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Roles for phthiocerol dimycocerosate lipids in *Mycobacterium tuberculosis* pathogenesis

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Abstract

The success of *Mycobacterium tuberculosis* as a pathogen is well established: tuberculosis is the leading cause of death by a single infectious agent worldwide. The threat of multi- and extensively drug-resistant bacteria has renewed global concerns about this pathogen and understanding its virulence strategies will be essential in the fight against tuberculosis. The current review will focus on phthiocerol dimycocerosates (PDIMs), a long-known and well-studied group of complex lipids found in the *M. tuberculosis* cell envelope. Numerous studies show a role for PDIMs in several key steps of *M. tuberculosis* pathogenesis, with recent studies highlighting its involvement in bacterial virulence, in association with the ESX-1 secretion system. Yet, the mechanisms by which PDIMs help *M. tuberculosis* to control macrophage phagocytosis, inhibit phagosome acidification and modulate host innate immunity, remain to be fully elucidated.

INTRODUCTION

Mycobacterium tuberculosis (Mtb), the etiological agent of tuberculosis (TB), has evolved alongside its human host, most likely for more than 70000 years [1]. Phylogenetic studies performed on Mtb genomes retrace the original lineages back to Africa, before human expansion out of the continent [1]. This long-time adaptation of the bacterium to its human host has allowed it to become a very efficient pathogen, still ranked the number one cause of death by a single infectious agent today [2]. Mtb is part of the *M. tuberculosis* complex (MTBC), which consists of pathogenic bacteria that cause TB in humans and animals (reviewed by [3]). Genome-based studies suggest that MTBC emerged from the same clonal group, which show similarities with *M. canettii* [4]. From this original strain, likely to have a recombinogenic genome, the MTBC common ancestor lost its ability for horizontal gene transfer (reviewed by [5]). Therefore, MTBC bacteria show low genetic diversity, mainly shaped by gene deletion and accumulation of specific mutations in genes coding for virulence factors. These long runs of genomic modifications finally determined the host-adapted specificity of the different MTBC strains.

Knowing what makes MTBC bacteria unique is essential for developing a strategy to ultimately eradicate them. Mtb exhibits a wide range of effectors essential for its infectious cycle; however, its most prominent feature is its cell envelope. Mtb is protected by a thick cell envelope containing an important amount of lipids, accounting for approximately 40% of the bacteria dry mass (reviewed by [6–8]). The innermost layer of the Mtb cell envelope is the cytoplasmic membrane (CM), surrounded by a thick layer of peptidoglycan covalently linked by phosphodiester bonds to long, ramified heteropolysaccharidic chains composed of arabinose and galactose termed arabinogalactan. The non-reducing extremities of the arabinogalactan chains are esterified by α-alkyl β-hydroxy long-chain fatty acids called mycolic acids, a signature of mycobacteria. Images obtained by cryo-electron tomography of vitreous sections (CEMOVIS), suggest that these long lipid chains (C70 to C90) are folded into a compact leaflet, forming the inner leaflet of a morphologically symmetrical lipid bilayer, the mycobacterial outer membrane (MOM). The MOM is asymmetrical in its lipid composition, however, as the outer leaflet consists of complex lipids and glycolipids [9, 10] (reviewed by [11]).

The different components of the mycobacterial envelope are schematized in Fig. 1a by the cryotomogram of Mtb $mc^2$6206 [12], which shows a symmetrical MOM with a similar density and thickness as the CM (Fig. 1a). This data supports the new model for the structure of the mycobacterial envelope, where the long chains of mycolic acids are folded and tightly packed

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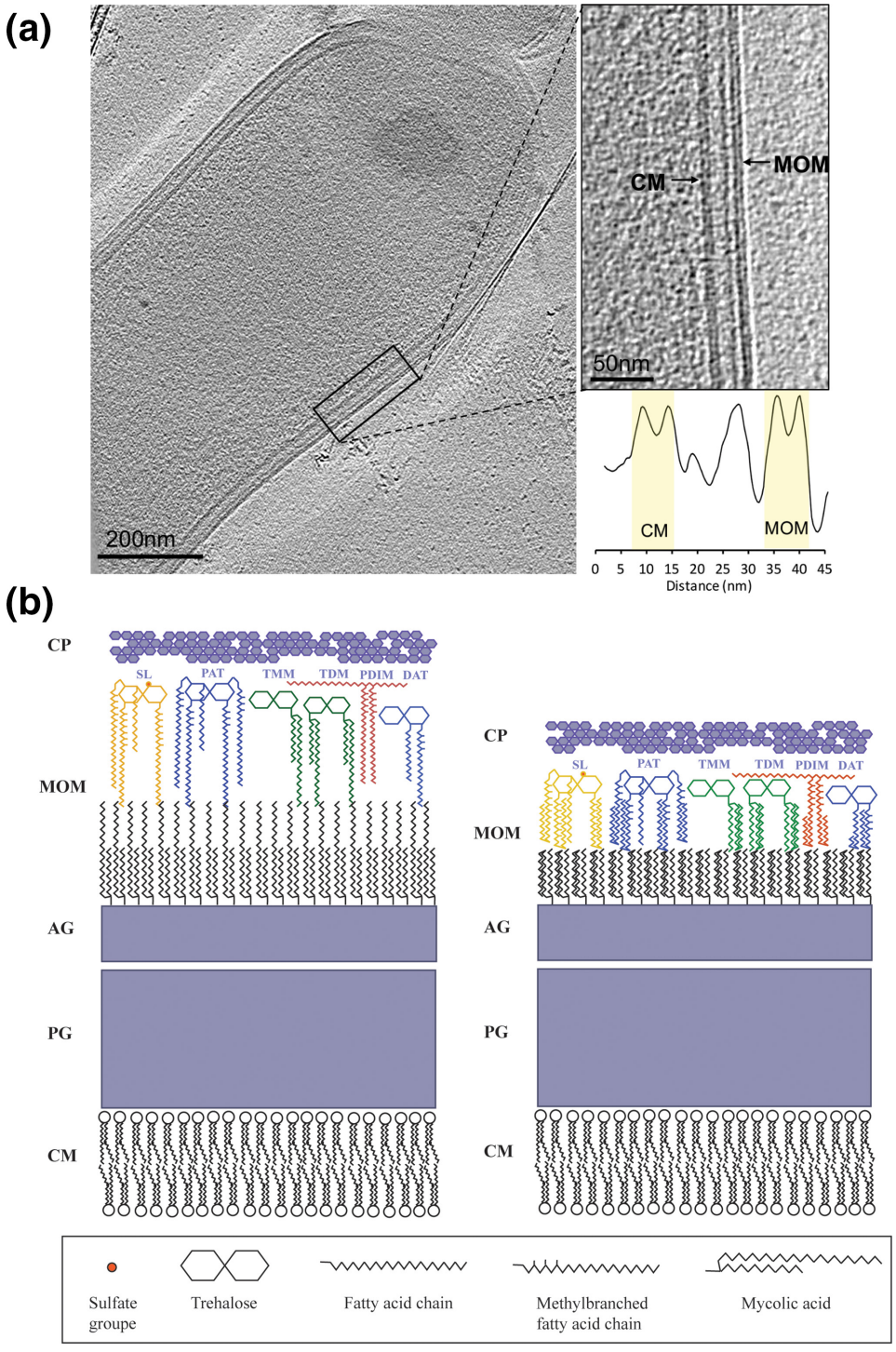

