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REVIEW

Innate immune responses to *M. tuberculosis* infection

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SUMMARY

A prerequisite for successful establishment of *Mycobacterium tuberculosis* in the host is its ability to survive after internalization in alveolar macrophages that they encounter after inhalation. The innate immune response protects some individuals to the extent that they remain uninfected. In others, the innate immune system is not sufficient and an adaptive immune response is generated. This is usually protective, but not sterilizing, and individuals remain latently infected. In susceptible individuals, *M. tuberculosis* successfully escapes immune surveillance. The interplay between the host innate immune response and the bacterial mechanisms in play to offset this response, is of considerable importance in dictating the course of the disease. In order to gain an understanding of this interplay it is of importance to analyze how *M. tuberculosis* interacts with innate immune receptors and makes its entry into macrophages, how it subverts the bactericidal effects of macrophages, and dampens processes required for protective immunity, including cytokine and chemokine induction. This review will focus on some of the Indian efforts in these areas, concentrating mainly on the interaction of *M. tuberculosis* with macrophages and dendritic cells (DCs). The role of the PE/PPE family of proteins in regulating the immune response, will not be discussed in this chapter. The genome-wide approaches of analyzing host-*M. tuberculosis* interactions will also be discussed elsewhere.

1. Lipoarabinomannan

Mycobacterial cell surface and secreted antigens are the predominant players involved in manipulation of the host immune system. The glycolipids lipoarabinomannan (LAM) and its precursor lipomannans (LMs) are recognized by innate pattern-recognition receptors (PRRs). LMs are composed of a mannosyl–phosphatidyl inositol membrane anchor to which is attached α (1 → 6) mannopyranosyls bearing single α (1 → 2) and α (1 → 3) branches.[1,2] The mannan core is linked to an arabinan domain which is capped by different motifs. *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and other slow growers such as *Mycobacterium kansasii* and *Mycobacterium avium* bear oligo mannose caps, therefore the name, ManLAM. *Mycobacterium smegmatis* LAM is capped with phospho-inositol (PILAM), whereas *Mycobacterium chelonae* LAM is uncapped (AraLAM). Recognition by distinct PRRs is attributable to capping motifs (or their absence). ManLAM binds to C-type lectin receptors, namely the mannose receptor on macrophages or Dendritic Cell-Specific ICAM-3 Grabbing Non-Integrin Receptor 1 (DC-SIGNR1) on dendritic cells (DCs).[3,4] LM and PILAM from *M. smegmatis* induce a proinflammatory response characterized by secretion of tumor necrosis factor (TNF) TNF-α, interleukin (IL)-8 and IL-12p40. ManLAM, on the other hand, inhibits IL-12p40 production from macrophages and DCs.[5] Subsequent work has shown that this involves repression of NF-κB signaling.

Pathogen-triggered innate immune responses involve surface-exposed and intracellular receptors of which the Toll-like Receptors (TLRs) are among the best studied.[6,7] TLRs dimerize and trigger a signaling cascade in which the proximal steps involve assembly of scaffolds dependent on homotypic interaction between the TLR intracellular domain and adapter proteins such as MyD88, TIR-domain-containing adapter-inducing IFN (TRIF) and TRIF-related adapter molecule (TRAM).[8] MyD88-dependent signaling is associated with the recruitment of the interleukin-1 receptor-associated kinase (IRAK) 4, followed by the phosphorylation of IRAK1 by IRAK4. Phosphorylated IRAK1 associates with TNF receptor associated factor (TRAF) 6 leading to TGF-beta activated kinase (TAK) 1-dependent phosphorylation of IκBα or MAP kinase kinase kinases (MKKs). In the classical NF-κB pathway, the degradation of IκBα leads to the nuclear translocation of NF-κB and induction of proinflammatory genes. A range of microbial pathogens subvert innate immunity by dampening the activation of NF-κB.[9,10] Virulent *M. tuberculosis* is also endowed with this ability. Work by Basu,

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Kundu and coworkers has shown that ManLAM inhibits IL-12p40 production by virtue of its ability to induce IRAK-M, a kinase-deficient IRAK family member which is a negative regulator of the classical NF-κB pathway.[11] The CATERPILLAR (CLR, also known as Monarch-1) protein has been independently reported to interfere with IRAK1 thereby functioning as a repressor of TLR signaling triggered by *M. tuberculosis* infection of macrophages.[12] These data suggest that virulent *M. tuberculosis* uses a variety of mechanisms to repress IRAK1 activation and inhibit the classical NF-κB pathway.

