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Review

A paucity of knowledge regarding nontuberculous mycobacterial lipids compared to the tubercle bacillus

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ABSTRACT

All mycobacteria, including nontuberculous mycobacteria (NTM), synthesize an array of lipids including phosphatidylinositol mannosides (PIM), lipomannan (LM), and lipoarabinomannan (LAM). While absent from *Mycobacterium tuberculosis* (*M. tb*), glycopeptidolipids (GPL) are critical to the biology of NTM. *M. tb* and some NTM also synthesize trehalose-containing glycolipids and phenolic glycolipids (PGL), key membrane constituents with essential roles in metabolism. While lipids facilitate immune evasion, they also induce host immunity against tuberculosis. However, much less is known about the significance of NTM-derived PIM, LM, LAM, GPL, trehalose-containing glycolipids, and PGL as virulence factors, warranting further investigation. While culling the scientific literature on NTM lipids, it's evident that such studies were relatively few in number with the overwhelming majority of prior work dedicated to understanding lipids from the saprophyte *Mycobacterium smegmatis*. The identification and functional analysis of immune reactive NTM-derived lipids remain challenging, but such work is likely to yield a greater understanding of the pathogenesis of NTM lung disease. In this review, we juxtapose the vast literature of what is currently known regarding *M. tb* lipids to the lesser number of studies for comparable NTM lipids. But because GPL is the most widely recognized NTM lipid, we highlight its role in disease pathogenesis.

1. Introduction

Tuberculosis (TB) remains one of the top ten causes of death worldwide. While active cases of TB are required to be reported to public health agencies, providers are not mandated to report infections due to nontuberculous mycobacteria (NTM); hence, the prevalence and deaths directly attributable to NTM lung diseases (NTM-LD) are likely to be grossly underestimated. A further complicating attribution is that species identification of mycobacteria is typically not performed in most parts of the world – evinced by the fact that a number of presumed TB cases have been shown to be due to NTM instead [1]. According to the American Lung Association, it is estimated that there are 100,000 people currently living with NTM-LD in the United States (U.S.). Between 1997 and 2007, the annual NTM prevalence for elderly individuals more than 65 years old increased 8.2% yearly and the annual prevalence of NTM-LD remains 10–100 times more than tuberculosis [2,3]. Moreover, NTM-LD cases are increasing globally [4,5] and associated with significant morbidity and mortality (*e.g.* 2990 deaths in the U.S. from 1999 to 2010) [1,6,7].

Due to its undeniable status as a global infectious disease threat, clinical, academic/research, and industry entities have historically invested unprecedented time, money, and energy to elucidating the biology, pathogenesis, and treatment for TB. However, now that the increase in NTM-LD cases have exceeded TB in many areas of the developed world, we believe a directional shift towards understanding the biology of NTM organisms is necessary to prevent new infections and reduce disease-associated mortality. To start, prior knowledge of the biochemistry of the cell envelope constituents of *Mycobacterium tuberculosis* (*M. tb*) and the techniques previously optimized and applied for TB-specific lipid studies should be applied to gain a better understanding of the NTM cell envelope and strengthen targeted drug development for NTM-LD. Herein, we review the current literature on

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mycobacterial cell wall lipids, their roles in disease pathogenesis, immune evasion, and induction of host immunity against *M. tb* and NTM as well as highlight the knowledge gap of the latter.

2. Differences in *M. tb* and NTM biology

Mycobacteria have unique cell wall components that differ from the typical Gram-positive and Gram-negative organisms, making mycobacteria resistant to acid-alcohol decolorization and their attribution as "acid-fast" bacteria. NTM species are generally divided into two categories: rapid-growing (RGM) and slow-growing mycobacteria (SGM). RGM such as *Mycobacterium abscessus, Mycobacterium chelonae,* and *Mycobacterium fortuitum* typically produce visible growth within one week [8]. Other than the RGM, all other NTM including SGM typically take two or more weeks to grow [9]. Despite being "slow-growing," these bacteria show high metabolic activity, synthesize a complex array of lipids, as well as a variety of phosphorylated lipid forms, that are fundamental to the normal function of the cell wall and organism viability. Using specialized biosynthetic pathways, mycobacteria dedicate more time and effort to cell wall synthesis and division compared to other types of bacteria [10]. Replication rates may contribute to the innate resistance of NTM to antibiotics [11]; however, a recent study showed no correlation between the generation rate of *Mycobacterium smegmatis* and its susceptibility to ethambutol (EMB), isoniazid (INH), or pyrazinamide (PZA) [12].

3. Mycobacterial cell wall and spanning lipids

The mycobacterial cell envelope is a complex structure comprised of the plasma membrane and a cell wall consisting of lipids containing fatty acids and lipoproteins (Fig. 1). The mycolyl-arabinogalactan-peptidoglycan (mAGP) structure spans the cell wall creating an insoluble core skeleton comprised of mycolic acids (MA) linked to arabinogalactan (AG) anchored to a layer of peptidoglycan (PPG) superficial to the plasma membrane [14]. mAGP plays an important role in the overall architecture and impermeability of this specialized cell wall. MA and their homologs, comprised of 2-alkyl, 3-hydroxy long chain fatty acids, are sine *qua non* structures of mycobacteria that maintain cell structure and form serpentine-like cords; hence, mycobacteria are also known as "cord-formers" [15]. Since MA are not found in humans, MA-targeting drugs such as isoniazid are effective against *M. tb*; however, among the clinically-relevant NTM, isoniazid is only recommended for *Mycobacterium kansasii* [16]. The high amounts of hydrophobic MA in the cell wall also contribute to the low permeability of mycobacteria to exogenous hydrophilic agents such as antibiotics, glucose, and glycerol [15]. MA are either unbound or esterified to the terminal penta-arabinofuranosyl units of AG forming the outer layer of the *M. tb* cell wall [15]. The heteropolysaccharide AG, comprised of arabinose and galactose, represents approximately 35% of the total cell envelope. AG is positioned between the plasma membrane and the outer MA layer and covalently links with the PPG layer to form an ultra-large polymer. Mycobacterial PPG forms the inner layer foundation of the cell wall and consists of alternating residues of β-(1,4) linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM), a structure referred to as the glycan chain [17]. PPG is the robust yet flexible mesh-like structure whose function is to surround, protect, and maintain bacterial integrity, withstand osmotic pressure, and anchor protein complexes and lipids [17]. The mycobacterial cell envelope also possesses a periplasmic space that separates the mAGP complex from the plasma membrane, as well as an outer capsular layer composed of glucans, proteins, and a modest amount of lipids.

The mycobacterial cell envelope also harbors additional components including acyl lipids such as trehalose 6,6′-dimycolate (TDM) as well as porins. Thus, the mAGP along with the plasma membrane and other constituents of the cell wall provide a lipid-rich, low-permeability barrier that contributes, in part, to the ability of mycobacteria to survive and colonize harsh environments [18]. In fact, the cell wall of *M. chelonae* and *M. smegmatis* are, respectively, 20 and 30 times less permeable to hydrophilic molecules than *Escherichia coli* [10]. Although small hydrophilic antibiotic drugs, such as β-lactams, gain access to the interior of mycobacteria using porins, the ability of most other small, hydrophilic agents to enter the cell is extremely low due to the high specific activity of porin molecules [19,20]. It is therefore necessary to understand both the physical and chemical properties of the