Fig. 1. Structure of the Mtb cell envelope. (a) Tomographic slice through an Mtb cell showing overall envelope architecture. Inset (upper right) reveals that the lipid bilayers of the cytoplasmic membrane (CM) and mycobacterial outer membrane (MOM) are clearly visible, along with several periplasmic layers. A density profile (lower right) of the cell envelope reveals two peaks within the CM and MOM (yellow), typical of lipid bilayers. Scale bar 200 nm, inset 50 nm. Cryotomograms were collected on a Titan Krios TEM microscope maintained at 300 kV and equipped with a Falcon 2 direct electron detector. Data were collected at 22.5x magnification,+/-60° with 1° oscillations, 100e^{-}/Å^{2} total dose and −4 µm defocus. (b) Two models showing lipid packing in the MOM of Mtb. The old model (left) suggests that the lipids in the MOM are in an extended conformation. Based on recent cryo-ET data, a new model (right) suggests that the long chains of the mycolic acids are folded and tightly packed with the complex MOM lipids. PG: peptidoglycan; AG: arabinogalactan; CP: capsule.

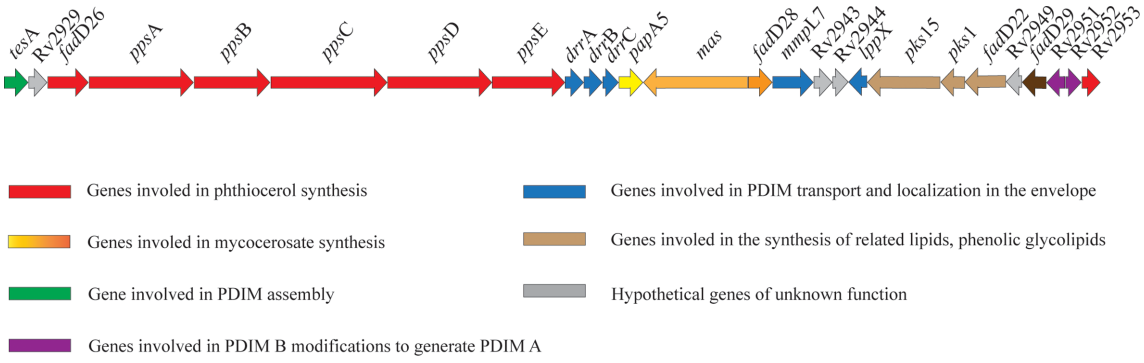

Fig. 2. The PDIM gene cluster. Colours depict the various gene functions in PDIM synthesis.

with complex lipids in the MOM (Fig. 1b right), as opposed to the former model, which proposed the mycolic acids lie in an extended conformation [13] (Fig. 1b left).

The outermost leaflet of the MOM contains a wide variety of complex lipids unique to mycobacteria, including trehalose mono-mycolate (TMM), trehalose di-mycolate (TDM), di-acyl trehalose (DAT), poly-acyl trehalose (PAT), sulfolipids (SL), phthiocerol di-mycocerosates (PDIMs), lipomannan (LM), lipoarabinomannan (LAM) and phosphatidylinositol mannoside (PIM). Due to their outward-facing presence in the bacterial cell envelope, these complex lipids in Mtb have multiple roles in infection, by modulating the host immune system, conferring drug resistance, and supporting intracellular survival (reviewed by [6]). TMM and TDM have a trehalose core esterified with mycolic acids and thus are linked to mycolic acid synthesis and transmembrane transport [6, 7]. TDM, also known as 'cord factor', was extensively studied for its multiple roles during infection such as in granuloma formation, resistance to antibiotics and evasion from killing by macrophages (reviewed by [14]). Other trehalose derived glycolipids such as DAT, PAT and SL, have a trehalose core esterified with methyl-branched long-chain fatty acids. These lipids are found only in pathogenic bacteria of the MTBC, with SL found only in Mtb, and play a role in the immune modulation of the host by the bacteria (reviewed by [15]). PDIMs are long-chain lipids, with a phthiocerol core esterified by two methyl-branched fatty acid long chains (reviewed by [16]). These lipids are found in several different slow-growing mycobacteria from the MTBC such as Mtb and *M. bovis* but also in other mycobacteria such as *M. marinum* [17]. LM, LAM and PIMs are lipoglycans built on a phosphatidylinositol backbone located in the cytoplasmic membrane. Following extensive glycosylation, these molecules are exported to the outermost layer of the cell envelope (reviewed by [18]). Their glycosylated extensions are very similar to polysaccharidic ramified chains reported in the Mtb outermost structure termed the capsule, mainly composed of polysaccharides, proteins and very few lipids (reviewed by [19]). These lipoglycans, in addition to their structural function are also involved in the pathogenesis by modulating the immune system, promoting bacterial uptake by phagocytes and intracellular survival [20]. Several of these lipids have already been extensively described in valuable reviews [6–8, 18].

PDIM lipids form a natural barrier, protecting the bacteria from antimicrobial compounds, and allow Mtb to escape detection by the early host immune response. Moreover, this component of the bacterial cell envelope is involved in essential steps in infection, such as bacterial entry into the macrophage, control of phagosome acidification, phagosomal escape, and use of host cholesterol as a carbon source [21–23]. More recently, PDIMs were shown to be essential for the proper functioning of the type VII secretion system ESX-1 and play a vital role in Mtb virulence [24, 25]. This review aims to summarize our increasing knowledge on PDIM lipids and clarify their multiple roles during Mtb infection.

PDIM BIOSYNTHESIS

PDIM synthesis relies on multiple effectors to efficiently produce and incorporate this important component in the MOM. Impairment of any of these effectors leads to a lack of PDIMs in the MOM. To better understand how these key players collaborate, this section will describe PDIM synthesis and export.

