replication while the immune evasion pathways counterattack antibacterial effector mechanisms. The immune regulation pathways that are activated dampen TLR2 signaling. The combinatorial effect of these functional responses is persistence of Mtb with minimal immunopathology.

Introduction

TLR2 is a key innate immune receptor that recognizes a diverse array of conserved ligands on pathogens and also endogenous “alarmins”. TLR2 signaling is initiated by heterodimerization with TLR1 or TLR6 and proceeds through a well-characterized signaling cascade. Dimerized receptors recruit the “bridging adaptor” Mal which facilitates the recruitment of MyD88 and assembly of the myddosome complex consisting of Mal, MyD88 and IRAK proteins. Activation of IRAK4 followed by IRAK1/IRAK2 and the subsequent recruitment and activation of TRAF6 and TAK1 culminates in the nuclear translocation of NFκB and AP1 to initiate transcription of cytokine and chemokine genes (Reviewed in [1]).

Mtb expresses a large repertoire of TLR2 ligands. The 19kDa lipoprotein (LpqH), a secreted antigen of Mtb, was the first Mtb ligand shown to signal through TLR2 [2]. Mycobacterial lipoproteins, LprA (Rv1270), LprG (Rv1411c) and PhoS1 are also TLR2 agonists [3–5]. In addition to lipoproteins, cell wall glycolipids, including lipoarabinomannan (LAM), lipomannan, and phosphatidyl-*myo*-inositol mannoside (PIM) also interact with TLR2 to initiate cellular activation (Reviewed in [6]). In this review, we will discuss three distinct

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outcomes that arise during Mtb infection from signals downstream of TLR2 activation: induction of i) host protection, ii) immune subversion and iii) immune regulation. We advance the paradigm that activation of these multiple mechanisms impacts Mtb replication and host immunopathology thereby facilitating Mtb persistence and host survival. In this review, we do not cover all the primary literature but refer to excellent reviews in the specific areas and mainly cite recent literature that highlights the multiple roles of TLR2 in handling Mtb infection.

TLR2 and Host Protection

The identification of a number of Mtb TLR2 agonists led to many *in vitro* investigations on the effect of TLR2 signaling on intracellular Mtb survival. In mouse macrophages, TLR2 activation enhances anti-mycobacterial activity in a NOS-2-dependent manner while in human macrophages it is via the upregulation of anti-microbial peptides [7], cathelicidin and beta defensin 4 [7,8]. The TLR2 agonist LpqH from Mtb also activates autophagy [9] and apoptosis [10] in macrophages, pathways that restrict intracellular Mtb growth. PE-PGRS33, a surface exposed protein of Mtb, induces TNF via TLR2 recognition and mediates macrophage apoptosis [11]. Mtb clinical strains with PE-PGRS33 deletions are attenuated in this pathway supporting the thesis that TLR2 induces anti-mycobacterial effector responses in macrophages [11]. Although these *in vitro* studies clearly demonstrated a role for TLR2 in host protection, however, the receptor was not found to have a critical role *in vivo*. Mice lacking TLR2 are not compromised in their ability to resist acute infection when infected with a low inoculum of Mtb [12–16]. These data, together with the report that TLR2/TLR4/TLR9-triple deficient animals had survival rates similar to wild type animals [17] led to the inference that TLR2 is not significant to host defense against Mtb infection.

Numerous studies have investigated polymorphisms in *TLR2*, *TLR1* and *TLR6* genes and their association with susceptibility to tuberculosis (TB). Although a few studies did not find a correlation between TLR2 polymorphism and TB susceptibility [18,19], the majority of studies, however, did find specific association in different ethnic populations between a particular single nucleotide polymorphism (SNP) and TB susceptibility [20–28]. These human genetic studies illustrate that immune responses regulated by TLR2 must contribute in some way to host defense and these mechanisms need to be identified. Indeed, in later investigations, a definite role for TLR2 in protection against chronic Mtb infection was demonstrated in the murine model of TB [12,16]. In these studies, low dose Mtb infection in mice lacking TLR2 resulted in increased lung bacterial burden during chronic infection. It is recognized that the adaptive immune response to Mtb does not induce sterilizing immunity and Mtb persists in the granuloma leading to chronic infection [29]. Furthermore, host immunity is important to maintain granuloma architecture and prevent reactivation. Together, these data indicate that TLR2 signaling is relevant to Mtb containment in the granuloma, and therefore the role of TLR2 in chronic Mtb infection needs to be further explored.

