Mitochondria: Powering the Innate Immune Response to *Mycobacterium tuberculosis* Infection

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ABSTRACT Within the last decade, we have learned that damaged mitochondria activate many of the same innate immune pathways that evolved to sense and respond to intracellular pathogens. These shared responses include cytosolic nucleic acid sensing and type I interferon (IFN) expression, inflammasome activation that leads to pyroptosis, and selective autophagy (called mitophagy when mitochondria are the cargo). Because mitochondria were once bacteria, parallels between how cells respond to mitochondrial and bacterial ligands are not altogether surprising. However, the potential for cross talk or synergy between bacterium- and mitochondrion-driven innate immune responses during infection remains poorly understood. This interplay is particularly striking, and intriguing, in the context of infection with the intracellular bacterial pathogen *Mycobacterium tuberculosis* (Mtb). Multiple studies point to a role for Mtb infection and/or specific Mtb virulence factors in disrupting the mitochondrial network in macrophages, leading to metabolic changes and triggering potent innate immune responses. Research from our laboratories and others argues that mutations in mitochondrial genes can exacerbate mycobacterial disease severity by hyperactivating innate responses or activating them at the wrong time. Indeed, growing evidence supports a model whereby different mitochondrial defects or mutations alter Mtb infection outcomes in distinct ways. By synthesizing the current literature in this minireview, we hope to gain insight into the molecular mechanisms driving, and consequences of, mitochondrion-dependent immune polarization so that we might better predict tuberculosis patient outcomes and develop host-directed therapeutics designed to correct these imbalances.

KEYWORDS LRRK2, *Mycobacterium tuberculosis*, cytosolic DNA sensing, innate immunity, macrophage, mtDNA, type I interferon

Macrophages are exquisitely well-evolved to sense bacterial pathogens via a vast array of extracellular, endosomal, and cytosolic pattern recognition receptors and respond to threats via activation of antibacterial gene expression programs (1). In fact, macrophages even mount robust immune responses when they sense pieces of damaged mitochondria in their cytosol, despite the fact that mitochondria have not been free-living bacteria for over a billion years. Although the ability of mitochondrion-derived damage-associated molecular patterns (mito-DAMPs) to activate a variety of innate immune responses in macrophages and other cells is fairly well-appreciated today, we still know almost nothing about how mitochondrion-triggered innate immune responses alter a cell's ability to sense and respond to an actual microbial threat.

Mounting evidence from a variety of disease models demonstrates that mitochondrial dysfunction and the release of mito-DAMPs can polarize innate immune responses and increase host susceptibility to certain pathogens (2–6). Furthermore, pathogens across kingdoms have been shown to interact with, and, in many cases, manipulate mitochondrial function to promote infection. Several recent reviews have

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tackled the vast microbe-mitochondrial interface literature and present valuable insights into unique and conserved strategies used by pathogens to manipulate mitochondrial metabolism and homeostasis (3, 5, 7, 8). Here, we will specifically focus on the surprising number of connections that have emerged between mitochondria and the important human pathogen *Mycobacterium tuberculosis* (Mtb). Mtb, the causative agent of tuberculosis, is an intracellular bacterial pathogen that survives and replicates within the harsh environment of a macrophage. Although long considered a strictly vacuolar pathogen, we now appreciate that Mtb permeabilizes or destabilizes its phagosome, accesses the macrophage cytosol, and triggers a variety of pathways downstream of cytosolic pattern recognition receptors. These same pathways, type I interferon expression, ubiquitin-mediated selective autophagy, and inflammasome activation/programmed cell death, are activated by damaged mitochondria when they release ligands like mitochondrial DNA (mtDNA) into the cytosol (9–11). If macrophages sense and respond to Mtb and mitochondrial damage in the same ways, what happens when a cell senses both simultaneously? How are these signals integrated into a cohesive innate immune response? Is such a response inherently pro- or antibacterial? Finally, has a pathogen like Mtb evolved to disrupt the balance of these responses to promote its own survival within a host?

This review focuses on three seemingly distinct but likely intimately connected observations related to the mitochondrial-Mtb interface: (i) macrophages sense and respond to damaged mitochondria and Mtb by activating the same innate immune responses, (ii) Mtb can directly damage mitochondria in infected macrophages, and (iii) mutations in mitochondrion-associated genes alter host susceptibility to mycobacterial infection. By understanding how mitochondrion-triggered innate immune responses influence tuberculosis disease severity, we might one day develop host-directed therapeutics designed to correct imbalances in these responses and promote positive tuberculosis patient outcomes. Likewise, because mitochondrial mutations that confer susceptibility to mycobacterial infection are also associated with several chronic diseases, including Parkinson's disease (PD) (12–15), Crohn's disease (16–19), inflammatory bowel disease (20–22), and cancer, understanding how they alter mitochondrial function and dysregulate immune responses will help illuminate common molecular mechanisms driving these otherwise disparate diseases.