PDIM lipids are composed of a phthiocerol core esterified by two mycocerosic acids. Enzymes involved in PDIM biosynthesis are encoded by genes clustered in a ~70 kb genomic region [7] shown in Fig. 2. Phthiocerol biosynthesis begins with the transfer of a fatty acid chain from fatty acid synthetase (FAS) I — exclusively involved in *de novo* synthesis of saturated lipids from acetyl-CoA — to a series of polyketide synthases (pks), PpsA to PpsE, which use malonyl and methylmalonyl building blocks to produce a complex lipid chain. The fatty acid precursor is transferred to the first polyketide synthase, PpsA, by FadD26, a fatty acyl-AMP ligase [26]. PpsA extends this precursor and adds two carbons from a malonyl-CoA building unit, which is recognized by the PpsA acyl transferase (AT) and ketoacyl ACP synthase (KS) domains. In turn, the ketoreductase (KR) domain of PpsA reduces the extended unit to yield a hydroxyacyl intermediate. Next, this intermediate undergoes the same process with the

second polyketide synthase, PpsB, which elongates it further by two carbons with an additional hydroxyl group on the acyl intermediate [27]. PpsC contains a dehydratase (DH) and an enoylreductase (ER) domain allowing a complete classical saturated elongation of the acyl intermediate from malonyl-CoA. PpsD also possesses a DH domain and appears to collaborate with Rv2953 for ER function [28]. PpsD AT and KS domains show affinity for methylmalonyl-CoA extension units, producing a methyl-branched intermediate. PpsE AT and KS domains are both able to use methylmalonyl-CoA or malonyl-CoA for elongation [27]. The resulting phthiodolone is released from PpsE by TesA, to be further esterified with two mycocerosates by PapA5 [26, 29]. Mas, another polyketide synthase, is involved in synthesizing these mycocerosates [30]. A FAS I fatty acid precursor, delivered by the FadD28 acyl-AMP ligase, is elongated by Mas by iterative cycles of methylmalonate addition to give a final methyl-branched lipid chain [26, 31]. The esterification of two mycocerosates on the phthiodolone, led by PapA5 [27], yields an initial PDIM product call PDIM B, which is subsequently reduced and methylated to generate the PDIM A form [32, 33].

PDIM A and B molecules cross the cytoplasmic membrane via two kinds of transporters, MmpL7 and DrrA-C, using an as yet unknown mechanism. MmpL7 was reported to interact with PpsE, suggesting that PDIM synthesis and transport are coupled [34]. Finally, the appropriate positioning of these lipids in the MOM requires a lipoprotein, LppX [35]. All steps in PDIM biosynthesis have been reviewed in more detail by Onwueme and colleagues [16].

ROLE OF PDIMS DURING MTB INFECTION

The presence of PDIMs in the Mtb envelope has proven to be essential for several key steps of the infectious cycle. Before detailing PDIM involvement in Mtb virulence, a brief summary of the infectious cycle is outlined below.

Mtb typically enters the host respiratory tract through aerosol droplets emitted by an infected person. In the distal airways of the lungs, bacteria are phagocytosed by alveolar macrophages [36]. Once inside the macrophage, Mtb blocks phagosomal acidification and subsequent fusion with lysosomes, thus avoiding intracellular destruction [37–39] (reviewed by [40]). Relatively recent *in vivo* studies revealed a more nuanced scheme showing that despite an initial inhibition of phagosome maturation by Mtb, the bacterium can be found in lysosomes where it is able to survive [41]. Eventually, after several weeks of infection, many subsets of immune cells present in the lung can be infected by Mtb (reviewed by [42]). Infected cells migrate to a proximal lymph node, triggering the immune response (reviewed by [42]). Phagocytosed bacteria are able to influence the immune response of the infected macrophage from within, for example, by releasing proteins that are subsequently exported then processed by uninfected cells, thus luring adaptive immune cells to these uninfected cells and preventing the detection of infected macrophages [43]. In order to avoid macrophage self-clearance, Mtb inhibits the cellular 'suicide' response, apoptosis, which is usually triggered by infected macrophages in a last effort to get rid of the intracellular bacteria [44] (reviewed by [45]). The host immune response typically leads to the formation of an organized structure containing the infected macrophage, called a granuloma – the pathological hallmark of TB. Oxygen and nutriment depletion in granulomas can induce Mtb entry into dormancy (reviewed by [46]): dormant bacilli stop replicating and show a low metabolic profile, allowing their long-term survival in the host. This stage of the disease is asymptomatic and usually considered to be latent. If the patient's immunity is weakened, granuloma integrity is compromised and bacteria can exit their dormant state, which is mainly associated with TB relapse.

Camacho and colleagues were among the first to describe the PDIM operon and the consequences of the loss of its genes for Mtb integrity and virulence [47, 48]. These authors screened a signature-tagged transposon mutant library for growth defects in mouse lungs and identified 16 genes involved in pathogenesis, with three genes, *fad*D26, *mmp*L7 and *drr*C, belonging to the PDIM locus [47]. Two of the PDIM-deficient mutants (Δ*mmp*L7 and Δ*drr*C) exhibited a growth defect in macrophages similar to what was observed for *M. bovis* BCG, with a tenfold decrease in intracellular bacteria after 8 days of infection [47]. A later study showed a similar loss of virulence with another Mtb mutant in a PDIM transporter, DrrA, which impaired intracellular growth [49]. Additional *in vivo* studies showed variations in the level of attenuation in these mutant strains, depending on the route of inoculation [50, 51]. Similarly, *ex vivo* growth in a macrophage infection model differed depending on whether the cell was activated or resting [51]. Ultimately, of the numerous experiments performed in mice with PDIM-deficient strains, each mutant consistently exhibited a decrease in virulence compared to the *wt* strain [47, 49–53]. This feature was used in the conception of a new vaccine using a live-attenuated Mtb strain with deletions of the *fad*D26 gene as well as the *phoP* gene. Thus far, this vaccine, currently in clinical trials, has been demonstrated to be as safe as the current BCG vaccine [54]. The lack of virulence of these mutants highlights the importance of PDIMs as virulence factors.

Various attempts have been undertaken to understand PDIMs' roles during Mtb infection. These waxy lipids were first reported to act as a hydrophobic barrier, contributing to the impermeability of the mycobacterial envelope [48]. Indeed, each of the PDIM negative strains, Mtb Δ*fad*D26, Δ*mmp*L7 and Δ*drr*C, are more permeable to hydrophobic probes such as chenodeoxycholate, with Mtb Δ*fad*D26 exhibiting increased sensitivity to detergents [48]. Analogously, PDIMs are also linked to natural or acquired drug resistance. A PDIM-depleted *M. marinum* Δ*tes*A strain displayed increased susceptibility to several antibiotics [29]. Furthermore, a PDIM-depleted Mtb strain lacking *pps*E was reported to be ~100× more susceptible to vancomycin than the corresponding *wt* strain, an indication that these lipids may confer natural protection against some antibiotics [55]. However, the increased susceptibility of PDIM-depleted strains to antibiotics appears to be drug-dependent as

Camacho and colleagues did not observe any drug susceptibility with the drugs they tested [48]. Alternatively, mutant-dependent variability could also be a possibility, as Mohandas and colleagues demonstrated varying drug susceptibilities depending which PDIM gene was knocked out to produce the PDIM-negative phenotype in *M. marinum* mutants [56]. Interestingly, rifampicin-acquired resistance is associated with overexpression of *pps*A-*pps*E and *drr*A in *rpo*B mutant strains, resulting in overproduction of PDIMs [57, 58].

