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Mini Review

The Waxy Cell Envelope of Pathogenic and Non-Pathogenic Mycobacteria: Resistance to Antibiotics and Protection against Host Response. An Overview

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Abstract

Tuberculosis, an ancient infectious disease caused by Mycobacterium tuberculosis (MTb), a Gram-positive bacillus, is a public health problem because 95% of infected people develop latent tuberculosis, while 5% develop active disease. The situation is worsen by the increase in multidrug resistance (MDR), co-morbidities and the lack of a more effective vaccine, which prolongs and induces long-lasting memory immune responses in young older adults. The development of pharmacological and immunotherapies depends on an understanding the spectrum of the disease, the genetic susceptibility to mycobacterial infection and evasion the mechanisms developed by Mtb against the microbicide action of the host. The three-layered mycobacterial envelope is roughly seen from the outside to the inside: -A surface layer, -the capsule, composed of polysaccharides and proteins, present only in the pathogenic species. The mycomembrane (MM), composed of proteins and long fatty acids (long chain mycolic acids from C60-C90) with free intercalated glycolipids and covalently linked to arabinogalactan and peptidoglycan layer, similar to those of Gram-negative bacteria, and the phospholipid bilayer inner classical plasma membrane. These components are pathogen-associated molecular patterns (PAMPs) that are recognized by patterns of receptor recognition (PPRs) on the surface of antigen-presenting cells (macrophages, dendritic cells). This is the first interaction between host and pathogen that results in effective activation and immune modulation of the host response. The pathways involved in the biosynthesis of the outer and inner membranes, including the waxy cell wall of the mycomembrane, represent immunological or pharmacological targets for diagnosis and treatment. Here is an overview of the characteristics of the mycobacterial cell envelope.

Keywords: Waxy Cell Envelope; Pathogenic; Non-Pathogenic; Mycobacteria; Antibiotics; Protection against

Introduction

Tuberculosis (TB) is an infectious disease caused by the etiologic Gram-positive bacillus *M. tuberculosis* (*Mtb*). Tb is a threat in both underdeveloped countries (poor or inadequate sanitation or inaccessible treatment and detection methods) and developed countries (co-morbidities) [1,2]. A quarter of the world's population has latent *M. tuberculosis*, while 5% develop active disease. Reactivation of the infection occurs in 3-10% of infected individuals [3] and approximately one to five million deaths each year [1,2] are due to chronic TB. The situation is worsened by the increasing emergence of multidrug-resistant (MDR), extensively resistant (XDR) and super-resistant (SDR) strains. Intensive and continuous efforts are being made to find other therapeutic alternatives that increase memory and protection against *M. tuberculosis* (*Mtb)* [4,5]. Current prophylactic measures include the *Mycobacterium bovis Bacillus Calmette-Guerin* (BCG) vaccine, the only effective and officially licensed prophylaxis against *M. tuberculosis*. However, intensive and continuous efforts are being made to improve and enhance BCG immunity or to develop novel drugs that can inter-

fere with the growth and proliferation of mycobacterial strains [4-8]. In the context of comorbidities, autoimmune diseases or other chronic infections may predispose and favor *Mtb* infection [9,10]. Natural mutations in genes involved in the innate and/or adaptive immune response confer genetic susceptibility to mycobacterial infections [11].

The immune system in individuals with mutations and epigenetic regulation is impaired in receiving signals from the external environment that can influence the transcription and expression of genes that play a key role in all differentiation processes of the innate and adaptive immune system [12]. Antibiotic resistance in mycobacteria is increasing at an alarming rate, among other things, there is an overuse of drugs, the lack of accurate and sensitive diagnostics, accessible to the more vulnerable and elderly people. Therefore, it is a priority to improve BCG vaccine to control and stop TB transmission [8,13-18]. The use of molecular methods (real-time PCR), whole genome sequencing, single cell RNAseq and multi-omics has also contributed to the discovery of biomarkers of disease course and progression [19-21]. Indeed, lipid omics, combined with other classical chromatographic techniques (TLC) and ultramicroscopy have provided insight into the precise location and organization of the capsular lipids of pathogenic mycobacteria, providing thus targets for development of pharmacological and immunological treatments [23-26].

