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Fishing for Answers in Human Mycobacterial Infections

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Abstract

Two recent studies (Cambier et al., 2017; Madigan et al., 2017) reveal in vivo functions for specific phenolic glycolipids (PGLs) in the mycobacteria that cause tuberculosis or leprosy. *M. tuberculosis* (and *M. marinum)* PGL promotes bacterial spread to growth-permissive macrophages, while *M. leprae* PGL-1 induces macrophages to cause nerve demyelination characteristic of human leprosy.

Phenolic glycolipids (PGLs) have been known as components of mycobacterial cell walls for decades (Hunter and Brennan, 1981), yet their biological roles have been incompletely characterized. Two recent studies from the Ramakrishnan group (Cambier et al., 2017; Madigan et al., 2017) now reveal important in vivo roles for the PGLs of certain major lineages of *M. tuberculosis* and of *M. leprae*, the causative agents of tuberculosis (TB) and leprosy, respectively. Mycobacterial PGLs have a common core structure, but differ in their species-specific glycosylation and carbohydrate modifications. Ramakrishnan and colleagues provide insight into the functional importance of the species-specific PGL structures.

Understanding the earliest events in in vivo infection with *M. tuberculosis* has been constrained by the technical difficulties of studying events after a low bacterial inoculum reaches the depths of the lungs. Therefore, while it is known that *M. tuberculosis* infects diverse myeloid cells in host tissues (Wolf et al., 2007), it has been challenging to examine how the mycobacteria escape their earliest captors, presumed to be resident alveolar macrophages, and subsequently infect other cell types that are recruited to the site of infection. Cambier et al. (2017) use optically transparent zebrafish larvae and *M. marinum*, the cause of fish tuberculosis, to address this question, revealing how this pathogen actively creates a niche for itself very early in infection by manipulating growth-restrictive resident macrophages (ResM) to attract growth-permissive monocytes into which the mycobacteria can escape and thrive. They report a critical role for *M. marinum* PGL in this process, and activation of the STING cytosolic sensing pathway in ResM to induce expression of the

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chemokine CCL2 and subsequent recruitment of chemokine receptor 2 (CCR2)-expressing monocytes from the blood.

In an earlier study, the authors utilized zebrafish larvae to study how mycobacteria evade microbicidal macrophages and found that mycobacteria used phthiocerol dimycoceroserate (PDIM) lipids on their surface to mask pathogen-associated molecular patterns (PAMPs) (Cambier et al., 2014). Without this shielding of PAMPs, reactive nitrogen-producing macrophages were recruited via a Toll-like receptor (TLR)-dependent pathway. This study also revealed a role for PGL in promoting recruitment of permissive macrophages via host CCR2.

In the present paper in *Immunity* (Cambier et al., 2017), the authors distinguish between zebrafish larvae brain-ResM that can be recruited to the hindbrain ventricle infection site and peripheral monocytes. The distinction is made by injection of Hoechst 33342 dye that does not cross the blood-brain barrier and thus labels circulating monocytes but not ResM. This approach revealed that the first responders to *M. marinum* in the zebrafish hind brain ventricle are ResM (Figure 1), followed by monocytes. While recruitment of circulating monocytes was dependent on both PGL and CCR2, as determined using PGL-deficient mycobacteria and CCR2-deficient zebrafish, the recruitment of ResM to the site of infection was independent of these factors. The authors first showed that an initial encounter with ResM is absolutely required for subsequent monocyte recruitment. Mechanistically, this recruitment is dependent on expression of *Ccl2* by ResM, which is induced by mycobacterial PGL (Figure 1). Notably, depletion of the cytosolic sensor STING, known to be activated by cyclic dinucleotides, abrogated the induction of CCL2 by *M. marinum*, and concomitantly, monocyte recruitment to *M. marinum*-in-fected macrophages. Whether PGL interacts directly with STING as an alternative agonist, or whether one or more intermediate steps are involved in the activation of STING by PGL awaits further investigation; in either case, it will be important to understand how PGL reaches the host cell cytosol (Figure 1). Mycobacterial vesicles that are released in and by infected cells (Athman et al., 2015; Prados-Rosales et al., 2011; Srivastava et al., 2016) would be worthwhile candidates to study in this regard.