Multiple studies have explored how PDIMs contribute to Mtb virulence, and a wide variety of data implicates these lipids at different steps during the course of infection; however, the exact role of PDIMs remains unclear. We have summarized findings from these various studies below, following in chronological order different key steps in the Mtb infectious cycle.

The role of PDIMs during initial contact with the host

Aerosol droplets carrying Mtb bacilli emitted by an infected person are usually the main mode of transmission. After entering the host airways, Mtb reaches the lung alveoli where the bacteria interact and are taken up by alveolar macrophages. The pulmonary surfactant present in alveoli modulates this interaction between the bacteria and the phagocyte. In addition to its biophysical function in lowering alveolar surface tension, lung surfactant is part of the innate immune response. This secreted substance is mainly composed of lipids (90%) and contains proteins able to bind specific bacterial structures, increasing bacterial opsonisation and subsequent phagocytosis by macrophages [59].

Previous studies showed that lipids in Mtb's MOM inhibit the proper function of the surfactant by disturbing surface tension in alveoli [60, 61]. These publications mostly reported the effect of mixed waxes or TDM alone on biophysical functions of surfactants. A later study showed that incubation of Mtb for 2 h with whole lung surfactant led to an upregulation of genes from the PDIM gene cluster (*tes*A, *fad*D26, *pps*A-E, *drr*B-C, *pap*A5, *mmp*L7, *lpp*X) [62].

The impact of Mtb lipids *in vivo* on pulmonary surfactant layer physiology is still unknown. Perhaps through localized tissue irritation, innate immune cells such as alveolar macrophages are drawn towards Mtb. Indeed, during the early stages of infection, the response to Mtb is mediated by innate immunity. Intracellular Mtb growth is increased in mice impaired in mechanisms used by the innate immune system (NOS2-/-, INF-γ-/- and MyD88-/- mice). By contrast, no differences were observed in Mtb survival during the two first weeks of infection in mice deficient in the long-term adaptive immunity (Rag1-/-) when compared to WT mice [53]. A PDIM-negative Mtb strain exhibited decreased survival during this early innate immune response [53]. This strain was shown to have a similar rate of division when compared to the *wt* strain but exhibited an increase in its death rate thus impacting its survival [53]. Interestingly, this initial decrease in survival of Mtb PDIM-depleted strains did not impair their long-term persistence [51, 53]. Rousseau and colleagues reported that a Δ*fad*D26 Mtb strain was still present in mouse lungs 4 months after infection [51]. Although PDIMs appear to be important for Mtb survival during the first encounter with the innate immune system, growth of Mtb PDIM-depleted strains was the same in mice lacking NOS2, INF, MyD88 or CCR2, as compared to WT mice [49, 52, 53]. Mtb mutant growth was not improved in these KO mice although they were deficient in their innate immune response, implying a more complex role for PDIMs during this first encounter with the host.

Using an *M. marinum* strain depleted for PDIMs in a zebrafish infection model, Cambier *et al.* suggested a mechanism for how PDIMs contribute to evade the innate immune response [63]. PDIMs might help to mask bacterial pathogen-associated molecular patterns (PAMPs) and thus result in evasion of bacterial detection by toll-like receptors (TLRs) on innate immune cells [63]. TLR binding to PAMPs triggers various intracellular pathways resulting in a panel of cellular responses, from pro-inflammatory cytokine secretion to an increase in the production of reactive oxygen species (ROS) by the mitochondria for bactericidal purposes (reviewed by [64]). This study, performed on zebrafish larvae infected with *M. marinum,* showed that macrophage recruitment to the site of infection was not TLR-dependent [63]. By contrast, PDIM-negative strains of *M. marinum* (Δ*mmp*L7, Δ*mas*, Δ*drr*A) were able to trigger macrophage recruitment through TLR activation. Macrophages triggered by these mutant strains significantly increased inducible nitric oxide synthase (iNOS) expression (an enzyme synthesizing reactive nitrogen species, RNS) compared to infection with WT. This suggests that PDIMs promote bacteria evasion from TLR detection during the first encounter with immune cells, avoiding an escalated recruitment of macrophages and production of RNS [63]. Of note, these studies were performed mostly with *M. marinum,* which utilizes different mechanisms for survival in its host compared to *Mtb*; thus, it may be speculative to draw conclusions from this model. Numerous experiments performed on mice using fluorescence-activated cell sorting (FACS) seem to be in conflict with the findings of Cambier and colleagues [63]. Recruitment of immune cells in the lung occurs several weeks post-infection and promotes late innate immune response (reviewed by [42]). Earlier time points are difficult to observe *in vivo* due to FACS technical limitations [42]. Nonetheless, a recent study used microscopy to localize a fluorescent Mtb in alveolar macrophages as early as 48 h post-infection, confirming these cells to be the first immune cells to encounter the bacteria in mice lungs [36]. Alveolar macrophages are more permissive to Mtb intracellular growth, being the main bacterial reservoir during early infection [36, 65, 66]. Perhaps, the immunomodulatory role of PDIM, reported by Cambier and colleagues, is effective only during this first encounter with the host, protecting the bacteria from host innate immune response, and delaying the recruitment of immune cells and adaptative response.

Intracellular Mtb infection

Mtb enters the macrophage primarily via opsonization of specific Mtb PAMPs, leading to bacterial phagocytosis through selected cell receptors (reviewed by [67, 68]). PDIMs are suggested to be key contributors for the efficient phagocytosis of Mtb. Astarie-Dequeker and colleagues reported the impaired ability of a Δ*pps*E PDIM-negative strain, PMM56, to infect macrophages compared to the *wt* strain, both with and without opsonization [21]. Even though this mutant strain was shown to bind macrophages as efficiently as the *wt* strain, less bacilli were ultimately taken up by the macrophage. Coating the PMM56 strain with purified PDIMs, but not with any other apolar lipids, restored this strain's infectivity. Based on this work, recent studies suggested that PDIM lipids insert inside the macrophage phospholipid bilayer, inducing a re-organization of the plasma membrane to subsequently promote bacterial phagocytosis [69, 70] as illustrated in Fig. 3.