ManLAM has also been suggested to function as a modulator of macrophage apoptotic pathways. Considering that *M. tuberculosis* resides principally in macrophages, the simplest model would suggest that the pathogen would benefit by the containment of apoptosis at least at the initial stages to allow establishment of the bacterium within its intracellular niche. The mitochondrial apoptotic pathway requires the formation of hetero-oligomeric complexes of the proapoptotic Bcl-2 family members and mitochondrial pore formation.[13] This precedes the release of cytochrome c and formation of an apoptosome involving caspase 9. The proapoptotic Bcl-2 family members such as Bax and Bak are kept in check by forming complexes with a number of anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-xL and Bad. Anti-apoptotic molecules such as Bcl-2 are themselves complexed by proapoptotic Bcl-2 proteins such as Bad. Bad phosphorylation enables dissociation of the Bad-Bcl-2 complex and is therefore an anti-apoptotic event.[14] ManLAM leads to phosphorylation of Bad in an AKT-dependent manner.[15] Taken together with its ability to inhibit Ca^{2+} accumulation in macrophages,[16] these results support the contention that ManLAM modulates the immune response of infected macrophages using multiple mechanisms (Figure 1.).

2. ESAT-6/CFP-10

Comparative genomics of different virulent and avirulent strains of the *M. tuberculosis* complex has enabled identification of regions of difference (RD) between the various organisms.[17,18] The five ESAT-6 (or *esx*) gene clusters present in *M. tuberculosis* H37Rv but absent in *M. bovis* BCG (and other attenuated strains) are defined by region 1 (Rv3866–Rv3883c), region 2 (Rv3884c–Rv3895c), region 3 (Rv0282–Rv0292), region 4 (Rv3444c–Rv3450c) and region 5 (Rv1782–Rv1798).[19] Region of difference antigens have been implicated in virulence mechanisms of *M. tuberculosis* H37Rv,[20–22] the most prominent being ESAT-6 (EsxA) and CFP-10 (EsxB) which are secreted proteins that lack classical signal sequences and form a heterodimer.[23] Indian laboratories have been actively pursuing efforts to understand how these antigens modulate signaling in macrophages and dendritic cells.

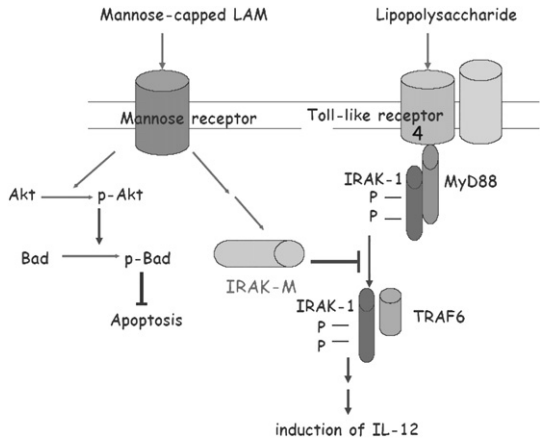

Figure 1. ManLAM attenuates inflammatory cytokine production and apoptosis in macrophages. The figure depicts the likely mechanisms involved in ManLAM-mediated inhibition of IL-12 production and apoptosis.

Basu, Kundu and coworkers have demonstrated that exogenous ESAT-6 downregulates MyD88-dependent TLR signaling.[24] ESAT-6 by virtue of its ability to attenuate MyD88-IRAK4 interaction, inhibits TLR-dependent NF-κB activation. The Sharma laboratory have in addition, reported that ESAT-6 and CFP-10 downregulate reactive oxygen species (ROS) linked to LPS/TLR4-dependent NF-κB transactivation, ERK1/2 and c-myc activation.[25,26] ESAT-6 has further been reported to downregulate ERK1/2 phosphorylation in the nucleus, possibly by activating phosphatase activity.[27] Earlier work from the Sharma laboratory had indicated that pretreatment of macrophages with CFP-10 could lead to marked reduction in their ability to produce nitric oxide (NO)[28,29] as well as ROS[30] resulting in extensive dephosphorylation of host proteins, dampening of host cell signaling and downregulation of transcription of several genes essential for macrophage function.[30]

