Broadening Our View About the Role of *Mycobacterium tuberculosis* Cell Envelope Components During Infection: A Battle for Survival

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1. Introduction

TB is an ancient disease, however, it is still a major health problem in the world (Centers for Disease Control and Prevention, 2006). The number of new cases of TB worldwide roughly correlates with economic conditions: the highest incidences are seen in those countries with the lowest gross national products (countries mainly in Africa, Asia, and Latin America). WHO numbers indicate that 8 million people are newly infected and nearly 2 million people die of TB every year; translating to one person infected every 4 seconds and one person dying every 18 seconds (WHO, 2007). WHO estimates that by year 2020 up to 36 million people will die of TB every year (WHO, 2010). The current TB burden in the world is strictly associated to *Mycobacterium tuberculosis* (*M.tb*) co-infection with HIV, and the recent emergence of practically untreatable extensive-, extremely- and totally-drug resistant *M.tb* strains (XDR-, XXDR- and TDR-) in endemic areas. In this context, a person infected with an XDR/XXDR *M.tb* strain currently has a survival rate between 36-50%, however, for people co-infected with HIV, their survival rate drops to ~15% with a life span of 16 days upon XDR/XXDR *M.tb* infection (Gandhi et al., 2006). XXDR-*M.tb* strains are resistant to all first and second line of drugs currently available, and TDR-*M.tb* strains are even resistant to the new developed drugs currently in clinical trials (Andrews et al., 2010; Basu et al., 2009; Gandhi et al., 2006, 2010). Very little is known about the cell wall composition of XDR/XXDR/TDR *M.tb* strains. The presence of phenolic glycolipids and triglycerides in the *M.tb* cell wall has been directly related to the hypervirulence observed in some strains (Reed et al., 2004, 2007). However, in the case of XDR/XXDR/TDR *M.tb* strains, it still is unknown which bacterial and host factors are involved in the induction of the overwhelming host immune response generated by these strains.

Initial interactions between *M.tb* and the host mark the pathway of infection and the subsequent host inflammatory response that defines disease outcome. Many studies have been performed analyzing the constitution of the cell wall of *M.tb*, where structural-biological function relationships for the majority of the cell wall constituents are still being elucidated. The majority of the cell wall of *M.tb* is comprised of carbohydrates and lipids,
and there is increasing evidence that microbial determinants readily exposed to the host immune system play critical roles in disease pathogenesis. Recent studies have been focused on depicting how \textit{M.\textit{tb}} adapts to the host by mimicking its cell envelope to mammalian glycoforms. Of particular interest is the fact that some \textit{M.\textit{tb}} strains are characterized by the presence of mannose-containing biomolecules, whose terminal epitopes closely resemble those on host mannoproteins. In this scenario, it is thought that \textit{M.\textit{tb}} may use this resemblance to the host to its advantage gaining entrance and establishing its particular intracellular niche within the host; thus, the initial \textit{M.\textit{tb}}-host interface may dictate the pathway of infection and the successful outcome of the disease. Many factors are involved in this interface. First the constitution of the \textit{M.\textit{tb}} cell wall, which is strain dependent. Here we will discuss the differences in the cell wall among the widely used laboratory strains (\textit{M.\textit{tb}} H\textsubscript{3}7\textsubscript{R}v and \textit{M.\textit{tb}} Erdman) and several relevant \textit{M.\textit{tb}} clinical isolates in endemic TB areas, including hypervirulent, MDR-, XDR, XXDR- and TDR- \textit{M.\textit{tb}} strains. We will discuss their cell wall constitution in relation to their infection outcome. Second, we will focus on the host cell that acts as a niche, the alveolar macrophage, and we will discuss the innate immunofactors present on the host cell that contribute to control or alternatively can favor the infection. Importantly, we will introduce our new results in an area frequently bypassed in many forums, the host environment and how this may challenge the old dogma of the real constitution of the \textit{M.\textit{tb}} cell wall during infection. We will discuss the alveolar microenvironment that \textit{M.\textit{tb}} encounters during infection, and how these may determine/contribute to the pathway of infection and disease outcome.

2. The cell wall of \textit{M. tuberculosis} and its biological functions

A great effort has been made by many research groups to depict the \textit{M.\textit{tb}} cell wall structure and biosynthesis. The main distinctive feature of the \textit{M.\textit{tb}} cellular envelope cited in all books is the thick and waxy cell wall. This complex structure contributes to the main characteristics that distinguish mycobacteria, such as the acid fast staining properties, the low permeability of the cell wall, the resistance to harsh environments, and the intrinsic resistance to many hydrophobic antibiotics (Brennan & Nikaido, 1995;Jarlier & Nikaido, 1994). The properties of the cell wall barrier also contribute to the intracellular survival of the organism by acting as a direct modulator in the immunological reaction between the host and mycobacteria (Barry & Mdluli, 1996;Lederer \textit{et al.}, 1975). \textit{M.\textit{tb}} is one of a small group of species able to survive inside the phagocytic cells of a host, so it is likely that its cell wall has special properties defending the bacterium against host microbicidal processes. Within the cell wall of \textit{M.\textit{tb}} may lie all of the elements associated with TB, including the factors responsible for caseation and other features of hypersensitization, the antigens responsible for humoral immunity, the agents of toxicity, and thus, the very antigens implicated in protective immunity (Brennan, 1988). A detailed electron microscopy study has not yet, however, identified any special features in \textit{M.\textit{tb}} compared to other non-pathogenic mycobacteria. The envelope consists of three distinct parts, the plasma membrane, the wall, and around it, the outer material. These parts are involved in providing mechanical support and osmotic protection plus transport exchange of ions and molecules with the micro-environment(s) surrounding the bacillus during the different stages of infection. Here we will discuss \textit{M.\textit{tb}} cell wall components in terms of their role in \textit{M.\textit{tb}} pathogenesis, focusing on the \textit{M.\textit{tb}} peripheral lipid layer constituents that are involved in \textit{M.\textit{tb}}-host cell recognition and pathogenesis.
2.1 The plasma membrane

Defined as a classical bilayer (Silva & Macedo, 1983), the M.tb membrane does, however, have some distinctive components, notably the lipoglycoconjugates mannose-capped lipoarabinomannan (ManLAM), lipomannan (LM), and phosphatidyl-myo-inositol mannosides. Integral membrane proteins embedded in the layers of the cell wall have also been described (Brennan & Draper, 1994). Analyses of the proteome of the plasma membrane of M.tb suggests that the plasma membrane of M.tb is likely to be rich in proteins comprising several essential enzymes, receptors and transporters (Sinha et al., 2002), like other prokaryotic cell membranes (Sigler & Hofer, 1997). Bioinformatic analysis of the M.tb genome predicts more than 600 ‘putative’ membrane-associated proteins with different numbers of transmembrane hydrophobic segments. These proteins undoubtedly play a role in the uptake and effects of various metabolites, peptides, drugs and antibiotics. Nonetheless, the real location, expression patterns, and function for the majority of these transmembrane proteins remain relatively unexplored (Lee et al., 1992; Yokoyama & Shimizu, 2002; D.B. Young & Garbe, 1991).

2.2 The cell wall

The shape-forming properties of the wall are attributable to the peptidoglycan, whose chemical structure in M.tb closely resembles that found in other bacteria. Mainly, the cell wall is defined as a skeleton formed by a covalently linked structure of peptidoglycan, with a branched-chain polysaccharide, the arabinogalactan, attached by phosphodiester bonds. The arabinogalactan distal ends are esterified with high-molecular weight fatty acids, the mycolic acids, of sizes and structures unique to mycobacteria. This cell wall skeleton receives the name of the mycolyl-arabinogalactan-peptidoglycan complex (mAGP) (Besra et al., 1995; Daffe et al., 1990). The wall is constructed of three layers. With conventional staining using electron microscopy, their appearance is defined with an inner layer of moderate electron density, a wider electron-transparent layer, and an outer electron-opaque layer of extremely variable appearance and thickness. The outer opaque layer probably contains the outer material. The electron-transparent layer appears to be mycolated arabinogalactan, which forms a large part of the wall. Finally, the inner layer is speculated to contain both peptidoglycan and arabinogalactan (Draper, 1971).

There are many models of the mycobacterial cell envelope (Bhamidi et al., 2011; Brennan and Besra, 1997; Crick et al., 2003; Dmitriev et al., 2000; Domenech et al., 2001). Interactions of the asymmetric plasma membrane, peptidoglycan, and covalent attached arabinogalactan together with LAM and PIMs have been speculated, at least some of which are known to be associated with the plasma membrane. Mycolic acids are known to be attached to the majority of the terminal and penultimate arabinose residues of the arabinogalactan (Barry et al., 1998). Since the mycolates possess two hydrocarbon chains of unequal lengths, which form an irregular monolayer, it is proposed that these are complemented by two different classes of polar lipids with medium (e.g. mycocerosates) and short (e.g. acylglycerols) fatty acyl chains, respectively (Barry et al., 1998). There is also evidence for a small number of porins in the envelope, presumably within the outer hydrophobic bilayer (Senaratne et al., 1998; Trias et al., 1992). A recent study had committed efforts to solve the enigma involving the spatial organization of the mycobacterial cell envelope. This study compared bacteria grown in vivo (i.e. Mycobacterium leprae) vs. grown in vitro (i.e. M.tb) showing that bacilli
grown in vivo had a more compact cell envelope with more mycolic acids and more but shorter arabinogalactan molecules per peptidoglycan (Bhamidi et al., 2011). This differential cell envelope spatial conformation may differentially impact the rearrangement of the outer surface exposed cell envelope components that have a critical role in M.tb-host recognition.

The barrier for the influx of solutes such as nutrients or drugs is associated with the parallel alignment of mycolic acids (Liu et al., 1995; Liu et al., 1996). As a consequence, mycobacteria develop aqueous channels formed by porin molecules in the cell wall structure. Other distinguishing cell wall components of M.tb include ManLAM, LM, PIMs, and a peripheral layer of lipids such as trehalose mycolates (trehalose dimycolate or TDM, trehalose monomycolate or TMM), lipoooligosaccharides (LOSs), phenolic glycolipids [PGLs, described in some M.tb clinical isolates (Reed et al., 2004; Torrelles et al., 2008b)], acyl trehaloses (diacyl- or DAT and triacyl- or TAT), triglycerides and sulfolipids (SLs) (Brennan & Nikaido, 1995; Muñoz et al., 1997a, 1997b). Recently, the role for individual components of the cell wall has been elucidated, and much emphasis has been placed on the identification and characterization of various genes that encode enzymes involved in the synthesis of the cell wall constituents. A better understanding of the cell wall components will lead to a better understanding of the relationship/symbiosis between M.tb and the infected host, which will lead to the identification of new drug targets and permit the development of new antituberculosis drugs targeting enzymes involved in the biosynthesis/maintenance of the cell wall within the host during infection.