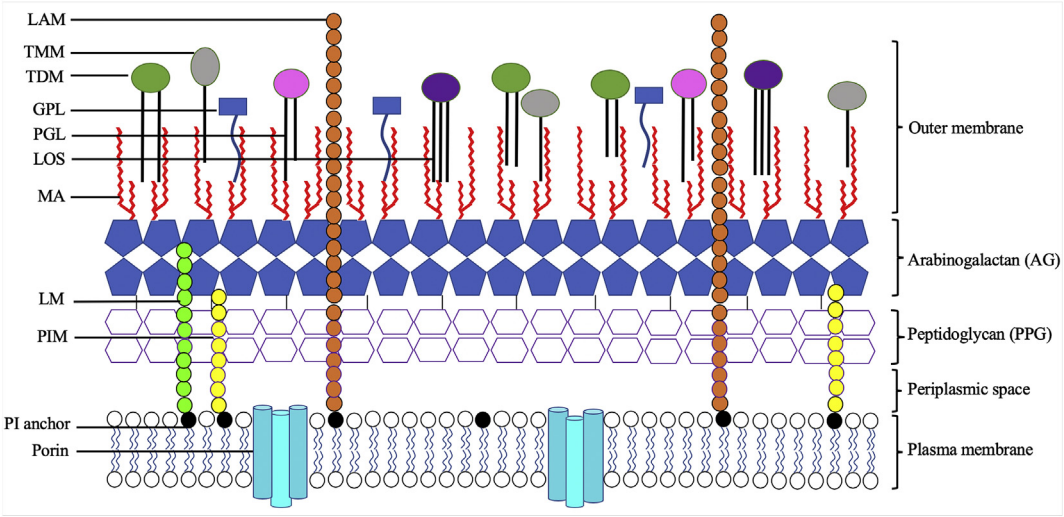


Fig. 1. Cartoon of the mycobacterial cell wall. The mycobacterial cell wall is comprised of parallel arrangement of mycolic acid (MA) chains (red) linked to middle layer of arabinogalactan (AG, blue hexagon) which in turn covalently attaches to the inner layer of peptidoglycan (PPG, purple hexagon). MA, AG, and PPG together form the mAGP complex which provides structure and contributes to the impermeability of mycobacterial cell wall. mAGP complex is separated from the lipid bilayer plasma membrane by a periplasmic space. The major lipids, phosphatidylinositol (PIM, yellow), lipomannan (LM, light green), and lipoarabinomannan (LAM, orange) are attached to the plasma membrane through a common phosphatidylinositol (PI) anchor (black). Other outer membrane/surface exposed-glycolipids include phenolic glycolipid (PGL, pink) and lipooligosaccharides (LOS, purple). Additional components such as trehalose 6,6′-dimycolate (TDM, dark green), trehalose 6-monomycolate (TMM, grey), porins (light blue), and glycopeptidolipid (GPL, blue rectangle) are shown. Outer capsular layer, which varies considerably among the mycobacteria, is not shown. Drawing not to scale. Adapted from Ref. [13]. MarvinSketch (ChemAxon) was used to generate the chemical structures in this and subsequent figures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mycobacterial envelope components and how these factors facilitate invasion and survival in the human body.

4. Role of mycobacterial lipids in immune evasion

Scattered among the mAGP macromolecule layer are phosphatidylinositol (PI), phosphatidylinositol mannosides (PIM), lipomannan (LM), lipoarabinomannan (LAM), trehalose-based lipooligosaccharide (LOS), phenolic glycolipids (PGL) and in the case of NTM, glycopeptidolipids (GPL) that are non-covalently attached to the mycobacterial plasma membrane. These lipids show widely different glycosylation patterns and are involved in various biological activities including cellular adhesion and signaling, but also act as ligands in cellular receptor-mediated uptake of mycobacteria [21]. More specifically, the hexamannosylated PIM_6 of *Mycobacterium bovis* shows high affinity binding to the dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), a major C-type lectin receptor on dendritic cells [22]. Whether NTM contain homologous PIM_6 molecules that also bind DC-SIGN is unknown. There is accumulating evidence that PI/PIM, LM, LAM, LOS, and PGL also serve as important virulence factors for *M. tb* and NTM [23]. PIM, LM, and LAM molecules exposed on the bacterial surface can be released extracellularly, acting as immune decoys while inhibiting mycobacterial uptake by macrophages [21,24].

Favoring for survival against host immune responses, pathogenic *M. tb* inhibit phagosome maturation, apoptosis, and cytokine production – activities attributed, in part, to its bacterial-specific lipids [25]. Herein, we tabulate and dissect each of the major *M. tb* lipids as pathogenic determinants and also include information for comparable NTM lipids.

5. Mycobacterial phosphatidylinositol (PI)

PIM, LM, and LAM are major lipids bound to the mycobacterial plasma membrane through a common PI moiety with different glycosylation patterns at C2 and C6 (Fig. 2, **black arrows**). The PI of mycobacteria and eukaryotes are similar consisting of a diacylglycerol backbone and a phosphodiester linkage to an inositol ring – a 6-carbon polycyclic alcohol. PI inositol assumes a "chair" conformation with five of its six –OH groups being equatorial at carbon positions 1, 3, 4, 5, and 6, and axial at carbon 2 [27]. Numerous studies show that PI is phosphorylated at its 3, 4, or 5 positions (Fig. 2) while serving as important regulatory molecules in eukaryotic cells [26] and marking cellular organelles and membranes for subsequent trafficking events [26,28,29]. PI additionally serves important roles in intracellular signaling by acting as a substrate for modifying enzymes including PI3-kinase, phosphatases, and phospholipases [26].

Mycobacterial PI inserts into the plasma membrane and maintains viability [18,21]. In fact, when PI concentrations and concentrations of PI synthases (enzymes that synthesize PI) decrease, mycobacterial viability concomitantly reduces indicating their essentiality to.

Mycobacterial survival, but also reveal them as potential targets for drug development [30]. PI also plays an important role during infection by mediating the binding of *M. tb* to macrophages because in the absence of PI, *M. tb* demonstrates decreased binding [21]. However, more studies are needed to understand and identify the mechanisms by which mycobacterial PI influences host immunity in addition to describing the activities of the enzymes involved in PI synthesis to improve their applicability as potential therapeutic targets [27]. While the aforementioned data focused on *M. tb*, very little data are available regarding the role of PIs in NTM biology. Overall, we found nine published studies of *M. tb* PI compared to one general study for both *M. tb* and NTM PI (Fig. 12).

6. Mycobacterial phosphatidylinositol mannoside (PIM)

PIM is found in both the inner and outer membranes of the mycobacterial cell envelope (Guerin et al., 2009; [23]. PI that is covalently attached to mannose residues at carbon positions 2 and 6 of the inositol moiety comprise PIM (Fig. 3, black arrows). When hyperglycosylated, PIM functions as the biosynthetic precursor of LM and LAM [23]. In *M. bovis,* PI and PIM represent approximately 56% of all phospholipids in the cell wall and approximately 37% of those in the plasma membrane [31]. PIM can also intercalate into endomembrane organelles within infected macrophages to interfere with phagosome maturation [26,32] and enhance fusion between early endosomes and phagosomes [10].

PIM with one mannose residue form phosphatidylinositol monomannoside (PIM_1), but can possess up to six mannose residues to form phosphatidylinositol hexamannoside (PIM_6) and up to four acyl chains [33], with mono- and diacylated phosphatidylinositol dimannoside (Ac_1PIM_2 and Ac_2PIM_2) and $AcPIM_6$ (acylated PIM_6) being the predominant species (Fig. 3, left). PIM_2 carries two mannose residues – each at C2 and C6, while PIM_6 carries six mannose residues – one at C2 and five at C6 (Fig. 3). In the biosynthesis of Ac_1PIM_6, Ac_2PIM_6, LM, and LAM, PIM_2 and its other acylated versions, Ac_1PIM_2 and Ac_2PIM_2, are generally considered to be both the intermediates and metabolic end products [23,33] (Fig. 4). PimA and PimB mannosyltransferases (MST) catalyze the addition of the first and second mannose residues, respectively [23]. Acyltransferases that catalyze the addition of acyl chain as well as other MST that catalyze the formation of higher PIM remain to be discovered [33].

PimA MST initiates the biosynthesis of PIM_1, by transferring a GDP-mannose to C2 of the PI inositol ring (Fig. 4, green arrow) [33]. The PimB MST catalyzes the transfer of the second GDP-mannose to C6 of the PIM_1 inositol ring giving rise to PIM_2 (Fig. 4, orange arrow). It is suggested that the initial steps of catalyzing the additions of mannose residues occur on the cytoplasmic side of the plasma membrane because

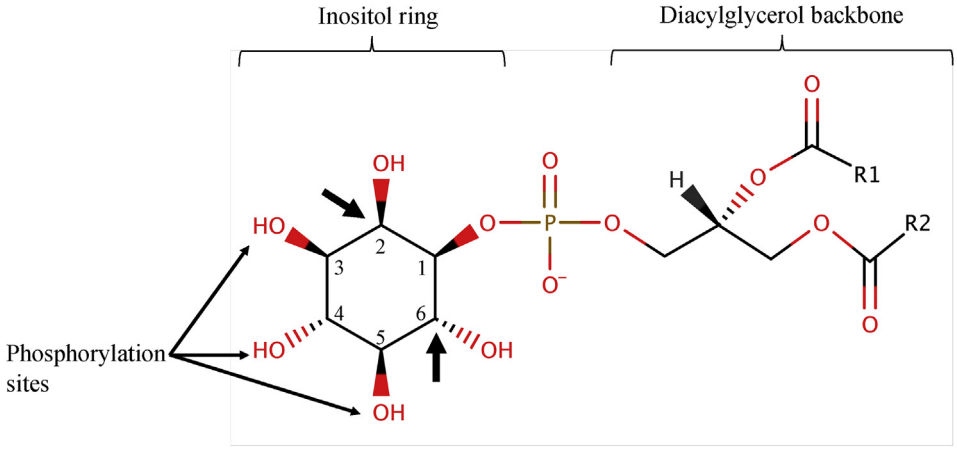


Fig. 2. Chemical structure of phosphatidylinositol (PI). PI consists of a diacylglycerol backbone that is phosphodiesteried to an inositol with five of its six –OH substituents being equatorial at C1, 3, 4, 5, and 6, and one remained being axial at C2. Carbon atoms 3, 4, and 5 are alternative phosphorylation sites. Additionally, different glycosylation can occur at C2 and C6 (black arrows). Adapted from Ref. [26].