MITOCHONDRIAL DAMPs AND Mtb TRIGGER MANY OF THE SAME INNATE IMMUNE RESPONSES

Mitochondria are membrane-bound organelles that are involved in myriad biological processes. Unlike the bean-shaped organelles depicted in many textbooks, mitochondria actually form a highly dynamic, branched network that continually undergoes cycles of fission, fusion, transport, and degradation (23–25). The cycle of fission and fusion plays a critical role in maintaining cellular homeostasis, with mitochondrial fusion promoting energy production in proliferating cells via oxidative phosphorylation (26) and mitochondrial fission being required for clearance of damaged pieces of the network and programmed cell death via apoptosis (27, 28). While mitochondria are most well-known for their prominent role in generating energy in the form of ATP through oxidative phosphorylation, they also contribute to a number of other metabolic processes in the cell, including fatty acid metabolism and biosynthesis of macromolecules such as nucleotides, lipids, heme, and iron sulfur clusters (29). Beyond metabolic and catabolic roles, mitochondria are key players in regulation of the cell cycle, cell proliferation, cellular differentiation, and programmed cell death (30–32). In addition to these crucial roles of making energy, controlling cell proliferation, and regulating cell death, mitochondria are being increasingly appreciated as critical players in immunity, and there is growing evidence that the metabolic/bioenergetic state of an immune cell influences its ability to detect and respond to pathogens (33–35). To date, most of what we know about mitochondrion-driven innate immunity relates to the ability of mito-DAMPs (e.g., mtDNA, cardiolipin, formyl-methionine-labeled peptides,

and cytochrome c) to engage pattern recognition receptors and trigger cytokine/chemokine production and/or cell death. Because the role of mito-DAMPs in eliciting immune responses has been reviewed extensively elsewhere (9, 36–38), we will focus here on mtDNA, the mito-DAMP that we currently know the most about during *Mtb* infection.

CYTOSOLIC DNA SENSING IS ACTIVATED BY mtDNA AND *Mtb*

The 16.6-kb mitochondrial genome is a double-stranded circular molecule that is rich in unmethylated CpG motifs and susceptible to oxidative stress (making it look more bacterial than eukaryotic) (39, 40). It is maintained independently of the eukaryotic genome by the mitochondrial DNA polymerase gamma (POLG). Organization of the mitochondrial genome is preserved by the mitochondrial transcription factor A (TFAM), which packs mtDNA into nucleosome-like protein-DNA complexes called nucleoids (41, 42). There are hundreds to thousands of copies of mtDNA present in every cell of the human body, with the exception of erythrocytes, which lack mitochondria.

Until somewhat recently, the role of the mitochondria in regulating immunity was mainly studied in the context of cell death and/or antiviral RNA sensing (for which mitochondria serve as an important signaling hub) (43–46). The first major study to implicate a mitochondrial ligand in directly eliciting inflammatory gene expression came from West et al., who used a *Tfam*^{+/-} model to study the role of mtDNA in innate immune responses (11). They found that TFAM heterozygosity is sufficient to destabilize the mitochondrial genome and cause mtDNA leakage into the cytosol. They went on to demonstrate that in *Tfam*^{+/-} cells, cytosolic mtDNA is recognized by the cytosolic DNA sensor cGAS (cyclic GMP-AMP synthase), which promotes chronic type I IFN expression and potentiates interferon-stimulated gene (ISG) expression upon cytosolic nucleic acid challenge. Thus, *Tfam*^{+/-} mouse embryonic fibroblasts (MEFs) are resistant to herpes simplex virus 1 (HSV-1) and vesicular stomatitis virus (VSV) infection. A diagram of the canonical cGAS signaling cascade, whereby double-stranded DNA (dsDNA) sensing by cGAS generates cGAMP (cyclic GMP-AMP or 2'3'-cyclic GMP-AMP), which activates STING (stimulator of interferon genes), which recruits and activates TBK1 (Tank-binding kinase), which phosphorylates the transcription factor IRF3 (interferon regulatory factor 3), is shown in Fig. 1. This paradigm-shifting study from West et al. elevated the mitochondrion to an active player in triggering type I IFN expression via cytosolic DNA sensing. Subsequent studies have confirmed that several other types of mitochondrial damage can spill mtDNA into the cytosol and elicit type I IFN expression downstream of cGAS, including pore formation via the apoptosis proteins Bak/Bax (47–49) and gasdermins (50, 51). While cGAS is generally accepted as the major sensor of cytosolic mtDNA, mtDNA also has been shown to trigger type I IFN expression via toll-like receptor 9 (TLR9), although this phenomenon has mostly been observed in dendritic cells (Fig. 1) (9, 52).

Because *Mtb* resides within a membrane-bound compartment, termed the *Mtb* phagosome, following phagocytosis by macrophages, *Mtb* is traditionally categorized as a vacuolar pathogen. While visually, *Mtb* bacilli are mostly membrane-bound within macrophages, if one considers *Mtb*'s intracellular lifestyle in terms of what innate immune responses it elicits, the distinction between vacuolar and cytosolic pathogen becomes blurred. Indeed, early work from the Cox laboratory revealed that the transcriptional signature of *Mtb*-infected macrophages is almost identical to that of macrophages infected by *Listeria monocytogenes* (53), which famously completely breaks down its phagosome via secretion of a pore-forming toxin, listeriolysin O (LLO) (54, 55). More surprisingly, the transcriptional response elicited by both of these pathogens in many ways resembles the characteristic antiviral type I IFN response of a cytosolic DNA virus. Expression of this type I IFN signature during infection with both pathogens requires phagosomal permeabilization (via ESX-1 for *Mtb* and LLO for *L. monocytogenes*) and the STING/IRF3 axis (53) (Fig. 1). In the case of *L. monocytogenes*, work from

FIG 1 Both mtDNA and Mtb DNA engage cytosolic DNA surveillance pathways to activate type I interferon expression. Mtb infection can stimulate type I IFN expression by activating both cGAS, via Mtb dsDNA, and STING, via secretion of cyclic-di-AMP. To date, mtDNA has been shown to engage cGAS and TLR9. While TLR9 has been shown to play a role in determining Mtb infection outcomes *in vivo*, it remains unclear whether the endosomal receptor actually directly binds to Mtb-derived dsDNA during Mtb infection of macrophages (represented by the question mark). The figure was created with BioRender.