After uptake by macrophages, Mtb is known to block the phagosome maturation process to promote its intracellular survival. Typically, phagocytosed bacteria are kept in the phagosome, which undergoes progressive acidification through the accumulation of proton pumps or H+-ATPase in the phagosomal membrane. The acidified phagosome ultimately fuses with lysosomes, which release a series of hydrolytic enzymes leading to bacterial breakdown. Mtb was reported to block this process from the very first step, by inhibiting phagosome acidification and phagolysosome fusion [37, 38](reviewed by [40]). PDIM lipids were reported to be involved in the regulation of phagosome acidification by Mtb but not in the inhibition of phagolysosome fusion. In a study by Rousseau *et al.*, no differences in phagolysosome fusion were observed between an Mtb Δ*fad*D26 mutant strain and the *wt* strain after one to 3 days of infection [51]. Astarie-Dequeker *et al.* showed later that the PMM56 strain lost the ability to block phagosome acidification, suggesting a role for PDIMs in phagosomal maturation. Indeed, *wt* Mtb was able to inhibit vacuolar-type H+-ATPase phagosomal recruitment, however, the PMM56 strain was unable to do so [21]. In agreement with previous results [51], both PMM56 and *wt* strains did not colocalize with CD63, a marker of the lysosomal compartment, indicating that while PDIMs might help block phagosomal maturation, they are not involved in evading fusion with lysosomes [21] (Fig. 3).

After phagocytosis by the macrophage, Mtb escapes the phagosome to reach the cytosol (reviewed by [71]). Usually, the presence of bacteria in the cytosol triggers autophagy as a cell defence mechanism. The specific process of autophagy directed against bacteria and other pathogens is termed xenophagy. In this process, bacteria are usually ubiquitinylated, triggering the recruitment of key cellular factors that lead to the internalization of the bacteria into an autophagosome. As with phagosomes, autophagosomes ultimately fuse with lysosomes to successfully get rid of the bacteria. Mtb is able to avoid xenophagy by modulating the macrophage response to cytokines; for example, the production of IL-10 during infection induces an increase in Akt/mTOR/p70S6K pathway activity, leading to inhibition of autophagy [72] (reviewed by [73]). In a last attempt to control the infection, infected macrophages can trigger apoptosis. Apoptosis and necrosis are two different forms of cell death. Apoptosis is a programmed cell death, triggered by cells in response to extrinsic or intrinsic stimulation, as well as cell infection. Necrosis is a non-programmed cell death following extensive and irreversible damage to the cell (reviewed by [74]). Macrophages infected with Mtb receive pro-apoptotic stimuli from both extrinsic effectors such as the cytokine TNF-α or from intrinsic stimuli, such as an increase in intracellular ROS. Although these stimuli are usually sufficient to trigger apoptosis and contain the bacteria, allowing subsequent elimination by surrounding immune cells, macrophages infected with Mtb fail to successfully complete the programmed death process and instead undergo necrosis, favouring bacterial dissemination and survival [44] (reviewed by [45]). Recent publications show that PDIMs may be involved in the modulation of phagosomal escape, autophagy and apoptosis. Quigley *et al.* studied an Mtb mutant depleted for *rv3167c*, a tetracycline repressor (TetR)-like transcriptional regulator [22]. During macrophage infection, this hypervirulent mutant showed an increase in phagosomal escape, autophagy and necrosis compared to WT. RNAseq analysis performed on Mtb *wt* versus Δ*rv3167c* showed that among the 442 genes differentially regulated by Rv3167c, the entire PDIM operon was upregulated in the mutant. Thin-layer chromatography (TLC) results confirmed an increase in PDIM lipids in Mtb Δ*rv3167c*. In order to investigate the involvement of PDIMs in the Mtb Δ*rv3167c* phenotype, these authors used a PDIM-deficient strain with the *mmp*L7 gene deleted. They observed that the mutant was impaired for phagosomal escape, autophagy and necrosis with less bacteria measured in the cytosol of infected macrophages, and consequently less autophagy and necrosis compared to the *wt* strain. This study elegantly showed the effects of an Mtb strain overproducing PDIMs and a strain lacking PDIMs in its cell envelope, demonstrating an inversion of phenotypes in these two strains compared to Mtb WT. Similar results were obtained in another study performed on primary human lymphatic endothelial cells (hLECs) infected with Mtb [75]. PMM100, an Mtb strain closely related to PMM56 but lacking the kanamycin resistance cassette, was shown to cause less damage to the phagosomal membrane and subsequently less autophagy than the *wt* strain. These authors proposed that the lower infectivity of PMM100 and its lower intracellular survival, compared to the *wt* strain, were due to an increase in phagolysosome fusion, a process that the mutant is not able to avoid in hLECs as in macrophages. Using electron microscopy, they calculated that 50% of *wt* Mtb reached the cytosol compared to only 21% for the PMM100 strain. Similarly, immunofluorescence labelling was used to measure bacterial association with well-known markers of autophagy such as ubiquitin, p62 and NDP52:

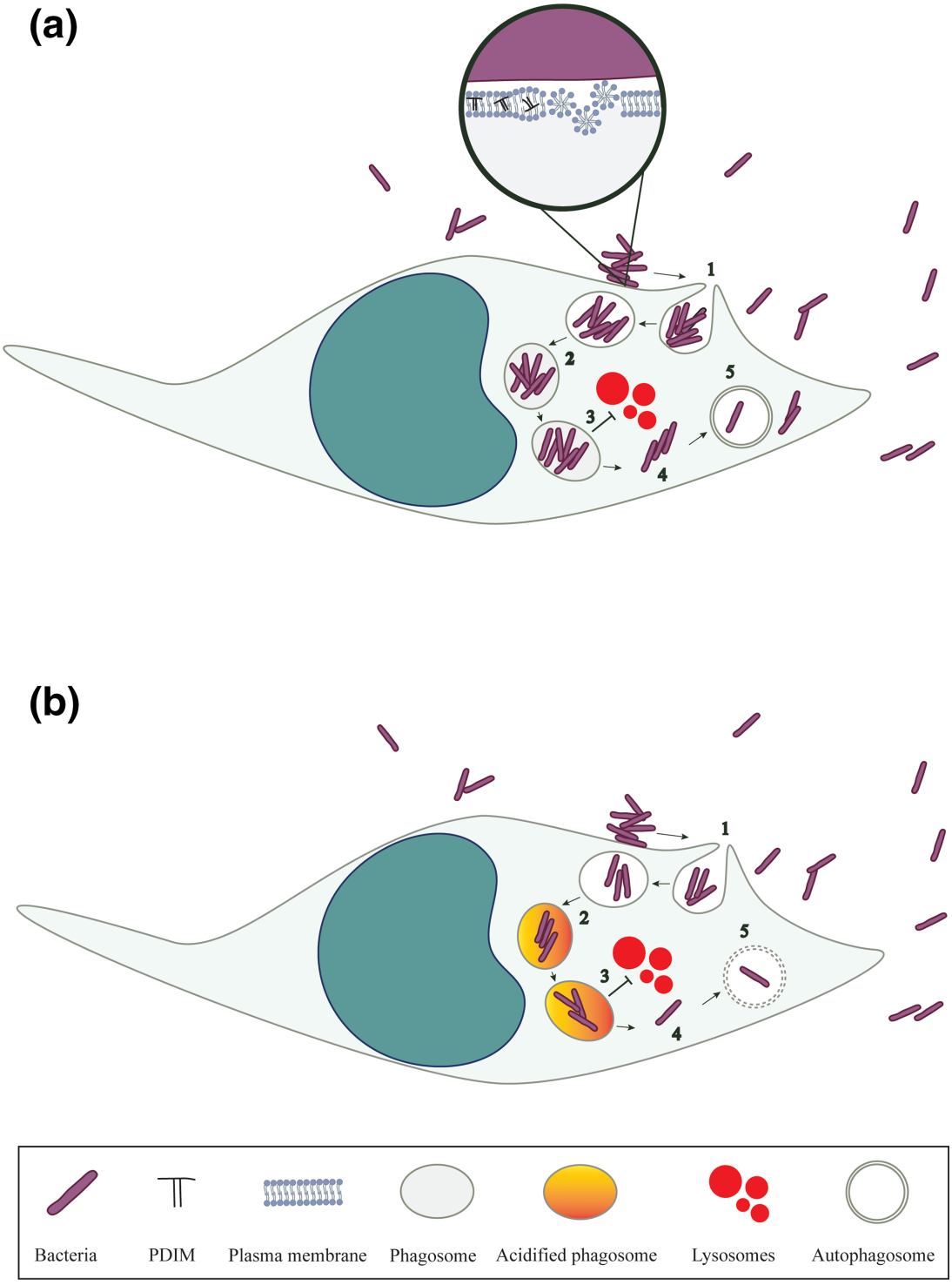

Fig. 3. Fate of Intracellular Mtb. The stages of Mtb phagocytosis include: entry into the macrophage (1), phagosome acidification (2), phagolysosome fusion (3), escape from the phagosome (4) and xenophagy (5). (a) WT, Mtb PDIM-positive strains increase phagocytosis through PDIM-mediated remodelling of macrophage plasma membrane. Inside the macrophage, Mtb avoids phagosome acidification and subsequent fusion with lysosomes. PDIM-positive cells can further escape the phagosome to reach the cytosol. Through modulation of the host response, autophagic triggering pathways are inhibited and very few bacilli will undergo autophagy. (b) Mtb PDIM-depleted strains enter macrophages around 50% less efficiently than *wt* strains. Inside the macrophage, the phagosomes containing bacteria will undergo progressive acidification, yet similar levels as *wt* will block fusion with lysosomes. Less bacilli are able to reach the cytosol without PDIM and subsequently, around 50% less will be targeted by xenophagy.

these experiments showed that 5–15% of *wt* Mtb bacilli underwent autophagocytosis during the course of infection compared to only 1–5% of the PMM100 strain [75]. PDIM-depleted bacteria were not able to reach the cytosol efficiently, resulting in less of these bacteria undergoing autophagy, confirming previous results [22] (Fig. 3).

GRANULOMA AND DORMANCY

Once inside the macrophage, Mtb lies in a nutrient-deficient vacuole, and hijacks host derived lipids as a carbon source; this process is essential for Mtb to replicate intracellularly and persist in the host [76]. Indeed, the *mce4* operon in mycobacteria encodes for an ATP-driven transport system involved in host lipid uptake by the bacteria, and is associated with long-term survival of Mtb [77]. In an *in vitro* granuloma model, infected macrophages accumulated cytosolic lipid bodies (LB) and turned into foamy macrophages [76]. Inside these foamy macrophages, bacilli-containing phagosomes colocalized with LB, and soon afterwards, lipid inclusions were observed in the bacterial cytosol [76]. Bacteria containing these lipid inclusions acquired characteristics of dormant Mtb, like the loss of acid fastness and drug tolerance [78–80]. Mtb cell envelope lipids such as TDM and oxygenated forms of mycolic acids were shown to be secreted by infected macrophages, further triggering the transformation of surrounding infected and uninfected macrophages into foam cells. These foamy macrophages were reported to be part of the granuloma histological structure, perhaps allowing bacterial transition into dormancy [76, 81–84].

Cholesterol is a host lipid used by Mtb during the course of infection as a host-derived carbon source [77, 85, 86]. Host cholesterol uptake was shown to be associated with PDIMs synthesis. Indeed, when cholesterol is the sole carbon source, bacilli increase the production of PDIMs *in vitro* [87]. To understand how PDIMs synthesis is linked to cholesterol catabolism, this last process should be briefly described.

Cholesterol import in bacteria is mediated by transporters, such as those encoded by the *mce*4 operon [77]. Once taken up, cholesterol is then catabolized by β-oxidation and sterol ring degradation to generate acetyl-CoA, propionyl-CoA, succinyl-CoA and pyruvate [88] (reviewed by [89]). Propionyl-CoA can be toxic for the cell, so the bacteria use two main processes to detoxify propionyl-CoA accumulation: (1) condensation with oxaloacetate to obtain pyruvate and succinate that will further be incorporated into bacterial metabolic pathways [90], (2) transformation into methylmalonyl-CoA via the methylmalonyl pathway (MMP) [91]. Methylmalonyl-CoA undergoes intramolecular rearrangement with succinyl-CoA to fuel bacterial global metabolism or is incorporated into methyl-branched lipids [92]. Mtb methyl-branched lipids are PDIMs, SL, DAT and PAT (reviewed by [93]). Bacteria grown *in vitro* on propionate or cholesterol as a sole carbon source were shown to incorporate more methyl units into their PDIM and SL mycocerosic moieties, producing longer PDIM and SL molecules [87]. These longer lipids result in a high mass form of PDIM and SL such as found *in vivo* [94]. PDIMs and other methyl-branched lipids are thus involved in the propionyl-CoA detoxification process consequent to cholesterol consumption by the bacteria.