2.2.1 Cell wall core

One distinguishing characteristic of the mycobacterial cell wall is the absence of lipoteichoic acids and lipopolysaccharides, typical structures of gram-positive and -negative bacteria, respectively. Instead, the M.tb cell wall has a cell wall core which is a covalently linked skeleton of the mAGP (Daffe et al., 1990). This structure is composed of the peptidoglycan, which is covalently linked to AG chains via phosphoryl-N-acetylglucosaminyl-rhamnosyl linkage units (α-L-Rhap(1→3)-D-GlcNAc-P-) (Daffe et al., 1990; Mikusova et al., 1996). AG non-reducing ends are esterified to a variety of α-alkyl, β-hydroxy mycolic acids. Lipoglycans, lipoproteins and especially free lipids are found to be associated with the mAGP complex (Andersen & Brennan P.J., 1994; Brennan & Nikaido, 1995).

2.2.1.1 The peptidoglycan

Peptidoglycan (PG) classes are identified by the type of peptide cross-linking that they display, the PG structure in M.tb is of the common A1γ type, although it does have some distinguishing features (Schleifer & Kandler, 1972). The insoluble PG consists of alternating units of N-acetylglucosamine (GlcNAc) and modified muramic acid residues [N-acetyl-β-D-glucosaminyl-(1→4)-N-acetylmuramic acid]. The typical N-acetyl groups in the muramic acid of bacterial PG are further oxidized to N-glycolyl groups in M.tb [N-glycolylmuramic acid]. The muramic acid residues are also modified by tetraketide [L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine] side chains (Schleifer & Kandler, 1972). Cross-linking can occur between two meso-diaminopimelic acid (DAP) residues as well as between DAP and D-alanine residues (Crick et al., 2001). The free carboxyl groups of the glutamate and DAP in the murein peptides from M.tb can be amidated in essentially any combination and a small percentage of the D-glutamate residues are substituted with a
glycine. PG serves as a foundation structure forming the backbone of mAGP and provides shape, strength, and rigidity to the M.tb cell wall. Although the role of PG in pathogenesis has been shown for other bacterial species (Boneca, 2005), in the case of M.tb is still unclear (Table 1). However, recent studies have been focused on the role of nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (Franchi et al., 2008; Sirard et al., 2007) in the recognition of M.tb PG fragments. In particular the NLR Nod2, which resides within the phagocyte cytosolic compartment, is shown to recognize the M.tb PG fragment muramyl dipeptide (MDP), where the replacement of the N-acetyl group of the muramic acid of MDP with a N-glycolyl moiety seem to significantly increase the potency of this compound as a Nod2 agonist (Coulombe et al., 2009). Other studies confirmed that during infection, intraphagosomal M.tb is capable of stimulating the cytosolic Nod2 pathway, and this event requires membrane damage that is actively inflicted by the bacillus (Pandey et al., 2009). Moreover, M.tb bacilli recognized by Nod2 trigger NF-kB activation (Ferwerda et al., 2005) and production of IFN-α/β with the subsequent transcription of CCL-5 (or RANTES) via Nod2, Rip2, Tbk-1, Irf3 and Irf5 cascade (Pandey et al., 2009). Recent studies by Brooks et al. using human macrophages showed that Nod2 plays a role in controlling pro-inflammation and M.tb intracellular growth (Brooks et al., 2011). This is in accordance with recent human polymorphisms studies linking Nod2 mutations to susceptibility to M.tb infection.

2.2.1.2 The arabinogalactan

The arabinogalactan (AG) is a heteropolysaccharide chain of furanoid arabinose (Araf) and galactose (Galp) (Daffe et al., 1990; McNeil et al., 1987). The furanosyl residues are arranged into three differential regions; the galactan core, the arabinan, and the non-reducing terminal segments of arabinan. Structural analysis of the AG shows that the galactan core of AG is composed by 5- and 6-linked β-D-Galp residues (Daffe et al., 1990). The arabinan chains consist of linear 5-linked α-Araf residues with branching introduced by 3,5-Araf residues (Daffe et al., 1990). The linkage of arabinan chains to the galactan core occurs at the C-5 of some of the 6-linked β-D-Galp residues. Clusters of four mycolic acids are then attached to the terminal arabinofuranosyl motifs of non-reducing ends of the arabinan chain via ester linkage. Approximately two-thirds of the non-reducing ends of arabinan are mycolated at the 5 position of Araf residues (Daffe et al., 1993), one-third with succinylic and one-third with glucosaminosyl residues (Bhamidi et al., 2008). There are approximately 2-3 arabinan chains attached to the galactan core (Baulard et al., 1998; Brennan and Nikaido, 1995). Finally, the galactan core of AG, in turn, attaches to the C-6-position of muramic acids of the PG via a phosphodiester linkage of α-L-rhamnopyranose (Rhap)-(1→3)-D-N-acetylglucosamine (GlcNAc)-(1→phosphate) (McNeil et al., 1990; Mikusova et al., 1996). Although M.tb AG has serological activity (Kotani et al., 1971; Misaki et al., 1974), its capacity to generate the innate immune response is unknown (Table 1).

2.2.1.3 Mycolic acids

Mycolic acids are complex hydroxylated branched-chain fatty acids with characteristic carbon numbers (60-90 carbon atoms) (Barry et al., 1998). They may also contain diverse functional groups such as methoxy, keto, epoxy ester groups and cyclopropane rings (Asselineau & Lederer, 1950). Mycolic acids found in M.tb are composed of an α-branch and a meromycolate branch, where the latter defines the heterogeneity in the mycolic acids, although the α-branch length frequently also generates variation (Barry et al., 1998).
possible functional groups (unsaturations, methyl branches and cyclopropanes) and polar moieties (ketones, methoxy groups) are localized only in the meromycolate branch (Barry et al., 1998). In particular for *M. tb*, unsaturated mycolates containing cyclopropanes (either cis/trans with sometimes an adjacent methyl branch), are known as α-mycolic acids. Mycolic acids containing a methoxy group with double bond or cyclopropane ring are known as methoxymycolic acids; similarly, mycolic acids containing an α-methyl-branched ketone are known as ketomycolic acids (Takayama et al., 2005). The α-mycolate is the most abundant form found in the *M. tb* cell wall [65-70%] followed by methoxy- and keto-mycolates [8-15%] (Qureshi et al., 1978). The majority of the mycolic acids are localized in the inner leaflet of the *M. tb* cell wall covalently bound via carboxylate ester to form the non-extractable tetramycolyl-pentarabinosyl unit (McNeil & Brennan, 1991). However, mycolic acids can also be found loosely forming extractible lipids mainly in the form of TDM and TMM (Minnikin, 1982). In this case, it is thought that TDM and TMM stabilize their position within the cell wall by associating their mycolic acid lipid tails with the AG-covalent linked mycolates. In general, mycolic acids are involved in maintaining a rigid cell shape but they also contribute to the resistance to chemical injury and to the protection of the *M. tb* bacillus against hydrophobic antibiotics (Barry et al., 1998).

The importance of the mycolic acids in the *M. tb* cell wall is defined by the action of isoniazid, which inhibits their biosynthesis and is an efficient antimycobacterial agent (Winder & Collins, 1970). Furthermore, looking at the biological functions described for mycolic acids (Table 1), it is noticeable to point out that these were the first known CD1-presented antigens capable of stimulating and activating CD1b-restricted T cells (Beckman et al., 1994; Montamat-Sicotte et al., 2011; Moody et al., 1999). Moreover, mycolic acids per se are shown to be immunomodulatory, where their structural nature (i.e. presence of determined functional groups) may determine the degree of virulence of a *M. tb* strain (Barry, et al., 1998).

### 2.2.2 The peripheral lipid layer in the *M. tuberculosis* cell wall

The study of mycobacterial lipids was initiated more than 70 years ago under the direction of Anderson in 1939 (Anderson, 1938). This field is still an active source of research due to the fascinating diversity of their structures and biological activities. The glycolipids are major *M. tb* cell wall constituents, known for their toxic or immunological properties (Brennan and Nikaido, 1995). They comprise the acyl trehaloses [mono- and dimycolyl trehalose (TMM, TDM), di- and triacyltrehalose (DAT, TAT) and sulfolipid (SL)], oligosaccharides containing lipids (lipooligosaccharides (LOSs), phenolglycolipid (PGL), apolar lipids such as the phthiocerol dimycocerosate (DIM), and the glycosyl derivatives of phosphatidyl-myco-inositol.

Trehalose-containing glycolipids share a common α-D-Glcp(1→1′) α-D-Glcp unit and are the class of *M. tb* lipids that have been most extensively studied and still fascinate the majority of lipidologists and mycobacteriologists. TDM (or cord factor) was first obtained by Bloch (Bloch, 1950) after a petroleum ether extraction from cells of a virulent strain of *M. tb*. The resulting extract was toxic when injected into mice and a drastic disorganization of the cords that *M. tb* formed at the culture medium surface was also observed. The toxic compound present in the extract was shown to be 6,6′-dimycoloyl-α-D-trehalose (Noll, 1956). TDM toxicity is due to an increase of the tissue specific nicotinamide adenine dinuclease activity decreasing the levels of NAD in several tissues by blocking the electron flow along the...
mitochondrial respiratory chain and thus oxidative phosphorylation (Artman et al., 1964; Barry et al., 1998; Brennan, 2003). During infection and when inside of the phagosome, *M. tb* is shown to produce large quantities of TDM (Fischer et al., 2001). From many studies done on TDM, it is remarkable that TDM induces lung granulomas and has immunostimulating properties (Bekierkunst, 1968) that are probably at the origin of its antitumoral activity (Bekierkunst et al., 1971a) (Table 1). TDM has also been shown to have adjuvant properties generating an optimal antibody response (Bekierkunst et al., 1971b) and a non-specific immune response against bacterial infections and parasitic infections (Bekierkunst et al., 1971b; Parant et al., 1977, 1978; Ribi et al., 1976; Yarkoni & Bekierkunst, 1976). Recent studies uncover that TDM is actively participating in blocking the mycobacterial phagosome maturation (Indrigo et al., 2003). Inhibition of the phagosome maturation is observed after phagocytosis of virulent strains of *M. tb*, allowing the bacillus to survive within the phagocyte (Schlesinger LS et al., 2008). Recently, Mincle (macrophage-inducible C-type lectin) (Yamasaki et al., 2008) on the macrophage surface, has been shown to recognize *M. tb* TDM, and working together with the Fcγ receptor transmembrane segment induces pro-inflammation (Ishikawa et al., 2009; Schoenen et al., 2010). In the case of *M. tb* TMM, this is shown to be used by the bacillus to transfer mycolic acids towards molecules like the wall-linked AG. In agreement with this fact, the known secreted immunogenic Ag 85 complex has been identified as a trehalose mycolyltransferase in *M. smegmatis* (Sathyamoorthy & Takayama, 1987) and later in *M. tb* (Belisle et al., 1997). TMM is also shown to have lethal toxicity, adjuvant activity, and capable of stimulating tumour necrosis factor via activation of the protein kinase C pathway (Numata et al., 1985) (Table 1).

