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Cell-Autonomous Effector Mechanisms against *Mycobacterium tuberculosis*

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Abstract

Few pathogens run the gauntlet of sterilizing immunity like *Mycobacterium tuberculosis* (*Mtb*). This organism infects mononuclear phagocytes and is also ingested by neutrophils, both of which possess an arsenal of cell-intrinsic effector mechanisms capable of eliminating it. Here *Mtb* encounters acid, oxidants, nitrosylating agents, and redox congeners, often exuberantly delivered under low oxygen tension. Further pressure is applied by withholding divalent Fe^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}, as well as by metabolic privation in the form of carbon needed for anaplerosis and aromatic amino acids for growth. Finally, host E3 ligases ubiquinate, cationic peptides disrupt, and lysosomal enzymes digest *Mtb* as part of the autophagic response to this particular pathogen. It is a testament to the evolutionary fitness of *Mtb* that sterilization is rarely complete, although sufficient to ensure most people infected with this airborne bacterium remain disease-free.

Cells constitute the basic unit in biology. They safeguard and transmit genetic information, exchange gases, assimilate nutrients, and compartmentalize their enzymatic machinery to concentrate activities that share a common function. Beyond these servoregulatory duties, cells also deploy host defense factors to monitor their structural integrity and protect their inner sanctity from infection by the outside world. This universal system of self-defense is known as cell-autonomous immunity (Beutler et al. 2006; Kim et al. 2012a; MacMicking 2012; Randow et al. 2013).

Cell-autonomous immunity operates across all three domains of life where it defends against facultative and obligate intracellular pathogens (Randow et al. 2013). It is particularly effective against microorganisms engaged in accidental or fleeting interactions with their host; however, durable pathogens necessitate multiple intracellular defense programs to overcome microbial counterstrategies (Staskawicz et al. 2001). One such example is *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB). It survives much of the microbicidal onslaught triggered inside human phagocytes via numerous adaptive and exaptative mechanisms (MacMicking 2008). These mechanisms reflect a close

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and long-standing association with its host. Indeed, paleogenetic evidence suggests *Mtb* has evolved alongside as well as within humans for at least 70,000 years (Comas et al. 2013).

Such tactics currently enable *Mtb* to persist within an estimated third of the world's populace. Here it causes disease in ~10% of people infected. For the remaining 90% of cases, however, host immunity likely contains and, in some cases, naturally eradicates *Mtb* (McKinney et al. 2001; Cobat et al. 2009). Autopsy studies conducted in the preantibiotic era, for example, found pulmonary lesions were often sterilized in latently infected individuals dying of nontuberculous causes (see McKinney et al. 2001 and references therein). Similar outcomes were recently reported for cynomolgus macaques (Lin et al. 2014), which together with Metchnikoff's original observations in rat macrophages and Lurie's classic studies of inbred rabbits encapsulate the idea that mycobactericidal activities may exist across several mammalian species (Metchnikoff 1905; Lurie 1964). How microbial killing is enacted and what molecular mechanisms are used for *Mtb* clearance or stasis remain central questions in mycobacterial pathogenesis. Potential antitubercular mechanisms are discussed below.

Cell-Autonomous Defense Against *Mtb*

Constitutive versus Inducible Programs

A basal repertoire of host defense factors provide some measure of protection against *Mtb*; however, resistance is often more effective in cells preactivated by cytokine or Toll-like receptor (TLR) signaling (Fig. 1). Here multiple defense genes are induced within 0.5–6 h of stimulation (MacMicking 2012). Notably, the order of activation and infection appears critical, because resting or naïve macrophages infected before immune stimulation allows *Mtb* time to interfere with host signaling events such as STAT1 binding its transcriptional coactivators, CBP and p300 (Ting et al. 1999), or blocking PI3K-dependent trafficking (Vergne et al. 2003). Both are needed to inhibit mycobacterial replication.

Likewise, the magnitude and success of cell-autonomous immunity depends on which immunoreceptor is engaged. Stimulating C-type lectin receptors dectin-1 or Mincle with *Mtb* mannosylated lipoarabinomannan or trehelose dimycolate, respectively, induces a limited defense repertoire that fails to control the pathogen (Ishikawa et al. 2009; Betz et al. 2011; Marakalala et al. 2011; Das et al. 2013; Heitmann et al. 2013). In contrast, activation with interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α), elicits hundreds of genes that can curtail mycobacterial growth (Ehrt et al. 2001; Nau et al. 2002; Tailleux et al. 2008; Vogt and Nathan 2011).