An elegant *in vivo* study released recently demonstrated that Mtb residing inside macrophages reroute propionyl-CoA into the MMP pathway, increasing PDIM production supported by upregulation of the PDIM operon [95]. The production of PDIMs and other methyl-branched lipids by Mtb in macrophages appears to be tightly regulated. After 2 days in macrophages, Mtb exhibits a clear increase in its PDIM lipid composition [23]. However, the biosynthesis of all of these complex methyl-branched lipids is not regulated simultaneously. WhiB3, a redox transcriptional regulator, was reported to be involved in methylmalonyl-CoA incorporation into SL, DAT and PAT instead of PDIMs [23]. An Mtb Δ*whi*B3 strain exhibited an increase in PDIMs and TAG after 2 days inside the macrophage with a dramatic decrease in SL, DAT and PAT. Intraphagosomal microarray showed a synchronized upregulation of *whi*B3 with genes involved in SL, DAT and PAT synthesis, soon after bacterial phagocytosis [96]. Surprisingly, the overproduction of PDIMs in the Mtb Δ*whi*B3 strain was not caused by any change in PDIM operon expression [23]. Perhaps post-translational modifications, such as phosphorylation, are involved in PDIM synthesis and transport regulation, as previously suggested by several publications [97–99]. WhiB3 appears to regulate lipid anabolism during Mtb infection, increasing the synthesis of some complex lipids over others. In addition to regulating metabolism, cholesterol uptake can also impact the distribution of methylmalonyl-CoA among the different types of branched lipids. Transporters encoded by the *mce4* operon are involved in cholesterol uptake by the bacteria. Indeed, overexpression of cholesterol transporters encoded by the *mce4* operon resulted in increased SL and PDIMs in the mycobacterial envelope, with corresponding overexpression of *pps*A and *pps*B genes encoding for PDIM synthesis enzymes [100].

PDIM AND ESX-1 SECRETION SYSTEMS

Although multiple studies have shown PDIMs' involvement in Mtb virulence, little is known as to how these lipids effectively impacts bacterial virulence. Recent publications have brought some insight into this conundrum, linking PDIMs to a long-known virulence effector, ESX-1 [24, 25]. ESX is a type VII secretion system, with five homologues present in Mtb (ESX-1 to ESX-5). ESX-1, -3 and -5 are involved in Mtb virulence, and export important effectors essential for pathogenesis (reviewed by [101]). ESX-1 has been extensively described along with its secreted proteins, such as the heterodimer composed of ESAT-6 and CFP-10, also called EsxA and EsxB, respectively. The ESX-1/EsxA system was shown to be involved in phagosomal escape through membrane permeabilization (reviewed by [101]). Similarly, PDIMs were shown to be important for Mtb phagosomal escape [24].

M. bovis BCG is a good model to study PDIMs' collaboration with ESX-1 in the context of phagosomal escape as this strain is PDIM positive but naturally deficient in the ESX-1

secretion system due to deletion of a genomic region termed RD1 containing the ESX-1 operon. Although *M. bovis* BCG displays PDIMs in its envelope, this strain cannot escape from the phagosome to reach the cytosol. Augenstreich and colleagues showed that transforming PDIM positive *M. bovis* BCG with the missing RD1 region confers the ability to escape phagosomes. By contrast, PDIM-depleted strains of *M. bovis* BCG transformed with the RD1 region could not escape phagosomes [24], thus showing that PDIMs and ESX-1 are jointly required to achieve phagosomal escape by mycobacteria.

A recent study showed that PDIMs and ESX-1 share a common multiparametric phenotype of infection as transposon mutants for the two systems were found to be clustered [25]. In agreement with these predictions, PDIM-depleted strains were shown to secrete several ESX-1-dependent effector proteins less efficiently. In all Mtb mutant strains tested (Δ*pps*D, Δ*mas*, Δ*drr*C, Δ*hrp1* and Δ*rv0712*), the secretion of EsxA was decreased, yet the secretion of its co-dimer unit, EsxB, was decreased only in Mtb Δ*mas* and Δ*drr*C mutants and increased in the Mtb Δ*pps*D strain [25]. This observation points to a decrease in secretion of virulence factors in PDIM-depleted strains, either carrying mutations in genes in the PDIM operon or in unrelated genes impacting PDIM production (Mtb Δ*hrp1* and Δ*rv0712*). Yet there are some variations in ESX-1 secreted proteins from one PDIM-depleted strain to another [25]. These strain-related variations can explain the observation by Quigley *et al.* that Mtb Δ*mmp*L7 mutant is efficient in secreting EsxA [22]. Additionally, Barczak *et al.* suggested that PDIMs are associated with other ESX systems. Indeed, a decrease was observed in the ESX-5 secretion of PPE41 and EsxN in Mtb Δ*pps*D and Δ*drr*C [25]. These results give interesting insights in PDIM cooperation with ESX systems.

This intricate relationship of PDIMs with secretion systems involved in virulence clarifies some of the roles of PDIMs during the course of infection. The role of PDIMs in phagosomal escape might be associated with the depletion of secreted virulence factors, such as EsxA, known to be essential for phagosome plasma membrane lysis [102, 103]. Additionally, Augenstreich *et al.* suggested a collaboration between PDIMs and EsxA for lytic activity on cell membranes [69, 70]. The association between PDIMs and ESX-1 appears to also impact the host immune response during the course of the disease. PDIM-depleted Mtb strains were unable to trigger a type I IFN response, associated with active TB [25]. As PDIMs are not immunogenic by themselves [51] and ESX-1-depleted strains also lack a type I IFN response [104], this suggests that reaching the macrophage cytosol might be important for the initiation of an IFN response. Indeed, the expression of listeriolysin O in a PDIM-depleted strain permeabilized the phagosome membrane and allowed the bacteria to reach the macrophage cytosol, restoring the type I IFN response in infected macrophages [25]. This observation suggests that PDIMs by themselves do not trigger an immune response but by their intricate link with ESX-1, facilitates Mtb localization in the macrophage cytosol, which triggers the classical type I IFN response seen during the course of the disease. Surprisingly, these results suggest a role of PDIM on the type I IFN response of infected macrophages, which somehow contradicts previous studies showing no impact of PDIM on cytokine production [51, 53]. Future research might be needed to fully understand PDIM impact on type I IFN response.

The mechanism by which PDIMs and the ESX secretion system function together in virulence still needs to be fully understood. Some other features appear to link these virulence effectors together. First, several transcription factors such as EspR, Lsr2 and SigM are known regulators of genes in the PDIM operon as well as genes in the RD1 region, suggesting co-regulation during infection [105–107]. Furthermore, Joshi *et al.* showed in a *M. marinum* model that EccA1, an ATPase component of the ESX1 system involved in transport of ESX1 substrates, binds PpsD and PpsE when its ATPase domain is disrupted [108]. These results show the interaction of an ESX-1 component with two enzymes involved in the last step of PDIM phthiocerol synthesis. Mycobacteria are also known to grow and elongate by their poles; this is a position potentially shared by the PDIM production machinery and the ESX-1 secretion system, as polar localization of ESX-1 was previously reported [109]. PDIMs' synthesis and ESX-1 substrate secretion might be co-dependent processes (Fig. 4). Perhaps other interactions exist between ESX-1 effectors and proteins involved in PDIMs synthesis and transport, explaining the variability observed in EsxA secretion from one Mtb PDIM-depleted strain to another. Finally, the possible interaction of PDIMs with other Mtb type VII secretion systems should not be excluded as ESX-5 function was shown to be impacted by PDIMs absence [25]. For further reading, the connection between the mycobacterial envelope and ESX-1 has been reviewed by Bosserman and Champion [110].

