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Diversity in *M. tuberculosis* mannosylated cell wall determinants impacts adaptation to the host

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Summary

Mycobacterium tuberculosis (the causal agent of TB) has co-evolved with humans for centuries. It infects via the airborne route and is a prototypic highly adapted intracellular pathogen of macrophages. Extensive sequencing of the *M. tuberculosis* genome along with recent molecular phylogenetic studies is enabling us to gain insight into the biologic diversity that exists among bacterial strains that impact the pathogenesis of latent infection and disease. The majority of the *M. tuberculosis* cell envelope is comprised of carbohydrates and lipids, and there is increasing evidence that these microbial determinants that are readily exposed to the host immune system play critical roles in disease pathogenesis. Studies from our laboratory and others have raised the possibility that *M. tuberculosis* is adapting to the human host by cloaking its cell envelope molecules with terminal mannosylated (*i.e.* Man-α-(1→2)-Man) oligosaccharides that resemble the glycoforms of mammalian mannoproteins. These mannosylated biomolecules engage the mannose receptor (MR) on macrophages during phagocytosis and dictate the intracellular fate of *M. tuberculosis* by regulating formation of the unique vesicular compartment in which the bacterium survives. The MR is highly expressed on alveolar macrophages (predominant C-type lectin on human cells) and functions as a scavenger receptor to maintain the healthiness of the lung by clearing foreign particles and at the same time regulating dangerous inflammatory responses. Thus *M. tuberculosis* exploits MR functions to gain entry into the macrophage and survive. Key biochemical pathways and mycobacterial determinants involved in the development and maintenance of the *M. tuberculosis* phagosome are being identified. The phylogenetic diversity observed in *M. tuberculosis* strains that impact its cell wall structure together with the genetic diversity observed in human populations, including those elements that affect macrophage function, may help to explain the extraordinary evolutionary adaptation of this pathogen to the human host. Major developments in these areas are the focus of this review.

Keywords

Tuberculosis; mannose-capped lipoarabinomannan; trafficking; macrophage; mannose receptor

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Conflict of Interest

The authors have not financial and personal conflict of interest.

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Introduction

TB accounts for more than one quarter of all preventable adult deaths in the world[1]. *M. tuberculosis* is an intracellular pathogen that is highly adapted to its natural host; the human. Its major host cell reservoir is the mononuclear phagocyte (monocytes and macrophages). Given its potent microbicidal mechanisms, *M. tuberculosis* has adapted strategies to manipulate the host cell response during and after its entry into the macrophage[2]. The outermost components of the *M. tuberculosis* cell wall, predominately lipids and carbohydrates, are the first to contact host molecular constituents and play a major role in facilitating host cell recognition and modulation of host responses[3].

Virtually all *M. tuberculosis* infections occur by airborne transmission of droplet nuclei containing a few viable organisms. The first interaction between *M. tuberculosis* and the human host takes place in the lung. The respiratory epithelium is actively involved in inflammation and host defense in multiple ways: providing a physical barrier, constituting the structural basis of mucociliary clearance; recognizing pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) expressed on epithelial and myeloid cells, and secreting a variety of pro- and anti-inflammatory mediators [reviewed in [4]]. When *M. tuberculosis* bacilli reach the alveolar space, resident alveolar macrophages (AMs) within the surfactant monolayer along with recruited monocytes, neutrophils and lymphocytes represent the array of immune cells that participate in host defense.

AMs are at the interface between air and lung tissue, and represent the first line of defense against inhaled *M. tuberculosis* [5]. In general, their primary function is the intracellular breakdown and disposal of particulate elements [6,7]. AMs are uniquely positioned within the alveolar surfactant film, the latter of which is composed of lipids and proteins produced by type II epithelial cells [8]. In a normal healthy individual, AMs represent more than 90% of the cells in the bronchoalveolar lavage fluid [9]. Many studies have demonstrated that resident AMs can phagocytose large numbers of microbes through both opsonic and non-opsonic receptors [5,10,11]. Though AMs have high phagocytic and clearance activity, their microbicidal capacity is less well-defined. Efficient microbial phagocytosis followed by slow intracellular killing may be sufficient to control infection by many routinely encountered extracellular pathogens. Intracellular pathogens like *M. tuberculosis*, however, appear to take advantage of the reduced microbial activity of AMs by residing and multiplying within these cells [12,13]. The participation of AMs in host defense, inflammatory processes and immune mechanisms has been amply documented [14].

The importance of host and microbial glycans in health and disease

Glycans play a major role in the host response to infection. Glycosylation produces an abundant, diverse, and highly regulated repertoire of cellular glycans that are frequently attached to lipids and proteins. Decades of research on glycan function have revealed that the enzymes responsible for glycosylation (glycosyltransferases and glycosidases) are essential in the development and physiology of living organisms [15,16]. Glycans participate in many key biological processes including cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis [17]. There is increasing attention being given to the importance of surface glycosylation motifs in microbial pathogens [Reviewed in [18]]. The main structure that defines a bacterium is its cell envelope. Its complexity level differs among bacteria; however, in all cases the cell envelope contains surface-exposed and solvent-extractable non-covalently bound lipids and carbohydrates associated with the peptidoglycan (in the case of gram positive and negative bacteria) or to the mycolyl-arabinogalactan-peptidoglycan complex in the case of mycobacteria [19]. The cell envelope is the microbe armor conferring a relative protection by endowing the microbe

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with innate resistance to therapeutic agents and host defenses. In order for a microbe to survive within the host, it responds to external stimuli by modulating its metabolism and cell envelope to adapt to its surrounding environment. In addition, microbial surface components may be prone to release, shedding, and/or cleavage upon environmental stress (*i.e.* drug treatment). Of particular interest to us are the mannose-containing biomolecules present in the cell envelope of *M. tuberculosis* and how these are implicated in the intracellular survival of *M. tuberculosis* in the macrophage.