Mtb-dependent signaling further enriches the host defense repertoire, either through direct transcription of effector genes by pattern-recognition receptor (PRR) ligation or after autocrine TNF-α and interleukin-1 beta (IL-1β) signaling (Ehrt et al. 2001; Nau et al. 2002; Vogt and Nathan 2011). Human monocytes and mouse macrophages regulate approximately 300–700 genes within 12–24h of *Mtb* exposure (Ehrt et al. 2001; Ragno et al. 2001; Nau et al. 2002). Similar transcriptional plasticity occurs in human alveolar macrophages (AMs) and dendritic cells (Tailleux et al. 2008). This expression profile may reach as many as 1300 genes when IFN-γ is also present (Ehrt et al. 2001). Thus *Mtb* plus classical Th1 cytokines

like IFN-γ are potent synergistic stimuli for remodeling the transcriptome of phagocytic cells. In combination they enlarge the suite of host proteins available for cell-autonomous defense, a recurring theme for many inducible programs directed against major human pathogens (MacMicking 2012). Indeed, resistance is most effective when multiple activating stimuli are present, consistent with the idea that a single stimulus may be necessary although not always sufficient to eradicate *Mtb* (Shi et al. 2003; Fabri et al. 2011; Vogt and Nathan 2011).

IFN-γR-Induced Programs

Discovery of IFN-γ as the principal macrophage-activating factor (Nathan et al. 1983, 1984; Pace et al. 1983) soon led to reports of its effectiveness against *Mtb* in monotypic cell culture (Rook et al. 1986a; Flesch and Kaufmann 1987). These in vitro activities likely operate in vivo given loss-of-function mutations in the IFN-γ signaling cascade confer profound susceptibility to *Mtb* and, more commonly, disseminated nontuberculous mycobacteriosis in both humans and mice (Cooper et al. 1993; Flynn et al. 1993; Casanova and Abel 2002). A congenital syndrome termed Mendelian susceptibility to mycobacterial disease (MSMD) reveals impaired antimycobacterial immunity in patients with hypomorphic or nullizygous IFNGR, STAT1, and IRF8 alleles, as do mutations in loci that encode IFN-induced effectors such as ISG15 or CYBB (Zhang et al. 2008; Bustamante et al. 2011; Hambleton et al. 2011; Bogunovic et al. 2012).

Genetic lesions in the IFN-γ signaling cascade provide strong support for its role in antitubercular defense. Pharmacologic complementation further reinforces it. Here aerosolized recombinant IFN-γ can alleviate bacillary burdens in patients with antibiotic-resistant *Mtb* strains and mobilize bacteriostatic programs in human alveolar macrophages ex vivo (Condos et al. 1997; Bonecini-Almeida et al. 1998). Concordant results are seen in long-term differentiated human PBMCs activated with IFN-γ under low physiologic oxygen (5%– 10%) that may begin to approach hypoxic conditions found in some pulmonary and extrapulmonarytissue granulomas (Vogt and Nathan 2011). Importantly, the tuberculostatic activities of IFN-γ can be augmented by other stimuli. These include TNF superfamily members (TNF-α, CD40L), IL-1R/TLR, and NLR (NOD-like receptor) agonists or bioactive metabolites such as vitamin D_3 (Fabri et al. 2011; Vogt and Nathan 2011; Pilli et al. 2012; Jayaraman et al. 2013; Klug-Micu et al. 2013). Their synergistic effects are discussed below.

TNFR-Induced Programs

Besides their crucial role in granuloma formation (Flynn et al. 1995; Bean et al. 1999), members of the TNF superfamily directly stimulate cell-autonomous immunity to help contain *Mtb* (Chan et al. 1992; Klug-Micu et al. 2013). TNF-α enhances IFN-γ-induced macrophage programming for *Mtb* clearance, a synergy which provides several advantages: (1) diversifying the host gene repertoire through alternate transcription factor usage; (2) heightened gene expression from promoters harboring transcription factor-binding sites for both pathways; and (3) lowering the immune activation threshold, a change brought about by shared preexisting components (Fig. 1). This latter point is especially germane given the relative paucity of IFNGRs (4000–12,000/cell[21]) on the human macrophage surface (Pace et al. 1983; Finbloom et al. 1985). TNFR1 is likewise expressed at low levels for capturing

soluble TNF-α to restrict *Mtb* growth rather than the nonprotective TNFR2 isoform that instead engages membrane-bound ligand (Jayaraman et al. 2013).