The mycobacterial cell envelope. The three layers of the mycobacterial envelope are roughly seen from the outside to the inside: -A surface layer, -the capsule, composed of polysaccharides and proteins, present only in the pathogenic species [14,16,26-30]. The outer membrane, or the mycomembrane (MM) is formed with proteins and long fatty acids (long chain mycolic acids of C60-C90) with free intercalated glycolipids. These components are covalently linked to arabinogalactan and the peptidoglycan layer, which is similar to that of Gram-negative bacteria, and the phospholipid bilayer of the inner classical plasma membrane [8,15,28-30] (Figure 1). The mycobacterial cell wall contains up to 60% lipids [22,25,26,30], which are mostly phospholipids (PL) in fatty acid (FA) chains linked to glycerol phosphate derivatives, structures that vary between bacterial species. Among the most abundant surface-exposed glycolipids is TDM, which is biosynthesized from its precursor TMM by the mycolyl transferase [(activity of antigen 85B (Ag85)]. Many biological functions have been attributed to these glycolipids, such as TMM, TDM, and GMM. TDM may affect the survival of mycobacteria in the host and possibly their virulence. Trehalose monomycolates (TMM) or trehalose dimycolates (TDM). Trehalose monomycolate (TMM) or trehalose dimycolate (TDM). TDM also known as cord factor, a factor of virulence, and it is essential for *Mtb* growth and survival [20,22,25-27,30,31].

Mycolic acids (MAs) on the waxy cell wall. MAs are long-chain α-branched β-hydroxylated fatty acids (FAs) with long-chain α-alkyl side chain ranging from C70 to C90 [32,33]. MAs are anchored to the outer cell wall membrane and esterified to sugars such as trehalose to form glycolipids [8,15,16,29,30] (Figure 1). The MAs core consists of a long merochain synthesized by a type II multienzyme fatty acid synthase (FAS-II) by elongation of an ACP-bound acyl primer. MAs are a mixture of structurally related molecules that differ primarily in the chemical nature groups at the "proximal" and "distal" positions of the main core (meromycolic) chain, resulting in chemical diversity in *Mtb* that leads to three subclasses of mycolates (alpha, methoxy, and keto). In addition, two position-specific cis-double bonds on the merochain are substrates for subsequent modifying enzymes that introduce cyclopropane rings, keto groups, or methoxy groups. The introduction of these double bonds is thought to occur during merochain elongation by the FAS II enzyme complex. These modifications play a key role in the pathology of granuloma formation and are essential for pathogenesis and persistence [32-37]. The waxy cell wall of *Mtb* is structured of three structural types of MAs: 1) the alpha-, 2) the methoxy and c) the keto mycolic acids (alpha-, M- and K-MAs). However, under *in vitro* growth conditions, it does not contain the epoxymycolic acids (E-MAs) found in *Mycobacterium smegmatis* (*M. smegmatis*) [37]. The peculiarity of these types of MAs is that both the keto and methoxy derivatives not only enhance the pathogenicity of *Mtb*[38; 39] but also allow it to modulate the host immune response [40-42] (Figure 2A). It is important to note that a three-type pattern of MAs is commonly found in *Mtb,* and phylogenetically related species, as well as in several slow-growing mycobacteria: The least polar, α-mycolates of *Mtb* are composed of C76-C82 fatty acids and contain two cis-cyclo-propyl groups, the more polar mycolates are composed of C80-C89. They both have a cis or trans (with a methyl group on the vicinal carbon atom) cyclo-propyl group in the prox-