The mannosylated cell envelope components of *M. tuberculosis*

The *M. tuberculosis* cell envelope is characterized by the presence of a variety of unique complex lipids, constituting 60% of the bacillus total weight. This lipid-rich low permeability matrix contributes to the difficulty in combating mycobacterial diseases by endowing the organism with innate resistance to therapeutic agents and host defenses. The complex *M. tuberculosis* cell envelope can be divided into two major structures, the cell wall and the capsule-like outermost structures [Reviewed in detail in [20]]. The outermost components are solvent-extractable non-covalently bound free lipids, carbohydrates and proteins associated with the mycolyl-arabinogalactan-peptidoglycan complex (cell wall core) [21]. These surface components may be prone to release, shedding, and/or cleavage upon contact with the host cell or within an appropriate intracellular environment of the cell. The surface of *M. tuberculosis* is particularly rich in mannose-containing biomolecules, including mannose-capped lipoarabinomannan (ManLAM), the related lipomannan (LM), phosphatidyl-*myo*-inositol mannosides (PIMs), arabinomannan, mannan and manno-glycoproteins (Fig. 1). PIMs, LM and ManLAM are incorporated into the plasma membrane and also exposed on the *M. tuberculosis* cell surface [22,20]. They act as ligands for host cell receptors and contribute to the pathogenesis of *M. tuberculosis* [23,24,25,26,27].

Biological functions of *M. tuberculosis* mannosylated cell envelope components ManLAM

One of the most abundant mannose-containing macromolecules of the *M. tuberculosis* cell envelope is ManLAM [19], which is implicated as a key molecule in immunopathogenesis and virulence of the bacterium [19,28,23]. ManLAM is expressed on the *M. tuberculosis* surface [29], and is in an ideal position to mediate interaction between *M. tuberculosis* and phagocytes. In the case of slow-growing mycobacteria like *M. tuberculosis*, *M. leprae*, *M. bovis* BCG and *M. avium* among others, ManLAM is an extremely heterogeneous lipoglycan with a defined tripartite structure: a carbohydrate core (*i.e.* D-mannan and D- arabinan), a mannosyl-phophatidyl-*myo*-inositol (MPI)-anchor and various mannose-capping motifs. These mannose-capping motifs are surface exposed mannooligosaccharides linked to the non-reducing end of the D-arabinan and define the characteristic ManLAM of *M. tuberculosis.* They are not found on LAM from fast-growing mycobacteria which have phospho-*myo*-inositol caps (PILAM) or are uncapped (AraLAM). The mannose caps bind to the macrophage MR and mediate phagocytosis of bacteria by human macrophages [30,24]. ManLAMs from different *M. tuberculosis* strains vary in the degree to which they bind to the MR pointing to a potential relationship between the length and/or presentation of the mannose-caps and their affinity for the MR [25]. The ManLAM caps also bind to DC-SIGN present on dendritic cells [26,27,31]. Thus, terminal components of ManLAM are very important in host cell recognition. Apart from ManLAM, the outermost layer of *M. tuberculosis* also contains manno-proteins (*i.e.* 45KDa) and other major mannose-containing polysaccharides, arabinomannan and mannan, whose mannan structures appear to be identical to that of ManLAM and LM, respectively, except for absence of the lipid anchor [32].

The macrophage immunomodulatory responses to ManLAM differ from those due to AraLAM and PILAM. ManLAM reduces macrophage microbicidal activities by negatively

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modulating the production of nitric oxide, oxygen radicals, inflammatory cytokines and inhibits *M. tuberculosis* induced-apoptosis through altering Ca^{2+}-depending signaling [28,33,34,35]. In contrast, PILAM generally induces pro-inflammatory responses. Using immunoelectron microscopy, ManLAM has been shown to traffic away from the mycobacterial phagosome in dense intracellular vesicles into the membrane-trafficking network of the macrophage[36]; however, this study could not address if it was intact ManLAM or ManLAM metabolites derived from intracellular processing. ManLAM has been found in the MHC class II antigen loading compartment of macrophages where it is loaded onto CD1 molecules for presentation to T cells [37]. It has been suggested that ManLAM undergoes intracellular processing to be accessible to the CD1 binding groove [38]. Lending support to this idea is the recent discovery of a single smaller ManLAM variant with specific structural characteristics that is uniquely involved in the presentation to T cells via CD1 [39].

Following phagocytosis of most bacteria, bacterial phagosomes rapidly mature to phagolysosomes via a series of fusion steps with vesicles of the endolysosomal pathway. In contrast, *M. tuberculosis* modifies the phagosomal environment to support its survival inside macrophages by limiting phagosomal acidification and phagosome-lysosome fusion. Recently, we demonstrated that engagement of the MR by ManLAM directs *M. tuberculosis* to its initial phagosomal niche enhancing its potential for survival in human macrophages [40]. The biochemical mechanisms underlying phagosome-lysosome fusion inhibition and where ManLAM or its metabolite(s) appear to be directly involved are being elucidated [41,42,43]. In this regard, ManLAM blocks the increase of macrophage cytosolic Ca^{2+} and thereby inhibits interaction of the phosphatidylinositol 3-kinase (PI3K), hVPS34, with cytosolic calmodulin, a step necessary for the production of phosphatidylinositol 3-phosphate (PI3P) which, in turn, is required for the recruitment of the Rab5 effector Early Endosome Antigen 1 protein (EEA1) to phagosomes [42]. EEA1, in combination with Syntaxin 6, is necessary for the delivery of lysosomal components from the trans-Golgi network to the phagosome and regulates fusion of phagosomes with vesicles of the endosomal-lysosomal pathway [43]. However, recently, a study showed that ManLAM does not induce the phagosomal maturation block through activation of p38 MAP kinase, contradicting some previous suggestions [44].

