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Immunoevasion and immunosuppression of the macrophage by *Mycobacterium tuberculosis*

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Summary: By virtue of their position at the crossroads between the innate and adaptive immune response, macrophages play an essential role in the control of bacterial infections. Paradoxically, macrophages serve as the natural habitat to *Mycobacterium tuberculosis* (Mtb). Mtb subverts the macrophage's mechanisms of intracellular killing and antigen presentation, leading ultimately to the development of tuberculosis (TB) disease. Here, we describe mechanisms of Mtb uptake by the macrophage and address key macrophage functions that are targeted by Mtb-specific effector molecules enabling this pathogen to circumvent host immune response. The macrophage functions described in this review include fusion between phagosomes and lysosomes, production of reactive oxygen and nitrogen species, antigen presentation and major histocompatibility complex class II expression and trafficking, as well as autophagy and apoptosis. All these are Mtb-targeted key cellular pathways, normally working in concert in the macrophage to recognize, respond, and activate 'proper' immune responses. We further analyze and discuss major molecular interactions between Mtb virulence factors and key macrophage proteins and provide implications for vaccine and drug development.

Keywords: *macrophages, bacterial, protein kinases, phagocytosis*

Introduction

Despite global efforts to halt the spread of tuberculosis (TB), the number of annual deaths caused by this disease remains almost unchanged, 1.5–2 million per year, making it the most prevalent disease caused by bacterial infection (1). Today, TB is more worrisome than ever due to the rapid spread of drug-resistant TB, the recent emergence of totally drug-resistant TB, and the low efficacy of the current bacilli Calmette-Guerin (BCG) vaccine.

Mycobacterium tuberculosis (Mtb), the pathogen responsible for TB, is unanimously recognized as one of the most successful human pathogens because of its ability to persist and survive in the macrophages of immunocompetent individuals. Macrophages are key components of the immune system that neutralize intracellular pathogens and initiate protective adaptive immune responses via antigen presentation to T cells. Recent advances in cellular mycobacteriology demonstrate that Mtb uses a plethora of complex strategies to evade the major antimicrobial mechanisms of macrophages, including

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phagosome-lysosome fusion, the recruitment of potent hydrolytic lysosomal enzymes, the production of reactive oxygen and nitrogen species, antigen presentation and major histocompatibility complex class II (MHC II) expression and trafficking, autophagy, and apoptosis. Disruption of these macrophage functions in turn disrupts innate and adaptive immune responses and impairs the macrophage's ability to properly recognize, respond, and react to infection.

Entry of Mtb into the macrophages

Entry of Mtb into alveolar macrophages can be mediated by either non-specific pinocytosis or through a well-defined variety of receptors. Characteristics and molecular markers associated with each one of these modes of entry are illustrated in *Table 1*. Non-specific mechanisms of Mtb internalization by pinocytosis or macropinocytosis to the macrophage need to be better characterized (2), yet recent work by the Pieters' laboratory (3) has shown that inflammatory stimuli can induce changes to the endocytic machinery from receptor-mediated phagocytosis to macropinocytosis. This occurs through protein kinase C-mediated phos phorylation of coronin 1 and subsequent activation of the phosphoinositol (PI)-3-kinase (PI3K) activity necessary for macropinocytosis (3). Pinocytosis has also been shown to be characteristic of Mtb invasion of non-phagocytic cells such as A549 human pneumocytes (4).

Phagocytosis of Mtb can also occur through a variety of receptors including, Fcγ receptors (FcγRs), complement receptor type 3 (CR3), and lectin receptors. Receptor-mediated phagocytosis can either be opsonic or non-opsonic (5). Opsonic uptake of Mtb involves the recognition of coated (opsonized) bacteria with complement factors, antibodies, and/or surfactants (6). FcγRs receptors on the surface of macrophages recognize the microorganisms coated with immunoglobulin G (IgG), while CR3 recognizes bacteria coated with C3bi, leading to different mechanisms of phagocytosis (7). As early as 1975, D'Arcy Hart and Armstrong (8) noted that coating of Mtb with rabbit serum influences phagocytosis outcome by increasing the prevalence of fusion between phagosomes containing opsonized Mtb and lysosomes, in comparison to non-opsonized Mtb. Although it is widely accepted that Mtb ingestion by the macrophage is mainly non-opsonic and thought to be more important during early stages of infection (6, 9–11), analysis of bronchoalveolar lavage fluids reveal abundant immunoglobulins (12) and complement components (13),

Table 1. **Mtb modes of entry to the macrophage**

Type of recognition	Receptors involved	Type of phagocytosis	Description	Markers	Contribution	References
Specific						
Opsonic	FcγRs	Type I phagocytosis	Recognizes IgG coated bacteria. Involves pseudopod formation	Activates pro-inflammatory mediators. The GTPases Rho, Rac and Cdc42 are required. Activation of the Hck, and Syk, and Pyk2 tyrosine kinases	Opsonized Mtb promotes phagosome-lysosome fusion	(1–4, 6, 7, 10, 12)
	CR3	Type II phagocytosis	Recognizes C3bi coated bacteria. Sinking into macrophage. No pseudopodia	Rho GTPase is required. Lack of inflammatory mediator production		
Non-opsonic	C-type lectin (CR3, Mannose)	Type I phagocytosis (CR3 mediated)	PRRs recognize PAMPs. CR3 is involved in non-opsonic type I phagocytosis	Phagocytosis via CR3 is mediated by capsular polysaccharides of Mtb. Phagocytosis via Mannose receptor activates anti-inflamatory signals: inhibition of IL-12, and activation of IL-1ra, IL-1RII, and IL-10	ManLAM binding to mannose receptors is key for the inhibition of phagosome-lysosome fusion	(7, 10, 13, 15–20)
	TLR, CD14, scavenger receptors, cytosolic DNA-sensors, among others	–				
Non-specific		Macropinocytosis	Internalization of large amounts of fluid and solutes	Lack of specific molecular markers	Coronin 1 activates the phosphoinositide 3-kinase to activate macropinocytosis	(22, 23)

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suggesting that opsonic uptake might be biologically relevant even during early stages of infection.

