Mini-Review

Pathogenic and immunosuppressive properties of mycobacterial phenolic glycolipids

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ABSTRACT

Phenolic glycolipids (PGL) are polyketide synthase products that are uniquely produced by a subset of pathogenic mycobacteria and are displayed at the bacterial cell surface, in a strategic position to interfere with host immune cells. Their expression has been associated with enhanced mycobacterial virulence *in vivo,* and suppression of the inflammatory responses of host phagocytes *in vitro*. In this review, we will present our current understanding of the mode of operation of PGL, along with functional evidence that demonstrates the evolutionary advantage conferred by PGL production for host cell invasion, intracellular persistence and evasion of host immune and bactericidal responses.

1. Introduction

Mycobacterium is a genus of *Actinobacteria*, the sole member of the *Mycobactericeae* family in the order *Actinomycetales* (1). Mycobacteria can be subdivided into fast- and slow-growing species, a subdivision that is recapitulated by 16S rRNA sequence homology analysis across the genus (2, 3) (Figure 1). Most mycobacterial species are innocuous saprophytes living in the soil, water, plants and in the air, however the slow-growing species subfamily contains major pathogens for humans. *Mycobacterium tuberculosis* and the genetically-related members of the *M. tuberculosis* complex (*M. canetti, M. africanum, M. microti* and *M. bovis*) cause tuberculosis in humans and other animals (4). *M. tuberculosis* is considered to be one of the most successful pathogens on the planet, with about one third of the human population infected worldwide (5). *M. leprae* causes leprosy, the second most common mycobacterial disease in humans. Of note, the anti-tuberculous vaccine *M. bovis* Bacille Calmette Guérin (BCG) was generated empirically by Calmette and Guérin by serial passages of a virulent strain of *M. bovis* in bile salts (6-8). Aside from its use as a live vaccine, and a treatment for superficial bladder cancer, BCG represents a useful vector for the delivery of heterologous antigens *in vivo*. It also provides researchers with a genetically engineerable reference strain for the study of species-specific products, such as phenolic glycolipids (PGL) (9).

The mycobacterial cell wall is unique among bacteria due to its lipid-rich, exceptionally impermeable structure (10-12). From interior to exterior, this cell wall consists of a plasma membrane and an outer, Gram-negative-like membrane (called mycomembrane) containing mycolic acids (MA). Besides MA, the mycomembrane also contains non-covalently bound lipids such as phthiocerol dimycocerosates (DIM), polyacyl trehaloses (PAT), diacyltrehaloses (DAT), sulfoglycolipids (SGL), lipomannan (LM) and lipoarabinomannan (LAM). In addition, the mycomembrane is coated by a detergent-labile capsule containing arabinomannan, alpha-glucan and oligomannosyl-capped glycolipids and proteins, which together with the cell wall forms the mycobacterial cell envelope. While the architecture and

composition of this envelope is largely conserved within the Mycobacterium genus, individual lipid constituents can differ across strains or clinical isolates. This is well exemplified by PGL, a family of polyketide synthase-derived, cell surface-displayed lipids that are only found in a limited subset of pathogenic species (Figure 1) (13, 14). Structurally, mycobacterial PGL all contain a phenol ring that is linked to a DIM-related lipid backbone on the one hand, and an oligosaccharide moiety on the other hand (Figure 2). While the lipid core is highly conserved across PGL-producing species, the oligosaccharidic domain that is attached to the phenol ring varies (15, 16). PGL-bovis, PGL-mar and PGL-ulc from *M. bovis*, *M. marinum* and *M. ulcerans*, respectively, display glycosidic domains that are restricted to a single monosaccharide. Most strains of *M. tuberculosis*, including the laboratory strain H37Rv, do not produce PGL due to a frameshift mutation in the polyketide synthase gene *pks15/1* (17). However, strains of the W-Beijing *M. tuberculosis* family have an intact *pks15/1*, and are known producers of PGL (hereafter named PGL-tb) (18, 19). As shown in Figure 2, PGL-tb and PGL-1 of *M. leprae* contain extended, structurally distinct trisaccharidic domains.