Although the effects of ManLAM on phagosome biogenesis are being defined biochemically from the host cell perspective, there is essentially nothing known about the biochemistry of ManLAM itself in this process. For example, is ManLAM or a metabolite(s) shed from *M. tuberculosis* in the phagosome and intercalated into the phagosomal wall via its MPI-anchor? Questions regarding the nature of ManLAM within the host and how it directly participates in regulating vesicular fusion remain unanswered. Our studies using the bead model of ManLAM uptake by human macrophages via the MR show that over time the carbohydrates of ManLAM are increasingly exposed on the cytoplasmic face of the phagosomal compartment, suggesting that ManLAM (or a metabolite) is being physically intercalated within the phagosomal membrane and flipped outside of the phagosomal compartment (Fig. 2A, unpublished results). Our findings are supported by other reports using free ManLAM in human macrophage cultures, where it was incorporated into membrane rafts of the macrophage cell membrane via its MPI-anchor and this incorporation was critical in reducing phagosomal maturation [45,44]. Similarly, studies on human lymphocytes showed that ManLAM localized to membrane rafts of the lymphocyte membrane interfering with signaling pathways and subsequently affecting cytokine production [46]. Thus, further work is necessary to answer the role of intact ManLAM or a metabolite(s) influencing the fluidity of the phagosome membrane and trafficking. Intracellular processing of ManLAM may be a critical step in directing the outcome of *M. tuberculosis* infection. Does ManLAM intracellular processing really occur? Our laboratory

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has begun to address this issue by incubating intact metabolically radiolabeled ManLAM with human macrophage lysates (cytosol + membranes). Our results indicate significant degradation of ManLAM by macrophage enzymatic activities, consistent with the notion that intracellular processing of mannosylated biomolecules present in the cell envelope of *M. tuberculosis* may occur during infection *in vivo* (Fig. 2B, unpublished results). Thus, an important question arises from these studies; does intact ManLAM or its metabolite(s) traffic to the cytosol and engage host cell molecules? These events are likely to influence not only phagosome biogenesis but also a number of immune and metabolic processes of the macrophage.

LM and the PIMs

Other important mannose-containing biomolecules present on the *M. tuberculosis* surface are LM and PIMs. LM is present in all mycobacterial species with structural differences among some pathogenic mycobacterial strains [47,48,49]. Both LM and PIMs regulate cytokine, oxidant and T cell responses [50,51,52]. LM associates with DC-SIGN but not with the MR [53] and induces apoptosis and a pro-inflammatory response through TLR2 [49,54,55,56,57]. PIMs are divided in two distinct groups depending on their number of mannoses. Lower- and higher-order PIMs contain 1 to 4 mannoses and 5 to 6 mannoses, respectively [58]. Lower order PIMs [$PIM_{2\text{ to }4}$] have a terminal α(1→6)-mannose and participate in phagocytosis events through complement receptor (CR) 3 and also facilitate fusion with early endosomal compartments [59,60,61]. Conversely, higher-order PIMs [$PIM_{5\text{ to }6}$] have the same α(1→2)-mono or dimannoside termini characteristic of the mannose-caps of ManLAM and participate in phagocytosis events through the MR limiting phagosome-lysosome fusion events [53]. Importantly, for higher-order PIMs, the degree of acylation is critical for host recognition via the MR, where only triacylated forms of PIM_6 [*i.e.* Ac_1PIM_6] efficiently bind to the MR [53]. Additional studies have shown that processing for antigen presentation via CD1 to T cells [62]. This intracellular sCD1e is involved in PIM_6 is in line with previous studies showing that CD1 loading of biomolecules containing terminal α(1→2)-mannose occurs via the MR [38] and thus highlights the critical importance of both the ManLAM/MR and higher-order PIMs/MR phagocytic pathways in limiting phagosome-lysosome fusion and antigen presentation. Additional studies using different PIM sources and mammalian cell lines expressing DC-SIGN also showed differences in the degree of PIM recognition by DC-SIGN [53,63].

Mannan and arabinomannan

Daffe and colleagues showed that the mannose-containing biomolecules mannan and arabinomannan are exposed on the surface of *M. tuberculosis* forming part of the so called outer material or capsule [64,65]. Although there is no direct evidence for it, capsular mannan and arabinomannan are thought to relate to LM and ManLAM, respectively, following loss of their MPI anchor due to their similar glycosidic structure [33]. Based on antibody recognition assays, the surface expression of arabinomannan appears to change with culture age during bacterial growth *in vitro* but this phenotype seems to be strain dependent [66]. In the same study arabinomannan was shown to be produced by bacteria grown *in vivo*, where the amount of *in vivo*-detected arabinomannan depended on the number of bacteria in the infected organ [66]. In general, arabinomannans appear to be immunosupressive components that can affect macrophage-dependent antigen-induced TH1 cytokine production by human and murine lymphocytes [67,68,69]. Interestingly, a recent study using purified arabinomannans from virulent and non-virulent *M. avium* strains showed that the degree of acylation of arabinomannan (additional acylation independent of the MGI-anchor) is a prerequisite for the effective stimulation of antigen presenting cells [70].

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Mannosylated proteins

M. tuberculosis is reported to produce both acylated and glycosylated proteins [71,72,73,74,75,76]. Among the mannosylated proteins, the most intensely studied are the 19 KDa and 45 KDa proteins. The 19 KDa protein is an abundantly expressed cell wall-associated and secreted glycolipoprotein that has biological activity attributable to its interaction with mammalian Toll-like receptors, especially with TLR2 [77,78]. However, the importance of the glycosylation units on the 19 KDa protein in *M. tuberculosis* pathogenesis is still not clear, since recombinant 19 KDa lacking posttranslational modifications is still capable of generating the pro-inflammatory response described for the native protein [79]. The 45 KDa glycoprotein was first identified as having three distinct glycoforms of 55, 50 and 38 KDa within the culture filtrate proteins of *M. tuberculosis* [71]. Later it was demonstrated that these three bands represented the same 45 KDa protein [80,81]. A clear structure-function relationship for the glycosylation of *M. tuberculosis* proteins is still largely unknown. It is speculated that glycosylation may be involved in protein export through the mycobacterial membrane [82] or that it increases the stability of glycosylated proteins in the intracellular environment in which *M. tuberculosis* normally resides [76] due to the fact that *O*-glycosylated proteins are more resistant to intracellular proteolytic activities [83,84]. The direct contribution of the glycosyl units to the immunoreactivity of *M. tuberculosis* glycoproteins and the role of these glycoproteins in pathogenesis was shown in one study where *M. tuberculosis* recombinant proteins with significant changes in their mannosylation types had little or no ability to elicit a DTH reaction in BCG pre-immunized guinea pigs[85].