TLR2-mediated immune subversion by Mtb

Innate and adaptive immunity generated in the host following Mtb infection does not lead to sterilizing immunity. However, they contribute to granuloma integrity and Mtb persistence within these structures. [30]. There is evidence that Mtb exploits its gamut of TLR2 ligands to generate multiple mechanisms for avoiding macrophage effector functions thereby facilitating its persistence. Mtb-infected macrophages have reduced MHC Class II expression resulting in compromised ability to present antigen to T cells. Subsequent studies demonstrated that this inhibition could also be mediated by prolonged exposure of Mtb TLR2 agonists LpqH, LprG and LprA. Continuous TLR2 signaling also blocks macrophage responsiveness to IFN γ (Reviewed in [6]). The molecular basis for the unresponsiveness was examined in macrophages exposed for an extended period with Mtb lipoproteins. The lipoprotein-treated macrophages did not respond to IFN γ . The expression of the class II transactivator (CIITA) and MHC-II, downstream targets of IFN γ signaling, were not upregulated in these macrophages, and MHC-II-restricted antigen presentation was inhibited, as well. (Reviewed in [6]). The findings from these *in vitro* studies imply that prolonged TLR2 signaling might allow for Mtb persistence in chronic infection.

Beginning with the seminal study by Hart [31], a number of successive studies have closely charted the interaction of Mtb with the macrophage, and the overall finding from these studies was that Mtb arrests phagolysosomal fusion and survives and replicates within macrophages [32]. Follow-up studies have proven autophagy [33], a macrophage defense response, as an additional mechanism contributing to the intracellular restriction of Mtb. TLR2 signaling is also engaged by Mtb to inhibit autophagy. UV irradiation resistance-associated gene (UVRAG) promotes activation of the Beclin1–PI(3)KC3 complex which has a central role in the induction of autophagy ([34] and Reviewed in [35]). Mtb via TLR2 signaling induces expression of MicroRNA-(miR)125a, which targets UVRAG to hamper autophagy activation. Inhibitors of miR125a enhanced autophagy and significantly restricted Mtb survival in macrophages. Furthermore, addition of activators of autophagy such as rapamycin to Mtb-infected macrophages inhibited miR125a expression and concomitantly increased expression of UVRAG and attendant activation of autophagy [36].

Foamy macrophages accumulate in the granulomas during chronic infection and the evolution of macrophages to the foamy phenotype is characterized by increasing accumulation of lipid droplets [37]. Lipid droplets are a rich source of nutrients for Mtb and the foamy macrophages present in the granulomas in chronic infection provide Mtb an intracellular environment that is permissive to its growth [38–40]. The TLR2 agonist LpqH upregulates the expression of peroxisome proliferator-activated receptor (PPAR) γ [41] which is a key transcriptional regulator of lipid droplet accumulation in macrophages. In another study, CD36 was shown to be required for TLR2-mediated upregulation of PPAR γ and lipid bodies in mycobacteria-infected macrophages [42]. These studies indicate that Mtb usurps TLR2 to modulate macrophage biogenesis and creates an environment conducive for survival in chronic infection.