Coating Mtb with IgG leads to type I phagocytosis, which involves macrophage membrane extensions, termed pseudopods, which surround the invading microorganism. Pseudopod formation requires the small GTPases, Rac, and Cdc42. Type I phagocytosis also activates the production of pro-inflammatory mediators like the NADPH oxidase (NOX2)-dependent superoxide, arachidonic acid metabolites, and tumor necrosis factor α. It also activates the Hck, Syk, and Pyk2 tyrosine kinases (14). On the other hand, the uptake of bacteria coated with C3bi leads to a type II phagocytosis, which consists of sinking the microorganism into the macrophage cell membrane with the participation of the enzyme Rho A, but without the involvement or activation of NOX2-dependent respiratory burst or even inflammatory signals (6, 7, 15–18).

Studies done on Chinese hamster ovary (CHO) cells transfected with CR3 indicate that opsonized Mtb may use CR3 to enter the macrophage (15). CR3 has a lectin domain that allows also non-opsonic uptake of bacteria, which involves type I phagocytosis (see below). Studies in CR3-deficient mouse macrophages (Cd11b knockout mice) show that CR3 account for 40–50% of non-opsonic binding of Mtb to the macrophage (19). *In vivo* experiments confirmed these studies and show that CR3 mediates 50–60% of opsonic binding of Mtb to the macrophage of CR3-deficient mice (19). Nevertheless, Mtb survival was not affected by CR3 deficiency, suggesting that other uptake mechanisms can compensate for the lack of CR3 (20).

Non-opsonic receptors recognize highly conserved patterns that are found on pathogens. These patterns are defined as pathogen-associated molecular patterns (PAMPs), and their receptors are denoted as pattern recognition receptors (PRRs) (21). Some of the macrophage PRRs used for recognition of Mtb are C-type lectin receptors, Toll-like receptors (TLRs), Nod-like receptors (NLRs), scavenger receptors, CD14, and cytosolic DNA sensors (5). C-type lectin receptors recognize carbohydrate-rich molecules. The mannose receptor (MR), a type of C-type lectin receptor, recognizes lipoarabinomannan (LAM), and mannosylated LAM (ManLAM), abundant glycolipids expressed on the surface of mycobacteria (22, 23). ManLAM has been shown to enhance mycobacterial phagocytosis (24), and ManLAM-dependent phagocytosis is mediated at least in part by CR3 (25) and heterodimer (CD18/CD11b) β_2 integrins. Non-opsonic entry of Mtb to the macrophage results in higher intracellular survival, which suggests that entry of the bacteria through specific receptors is needed for phagosome-lysosome manipulation (9, 26). For instance, studies by Kang *et al.* (27) show that binding of Mtb ManLAM to mannose receptors strongly inhibits phagosome-lysosome fusion.

The main signaling pathways associated with ManLAM involve the membrane receptors CD14 and TLR2 but not CR3 (28, 29). Thus, we investigated whether CD14, TLR2, and CR3 cooperate to bring about optimal uptake of mycobacteria, and if so, how they may be linked. Using transfected THP-1 and CHO (CR3 and CD14 positive) cells, we show that CD14 plays a major role in phagocytosis of Mycobacteria requiring serum factors such as the lipopolysaccharide-binding protein (LBP) (30). These findings are consistent with other reports showing that CD14 mediates uptake of mycobacteria by monocyte-derived microglial cells (31). Our findings contradict a previous report suggesting that CD14 may not be involved in mycobacterial ingestion (32). However, prior findings can be explained by the absence of serum and LBP in the experimental setting. We were able to show that binding of mycobacteria to CD14 induced an inside-out signaling pathway involving TLR2, PI3K, and the adapter protein cytohesin-1, leading to enhanced CR3-dependent bacterial internalization (*Fig.* 1). Cytohesin-1 is an adapter protein that interacts with the cytoplasmic tail of CD18 leading to changes in the functional properties of β_2 integrins. Cytohesin-1 binds to the

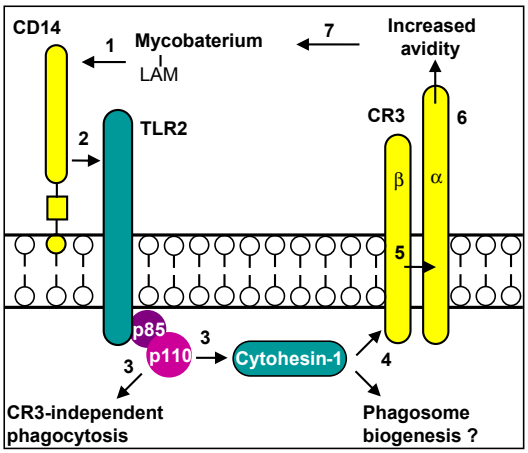

***Fig.* 1. Cross-talk between CD14 and CR3 mediates optimal ingestion of mycobacteria.** Signaling through membrane-bound CD14 initiated by mycobacteria or LAM leads to TLR2-mediated activation of PI3K. The latter is responsible for triggering two pathways for bacterial internalization: a unique CD14 pathway that can function in the absence of CR3 and a cytohesin-1-regulated CR3-dependent pathway that together with CD14 results in optimal phagocytosis of the tubercle bacilli. Reprinted from Sendide *et al.* (30) with permission from the American Association of Imunologists, Inc. Copyright 2005.