LOSS OF PDIMS *IN VITRO*

Mtb laboratory strains tend to be PDIM-depleted [111]. A heterogeneous population of PDIM-depleted and PDIM-positive bacteria coexist both *in vivo* and *in vitro* [111, 112]. Subculture *in vitro* tends to increase the proportion of PDIM-depleted bacteria, as PDIMs are non-essential *in vitro*, giving rise to misread experimental results [52, 53, 111, 113]. Additionally, the thick cell envelope of Mtb makes these bacteria difficult to transform [114]. PDIM-depleted bacteria might be more permissive to transformation considering the increased permeability of the MOM of these strains. *In vitro* culturing and transformation might be two processes favouring PDIM-depletion among lab strains. This bias induced by the artificial culture environment is a concealed threat limiting experimental reproducibility and reliability.

One solution is to consider alternative media for *in vitro* subculture: for example, the *M. bovis* attenuated BCG strain was obtained after 230 passages *in vitro*, but still synthesizes PDIMs. Calmette and Guerin used a bile potato media for

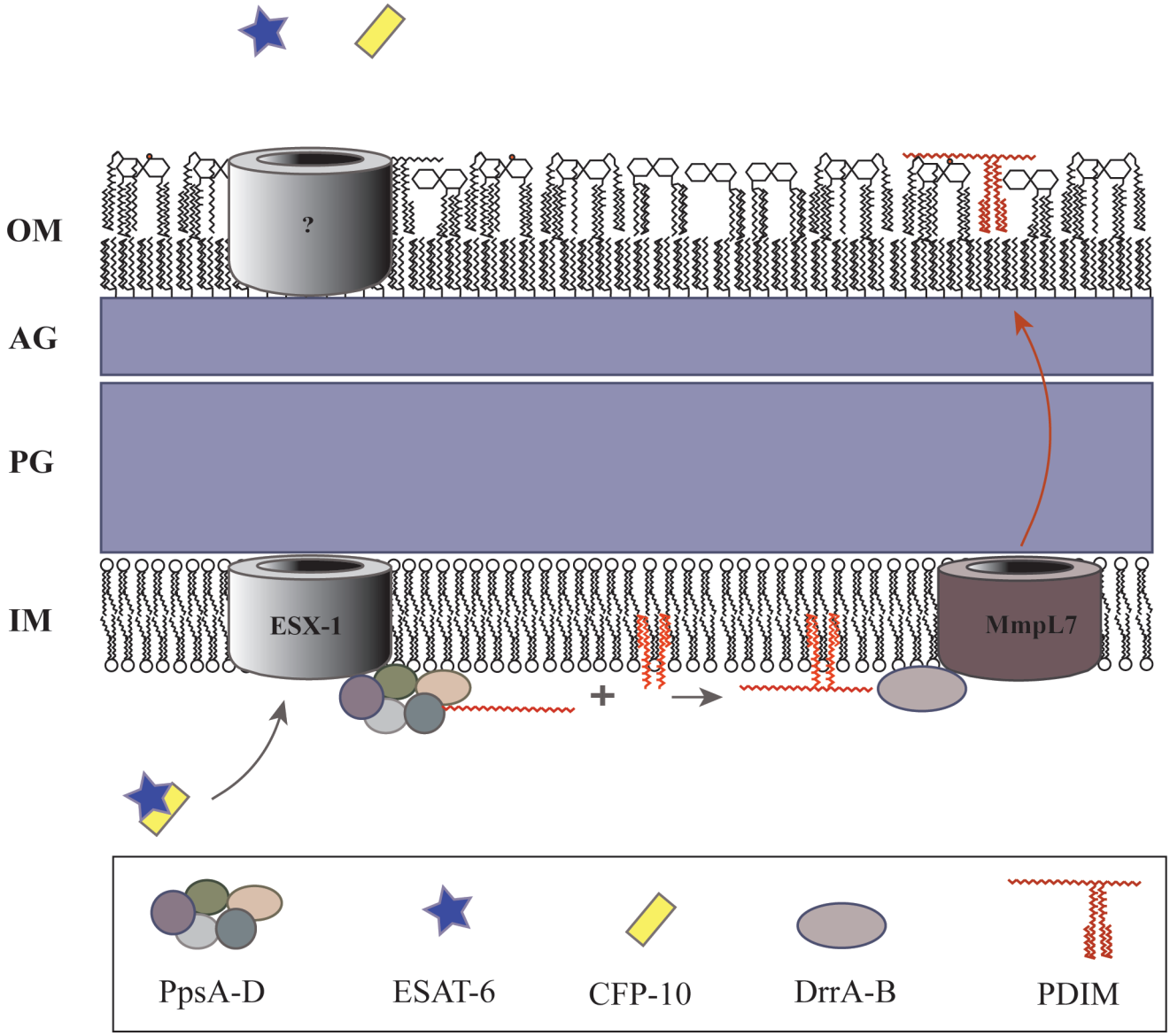

Fig. 4. Model of a co-dependent association between the ESX-1 secretion system and PDIMs. The efficient secretion of ESX-1 dependent virulent factors, such as EsxA and EsxB, rely on the functional synthesis and export of PDIM lipids.

their experiments, growing the bacteria on a slice of potato held in a glass tube and dipped on one end in a bile glycerate media [115]. This media composition and type may favour PDIMs' preservation. Perhaps favouring solid over liquid media may force the bacteria to grow in a biofilm-like structure, which requires the efficient production of PDIMs [56]. Thus, using solid media for passages may allow the preservation of PDIM positive strains.

Dedicated methods to test lab strains for the presence of PDIMs are thin-layer chromatography and mass spectrometry, both of which can be coupled with NMR spectroscopy for complete characterization [48, 116]. For an easier and relatively faster routine procedure, lab strains could be tested indirectly for the presence of PDIMs by drug-susceptibility assay, using drugs shown to be more potent on PDIM-depleted strains such as vancomycin [55]. Considering the numerous Mtb virulence pathways impacted by the absence of PDIMs, ensuring lab strain reliability is essential.

CONCLUSION

Numerous studies have implicated PDIMs in Mtb virulence, at different steps of the Mtb infectious cycle. How these lipids could perform so many roles in virulence is still unclear. Recent studies show a more nuanced role for PDIMs in supporting an extensively studied virulence factor, ESX-1. Perhaps the role of PDIMs in virulence is partly indirect and

the presence of these lipids in the mycobacterial envelope is a requirement for the proper function of other virulence factors. Moreover, PDIMs are required for virulence but are not sufficient by themselves, as illustrated by the *M. bovis* BCG strain, which is PDIM positive yet avirulent. Future studies will clarify the role of PDIMs as virulence factors and their involvement in pathogenesis.

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Conflicts of interest
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