A20 is a zinc finger protein that blocks TLR-mediated signaling by deubiquitylating TRAF6. A recent study showed that the microRNA, miRNA-let-7f, that targets A20, was

downregulated in macrophages infected with Mtb, and this was associated with increased bacterial survival [43]. Expression of miRNA-let-7f decreased while that of A20 increased as Mtb infection progressed in mice. Macrophages derived from myeloid-specific A20 deficient mice had increased proinflammatory cytokine response and restricted Mtb growth [43], providing direct evidence that A20 negatively regulates macrophage effector responses against Mtb. The modulation of miRNA-let-7f by Mtb could be recapitulated with ESAT-6 [43], a known TLR2 ligand [44].

Induction of IL-10 in macrophages in response to Mtb infection is TLR2-dependent [45] and the ERK pathway involved in IL-10 production is also activated in Mtb-infected macrophages in a TLR2-dependent manner. Consistent with this, macrophages treated with an ERK inhibitor or macrophages deficient in Tpl2, a kinase upstream of ERK kinase, did not make IL-10. These cells, however, had increased levels of IL-12 [46]. These changes in IL-10 and IL-12 levels in the ERK deficient Tpl2^{-/-} macrophages enhanced their antigen-presenting capacity which led to greater Th1 polarization and IFN γ production from T cells [46]. However, contrasting outcome was observed *in vivo* in mice lacking Tpl-2-ERK signaling pathway. Mtb infection of Tpl-2 deficient mice resulted in excessive production of type I IFNs and disease exacerbation [47]. Type I IFNs inhibited the production of pro-inflammatory cytokines in Mtb-infected macrophages and diminished macrophage responsiveness to IFN γ and subsequent Mtb killing. Mechanistically, modulation of macrophage functions by type I IFNs was found to be mediated by their ability to enhance IL-10 production [47], a cytokine that has been previously reported to exacerbate chronic Mtb infection [48–50]. A reason for the discrepant results between the *in vitro* and *in vivo* studies could be that in Tpl2-deficient mice, Tpl-2-ERK signaling is ablated not only from macrophages but also other cell types that contribute to the granulomatous response, including dendritic cells, inflammatory monocytes, T cells and epithelial cells. This suggests that studies in mice generated to have macrophage targeted depletion of Tpl2 are warranted in order to translate the *in vitro* findings of enhanced APC functions of Tpl2-deficient macrophages to determine whether this is beneficial during *in vivo* infection.

Overall, these findings indicate that Mtb has developed several strategies to harness the TLR2 signaling pathway for modulating macrophage effector functions and establishing chronicity in the host.

TLR2 and immune regulation

Concurrent with activation of TLR signaling, a number of anti-inflammatory pathways are also set in motion that can curtail inflammation and limit collateral tissue damage (Reviewed in [51]). An in-depth review by Murray and Smale [52] details the layers of endogenous and exogenous mechanisms that are integrated to fine tune TLR signaling. Constitutively expressed proteins such as IRAK-M, A20, ABIN1, I κ B α , Toll-interacting protein (TOLLIP) and DAP12 rapidly restrict TLR signaling. Expression of A20 and I κ B α are upregulated following TLR engagement, and this further limits TLR signaling. Downstream of TLR signaling, the activation of MAPK signaling is attenuated by Dual specificity phosphatases (DUSP). Key inflammatory mediators such as IL-10 and type 1 IFNs are additional molecular feedback control devices of the TLR signaling pathway (Reviewed in [52]).

Below we highlight what is known regarding the involvement of these anti-inflammatory pathways and the mechanisms following Mtb recognition by TLR2.

In mice infected with low dose Mtb inoculum, TLR2 signaling was shown to be critical for the recruitment of Foxp3+Tregulatory (Treg) cells to the granuloma, thereby maintaining its integrity. In the absence of TLR2, there was a conspicuous lack of Tregs in the granuloma, the lungs exhibited hyper-inflammation and bacterial numbers were significantly increased [16]. Adoptive transfer of WT macrophages to TLR2^{-/-} mice led to enhanced accumulation of Tregs in the lungs and restored granuloma integrity. Of note, increase in Tregs was not accompanied by reduction in bacterial numbers, indicating that TLR2's role in modulating immunopathology can be functionally uncoupled from its role in restricting Mtb growth during chronic infection. This data points to the complexity and multifunctional role for TLR2 signaling within the tubercle granuloma.