2. PGL and pathogenesis of mycobacterial infections

The first indication that PGL production may confer additional virulence to mycobacteria came in 2004 with the observation that clinical isolates of *M. tuberculosis* belonging to the W-Beijing family (which express PGL-tb) show a highly lethal phenotype in animal models of infection (18). While hyper-pathogenicity was lost upon disruption of PGL production in these isolates, it was not observed with a H37Rv strain engineered to produce PGL-tb (20). This suggested that PGL production is required but not sufficient for *M. tuberculosis* hyper-pathogenicity. Moreover, it implied that the mycobacterial genetic background interacts with PGL to increase virulence. Notably, ectopic expression of PGL-tb by *M. tuberculosis* efficiently downregulated the production of inflammatory cytokines in infected monocytes or macrophages, irrespective of the strain used (18, 20). Studies in zebrafish showed that PGL expression by *M. marinum* favors the recruitment of macrophages to the site of infection, through

a host chemokine receptor 2 (CCR2)-mediated pathway (21). Yet, DIM and PGL-mar both contributed to *M. marinum* virulence in infected zebrafish (22). By applying signature-tag mutagenesis to *M. bovis* and infection of guinea pigs, Collins *et al.* identified attenuated mutants with mutated *pks1*, a gene that is essential for PGL biosynthesis (23). PGL-1, made by *M. leprae*, is detected in all clinical isolates and considered a key player in leprosy pathogenesis (24, 25). PGL-1 was indeed shown to mediate the selective tropism of *M. leprae* to Schwann cells, through selective binding of its trisaccharidic moiety to Laminin-2 expressed at the basal lamina (26). More recently, PGL-1 was shown to interact with complement receptor (CR3), either purified and immobilized or displayed at the surface of phagocytes (9, 27). Altogether, these studies thus suggest that PGL production contributes to mycobacterial pathogenicity, by directly interfering with host cell invasion and/or by suppressing the generation of innate immune responses by the host. The next section reviews the state of the art with regard to PGL recognition by the innate immune system, and mode of operation.

3. Molecular mechanisms governing PGL recognition and biological activity

3.1 PGL and CR3 signaling

CR3, also known as Macrophage-1 antigen (MAC-1) is a heterodimeric complex composed of an alpha (CD11b) and beta subunit (CD18). CR3 belongs to the β2-integrin family, which also comprises CR4 (CD11c/CD18) and the lymphocyte function-associated antigen 1 (CD11a/CD18) (28). CR3 is a widely expressed receptor found on monocytes, tissue resident macrophages, dendritic cells, neutrophils, NK cells, basophils, eosinophils and platelets. It is also detected on activated CD8 cytotoxic T cells during acute viral infection (29, 30) and CD4 T cells (31). Its major functions are cellular adhesion, intracellular signaling and phagocytosis. Notably, CR3 has three conformational states of low-, intermediate- and high-affinity for ligands, co-existing in dynamic equilibrium that can be altered by the activity of other cellular receptors through a process called inside-out signaling. For instance, TLR2 and TLR5 ligation on leukocytes signal to augment β2-integrin affinity, resulting in increased cellular adhesion to

immobilized ICAM-1 and fibronectin (32). CR3 is also able to induce phagocytosis of opsonized and non-opsonized particles, with its I-domain (which binds ICAM-1, fibrinogen and complement component iC3b) and a carbohydrate-binding lectin domain (33, 34).

Phagocytosis of mycobacteria is known to occur in both opsonic and non-opsonic conditions (35), with CR3 being responsible for 40-50% of non-opsonic binding and 50-60% opsonic binding of *M. tuberculosis* to macrophages infected *in vitro* (36). CR3 also mediates the phagocytosis of *M. leprae* by monocyte-derived macrophages in both conditions (37). Also, it was shown that *M. bovis* BCG promotes its own phagocytic uptake by macrophages, through an inside-out activation of CR3 involving TLR2 (38). While CR3-mediated phagocytosis of mycobacteria occurs irrespective of PGL production, biochemical and cellular evidences suggest that PGL-1 promotes mycobacterial usage of CR3 for phagocyte infection. By designing a recombinant BCG producing PGL-1, Tabouret *et al.* indeed showed that PGL-1 increases the CR3-dependent uptake of mycobacteria. In line with these findings, PGL-1 was then found to bind CR3 *in vitro*, through interaction of its sugar moiety with the lectin domain of the receptor (27). Moreover, Lyn, a Src-family tyrosine kinase mediating β2-integrin signaling in phagocytes (39), was recently involved in the PGL-1-dependent increase in macrophage invasion (27). Binding to CR3 and exploitation of CR3-mediated phagocytosis was unique to PGL-1, and was not observed with PGL from *M. tuberculosis, M. bovis* or *M. ulcerans*. Whether this mechanism involves CD14, TLR2 and associated inside-out activation of CR3 remains unknown.