Thus, *M. tuberculosis* uses its mannosylated biomolecules to enter macrophages through defined receptor-mediated pathways [reviewed in [86,87]], signals the cell during and potentially after entry, and regulates a number of immune processes. Interestingly, these mannosylated biomolecules are apparently not transported out of the cell like some mycobacterial lipids [88] suggesting that the terminal fate of these mannosylated biomolecules is within the macrophage. However, a recent study using immunoblotting suggested that *M. tuberculosis* and/or *M. bovis* BCG infected macrophages or monocytic cell lines released the 19 KDa protein and ManLAM into the media via exosomes [89]. Such blotting techniques cannot distinguish between intact molecules or those that are processed. A more recent study by the same group showed that the antigen 85 protein is the major protein antigen in the exosomes [90].

Microbial carbohydrates and immunity: The importance of carbohydrate modifications in the host

Several microbes contain surface carbohydrates that are similar in structure to mammalian surface glycoproteins and glycolipids. As a result, these pathogens can evade immune surveillance due to a tolerance towards those structures in their niche [91,92]. The mannose capping of several *M. tuberculosis* surface structures fits this concept. Important conceptually, however, is whether the mannosylation motifs produced on the *M. tuberculosis* surface are different within the macrophage as well as during different stages of tuberculosis. It is well established that interactions with host intracellular enzymes and carbohydrates can lead to modification(s) of other microbial surface carbohydrates [93].

Immunologically active complex biomolecules like the ones present on the *M. tuberculosis* surface are also present on the surface of many other pathogens. Two examples of such molecules are gram-negative bacterial LPS and the lipophosphoglycan (LPG) of *Leishmania spp*. Evidence for intracellular modifications has been reported for both. A unique macrophage acyloxyacyl hydrolase (AOAH) detoxifies LPS by selectively removing two of its six fatty acyl chains [94]. Enzymatically deacylated LPS is about 0.2–1% as potent as an

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endotoxin as fully acylated LPS, as demonstrated in cell activation assays *in vitro* [95] and in an assay of tissue toxicity *in vivo* [96]. LPS deacylation, which occurs over several hours post internalization, is inhibited by agents that reduce lysosomal (endosomal) acidification, suggesting that LPS moves at least transiently into an acidic intracellular compartment [97]. Thus, enzymatic deacylation of LPS by AOAH is an intrinsic, regulated mechanism by which the macrophage may modulate host responses to this potent bacterial agonist [98]. This is not the case for the *M. tuberculosis* mannose-containing biomolecules like ManLAM, LM and PIMs, where their MPI-anchor is resistant to the action of AOAH (unpublished results). However, this does not preclude the involvement of other intracellular hydrolases in the deacylation of these molecules.

LPG is a key determinant of *Leishmania* invasion into macrophages and survival in vertebrate and invertebrate environments [99]. LPG undergoes specific trafficking inside infected macrophages [99]. Antibodies against LPG display a broad band of 50 to 110 KDa, however, in lysates of macrophages infected for 24 h with *Leishmania major*, an extra band of 21 KDa is detected indicating LPG processing within the host cell [99]. In addition to LPS and LPG, zwitterionic capsular polysaccharides from *B. fragilis* are processed and presented in the context of MHC class II proteins without a protein or peptide carrier and these metabolized polysaccharides activate CD4+ T cells [100]. The requirement for processing glycolipid antigens in T cell recognition has been shown for the disaccharide glycosphingolipid Gal-(α1→2)-GalCer, which involves a lysosomal α-galactosidase and lipid transfer proteins known as saposins [101,102]. Glycosphingolipid intracellular processing was later shown to be further enhanced by the addition of anionic lipids into substrate carrying liposomes [103]. Thus, macrophages not only process phospholipids, protein and RNA, but they can also process various carbohydrates including mannosylated biomolecules and this processing appears to be important in *M. tuberculosis* pathogenesis although more work needs to be done in this area.

Little is known regarding the potential of macrophages to modify the covalent structure of mycobacterial lipid antigens. Current evidence suggests that either exogenous (*i.e.* taken up by phagocytosis or endocytosis) or endogenous (*i.e.* produced by intracellular *M. tuberculosis*) lipid antigens can enter the CD1 antigen metabolic processing route and be presented to CD1-restricted T cells. A recent study showed that soluble CD1e allows for the intracellular processing of *M. tuberculosis* glycolipids with a large carbohydrate component [62]. This study provides evidence that mannosylated-glycoconjugates biosynthetically related to ManLAM (*i.e.* PIM_6) are processed intracellularly to smaller-size components (*i.e.* PIM_2) prior to CD1 presentation to T cells, indicating that carbohydrate degradation or editing of *M. tuberculosis* glycolipids and lipoglycans is important in enhancing immunogenicity. Whereas for some mycobacterial lipid antigens (*i.e.* trehalose-6,6′-dimycolate) intracellular processing appears to be less important for antigen loading to CD1 [104], for ManLAM, which has a very large, bulky polysaccharide component, enzymatic processing in the macrophage appears to be critical in order to reduce the antigen to a smaller core structure for optimizing antigen presentation [37,38]. It is unknown whether intracellular processing plays a role in enabling two different *M. tuberculosis* antigens to limit phagosome-lysosome fusion events (*i.e.* ManLAM and Ac_1PIM_6) [40,53]. Even less is known about *M. tuberculosis* carbohydrates and lipids produced endogenously, *i.e.* within the phagosome, however, interaction between host and pathogen metabolic pathways provides a mechanism for the immune response to pathogenic mycobacteria that have productively infected tissues [105].