To extend the significance of these findings and allow assessment of the outcomes of infection, the authors infected larvae with a low inoculum of 1–3 *M. marinum*. Time–lapse confocal microscopy confirmed that ResM arrive at the infection site early and are the predominant cells that phagocytize wild-type *M. marinum*, contrasting with PDIM-deficient *M. marinum* that are taken up by both ResM and monocytes. Imaging of different groups of larvae over the span 4.5 days after infection confirmed bacterial transfer from infected ResM to recruited monocytes, which peaked 66–72 hr after infection (Figure 1). Transfer events were independent of uptake of apoptotic vesicles and were also not observed upon infection with PGL-deficient mycobacteria. PGL-replete *M. marinum* rapidly spread to monocytes, while PGL-deficient mycobacteria dwelled for a longer time in ResM, and then were cleared, suggesting that ResM are more microbicidal than CCL2-recruited monocytes. Indeed, infection with PGL-deficient *M. marinum* resulted in a greater number of inducible nitric oxide synthase (iNOS)-positive cells than infection with wild-type mycobacteria, but only when infection occurred via the hindbrain ventricle. Thus, not only are ResM more

microbicidal than recruited monocytes, but also myeloid cells recruited via the CCL2-CCR2 axis are less microbicidal than when they are recruited in a TLR-dependent fashion, such as upon infection with PDIM-deficient *M. marinum*.

That ResM are more microbicidal than peripheral monocytes is not a completely new concept, because human alveolar macrophages are superior to peripheral blood monocytes in killing avirulent and attenuated mycobacteria in ex vivo assays Hirsch et al. (1994), Rich et al. (1997). In this regard, the zebrafish model resembles the human situation. Cambier et al. (2017) further show that human alveolar macrophages rapidly produce CCL2 upon infection with *M. marinum* and that this is dependent on PGL expression by the mycobacteria, thus showing conservation of this mechanism of immune evasion and bacterial dispersal in humans.

The versatility of PGL in bacterial infection is futher illustrated in a recent study published in *Cell*, which reports a role for the *M. leprae* version of PGL, PGL-1, in mediating nerve damage in leprosy via infected macrophages. In elegant confocal microscopy and transmission electron microcopy studies of transgenic zebrafish larvae infected with fluorescent *M. leprae*, Madigan et al. (2017) show that infection of macrophages with PGL-1+ *M. leprae* results in the production of excess nitric oxide; these macrophages in turn damage the mitochondria of adjacent nerves, leading to demyelination (Figure 1). *M. leprae* PGL-1 differs from that of *M. tuberculosis* or *M. marinum* by a unique trisaccharide, and this is a functionally important difference as infection with *M. marinum* engineered to produce *M. leprae* PGL-1 instead of its own PGL led to similar demyelination as that seen upon infection with *M. leprae*. These in vivo findings provide unique insight into the molecular and cellular mechanisms underlying *M. leprae* induced peripheral nerve damage, a characteristic of leprosy.

Taken together, these findings provide unique insights into the biology of tuberculosis and leprosy and into the functional relevance of mycobacterial PGL in infection, immune evasion, and the pathogenesis of disease. Like any exciting work, the results presented raise questions and provide directions for future research. *M. tuberculosis* PGL is an unusual virulence factor in that it is absent from some strains and lineages that are still fully capable of causing disease. While it is clear that PGL confers measurable differences on mycobacteria, how do strains that lack PGL cause disease? Do they have other factors that compensate for the absence of PGL, or do they have different mechanisms of causing disease? Are the manifestations of infection with a PGL-competent strain different than with a PGL-deficient strain? Do PGL-deficient strains require a higher inoculum, or do they only target certain individuals? Because the two phylogenetic lineages of the *M. tuberculosis* complex that are most successful globally differ in their capacity to make PGL (Gagneux et al., 2006), there are rich opportunities to better understand the diverse mechanisms of virulence and pathogenicity in human tuberculosis. Mechanistically, it will be interesting to determine how *M. tuberculosis* PGL activates STING. Because drugs that target STING are already in the clinical pipeline, it will be worthwhile to determine whether they might be adjuncts to modulate the pathogenicity of TB in individuals infected with PGL-producing strains. Moreover, the findings of distinct populations of macrophages that differ in their ability to restrict or support growth of mycobacteria are sure to prompt additional efforts to

understand their differences, with the hope of being able to skew cells toward a functional state where they can eliminate intracellular bacteria and thereby accelerate immune clearance of infection.

Madigan et al. (2017) present a tractable in vivo model for understanding the pathogenesis of nerve damage in leprosy, and for screening new drugs targeting this pathology, which might benefit people infected with *M. leprae* who suffer from “reversal reactions,” wherein heightened inflammatory responses can be accompanied by rapidly progressive nerve damage and loss of function. The zebrafish model will also enable addressing basic mechanistic questions that were previously unapproachable due to the lack of a tractable system, such as whether the findings by Madigan et al. (2017) apply only to multibacillary leprosy, where there are numerous bacteria and infected macrophages, or also to paucibacillary leprosy, where few bacteria or infected macrophages are present. Furthermore, because antibody responses to PGL-1 are prominent in patients, especially those with multibacillary leprosy, might those antibodies actually protect from pathology or might they be pathogenic? Future studies in the zebrafish model might provide answers to these questions.