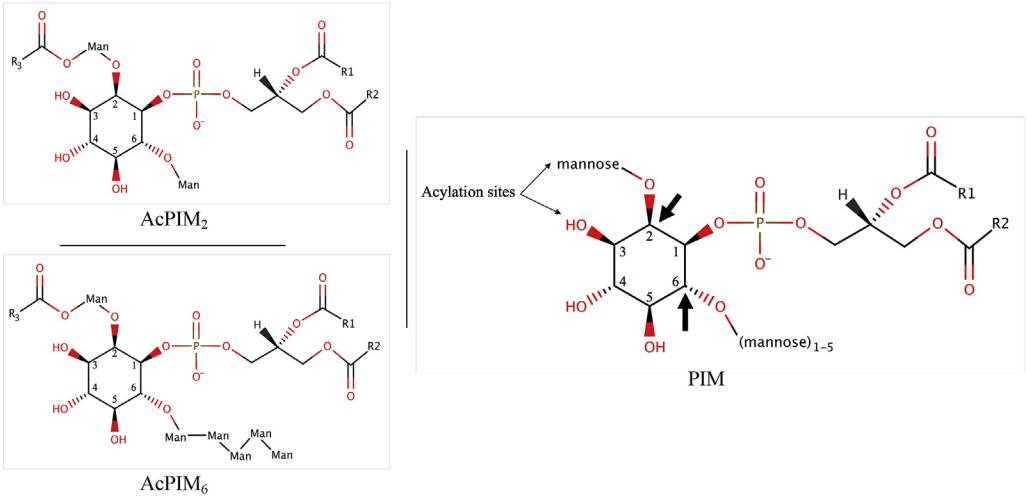


Fig. 3. Chemical structure of phosphatidylinositol mannoside (PIM). PIM has the identical core structure as PI, but with mannose residues covalently attached to carbon positions 2 and 6 (black arrows). Additional acyl chains can be added to the 2-OH and/or 3-OH inositol ring to form $AcPIM_2$/Ac_2PIM_2 and $AcPIM_6$/Ac_2PIM_6. Adapted from Ref. [23].

both PimA and PimB utilize the soluble sugar nucleotide GDP-mannose and lack recognizable signal sequence in their gene sequences [23]. PIM_1 and PIM_2 are acylated to form Ac_1PIM_1 and Ac_1PIM_2 by acyltransferase AcylT1 [33]. The acylation occurs at C6 of the mannose ring transferred by PimA (Fig. 4, blue arrow). The –OH of C3 of the inositol ring can be further modified with an addition of acyl chain to form Ac_2PIM (Fig. 4, pink arrow). Boldrin et al. demonstrated that: *(i)* four enzymes participate in the synthesis of PIM – PgsA1, PimA, PimB, and AcylT and are essential for *M. smegmatis in vitro*, *(ii)* a strong correlation between undetectable PimA levels and growth arrest of *M. tb*, and *(iii)* a drastic decrease in *M. tb* viability when PimA is depleted suggesting PimA is essential for bacterial replication and viability. A third mannose unit can be added onto the growing PIM_2 to form PIM_3 by PimC [34]. The initial steps of PIM, LM, and LAM biosynthesis are similar, however, the biosynthesis of LM and LAM diverges from that of $AcPIM_6$ at $AcPIM_4$. PimE (MSMEG_5136) commits AcPIM4 to the AcPIM6 pathway. Deletion of PimE in mutant *M. smegmatis* strain showed a complete absence of $AcPIM_6$, while it had no apparent effects in LM and LAM biosynthesis [35].

From the drug development perspective, PimA may be an appealing anti-mycobacterial target since there is now greater understanding of the biochemistry and biological significance of PimA for growth and pathogenesis of mycobacteria and the lack of a human homolog [36]. The demonstration of the importance of PIM in facilitating the survival of mycobacteria in host cells was largely based on reports regarding *M. tb* and *M. smegmatis*. While we found 17 prior studies related to PIM of *M. tb*, we found only two PIM studies for NTM, one each for *M. smegmatis* and *Mycobacterium abscessus* (Fig. 12).

7. Mycobacterial lipomannan (LM)

LM is structurally similar to PIM but contains 21–34 mannose residues and is a precursor of LAM (Fig. 5). $AcPIM_4$ was identified as the attachment point for the synthesis of the mannan backbone, a common

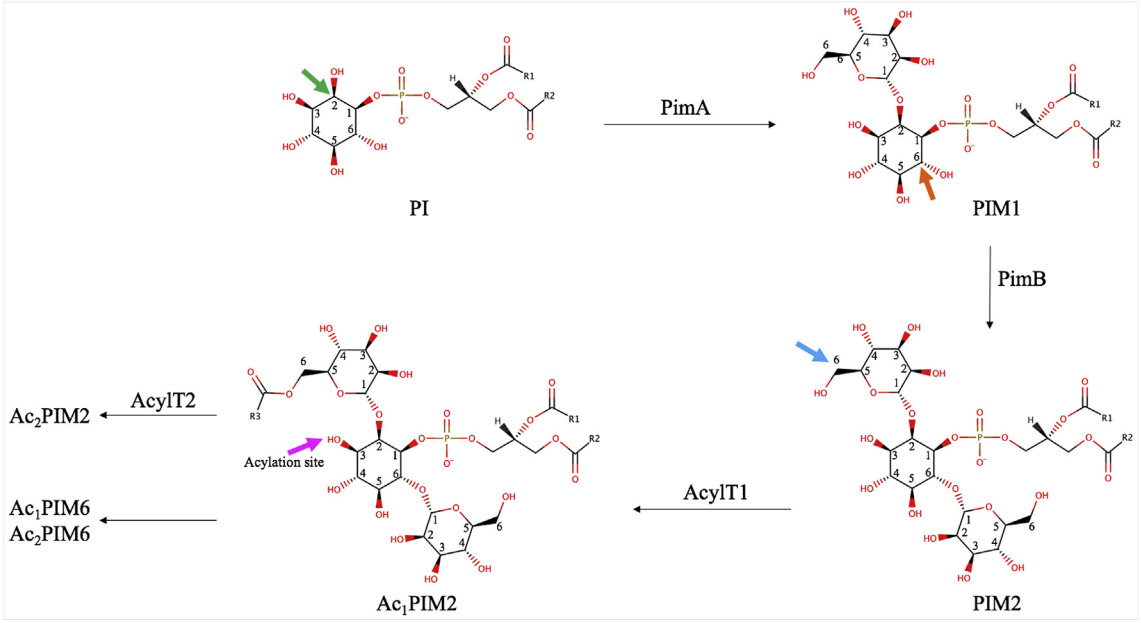


Fig. 4. Biosynthesis pathway of phosphatidylinositol mannoside (PIM). PimA mannosyltransferase (MST) catalyzes the addition of first mannose residue to C2 of the PI's inositol ring forming PIM_1 (green arrow). PimB MST catalyzes the transfer of second mannose residue to C6 of the PIM_1's inositol ring giving rise to PIM_2 (blue arrow). PIM_2 and PIM_6 are acylated by acyltransferase AcylT1 to form Ac_1PIM_2 and Ac_1PIM_6 at C6 of the mannose ring that was transferred by PimA, only Ac_1PIM_2 pathway was shown. Second acyl chain can be added at the hydroxyl group of C3 of the inositol ring (purple arrow). Adapted from Refs. [23,33]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