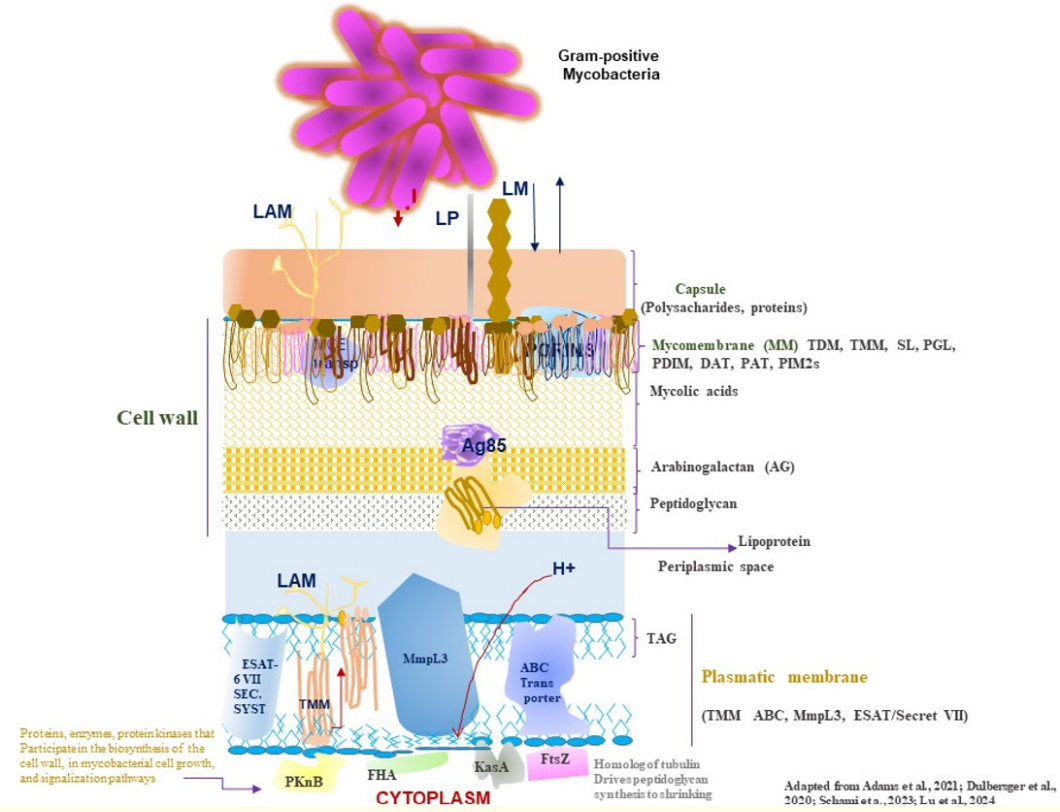

Figure 1: Scheme of the structural organization and composition of the mycobacterial cell envelope. A. The mycobacterial cell envelope in non-pathogenic and pathogenic strains is a characteristic three-layered structure with an unusual and abundant complex of lipids and proteins, all of which play a role in the molecular mechanism of pathogenicity of micobacteria of the M. tuberculosis complex. The first step is the recognition of the carbohydrates and the glycosylated part of the proteins. Among the proteins, as it has been recently described, the mannosyltransferase PimB, the galactofurnosyltransferase GIFT2, the cytochrome p450 and the ABC transporter VjiF, as well as the MmpL3 in the PM, the antigen 85 (Ag85) complex, porins and the putative transporter MCE protein family, mostly found in the native myco-membrane (MM). The capsule consists mainly of proteins and polysaccharides. In the outer leaflet of the MM are the peripheral lipids TDM, trehaholse dimycolate cell wall. TMM. Trehalose monoycollates, DAT, diacyl trehalose, PAT, pentaacyl trehalose; PIMS, glycolipids, such as phenolic glycolipids (PGL), lSL, sulfolipids, lipoglycans (ie. Mycobacterial lipoarabinomannan (ML) phosphoglycerol phosphatidyl-myo-inositol mannosidase (PIM2), and glycolipids containing MA esterifying arabinogalactan (PDIM2), glycoglycerolipids, glycosphingolipids and glycosylphosphatidylinositol. PAT, pentaacyl trehalose; PIMs, phosphatidyl-myo-inositol mannosides; SL, sulfolipids; PDIM, phthiocerol dimycocerosates.