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PI3K metabolite PtdIns-3,4,5-P_3, leading to changes in properties of the protein (33, 34). These findings suggest a role for PI3K in regulating the activity of LFA-1 β_2 integrin (35, 36). Our study shows that PI3K has a role in regulating CR3 as well. The clinical ramifications of Mtb interacting with CD14 are broad. For example, vitamin D pretreatment has been shown to restore macrophage responses to Mtb infection by increasing surface expression of CD14 (37).

It is interesting to view the CD14/CR3 cross-talk in the context of findings made by Tailleux *et al.* (38), who reported that Mtb enters human dendritic cells (DCs) after binding to DC-SIGN (DC-specific intercellular adhesion molecule-3 grabbing non-integrin) and found that CR3 was not involved in mycobacterial binding to DCs. Given that DCs gradually lose their surface CD14 during the maturation process (39), it is reasonable to consider that CR3 remains inactive in these cells in the absence of a CD14-dependent regulatory mechanism, in contrast to our findings with $CR3^+$ $CD14^+$ THP-1 cells.

The relative contribution of each receptor for Mtb uptake into the human macrophage is difficult to assess, and it is unknown if engagement of specific receptors determines the fate of the phagosome *in vivo* or the course of infection. Yet, it is clear that they work in cooperation and can compensate for the lack of other receptors, leading to activation of the macrophage endocytic pathways (6).

Blockage of early events of phagocytosis

Macrophages utilize a plethora of killing and digestion processes which destroy engulfed invading microorganisms. Specifically, as illustrated in *Fig.* 2, macrophages undergo phagosome maturation, an essential cellular process in which phagosomes interact with endosomes and lysosomes allowing the exchange of endocytic solute materials and membrane components between these organelles. These fusion events result in the acidification of phagosomes through the acquisition of vesicular-Hþ-ATPase pumps (V-ATPase) and proteolytic enzymes (lysosomal hydrolases) as well as the production of reactive oxygen intermediates (40).

Mtb is able to circumvent the macrophage killing machinery, managing to replicate and persist in macrophages. Mtb

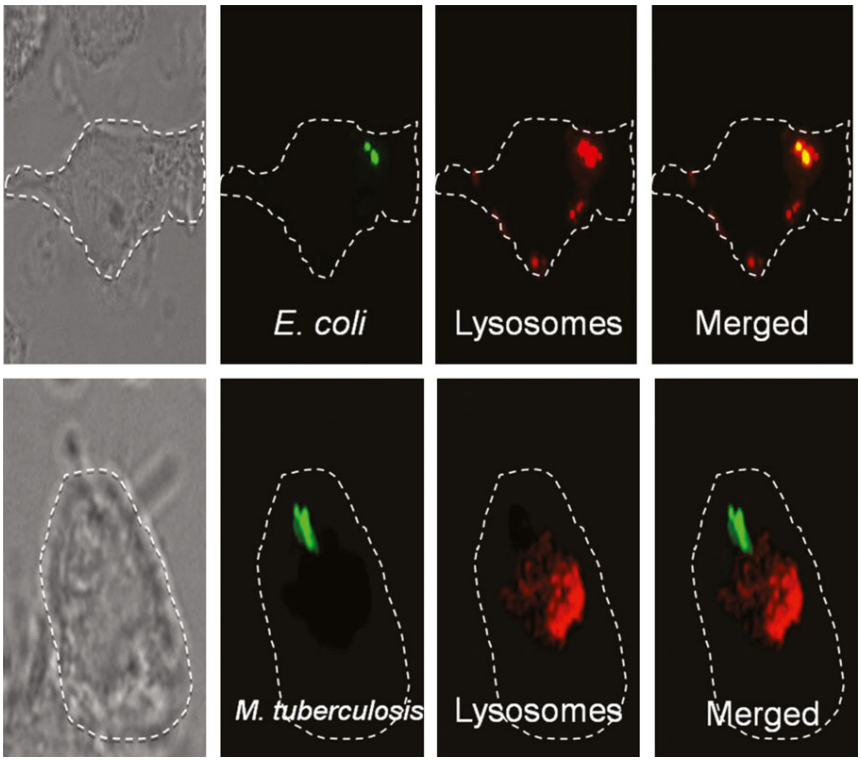

Fig. 2. Confocal microscopy of THP-1 macrophages infected with *E. coli* or Mtb. Localization of *E. coli* or Mtb with lysosomes is detected with immunofluorescence staining. Lysosomes are stained with Lysotracker Red dye (Red), and bacteria are labeled with fluorescein isothiocyanate (FITC) (green) before infection. While Mtb-containing phagosome does not fuse with the lysosomes (Bottom Panel), the *E. coli*-containing phagosome co-localize with the Lysotracker Red dye, indicating phagosome-lysosome fusion event (Top Panel). Courtesy of Dr. Dennis Wong.