The Natarajan laboratory has focused on how CFP-10 interacts with DCs to modulate the immune response. This group has shown that CFP-10 induces differentiation of DC precursors into immature DCs,[31] and maturation of GM-CSF-differentiated bone marrow or splenic DCs which have suppressive capacity.[32,33] The CFP-10 matured DCs (CFP-10 DCs) fail to respond to secondary challenge with antigens or *M. tuberculosis* cell extracts. CFP-10 DCs also mount a poor oxidative burst facilitating bacterial proliferation and show reduced secretion of proinflammatory chemokines. The Natarajan group has further shown that adoptive transfer of RANTES and IP-10 conditioned CFP-10 DCs clears established *M. tuberculosis* infection in mice.[34] It would be of interest to use a CFP-10 knockout of *M. tuberculosis* to ascertain whether the effects of CFP-10 on DC maturation are dominant or not.

This body of work strengthens the contention that ESAT-6 and CFP-10 influence the in vivo interaction between mycobacteria and the host immune system. Other work has established that *M. bovis* BCG carrying the RD1 region (BCG::RD1) has an enhanced capacity to recruit $CD11c^+$ cells to the lungs of infected mice compared to *M. bovis* BCG.[22] Parenchymal $CD11c^+$ cells from mice infected with BCG::RD1 also show enhanced expression of a set of inflammatory cytokines and chemokines.[22]

3. Differentially expressed antigens in infection

While much work has been done on the role of identified virulence factors in modulating the immune response, less studies have used unbiased approaches to track expression of *M. tuberculosis* antigens at different time points following infection of macrophages.[35] The Natarajan group has utilized this approach to zoom in on 5 genes (Rv1238, Rv2463, Rv3723, Rv0082, Rv1483) enriched 24 h post-infection (Day 1) and 5 genes (Rv2391, Rv3911, Rv0981, Rv3416 and Rv0353) enriched 120 h post-infection (Day 5). They have also elucidated the effects of some of the expressed antigens on protective immunity by ex vivo studies using recombinant proteins. Day 1 antigens enhanced *M. tuberculosis*-mediated activation of DCs with Rv2463 being the most potent. By contrast, several of the Day 5 antigens down regulated MHC class I and II and CD54 molecules. The same held true for macrophages as well. Day 1 and Day 5 antigens also downregulated LPS-induced IL-12 production. For several antigens, this was dependent on subversion of LPS-induced inducible nitric oxide synthase expression.

In summary, different antigens probably act in concert through distinct mechanisms to downregulate macrophage priming during the course of infection. Taken together, the large body of work in Indian laboratories has helped to give an improved understanding of the crucial role of surface-exposed and secretory antigens in subverting the innate immune response against *M. tuberculosis*.

4. Regulation of interferon γ (IFN-γ), suppressors of cytokine signaling (SOCS) and voltage-gated calcium channels (VGCC)

Interferon γ (IFN-γ) is necessary for microbicidal functions of macrophages leading to the eradication of *M. tuberculosis*.[36,37] IFN-γ has been detected at infectious foci.[38] In order to survive, *M. tuberculosis* is therefore likely to dampen macrophage responsiveness to IFN-γ. It has been established that *M. tuberculosis* inhibits macrophage responses to IFN-γ [39] resulting in attenuated killing of *M. tuberculosis* and class II antigen presentation.[40] IFN-γ binds to its heterodimeric receptor consisting of a ligand-binding subunit IFN-γR1 and a signaling subunit IFN-γR2. Prasad and coworkers have demonstrated that *M. tuberculosis* dampens macrophage responsiveness to IFN-γ by down-regulating IFN-γR1 expression.[41] Suppressor of cytokine signaling (SOCS) family members function as negative regulators of IFN-γ-stimulated JAK/STAT signaling in macrophages.[42,43] Hypervirulent *M. tuberculosis* strains upregulate members of the SOCS family of proteins.[44] TLR2/MyD88 signaling has been reported to activate Notch1 expression and Notch target genes involved in pro-inflammatory responses.[45] A link between TLR2, Notch 1 and SOCS3 in mycobacterial infection, has been established by Balaji and coworkers. This group has demonstrated that *SOCS3* transcription involves TLR and NOTCH1 signaling, giving insight into one of the mechanisms of anti-inflammatory gene expression in mycobacterial infection.[46] The crosstalk between Notch1 and TLR2 signaling is mediated by NO.[47]