The sulfated trehaloses (sulfolipids, designated by SL) (Middlebrook et al., 1959) are also present in virulent strains of *M. tb*, specifically SL-1 (Goren, 1970a, 1970b). SL-1 can be acylated by 2 to 4 very long (up to C64) saturated and unsaturated, highly branched fatty acids. Sulfate derivatives are rare in natural substances, and some of the acyl chains of SL-1 are also uncommon, since they are mainly highly branched in their carboxyl end (Goren & Mor, 1990; Leigh & Bertozzi, 2008). The SLs have attracted much interest since it was shown that, like TDM, they seem to inhibit phagosome-lysosome fusion in macrophages (Goren et al., 1976), and are cytotoxic (Kato & Goren, 1974a, 1974b). However, the role of SL-1 in *M. tb* pathogenesis seems to be dependent of the model system studied. In this context, results obtained from *in vitro* and *in vivo* studies (the latter using different animal models and *M. tb* strains) dispute the role of SL-1 in *M. tb* pathogenesis (Brozna et al., 1991; Gangadharam et al., 1963; Goren et al., 1974, 1982; Pabst et al., 1988; Rousseau et al., 2003; L. Zhang et al., 1988, 1991). While SL-1 has been shown to induce specific host cell responses, such as inhibition of phagocyte priming/activation (Brozna et al., 1991; Pabst et al., 1988), its mechanism of action is still unclear; although a role of a guanine nucleotide binding protein in both priming and direct activation of neutrophils by SL-1 has been suggested (L. Zhang et al., 1991) (Table 1).

Diacyltrehalose (DAT, a 2, 3-diacyltrehalose) and triacyltrehalose (TAT, a 2, 3, 6-triacyltrehalose) (Gautier et al., 1992), who’s acyl groups are mainly branched polymethyls, are also present in the *M. tb* cell wall. The main use of these glycolipids is in *M. tb* serodiagnosis (Muñoz et al., 1997a). However, a study recently showed that *M. tb* mutants lacking DATs and sulphoglycolipids cannot block phagosome maturation and thus, revealing the importance of these molecules in the *M. tb* pathogenesis (Table 1) (Brodin et al., 2010). In *M. tb*, other trehalose-based lipids are lipooligosaccharides (LOSs), which contain a polyacylated trehalose with long chain fatty acids and an oligosaccharide. It contains 2 or 3
straight or methyl-branched chains. In some LOSs, acyl residues can be distributed between the two glucose residues of the trehalose end of the polymer. Depending on the species, an oligosaccharide (2 to 6 sugar residues) is linked either on carbon 3, 4 or 6 of the trehalose end (Gilleron et al., 1994; Hunter et al., 1985). Pyruvic acid residues (carboxyethylidene) can also be present, giving an anionic character to the molecule. LOSs have been considered to be immunogenic and also phage receptors (Besra & Chatterjee, 1994), suggesting that they are located in the \textit{M.tb} cell wall surface. Other \textit{M.tb} lipids containing oligosaccharides are the phenolic glycolipids (PGLs). These have been extensively studied in \textit{Mycobacterium leprae} (PGL-I) (Hunter et al., 1983; Hunter & Brennan, 1981). Leprosy patients have antibodies against this molecule, and therefore it is a useful diagnostic tool (Cho et al., 1983). PGLs have been found in the Canetti strain of \textit{M.tb} (Daffe et al., 1987) (PGL-Tb), however, serodiagnostic studies have shown that there were large variations among tuberculous patients in the response to this antigen (Daffe et al., 1991). This is likely due to large differences in phenolglycolipid amounts produced by different \textit{M.tb} strains (Cho et al., 1992; Torrelles et al., 2008b). Recently, the hypervirulent phenotype observed in several strains of \textit{M.tb} (i.e. strain HN878) has been associated with the presence of PGL in their cell wall (Reed et al., 2004).

There are many apolar lipids described forming part of the cell wall of \textit{M.tb}, however, of particular importance is the phthiocerol dimycocerosate (or PDIM/DIM). PDIM is a major apolar lipid present in the cell wall of \textit{M.tb}. PDIM is considered a wax containing multiple methyl and/or methylene groups (Brennan, 2003). Several studies have tied, as in the case of TDM and SL-1, the presence of PDIM to \textit{M.tb} virulence (Goren & Brennan, 1980). In \textit{in vivo} studies using \textit{M.tb} strains depleted of PDIM show attenuation in the growth of these strains in mice (Cox et al., 1999; Ferwerda et al., 2007), in accordance with previous studies in Guinea pigs using a \textit{M.tb} clinical isolate lacking PDIM in its cell wall (Goren & Brennan, 1980) (Table 1). Recent studies using a genetically engineered \textit{M.tb} PDIM mutant concluded that PDIM inserts into the host membrane and participates both in the receptor-dependent phagocytosis of \textit{M.tb} and the prevention of phagosomal acidification (Astarie-Dequeker et al., 2009). Using PDMI mutants also is shown that these are required for \textit{M.tb} resistance to an IFN-\(\gamma\)-mediated immune response that is independent of NOS2 (Kirksey et al., 2011).

Glycosyl derivatives of phosphatidyl-\textit{myo}-inositol in the \textit{M.tb} cell wall are the phosphatidyl-\textit{myo}-inositol (PI) and its mannosylated derivatives known as phosphatidyl-\textit{myo}-inositol mannosides (PIMs), phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, cardiolipin, and glycosylphosphopolyisoprenols. PI and its mannosylated derivatives are important lipids in the cell wall of \textit{M.tb}, both as key membrane constituents and as participant in essential \textit{M.tb}-host interactions and metabolic processes. PI is an acidic (anionic) phospholipid that in essence consists of a phosphatidic acid backbone, linked via the phosphate group to inositol (hexahydroxycyclohexane). In mycobacteria, the stereochemical form is \textit{myo}-D-inositol. As early as 1930’s, it was recognized by Anderson that the phospholipidic fraction extracted from \textit{M.tb} and related mycobacteria contained inositol and mannose (Anderson, 1938). Besides phosphatidylethanolamide, PIMs are the major phospholipid components of the \textit{M.tb} cell wall (Brennan, 2003). PIMs are found as a mixture of compounds differing one from the other by the number of mannosyl residues and fatty acids. Their structures consist of a mannosyl unit attached to position C-2 of the \textit{myo}-inositol of a PI anchor. Position C-6 of \textit{myo}-inositol is further substituted by an \(\alpha\)-D-
mannosyl or a linked trimannosyl unit, giving PIM$_2$ and PIM$_4$, respectively. PIM$_4$ may be further substituted at position C-2 by a $\alpha$-D-mannosyl leading PIM$_5$, which can also be further substituted at the same position leading PIM$_6$ \([\alpha$-D-Manp(1→2)$\alpha$-D-Manp(1→2)$-\alpha$-D-Manp(1→6)$-\alpha$-D-Manp(1→6)$-\alpha$-D-Manp(1→6)-myo-inositol], the higher PIM encountered in mycobacteria (Torrelles & Schlesinger, 2010). Studies have shown that PIMs, which are known to interact with the plasma membrane, are also present on the \textit{M.tuberculosis} cell wall surface (Ortalo-Magné \textit{et al.}, 1996). The intrinsic heterogeneity of PIMs is evident looking at their carbohydrate constitution (PIM to PIM$_6$). A difficulty is added, however, when we look at the acylation sites of the PIMs. Many studies have shown evidence for multiacylated forms of PIMs in \textit{M.tuberculosis} [reviewed in (Torrelles & Schlesinger, 2010)]. Differences in the degree of acylation and the kind of fatty acid linked was studied in detail by Khoo \textit{et al.} (Khoo \textit{et al.}, 1995), who confirm the existence of triacylated PIMs (Ac$_1$PIM$_x$) esterified by palmitic (16:0) and tuberculostearic (TBST or 10-methyl-octadecanoic) acids, and discussed the presence of tetracylated PIMs (Ac$_2$PIM$_x$), where an additional fatty acyl could be carried in the myo-inositol ring. This fact was later corroborated by Gilleron \textit{et al.} (Gilleron \textit{et al.}, 1999) showing an unambiguous localization of a fourth fatty acid on the C-3 of the myo-inositol beside the fatty acids on C-1 and C-2 position of the glycerol and on the C-6 position of the (1→2) linked mannose. PIMs can be grouped in lower- and higher-order depending of the number of mannoses, where lower-order PIMs contain 1 to 4 mannoses and higher-order PIMs contain 5 to 6 mannoses (Torrelles \textit{et al.}, 2006). The most common PIMs found are Ac$_1$PIM$_2$ and Ac$_1$PIM$_5$ (di- and triacylated PIM$_2$) and Ac$_1$PIM$_6$ and Ac$_1$PIM$_6$ (di-and triacylated PIM$_6$) (Khoo \textit{et al.}, 1995). Lower-order PIMs have a terminal $\alpha$(1→6)-mannose and are shown to participate in the phagocytosis process through association with the non-opsonic domain of complement receptor-3 (Villeneuve \textit{et al.}, 2005), and also participate in trafficking processes within the phagocyte by facilitating early endosomal fusion with phagosomes (Vergne \textit{et al.}, 2004). Higher-order PIMs have a terminal $\alpha$(1→2)-mono- or di-mannoside (Ac$_1$PIM$_5$ or Ac$_1$PIM$_6$, respectively) similar to the mannose caps of mannose-capped lipoarabinomannan [see section 2.2.2.1 for details]. Only triacylated forms of higher-order PIMs are shown to interact with the mannose receptor (MR) and interfering with trafficking pathways by limiting phagosome-lysosome fusion (Torrelles \textit{et al.}, 2006). Moreover, several studies have also shown that all \textit{M.tuberculosis} PIMs interact with dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) (Torrelles \textit{et al.}, 2006), although differences in DC-SIGN PIM recognition specificity may be species dependent (Driessen \textit{et al.}, 2009). Additional studies have also shown that cytosolic soluble CD1e is involved in PIM$_6$ processing and presentation via CD1 with subsequent T cell activation (de la Salle \textit{et al.}, 2005) (Table 1).