3.2 PGL and TLR signaling

TLR2, TLR4 and TLR9 are activated by mycobacterial products, and functionally important for the control of mycobacterial infection (40). TLR2 KO mice have increased susceptibility to high dose *M. tuberculosis* infection, develop chronic pneumonia and display disrupted granuloma formation (41). In humans, TLR2 receptor polymorphisms are associated with an increased susceptibility in developing

tuberculosis disease (42, 43). TLR4-deficient mice had increased mortality during chronic infection with *M. tuberculosis*, which was accompanied by reduced IL-12p40, TNF and MCP-1 cytokine production along with reduced macrophage infiltrate in infected lung tissue (44). *M. leprae* stimulates TLR4 in human macrophages that lack TLR2 expression, and blocking TLR4 with neutralizing antibodies before stimulation with killed *M. leprae* resulted in a reduction in CXCL10, IL-6 and TNF-α production (45). Finally, *in vivo* studies revealed that TLR9 signaling contributes to a regulation of Th1 responses during *M. tuberculosis* infection (46).

TLR2 recognizes various components of the mycobacterial cell wall, including lipoproteins and glycolipids such as mannose capped-LAM and LM (40, 47). TLR4 recognizes mycobacterial 38 kDa glycoprotein and heat shock protein 60/65, while TLR9 detects unmethylated cytosolic mycobacterial CpG DNA (46). TLR2/4/9 signaling uses the Myeloid Differentiation Primary Response 88 (MyD88) as a common adaptor protein. Other adaptors used by these TLR include TIR domain-containing adaptor protein-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and TIR-associated protein (TIRAP) (48). The MyD88-dependent pathway signals downstream to activate the transcription factors NF-κB and AP-1, which leads to the transcription of inflammatory cytokines (49). A critical MyD88-independent pathway involved in TLR3 and TLR4 signaling utilizes the adaptor protein TRIF to signal downstream to cause the dimerization and activation of IRF3 (50), which results in the transcription of IFN-β. TRIF also activates the transcription factors AP-1 and NF-κB (51, 52), a critical requirement for the transcription of inducible nitric oxide synthase (53). Notably, MyD88-deficient mice are able to mount an adaptive immune response to *M. tuberculosis* infection, but succumb more quickly than wild-type mice (54). Although IL-1 receptor signaling is the primary inducer of MyD88-dependent immune responses during the early control of *M. tuberculosis* infection (55), studies in TLR knock-out mice showed that TLR-mediated signals are required for the long-term control of infection, through

initiating bactericidal functions and triggering the production of pro-inflammatory cytokines (41, 44, 46).

Notably, PGL-1 and PGL-tb analogues missing part or all of the lipid core inhibited the activation of NFκB and downstream production of inflammatory cytokines by TLR2-stimulated macrophages (27, 56, 57). In line with this result, native PGL-1 was recently found to bind TLR2 *in vitro,* through its trisaccharidic epitope (27). Differently from CR3-mediated phagocytosis, PGL-1-mediated inhibition of TLR2 stimulation was not affected by Lyn silencing. Moreover, while PGL-1 was unique among PGL in its capacity to promote CR3-mediated phagocytosis, TLR2 signaling was comparably inhibited by PGL-1 and PGL-tb. In contrast, monosaccharide-bearing PGL did not display such an effect. Together, these observations thus suggest that PGL-1 and PGL-tb inhibit TLR2 activation independently of CR3 signaling.

Parallel to this, it was shown that *in vitro* exposure of monocytes from healthy donors to PGL-1 interferes with their production of TNF-α following stimulation with the TLR4 agonist LPS and IFN-γ (58). Tabouret *et al.* reported previously that bacterially-expressed PGL-1 alters the induction of NF-κB and subsequent TNF-α production by infected macrophages, in a CR3-dependent manner (9). More recently, we found that PGL-1 (and PGL-tb) also impairs the induction of nitric oxide synthase (iNOS), and subsequent production of bactericidal nitric oxide (NO) by macrophages stimulated with LPS and IFN-γ, in a CR3-dependent manner (unpublished observations). We found that PGL-1 and PGL-tb operate by downregulating the endogenous levels of TRIF in macrophages. Notably, PGL-mediated decrease in TRIF protein levels occurred post-transcriptionally and independently of Src-family tyrosine kinases, lysosomal and proteasomal degradation. We observed a selective impairment of the induction of TRIF-dependent gene products, including NO synthase and inflammatory mediators IFN-β and CXCL10, upon cell stimulation of TLR4. With TRIF being situated at the crossroads of NO and cytokine production, this observation thus provided a unifying mechanism for the capacity of PGL to

promote mycobacterial intracellular persistence while dampening inflammatory signaling. To conclude, PGL-1 and PGL-tb are capable of suppressing TLR2 and TLR4 signaling in CR3-independent and CR3-dependent manners, respectively. Our observation that PGL-1 and PGL-tb induce TRIF downregulation suggest that they may also affect TLR3 signaling, and therefore the host response during viral co-infection.