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Importance of mannose on the surface of the *M. tuberculosis* cell envelope: A concept

M. tuberculosis mannosylated biomolecules like ManLAM are key microbial virulence determinants in the *M. tuberculosis*-macrophage interaction. Great efforts are being made by several laboratories to resolve the complicated biosynthetic pathways that involve ManLAM, LM and PIMs production [reviewed in [106]]. Several studies, including ours, have shown that altering the presence of mannose on the surface of *M. tuberculosis* has relevant biological consequences. This is the case for PimB since disruption of this mannosyltransferase decreases surface exposed ManLAM and LM by ~60% and results in faster intracellular replication and increased macrophage death [107]. Conversely, increasing surface mannosylation of *M. smegmatis* by over-expressing ManB, a phosphomannomutase involved in the biosynthesis of GDP-mannose (a major mannose donor in ManLAM biosynthesis), results in a greater association of mycobacteria with human macrophages in a mannan-inhibitable fashion [108]. The essentiality of mannose on the mycobacterial cell envelope is also supported by other studies where it is speculated that mannose-containing biomolecules have a critical role in regulating septation and cell division without perturbing other pathways of lipid biosynthesis [109]. Although these studies support the importance of mannose on the surface of *M. tuberculosis* for host recognition, other studies have pointed out that this may not be the case for other mycobacterial species. For example, using *in vitro* and *in vivo* models, Dinadayala et al. reported that a mutation in Rv1635c, the gene responsible for the mannose capping of ManLAM in *M. tuberculosis* [110], did not attenuate *M. bovis* BCG [111]. Similarly, another study showed that *M. bovis* BCG lacking surface exposed mannose did not influence the immune response [63]. Thus, these studies indicate that the relative importance of surface mannose varies among mycobacterial species. In the case of *M. tuberculosis* there is evidence that surface mannosylated biomolecules play a critical role in the recognition, intracellular survival and nature of the immune response of the bacillus in the host. In this context, we recently showed that clinical isolates of *M. tuberculosis* deficient in surface mannosylation were defective in phagocytosis by primary human macrophages when compared to the heavily mannosylated standard laboratory strains (*i.e. M. tuberculosis* $H_{37}R_v$ and Erdman strains) although those bacteria that did enter macrophages had a short doubling time under some conditions [112].

Our recent studies [40,53] have led to the conclusion that *M. tuberculosis* is adapting to the human host by cloaking its cell wall molecules with terminal Man-α [1→2]-Man oligosaccharides that resemble the glycoforms of mammalian mannoproteins [113] (Fig. 3A). Continued efforts to define the molecular events in the early interaction between *M. tuberculosis* and the human macrophage are necessary to further our understanding of the immunopathogenesis of TB and disease outcome. To identify a relationship between a group of clinical isolates of a distinct genetic lineage of *M. tuberculosis* and their phenotype with regard to cellular interactions is a first step to understanding how *M. tuberculosis* is evolving to adapt to the human host. Phylogenetic studies have grouped clinical isolates that were found associated with large cluster outbreaks [114] in geographical areas of high TB incidence [115]. Some of these clinical isolates are hypervirulent in animal models [116,117,118] and better able to bypass the protection afforded by the BCG vaccine [119]. They may represent “ancestor strains” [120] within distinct phylogenetic lineages that have evolved in genetic isolation with little effective horizontal gene transfer [114,115]. We have studied a few strains from these phylogenetic groups and found that some within the principal genetic group (PPG)-1 [120] have a marked reduction in macrophage phagocytosis. This reduction resulted from significant alterations in *M. tuberculosis* cell envelope components as determined at the molecular level (limited exposed mannose and the presence of phenolic glycolipid and triacylglycerols) that impacted recognition by macrophage receptors and bacterial

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intracellular survival [112]. We have speculated that these clinical isolates may be less adapted to the human host (more prone to disease development following infection). We propose a new model for the phagocytosis and host response of *M. tuberculosis* strains, where the amount and nature of mannose exposed on the surface are major determinants (Fig. 3). *M. tuberculosis* strains with less surface mannosylation do not use the MR during phagocytosis by the human macrophage. Such strains have reduced phagocytosis, relying primarily on C3 opsonization and the more primitive CR3 pathway for entry. These strains are "hypervirulent" in part due to the presence of other surface exposed cell envelope components [*i.e.* phenolic glycolipid and triacylglycerols] [121,122], which regulate the cytokine response, and demonstrate rapid intracellular growth and marked tissue damage [116,117]. Conversely, *M. tuberculosis* strains with abundant mannose on their surface have become more host-adapted in part by increasing surface mannosylation with mannans that resemble the glycoforms of eukaryotic mannoproteins that are normally removed from circulation by the homeostatic macrophage MR to maintain a healthy state [113]. In support of this concept, *M. tuberculosis* was recently found to contain a mammalian mannosyltransferase homologue [123]. Thus, more host-adapted *M. tuberculosis* strains may expose a large and heavily mannosylated ManLAM and greater amounts of higher-order PIMs that bind to the MR and other C-type lectins. Such strains are optimized in phagocytosis by cooperatively engaging the MR and complement receptors. Use of the mannose-containing biomolecule/MR pathway provides a safe portal for *M. tuberculosis* within the macrophage by regulating the trafficking of bacteria and cytokine response. These strains grow more slowly in the macrophage and cause less tissue damage during infection. We speculate that such host-adapted strains would be highly successful in establishing an infection in humans but would more likely lead to the latent state rather than to an active disease state following infection. The concept that *M. tuberculosis* has evolved by increasing surface mannosylation as an adaptation to the host has support, but will require additional investigation. However, using biochemical and immunologic approaches, it is now possible to categorize genetically defined groups of *M. tuberculosis* for the potential relationship between strain genotype and disease phenotype.