the Portnoy laboratory showed that the major host determinant for type I IFN expression during *L. monocytogenes* infection is STING, as *Listeria* bacilli secrete c-di-AMP (cyclic di-3',5'-AMP) via a bacterially encoded di-adenylate cyclase, effectively bypassing a cytosolic DNA sensor like cGAS (56, 57). During Mtb infection, *ex vivo* genetic studies suggest that the majority of type I IFN expression occurs downstream of cGAS, as the response is completely ablated in Mtb-infected *Cgas*^{-/-} human and murine macrophage cell lines and primary murine macrophages (58–60). Curiously, in spite of this strong phenotype, loss of cGAS in mouse models of disease has a limited impact on type I IFN and Mtb *in vivo* infection outcomes (58, 60). More recent work found that STING is also dispensable for controlling Mtb infection in mice (61), despite earlier studies that identified and characterized a role for two Mtb adenylate cyclases, DisA and DacA, in stimulating type I IFN expression downstream of STING (62). These surprising observations suggest that type I IFN expression can be elicited downstream of one or more additional sensors *in vivo* or that the loss of these factors simultaneously impacts both pro- and antibacterial pathways such that phenotypes are obscured. Although the nature of these additional sensors remains elusive, several studies suggest TLR9, an endosomal sensor of CpG DNA, contributes to host resistance to Mtb (63–65), and RNA sensing pathways have been shown to contribute to type I IFN responses *in vivo* (66).

Even though we do not fully understand the contribution of specific host factors to eliciting type I IFN responses, the aforementioned studies and many others (67) strongly suggest that the ability to induce host type I interferon is a conserved

FIG 2 Cross talk between *Mycobacterium tuberculosis* and mitochondria can influence cytosolic sensing pathways. Schematic representation of molecular interplay between Mtb and mitochondria in infected cells. Yellow question marks indicate several phenomena whose mechanisms/consequences are unclear, namely, whether PDIM (phthiocerol dimycocerosates) and/or ESAT-6 (6-kDa early secretory antigenic target 6) directly alter the mitochondrial membrane, what secretion systems introduce mitochondrion-modulating Mtb effectors into host cells, how Mtb infection generally affects mitochondrial metabolism, and whether mtDNA engages with cGAS (cyclic GMP-AMP synthase) and AIM2 (absent in melanoma) simultaneously (and, if so, what the outcomes for an Mtb-infected cell are). Secreted Mtb virulence factors that have been shown to alter mitochondrial homeostasis are colored in shades of purple. The figure was created with BioRender.

intracellular bacterial adaptation shared by Mtb, *L. monocytogenes*, *Legionella pneumophila*, *Chlamydia trachomatis*, *Brucella abortus*, *Coxiella burnetii*, and *Francisella tularensis* (67). To date, most research has failed to define the nature of the cytosolic DNA ligand in cells infected with intracellular bacterial pathogens. We will address the potential relative contributions of bacterial (Mtb) and mitochondrial dsDNA to cytosolic DNA sensing in the next section.

MITOCHONDRIAL DAMPs AND Mtb PAMPs CAN MEDIATE IL-1 β RELEASE AND TRIGGER PYROPTOSIS VIA ACTIVATION OF THE INFLAMMASOME

Inflammasomes are multisubunit, cytoplasmic protein complexes that consist of receptor/sensor molecules, the adaptor protein ASC, and the inflammatory cysteine protease caspase 1. Inflammasome assembly triggers caspase 1 activation, leading to the cleavage of pro-interleukin-1 β (pro-IL-1 β) and pro-IL-18 into mature, secreted forms of the cytokines (68, 69) and an inflammatory form of programmed cell death, called pyroptosis. Upon cellular stress or in the face of mitochondrial dysfunction, release of mito-DAMPs, including mitochondrial reactive oxygen species (ROS) (70), cardiolipin (71, 72), and extracellular ATP (73), can activate the NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome. In addition to triggering type I IFN expression through engaging cGAS, mtDNA also directly activates the NLRP3 and AIM2

(absent in melanoma) inflammasomes (Fig. 2) (74, 75). Because mtDNA can seemingly serve as a ligand for both cGAS and AIM2/NLRP3, an obvious unanswered question is whether mtDNA activates each of these cytosolic sensing pathways with similar efficiency and at the same time. One characteristic of mtDNA that could influence its ability to stimulate these two pathways independently is its oxidation state. Oxidized mtDNA has been shown to activate the NLR family pyrin domain-containing 3 (NLRP3) inflammasome (75) and has been shown to be a poor substrate of the cytosolic DNase TREX1 (three prime repair exonuclease 1) (76). Thus, oxidized mtDNA may persist longer in the cytosol, thereby affecting its ability to engage different PRRs. Likewise, the sequence, length, and subcellular localization of an mtDNA fragment may influence its ability to bind to/engage PRRs, although the effect of these variables has yet to be experimentally tested.