Apart from the mannoses of phosphatidylinositol, there are other mycobacterial phosphodiacylglycerol, whose origins are based on phosphatidic acid. These are phosphatidylglycerol, diphasphatidylglycerol (DPG) and phosphatidylethanolamine. Although the role of these \textit{M.tuberculosis} cell wall phospholipids during infection is uncertain, \textit{M.tuberculosis} cardiolipin (a DPG molecule) is shown to be processed into lysocardiolipin by the lysosomal phospholipase A$_2$ during \textit{M.tuberculosis} infection (Fischer \textit{et al.}, 2001). Antibodies against \textit{M.tuberculosis} cardiolipin are also found in sera from TB patients (Santiago \textit{et al.}, 1989), and their production is shown to be strictly related to IL-4 and T cells (Fischer \textit{et al.}, 2002). Anti-
cardiolipin antibodies are also capable of activating complement (Santiago et al., 1991). Small quantities of glycosylphosphopolyisoprenols, which are involved in \( \text{M}.\text{tb} \) cell wall biosynthesis, have also been isolated from the cytoplasmic membranes of \( \text{M}.\text{tb} \), where ribosyl-, mannosyl- and arabinosyl-phosphopolyisoprenols have been characterized (Takayama et al., 1973; Takayama & Goldman, 1970; Wolucka & De Hoffmann, 1995).

In summary, \( \text{M}.\text{tb} \) elaborates a great variety of glycolipids of rather unusual structure. Some of these lipids are abundant in the inner cell wall and others are exposed on the bacillus surface. These include acylglucosides, sulfatides, lipooligosaccharides, phenolic glycolipids, dimycocerosates, and the ubiquitous phosphatidyl-myoinositol-mannosides. Some of these glycolipids are described as virulence factors helping \( \text{M}.\text{tb} \) to survive as intracellular ‘parasites’ that infect and reside in the host cell. The biological activities attributed to these surface-exposed glycolipids may derive, at least in part, from the modulation of cell functions through the interactions between host membranes and them, whose structures are different from those of mammalian cell membrane components. Biologically active glycolipids have been shown to profoundly affect the physical and functional properties of biologic membranes (Brandley & Schnaar, 1986) as well as inhibit both macrophage antimicrobial activities and lymphocyte proliferation (Vergne & Daffe, 1998). Therefore, the enzymes involved in their biosynthesis may represent potential drug targets (Kaur et al., 2009). Nevertheless, for some of these lipids confirmation of their role in \( \text{M}.\text{tb} \) pathogenicity is still lacking, opening the necessity to genetically manipulate \( \text{M}.\text{tb} \) to obtain glycolipid deficient mutants. These mutants may be unable to elaborate determined glycolipid thought to be involved in \( \text{M}.\text{tb} \) pathogenesis. Thus, the lack of specific glycolipids in virulent strains of \( \text{M}.\text{tb} \) may help us to understand their real implication in pathogenesis.

### 2.2.3 Lipoglycoconjugates of the \( \text{M}.\text{tuberculosis} \) cell wall

An extensive study of the mannose-capped lipoarabinomannan (ManLAM) has been performed in \( \text{M}.\text{tb} \) [reviewed in (Torrelles & Schlesinger, 2010)]. \( \text{M}.\text{tb} \) ManLAM is an extremely heterogeneous lipoglycan with a defined tripartite structure that possesses a carbohydrate core, a mannosyl-phosphatidyl-myoinositol anchor (MPI) and various capping motifs. Following the earlier work performed by Chatterjee and co-workers (Chatterjee & Khoo, 1998), a series of detailed structural analyses have produced evidence of this tripartite structure, in which ManLAM was distinguished from the related lipomannan (LM) by virtue of having an additional immunodominant arabinan domain that extends from a common phosphatidyl-myoinositol mannan core in an as yet undefined manner. The polysaccharide core of \( \text{M}.\text{tb} \) ManLAM consists of two very well differentiated polymers, a D-mannan and a D-arabinan. The D-mannan structure consists of a linear \( \alpha(1\rightarrow6) \) linked mannopyranosyl backbone that is linked to a phosphatidyl-myoinositol anchor, and presents substitutions/ branches on their C-2 with another single mannose (\( \alpha-D-\text{Manp}(2\rightarrow1)\alpha-D-\text{Manp} \)). The D-mannan size and the degree of branching can vary among \( \text{M}.\text{tb} \) strains. This mannosyl backbone carries an unknown number of branched arabinosyl side chains, which form the D-arabinan. To date, the linkage between the arabinan polymer and the D-mannan core is still not determined. The arabinan is based on the rare \( \alpha-D-\text{arabinofuranose} \) (Araf), and consists of a branched linear \( \alpha(1\rightarrow5) \) linked Araf backbone. Branching residues carry an additional \( \alpha(1\rightarrow3) \) linked Araf. At its non-reducing end we can
find two types of arrangements or motifs, a linear tetraarabinofuranoside (Ara4) defined as $\beta$-D-Araf(1→2)-$\alpha$-D-Araf(1→5)-$\alpha$-D-Araf(1→5)-$\alpha$-D-Araf and a branched hexaarabinofuranoside (Ara5) defined as $[\beta$-D-Araf(1→2)-$\alpha$-D-Araf(1→5)]2-3 and 5-$\alpha$-D-Araf(1→5)-$\alpha$-D-Araf. In the case of slow-growing mycobacteria like M.tb, M. leprae and M. bovis BCG, some of the terminal arabinan motifs are extensively capped at C-5 with one or more $\alpha$-mannoses attached to these $\beta$-Araf termini. The mannoooligosaccharides linked to the terminal $\beta$-Araf thus define ManLAM. The manno caps are defined as a single Manp, a dimannoside ($\alpha$-D-Manp-(1→2)-$\alpha$-D-Manp) or a trimannoside ($\alpha$-D-Manp-(1→2)-$\alpha$-D-Manp-(1→2)-$\alpha$-D-Manp). These units are located in both tetra- and hexaarabinoanysyl motifs of the arabinan non-reducing terminal (Chatterjee et al., 1993). Data reported from different studies shown that the disaccharide unit is the cap most frequently found in both linear (Ara4) and branched (Ara5) termini. Man$_2$Ara$_4$ and Man$_4$Ara$_6$ are then the most frequent motifs in all ManLAM studied (Chatterjee et al., 1993), however, trimannoside caps in the linear Ara4 have also been found (i.e. Man$_3$Ara$_4$), and in the branched Ara$_6$, all three combinations of mannose caps have been found, i.e. (Man$_{[1 \text{ to } 3]}$Ara$_{[2]}$). The degree of ManLAM capping varies according to the M.tb strain studied, where M.tb Erdman is the most capped when compared to M.tb H$_3$7R$_v$ and H$_3$7R$_a$ strains [reviewed in (Torrelles & Schlesinger, 2010)]. The anchor structure in M.tb ManLAM is similar to the one in PIMs, and consists in an ns-glycerol 3-phospho-(1-D-myo-inositol) unit with a $\alpha$-D-mannopyranosyl residue at C-2 of the myo-inositol (MPI). In the C-6 position of this myo-inositol there is O-linked the mannan polymer described previously (Chatterjee & Khoo, 1998). Some of the heterogeneity that characterizes ManLAM occurs through the number, the location, and the nature of the fatty acids esterifying the PI anchor. The characteristic fatty acids described in the ManLAM anchor are 16:0 and TBST (Hunter et al., 1986). However, traces of stearic (18:1), myristic (14:0), heptadecanoic (17:0), 10-methyl-heptadecanoic, 12-O-(methoxypropieryl)-12-hydroxy-stearic and 12-hydroxy-tuberculostearic acids have also been described (Leopold & Fischer, 1993; Nigou et al., 1997). The average number of fatty acids per ManLAM molecule cannot be generalized. Some studies confirmed an average of 3 fatty acids per molecule of ManLAM in positions 1 and 2 of the ns-glycerol and position 6 of the Manp unit linked to C-2 of the myo-inositol (Khoo et al., 1995). Studies by Chatterjee and colleagues performed in an ethambutol resistant strain of M.tb supported the existence of tetraacylated ManLAM as the most common molecular form (Torrelles et al., 2004). Thus, the only fact that can be generalized is that, with the exception of the lysoforms of ManLAM (only one fatty acid in the ManLAM anchor), ManLAM at least has two fatty acids, with both fatty acids in the ns-glycerol unit, where 16:0 and TBST are at position 1 and at position 2, respectively.

The presence of additional acyl groups on ManLAM has been reported by several authors. Hunter et al. reported the existence of succinates and lactates (Hunter et al., 1986). Delmas et al. used nuclear magnetic resonance spectroscopy to locate the succin groups (1 to 4 per molecule) in the C-2 of the 3,5-$\alpha$-D-Araf and/or 5-$\alpha$-D-Araf residues in ManLAM from different Mycobacterium bovis BCG strains (Delmas et al., 1997). Later, studies performed by Chatterjee and colleagues analyzing the content of succinates in ManLAMs from different mycobacterial species and strains showed that Mycobacterium leprae, the M.tb laboratory strain H$_3$7R$_v$, and a M.tb clinical isolate (CSU 20) had also succinates (Torrelles et al., 2004); where ManLAM from M. leprae, the laboratory strain H$_3$7R$_v$ and CSU20 had an average
number of 7, 2 and 4 succinates, respectively. The succinates biological function in ManLAM is still a question to be resolve. Our recent studies show that a biosynthetically related lipoglycan to ManLAM, the lipomannan, also contains succinates, where succinates seem to influence CD1-Ag presentation to T cells and subsequent T cell activation (Torrelles et al., 2011). Recently, Treumann et al. using nuclear magnetic resonance spectrometry defined a new terminal sugar located in the caps of M.tb ManLAM (Treumann et al., 2002). This sugar consisted in a 5-deoxy-5-methylthio-α-xylofuranosyl (MTX), and may be involved in M.tb-host interactions battling the effects of reactive oxygen species by adding to the antioxidant properties of ManLAM (Turnbull et al., 2004). The orientation of LAM in the M.tb cell wall is still unresolved. There are many hypotheses, but the most accepted is that ManLAM is anchored by its lipidic anchor into the plasma membrane, and projects through the thickness of the wall so that its terminal arabinose or mannose-capped arabinose units are accessible to the outside (McNeil & Brennan, 1991). Other possibilities are that ManLAM is interacting by its lipid anchor with the mycolic acid layer and with other polar wall associated lipids (Rastogi, 1991), or that ManLAM has a non-permanent location in the cell wall, and is essentially a secreted molecule in transit through the envelope. The many studies carried out on ManLAM have led to data that supports each of these hypotheses. For example, Lemassu and Daffe demonstrate the existence of non-PI containing mannose-capped arabinomannan in the so called capsular/outer material polysaccharide associated with M.tb (Lemassu & Daffe, 1994). Other studies, subdivided ManLAM into two different kinds, the parietal ManLAM and the cellular ManLAM. Both had similar core structure presenting remarkable differences in the degree of mannose-capping and the acylation of the PI-anchor (Gilleron et al., 2000). The fact that the parietal LAM is obtained without cell disruption reinforces the hypothesis of two different locations for ManLAM. Thus, ManLAM may be firmly, but not covalently, attached to the M.tb cell wall and it may also be anchored to the plasma membrane. The biological function of M.tb ManLAM is discussed later in this chapter (see also Table 1), and information about its biosynthesis pathway(s) can be found elsewhere (Kaur et al., 2009).