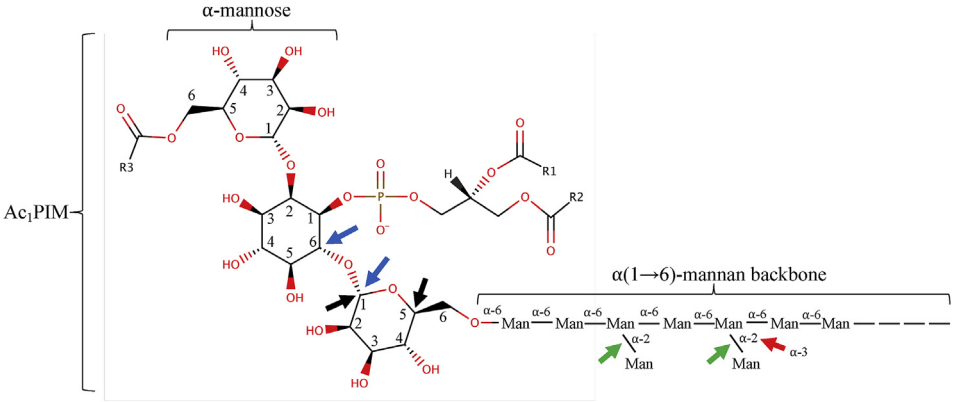


Fig. 5. Chemical structure of lipomannan (LM). LM is structurally similar to PIM but longer with additional ~26–44 mannose residues. Ac_1/Ac_2PIM_2 serves as the attachment point for the synthesis of mannan backbone. The mannan backbone consists of 21–34 α(1 → 6) linked mannopyranosyl (Manp) residues and decorated by 5–10 α(1 → 2) linked Manp units (green arrows) leading to the formation of LM. LM of *M. chelonae* distinctly shows α(1 → 3) linked Manp residues (red arrow). Adapted from Ref. [37]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

component in both LM and LAM [38]. The mannan backbone consists of 21–34 α(1 → 6) linked mannopyranosyl (Manp) residues decorated by 5–10 α(1 → 2) linked Manp units (Fig. 5, green arrows) leading to the formation of lipomannan (Fig. 5) [34,37]. The α designate substituents on C1 and C5 that lie on opposite sides of the mannose ring's plane (*trans* arrangement) (Fig. 5, black arrows) and 1 → 6 designates the two carbons that participate in the glycosidic bond linking the two monosaccharides (Fig. 5, blue arrows). In contrast to LM of *M. tb*, and most other mycobacteria such as *Mycobacterium leprae* and *M. smegmatis*, LM of *M. chelonae* distinctly shows α (1 → 3) linked Manp residues (Fig. 5, red arrow) [37].

For the LM and LAM biosynthesis pathway, it was identified that the synthesis of mannan backbone is performed by elongating α(1 → 6)-MST (MSMEG_4241) and branching α(1 → 2)-MST (MSMEG_4247) [35]. Deletion of MSMEG_4241 resulted in an LM intermediate with 5–20 Manp residues compared to the wildtype LM with 21–34 Manp residues suggesting the presence of another unidentified elongating α(1 → 6)-MST involved in the mannan extension. Deletion of MSMEG_4247 led to the formation of branchless LAM and the complete absence of LM [35]. Additionally, LM and LAM were found to be absent from a mutant strain of *M. smegmatis* (MSMEG_4245) defective in a conserved gene for a putative glycosyltransferase suggesting MSMEG_4245 is a key MST for proper elongation of the mannan backbone during the biosynthesis of LM, as well as subsequent synthesis of LAM [38]. Further studies are required to examine the effects of these mutated MST on the mycobacterial growth, survival, and ability to invade host immune system.

LM elicits a strong proinflammatory cytokine response from host cells, including IL-12 production in macrophages, and apoptosis of macrophage cell lines [39]. Other studies utilizing bone marrow-derived macrophages from MyD88-and Toll-like receptor (TLR)-deficient mice show that LM from several mycobacterial species, including *M. chelonae*, *M. kansasii*, and *M. bovis* perform a dual function by both stimulating and inhibiting pro-inflammatory cytokine production via different pathways [40]. The pro-inflammatory effect of LM with TNFα and IL-12 production is mediated by MyD88 and TLR2 while the anti-inflammatory effect of LM on lipopolysaccharide (LPS)-induced TNFα production was independent of TLR2 and MyD88 and likely mediated through other pattern recognition receptors [40]. More detailed characterization of LM's pro- and/or anti-inflammatory properties as well as the pathways involved in transmitting the signals is likely to provide better insights into the immunomodulatory signals induced during infection with pathogenic mycobacteria. These studies provide evidence that LM carry structural motifs that interact with different host recognition receptors. Similar to prior lipids discussed, more published studies were found for *M. tb* LM (n = 5) compared to NTM LM (n = 1) (Fig. 12).

Future studies of mycobacterial LM motifs with a concerted effort to learn more about NTM LM will likely reveal important differential determinants of virulence, allowing for new drugs designed to target LM-modifying enzymes.

8. Mycobacterial lipoarabinomannan (LAM)

LAM is essentially a “mature” LM that is heavily glycosylated with extensively branched D-arabinan and D-mannan chains [37] (Fig. 6). Structural defects in the LM and LAM of *M. smegmatis* results in the loss of acid-fast staining, increased sensitivity to β-lactam antibiotics, and faster elimination by human THP-1 macrophages [35]. The arabinan polymer of LAM consists of a linear backbone of α (1 → 5)-linked arabinofuranosides with branched hexa-arabinofuranosides and linear tetra-arabinofuranosides [37,41] (Fig. 6). LAM is classified into three groups based on structural differences in the capping motifs present in the arabinosyl side chains' non-reducing termini (Fig. 7A–C). The first group – ManLAM – is characterized by the presence of mannosyl caps on the distal ends of some arabinan polymers and is present in pathogenic species such as *M. tb, M. kansasii, Mycobacterium avium,* and *M. leprae*, but also in opportunistic species such as *Mycobacterium marinum* [42–45] (Fig. 7A). The second group, designated PILAM, is present in RGM such as *M. smegmatis* that have branches of arabinan with terminal phosphor-myo-inositol caps [46] (Fig. 7B). Arrest of the branching enzyme MSMEG_4250 of *M. smegmatis,* which functions to add α(1 → 2) branches to the mannan backbone, hindered formation of native LM and LAM and altered growth [47]. The third group, AraLAM, is a LAM molecule that lacks both mannosyl and phosphor-myo-inositol caps and identified in *M. chelonae* (Fig. 7C) [37].

LAM exerts various biological activities as a virulence factor and immune modulator [39]. As a virulence factor, LAM interferes with phagosome procurement of late endosomal constituents [37]. TNF-α secretion from peritoneal or bone marrow-derived macrophages are abrogated when LAM is deacylated by strong base treatments, indicating that the fatty acyl groups associated with PI components of LAM are critical for activity [41]. As an immune modulator, LAM plays a significant role in the downregulation of innate immune response to *M. tb* and acts as a ligand in the interaction between *M. tb* with antigen presenting cells (Chatterjee & Khoo; [48]. Additionally, LAM has the capacity to affect host macrophages and dendritic cells by both inhibiting and stimulating apoptosis and IL-12 production, as well as inducing nitric oxide (NO) production [39,49,50]. As an anti-inflammatory molecule, ManLAM inhibits the production of IL-12, TNF-α, and apoptosis induced by bacterial infection or LPS stimulation, and increases IL-10 production by dendritic or monocytic cell lines [39,40]. In contrast, PILAM acts as a pro-inflammatory molecule by stimulating the production of TNF-α and IL-12 [39]. ManLAM activates macrophages by binding to the mannose receptor, whereas the pro-inflammatory effects of PILAM occur through NF-κB activation [40]. As a result, structural differences of LAM impact the ability of different mycobacterial species to change the pro- and/or anti-inflammatory