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does so mainly by (i) preventing phagosome acidification and (ii) blocking the fusion of the mycobacteria-containing phagosomes with lysosomes. As illustrated in *Fig.* 2, macrophages digesting an invading microorganism go through rapid fusion with lysosomes; however, this is not the case of macrophages that phagocytize Mtb. Approximately 70% of phagosomes containing Mtb do not fuse with lysosomes (41), and some even escape the phagosomal milieu (42). The blockage of phagosome maturation allows Mtb to avoid proteolytic degradation and late immunological events, such antigen presentation required to initiate an adaptive immune response.

Several studies have shown that Mtb actively blocks the phagosome maturation machinery by secreting various macromolecules that interfere with this process. For example, two phosphatases, PtpA and SapM, contribute to the blockage of phagosome maturation (43, 44).

PtpA, a secreted protein tyrosine phosphatase (45, 46), is essential for Mtb pathogenicity within the macrophage (43). Because it is a secreted protein, it was proposed that PtpA's primary substrate is a host macrophage protein (45). Indeed, we identified PtpA substrate to be the vesicle trafficking protein vesicular protein sorting 33B (VPS33B) (43). We used a substrate 'trapping' approach where we replaced the catalytic amino acid, aspartic acid (D126), with alanine residue and incubated the recombinant SDM protein with macrophage cellular extract. This enabled us to capture cognate host substrates. PtpA binding to VPS33B was verified *in vitro* by α-screen and *in vivo* by immunoprecipitation methods, and the activity was determined using combined kinase-phosphatases assays (43). More recently, we confirmed that VPS33B is a genuine substrate for PtpA by using two dimensional gel electrophoresis analysis of macrophage extract treated with recombinant PtpA (47). Furthermore, we were able to identify another catalytic substrate for PtpA, the host signaling protein GSK3α (47).

VPS33B is involved in the regulation of membrane fusion in the endocytic pathway (48). VPS33B dephosphorylation by PtpA leads to phagosome maturation arrest (43). We have shown that the arrest of phagosome maturation is dependent on both active PtpA and intact host VPS33B, using siRNA against the native VPS33B combined with native and mutant forms of VPS33B, introduced by transfection. VPS33B is present in a complex termed the Class C vacuolar protein sorting (VPS) complex. Class C VPS complex binds to the protein subunits VPS41 and VPS39 to form another multimeric protein complex, the homotypic fusion and protein sorting (HOPS) complex, which regulates lysosomal trafficking (49). Class C VPS complex also mediates another large protein superfamily (SNARE) dependent vesicle fusion during phagocytosis (50, 51).

PtpA has been shown to bind to subunit H of the macrophage V-ATPase in a non-catalytic manner (52). As such, PtpA disrupts phagosome pump assembly during early stages of infection (52). The V-ATPase pump is a protein complex that controls phagosome acidification by transporting protons across membranes (53). During phagosome maturation, lysosomes fuse with and deliver the V-ATPase pump to the phagosome. The recruitment of the pump generally results in a reduction in phagosomal pH from 6.5 to approximately 4.5 (54).

The binding of Mtb PtpA to subunit H of the macrophage V-ATPase pump during phagosome maturation blocks phagosome acidification and thus prevents proper elimination of the invading pathogen by the host macrophage (52). This finding provides the mechanistic explanation to a more than two decade long observation showing a lack of acidification in Mtb phagosomes (40). The central role of PtpA in Mtb intracellular pathogenesis has led to industrial and academic efforts to develop new interventions against TB utilizing PtpA as a target for drug discovery programs (55).

The blockage of phagolysosome biogenesis is one of the most important virulence traits described for Mtb (56). Prevention of the fusion between phagosome and lysosomal compartments enables Mtb to avoid exposure to lysosomal hydrolases, and also influences the ability of the macrophage to proteolytically digest Mtb proteins and subsequently load processed peptides in the context of MHC class II molecules (see below). Although Mtb uses multiple ways to inhibit phagosome-lysosome fusion, failure to incorporate proton pumps (40), and the retention of coronin 1 (TACO) on phagosomes, contributes significantly to the inhibitory effect (57). It is widely accepted that phagolysosome biogenesis is crucial for the normal processing and presentation of microbial proteins as antigens to evoke adaptive immunity. In this context, we have shown that a Mtb protein, lipoamide dehydrogenase (LpdC), mediates the retention of coronin 1 on BCG vacuoles leading to phagolysosome fusion arrest (58). As described earlier, we have also shown that PtpA dephosphorylates VPS33B leading to the inhibition of phagosome-lysosome fusion and subsequent antigen presentation (43). Another Mtb protein that seems to be needed for antigen presentation is the mycobacterial zinc metalloprotease 1. Johansen *et al.* (59) showed that infection of murine bone marrow-derived DCs with a mycobacterial mutant deficient in the *zmp1* gene resulted in increased

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presentation of MHC II-restricted antigens. ZMP1 is known to interfere with inflammasome activation leading to defective phagosome maturation and subsequent antigen presentation. Thus, Mtb alteration of the intraphagosomal milieu directly affects antigen presentation activity in macrophages and DCs interacting with subsets of $C4^+$ T cells.

NADPH oxidase-mediated ROS production

Reactive oxygen species (ROS), which were identified over 30 years ago as powerful microbicidal agents against a variety of intracellular pathogens (60), are produced by the phagocyte NADPH oxidase (NOX2). Recently, it has been suggested that ROS are also involved in controlling mycobacterial infection (61). Clinical evidence for the contribution of NOX2 to innate immunity comes from indications of higher susceptibility of chronic granulomatous disease (CGD) patients to opportunistic pathogens (62, 63). These data were confirmed experimentally using mouse models of CGD (61, 64).