Another remarkable lipoglycan in the M.tb cell wall is lipomannan (LM). The α (1→6) mannose polymer of LM presents identical characteristics to the mannan backbone of ManLAM. The mannan of LM is directly attached to position C-6 of the myo-inositol of its MPI anchor. The M.tb ManLAM and LM MPI anchor is indistinguishable from the M.tb dimannosylated phophatidyl-myo-inositol (Ac,PIM2), the structure of which was established by Lee and Ballou (Lee and Ballou, 1965). LMs are considered multimannosylated forms of PIMs by the fact that both types of molecules have an elaborated anchor in common (Gilleron et al., 1999). Their common structure with ManLAM also enforces the hypothesis that LM is a precursor of ManLAM (Besra et al., 1997). However, it seems that LM could also be a co-lateral final product in the biosynthetic pathway of ManLAM (Besra et al., 1997). Few biological properties of M.tb LM have been described, mainly because this molecule is still understudied (Table 1).

Mycobacterial LM is shown to regulate cytokine, oxidant and T cell responses (Barnes et al., 1992; Chan et al., 2001; Gilleron et al., 2001). M.tb LM is shown to associate with DC-SIGN and not with the MR (Torrelles et al., 2006), and to induce apoptosis and a pro-inflammatory response through TLR2 (Dao et al., 2004; Nigou et al., 2008). However, recent studies showed that although M.tb LM is capable of activating macrophages via TLR2 inducing
signaling cascades required for TNF mRNA expression, the TNF mRNA produced is poor translated and faster degraded (Rajaram et al., 2011).

<table>
<thead>
<tr>
<th>M. tb cell wall location</th>
<th>M. tb cell wall component</th>
<th>Host Cell Receptor(s)</th>
<th>Phagosome maturation blockade</th>
<th>Host Immune Response</th>
<th>Seroactivity</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Material</td>
<td>α-Glucan</td>
<td>DC-SIGN, CR3?</td>
<td>No</td>
<td>Anti-inflammatory</td>
<td>Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>Cell wall Core</td>
<td>Peptidoglycan (PG)-MDP</td>
<td>Nod2</td>
<td>ND</td>
<td>Pro-inflammatory</td>
<td>Unknown</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Arabinogalactan (AG)</td>
<td>ND</td>
<td>ND</td>
<td>Pro-inflammatory</td>
<td>Unknown</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Mycolic Acids</td>
<td>CD1 (in Ag-presentation)</td>
<td>ND</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Peripheral lipid layer</td>
<td>Trehalose dimycolate (TDM)</td>
<td>Mincle-FcγR TLRs</td>
<td>Yes</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Trehalose monomycolate (TMM)</td>
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<td>ND</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Sulfolipid-1 (SL-1)</td>
<td>ND</td>
<td>Yes</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Diacyl- and Triacyl-trehalose (DAT &amp; TAT)</td>
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<td>Yes</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Lipoooligosaccharides (LOSs)</td>
<td>ND</td>
<td>ND</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Phenolic glycolipid (PGL-TB)</td>
<td>CR3?</td>
<td>ND</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td></td>
<td>Triglycerides</td>
<td>TLRs</td>
<td>ND</td>
<td>Pro-inflammatory</td>
<td>Unknown</td>
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</tr>
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<td></td>
<td>Phthiocerol dimycocerosate (PDIM)</td>
<td>Direct insertion into host Mbrs</td>
<td>Yes</td>
<td>Pro-inflammatory</td>
<td>Unknown</td>
<td>Yes</td>
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<tr>
<td></td>
<td>Lower-order phosphatidyl-myoinositol mannosides (PIMs)</td>
<td>CR3, TLRs, DC-SIGN</td>
<td>No</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Higher-order phosphatidyl-myoinositol mannosides (PIMs)</td>
<td>MR, DC-SIGN</td>
<td>Yes (through the MR only)</td>
<td>Anti-inflammatory</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Lipomannan (LM)</td>
<td>TLRs, DC-SIGN</td>
<td>No</td>
<td>Pro-inflammatory</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Mannose-capped lipoarabinomannan (ManLAM)</td>
<td>MR, DC-SIGN</td>
<td>Yes (through the MR only)</td>
<td>Anti-inflammatory</td>
<td>Yes</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mbrs: Membranes.

Table 1. M. tb cell wall components and their interaction with the host outcome.
Due to the complexity of the cell wall of *M. tb*, we may need to be careful when assessing the essentiality of a specific cell wall component thought to be a virulence factor. Current strategies are directed in creating isogenic strains of *M. tb* deficient in the production of a specific virulent factor. We may not obtain the real answer by just depleting the presence of a potential virulent factor in the cell wall of *M. tb*. In this context, efforts to discern the enzymes involved in the biosynthetic pathways of the *M. tb* lipids are critical to address their essentiality in *M. tb* survival. These will allow us to uncover novel drug targets. However, when evaluating the role of the omitted/mutated lipid in *M. tb* pathogenesis, we need to be careful in considering the rearrangement that the *M. tb* cell wall may suffer upon the lack of a specific lipid. Depending of the structural nature of the lipid depleted from the *M. tb* cell wall (*i.e.* size, charge, hydrophobicity, etc.), we may find that the bacterial cell wall is altered in a way that the absence of the lipid is unexpectedly compensated. This is the case for SL-1, where the lack of this lipid in *M. tb* isolates is being linked to TB pathogenesis, however, studies performed by Jackson and colleagues clearly showed that when using an isogenic SL-1 mutant, SL-1 deficiency did not affect *M. tb* virulence (Rousseau *et al.*, 2003). As Jackson and collaborators stated in this study, there are several explanations behind this observed discrepancy, but one of them is related to the presence of a potent attenuator lipid in the clinical isolates lacking SL-1 that compensated the SL-1 phenotype (Goren *et al.*, 1982). Other factor to account for is the synergy between *M. tb* lipids; this is also observed for SL-1 and TDM, where purified SL-1 alone at high doses was innocuous, but when administered simultaneously with TDM, a synergistic increase in the TDM cytotoxicity was observed (Kato & Goren, 1974a, 1974b). Thus, indicating that the lack of a specific lipid may also significantly alter the cytotoxic properties of other *M. tb* cell wall components. Finally, we will need to consider evaluating the constitution of the *M. tb* cell wall during infection. Are the properties of the *M. tb* cell wall altered during infection? Some studies indicate that this may happen in the case for TDM, which is overproduced during *M. tb* infection (Backus *et al.*, 2011; Fischer *et al.*, 2001). Why do clinical isolates of *M. tb* present different cell wall rearrangements than the widely studied *M. tb* laboratory strains Erdman, H₃₇Rᵥ, and H₃₇Rₐ? In this context, hypervirulent *M. tb* strains of the Beijing family (Tsenova *et al.*, 2005) are shown to contain large amounts of triglycerides (Reed *et al.*, 2007), and some of them also contain the PGL-TB (Reed *et al.*, 2004). *M. tb* clinical isolates deficient in ManLAM and PIMs surface exposure, but presenting in their cell wall large quantities of triglycerides, PGL-TB, and dimycocerosates, are also shown to have reduced phagocytosis but faster intracellular growth rate in human macrophages (Torrelles *et al.*, 2008b). These studies performed by Schlesinger and colleagues concluded that the clinical spectrum of TB is not only dictated by the host but also it may be related to the amounts and ratios of specific surface-exposed *M. tb* adherence factors defined by *M. tb* strain genotype (Torrelles *et al.*, 2008b; Torrelles & Schlesinger, 2010). Is this *M. tb* genotypic/phenotypic adaptation due to their multiple passages through the host? Triglycerides and DIMs are a major part of the peripheral lipid layer in the *M. tb* cell wall; however, their role in pathogenesis has been until recently overlooked due the presence of other hydrophilic and hydrophobic cell wall components more attractive to TB researchers due to their potential (or already established) role in dictating *M. tb*-host cell interactions. Other questions demanding answers refer to the structural properties of the cell wall of MDR-, XDR-, XXDR, and TDR-*M. tb* strains. What is the cell wall constitution of these strains? Studies performed using transmission electron
and atomic force microscopy techniques started to dig into this question showing that MDR-, XDR-, XXDR- and TDR- strains have thicker cell wall and rougher cell surface (supposedly produced by the progressive erosion of their cell wall by the action of the drugs) with tubular extensions than susceptible strains (Velayati et al., 2009a, 2010). Because some XDR- and TDR-M.tb strains are related to the Beijing family (Velayati et al., 2009b), which are shown to have their cell wall overpopulated with triglycerides, it is plausible to question any relationship between the abundance of a specific hydrophobic lipid on the M.tb cell wall and drug resistance. Many of these questions remain unanswered.

3. M.tb-Host interface

The initial recognition of M.tb by the host is quite complex and involves alveolar resident cells and many of their surface receptors. The concept of studying the contribution of a specific receptor(s) in the M.tb recognition and/or uptake is critical to our understanding of the pathway(s) that the bacillus exploits to gain entrance into the host cell minimizing or triggering the immune response. However, it is important to link the results obtained studying a specific receptor to the existence of other receptors that may also participate at the same time in recognizing M.tb generating a completely different outcome. Normally the outcome of M. tuberculosis-host recognition is beneficial for the host, triggering the innate immune response; however, engagement of M.tb with specific phagocytic receptors is shown to be beneficial for M.tb leading to a pathway of survival and subversion of the immune response. Here we will describe the host phagocytic and signaling receptors [some of them known as a pattern recognition receptors, PRRs, for their unique capability to recognize specific motifs on the M.tb cell surface (these motifs are also known as pathogen/microbial-associated molecular patterns, PAMPs/MAMPs)] involved in M.tb recognition and the subsequent inflammatory response attending in our discussion to the fact that M.tb recognition simultaneously includes multiple receptors.

3.1 Phagocytic receptors

The encounter of M.tb with the host triggers the phagocytosis process. This process depends of two important factors; one is the constitution of the cell wall of M.tb (which is strain dependent) and the surface receptor repertoire present on the phagocyte (which is host cell dependent). Mainly M.tb infections occur by airborne transmission of droplet nuclei containing few viable bacilli. The first contact between M.tb and the human host cell is within the alveolar space of the lung. When M.tb reaches the alveolar space, resident alveolar macrophages (AMs), and alveolar epithelial cells together with recruited monocytes, neutrophils, lymphocytes and fibroblast represent the array of immune cells that participate in host defense. Phagocytic receptors involved in M.tb recognition by the host mainly are: the mannose receptor (MR), DC-SIGN, and complement receptors (CRs). M.tb uptake by these receptors leads to the formation of an M.tb containing phagosome with different outcomes as noted below (Table 1).