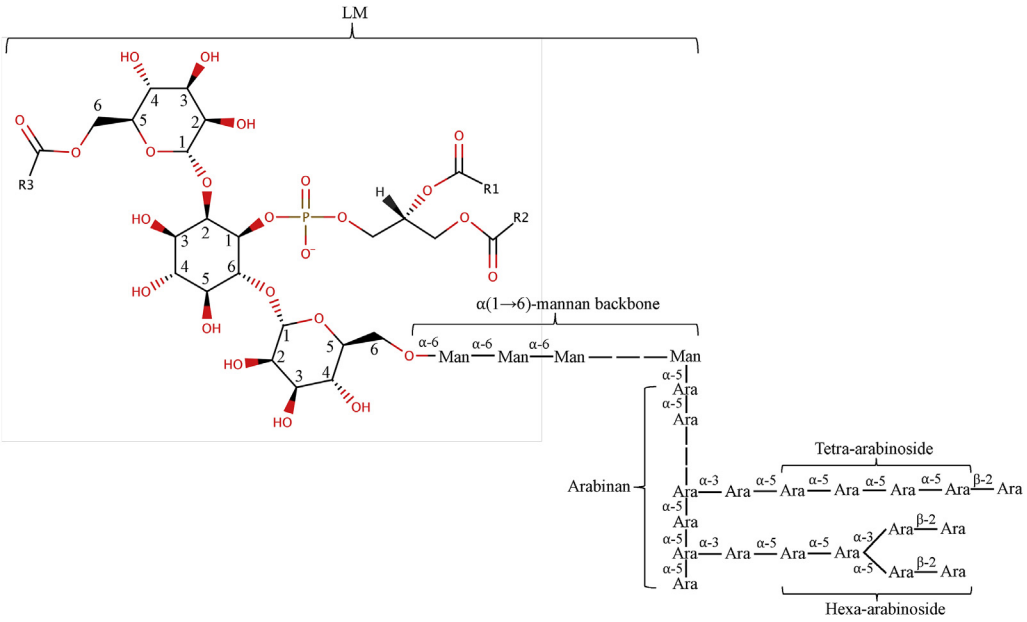


Fig. 6. Chemical structure of lipoarabinomannan (LAM). LAM has same core structure as LM but is modified with an arabinan and mannose or phosphate inositol capping structures. LAM is essentially a "mature" LM glycosylated with arabinan. The arabinan polymer of LAM consists of a linear backbone of α(1 → 5)-linked arabinofuranosides with branched hexa-arabinofuranosides and linear tetra-arabinofuranosides. Adapted from Ref. [37].

response on macrophages and dendritic cells [39].

Together PIM and LAM play important roles in intracellular signaling and trafficking in mammalian cells, as well as in the biogenesis and maturation of phagosomes. The viability of *M. tb* in phagocytic cells relies on the balance between the ability of the bacilli to arrest phagosome maturation and the need to access nutrients while residing in macrophages [26,51]. PIM stimulates fusion between phagosomes and early endosomes *in vitro* [26]. As a result, mycobacterial vacuoles gain access to the nutrients within endosomes, including iron that is critical for intracellular survival of *M. tb*. In contrast, LAM inhibits effectors that normally promote delivery of acid hydrolases, thus inhibiting phagosome maturation [8]. EEA1, an endosomal tethering molecule recruited by phosphatidylinositol 3-phosphate (PI3P), is necessary for the formation of phagolysosomes by connecting vesicles to their target membranes [26]. However, LAM inhibits the recruitment of EEA1 to the phagosome, interrupting the transport of lysosomal hydrolases and ATP-dependent proton pumps, resulting in phagosome maturation arrest [26]. Hence, maturation arrest of phagosomes by LAM prevents *M. tb* degradation in host macrophages [52]. Although the LAM-mediated block of phagosome maturation is considered an advantage to the bacteria, recruitment of tethering molecules (*e.g.,* EEA1) necessary for mycobacteria to acquire nutrients are reduced. However, the stimulatory effect of PIM on early endosomal fusion most likely surpasses the negative effect of LAM, generating a shunt for fusion with early endosomes [26]. These findings demonstrate the virulence roles of PIM and LAM of *M. tb* in macrophages, but whether NTM PIM and LAM results in similar effects is unknown.

While we found 24 studies related to LAM of *M. tb*, we found only one *M. avium*-specific LAM study (Fig. 12). This study showed that ManLAM has the highest affinity for the carbohydrate recognition domain of human mannose-binding protein, followed by LM, PIM, AraLAM, and TDM. To date, no other work has been conducted to analyze the types and structures of LAM in NTM species.

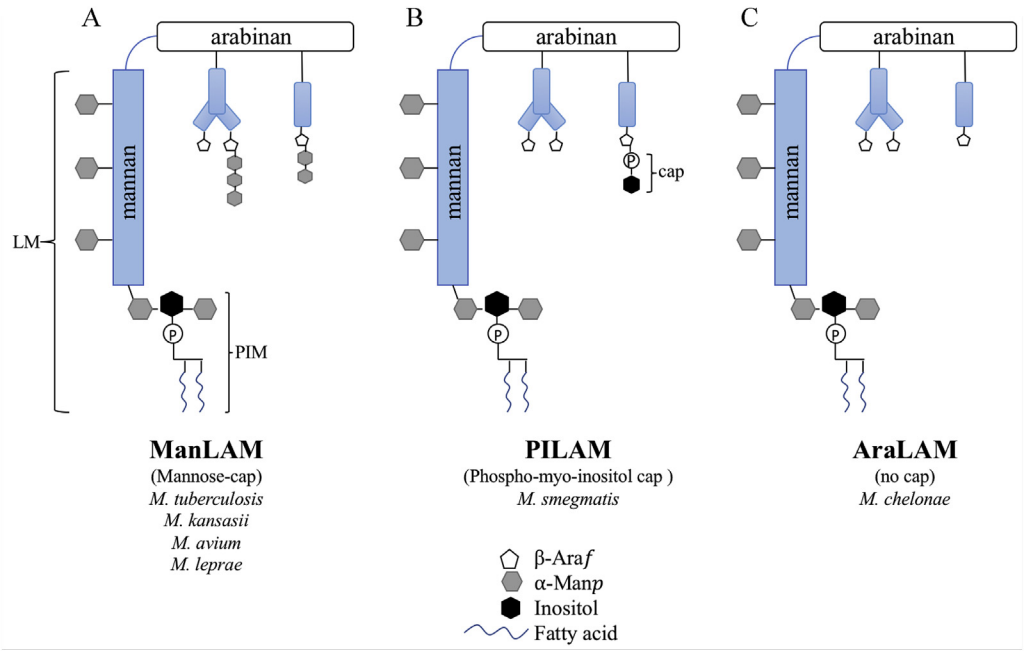


Fig. 7. Structures of the three representative families of LAM molecules: (A) ManLAM, (B) PILAM, and (C) AraLAM. ManLAM and PILAM show branches of arabinan with a mannosyl cap or a terminal phosphate inositol cap, respectively. AraLAM lacks both types of caps. Adapted from Ref. [39]. Species listed are only representative.

9. Mycobacterial trehalose-containing glycolipids

Mycobacteria contain large amounts of nonreducing disaccharide α-D-trehalose in free form that can also be incorporated within various immunologically related glycolipids. For example, approximately 1.5–3% of *M. smegmatis* dry weight is trehalose [53]. Trehalose has been implicated in thermotolerance, prevention of protein aggregation, osmoprotection, and is a biosynthetic precursor of glycolipids found in the mycobacterial cell wall [53]. Trehalose is also implicated in virulence, sliding motility, biofilm formation, modulation of phagocytosis, and infection of host macrophages, and yet its breadth of biological functions have only been partially characterized [54–56]. The mycobacterial cell envelope has been shown to be rich in trehalose-containing glycolipids including lipooligosaccharides (LOS), TDM, and trehalose monomycolate (TMM) [57]. Since trehalose is not synthesized in mammals, agents that target the biosynthetic pathway of trehalose and/or trehalose-based glycolipids are plausible candidate antimicrobial compounds.

LOS is important for mycobacterial cell wall structural integrity and virulence. *M. kansasii* produces seven subclasses of LOS, all of which share a common tetraglucose core: β-D-Glcp (1 → 3) – β-D-Glcp (1 → 4) – α-D-Glcp (1 → 1) – α-D-Glcp where Glcp is glucopyranose (Fig. 8). The tetraglucose core itself contains a unique α,α-trehalose substituent (Fig. 8). Thus, trehalose is a major component of LOS. An additional feature of *M. kansasii* LOS is that its oligosaccharides are acylated with 2,4-dimethyltetradecanoate (Fig. 8), which can be saturated (*Mycobacterium canettii*), or unsaturated (*M. smegmatis*) [60]. Unlike the surface-exposed LOS in several mycobacterial species as seen in *M. canettii*, *M. marinum,* and *M. kansasii*, LOS produced by *M. smegmatis* is found in a deeper layer of the cell envelope [60]. A study in *M. smegmatis* mutant strain showed that the disruption of gene MSMEG_4727, which codes for a putative Mas-type Pks (polyketide synthase), abrogates the production of both LOS and polymethyl-branched fatty acids [60]. Within the same cluster of gene MSMEG_4727, two genes immediately adjacent to MSMEG_4727 are posited to encode enzymes associated with Pks in the synthesis of polymethyl-branched fatty acid: MSMEG_4731 (FadD-activates Pks substrate) and MSMEG_4728 (Pap-putative acyltransferase), whereas two other genes MSMEG_4733 (gap 2) and MSMEG_4741 (mmpL) are involved in the synthesis and transport of glycolipids such as LOS, GPL, and sulfolipid-1 [60–62]. Previous work illustrated the distinctly amphipathic properties of LOS in which the fatty acids appear to be restricted at one terminus allowing interactions with the MA-AG polymer, while the oligosaccharide moiety at the other end extend into the hydrophilic milieu [58].