A final intriguing concept is the potential importance of the content and location of mannose present in different mycobacterial species. GDP-mannose has been described as the universal donor for the biosynthesis of mannose-containing biomolecules in mycobacterial species. Interesting to us is the knowledge that fast-growing, non-pathogenic mycobacteria contain cytosolic methylated-mannose polysaccharides (MMPs). Conversely, to date, the production of these polysaccharides in slow-growing mycobacteria like *M. tuberculosis* has not been reported [reviewed in [124]]. Rather, pathogenic mycobacteria contain cytosolic methylated-glucose polysaccharides (MGPs) [125,126]. Thus, the relative amount of mannose retained and/or sequestered in the cytosol *versus* that available for building mannosylated biomolecules in the mycobacterial cell envelope may also be an important evolutionary attribute. How GDP-mannose is used is likely to be linked to the species-specific expression of carbohydrate biosynthetic enzymes in different environments. In this context, there are still many unanswered questions about the location and functionality of the mannose-containing biomolecules produced during infection *in vivo*.

Based on the ideas raised in this review, we propose a model (Fig. 3B) whereby mycobacterial strains differentially mannosylate their surface with α (1→2)-Man*p* oligosaccharides mimicking eukaryotic glycosidic forms. This mannosylation depends on the amounts of cytosolic GDP-Man and polyprenol-phosphate-mannose (PPM) and the presence of the required glycosyltransferases to construct the mannose-containing biomolecules located on the surface of pathogenic mycobacterial strains. Only non-pathogenic mycobacterial species produce cytosolic MMPs. However, the role of these biomolecules is still unclear. It is possible that GDP-mannose produced by non-pathogenic

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mycobacteria is used mainly by its mannosyltransferases to produce lower-order PIMs and MMPs, the latter serving to store/sequester cytosolic mannose. This storage may limit the amount of PPM produced which serves as the mannose donor for mannosyltransferases involved in the production of cell envelope mannosylated biomolecules (*i.e.* higher-order PIMs, LM and ManLAM). The accumulation of lower-order PIMs and MMPs in non-pathogenic mycobacteria may also be related to the efficiency of their *ppm1*/*ppm2* complex to generate PPM. Heavily mannosylated strains are optimally phagocytosed by human macrophages using both the MR and complement receptors resulting in an intracellular bacterial survival program that favors latency. In contrast, other *M. tuberculosis* strains have a cell envelope characterized by poor surface mannosylation and the presence of other virulence determinants such as phenolic glycolipid and triacylglycerols. These strains do not associate with the MR, but instead with CR3 and other receptors that lead to the induction of progressive lung pathology and a poor protective TH1 response [118]. These strains have a "hypervirulent" phenotype which favors progression from latency to active TB disease (Fig. 3B).

Despite numerous reports regarding the effects of mannose-containing biomolecules on the immune response of macrophages, the precise structural motifs that mediate these responses remain largely unclear. These molecules have been studied mostly with *in vitro* systems, particularly with rodent cells, which differ in many respects from human cells. Further biochemical characterization of these molecules, including their production and metabolism inside macrophages and tissues, will further our understanding of their interactions with macrophages and their role in immunopathogenesis. Further definition of carbohydrate production and processing pathways *in vivo* that impact the immune response will also aid in the development of new molecular targets for diagnosis, therapy and vaccine development.

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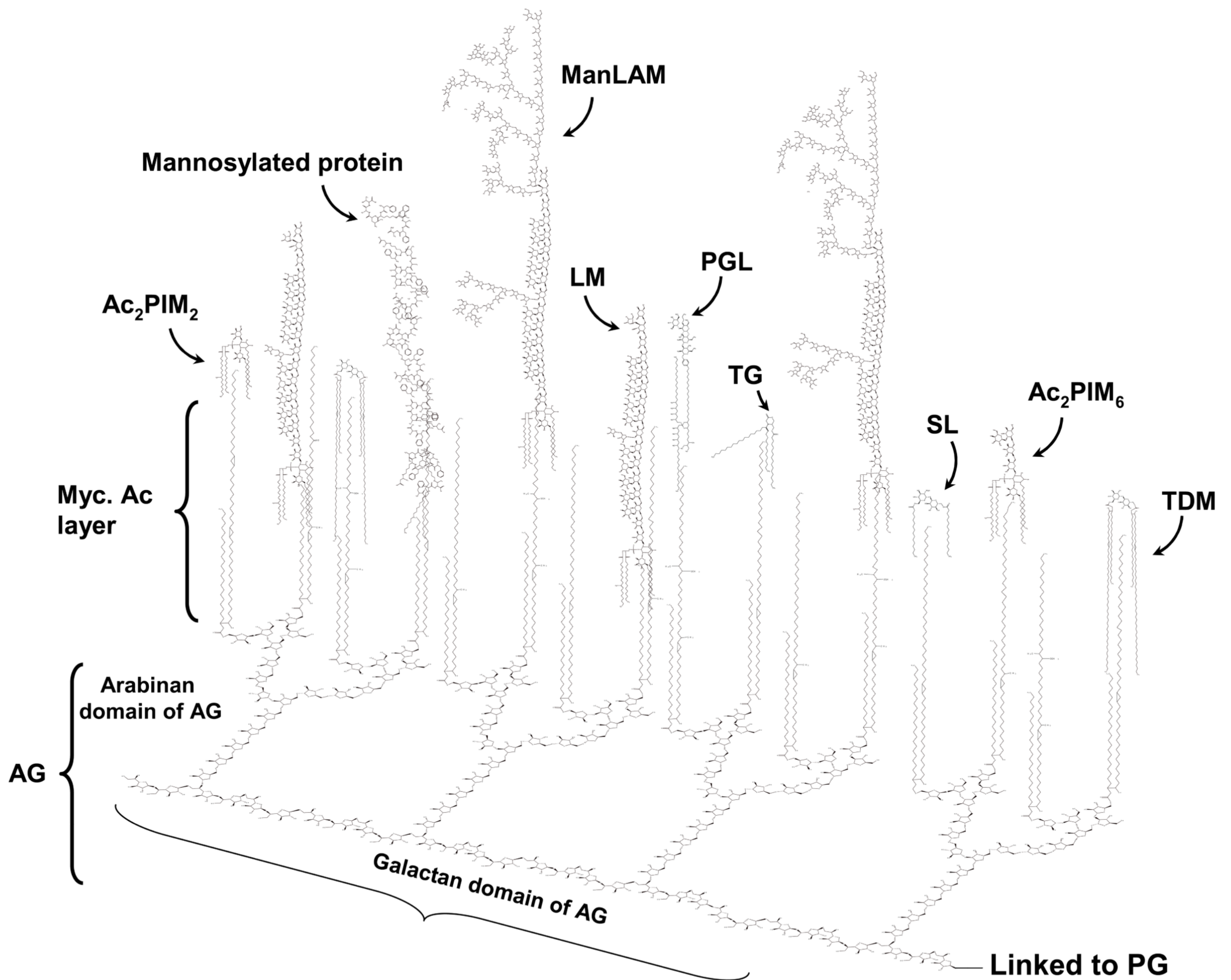