3.1.1 The mannose receptor

The cell wall of certain M.tb strains has been characterized to be heavily mannosylated with molecules exposing their α(1→2)-Manp termini on the bacterial surface acting as ligands for
host cell receptors contributing to \(M.\text{tb}\) pathogenesis (Torrelles & Schlesinger, 2010). These molecules are ManLAM, LM, PIMs, arabinomannan, mannan, and mannosylated glycoproteins. The MR is conceived as a homeostatic receptor, whose main function is the recycling of endogenous highly N-mannosylated glycoproteins normally generated during inflammation (Martinez-Pomares et al., 2001). Studies by Schlesinger and colleagues have suggested that \(M.\text{tb}\) may be capable of using its surface mannose coating to gain entrance and survive within the host cell by associating with the MR. In this context, we demonstrated that \(M.\text{tb}\) can use two of its mannosylated cell wall components, ManLAM and higher-order PIMs, to associate with the MR, leading to a pathway of intracellular survival within the host by blocking phagosome acidification (Kang et al., 2005; Torrelles et al., 2006). Association with the MR has also been shown to reduce microbicidal activities by down-regulating the generation of pro-inflammatory cytokines, nitric oxide, oxygen radicals and by blocking \(M.\text{tb}\)-induced Ca\(^{2+}\)-depending apoptosis[reviewed in (Torrelles et al., 2008a)]. In particular, \(M.\text{tb}\) ManLAM has been shown to interact with the MR triggering an anti-inflammatory response by blocking the production of inflammatory cytokines such as TNF and IL-12, and inducing the generation of IL-10 and TGF-\(\beta\) (Astarie-Dequeker et al., 1999; Chieppa et al., 2003; Nigou et al., 2001)(Table 1). Recently, studies by Schlesinger and colleagues showed that engaging of the MR by ManLAM and/or virulent \(M.\text{tb}\) upregulates the peroxisome proliferator-activated receptor-gamma (PPAR-\(\gamma\), a transcription factor showed to be important in regulating the inflammatory response) leading to a simultaneous increase in the generation of CXCL-8 (or IL-8), expression of cyclooxygenase 2 (COX2), and production of prostaglandin 2 (PGE\(_2\)) (Rajaram et al., 2010). Moreover, this study depicts how \(M.\text{tb}\) negatively regulates protective inflammatory modulators through the MR, where engaging of the MR down-regulates TNF levels via PPAR-\(\gamma\) (Rajaram et al., 2010). In addition to \(M.\text{tb}\) ManLAM blocking the generation of TNF via the MR, Schlesinger and colleagues also identified a novel molecular and cellular mechanism underlying the ability of another major \(M.\text{tb}\) cell wall component, the LM, to block TLR2 induced biosynthesis of TNF in human macrophages, thereby allowing \(M.\text{tb}\) to subvert the host immune response and potentially increase its virulence (Rajaram et al., 2011).

### 3.1.2 DC-SIGN

\(M.\text{tb}\) is shown to associate with dendritic cell-specific ICAM-3-grabbing non-integrin (or DC-SIGN) (Geijtenbeek et al., 2000) through is cell surface cell wall components ManLAM, LM and PIMs [reviewed in (Ehlers, 2009)](Table 1). Recently, \(\alpha\)-glucan was also described as another \(M.\text{tb}\) cell wall ligand for DC-SIGN (Geurtsen et al., 2009). Binding of \(M.\text{tb}\) to DC-SIGN in DCs leads to bacterial killing by acidification of the \(M.\text{tb}\) phagosome (Geijtenbeek et al., 2003). However, the implication of DC-SIGN in triggering the immune response is still controversial. On one hand, engaging of \(M.\text{tb}\), mannosylated cell wall components, or \(\alpha\)-glucan has been shown to induce generation of anti-inflammatory modulators such as IL-10 (Ehlers, 2009; Geurtsen et al., 2009). These findings were supported by in vivo studies using mice expressing human DC-SIGN homologues (McGreal et al., 2005; Park et al., 2001; Powlesland et al., 2006) or transgenic mice expressing human DC-SIGN, showing that DC-SIGN may act damping the immune response, and thus, promote host protection by limiting tissue damage (Schaefer et al., 2008; Tanne et al., 2009; Wieland et al., 2007). On the other hand, another study concluded that the ManLAM-PIM/DC-SIGN pathway may not
be significantly involved in regulating cytokine secretion using an engineered \textit{M. marinum} strain lacking essential mannosylated components (Appelmelk \textit{et al.}, 2008).

### 3.1.3 Complement receptors

Complement receptors are described on the surface of all mononuclear phagocytes. In \textit{M.tb} phagocytosis CR1, CR3 and CR4 have been implicated (Fenton \textit{et al.}, 2005). Several studies have established the role of the complement component 3 (C3) in \textit{M.tb} opsonization. C3 deposition in the form of C3b and C3bi happens quickly via covalent linkages with cell wall components located on the \textit{M.tb} surface (Ferguson \textit{et al.}, 2004). As C3 opsonization depends of serum levels in the tissue, it is still unknown how C3 opsonization varies in form (classical and/or the alternative pathways) and amount among different stages of \textit{M.tb} infection or tissue sites. \textit{M.tb} surface-exposed lower-order PIMs (\textit{i.e.} PIM$_2$) and specific polysaccharides have been shown to directly interact with the lectin domain of CR3 (Cywes \textit{et al.}, 1997; Hoppe \textit{et al.}, 1997; Villeneuve \textit{et al.}, 2005) and thus, presumably mediate \textit{M.tb} uptake by macrophages (Table 1). PGL-TB from \textit{M.tb} may also interact with CR3, as this is the case for the structurally related PGL-1 from \textit{M. leprae} (Tabouret \textit{et al.}, 2010). Although CR3 seems to drive \textit{M.tb} uptake under opsonic and non-opsonic conditions, \textit{in vitro} and \textit{in vivo} studies using wild type and CR3-deficient mice did not show differences in lung pathology and bacterial burden (Hu \textit{et al.}, 2000), and thus CR3 role in \textit{in vivo} infections remains unanswered. Less attention has been put into CR4, which together with the MR, is highly expressed on AMs and other cells involved in \textit{M.tb} uptake (Hirsch \textit{et al.}, 1994; Zaffran \textit{et al.}, 1998; Schlesinger \textit{et al.}, 2008), and thus may be playing a major role in the uptake of \textit{M.tb} by the naive host in early stages of infection.

### 3.2 Signaling receptors

Apart from the phagocytosis process, \textit{M.tb} also is shown to signal through specific signaling receptors located on the host cell surface and/or cytosol. The main signaling receptors for \textit{M.tb} are Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain-like (NODs) receptors.

#### 3.2.1 Toll-like receptors

TLRs are a set of PPRs expressed on many cell types but their function on phagocytes is particularly important [reviewed in (Kawai & Akira, 2010)]. On macrophages TLRs are either expressed on the surface (like TLR2 and 4) or inside cell compartments (like TLR8 and 9) (Kawai & Akira, 2010). TLRs detect a wide range of PAMPs on \textit{M.tb}, which activates the innate immune response and enhance adaptive immunity by mediating the secretion of various pro-inflammatory cytokines along with other anti-bacterial modulators (Table 1). TLRs shown to be key players in triggering immunity against \textit{M.tb} infection are TLR2 (alone or as a heterodimer with TLR1 or TLR6), TLR9, and probably TLR4 (Harding & Boom, 2010). TLR2 alone or dimerized with TLR1 or TLR6, is shown to trigger a strong pro-inflammatory response by recognizing \textit{M.tb} 19 KDa lipoglycoprotein, lower- and higher-order PIMs, LM and TDM [reviewed in (Jo \textit{et al.}, 2007)]. This pro-inflammatory response via TLR2 is shown to be mediated through its adaptor protein myeloid differentiation primary-response protein 88 (MyD88)(Quesniaux \textit{et al.}, 2004), triggering a nuclear factor kappa-light chain-
enhancer of activated B cell (NFκB) signaling cascade through the recruitment of MyD88 and TIRAP (toll-interleukin 1 receptor [TIR] domain containing adaptor protein) (Kawai & Akira, 2010). Of no surprise the intensity of the immune response observed via TLR2 depends on the \textit{M.\textit{tb}} ligand and the nature of the host cell studied (Thoma-Uszynski et al., 2001; Underhill et al., 1999). Surprisingly, prolonged TLR2 signaling can also benefit \textit{M.\textit{tb}}. Studies implicating a prolonged stimulation of TLR2 showed an inhibition of antigen presentation due to the down-regulation in the expression of the major histocompatibility complex (MHC) class II in macrophages infected with \textit{M.\textit{tb}} (Harding & Boom, 2010). Other studies also show that \textit{M.\textit{tb}} is capable of inhibit MHC-I antigen cross processing and presentation to CD8+ T cells via TLR2 signaling (Harding & Boom, 2010). TLR2-dependent inhibition of TLR9-dependent IFN-α/β expression, thus leading to a decrease of IFN-α/β-dependent MHC-I cross processing is also shown in DCs (Simmons et al., 2010). In this regard, it is unknown if these described mechanisms of MHC-I and -II inhibition via TLR2 will be beneficial for \textit{M.\textit{tb}} by passing the host immune response, or will be beneficial for the host by limiting the harmful effects of excessive inflammation. Thus, it is not unreasonable to search for a regulatory mechanism(s) among TLRs signaling networks necessary to control inflammation during \textit{M.\textit{tb}} chronic infection (Drennan et al., 2004; Simmons et al., 2010). TLR9 recognizes unmethylated CpG (cytosine phosphate guanosine motif) found in \textit{M.\textit{tb}} DNA (Kawai & Akira, 2010). Activation of TLR9 induces IFNα/β and MHC-I antigen cross processing (Simmons et al., 2010). The role of TLR4 in \textit{M.\textit{tb}} infection is unclear as only a few \textit{M.\textit{tb}} ligands for TLR4 have been described. Recently, recombinant \textit{M.\textit{tb}} heat shock protein (hsp) 65 was shown to induce the generation of TLR4-dependent NFκB via MyD88-, TIRAP-, TRIF- (TIR-domain-containing adapter-inducing interferon-β) and TRAM- (TRIF-related adaptor molecule)-dependent signaling pathways (Bulut et al., 2005).