The other two benefits of this synergy emerge when regulation of the antitubercular enzyme, inducible nitric oxide synthase (NOS2), is considered (Chan et al. 1992; MacMicking et al. 1997a; Bekker et al. 2001; Nathan 2006). *NOS2* transcription requires IFN-γ-induced STAT1 binding to g-activated sites (GASs) that is increased by NF-κB docking to its own sites within the NOS2 promoter after TNF-α or lipoarabinomannan stimulation (MacMicking et al. 1997a; Chan et al. 2001. In addition, TNF-α elicits GTP cyclohydrolase 1 that furnishes tetrahydrobiopterin (BH_4) as an essential cofactor for NOS2 catalysis and induces argininosuccinate synthetase 1 and cationic amino acid transporter 2 (CAT-2) to regenerate and import the NOS2 substrate, L-arginine, respectively (Bogdan 2001; Qualls et al. 2012). Thus cooperation between TNF-α and IFN-γ regulates transcriptional and posttranslational events for antitubercular defense at the level of the individual cell.

Macrophage reprogramming by another TNF superfamily member, CD40L, also impacts *Mtb* survival. Human monocytes stimulated with CD40L, alone or in combination with IFN-γ, induce CYP27b1-hydroxylase, which converts the vitamin D_3 precursor, 25-hydroxy-vitamin D_3 ($25D_3$), into bioactive 1,25-dihy-droxyvitamin D ($1,25D_3$) (Klug-Micu et al. 2013). Subsequent 1,25D binding to the vitamin D receptor (VDR) elicits antimicrobial peptide expression and autophagy, both potent effector mechanisms targeting mycobacterial replication (Thoma-Uszynski et al. 2001; Gutierrez et al. 2004; Liu et al. 2006, 2007; Singh et al. 2006; Yuk et al. 2009; Ponpuak et al. 2010; Fabri et al. 2011; Pilli et al. 2012).

IL-1R, TLR-, and NLR-Induced Programs

Members of the IL-1/TLR family stimulate distinct intracellular programs following cognate ligand interaction to combat TB. For IL-1 receptor (IL-1R) signaling, both IL-1β; and IL-1α limit mycobacterial growth in murine macrophages via a MyD88-dependent pathway (Jayaraman et al. 2013). A similar IL-1β-dependent profile emerges within human monocyte-derived macrophages. Here IL-1β up-regulates TNFR1 and promotes autocrine signaling via TNF-α release, along with caspase 3–dependent apoptosis (Jayaraman et al. 2013). IL-1β can also elicit autophagic killing via a Tank-binding kinase-1 (TBK-1)-dependent pathway (Pilli et al. 2012), suggesting multiple effectors are deployed downstream of IL-1R to restrict *Mtb* growth. Such effects presumably operate within intact hosts because IL-1β- and IL-1R1-deficient mice display marked vulnerability to aerogenic *Mtb* challenge (Mayer-Barber et al. 2010).

Like the IL-1R cascade, TLRs 1, 2, and 4 mobilize multiple effectors as part of the anti-tubercular arsenal. Engagement of TLR1/TLR2 heterodimers with the 19-kDa *Mtb*-derived lipoprotein promotes a novel NOS2- and TNF-independent pathway in human macrophages, whereas the same lipopeptide induces a NOS2-dependent mechanism in mouse cells (Thoma-Uszynski et al. 2001). This novel human pathway involves CYP27b1-hydroxylase to produce $1,25D_3$ for binding the VDR, which in turn elicits cathelicidin (hCAP-18/LL-37) and defensin-4 (DEF4) antimicrobial peptides for tuberculostasis (Liu et al. 2006, 2007). Because the latter cascade also induces autophagy, it may overlap with signals provided by

IFN-γ, CD40, and IL-1β at a common juncture downstream (Fabri et al. 2011; Klug-Micu et al. 2013).

Autophagic clearance of *Mtb* invoked by TLR4 stimulation in human and mouse macrophages requires TRIF (Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon-β) adaptor signaling, unlike IL-1β that relies on MyD88 (Xu et al. 2007). Use of a different adaptor helps widen the TLR transcriptional response to encompass IRF3-dependent genes. In humans, genetic mapping has unearthed numerous *TLR4* SNPs that segregate with TB in ethnically diverse subjects, suggesting this TLR4-TRIF pathway operates across a broad swathe of the global population (Velez et al. 2009; Shah et al. 2012). Genetic TB associations have also been noted for TLRs 1 and 2 along with their signaling adaptors (Hawn et al. 2006; Thuong et al. 2007).