4. Conclusion

Thanks to recent advances in the genetic engineering of mycobacteria and in synthetic glycobiology, our understanding of the molecular mechanisms underlying the pathogenic and immunosuppressive properties of PGL has significantly progressed over the past few years. Studies using synthetic subunits of PGL and recombinant BCG expressing species-specific PGL have allowed to show that certain PGL interact with multiple pattern recognition receptors expressed by host phagocytes and alter their intracellular dialogue (Figure 3). The unique oligosaccharide domain of PGL-1, made by *M. leprae,* was shown to hijack CR3 for optimal invasion of host phagocytes. In addition, PGL-1 and PGL-tb (produced by highly pathogenic clinical isolates of *M. tuberculosis*), but not PGL-bovis or PGL-ulc, shared the capacity to block agonist-driven activation of TLR2. Additionally, these PGL downregulated TRIF-dependent TLR4 signaling in macrophages, producing relatively less inflammatory and bactericidal responses during infection. Since *M. tuberculosis* and *M. leprae* are the most successful pathogens in the Mycobacterium genus, it is tempting to speculate that they have evolved the biosynthesis of PGL with extended saccharidic domains to escape from the host immune system. Much remains to be elucidated, such as the fine structural specificities of PGL binding to CR3 and TLR2, the contribution of inside-out signaling in PGL binding to CR3, and the CR3-dependent mechanism that leads to TRIF downregulation. The answers to these questions will not only improve our understanding of mycobacterial pathogenicity, but will also undoubtedly reveal novel mechanisms that regulate inflammation by modifying the crosstalk between innate immunity receptors.

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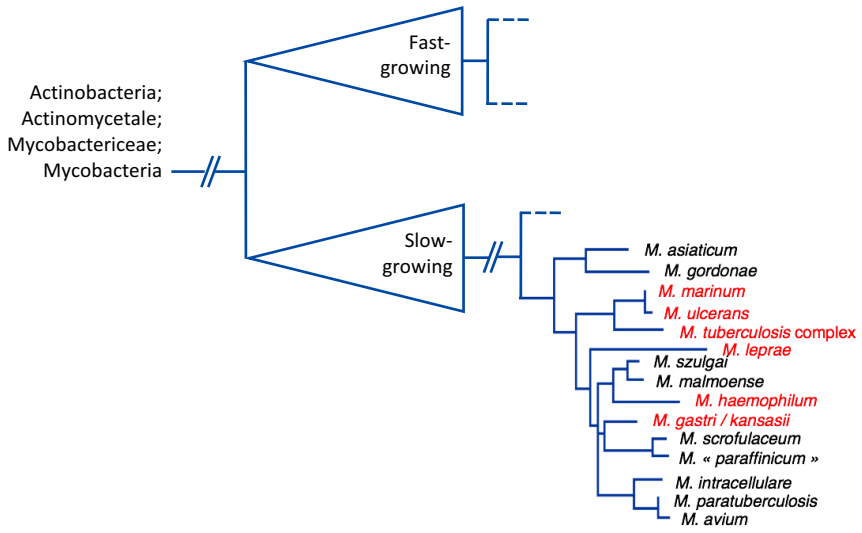

Figure 1: Phylogenetic position of the PGL-producing species within the genus Mycobacterium. PGL-producing species are shown in red, in the context of their phylogenetic branch, in the subfamily of slow-growing mycobacteria. Adapted from Springer *et al.*, 1996 (2, 3).