TOLLIP inhibits IRAK phosphorylation and was initially discovered as a regulator of the IL-1R pathway [53]. TOLLIP also directly associates with TLR2 and suppresses the phosphorylation and kinase activity of IRAK1 and thereby facilitates termination of TLR2 signaling [54]. Interestingly, polymorphisms in the human TOLLIP gene are observed to be associated with susceptibility to TB [23,55]. SNPs in rs5743899 led to decreased mRNA levels of TOLLIP and a subsequent increased production of IL-6 cytokine in peripheral blood monocytes stimulated with Mtb lysate. Additionally, rs5743899 polymorphism was observed in patients with pulmonary tuberculosis and tuberculosis meningitis [23].

Consistent with the idea that increasing inflammation has poor outcomes in TB, TOLLIP deficiency is associated with an increased risk of TB while the polymorphism rs3750920 that results in increased mRNA levels of TOLLIP is associated with TB protection [23]. Mechanistically, these variable outcomes with the two SNPs is likely at the level of IL-10 since TOLLIP knock-down in peripheral blood monocytes increased expression of pro-inflammatory cytokines while IL-10 production was abrogated. Regulation of TLR2 may also occur in other cell types that contribute to pulmonary pathogenesis in TB. Kaufmann and colleagues found that neutrophil-driven destructive inflammation in pulmonary tuberculosis requires TLR2-mediated CXCL5 secretion from epithelial cells [56]. That TOLLIP may also regulate TLR2 signals in lung epithelial cells during Mtb infection is suggested by the observation that primary alveolar epithelial cells respond strongly to TLR2 and have low TOLLIP expression while nasal epithelial cells do not respond to TLR2 and have high TOLLIP expression [57]. Understanding TLR2 regulation in epithelial cells is a rich area for further investigations.

Role of E3 ubiquitin ligases are now being studied in the regulation of TLR signaling. Pellino proteins, a type of E3 ubiquitin ligases, are known to regulate TLR signaling cascade by ubiquitylating lysine residues of IRAK1. While some of these modifications on IRAK1 by Pellino proteins leads to degradation of IRAK1 [58], other events enhance the downstream TLR signaling [59]. A recent study has demonstrated that Pellino 3 negatively regulates TLR2 signaling. Overexpression of Pellino 3 in monocytes led to a decrease in the TLR2 induced NF- κ B activation and chemokine production. The reverse was observed in human monocytic THP-1 cell line that was transduced with Pellino 3 shRNA [60]. Further

studies are required to understand the role of Pellino 3 and other Pellino proteins in regulating TLR2 signaling during Mtb infection.

In sum, there is growing evidence that TLR2 signaling is highly regulated during Mtb infection to prevent cell and tissue destruction in chronic infection.

Concluding remarks and future research

It has generally been assumed that TLR2 plays little role in Mtb immunity. However, recent data have provided new insights into its functions. TLR2 signaling triggers broadly three functional responses in Mtb infection: protection, evasion and regulation. These responses are nonessential to control acute Mtb infection but may have non-redundant requirement during chronic infection. The TLR2-triggered pro-inflammatory cytokines initiate protective mechanisms and keep Mtb in check while the immune evasion mechanisms that are triggered allow Mtb to resist antibacterial effector molecules. The immune regulation pathways that are activated restrain TLR2 signaling. The combinatorial effect of the multifactorial functional response is persistence of Mtb with minimal immunopathology and collateral damage to host tissue. TLR2 signaling in chronic infection benefits both host and bug. (Figure 1)

Several areas stand out for future research.

NOD2 (member of NOD-like receptor (NLR) family), Dectin-1, Dectin-2 and Mincle (C-type lectin-like receptor (CLR) family) sense Mtb ligands [61]. Whether these PRRs, in the absence of TLR2, can serve as alternate pathways for activating innate responses in acute infection needs to be investigated.