Recent studies have shown members of the NLR family may mobilize intracellular immunity to control *Mtb* growth. NOD2 detects muramyl dipeptide (MDP) that is *N*-glycolylated in mycobacteria to elicit TNF-α secretion as part of the defense profile in mouse macrophages (Coulombe et al. 2009). Human AMs stimulated with MDP also elicit IL-1β and TNF-α plus the antimicrobial peptide cathelicidin and autophagy-related defense protein, immunity-related GTPase M (IRGM) (Juárez et al. 2012). Here MDP promotes IRGM, ATG16L, and LC3B recruitment to *Mtb* phagosomes, suggesting NOD2 signaling governs both transcriptional and autophagic defense to this pathogen. Gain-of-function *NLRP3* and *CARD8* mutations cooperatively limit *Mtb* in human macrophages (Eklund et al. 2014). Hence several NLRs and related adaptors contribute to bacterial restriction within infected cells.

VDR- and P_2XR-Induced Programs

Besides classical cytokine and PRR signaling, two other inducible programs deserve mention within the context of cell-autonomous immunity to *Mtb*: VDR and purinergic receptor signaling. Early studies reported that $1,25D_3$ was crucial for IFN-γ-induced tuberculostasis in human macrophages; this effect was attributed to $1,25D_3$ promoting macrophage differentiation (Rook et al. 1986b; Crowle et al. 1987). Subsequent work, however, has delineated a circuitry whereby CYP27b1-hydroxylase expression and $1,25D_3$ production are induced by IL-15 following IFN-γ or TLR1/2 stimulation (Fabri et al. 2011). VDR signaling in turn elicits cathelicidin to activate Beclin-1 and Atg5 for autophagosomal capture of mycobacteria (Yuk et al. 2009). Such activities may impact TB outcomes in people because lower levels of serum 1,25D correlate with susceptibility, with VDR disease-related polymorphisms in linkage for some African and Asian populations (Bellamy et al. 1999; Wilkinson et al. 2000).

ATP-dependent stimulation of P2 purinergic receptor subtypes has likewise been strongly implicated in macrophage-mediated *Mtb* killing. In human macrophages, P2X7 activates cytosolic Ca^{2+} release and phospholipase D for maturation of *Mtb* phagosomes (Kusner and Adams 2000; Fairbairn et al. 2001; Kusner and Barton 2001). Subtypes other than P2X7, however, appear to stimulate NOS2-mediated killing in murine cells (Sikora et al. 1999). Because a number of *P2X7R* polymorphisms associate with TB susceptibility in the human

population, this pathway likely influences disease development in its natural setting (Fernando et al. 2007; Sharma et al. 2010; Areeshi et al. 2013).

In sum, multiple receptors induce intracellular programs to protect the host cell from *Mtb* infection. These signals originate from IFN-γR, TNFR, IL-1R, TLR, NLR, VDR, and P2XR complexes after ligand binding primarily at the plasma membrane or in the cytosol. How their downstream effectors subsequently operate and the mechanisms they deploy are discussed below.

Cell-Autonomous Arsenal for Defense Against *Mtb*

Cytotoxic Gases: Reactive Oxygen Species and Nitrogen-Centered Free-Radical Species

Production of cytotoxic gases is a major component of antimicrobial arsenals in all aerobic organisms (Nathan and Shiloh 2000). Reactive oxygen species (ROS) and nitrogen-centered free-radical species (RNS) show distinct chemical properties—oxidizing power, penetrative diffusion limits, lipophilicity—well-suited for killing a variety of facultative bacteria including *Mtb* (Klebanoff 1999; Nathan and Shiloh 2000). ROS and RNS typically possess low Stokes radii, are highly lipophilic, and electroneutral; thus they are small enough to bypass structural barriers that impede the access of larger host macromolecules and sufficiently permeable to diffuse across the bacterial membrane (Klebanoff 1999). Other chemical properties, notably extended diffusion limits for some RNS, allow them to protect neighboring cells (MacMicking 2012). Carbon monoxide (CO) has also been recently touted as an emerging bactericidal agent, although whether it is generated in sufficient amounts to act as a genuine mycobacterial poison in addition to its role as a sensory cue (Shiloh et al. 2008) has yet to be determined.