The NOX2 complex consists of two constitutively associated transmembrane proteins, $gp91^{phox}$ and $gp22^{phox}$, and four cytosolic subunits, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac1, which is a small GTPase (65). Fully functional NOX2 requires membrane translocation of $p40^{phox}$, $p47^{phox}$, active Rac1 (GTP-bound form) and $p67^{phox}$, and their assembly around $gp91^{phox}$ and $gp22^{phox}$ subunits (66). NOX2 assembly leads to $gp91^{phox}$ activation to generate superoxide through a redox chain by transferring electrons from cytosolic NADPH to phagosomal oxygen (65). The production of superoxide in turn is converted into several other microbicidal molecules, such as hydrogen peroxide and hydroxyl radicals, along with peroxynitrite when combined with nitric oxide radicals (65). While the role of NOX2 in innate immunity is well established, several reports also suggest that it might act beyond the control of intracellular infections to trigger macrophage apoptosis (67, 68), a central event that paves the road to adaptive immunity (69–71).

Previous results from our laboratory identified Mtb nucleoside diphosphate kinase (Ndk) as a GTPase-activating protein (GAP) acting on macrophage Rab5 and Rab7 GTPases, leading to reduced phagolysosome fusion (72, 73). We further found that Mtb Ndk interacts with Rac1 and inactivates it, leading to inhibition of NOX2 assembly. We also established a link between Ndk-dependent NOX2 attenuation and inhibition of apoptosis response to Mtb (*Fig.* 3). Consistent with these findings, Ndk knockdown significantly reduced Mtb survival *in vitro* and *in vivo*. Noteworthy is the finding that another Mtb protein, *nuoG*, is a potential virulence factor operating at the level of NOX2 by mechanisms yet to be defined (74).

Antigen presentation in Mtb-infected macrophages

Attenuation of macrophage antigen presentation to T-helper cells ($CD4^+$ T cells) is one of the main strategies used by Mtb to escape host immune surveillance (75, 76). Antigen presentation to T-helper cells is mediated by surface expression of MHC II loaded with antigenic peptides. This induces T-cell activation and successive secretion of various cytokines leading to enhanced macrophage microbicidal activities and recruitment of pro-inflammatory leukocytes (77). Effector T cells control intracellular infection by secreting cytokines and also through contact-dependent cytolysis of infected cells. In this regard, Srivastava and Ernst (78), used MHC II mixed bone marrow chimeras to compare infection levels of lung myeloid cells that were capable (MHC $II^{+/+}$) or not (MHC $II^{-/-}$) of being recognized by $CD4^+$ T cells. Interestingly, their results showed that (i) MHC $II^{+/+}$ cells had lower bacterial burdens than did MHC $II^{-/-}$ cells and that (ii) $CD4^+$ T-cell depletion increased the number of bacteria associated with MHC $II^{+/+}$ cells but not MHC $II^{-/-}$ cells. These experiments clearly demonstrated that in addition to cytokine-mediated macrophage activation, direct recognition

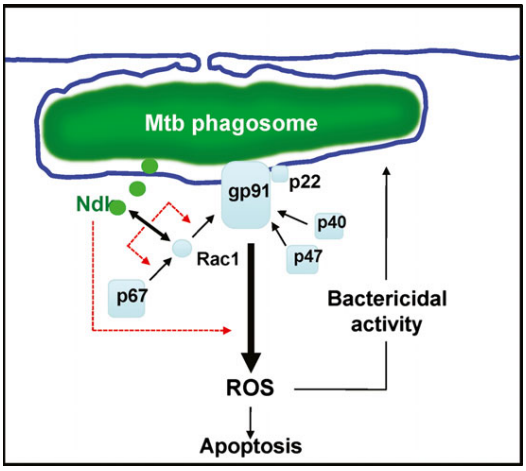

Fig. 3. Mechanism by which Mtb neutralizes NOX2 dependent oxidative burst. Ndk binds to and inactivates the small GTPase Rac1 in the macrophage. This results in the exclusion of the Rac1 binding partner p67(phox) from phagosomes containing Mtb. Exclusion of p67 (phox) is associated with a defect of both NOX2 assembly and production of ROS in response to Mtb. Inhibition of NOX2-dependent ROS production is associated with attenuation of apoptosis in infected cells.

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of infected cells by $CD4^+$ T cells is required for control of intracellular Mtb (78–81). Since the ability of Mtb to evade the host immune response contributes largely to its success as a pathogen, the mechanisms underlying attenuation of macrophage antigen presentation function are of significant interest. Thus, we discuss here key strategies used by Mtb to downmodulate MHC class II-mediated antigen presentation.

Macrophage antigen presentation involves a series of highly organized multi-step events including the identification, uptake, and delivery of the pathogen to the pertinent intracellular compartments followed by the enzymatic processing and loading of the processed antigen into MHC II molecules (82, 83). Loaded molecules then get exported to the cell surface where they present antigenic peptides to the appropriate T-cell subsets (75). Many steps of this highly integrated pathway can be targeted by Mtb to hide its antigen repertoire from being recognized by T-helper cells (84). In fact, Mtb has evolved various strategies to downmodulate the macrophage's ability to process and present antigen to T cells (85–88).