On similar lines, the Natarajan group recently investigated regulation of TLR2 and DC-SIGNR1 mediated SOCS1 expression in DCs during *M. tb* infection.[48] They showed that compared to TLR2, stimulating DC-SIGNR1 on DCs induces higher SOCS1 expression and lower IL-12 production. Co-stimulating DC-SIGNR1 and TLR2 differentially regulates SOCS1 expression depending on the relative concentration of their ligands. Stimulating DC-SIGNR1 with *M. tuberculosis* increases SOCS1 expression, while stimulating TLR2 with *M. tuberculosis* reduces SOCS1 expression. Knockdown of SOCS1 in DCs by siRNA enhances IL-12 transcription and protein expression upon DC-SIGNR1 stimulation. Raf-1 and Syk differentially regulate TLR2 and DC-SIGNR1 mediated SOCS1 expression. In addition, DC-SIGNR1 shows greater association with SOCS1 when compared with TLR2. Interestingly, compared to healthy asymptomatic individuals, PBMCs of patients with active TB disease showed higher expression of SOCS1, which was reduced following chemotherapy. Similarly, stimulating DC-SIGNR1 on DCs from *M. tuberculosis*-infected TLR2$^{-/-}$ mice enhanced SOCS1 expression that was reduced following chemotherapy. Further, knockdown of SOCS1 in mouse DCs or human PBMCs resulted in increased killing of virulent *M. tuberculosis*. These results indicate that TLR2 and DC-SIGNR1 differentially regulate SOCS1 expression during *M. tuberculosis* infection. This in turn regulates *M. tuberculosis* survival by governing key cytokine expression.

A key mechanism linked to the immune response is calcium mobilization. Inhibition of L-type and R-type voltage gated calcium channels (VGCC) leads to increased calcium influx and expression of proinflammatory cytokines. PBMCs from tuberculosis express higher levels of VGCCs compared to healthy controls and these levels go down with chemotherapy. Natarajan and coworkers have suggested that VGCC-blocked DCs could activate T cells to augment killing of *M. tuberculosis* during infection.[49]

The interaction between alvelolar epithelial cells and *M. tuberculosis* remains far less understood than the interaction of the pathogen with classical antigen-presenting cells. The group of Mridula Bose has explored the role of pulmonary epithelial cells in the innate immune response in tuberculosis. Using the alveolar epithelial cell line A549 as a model system, this group has shown that A549 cells produce NO [50] and IFN-γ [51] in response to *M. tuberculosis* infection, warranting a detailed evaluation of the role of pulmonary epithelial cells in the innate immune response.

Taken together, the Indian perspective adds significantly to our knowledge of the innate immune response and the immune subversion mechanisms of *M. tuberculosis*.

5. Regulation of cellular death and/or survival pathways

The extrinsic pathway of apoptosis is initiated by the binding of ligands, to death receptors triggering assembly of an intracellular scaffold known as DISC (death-inducing signaling complex). Recruitment of procaspase 8 and/or procaspase 10 facilitates intermolecular proteolysis and activation of these caspases. These receptors interact with adapter proteins, which recruit and activate caspase 8 and/or caspase 10 to form the DISC This leads to the initiation of a caspase cascade, which ultimately leads to the activation of caspase 3 and other 'executioner' caspases, which digest important substrates in the cell to induce cell death. The various pathways linked to cell survival or death, namely autophagy, apoptosis and necrosis have been independently studied in the context of *M. tuberculosis* infection in laboratories across countries for a number of years.[52–55] At first glance, the literature in the field appears contradictory. More than a decade ago, it was reported that *M. tuberculosis* H37Rv triggers alveolar macrophage apoptosis.[56] Evidence of elevated macrophage apoptosis in human alveolar macrophages obtained after bronchoalveolar lavage of tuberculosis patients when compared to healthy subjects has also been documented.[57] Macrophages isolated from mice with resistant *sst1* (super-susceptibility to tuberculosis-1) locus undergo apoptosis in response to *M. tuberculosis* infection.[58] More recent studies show that virulent *M. tuberculosis* also triggers necrosis in macrophages.[59] In addition, *M. tuberculosis* uses a variety of mechanisms to inhibit apoptosis. Apart from ManLAM, *M. tuberculosis* anti-apoptotic factors include NuoG and SecA2.[60,61] The balance between cellular death and survival pathways likely depends on the cell type chosen for a particular study, the activation status of