3.2.2 Cytosolic receptor: NOD2

Cytosolic regulators known as NODs receptors (Franchi et al., 2008) are known to participate in the induction of pro-inflammation during \textit{M.\textit{tb}} infection. Specifically Nod2, which is found in epithelial cells and antigen presenting cells (Gutierrez et al., 2002; Inohara and Nunez, 2003; Ogura et al., 2001), is shown to regulate the production of inflammatory mediators in response to \textit{M.\textit{tb}} PG components such as muramyl dipeptide (MDP) (Brooks et al., 2011; Franchi et al., 2008; Sirard et al., 2007) (Table 1). Nod2 polymorphism studies in humans are linked to susceptibility to mycobacterial infection (Austin et al., 2008; F.R. Zhang et al., 2009). Studies done in vitro using different models and in vivo using the mouse model dispute the significance of Nod2 in controlling \textit{M.\textit{tb}} growth during infection (Divangahi et al., 2008; Gandotra et al., 2007). However, recent studies using human macrophages align with the human polymorphism studies showing that Nod2 plays a role in controlling pro-inflammation and \textit{M.\textit{tb}} intracellular growth (Brooks et al., 2011). How Nod2 intersects with signaling/taxicating networks starts to be uncovered (Pandey et al., 2009). Although, Nod2 can synergize with TLR-signaling pathways enhancing pro-inflammation (Ferwerda et al., 2005), its capacity to interfere/associate with phagocytic receptor trafficking networks is not well established. As cytosolic Nod2 appears to be associated with intracellular vesicles (Brooks et al., 2011), its role in triggering pro-inflammation may depend on vesicular fusion events controlled during \textit{M.\textit{tb}} phagocytosis and phagosomal maturation (Sasindran & Torrelles, 2011).
3.3 Other phagocyte receptors

Collectins such as surfactant protein –A and –D, and mannose binding protein, and their specific receptors have been shown to be important in \textit{M. tb} recognition by the host; and their contribution in \textit{M. tb} pathogenesis is discussed elsewhere (Sasindran & Torrelles, 2011;Torrelles & Schlesinger, 2010;Torrelles \textit{et al.}, 2008a). Other receptors involved in the recognition of \textit{M. tb} and inflammation are CD14, scavenger receptor-A, Fcγ-receptor, Mincle, and Dectin-1 (Sasindran & Torrelles, 2011). CD14 (Khanna \textit{et al.}, 1996) and the scavenger receptor SR-A (Zimmerli \textit{et al.}, 1996), are shown to participate in the uptake of non-opsonized bacilli by tissue-specific macrophages; where their role in inflammation varies depending on the species-specific cell type used. Dectin-1 (dendritic cell-associated C-type lectin 1), a β-glucan receptor, in combination with TLR2 has also been shown involved in the immune response against \textit{M. tb} (Yadav & Schorey, 2006). Recently, Mincle (macrophage-inducible C-type lectin) (Yamasaki \textit{et al.}, 2008) on the macrophage surface, has been shown to specifically recognize \textit{M. tb} TDM, inducing a pro-inflammatory response by working together with the Fcγ receptor transmembrane segment (Ishikawa \textit{et al.}, 2009;Schoenen \textit{et al.}, 2010)(Table 1). Conversely, Fcγ receptors do not play a role in the phagocytosis of \textit{M. tb} in the absence of specific antibody (Schlesinger \textit{et al.}, 1990).

In this type of studies we should carefully consider differences between model systems used. There are multiple examples of contradictions when comparing studies performed \textit{in vivo} vs. \textit{in vitro} and/or when comparing cells from an animal model \textit{vs.} human primary cells. In this context, it is plausible that depending of the model used (i.e. primary alveolar macrophage \textit{vs.} THP-1 cell; or human \textit{vs.} another mammalian host cell), a host cell may differentially express the targeted receptor on its surface, or this targeted receptor may be involved in triggering additional or different signaling and/or trafficking network(s). The same concept can be attributed when studying different strains of \textit{M. tb}. A clear example is the variable degree of mannosylation observed on the \textit{M. tb} surface among different strains (i.e. less ManLAM and PIMs and more triglycerides, PGL-TB and PDIM on the cell wall of \textit{M. tb} clinical isolates \textit{vs.} \textit{M. tb} laboratory strains H\textsc{37}R\textsc{v} and Erdman) (Torrelles \textit{et al.}, 2008b), and how this may impact the infection outcome (Torrelles \& Schlesinger, 2010). In light of these findings, we need to be careful in considering which cell wall components are heavily present on the surface of the \textit{M. tb} strain(s) studied and their implications in the host cell phenotype observed.

4. \textit{M. tuberculosis}-Host relationship with the alveolar environment(s) found during infection

It is thought that initial interaction between \textit{M. tb} and the host dictates the pathway and outcome of infection. When \textit{M. tb} infection occurs by airborne transmission, bacilli are deposited in the alveolar spaces of the lungs. The traditional view is that \textit{M. tb} is somewhat “static” during initial infection, does not induce an immune response, and it is taken up by non-activated AMs that serve as an important reservoir for infection. However, we envision that upon deposition in the alveolar space \textit{M. tb} may enter a dynamic phase where it encounters pulmonary surfactant that contains homeostatic and antimicrobial enzymes (Hawgood & Poulain, 2001;van Golde, 1985) (called hydrolases) which alter the \textit{M. tb} cell wall. Due to the dynamics of \textit{M. tb} infection (Chroneos \textit{et al.}, 2009), when \textit{M. tb} is initially
deposited in the terminal bronchioles and alveoli, as well as, following release from lysed macrophages and in cavities in reactivated TB, \textit{M.\textit{tb}} bacilli are in intimate contact with lung surfactant hydrolases. We recently demonstrated that hydrolases present in the human lung surfactant (Mason, 2006; Williams, 2003), at their relevant concentrations in vivo, dramatically alter the cell wall of \textit{M.\textit{tb}} during infection (Arcos \textit{et al}., 2011). As a result of these cell wall modifications, a significant decrease in association of \textit{M.\textit{tb}} with human macrophages was observed followed by an increase in phagosome-lysosome fusion (35%), which translated to a significant decrease in \textit{M.\textit{tb}} intracellular survival within these cells and an increase in inflammatory cytokine production leading to better control of infection (Arcos \textit{et al}., 2011). Importantly, we demonstrated that minimal contact time (15 min) with human lung surfactant hydrolases significantly reduced the cell surface exposure of two major \textit{M.\textit{tb}} virulence factors, ManLAM and TDM (Arcos \textit{et al}., 2011). As mentioned above, both, ManLAM and TDM have been shown to play important roles in the intracellular survival of \textit{M.\textit{tb}} in the host by blocking the phagosome maturation process (Axelrod \textit{et al}., 2008; Kang \textit{et al}., 2005). Thus, below we will address which are the sources of these hydrolases in the alveolar space.

\textbf{4.1 The \textit{M.\textit{tuberculosis}} infection pathway and the alveolar environment: The potential role of human lung surfactant hydrolases}

The first interaction between \textit{M.\textit{tb}} 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 aimed at the physical removal of inhaled bacteria; recognizing PAMPs/MAMPs by PPRs expressed on epithelial and myeloid cells, and secreting a variety of pro- and anti-inflammatory mediators, including a large variety of hydrolases (Nicod, 2005). When \textit{M.\textit{tb}} bacilli reach the alveolar space, AMs, monocytes, and neutrophils represent the array of innate immune myeloid cells that will participate in host defense.

\textbf{4.1.1 The alveolar macrophage}

The AM is the first professional phagocyte to encounter inhaled \textit{M.\textit{tb}} bacilli. AMs are placed in a unique location within the alveolar surfactant film, the latter of which is produced by type II alveolar epithelial cells and is composed of phospholipids and proteins (Jonsson \textit{et al}., 1986). AMs are at the interface between air and lung tissue, and represent the first line of defense against inhaled \textit{M.\textit{tb}} found in the air (Lohmann-Matthes \textit{et al}., 1994). AMs possess a high phagocytic and clearance potential. In a normal healthy individual, they represent more than 90% of the cells in bronchoalveolar lavage fluid (Reynolds, 1987).

Many studies have demonstrated that resident AMs can phagocytose large numbers of microbes through both opsonic and non-opsonic receptors (Fels & Cohn, 1986; Lohmann-Matthes \textit{et al}., 1994; Palecanda & Kobzik, 2001; Serrano-Gomez \textit{et al}., 2004; Stephenson and Shepherd, 1987; Tailleux \textit{et al}., 2005; Taylor \textit{et al}., 2002; F.X. Zhang \textit{et al}., 1999). Though AMs have high phagocytic activity, their microbicidal capacity is less well-defined. Efficient microbial phagocytosis followed by slow intracellular killing may be sufficient to control infection with many routinely encountered extracellular pathogens. Intracellular pathogens like \textit{M.\textit{tb}}, however, may take advantage of the reduced microbial activity of the AM by residing and multiplying within these cells (Ferguson & Schlesinger, 2000).
participation of AMs in host defense, inflammatory processes and immune mechanisms has been amply documented (Schlesinger, 1997). In general, their primary function is the intracellular breakdown and disposal of particulate elements. In this regard, they contain a wide variety of hydrolases such as abundant lipase, acid phosphatase, cathepsin, lysozyme, esterase, acid ribonuclease, and β-glucuronidase activities (on a specific activity basis) (Cohn & Wiener, 1963; Sorber et al., 1973). Interestingly, dead BCG-stimulated AMs exhibited up to a 4-fold increase in the activities for lipases, acid phosphatases and lysozyme compared to control (Cohn & Wiener, 1963) indicating an up-regulation of these hydrolases within the AMs in the presence of antigen. This observation was corroborated in AMs obtained from live BCG-vaccinated rabbits, where acid phosphatase, lipase and lysozyme activities increased up to 40-fold when compared to AMs from control rabbits (Sorber et al., 1973). How these hydrolases are regulated during *M.tuberculosis* infection and their role in redecorating the cell envelope of *M.tuberculosis* are currently unknown.

### 4.1.2 Monocytes and neutrophils in the alveolar space

Mononuclear phagocytes enter the lung both constitutively to maintain AM and dendritic cell populations, and during lung inflammation (Srivastava et al., 2005). The role of monocyte accumulation in the lung in acute and chronic pulmonary inflammation is largely unknown, although these cells are accessible by bronchoalveolar lavage (Maus et al., 2001). In the mouse, alveolar deposition of a stimulus provoked a significant influx of monocytes into the interstitium of the alveolar compartment along with a characteristic recruitment of neutrophils (Gunn et al., 1997; Li et al., 1998; Ulich et al., 1991). This was confirmed by studies showing that circulating leukocytes could be recruited across the endothelial and epithelial barriers into the alveolar space under both non-inflammatory and highly inflammatory conditions (Li et al., 1998; Maus et al., 2001). Given the capacity of monocytes to produce hydrolases, reactive oxygen species, or inflammatory cytokines (Van Furth, 1988), their accumulation has been implicated in several inflammatory diseases in the pulmonary system (Antoniades et al., 1992). As is the case for AMs, how monocytes and their secreted products affect the cell wall of *M.tuberculosis* within the alveolar space remains unknown.