Figure 1. The cell envelope of *M. tuberculosis* with an emphasis on exposed mannosylated cell envelope components

This scheme depicts the cell envelope "skeleton or core" determinants (mycolyl-arabinogalactan-peptidoglycan complex) and emphasizes the distribution of intercalated major mannosylated cell envelope components that are exposed on the *M. tuberculosis* surface. AG is covalently linked to PG via the galactan chain and the arabinan chain is in turn linked to the mycolic acids (Myc Ac) which are shown perpendicular to the plasma membrane. The polar groups (*i.e.* carbohydrate domains) of several mannosylated cell envelope components are exposed on the cell surface and their lipid domains are intercalated with the Myc Ac acid layer. These envelope components include ManLAM, LM, higher- and lower-order PIMs, and lipomannoproteins. Other known virulence factors described for *M. tuberculosis* that interact with the Myc Ac layer [*i.e.* TDM, SL; and TGs and PGL, the latter on some *M. tuberculosis* strains)] are also depicted. Not all Myc Ac are depicted interacting with cell surface components. Not shown are capsule-like components (*i.e.*, arabinomannan, glucan, mannan, and xylan). In order to maintain simplicity, molecular quantities depicted (relative number of molecules) do not accurately reflect experimental data. AG (arabinogalactan); PG (peptidoglycan); Myc Ac (mycolic acids); ManLAM (mannose-capped lipoarabinomannan); LM (lipomannan); PIMs (phosphatidyl-*myo*-inositol

mannosides); TDM (trehalose dimycolate); SL (sulfolipid); TGs (triglycerides); PGL (phenolic glycolipid).

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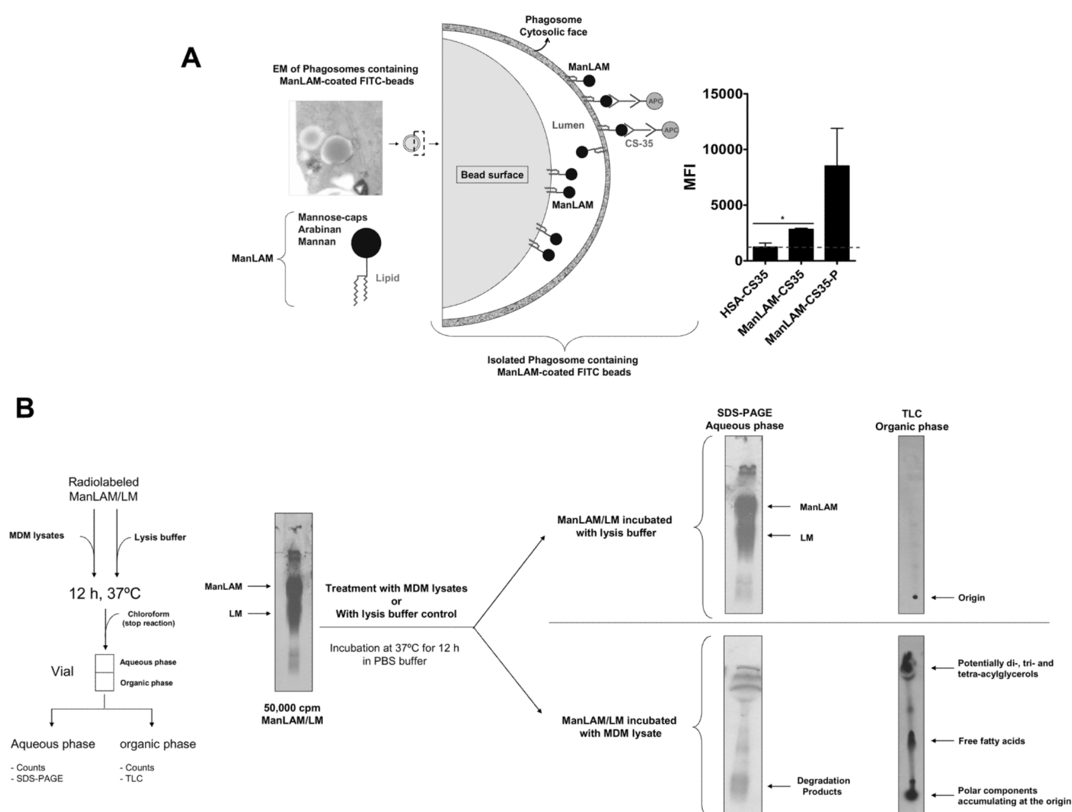