Mtb inhibits MHC II gene expression in the macrophage

Several studies have reported that Mtb-infected macrophages have diminished expression of surface MHC II molecules, decreased antigen presentation, and altered $CD4^+$ T-cell priming (76, 89–91). Some studies suggest that direct downmodulation of MHC II gene expression is the main strategy used by Mtb to inhibit antigen presentation (84). Inhibition of MHC II expression can be achieved by the addition of Mtb lysate to macrophages (92), suggesting that Mtb viability is not necessary to downmodulate antigen presentation. In this regard, purified mycobacterial lipoproteins such as LpqH (93), LprG (94), and LprA (95) strongly inhibit MHC II expression. These lipoproteins act as TLR2 agonists and induce excessive and prolonged signaling that results in the inhibition of MHC II expression. The most characterized of them, LpqH (encoded by *Rv3763*), is a triacylated cell wall lipoprotein (aka 19 kDa lipoprotein antigen), which suppresses the transcription of MHC II transcriptional transactivator (CIITA) through downmodulation of the transcription factor C/EBP (75, 96). These findings are supported by the observation that LpqH is shed from live intracellular mycobacteria into the phagosome and is exported toward the cytosol (97), making it available for chronic TLR2 stimulation and subsequent prolonged MHC II inhibition and antigen presentation. Mycobacterial cell wall glycolipids such as LAM, lipomannan (LM) and phosphatidylinositol mannoside (PIM), also act as TLR agonists (92, 98), which further amplify chronic TLR signaling.

Mtb interference with MHC expression extends beyond the macrophage to reach DCs, which express the highest levels of MHC II and other costimulatory molecules and thus are recognized as the most efficient antigen-presenting cells (99). Indeed, a recent study shows that Mtb uses a serine hydrolase, Hip1, to inhibit MHC II expression in DCs, a phenotype that was reversed in DCs infected with a *hip1* mutant strain (100).

Mtb interferes with maturation and trafficking of MHC molecules

Besides the inhibition of gene expression, other studies showed that Mtb also interferes with the endosomal trafficking and maturation of MHC II molecules, leading to defective antigen presentation (77). Newly synthesized class II α- and β-chains associate with invariant chain (Ii), then exit the endoplasmic reticulum, subsequently localizing to an acidic endosomal/lysosomal compartment referred to as the MHC class II compartment (MIIC) (101). In the MIIC, removal of Ii and peptide loading are critical for appropriate export of peptide-loaded class II molecules to the cell surface (102). The processing of Ii involves a coordinated action of different proteases generating Ii intermediates p22 and p10, down to the class II-associated Ii peptide (CLIP) (101, 103). The CLIP fragment is subsequently exchanged, under the catalytic effect of HLA-DM, with the peptide antigen to be presented at the surface of the macrophage. Our investigations have shown normal steady-state levels of αβ dimers and intracellular sequestration of a large proportion of class II molecules in cells infected with mycobacteria, indicating distal effects on class II expression likely involving intracellular sequestration that prevents transport to the cell surface (85, 104) (*Fig.* 4). We therefore suggested the possibility of defective maturational processing of class II molecules in cells harboring live mycobacteria (85, 104), and thereafter demonstrated that inhibition of MHC II surface expression is related to inhibition of Cathepsin S (Cat S) expression in infected cells (105). Cat S is the most important, if not the only, protease responsible for the late steps in Ii processing to CLIP (106). We showed that reduced Cat S activity is associated with reduced export of mature peptide-loaded MHC II molecules but also increased export of immature MHC II molecules associated with the invariant chain to the macrophage surface. Our study also showed

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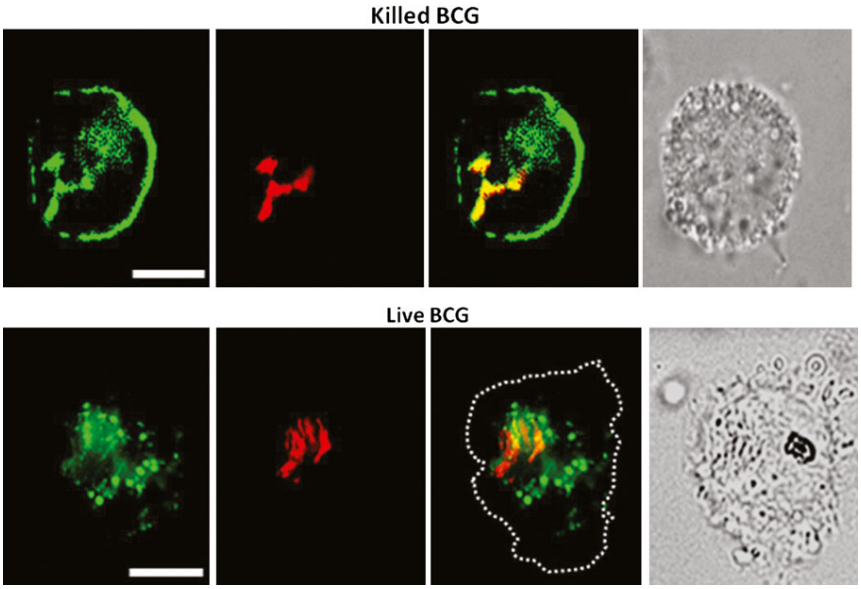

***Fig. 4.* Intracellular accumulation of class II molecules in BCG-infected cells.** Adherent THP-1 were infected with red fluorescent live or killed BCG for 24 h. Cells were then stimulated with IFN-γ for 24 h, fixed, permeabilized, and stained with anti-DR MAb and FITC-labeled secondary Ab. Labeled cells were analyzed with digital confocal microscopy and scanned for green and red fluorescence. The images are displayed in panels with green (HLA-DR), red (bacteria), and yellow signals, the latter depicting co-localization of green with red. Dotted lines represent the cell membrane delimitation deducted from bright-field images. Bar, 10 μm. Reprinted from Sendide *et al.* (104) with permission from the American Society for Microbiology.

that reduction in Cat S gene expression is associated with induction of IL-10 in infected cells (105). Addition of anti-IL-10 antibodies to culture media restored macrophage Cat S expression and the export of mature class II molecules to the surface of infected cells. IL-10 is an anti-inflammatory cytokine produced by Th2 cells (107), which is believed to balance the immune response that restricts Mtb growth by minimizing host-mediated collateral damage of delicate lung tissues (108). However, excessive IL-10 expression might allow Mtb to evade immune responses and mediate severe lung infections. In fact, IL-10 has been shown to be elevated in the lung and serum of active TB patients (109, 110).