The inflammasome also plays an important role in the immune response against *Mtb*, although certain aspects of the molecular mechanisms driving inflammasome activation and *Mtb*'s ability to manipulate this pathway within infected macrophages remain unclear. Activation of the inflammasome is certainly important during *in vivo* *Mtb* infection, as deletion of key inflammasome factors, including IL-18 (77), IL-1 β (78), and IL-1 receptor (IL-1R) (78, 79), confer susceptibility to *Mtb*. *Aim2*^{-/-} mice are also highly susceptible to *Mtb* infection and display impaired Th1 responses (80). In macrophages *ex vivo*, *Mtb* activates the AIM2 inflammasome in an ESX-1-dependent fashion (59), and a recently developed vaccine strain, BCG Δ ureC::hly, was also shown to stimulate the AIM2 inflammasome (81). BCG Δ ureC::hly is a bacille Calmette-Guérin (BCG) vaccine strain of *Mtb*, genetically engineered to express the *L. monocytogenes* LLO, which enables phagosomal permeabilization in what is otherwise an ESX-1 mutant (BCG lacks region of difference 1 [RD1], a region of the *Mtb* genome that encodes the ESX-1 secretion system). While AIM2 activation does appear to depend on the ESX-1 secretion system, we also know that AIM2 is not the only inflammasome induced by mycobacterial infection; significant levels of IL-1 β are released from *Aim2*^{-/-} bone marrow-derived macrophages, and nonvirulent strains of *Mtb* lacking RD1 can promote IL-1 β release via the NLRP3 inflammasome (59, 82). Further complicating matters is a report that *Mtb* can inhibit AIM2 inflammasome activation in an ESX-1-dependent fashion, as evidenced by the fact that caspase-1 cleavage and IL-1 β secretion are inhibited in *Mtb*-infected cells exposed to a secondary inflammasome trigger (82). Again, while these studies nicely demonstrate the involvement of the AIM2 and NLRP3 inflammasomes during *Mtb* infection of macrophages *ex vivo*, they only measure the final consequences and not the upstream drivers of inflammasome engagement. In all of these experiments, it is feasible that *Mtb*-derived ligands, mitochondrion-derived ligands, or both prime and/or trigger inflammasome activation. Indeed, the parallels between NLRP3 and AIM2 activation in response to *Mtb* infection and mitochondrial damage suggest an intriguing model whereby *Mtb* may mediate inflammasome activation through ESX-1-dependent perturbation of mitochondria and release of mtDNA/mito-DAMPs into the cytosol.

Mtb PERTURBS THE MITOCHONDRIAL NETWORK DURING MACROPHAGE INFECTION

Central to *Mtb*'s ability to modulate macrophage innate immune responses is its sec-independent type VII virulence-associated ESX-1 secretion system. One of the main consequences of ESX-1 secretion is destabilization/permeabilization of the *Mtb* phagosomal membrane, which allows for mixing of bacterial components from inside the phagosome with innate immune sensors in the host cytosol (Fig. 2). The prevailing paradigm asserts that permeabilization of the *Mtb* phagosome is achieved via cooperation between two major *Mtb* virulence factors, ESAT-6 (the major substrate of ESX-1) and surface glycolipids, called phthiocerol dimycocerosates (PDIMs) (83–87). ESX-1/ESAT-6 have been repeatedly implicated in destabilizing the *Mtb* phagosome (88–91). Although earlier work suggested that ESAT-6 formed pores in biological membranes, a *bone fide* pore-forming function for ESAT-6 has largely been dismissed as an artifact of

residual detergent in ESAT-6 purified protein samples (92). Instead, work from the Ramakrishnan laboratory supports a model whereby ESX-1-dependent hemolysis requires direct contact between bacteria and membranes and ESX-1 secretion more accurately causes gross membrane disruption than discrete pores (92).

Like ESX-1, PDIM is required to maximize cytosolic access of both Mtb (83, 84) and *M. marinum* (a common experimental model for Mtb that can infect zebrafish) (85, 93). A recent set of biophysical experiments from Augenstreich et al. examined how various Mtb strains affected membrane polarity. They found that wild-type (WT) BCG elicits changes in membrane polarity of a lipid bilayer, while a PDIM mutant did not (86). Using hemolysis as a readout for membranolytic activity, the same group showed that BCG expressing ESX-1 but lacking PDIM are less efficient at potentiating membrane lysis compared to WT BCG or BCG expressing ESX-1. PDIM has also been shown to decrease plasma membrane polarity of THP-1 cells during BCG infection (94). Taken together, these experiments convincingly link ESX-1/ESAT-6 and PDIM to destabilization of the Mtb phagosome. However, we still know very little about how these virulence factors interfere with other lipid bilayers, like the mitochondrial membrane, in Mtb-infected cells.