Oxidant Stress

Aerobic organisms use allotropic dioxygen, as a substrate for generating superoxide (O_2^-) via oxidoreductases and as a by-product of electron (e^2) transport in mitochondria to combat infection (Klebanoff 1999; MacMicking 2012). Single e^- additions yield, respectively, hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·), both powerful oxidants capable of damaging mycobacterial DNA, lipids, and hemoproteins (Chan et al. 1992; Nathan and Shiloh 2000; Vilchèze et al. 2013). Moreover, within phagolysosomes, elevated levels of O_2^- (Podinovskaia et al. 2013) undergo spontaneous dismutation at low pH to generate H_2O_2 that enhances lysosomal killing. Hypochlorous acid ($HOCl^2$) produced by myeloperoxidase-catalyzed oxidation of chloride by H_2O_2 in neutrophils could also target *Mtb* internalized from infected macrophages (Yang et al. 2012). Here *Mtb* expresses methionine sulfate reductases that may partly mitigate this damage (Lee et al. 2009). Indeed, *Mtb* detoxifying enzymes (e.g., catalases, SODs, peroxiredoxins) limit the overall effectiveness of ROS as a sole agent that could regain toxicity when other bactericidal agents help disable these antioxidant systems.

Large quantities of ROS are produced within phagocytic cells via the NADPH oxidase 2 (NOX2) isoform encoding the flavocytochrome$_{b558}$ heavy chain subunit (gp91^{phox}; CYBB gene) of the phagocyte oxidase, which generates the respiratory burst (Nathan and Shiloh

2000; MacMicking 2012) (Fig. 2). Congenital X-linked CYBB mutations lead to recurrent TB and "BCG-osis" in patients with defects preferentially manifest in macrophages rather than monocytes or granulocytes (Bustamante et al. 2011). Additional mutations in other NADPH oxidase subunits yield a collective syndrome—chronic granulomatous disease (CGD)—that also appears to predispose to TB (Lau et al. 1998). *Mtb* susceptibility is less pronounced in mice with targeted disruptions in either $gp91^{phox}$ or $gp47^{phox}$ subunits (Adams et al. 1997; Cooper et al. 2000); however, relative vulnerability increases when *Mtb* mutants lacking *KatG* are used, reinforcing the importance of pathogen detoxification systems (Ng et al. 2004).

NADPH oxidase is composed of two membrane-associated ($gp91^{phox}$, $p22^{phox}$) and three cytosolic subunits ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$). IFN-γ increases $gp91^{phox}$ and $p22^{phox}$ mRNA transcription and promotes NADPH oxidase assembly on mycobacterial phagosomes (Kim et al. 2011). An IFN-γ-induced GTPase, guanylate binding protein 7 (Gbp7), facilitates this assembly by recruiting cytosolic $p67^{phox}$-$p47^{phox}$ heterodimers to $gp91^{phox}$-$p22^{phox}$ membrane complexes surrounding the pathogen compartment (Kim et al. 2011 Kim et al. 2012) (Fig. 2). Whether natural mutations in this or other members of the human GBP family induced during TB (Berry et al. 2010; Maertzdorf et al. 2011) are linked to disease is a topic for future investigation.

TNF-induced ROS has also recently emerged in zebrafish models of TB (Roca and Ramakrishnan 2013). RIP1-RIP3 signaling elicits microbicidal activity that if left unabated can induce macrophage necroptosis for subsequent dissemination of bacilli. Notably, the primary source of ROS in this setting was mitochondria (Roca and Ramakrishnan 2013). It will therefore be interesting to see if mitochondrial ROS plays a defining role against *Mtb* like its reported anti-*Salmonella* activities (West et al. 2011).

Nitrosative Stress

RNS are a second class of oxidants capable of potent tuberculocidal activity. As little as 90 ppmofnitric oxide (·NO) gas—approaching the exhaled concentrations found for some TB patients (Wang et al. 1998)—kills .99% of plat-edorganisms (Long et al. 1999). ·NO shows molar potencies approaching many of the current antibiotics used to treat TB, and the tuberculocidal activity of some new drugs like bicyclic nitroimidazoles are ascribed to release of ·NO (Singh et al. 2008). Because it is a water- and lipid-soluble diatomic gas, ·NO traverses biologic membranes to react with O_2^- or other ROS to yield stable nontoxic anions (NO_2^-, NO_3^-) as well as intermediates with marked bactericidal properties. The latter include unstable dinitrogen oxides (N_2O_3, N_2O_4), compound peroxides ($ONOO^2$), and nitrosothiol adducts (RSNO) (Yu et al. 1999; Venketaraman et al. 2005) (Fig. 2).