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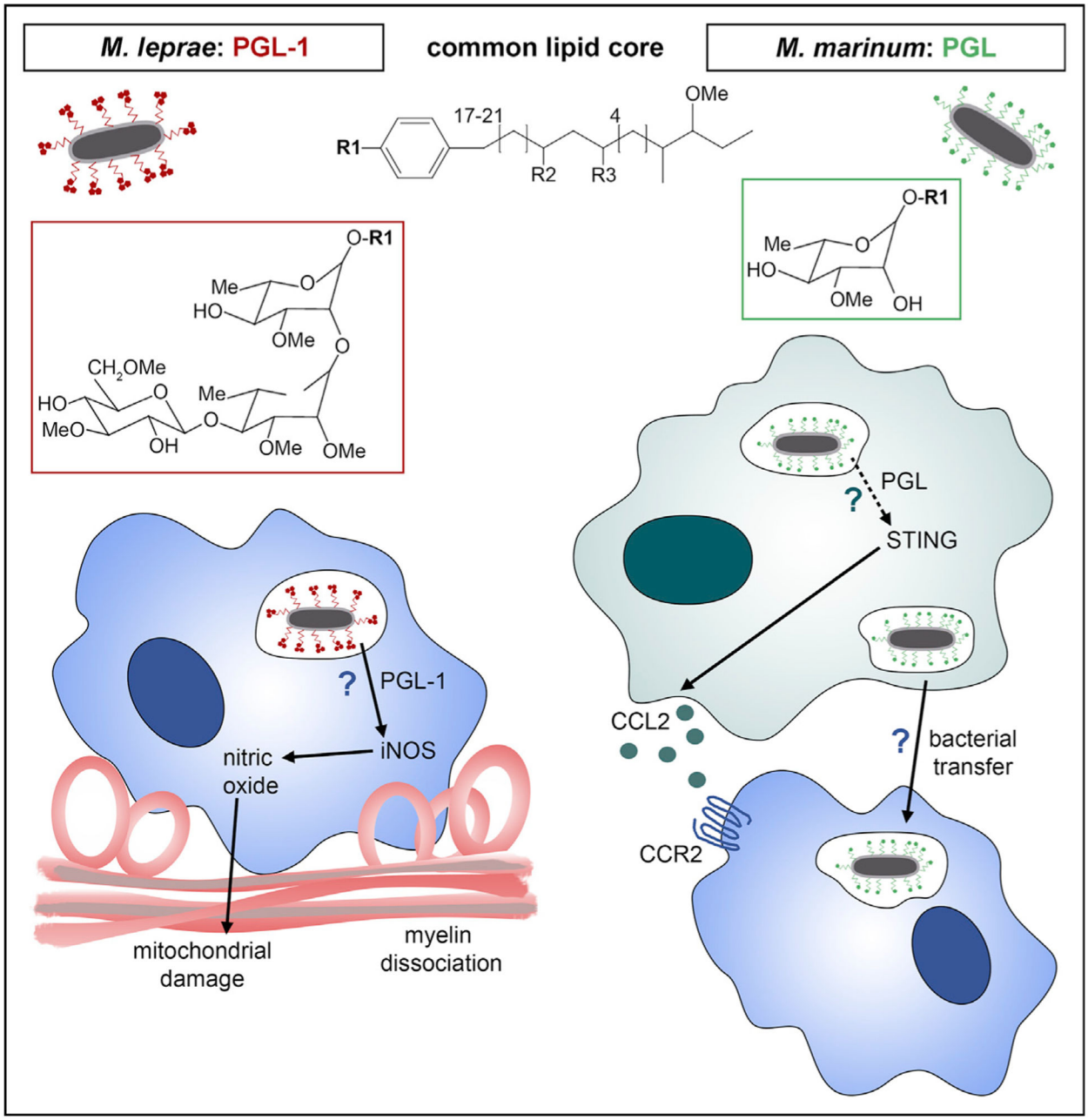

Figure 1. In Vivo Functions for Species-Specific Phenolic Glycolipids in Mycobacteria

Mycobacterial phenolic glycolipids (PGLs) have a common core structure, but differ in their species-specific glycosylation and carbohydrate modifications. *M. marinum* infection in the zebrafish hindbrain ventricle leads to recruitment of brain-resident macrophages (ResM) and phagocytosis of the mycobacteria. *M. marinum*-specific PGL depends on STING cytosolic signaling (directly or indirectly) to induce chemokine CCL2 expression in ResM. CCR2-expressing circulating monocytes are subsequently attracted and *M. marinum* escape into those more growth-permissive cells. *M. leprae* PGL-1 in infected monocytes induces nitric oxide synthase and pathologic amounts of nitric oxide, which leads to nerve damage caused

by mitochondrial damage and demyelination in areas of intimate contact of the infected macrophage with axons.