To date, most of what we know about how PDIM and ESX-1 secretion are linked to mitochondrial damage and innate immune regulation is correlative. As described above, work from the Cox laboratory was among the first to demonstrate that type I IFN expression during Mtb infection requires ESX-1 secretion (53, 95), and our subsequent studies identified cGAS as the major cytosolic DNA sensor driving type I IFN expression in response to Mtb-derived dsDNA (which we found bound to cGAS in Mtb-infected cells) (60). A recent transposon sequencing (Tn-seq) experiment designed to identify Mtb mutants defective in intracellular growth reported high correlation between mutants in ESX-1 and mutants in PDIM and showed that production and transport of PDIM is required for type I IFN expression during Mtb infection of macrophages (96). These data support a model whereby permeabilization of the Mtb phagosome allows for release of bacterial dsDNA into the cytosol, where it can engage with cGAS and elicit cytosolic innate immune sensing. However, due to growing interest in the involvement of cytosolic DNA of mitochondrial and/or nuclear origin in eliciting type I IFN expression during Mtb infection, studies have begun to challenge this model. Notably, Wiens and Ernst infected macrophages with three phylogenetically distinct strains of the Mtb complex and found that the level of type I IFN response correlates with a strain's ability to induce mitochondrial stress (as measured by mitoSOX, a fluorogenic dye used to measure mitochondrial superoxide in live cells) and induce cytosolic mtDNA accumulation (97). Likewise, using a collection of transposon mutants in ESX-1 genes in *M. marinum*, Lienard et al. were able to uncouple *M. marinum* phagosomal access from type I IFN expression (98). Their work shows that in *M. marinum*-infected bone marrow-derived macrophages (BMDMs), permeabilization of the phagosome is not sufficient to induce type I IFN production and that mtDNA, but not *M. marinum* dsDNA, accumulates in the cytosol in an ESX-1-dependent fashion. These authors conclude that an unidentified ESX-1-dependent function or secreted factor (not ESAT-6) is required to release host mtDNA into the cytosol. Critically, while both of these reports attempt to dismiss the contribution of Mtb dsDNA to driving type I IFN expression, it is possible that the amount of cytosolic Mtb dsDNA changes dynamically over the course of infection (both studies examine a single time point: 24 h postinfection). The same caveat could apply to cytosolic mtDNA levels, which one can imagine will increase over the course of infection as mitochondrial damage accumulates. Thus, it is possible that activation of type I IFN is initiated at very early time points after phagocytosis via bacteria-derived dsDNA and later is augmented and/or sustained through Mtb-dependent release of mtDNA. Experiments designed to alter the mtDNA content of cells and/or identify and characterize Mtb mutants that lack the ability to induce type I IFN altogether or fail to release mito-DAMPs will help tease apart the contribution of each dsDNA source to the type I IFN response during Mtb infection.

Beyond directly or indirectly affecting the release of mito-DAMPs, Mtb, ESAT-6, and ESX-1 secretion have also been implicated in generally altering mitochondrial function. A very recent paper from Yabaji et al. demonstrates that the expression of ESAT-6 upregulates superoxide dismutase (SOD2), an enzyme that detoxifies free radical by-products of mitochondrial respiration. Upregulated SOD2 activity, in turn, inhibits the acidification of the Mtb phagosome (99). Although the molecular mechanisms linking ESAT-6 to increased SOD2 activity remain elusive, loss of SOD2 by short interfering RNA knock-down is sufficient to limit BCG survival in J774.A.1 macrophages, supporting the idea that mitochondrial superoxide contributes to Mtb killing. Thus, ESAT-6 may facilitate mycobacterial survival by upregulating radical detoxifying enzymes in the mitochondria. Along the same lines, work from Cumming et al. demonstrated that a dramatic reprogramming of cellular respiration occurs in Mtb-infected cells (100). They show that a decrease in maximal respiration and a decrease in basal respiratory capacity are dependent on an intact ESX-1 secretion system (wild-type Mtb induced these changes while the BCG vaccine strain did not). Overall, these changes correspond to a quiescent energy phenotype, with bioenergetic metabolism further decelerating with increasing multiplicity of infection (MOI). These changes in mitochondrial respiration corresponded to changes in the dependence of mitochondria on different energy sources, whereby Mtb-infected macrophages are more dependent on exogenous fatty acids than are uninfected cells. While several outstanding questions remain, namely, how Mtb both up- and downregulates glycolysis at different time points during infection and how these changes benefit the pathogen, this research provides an important framework for understanding how intracellular bacterial pathogens reprogram mitochondrial metabolism.

Another very recent study suggests that one way Mtb infection restricts macrophage glycolysis is via induction of miR-21, which targets the phosphofructokinase muscle (PFK-M) isoform, an enzyme that controls a critical rate-limiting step of glycolysis (101). It will be interesting to determine whether these changes can be ascribed to the direct action of Mtb virulence factors versus the activation of various innate immune pathways, which themselves have been shown to reprogram macrophage energetics (102). Some potential candidates for mitochondrial metabolism-modulating Mtb effectors are LprG and PE-PGRS33 (Fig. 2). Treatment of monocyte-derived macrophages with recombinant LprG induces mitochondrial fission and decreases the basal respiratory rate, while addition of PE-PGRS33 induces mitochondrial fusion (103). Although both of these factors have predicted mitochondrial targeting sequences and PE-PGRS33 colocalizes with the mitochondrial network in transfected cells (104), the precise mechanisms for how they interfere with mitochondrial network dynamics and/or how they get from Mtb to the mitochondria have yet to be explored. Likewise, a recent large-scale protein-protein interaction study of Mtb effector and host proteins (105) identified several Mtb secreted proteins that had high-confidence interactions with mitochondrial proteins (e.g., Rv2469c, PE25, and Rv2941), although follow-up analyses on whether these factors modulate mitochondrial biology are still needed. As additional insights are made into the mechanisms through which Mtb effectors impact mitochondrial homeostasis, it will be interesting and important to compare and contrast with the strategies employed by myriad other pathogens across kingdoms.

Lastly, there are several mycobacterial proteins shown to regulate macrophage cell death via interacting with the mitochondrion. For example, heparin-binding hemagglutinin (HBHA), a major secreted antigen during Mtb infection, localizes to mitochondria and can induce macrophage apoptosis via caspase activation. HBHA treatment of RAW 264.7 macrophages causes Bax translocation to the mitochondria, cytochrome c release, and decreased mitochondrial membrane potential, suggesting a role for HBHA in triggering programmed cell death (106). An opposite, antiapoptotic function has been ascribed to a mycobacterial chaperone protein, Cpn60.2 (GroEL2), which was shown to interact with and inhibit mortalin, a mitochondrial protein and host mediator of apoptosis (107). Another Mtb-secreted complex, PE25/PPE41, can induce necrosis but not apoptosis in RAW 264.7 macrophages (108) (Fig. 2). While these cell death studies are