Within acidified phagolysosomes, ·NO equivalents can be retrieved from stably oxidized forms (e.g.,NO_{2-}) by protonation to $HNO_2$and subsequent dismutation (Nathan and Shiloh 2000) (Fig. 2). Indeed, acidified $NaNO_2$ was one of the earliest compounds used to show a tuberculostatic role for ·NO (Chan et al. 1992). Along with other RNS, ROS, and hypoxia, ·NO is sensed by *Mtb* to mobilize the dormancy regulon, underscoring the environmental pressure applied by both nitrosative and oxidative stress within tissue granulomas

(Schnappinger et al. 2003; Voskuil et al. 2003). RNS-mediated growth arrest may also involve nitrogenous products from *Mtb* itself: Reduction of nitrate (NO_3^- by the bacterial narG system yields nitrite NO_2^- that inhibits ATP consumption and induces a transcriptomic profile distinct from the ·NO dormancy regulon in human macrophages (Cunningham-Bussel et al. 2013).

The chief phagocytic source of RNS is the dimeric flavoenzyme, NOS2, which catalyzes the $5e^2$ oxidation of L-arginine to L-citrulline plus ·NO (MacMicking et al. 1997a) (Fig. 2). Robust NOS2 expression is elicited via cytokine (notably IFN-γ, TNFa, IL-1β) and microbial-based PRR signals (e.g., lipopeptides), although its induction in human monocytes may be more complex. Some human cells require at least three combined stimuli—IFN-γ, TNF-α, and IL-1β—to produce a relatively weak response caused by STAT1 and NF-κB acting at some distance (+ 5–8 Kb) from the transcriptional startsite (Ganster et al. 2001). Limited availability of the NOS2 cofactor, BH_4, may also contribute to the generally lower RNS production of human versus rodent phagocytes in cell culture (Bertholet et al. 1999).

Priming signals or cofactors that may be missing in vitro nonetheless appear present in vivo because robust NOS2 expression within *Mtb* granulomas is observed and macrophages isolated from inflamed lungs or peripheral blood of TB patients, or AMs from healthy donors subsequently infected with Mtbex vivo, produce mycobactericidal amounts of ·NO (Nicholson et al. 1996; Fachetti et al. 1999; Dlugovitzky et al. 2000; Means et al. 2001; Wangetal. 2001; Choi et al. 2002; Mattila et al. 2013). Thus NOS2 is likely to be active under native TB settings. Other evidence supporting an in vivo role for RNS includes NOS2 loss-of-function mutations or use of NOS inhibitors that lead to acute susceptibility in experimental mouse and zebra-fish models (Chan et al. 1995; MacMicking et al. 1997b, 2003; Flynn et al. 1998; Mogues et al. 2001; Scanga et al. 2001; Cambier et al. 2014). Finally, the acquisition of *Mtb* resistance proteins, including NoxR1, NoxR3, alkyl hydroperoxide reductase (AhpC), peptidyl methionine sulphoxide reductase (MsrA), dihydrolipoamide dehydrogenase (Lpd), and dihydrolipoamide succinyl-transferase (SucB), also suggest RNS have impacted the evolutionary course of TB (Nathan and Shiloh 2000; St. John et al. 2001; Bryk et al. 2002; Nathan 2012).

Acid Exposure and Lysosomal Killing

Acid as an antimicrobial defense was first proposed by Metchnikoff using litmus dyes on infected guinea pig macrophages (Metchnikoff 1905) and gained credence from two early observations: (1) that activated phagocytes acidify their phagolysosomes (Rous 1925), and (2) that achlorhydric hosts permit colonization of the stomach (Giannella et al. 1973).

For *Mtb*, additional evidence came from its marked sensitivity to pH, 6.5 in axenic media with growth arrest at pH 5.0, especially when examined under hypoxic or microaerophilic conditions (Chapman and Bernard 1962; Tan et al. 2010). Whether protonation itself or other components (e.g., low divalent cation concentrations) contribute to this axenic attenuation is unknown (Jackett et al. 1978; Chan et al. 1992; Piddington et al. 2000; Vandal et al. 2008). *Mtb* has evolved periplasmic mechanisms such as Rv3176c encoding a serine protease (Vandal et al. 2008) and putative transporters that could exclude protons (H^+)

acting directly on the bacterium (Pethe et al. 2004). Even so, because low pH embellishes both ROS and RNS (Fig. 2) coupled with the fact that lysosomal hydrolyses have pH optima in this range, acidification probably facilitates other tuberculocidal effectors to mediate killing inside the (auto)phagolysosomal compartment (Rohde et al. 2007).