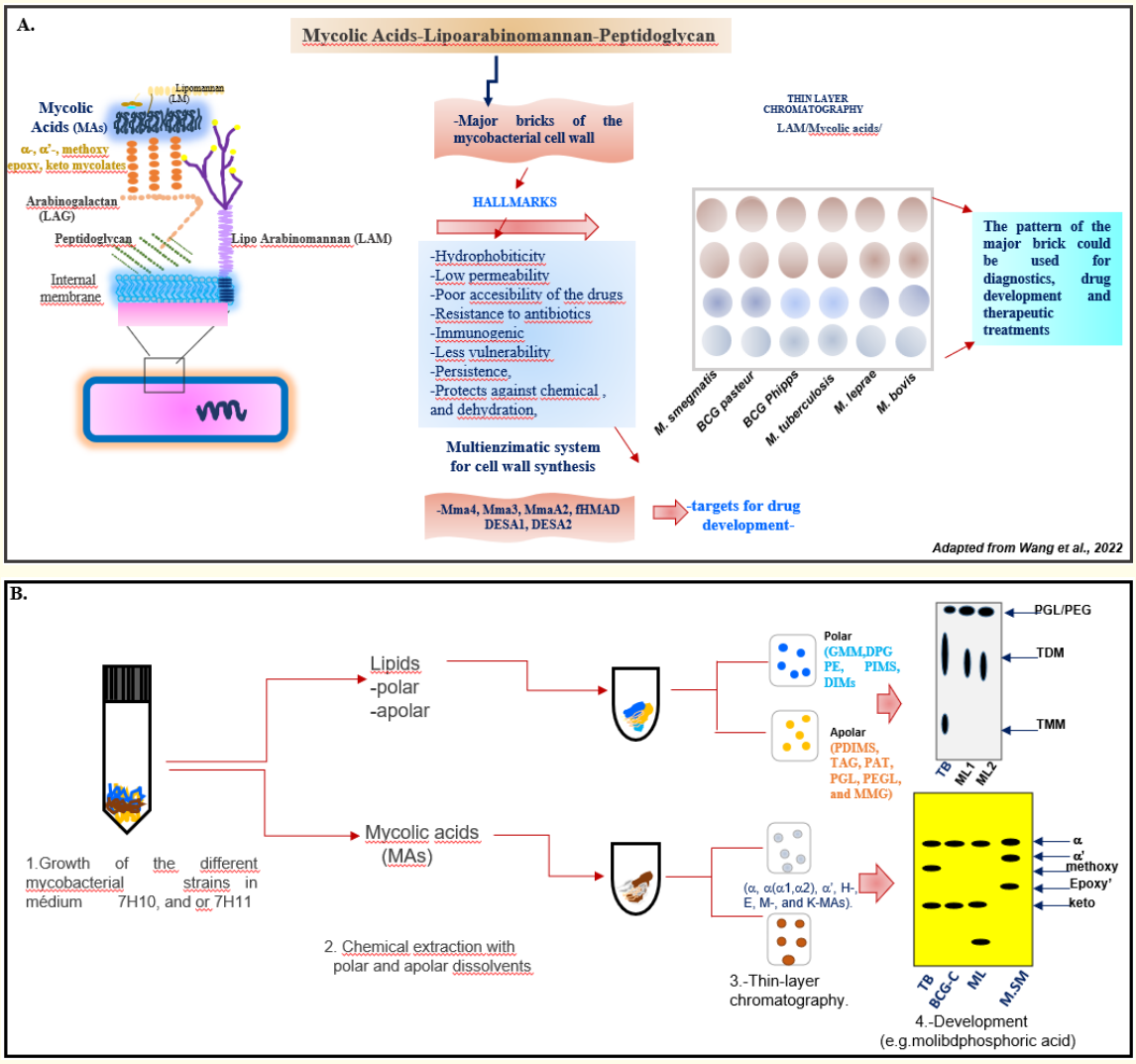

Figure 2: Analysis of the mycobacterial waxy cell wall. The mycobacterial cell envelope, from the inside to outside is a three layer of cytoplasmic membrane, a cell wall part of the mycomembrane and a capsuleis a three layered hydrophobic envelope from the inside to the outside comprised by lipids (Phospholipids) and by the presence of the three key molecular components (mycolic acids, arabinogalactan and peptidoglycan). The biosynthesis of these components involved several enzymes which can be targeted by drugs **(A)**. Mycolic acids (MAs) are the principal components of the outer mycomembrane, the cell wall, composed in addition of glycolipids including the trehalose dimycolate (cord factor). The spatial organization among mycobacteria species, can be studied and analyzed using different biochemical **(A)** and microscopy tools. One of them is classical thin layer chromatography, which with polar and apolar combinations, results in separation and identification of the different glycolipids (TMM; TDM) and MAs (alpha-, metoxy-, and ketomycolates) (B).

imal position. A methoxy or keto group with a methyl group on the vicinal carbon atom at the distal position [37-39]. In another human pathogen, *M. leprae*, the waxy cell wall is structured with mycolates that have the same structures as those of *Mtb*. Dicyclopropanated, alpha- and (Cis and Trans) monocyclopropanated keto- and methoxymycolates. Non-pathogenic mycobacteria such as *M. smegmatis* produce three subclasses, alpha-mycolates, shorter alpha-mycolates and epoxy-mycolates [37-39] (Figure 2A,b).

MAs are transported across the plasma (inner) membrane as trehalose monomycolates (TMM) (Figure 1). They are either covalently linked to the arabinose-galactan-peptidoglycan complex or incorporated into trehalose mono-/di-mycolates (TMM and TDM) located in the outermost leaflet of the mycomembrane [34-36]. The outer leaflet of the MM also contains other non-covalently associated lipids such as phthiocerol dimycoserates and sulfolipids (Figure 1). The arabinogalactan-linked mycolates are proposed to extend outwards and interact non-covalently with carbon chains of the so-called surface-exposed glycolipids, including trehalose 6-monomycolate (GMM), to form the hydrophobic cell wall [40-43]. GMMs are present at various levels in the mycobacterial cell wall. In addition to its role in cell wall barrier functions, GMM is a granuloma-forming agent in mice and a CD1b-presenting antigen in humans [38,39,44-46].