FIG 3 Mutations in functionally diverse mitochondrial proteins confer susceptibility to mycobacterial disease. Shown is a representation of proteins associated with leprosy/tuberculosis susceptibility via either GWAS or direct experimental investigation. From left to right, proteins are Presenilin-associated rhomboid-like protein (PARL), Parkinson's disease 2 (PARK2), PTEN-induced kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2), mitofusin 2 (MFN2), optic atrophy 1 (OPA1), fatty acid metabolism-immunity nexus (FAMIN), mitochondrial transcription factor A (TFAM), and mitochondrial DNA polymerase gamma (POLG). The figure was created with BioRender.

somewhat limited by the use of the RAW 264.7 macrophage cell line, which does not encode an active inflammasome, these findings demonstrate that *Mtb* has evolved multiple ways to modulate cell death via interacting with mitochondrial proteins, consistent with the pathogen's need to control how and when an infected macrophage dies.

MITOCHONDRIAL MUTATIONS CONFER SUSCEPTIBILITY TO MYCOBACTERIAL INFECTION

Additional insights into mitochondria/*Mtb* cross talk come from genome-wide association studies (GWAS) that repeatedly correlate human single-nucleotide polymorphisms (SNPs) in mitochondrial genes with susceptibility to mycobacterial infection. Because GWAS are generally more successful in genetically similar human populations, we know the most about mycobacterial susceptibility loci from experiments conducted with patients infected with *Mycobacterium leprae* (Mlep) the causative agent of leprosy, which persists in certain isolated, rural communities. Mlep and *Mtb* are highly related pathogens (both encode an analogous virulence-associated ESX-1 secretion system), and the severity of both leprosy and tuberculosis is highly correlated with type I interferon responses *in vivo* (109, 110). Numerous susceptibility loci have been associated with exacerbated leprosy in human patients, most of them in *bone fide* immune sensors and signaling molecules (e.g., NOD2, IL-6, RIPK2, TNFSF15, SOCS1, IL-12B, IL-18RAP/IL-18R1, and IL-23R) (111–119). However, a curious number of leprosy-associated mutations appear in mitochondrion-related genes, again pointing to a role for this organelle in regulating immune outcomes during mycobacterial infection.

One of the most studied genes associated with leprosy susceptibility and other inflammatory disorders, like Crohn's disease, is *LRRK2* (17, 19, 111, 115, 120–123), which encodes a massive, multifunctional protein with both GTPase and kinase activity (124) (Fig. 3). Mutations in *LRRK2* are the largest genetic contributor to familial PD (12, 125–127), and the best-characterized *LRRK2* mutation is *LRRK2*^{G2019S}, a gain-of-function allele with overactive kinase activity. While the *LRRK2*^{G2019S} SNP has not been specifically identified in GWAS to confer leprosy or tuberculosis susceptibility, an SNP in a nearby amino acid also in the kinase domain (M2397T) has been linked to both leprosy

and Crohn's disease (121). Because of LRRK2's strong links to PD, the vast majority of mechanistic studies have investigated its function, and the function of the *LRRK2*^{G2019S} allele, in neurons and other cells of the central nervous system (CNS). Through that research, we have learned that LRRK2 contributes to mitochondrial homeostasis, lysosomal acidification, and autophagy/mitophagy (128–131). However, in light of its numerous genetic links to mycobacterial infection and chronic inflammatory disorders, it has become clear that LRRK2 function is not relevant solely in the brain.

How might LRRK2 and mutations in LRRK2 alter peripheral inflammatory responses and confer susceptibility to infection? First, there are several established links between LRRK2 and the autophagy pathway, a conserved process that clears both damaged mitochondria and cytosolically exposed pathogens via a lysosome-dependent process. As autophagy is a dynamic process involving transport of vesicles, a number of Rab proteins (small GTPases that play key roles in endosomal vesicle transport) are directly involved in various stages of autophagy. A recent proteomics study identified RAB10 and several related Rab family members as substrates of LRRK2 (132), and a follow-up study found that RAB10 is recruited to depolarized mitochondria in a process that is dependent on the mitophagy factors PINK1 and Parkin (129) (which are also associated with susceptibility to bacterial infection; see below). These data directly link LRRK2 kinase activity to mitophagy and suggest that mutations in LRRK2 may impact the ability of macrophages to target Mtb and/or mitochondria for turnover in autophagosomes, resulting in the accumulation of cytosolically exposed immune ligands and dysregulated innate immune responses.

Second, LRRK2 has been implicated in regulation of endosomal dynamics and phagosomal maturation. Hartlova et al. showed that LRRK2 is required for recruitment of class III phosphatidylinositol 3-kinase (PI3K) and the Beclin-1 binding protein Rubicon to phagosomes and that loss of LRRK2 promotes acidification of Mtb-containing phagosomes and limits Mtb replication in macrophages. Notably, these changes are seemingly independent of selective autophagy, as LC3 recruitment to Mtb phagosomes is unaltered by loss of LRRK2 or by the presence of the *Lrrk2*^{G2019S} mutation. *In vivo*, they report that loss of LRRK2 restricts Mtb replication in the lungs at early time points and correlates with increased inflammatory cytokine expression (133). Subsequent work, also from the Gutierrez laboratory, showed that following pathogen-induced or sterile endomembrane damage, LRRK2 recruits Rab8A and components of the ESCRT (endosomal sorting complexes required for transport) repair complex to damaged endolysosomes, which accumulate in the absence of LRRK2 and Rab8A (134). Because the intracellular niches of both Mtb and Mlep need to be precisely regulated by both the host and pathogen, it makes sense that even slight defects in phagosomal maturation could impact the ability of these bacterial pathogens to survive and replicate. It will be interesting for future studies to determine how LRRK2 phenotypes in macrophages *ex vivo* translate to an *in vivo* model, i.e., does LRRK2's ability to control maturation of the Mtb phagosome inside macrophages explain the importance of the protein in regulating mycobacterial disease severity? One could begin these admittedly challenging experiments by determining whether macrophages are the major cells driving *Lrrk2*^{-/-} and *Lrrk2* mutant phenotypes *in vivo*.