There is growing evidence that alveolar epithelial cells contribute to the inflammatory network and to TB pathogenesis during chronic infection. However, the interaction of TLR2 with epithelial cells during chronic infection is not well understood. Data generated in a recent study on the transcriptomics of alveolar epithelial cells [62] should provide a useful platform to begin to dissect the pathways, transcriptional factors and networks that are modulated in epithelial cells by TLR2.

The mouse model is a useful tool for probing the role of TLR2 in protection against Mtb infection but it is important to note that the outcome of TLR2-Mtb interaction between humans and mice may be different. In human macrophages, TLR2 mediates anti-microbial activity through the engagement of the VDR pathway leading to the induction of cathelicidin expression and autophagy [63]. This interaction between TLR2 and VDR does not occur in mouse macrophages since mouse cathelicidin promoter does not contain the VDR response element. In mouse macrophages, anti-mycobacterial activity is upregulated by TLR2 [7], and so the final outcome of TLR2-Mtb interaction may be similar in humans and mice. Nonetheless, future studies could exploit *in vitro* granuloma models that are currently being developed [64] to test whether the critical functions for TLR2 identified in the mouse model are also valid in humans. Also, in humans, unlike TLR2KO mice, Mtb interaction with TLR2 SNPs may initiate signalosome assembly, albeit different from WT TLR2. So activation of TLR2 SNPs may lead to differential activation of kinases and transcription

factors and consequently altered cytokine response compared to WT TLR2. In this context, generation of knock-in mice carrying the different TLR2 SNPs would provide a valuable tool to mechanistically dissect how these polymorphisms affect host responses to Mtb infection and disease outcome. In addition, mycobacterial genotype may also influence the final outcome of TLR2 signaling. A study by Caws et al [65] found that enhanced bacterial dissemination was a combinatorial effect of TLR2 SNPs in the patients and the infecting Mtb strain. This finding adds to the complexity of TLR2 regulation and host defense and strongly suggests that multiple Mtb clinical strains need to be evaluated to fully understand Mtb interaction with TLR2.

Another area that needs to be queried is how Mtb TLR2 ligands are generated and recognized in the granuloma in chronic infection. A vesicular system in Mtb has been demonstrated and the released membrane vesicles (MVs) contain TLR2 agonists [66,67]. How these MVs interact with TLR2 and modulate host granulomatous response and pathogenesis in chronic infection needs to be studied. In cynomolgus macaques infected with Mtb, some lesions progress while others show sterilization [68]. The sterilized lesions demonstrate a balanced level of pro- and anti-inflammatory cytokines [69]. Whether released MVs interact with TLR2 to regulate this balance is unknown, but it does evoke the possibility that unraveling the complex interaction of Mtb TLR2 agonists with the TLR2 signaling pathway has the potential to inform on how to fine-tune the balance of mediators in the granuloma necessary to achieve sterilizing immunity with minimal pathology. Thus, understanding TLR2 and its downstream signaling components on immune and respiratory epithelial cells has the potential to unravel novel targets for host-directed therapy.

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HIGHLIGHTS

1. TLR2 does not contribute to protection against acute Mtb infection.
2. TLR2 has a significant role in protection against chronic Mtb infection.
3. TLR2 controls immunopathology in chronic Mtb infection.
4. Mtb hijacks TLR2 to evade macrophage effector mechanisms.
5. TLR2 signaling is regulated to minimize host immunopathology.

Figure 1. TLR2 mediated Immune Protection, Evasion and Regulation in Mtb infection

Stimulation of TLR2 by Mtb ligands leads to the activation of different transcription factors that initiate the gene transcription of several cytokines and regulatory molecules. These TLR2 induced molecules work in concert to initiate Immune protection, Immune evasion and Immune regulation pathways that allow Mtb to persist in the host with minimal immunopathology.