In another related study, we examined whether intracellular alkalinization due to mycobacterial urease could account for the defect in intracellular trafficking of class II molecules (104). By using a wildtype BCG strain as a surrogate pathogen and a urease-negative mutant strain (BCG ΔureC), we showed that macrophage infection with wildtype BCG but not BCG ΔureC is associated with secretion of ammonia intracellularly, which increased substantially upon addition of exogenous urea to the culture medium. Increased intracellular ammonia, due to degradation of urea by the bacterium, correlated with inhibition of class II surface expression. A direct cause–effect relationship between urease and class II molecule trafficking was established by showing that digestion of beads coated with urease result in increased ammonia levels and decreased surface expression of class II molecules. This study suggests that alkalinization of critical intracellular organelles by pathogenic mycobacteria is also contributing to the intracellular retention of class II dimers.

To explain some discrepancies between the above findings showing intracellular sequestration of class II molecules and other studies suggesting downmodulation of gene expression, Stewart *et al.* (84) have shown, using well-elaborated mathematical modeling, that modulation of multiple cellular processes (i.e. gene expression and maturational processing) may serve as an optimal strategy for Mtb to maintain continuous inhibition of antigen presentation.

Deciphering mechanisms of intracellular sequestration of class II molecules in infected cells has opened up opportunities for vaccine development. For example, Kauffman's group (111) developed a urease-deficient BCG strain expressing listeriolysin (Hly) of *Listeria monocytogenes* (BCG ΔureC::hly). In addition to increased surface expression of MHC II molecules (increased antigen presentation to T-helper cells) described above, phagosomes containing urease-deficient BCG provide an acidic intraphagosomal pH that allows optimal Hly activity. Activated Hly perforates the phagosomal membrane releasing mycobacterial antigens into the macrophage cytosol, resulting in optimal antigen class I-directed cross-presentation to $CD8^+$ T cells. Consistent with these *in vitro* observations, BCG ΔureC::hly was shown

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to induce significantly better protection than wildtype BCG in murine Mtb challenge model (111, 112). A less characterized vaccine is a BCG strain we engineered to express and secrete an active form of Cat S within the macrophage. This approach emanated from our observation of Cat S inhibition in BCG-infected macrophages (105). The novelty of this approach is in how it forces BCG to compensate for host diminished Cat S expression to reverse the phenotype of attenuated antigen presentation observed in cells infected with BCG. We found that infection with BCG-CatS induces surface expression of substantial levels of mature class II molecules and by doing so stimulates strong macrophage presentation of Ag85B to $CD4^+$ T cells, which is much better than conventional BCG (113).

Autophagy contribution to mycobacterial killing and antigen presentation

Autophagy is a homeostatic and inducible process whereby components of cytoplasm, including organelles and intracellular pathogens, are sequestered in an autophagosome and delivered to the lysosome for degradation (114). Some of the processed material becomes a nutrient source for starved cells (115), and some of it integrates into the pathway of class II-directed antigen presentation (116, 117). Autophagy has also been linked to MHC I mediated antigen cross-presentation (118, 119).

In the case of mycobacterial infection, it has been reported that Mtb impairs autophagy at the step of autophagosome-lysosome fusion through an ESX-1 mediated mechanism (120). Therefore, manipulation of autophagy is regarded as an efficient Mtb survival strategy in infected hosts. In this regard, IFNγ stimulation was shown to induce autophagy along with increased phagosome acidification and fusion with lysosomes leading to efficient control of mycobacterial infections (114, 121). Other studies showed that vitamin D3 treatment, as well as stimulation of TLR4 signaling via Toll–IL-1 receptor domain-containing adapter-inducing IFNβ, also induce autophagy-mediated intracellular killing of mycobacteria (114, 122, 123). In a recent collaborative work with Roberge's laboratory (124), we showed that the antiprotozoal drug nitazoxanide strongly stimulates macrophage autophagy via inhibition of the mammalian target of rapamycin complex 1 (mTORC1) signaling. We also identified the human quinone oxidoreductase NQO1 as a nitazoxanide target and propose that NQO1 inhibition is, at least in part, responsible for mTORC1 inhibition and enhanced autophagy. On the other hand, we also demonstrated that nitazoxanide directly targets Mtb in infected macrophages and inhibits its proliferation. Therefore, these findings open up opportunities to develop improved TB treatment based upon the dual action of nitazoxanide on both the bacterium and the host cell response to infection. In this line of investigation, rapamycin treatment was also shown to overcome the autophagic block induced by Mtb through increased IL-12 expression, which initiates Th1 response. The relevance of autophagy to the protection of humans against TB infection resulted from field observations that IL-1β promoter polymorphism is highly linked to susceptibility to develop TB disease (125). In fact, IL-1β was shown to promote autophagosome maturation and subsequent antigen presentation in Mtb-infected macrophages (126–128).