Third, the loss of LRRK2 can induce mitochondrial damage that leads to mito-DAMP release. Our laboratory recently found that loss of LRRK2 in human and mouse macrophages causes mitochondrial instability such that mtDNA accumulates in the cytosol, resulting in chronic cGAS stimulation and elevated basal type I interferon expression in resting macrophages (135). Consequently, these reprogrammed *Lrrk2*^{-/-} macrophages produce a blunted type I IFN response following Mtb infection. We found that mitochondrial dysfunction and mito-DAMP release are driven by a combination of two major stresses endured by *Lrrk2*^{-/-} cells: (i) hyperphosphorylation of the mitochondrial fission protein DRP1 and increased mitochondrial fragmentation and (ii) oxidative stress resulting from low levels of purine metabolites. These findings are reminiscent of those from an earlier study that connected LRRK2 mutations to altered

mitochondrial respiratory capacity and mtDNA damage in the rat primary midbrain (136), suggesting that chronic type I IFN expression is an underappreciated contributor to LRRK2-associated PD phenotypes in the CNS.

Moving forward, it will be critically important to translate these findings in *Lrrk2*^{-/-} macrophages to cells harboring actual human SNPs in LRRK2. Intriguingly, in parts of the world, 3 to 5% of the population are carriers of the *LRRK2*^{G2019S} mutation, suggesting that there is a selective advantage to harboring this allele (137, 138). One could not predict such selective pressure to be driven by associated with PD, since the disease afflicts individuals who are well beyond reproductive age, and the mutation is certainly not advantageous. To address this evolutionary curiosity, recent studies have used several infection models to probe the function of LRRK2 in immunity and pathogenesis. Shutinoski et al. found that in a sepsis model, where adult mice were intravenously injected with the gut pathogen *Salmonella enterica* serovar Typhimurium, that mice carrying the *Lrrk2*^{G2019S} allele better control infection, with reduced bacterial growth and increased animal survival times postinoculation (139). They also induced encephalitis by infecting newborn pups intranasally with reovirus and found that although mice harboring *LRRK2*^{G2019S} have lower viral titers, pups harboring the mutation experience significantly increased mortality (139). Together, this important study suggests that *Lrrk2*^{G2019S} mice are more prone to inflammation, which could protect against or promote disease, depending on the route of infection or type of pathogen.

Common variants of mitophagy-related factors, like PARK2 (Parkinson's disease 2), PARL (Presenilins-associated rhomboid-like protein), and PINK1 (PTEN-induced kinase 1) (Fig. 3), also increase leprosy risk. These three proteins in particular are critical for mitochondrial turnover via mitophagy. Briefly discussed earlier, mitophagy is a conserved mechanism that tags damaged mitochondria with the ubiquitin "eat me" signal for subsequent destruction in autophagolysosomes. Mitophagy is triggered when mitochondrial depolarization stabilizes the kinase PINK1 on the mitochondrial outer membrane, where it can recruit the E3-ubiquitin ligase Parkin (PARK2). Parkin-mediated ubiquitination of mitochondrial substrates, including BCL2 (B cell lymphoma 2), mitofusins, and VDAC (voltage-dependent anion channel), provides the "eat me" signal that recruits autophagy adaptors that link the tagged/damaged mitochondria to autophagosomes (140–142). Mitophagy is highly analogous to xenophagy, whereby cytosolically exposed pathogens are recognized, tagged with ubiquitin, and targeted to autophagolysosomes (143, 144). In fact, one study found that in the absence of PARK2, significantly fewer *Mtb* organisms are ubiquitinated and targeted to LC3⁺ autophagosomes (145). Remarkably, *Park2*^{-/-} mice are incredibly susceptible to *Mtb* infection (145). Because only about 30% of *Mtb* cells in any infected macrophage are ever tagged with ubiquitin and targeted to selective autophagy, this strong *in vivo* phenotype is actually at odds with the idea that Parkin simply restricts *Mtb* replication inside macrophages. While we can speculate that *Park2*^{-/-} mice succumb to *Mtb* infection because they fail to degrade damaged mitochondria, leading to mito-DAMP accumulation and hyperinduction of type I IFN and/or inflammasome activation, such a mechanism has yet to be directly tested.

SNPs in several proteins required to maintain proper mitochondrial network structure also show up in leprosy GWAS. OPA1 (optic atrophy 1) is a mitochondrial inner membrane dynamin-like GTPase required for maintaining normal mitochondrial morphology (Fig. 3). Researchers identified two *OPA1* variants that showed positive association with increased risk for lepromatous (i.e., more severe) leprosy. One allele (rs414237) correlated with lower *OPA1* mRNA expression (146), suggesting that the susceptibility phenotype is attributable to the loss of OPA1. The mitochondrial fusion regulator mitofusin 2 (MFN2) has been implicated in controlling *Mtb*'s ability to survive and replicate inside macrophages *ex vivo* and in a mouse model of infection, consistent with GWAS that identified human SNPs in *MFN2* associated with susceptibility to tuberculosis in a population of Han Chinese (147) (Fig. 3). While siRNA knockdown of *Mfn2* in primary BMDMs restricts *Mtb* replication (148), *Mfn2*^{n/n}-Cre-LysM mice are

susceptible to infection with both Mtb and *Listeria monocytogenes*, and expression of ESAT-6 has been shown to promote interaction between MFN2 and the NLRP3 inflammasome, leading to IL-1 β secretion (149). While discrepancies between *ex vivo* and *in vivo* *Mfn2*^{-/-} Mtb infection phenotypes need to be resolved and the precise nature of the mitochondrial damage driving these phenotypes remains unclear, these data highlight the diverse contributions that a mitochondrial outer membrane protein and fusion regulator can have on Mtb replication and downstream inflammatory responses.