Strains of *M. kansasii* that show smooth colony morphologies possess trehalose-based LOS surface antigens while rough strains are devoid of such surface antigens [63]. Of interest, studies from mice demonstrate that rough LOS-deficient *M. kansasii* survives longer to cause chronic pulmonary disease compared to smooth LOS-producing strains [63,64]. An *in vitro* study demonstrated that LOS stimulates expression of proteins on the surface of macrophages to form mature granulomas and restrict bacterial growth [56]. LOS also might be acting as antivirulence determinant that coats or “masks” other glycolipids that contribute to virulence such as LAM [57].

TDM (*a.k.a.,* “cord factor”) (Fig. 8) is recognized as an essential structural component that contributes to the low permeability of the mycobacterial cell wall (Fig. 1) and is associated with rough morphology [65,66]. Trehalose 6-monomycolate (TMM), a TDM precursor, is not only a major component of the mycobacterial envelope, but also fulfills a critical role in the formation of the MA envelope layer [59,66]. All known mycobacteria synthesize TDM, except *M. leprae*; however, different species synthesize TDM with different physical and chemical properties and antigenic structure which results in varied virulence [67,68]. Free TDM forms a monolayer at lower concentrations, whereas higher concentrations result in the formation of cord-like structures [69]. TDM exerts numerous immunomodulatory effects including granulomagenesis, stimulation of innate, humoral and cellular adaptive immunity, and induction of various cytokines (IL-12, TNF-α, IFN-γ, IL-4, IL-6, IL-10, and Il-12) and chemokines (MCP-1, IL-8, and MIP-1α) [70]. Similar to LAM, TDM inhibits the phagolysosome acidification system by modulating macrophage gene expression [71,72]. Moreover, TDM exerts potent immunostimulatory activities such as granuloma formation and tumor regression [72,73].

Although the structures of LOS subclasses have been studied, their biosynthetic pathways and roles in immunomodulation and antigenicity is still poorly understood. More studies are emerging, yet LOS remains among the lesser characterized glycolipids of NTM. Additionally, while TDM is the most commonly studied component of *M. tb* as evinced by the 43 studies found, there were significantly fewer NTM TDM studies (n = 12) (Fig. 12).

10. Mycobacterial phenolic glycolipid (PGL)

Phenolic glycolipid (PGL), derived from the enzymatic activity of a polyketide synthase, is non-covalently linked to the outer cell wall and functions as an important virulence factor for pathogenic SGM including *M. tb*, *M. marinum, M. leprae, M. bovis, and M. kansasii* [74]. Structurally, mycobacterial PGL contains a common lipid backbone at

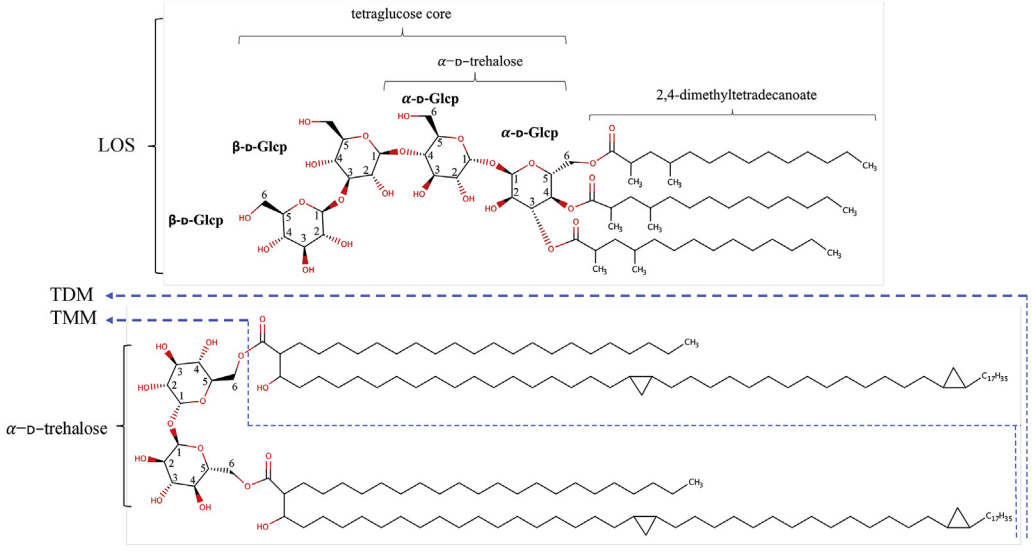
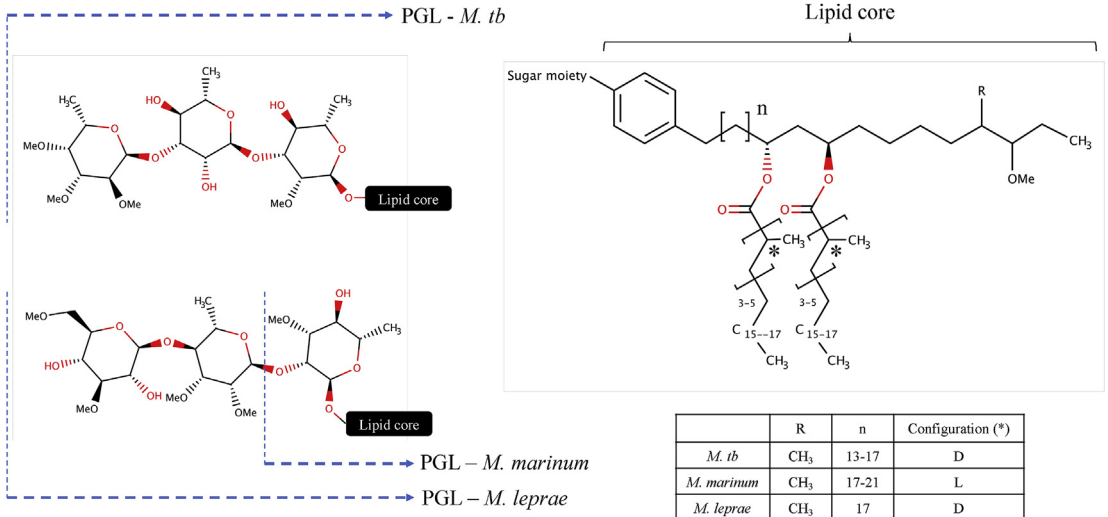


Fig. 8. Chemical structures of trehalose-containing glycolipids: LOS, TMM, and TDM. All LOS contain a common triacylated tetraglucose core in which itself contains a unique nonreducing α,α-trehalose disacharride. The tetraglucose core is composed of β-D-Glcp (1 → 3) – β-D-Glcp (1 → 4) – α-D-Glcp (1 → 1) – α-D-Glcp where Glcp is glucopyranose. Adapted from Refs. [58,59].



	R	n	Configuration (*)
M. tb	CH_3	13-17	D
M. marinum	CH_3	17-21	L
M. leprae	CH_3	17	D
M. kansasii	OCH_3	16	L

Fig. 9. Chemical structure of phenolic glycolipids (PGL). All PGL contain a common lipid core at one terminus and an oligosaccharide moiety at the other terminus. Lipid core is composed of glycol-containing long-chain phenolphthiocerol that is esterified by polymethyl-branched fatty acids. The configuration, indicated by asterisks, can be either D or L depending on the mycobacterial species. Adapted from Ref. [75].

one end and a sugar moiety at the other end, depending on the mycobacterial species (Fig. 9). The lipid core is composed of glycol-containing long-chain phenolphthiocerol esterified by polymethyl-branched fatty acids [76]. At the methyl branching chiral centers (Fig. 9, asterisks), either D or L configurations are used by various mycobacterial species. The configuration of D series corresponds to mycocerosic acids while configuration of L series corresponds to phthioceranic acids (Fig. 9). Previous studies from *M. marinum* showed that proteins FadD22 (p-hydroxybenzoate-AMP ligase/initiation module) and Pks15/1 (iterative type I Pks) are accountable for the production of *p*-hydroxyphenylalkanoate (PHPA) intermediates which are required for the biosynthesis of PGL [77,78]. PHPA are postulated to be further extended to form the long-chain phenolphthiocerol moiety of the PGL lipid core by the modular type I PKS system PpsABCDE [79]. Identifying and confirming the specific roles of these essential proteins for the biosynthesis of PGL might help to design pharmacological drugs for the control of mycobacterial infection.