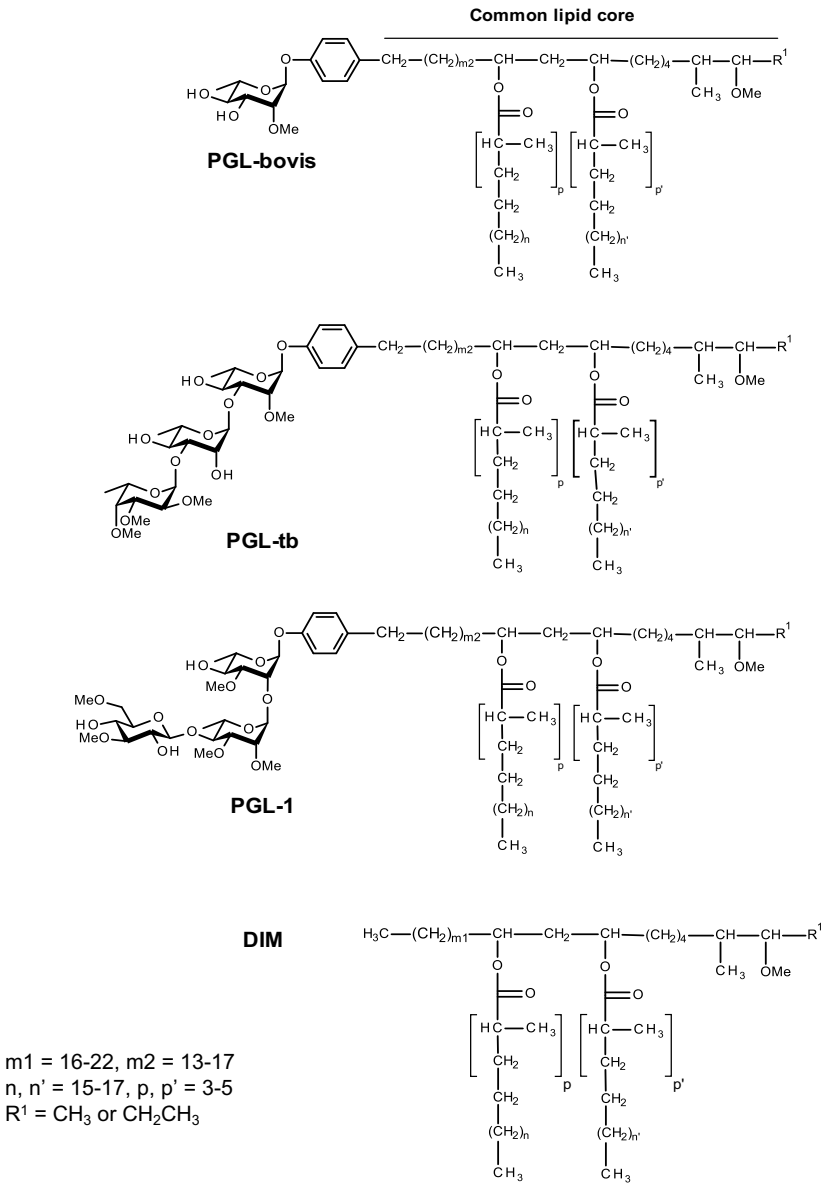

Figure 2: Structure of the major forms of PGL made by *Mycobacterium bovis* (PGL-bovis), *M. tuberculosis* (PGL-tb) and *M. leprae* (PGL-1), and of mycobacterial DIM. The common lipid core is composed of a long-chain β-diol esterified by polymethyl-branched fatty acids. Adapted from Arbues *et al.*, 2014 (15).

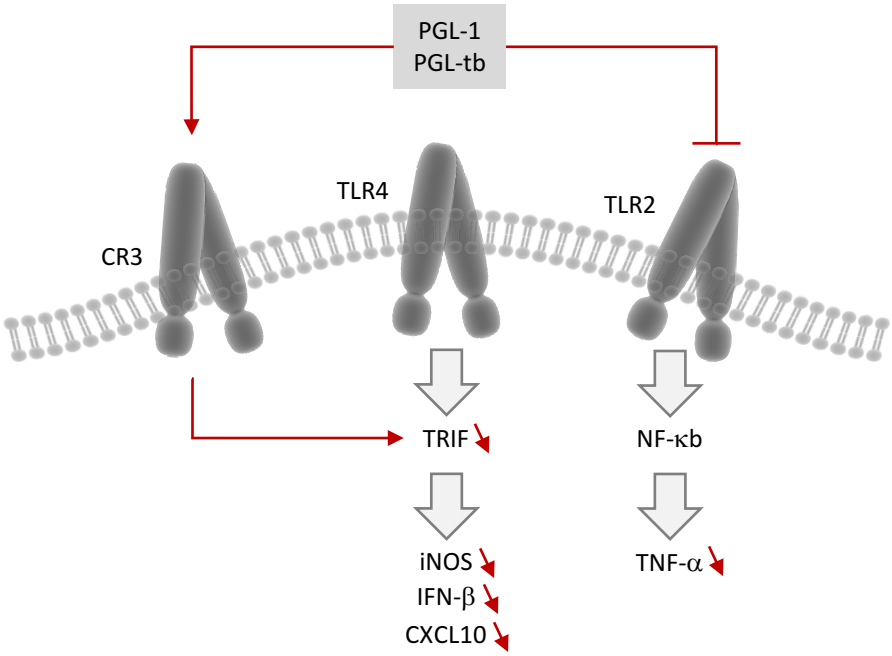

Figure 3: Schematic diagram recapitulating the mechanisms used by PGL-1 and PGL-tb to suppress the innate immune responses of macrophages