Mutations in factors required to replicate and maintain the mitochondrial genome are also associated with mycobacterial susceptibility. Mutations in TFAM, the mitochondrial transcription factor A, and POLG, the mitochondrial DNA polymerase, are associated with susceptibility to leprosy (150) (Fig. 3). In another study, knockdown of TFAM was shown to reduce *Mycobacterium bovis*-induced IFN- β production in BMDMs and J774A.1 macrophages (151), although these results are somewhat at odds with previously published findings whereby loss of TFAM increases cytosolic mtDNA and IFN- β expression in MEFs and BMDMs (11). It will be interesting to see whether human SNPs in TFAM result in destabilization of the mitochondrial genome and/or altered type I IFN expression in leprosy patients.

Lastly, very recent work discovered a role for FAMIN (encoded by *C13orf31* or *LACC1*) in regulating immunometabolism through controlling the bioenergetic state of macrophages. Loss of FAMIN decreases mitochondrial ROS production and impairs the ability of macrophages to control both BCG and *Salmonella* intracellular replication. Intriguingly, macrophages express the highest levels of *C13orf31* mRNA of any cell type (152), hinting at a connection between *C13orf31* SNPs and susceptibility to macrophage-dwelling bacteria like Mtb. Indeed, a missense mutation in *C13orf31* contributes to leprosy risk (153).

Together, these GWAS and related studies strongly argue that humans and/or mouse models harboring mitochondrial mutations have altered ability to control Mtb infection. But altered how? Do certain mutations protect against Mtb while others exacerbate disease? Do some mutations increase local inflammation such that Mtb replication is controlled (as is the case in *Lrrk2*^{-/-} mice), or does dysregulated release of mito-DAMPs create hyperinflammation that exacerbates disease (as we predict is the case of *Park2*^{-/-} mice)? A concerted comprehensive approach is needed to experimentally define how mitochondrial mutations affect release and accumulation of mito-DAMPs *in vivo* and the subsequent effects of these DAMPs on activating type I IFN expression, selective autophagy, and the inflammasome/pyroptosis. Only then might one be able to link certain mitochondrial phenotypes with tuberculosis patient outcomes, as in the example, high circulating mtDNA $\rightarrow$ increased type I IFN expression $\rightarrow$ acute tuberculosis disease.

TARGETING MITOCHONDRIAL FUNCTION THERAPEUTICALLY TO IMPROVE TUBERCULOSIS PATIENT OUTCOMES

Furthering our understanding of how mitochondrial dysfunction alters immune responses during infection introduces a variety of new opportunities for therapeutic intervention designed to target mitochondrial function (154). Abundant evidence suggests that modulation of mitochondria could alter Mtb infection outcomes and/or the efficacy of current treatment plans. One recent study that examined human blood RNA signatures over the course of tuberculosis treatment found that the strongest predictor of treatment failure is downregulation of mitochondrial gene expression in pretreatment patients (155). Gene expression studies in Mlep-infected macrophage cell lines and from patient nerve biopsies both revealed a global downregulation of mitochondrial gene expression associated with leprosy (156). These studies suggest that patients with compromised mitochondrial function experience more severe mycobacterial disease, but again, the molecular changes driving these outcomes are completely unknown.

Several studies have already tested mitochondria-modulating drugs during Mtb infection of cells *ex vivo*. Experiments designed to understand the role of cholesterol in modulating macrophage function found that treatment of THP-1 cells with a small molecule called M1 rescued mitochondrial membrane potential and restored the ability of cholesterol-treated cells to control Mtb replication (157). Likewise, treatment of Mtb-infected THP-1 cells with an inhibitor of the mitochondrial pyruvate carrier (resulting in a decrease of acetyl-coenzyme A) dramatically limited Mtb replication over a 48-h time course (158). While we are far from mechanistically understanding how such treatments alter the ability of Mtb to survive and replicate in macrophages, these types of experiments should help motivate additional studies on how we might target mitochondria to skew innate and adaptive immune responses to Mtb. Lastly, it is worth considering the consequences of tuberculosis antibiotic regimens on mitochondrial health and homeostasis. Most current tuberculosis treatment plans span between 6 and 9 months, providing ample opportunity for unintended side effects related to mitochondrial function. Rifampin, for example, has long been known to inhibit mitochondrial RNA polymerase and promote cytochrome *c* release (159). Pyrazinamide and isoniazid, two other first-line drugs, increase ROS production in *ex vivo* experiments (159). If tuberculosis treatment alters mitochondrial function in a way that promotes Mtb pathogenesis, we may be inadvertently interfering with the ability of patients' own immune cells to control infection and/or inflammation. As pathogens like Mtb continue to acquire resistance to many first-line antibiotics, there is a growing need to understand the intricacies of the host response to infection so that we can develop host-directed interventions, including strategies that modulate mitochondrial homeostasis, to improve patient outcomes.

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