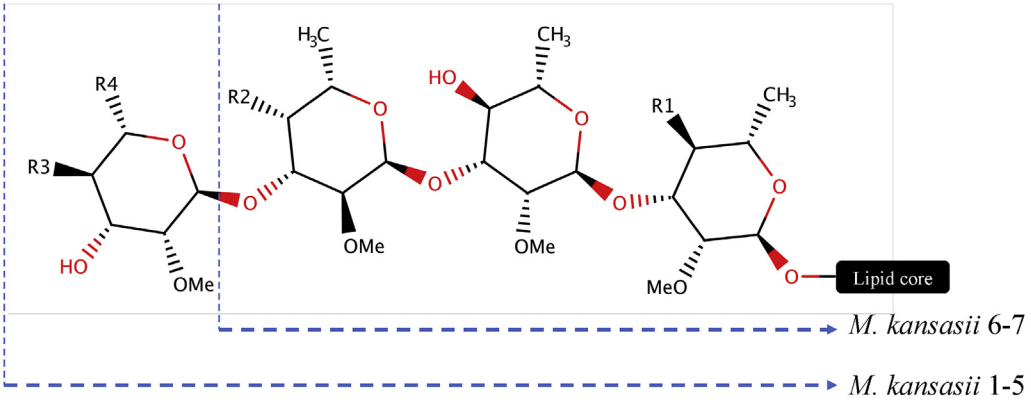
The immunomodulatory effects of *M. tb, M. bovis,* and *M. leprae* PGL have been demonstrated. For example: *(i)* disrupted PGL synthesis correlated with the loss of hyper-lethality associated with W-Beijing strain of *M. tb* in mice and increased release of proinflammatory cytokines TNF-α, IL-6, and IL-12, *(ii)* overproduction of PGL by *M. tb* lead to a dose-dependent inhibition of proinflammatory cytokines, and *(iii)* PGL served as a ligand for carbohydrate-recognizing receptors on macrophage surface [75,80,81]. Other studies utilizing synthetic PGL analogs from *M. tb, M. bovis,* and *M. leprae* demonstrate that PGL inhibits the release of NO, TNF-α, IL-6, IL-1β, and monocyte chemoattractant protein-1 (MCP-1) in a concentration-dependent manner via TLR2 (H. R [74,82]. Additionally, PGL-1 (the only *M. leprae*-specific antigen) and Man-LAM worked in concert to significantly inhibit either T-cell antigen receptor (TCR) alone or TCR/CD28 signaling, leading to the loss of T-cell mediated production of IL-2, IL-4, IL-1α, IL-1β, IL-6, IL-8, IL-10, IFN-γ, TNF-α, and GM-CSF [83]. A more recent study defined a critical role for PGL in engineering the escape of *M. tb* from macrophages by activating the macrophage Stimulator Interferon Genes (STING) cytosolic sensing pathway and CCL2 production that facilitates the transfer of bacteria to other uninfected macrophages [80]. Therefore, interventions to block or develop vaccines against PGL may potentially reduce disease burden.

M. kansasii produces seven different classes of PGL (Fig. 10) with differing effects on NO and proinflammatory cytokine release. To study the effects of PGL on host immunity, seven direct analogs of the native PGL were synthesized in addition to ten more analogs with different methylation and substitution patterns (H. R. H [84]. Using these synthetic analogs, the authors demonstrate the *(i)* immunomodulatory activity of PGL was mediated through TLR2, *(ii)* the PGL sugar moiety and differing methylation patterns contribute to inhibition of proinflammatory cytokines, and *(iii)* longer PGL sugar moieties show higher cytokine inhibition activity (H. R. H [84]. While only SGM species such as *M. tb, M. bovis, M. leprae,* and *M. kansasii* synthesize PGL, it might be worthwhile to investigate the significance of the lack of PGL in RGM species. The number of studies investigating NTM PGL (n = 8) is less than half of those published for *M. tb* PGL (n = 20) (Fig. 12).

11. NTM glycopeptidolipids (GPL)

Glycopeptidolipid (GPL) is a major class of mycobacterial lipids present on the outermost portion of the cell envelope of NTM species, but are absent from *M. tb* [85]. All GPL consist of a common fatty acyl chain *N*-linked (C_{26} – C_{34}) tetrapeptide-amino-alcohol core (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol) (Fig. 11) [87]. Within the fatty acyl-tetrapeptide core, D-*allo*-Thr is glycosylated with 6-deoxy-α-L-talose (6-d-Tal) via a glycosidic linkage (Fig. 11, black arrow), while L-alaninol is glycosylated with variable *O*-methyl-rhamnose (*O*-Me-Rha) (**purple arrow**), resulting in apolar or nonserovar-specific GPL (nsGPL) [85]. nsGPL produced from *M. smegmatis*, *M. chelonae*, and *M. abscessus* is O-acetylated at C3 and C4 of 6-d-Tal, and at C2, C3, and C4 or C3 and C4 of O-Me-Rha [87]. In contrast, *M. avium* nsGPL contains 6-d-Tal that is nonmethylated or O-methylated at C3 and O-Me-Rha that is O-methylated at C3 or C3 and C4 [88]. Because ethambutol-resistant *M. avium* serovar 1 strains contain nonpolar nsGPL, it is plausible that nsGPL plays a role antibiotic resistance [89]. nsGPL of the smooth morphotype of *M. abscessus* has been shown to overlie PIM in the cell wall, providing a cloaking mechanism that reduces recognition of such *M. abscessus* strains by macrophage TLR2, prevents inflammation, and delays deployment of host immunity. Moreover, spontaneous loss of nsGPL exposes PIM to engage TLR2, resulting in increased inflammation [90]. Whether nsGPL from other NTM species act similarly is yet to be determined.

In addition to nsGPL, *M. avium* and *Mycobacterium intracellulare* are SGM that produce a variety of polar and more complex GPL called serovar-specific GPL (ssGPL). ssGPL are produced when an additional

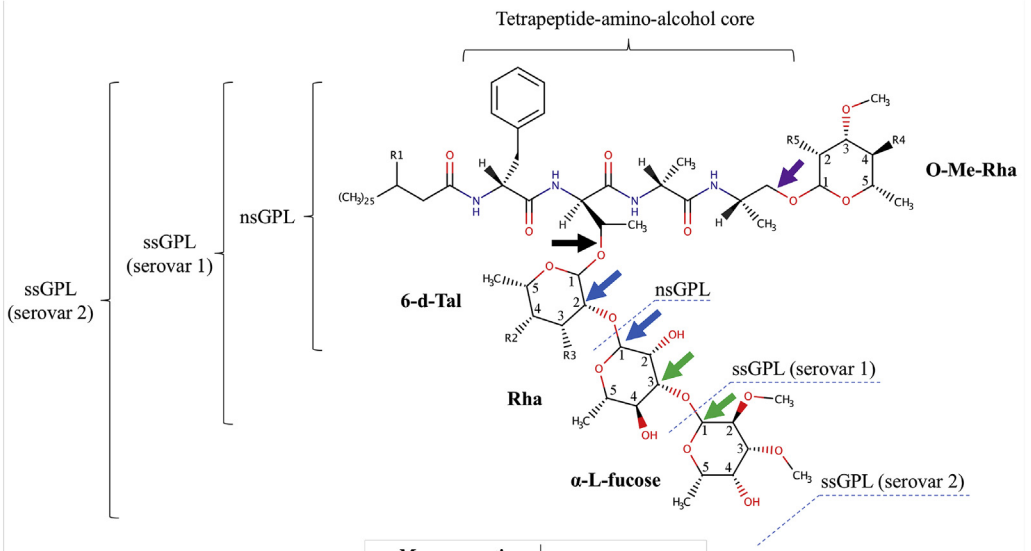


M. kansasii	R1	R2	R3	R4
1	OMe	OAc	OMe	Me
2	OMe	O-proprionyl	OMe	Me
3	OMe	OH	OMe	Me
4	OMe	OH	OMe	CH_2OH
5	OMe	OH	OH	CH_2OH
6	OH	OH	—	—
7	OMe	OH	—	—

Fig. 10. Chemical structures of *M. kansasii* subclasses phenolic glycolipids (PGL). Seven *M. kansasii* PGL have been isolated and characterized to date. All consist the same lipid core to that of other mycobacterial species (see Fig. 9). Carbohydrate moiety of *M. kansasii* PGL 1–5 is composed of a tetrasaccharide, differing from one another at R2, R3, and R4, whereas carbohydrate of PGL 6–7 is composed of a trisaccharide, differing from each other at R1. Adapted from (H. R. H [84].