Neutrophils may play an important role in controlling *M.tuberculosis* infection (Pedrosa et al., 2000; Seiler et al., 2003). In the bronchoalveolar lavage fluid, they normally represent less than 2% of all cells, however, during inflammation a massive influx of neutrophils occurs (Mizgerd, 2002; P. Zhang et al., 2000). Neutrophils eliminate microbes by a number of oxidative and non-oxidative mechanisms (P. Zhang et al., 2000) including secreted hydrolases such as N-acetyl-β-glucosaminidase, β-glucuronidase, α-mannosidases, and lysozyme. Neutrophils can kill *M.tuberculosis* through both oxidative and non-oxidative processes (Brown et al., 1987; Jones et al., 1990). Although neutrophils may interact with *M.tuberculosis* cell wall components during alveolar deposition [such as the 19-KDa lipoglycoprotein, SL-1 and PGLs (Faldt et al., 1999; Neufert et al., 2001; L. Zhang et al., 1991)], there is no information regarding how alveolar neutrophil-derived hydrolases affect the integrity of the cell wall of *M.tuberculosis* during infection.

### 4.1.3 Alveolar epithelial cells

Alveolar epithelium lines the alveoli air sacs of the lung and is comprised predominantly of two specialized cell types (type I and type II). Alveolar type I cells function in gas exchange.
These cells have an extremely thin cytoplasm extending away from the nuclear body and contain a large number of plasmalemmal invaginations termed caveolae (Gil et al., 1981; Williams, 2003). Caveolae regulate removal of endogenous and exogenous particulates from the alveolar space by regulating activities of receptors, hydrolase secretion to the alveolar lumen and signaling molecules (Gumbleton, 2001; Marx, 2001; Razani & Lisanti, 2001). Importantly, these caveolae contain lipid phosphate phosphohydrolase, a critical enzyme in hydrolyzing a variety of phospholipids to produce diacylglycerol (Nanjundan & Possmayer, 2003). Type I cells (as well as AMs) also produce carboxypeptidase which increases during bacterial deposition in the alveolar space and functions in the processing of many peptides (Skidgel & Erdos, 1998). Other epithelial cell enzymes are related to the regulation of ion transport to the alveolar space (Johnson et al., 2002).

Alveolar type II cells are considerably smaller than the type I cells and are richly endowed with organelles and microvilli on their apical membrane. These cells are located in the corners of the alveolus where their physiological functions include surfactant production, secretion and recycling (Fehrenbach, 2001). Surfactant is released to the alveolar space by exocytosis from intracellular storage organelles termed lamellar bodies which contain the majority of the components of the surfactant. During active secretion of contents from the lamellar bodies to the alveoli and during the surfactant recycling process, a variety of hydrolases have been related to these organelles (de Vries et al., 1985; DiAugustine, 1974; Edelson et al., 1988; Gilder et al., 1981; Hook & Gilmore, 1982; S.L. Young et al., 1993), many of which have lysosomal-type degradative functions. The presence of hydrolases within the lamellar bodies implies that if the contents of these structures are secreted, then hydrolases should also be secreted with surfactant. It has been shown that surfactant contains substantial quantities of hydrolases (Hook, 1978), where some hydrolases are highly active (i.e. α-mannosidase and β-N-acetylglucosaminidase) while others much less so (i.e. β-glucuronidase and arylsulfatase). Thus, the lamellar bodies provide a vehicle for the release of hydrolases into the alveoli, and their influence on host defense is being elucidated by our laboratory. In general, studies on epithelial cells have focused on phospholipases as second messengers in signaling, however, these same phospholipases are also degradative hydrolases with great potential to redecorate any microbial cell wall. For example, phospholipase A₂ can release the fatty acids from phospholipids (Dennis, 2000). The action of phophatidylinositol phospholipase C provides both diacylglycerol and inositol trisphosphate. Other hydrolases include secreted and extracellular membrane-associated phosphatidic acid phosphatases which act on 1,2-diacyl,sn-glycerol phosphate to produce diacylglycerol and inorganic phosphate (Brindley & Waggoner, 1998), however, the membrane-associated hydrolases also act on a variety of phospholipids to generate anionic and/or neutral lipids (Nanjundan & Possmayer, 2003). These hydrolases may also be active against the rich phospholipid content of the cell wall of M.tb. (i.e. PIMs, cardiolipid, phosphatidylethanolamine, etc.) (Arcos et al., 2011). To what extent M.tb bacilli directly interact with epithelial cells and their secreted products remains unknown. However, the location of the alveolar epithelium, as well as, the relatively large alveolar epithelial surface area estimated at 100 to 140 m², makes it likely that M.tb will interact with components of the alveolar space prior to and following its residence in the AM. The low alveolar fluid volume relative to the alveolar epithelial surface area (7-20 ml per 100 m²) likely increases the local concentration of released hydrolases when compared to other tissue compartments.
This, in turn, increases the probability that secreted hydrolases will impact the *M.tb* bacillus in the alveoli, altering its cell envelope and metabolism.

### 4.1.4 Pulmonary surfactant

Pulmonary surfactant prevents alveoli from collapsing at low lung volumes by reducing the surface tension in the alveolar space. Dipalmitoylphosphatidylcholine comprises almost 50% of total surfactant and is its major surface-active component (van Golde, 1985). The exact function of the remaining lipid components, such as unsaturated phosphatidylcholines, phosphatidylglycerols, phosphatidylethanolamines, phosphatidyl-inositols, and cholesterol are still uncertain (King, 1982;van Golde, 1985). The surfactant protein fraction comprises a highly variable amount of serum proteins, a wide variety of specific hydrolases (Griese, 1999) and four apoproteins (the surfactant proteins termed SP-A, -B, -C, and –D) that contribute to its specific function (Weaver & Whitsett, 1991). As mentioned above, surfactant contains several products secreted by alveolar myeloid and epithelial cells, some of them already defined in host defense, such as the bacteriolytic lysozyme (Haller *et al.*, 1992;Singh *et al.*, 1988). The wide variety of hydrolases secreted into surfactant resemble but are distinct from lysosomal enzymes (Hook and Gilmore, 1982). The surfactant resident hydrolases alter the *M.tb* cell wall (Arcos *et al.*, 2011).

There remain many fundamental questions about how the alveolar environment influences *M.tb* pathogenesis. We recently showed that secreted hydrolases involved in surfactant homeostasis affect the *M.tb* cell wall (Arcos *et al.*, 2011) and/or trigger *M.tb* endogenous hydrolases to modify its own cell wall prior to contacting its natural niche, the AM, or after its lysis from the cell. Exposure of ‘*de novo*’ motifs on the surface of *M.tb* after alveolar enzymatic processing will provide insight into the real nature of the *M.tb* cell envelope during infection. Since components of the *M.tb* cell envelope dictate the innate immune response against the bacillus via their interaction with surface receptors on myeloid cells, the identification of hydrolases that shape the surface of the *M.tb* cell envelope will enable more predictive *in vitro* models to be developed and novel drug targets to be identified.

### 5. Conclusions

The interface between *M.tb* and the host depends of many factors. *M.tb* strains differ in their cell wall components exposed on their surface. Even within the same strain, it is likely that some bacilli differ, and thus bacilli may interact different with the host. Thus, at a given infection we may find a mixture of events. On one hand, and depending on the cell wall components exposed on their surface, we may find bacilli that interact with a receptor inducing pro-inflammation and promoting *M.tb* killing, and on the other hand, we may find bacilli interacting with another receptor inducing anti-inflammation and *M.tb* intracellular survival. From the host perspective, genetic predisposition and living conditions dictate the predilection for *M.tb* infection. Even in the context of the same infected person and within the same host cell population, differences amongst cells in the expression of cell surface receptors, signaling, trafficking and innate and adaptive function exist. These differences in the host get enhanced even more when we compare host cells from different model systems. Our current studies on the impact of the alveolar space in the *M.tb* infection outcome also
indicate that environmental host factors, such as alveolar hydrolases, play important roles in the establishment of the infection. With this in mind, what are the necessary elements that result in a successful *M. tb* infection? Is *M. tb* infection just chance or a perfect combination of bacterial and host elements? Upon the successful establishment of *M. tb* infection, the ultimate goal of the host is to reduce inflammation and tissue destruction and in this scenario *M. tb* has learned to evolve, adapt, and survive.

What do we know about the *M. tb* cell wall adaptation to the host? The most revealing attribute of the *M. tb* cell wall is its complexity. Studies have been focused on depicting the composition, structure, biosynthesis, and the spatial conformation of the *M. tb* cell wall and its components for decades. Currently, researchers are focusing to reveal how the cell wall of *M. tb* is during infection (*in vivo* and *in vitro*). The development of new technologies and/or the use of known technologies already successfully applied in other fields (such as cancer research) are moving fast into the field of TB. Thus, studies using scanning electron and atomic force microscopy revealed that the *M. tb* cell wall from MDR- and XDR-strains differs from susceptible strains (Velayati *et al.*, 2009a, 2009b, 2010). Experiments analyzing infected granulomas by using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS-NMR) showed that we are capable of analyzing the cell wall of *M. tb* when inside of the granuloma without further manipulation (Somashekar *et al.*, 2011). The introduction of novel reporters that can be used for selective labeling of the cell wall of *M. tb* during infection *in vitro* and *in vivo* is already allowing us to see how the cell wall of *M. tb* is remodeling during infection (Backus *et al.*, 2011). Efforts in improving purification techniques are also allowing us to be able to purify *M. tb* directly from infected tissues. The use of novel *state-of-the art* mass spectrometry techniques such as LC/MS/MS (Sartain *et al.*, 2006, 2011), ESI/MS (Barry *et al.*, 2011) and MALDI-Tissue Imaging (Prideaux *et al.*, 2011) in drug discovery will allow us to obtain new information about the cell wall composition from a single *M. tb* bacillus isolated from tissue and also to see how *M. tb* cell wall components, and other biomarkers and drugs are distributed within the infected tissue. These are few of many other novel biotechniques that are starting to be applied in the field of TB. But this is only the beginning, more efforts improving the protocols and the development of new technology will allow us to move quickly to solve the “mystery” involving how *M. tb* adapted to the host and became such a successful infection currently affecting one third of the world population and taking away ~2 million lives every year.

6. Acknowledgements

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7. References


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Understanding Tuberculosis – Analyzing the Origin of Mycobacterium Tuberculosis Pathogenicity


Parant, M., Parant, F., Chedid, L., Drapier, J. C., Petit, J. F., Wietzerbin, J., & Lederer (1977) Enhancement of nonspecific immunity to bacterial infection by cord factor (6,6'-
Broadening Our View About the Role of Mycobacterium tuberculosis Cell Envelope Components During Infection: A Battle for Survival


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Mycobacterium tuberculosis in an attempt to understand the extent to which the bacilli has adapted itself to the host and to its final target. On the other hand, there is a section in which other specialists discuss how to manipulate this immune response to obtain innovative prophylactic and therapeutic approaches to truncate the intimal co-evolution between Mycobacterium tuberculosis and the Homo sapiens.

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