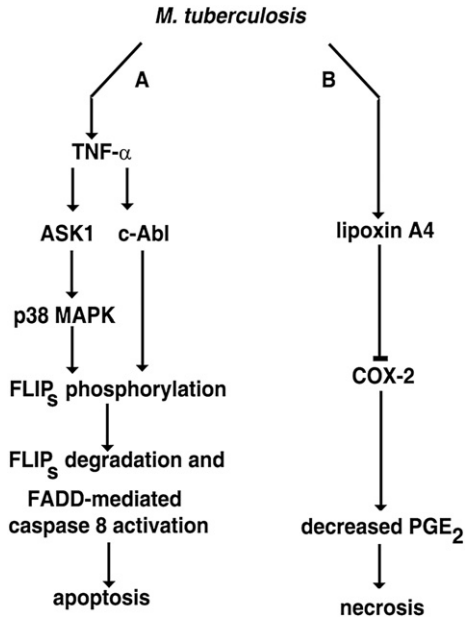

Figure 2. Interplay between apoptosis and necrosis in *M. tuberculosis*-infected macrophages. The *M. tuberculosis*-infected macrophage may undergo apoptosis (A) or necrosis (B). The apoptotic pathway predominates in the case of avirulent *M. tuberculosis*. Virulent *M. tuberculosis* undergoes apoptosis and necrosis, and also uses mechanisms to subvert death. The balance is dictated by factors such as bacterial burden and stage of infection.

the cell, the bacterial burden and other factors that may, as yet, await discovery. It is evident that we are far from understanding the interplay between these pathways in the context of infection. Work by Basu, Kundu and coworkers has shed new light on the role of an ASK1/p38 MAP kinase pathway in regulating caspase 8 activation and apoptosis of *M. tuberculosis*-infected macrophages. Their work has uncovered a novel role of this MAP kinase pathway along with the tyrosine kinase c-Abl (Figure 2). *M. tuberculosis* infection triggers the serine/tyrosine phosphorylation of the anti-apoptotic c-FLIP$_S$.[62] The phosphorylated c-FLIP$_S$ is recognized by the ubiquitin E3 ligase c-Cbl leading to its ubiquitination and subsequent degradation. This likely facilitates procaspase 8 recruitment to the DISC and its proteolytic activation. The serine/threonine kinase AKT facilitates survival of *M. tuberculosis* in macrophages.[63] Considering that AKT is an inhibitor of ASK1, an interplay of the AKT-dependent survival pathways and ASK1-dependent apoptotic pathway(s) could regulate the balance between macrophage survival and death in *M. tuberculosis*-infected macrophages.

A system-level analysis of the networks linked to autophagy, apoptosis and necrosis, and their crosstalk in *M. tuberculosis* infection, is likely to provide a better perspective of how the interplay of these pathways dictates the course of infection.

6. Concluding remarks

In spite of the large body of literature documenting various aspects of the immune response to *M. tuberculosis* infection, plausible therapeutic approaches targeting this response, require a more complete understanding of the processes involved. We require: (1) a system level understanding of the signaling processes triggered in classical antigen-presenting cells and the epithelial cells of the alveolar mileu during infection, and how these processes influence the temporal evolution of cytokines and chemokines as the host attempts to clear infection; (2) an understanding of the interplay between autophagy, apoptosis and necrosis, and their balance during infection; and (3) an understanding of the bacterial effectors and how they modulate these processes. The contributions from Indian laboratories has thrown light on many of these aspects in recent years.

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