Rha residue is glycosidically linked to the 6-d-Tal residue of an already formed nsGPL by rhamnosyltransferase gene (*rtf*A) products [85,91]. While *M. avium* serovar 1 produces ssGPL by the addition of Rha (1 → 2) linked 6-d-Tal (Fig. 11, blue arrows), serovar 2 produces ssGPL with additional 2,3-di-*O*-methylated α-L-fucose (1 → 3) linked Rha linked to serovar 1 (Fig. 11, green arrows) [87]. It was determined that the *rtf*A gene located in the *ser*2A locus of *M. avium* is responsible for the transfer of Rha to 6-d-Tal [85,91]. By varying GPL expression, certain NTM species transition between a smooth morphotype expressing GPL to a rough morphotype lacking GPL. For example, colony morphologies of *M. avium* complex (MAC) can include smooth opaque (SmO) and smooth transparent (SmT) forms which contain GPL, compared to rough opaque (Rg), and rough transparent (RgT) colonies that lack GPL [87]. The rough morphotype has been described for *M. avium* 2151 serovar 2 and *M. smegmatis mps* mutants, and postulated to increase virulence of RgT *M. avium* strains [92–95].

In contrast, *M. smegmatis*, *M. abscessus*, and *M. chelonae* are RGM that produce polar GPL formed by the addition of 3,4-di-*O*-methylated



M. smegmatis *M. chelonae* *M. abscessus*	MAC
R1: OH or OCH_3	R1: OH or OCH_3
R2: OAc	R2: OH
R3: OAc	R3: OH or OCH_3
R4: OCH_3	R4: OH or OCH_3
R5: OH or OCH_3	R5: OH

Fig. 11. Chemical structure of glycopeptidolipid (GPL). All GPL consist a common fatty acyl chain *N*-linked (C_{26} – C_{34}) tetrapeptide-amino-alcohol core (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol). Within the tetrapeptide core, D-*allo*-Thr is glycosylated with 6-deoxy-α-L-talose (6-d-Tal) via a glycosidic linkage (black arrow) forming nsGPL. MAC ssGPL serovar 1 strain is synthesized by the addition of Rha (1 → 2) linked 6-d-Tal (blue arrows), whereas ssGPL produced by serovar 2 strain has additional α-L-fucose (1 → 3) linked Rha of the serovar 1 strain (green arrows). RGM species including *M. smegmatis*, *M. abscessus*, and *M. chelonae* synthesize polar GPL by the addition of 3,4-di-*O*-methylated Rha (1 → 2) linked to another 3,4-di-*O*-methylated Rha attached to the terminal alaninol of the tetrapeptide core that are different from MAC's ssGPL. Adapted from Ref. [86]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

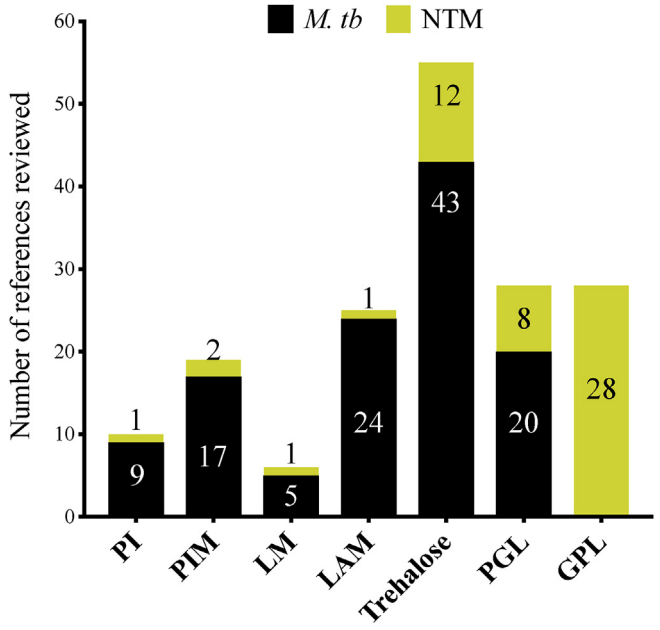


Fig. 12. Summary demonstrating the considerably less amount of knowledge regarding NTM lipids compared to *M. tb* lipids. The number of articles reviewed for *M. tb* (black bar) compared to NTM (red bar) are shown per mycobacterial lipid discussed. The number of studies per lipid is numerically denoted in the respective bars. Article discovery was conducted using Google Scholar and Pubmed searches utilizing lipids and NTM species names as search criteria. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Rha (1 → 2) linked to another 3,4-di-*O*-methylated Rha attached to the terminal alaninol of the tetrapeptide core (not shown) [87]. It is proposed that *M. abscessus* biofilm-forming smooth strains found in the environment initially colonize lung airways and subsequently transition to rough strains that are more invasive and elicit greater inflammatory responses [90]. However, identification of specific genetic abnormalities for the loss of GPL production makes switching back-and-forth between smooth and rough colonies in *M. abscessus* highly unlikely [96]. Despite several reports indicating the rough morphotype of *M. abscessus* as more virulent than the smooth morphotype [95,97], no clear correlation between colony morphology and mycobacterial virulence has been proven.

Because smooth *M. avium* 2151 spreads more easily on solid culture medium than rough strains, GPL play active roles in sliding motility [98]. Moreover, isogenic GPL-producing *M. smegmatis* strains show increased sliding motility compared to GPL-lacking strains [99]. It is posited that sliding motility is due to reduced friction between the exposed hydrophobic fatty acyl chains in GPL and the external hydrophilic environment [99]. GPL-producing *M. abscessus* strains also exhibit both sliding motility and biofilm formation as opposed to GPL-deficient rough strains [95]. Finally, a study by Davidson et al. demonstrated that respiratory epithelial cells infected with *M. abscessus* strains lacking GPL, but not those expressing GPL, show increased expression of IL-8 and human β-defensin mediated via TLR2 [100]. These data suggest that the presence of GPL facilitates the ability of smooth *M. abscessus* strains to colonize host cells by masking underlying cell wall lipids and avoid detection by the host immune response.

Extracted and purified GPL has been used to evaluate its immunomodulatory roles. GPL has been implicated in blocking phagosome-lysosome fusion to increase mycobacterial survival, while stimulating proinflammatory responses through MyD88 and TLR2 signaling [101–103]. ssGPL from smooth *M. avium* serovar 1 and 2, but not nsGPL, stimulates inflammatory responses in macrophages by releasing TNF-α via TLR2 as well as prostaglandins, IL-1, and IL-6 [40,104,105]. Yet, the same proinflammatory responses were not observed by ssGPL from *M. avium* serovars 4 and 20, suggesting ssGPL responses are structure specific [106]. Fig. 12 highlights the 28 studies we found regarding GPL of NTM. The biological and evolutionary reasons why GPL predominate in NTM species but are devoid in *M. tb* remain an unsolved mystery.

12. Conclusions

Knowledge of individual lipids that comprise the *M. tb* cell envelope is vast and comprehensive. This review highlights the significant knowledge gap regarding the basic biology of NTM cell envelope lipids. Additionally, differences in the presence and absence of lipids in different mycobacterial species were also observed: *(i)* PI, PIM, LM, LAM, and trehalose-containing glycolipids are present in both *M. tb* and NTM, *(ii)* PGL is absent from RGM, and *(iii)* GPL is only present in NTM. In conclusion, although PI, PIM, LM, LAM, trehalose-containing glycolipids, PGL, and GPL are regarded as the essential components of the mycobacterial cell wall, positive proof of an essential role of these molecules on the viability and/or virulence of NTM has yet to be conclusively determined. We assert that studies of NTM lipids are timely, urgently needed, and will potentially lead to the discovery of novel NTM lipid-directed therapies to reduce lung disease burden for those infected.

Author contributions statement

EC and JH contributed to the conception of the work. TT conducted the literature review and drafted the manuscript. AB (chemistry), EC (clinical mycobacterial lung disease), and JH (mycobacteriology) added their respective expertise and contributed to sections of the manuscript. All authors edited the manuscript as well as read and approved the final submitted version. JH supervised the work and also performed final corrections.

Conflicts of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.02.008.

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