The functionality of the mycobacterial *waxy cell wall* is contributed by the complex physicochemical composition, predominantly of mycolyl-arabinogalactan-peptidoglycan layer (mGAP), in particular, the MAs play a role in the formation of a strong barrier, impermeable to drugs, and are the target of the isoniazid treatment [28-30,32,33]. Indeed, it has been proposed that the variability, the spatial organization along the length of the cell and throughout the entire process of infection, allows *Mtb* among other things, to parasitize and invade the host macrophages, aided by the evasion mechanisms of *Mtb* [8,12,15,16,38,39,44]. Moreover, the mycobacterial cell envelope as a dynamic structure also plays a dynamic role due to its unusual lipid composition, rich in glycolipids, glycosylated phospholipids, and complex carbohydrates with mycolic acids or peptides, as surface-exposed lipids as PAMPs, allowing to mycobacteria to reach and actively interact with patterns of receptor recognition (PPRs) on the surface membrane of antigen-presenting cells (macrophages, dendritic cells), thereby exerting immunomodulatory properties [25,31,38,39,44-46]. In addition, the mycobacterial cell envelope serves as a route pathway for the transport and secretion of solutes and proteins through efflux pumps (transmembrane proteins), resulting in the development of resistance to antibiotics [8,15,16,30].

Study and analysis of the molecular components of the mycobacterial cell envelope. Recent in-depth insights into the definition and dissection of the mycobacterial cell envelope using biochemical tools and high-resolution microscopy combined with omics technologies have revealed its composition and arrangement [8,15,16-19,30] For example, exposed and ubiquitous lipids follow an organization as a general discriminator between pathogenic and non-pathogenic mycobacteria. Interestingly, phosphatidylethanolamine and phosphatidylinositol mannosidase have a similar pattern in non-pathogenic and pathogenic mycobacteria [25,26,30,31,40,41].