IFN-γ- or TLR-stimulated macrophages rapidly acidify mycobacterial phagolysosomes to a pH of ~ 4.5–5.5, where it remains for at least 24 h (Schaible et al. 1998; MacMicking et al. 2003; Vandal et al. 2008). Sustained acidification requires proton-pumping V-ATPases and enables processing of lysosomal cathepsins; both correlate with diminished *Mtb* growth (Schaible et al. 1998; Gomes et al. 1999; Ullrich et al. 1999; MacMicking et al. 2003). Agents that inhibit the V-ATPase (e.g., omeprezole) increase *Mtb* replication in human AMs and murine bone marrow–derived macrophages (Suzuki et al. 2000; MacMicking et al. 2003). Notably, the low luminal pH within phagolysosomes approaches the pK_a of nitrous acid (3.8), which may allow dismutation to ·NO and nitrogen dioxide (NO_2) as a way of reusing protonated nitrite (Vandal et al. 2009) (Fig. 2). This intraluminal RNS also overcomes phagosomal arrest normally imposed by *Mtb* and envelope lipids like trehalose dimycolate (Axelrod et al. 2008). Thus acidification cooperates with RNS to target intravacuolar *Mtb*. Low pH also promotes the activity of lysosomal proteases, lipases, glycosidases, and antimicrobial peptides— estimated at more than 200 in all (Trost et al. 2009)—that may disrupt and digest *Mtb* once it is delivered to this compartment.

Immune and Nonimmune GTPases—Delivery of *Mtb* to (auto)lysosomes relies on vesicular trafficking by immune and nonimmune GTPases. Members of the immunity-related GTPase (IRG) and GBP families orchestrate assembly of fusogenic SNARE proteins and autophagy components on mycobacterial phagosomes in IFN-γ-activated macrophages (MacMicking et al. 2003; Gutierrez et al. 2004; Singh et al. 2006; Tiwari et al. 2009; Kim et al. 2011, 2012a) (Fig. 3). Irgm1 targets the nascent phagocytic cup surrounding mycobacteria through carboxy-terminal amphipathic helical interactions with PI3,4,5P3 and PI3,4P2 generated by host lipid kinases on the plasma membrane (Tiwari et al. 2009). Once recruited, Irgm1 binds the SNARE and dynein motor adaptor, Snapin, for ATG14L-mediated endolysosomal fusion (MacMicking et al. 2003; Tiwari et al. 2009; Kim et al. 2012b). Human IRGM similarly facilitates trafficking of the autophagic machinery to *Mtb* vacuoles for lysosomal fusion and acidification (Singh et al. 2006). Such cell-autonomous activities also manifest in vivo: $Irgm1^{2/2}$ mice are profoundly susceptible to *Mtb* infection (MacMicking et al. 2003; Feng et al. 2004) (Fig. 3), and IRGM polymorphic variants associate with human TB in Ghanese, African American, Korean, and Chinese populations (Intemann et al. 2009; Che et al. 2010; King et al. 2011; Song et al. 2014).

For the GBPs, Gbp7 delivers antitubercular cargo in the form of NADPH oxidase to mycobacterial phagosomes for local ROS production (Kim et al. 2011) (Fig. 3). In addition, Gbp1 interacts with SQSTM1/p62 for transporting substrates that generate tuberculocidal peptides within lysosomes and $Gbp1^{2/2}$ mice are vulnerable to infection (Alonso et al. 2007; Kim et al. 2011). Last, RAB family proteins such as Rab20 recruit the Rab5 exchange factor Rabex-5 for transition to late endosomes in IFN- g-activated macrophages infected with mycobacteria (Pei et al. 2014), and Rab8 directs TBK1-mediated autophagic clearance (Pilli

et al. 2012). Together these different GTPase classes orchestrate lysosomal killing of *Mtb* following immune activation of host cells.

Ubiquitination and Autophagic Clearance

A second route to lysosomal degradation involves the detection of cytosolically exposed *Mtb* and subsequent sequestration by autophagy (Stanley and Cox 2013) (Fig. 4). It has been known for some time that *Mtb* communicates with the cytosol (Myrvik et al. 1984; McDonough et al. 1993). Access to or escape into this compartment departs from the historical view of bacilli being exclusively phagosome-bound inside human macrophages (Armstrong and Hart 1971). In fact both fates probably operate within a given cell where most *Mtb* destined for lysosomal killing remain “trapped” inside vacuoles, whereas a smaller number (12%–25%) may enter the cytosol, most notably after several days of infection (see Stanley and Cox 2013 and references therein).