Given the major role, it plays in eliciting protective adaptive immunity (129), autophagy induction is being examined as a novel strategy to enhance the efficacy of TB vaccines currently under development. In mice immunized with DCs infected with vaccine candidate Δ*fbpA* attenuated Mtb (130) and treated with rapamycin, T-helper cell-mediated protection was induced in response to virulent Mtb (131). The same study showed that enhanced immunogenicity of BCG overexpressing Ag85B is associated with increased macrophage autophagy. Another autophagy-based strategy currently under investigation involves incorporating an autophagy inducing element, the kinase defective mTOR (mTOR-KD) plasmid, and Ag85B into a DNA vaccine that appears to elicit higher production of IFN-γ and IL-2 in the spleen of immunized animal (132). Altogether, these studies suggest that vaccine efficacy can be significantly enhanced by augmenting autophagy-mediated antigen presentation.

Apoptosis and mycobacterial antigen presentation

Apoptosis is a tightly regulated form of cell death where the cytoplasmic contents of the dying cells are confined within membrane-bound structures, called apoptotic bodies, that express the so called 'eat me' signal; phosphatidyl serine on the surface leads to recognition and subsequent removal by professional phagocytes through a process termed efferocytosis (133, 134). Apoptosis was shown to contribute efficiently to intracellular pathogen removal by eliminating the favorable intracellular niche for replication. Moreover, apoptosis initiates efficient antigen cross-presentation leading to an effective immune response against the pathogen (71).

Mtb has evolved various mechanisms to actively block macrophage apoptosis (134–137), and in combination with various host cell subversion strategies, it helps the

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pathogen to gain a solid survival advantage over the host. Thus, by turning on the 'death' mode of the infected macrophage to necrotic fate while avoiding induction of apoptotic mode, Mtb is able to spread into neighboring cells (138). Mtb genes such as *secA2*, *nuoG*, *Ndk*, and *ptpA* have been implicated in blocking apoptosis. SecA2 directs the transport of superoxide dismutase (SodA) out of the bacterial cell. NuoG is a subunit of NADH dehydrogenase type I complex in the mycobacterial membrane. Infection with knockout strains in these genes results in a pro-apoptotic phenotype (70, 139, 140). As mentioned earlier, ingested Mtb encounters a hazardous intracellular environment created by a variety of reactive oxygen and nitrogen intermediates (141). Secretion of SodA bestows Mtb with the ability to detoxify reactive oxygen intermediates known to induce host cell apoptosis if induced at sufficiently high levels. Hinchey *et al.* (70) have shown that inactivation of the *secA2* gene causes impaired superoxide dismutase secretion leading to enhanced apoptosis associated with increased macrophage antigen presentation to $CD8^+$ T cells. Similarly, deletion of the *nuoG* gene in Mtb also reverses the phenotype of attenuated macrophage apoptosis resulting in significantly reduced virulence in mice (139). In this context, a recent study in our laboratory (140) involving protein–protein interaction experiments showed that mycobacterial Ndk is secreted within the macrophage and inhibits apoptosis. We found that Ndk binds to and inactivates Rac1, a small GTPase involved in phagosomal recruitment of NOX2 subunit $p67^{phox}$, leading to defective assembly and function of NOX2. Abolition of NOX2 mediated ROS production resulted in inhibition of apoptosis. Consistent with this finding, knock down of Ndk significantly attenuated survival of Mtb *in vitro* and *in vivo* (140).

We have shown more recently that PtpA also influences macrophage apoptotic responses (47). PtpA dephosphorylates GSK3α on amino acid Y279, which leads to modulation of GSK3α anti-apoptotic activity, promoting pathogen survival early during infection (47).

In experimental settings where macrophage apoptosis can be induced, viability of intracellular mycobacteria is severely attenuated (136, 142), thus shifting the host-pathogen equilibrium in favor of the host. Recent studies show that non-infected macrophages are able to recognize and engulf membrane-bound apoptotic bodies emanating from infected cells and also entire infected cells through a process called efferocytosis (133, 143). Thus, efferocytosis enables the host cells to overcome the phagolysosome biogenesis block exerted by Mtb, leading to mycobacterial killing. In addition to helping eliminate the favorable intracellular environment for pathogen replication, macrophage apoptosis also facilitates mycobacterial antigen cross-presentation through delivery of apoptotic blebs to non-infected DCs, which insure optimal antigen presentation to T cells, a process called 'the detour pathway' (133). This alternate mechanism of antigen presentation was also shown to facilitate DC presentation of mycobacterial lipid antigen through CD1 molecules (69, 75). Given that obstruction of apoptosis subverts macrophage antigen presentation capacity, numerous vaccine strategies currently include a proapoptotic feature in vaccine design and development (144).

Conclusion

TB pathophysiology is highly dependent on the bacilli's ability to subvert the macrophage innate immune response. As outlined above, Mtb uses multiple and even overlapping strategies to hide and replicate within the human host. These strategies include numerous and alternative modes of entry to the macrophage, blockage of phagocytosis biochemical and cellular events, as well as interference with cellular trafficking and immune recognition. Manipulation of the macrophage response to Mtb has been proposed as a promising new avenue for the treatment of infection and development of vaccines. However, these novel strategies face challenges given Mtb plasticity in developing coinciding independent strategies to target the macrophage response to infection. Further studies are needed to determine the best approach to prevent or arrest Mtb's ability to manipulate macrophage responses and enable better methods to combat TB.

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