By other hand, *using methanolysis and two-dimensional thin-layer chromatography, α-methoxy and keto mycolates were found in M. asiaticum, M. bovis, M. gastri, M. kansasii, M. marinum and M. tuberculosis. In contrast, a representative of M. thermoresistible contained lower molecular weight mycolates in addition to a-methoxy, and ketomycolates. M. fortuitum and M. giae contained a'- and epoxymycolates, and both serovars of M. simiae had a very characteristic pattern of a-, a'- and ketomycolic acids. M. fortuitum* and "*M. giae*" contained α′- and epoxymycolates, and both serovars of *M. simiae* had a very characteristic pattern of α-, α′- and keto mycolic acids. Moreover, in *addition to the three patterns (alpha, methoxy, and keto), ketomycolates also found to be associated to carboxymycolates and 2-eicosanol and homologous alcohols derived from wax esther mycolates in species representative of M. avium, M. intracellulare, M. nouchromogenicum, M. novum, M. paratuberculosis, M. scrofulaceum, M. terrae, M. xenopi, and Mycobacterium sp. MNC 165* [32-37,42,43].

Recently, the combination of MALDI-MS with thin-layer chromatography (TLC) has been reported to allow rapid spatially resolved screening of an entire TLC plate, making the method competitive with LC (MS) [43,*47-50*]. *In addition, TLC and high-performance TLC (HPTLC) allow reliable separation of the individual (phosphorus)*

lipid classes (Figure 2B). For the detection, visualization and quantification of lipids or the separated lipids (fatty acids to glycosphingolipids and phosphoinositide) by mass spectrometry, TLC or, after re-elution, the stationary phases, the associated solvent system plays a role. For example, a brief treatment of Mtb with detergents such as Tween 80 prior to the use of glass beads results in the gradual exposure of both the cord factor (dimycoloyl trehalose, lipid-arabinomannan) and other lipids on the cell surface of the bacilli [47-50].

Remarks

*In the mycobacterial cell envelope, the glycolipids represent 25% of the total dry weight, of which, 40% are mycolic acids bound to trehalose (a disaccharide of a-D-glucose formed by a-D-gluco-piranosil (1-1)-a-glucopyranose residues).*The glycosylated structures with O-glycosidic bonds have a dual role: a) structural like the polymers such as peptidoglycan and polysaccharides, and b) secondly, the glycosylated compounds act as pathogen-associated molecular patterns (PAMPs) that interact with the pattern of receptor recognition (PRR) of antigen-presenting cells (APCs). *Lipoglycans, lipoarabinomannans and lipomannans are characteristic PAMPS with a biologically important role in the mechanism of action of tubercle bacilli and t*he mannosidase residues and glycosylated -O-linked can even bind to Fc receptors and induce a strong host immune response. Indeed, these molecular fluidic glycosylated structures of the cell envelope protect from the microbicidal action of the host response. *The variations in TG triacylglycerols, diacylglycerols (DG) and monoacylglycerols (MG), the trehalosa,* called "cord factor", a polymer of about C80, classified as mycolates trehalose and sulpholipids of trehalosa. When it is acetylated it is called dimycoloyl trehalose (TDM) *may influence resistance or susceptibility to different antibiotics.*

The use of high-throughput sequencing technologies (RNAseq) and multi-omic approaches (lipidomics, proteomics) and TLC (2D TLC) has allowed insight into the architecture and macromolecular organization of the mycobacterial cell envelope, and subtle differences, variations in composition, and spatial organization, which have revealed a multi-enzymatic system involved in the synthesis of the outer and inner membranes and the composition of the cell wall (arabinomannans, peptidoglycans, shape determination), and of the capsule, all have implications as targets for pharmacological and immunological treatments.

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