Figure 2. Phagosomes contain ManLAM exposed on their cytosolic face

A. Purified phagosomes containing ManLAM-coated or human serum albumin (HSA)-coated (control) FITC positive beads were incubated with anti-ManLAM (CS-35) or mouse IgG for 20 minutes, washed, stained with secondary anti-mouse IgG -APC and analyzed on a LSRII flow cytometer. A subset of phagosomes was permeabilized (P) using reagents from BD Pharmingen (Fix/Perm kit) and stained and analyzed as described above for the non-permeabilized phagosomes. Analysis was performed using DIVA software. Gates were set around FITC positive phagosomes and MFI determined for the presence of ManLAM using anti-ManLAM antibody (IgG control antibody was subtracted out). HSA-CS-35: phagosomes-containing HSA-coated FITC beads stained with anti-ManLAM mAb (CS-35); ManLAM-CS-35: phagosomes containing ManLAM-coated FITC beads stained with CS-35; ManLAM-CS-35-P: permeabilized phagosomes containing ManLAM-coated FITC beads stained with CS-35. Mean ± SEM from 2 independent experiments, $^*p<0.05$, T-test using GraphPad Prism v.4.01 software. The EM photomicrograph on the left shows ManLAM-coated bead phagosomes within a macrophage prior to phagosome purification (× 40,000). Based in these results, the depicted scheme shows ManLAM leaving the bead and intercalating into the phagosome inner leaflet by its lipidic domain, to later become exposed on the phagosome cytosolic leaflet through yet an unknown mechanism. **B** Effects of enzymatic activities derived from human macrophages on ManLAM/LM. Radiolabeled ManLAM/LM (50,000 cpm) was incubated with lysis buffer (PBS) or with human macrophage lysate (cytosol and membranes in PBS) for 12 h at 37°C. Results show that ManLAM/LM treated with lysis buffer alone remained intact [SDS-PAGE and TLC (chloroform:methanol, 96:4, v/v)]; whereas ManLAM/LM treated with MDM lysate was hydrolyzed into smaller metabolites that migrated to the organic layer after chloroform-water partition as revealed by SDS-PAGE and TLC. Shown is a representative experiment of n=3.

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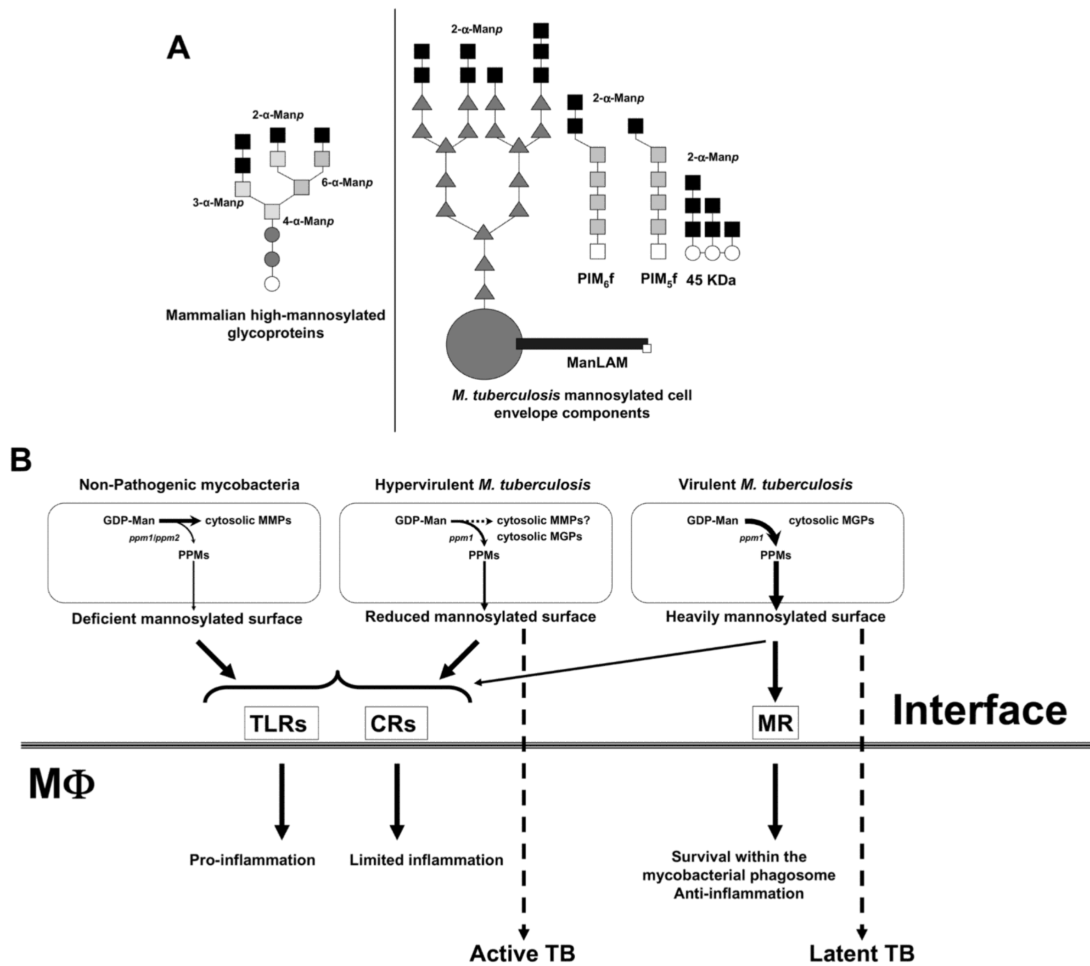

Figure 3. Mannosylated biomolecules present on the surface of *M. tuberculosis* and their contribution to pathogenesis

A. *M. tuberculosis* strains decorate their surface with various amounts of α (1→2)-Man oligosaccharides mimicking eukaryotic glycoforms that are normally removed from circulation by the homeostatic macrophage MR to maintain a healthy state. Among these bacterial mannose-containing biomolecules are ManLAM, LM, PIMs, arabinomannan, mannan, and several glycoproteins (*i.e.* 19 KDa and 45 KDa among others). **B.** A scheme depicting the contribution of *M. tuberculosis* surface mannosylation in directing *M. tuberculosis* to a pathway/niche for intracellular survival within human macrophages by limiting phagosome-lysosome fusion events and down-regulating the inflammatory response of the host. Details are found in the text. It is unknown whether the *M. tuberculosis* hypervirulent strains produce MMPs. CRs (complement receptors); MGPs (methyl glucose polysaccharides); MMPs (methylated mannose polysaccharides); MR (mannose receptor); MTs (mannosyltransferases) PPMs (polyprenol-phosphate-mannoses); *ppm1*/*ppm2* (polyprenol phosphate mannose syntheses); TLRs (Toll-like receptors).