Rupture or permeabilization of phagosomal membranes appears to rely on the type VII secretion system ESX-1 as first shown by examining *Mtb* RD1 mutants via electron microscopy (van der Wel et al. 2007; Houben et al. 2012). Permeabilization leads to mycobacterial DNA leakage detected by cytosolic surveillance pathways including the DNA and cyclic dinucleotide sensor, STING (Manzinillo et al. 2012; Watson et al. 2012). STING activation initiates a cascade involving TBK1 that engages ubiquitin-binding receptors downstream (Watson et al. 2012) (Fig. 4). Cytosolic mycobacteria are ubiquitinated primarily on K63 chains for detection by p62/SQSTM1 and NDP52, both of which contain LC3-interacting regions (LIRs) for binding to the autophagic membrane proteins, LC3B and LC3C, respectively (von Muhlinen et al. 2012; Watson et al. 2012).

The E3 ligase, PARKIN, mediates ubiquitination and recruitment of downstream Ub receptors (Fig. 4) and PARK2-deficient mice are moderately susceptible to *Mtb* challenge (Manzanillo et al. 2013). This differs from the highly susceptible phenotype of $Atg5^{2/2}$ animals, suggesting additional roles for some autophagy-related proteins (Watson et al. 2012). In this respect, SQSTM1/p62 also participates in delivering unbiquitinated substrates (e.g., ribosomal subunits) to autolysosomes for generating tuberculocidal peptides in IFN-γ-activated macrophages (Alonso et al. 2007; Ponpuak et al. 2010), a process that may require interaction with GBP1 (Kim et al. 2011). A human IRGMd isoform likewise induces mitochondrial Bax/ Bak-dependent cell death during mycobacterial infection as an IFN-γ-induced mechanism distinct from its autophagic activities (Singh et al. 2010). Hence, unconventional roles may be subsumed by both autophagy-related and ubiquitin-binding proteins to confer antitubercular defense.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are evolutionarily ancient constituents of lysosomes, secretory granules, and inflammatory exudates (Ganz 2003; Zanetti 2004; Lai and Gallo 2009). At least 1700 AMPs exist in metazoans and unicellular organisms which show considerable sequence diversity (http://aps.unmc.edu/AP/). Nonetheless, AMPs share certain structural features, namely, an ~ 15–30 amino acid amphipathic core composed largely of cationic and hydrophobic residues. Both features likely contribute to mycobacterial killing.

Electrostatic interactions between positively charged amino acids and negatively charged *Mtb* cell wall phospholipids may provide initial contact, whereas hydrophobic residues integrate into the bacterial membrane for disruption (Ganz 2003). These features—size, amino acid composition, helical or extended loop conformations, and disulfide linkage—help classify the lytic intracellular AMPs into three major categories: a-defensins, b-defensins, and cathelicidins (Ganz 2003; Zanetti 2004; Lai and Gallo 2009).

More than 50 mammalian a-defensins, 90 b-defensins, and 30 cathelicidins are known. To date, evidence exists for b-defensins and cathelicidins in macrophage immunity to *Mtb* although neutrophil granules also kill bacilli via uncharacterized peptides (Tan et al. 2006). IFN-γ elicits b-defensin 4 and the single processed cathelicidin peptide, hCAP18/LL-37, in human macrophages to promote 1,25-dihy-droxyvitamin D_3-dependent autophagic killing of tubercle bacilli (Fabri et al. 2011). In mice, IFN-γ elicits ubiquicidin-like peptides, which, when isolated from macrophage lysosomal fractions or synthetically reconstituted, kill *Mtb* at low micrometer concentrations (Alonso et al. 2007). Like the AMPs, Ub-like peptides (e.g., Ub2) have been shown to insert into the bacterial membrane to disrupt integrity and equilibrate the transmembrane potential (Foss et al. 2012).

Nutritional Immunity: Competition for Cations

Macrophages and other phagocytic cells constitute rich nutritional sources of amino acids, lipids, sugars, and transition metals. Competition for cations is one of the main contests fought between *Mtb* and its host cell with the pathogen coming well-equipped in terms of siderophores (e.g., mycobactins, exochelins) that sequester transition metals like iron (Schaible and Kaufmann 2004). Divalent zinc (Zn^{2+}), manganese (Mn^{2+}), magnesium (Mg^{2+}), and copper (Cu^{2+}) are other cations nutritionally contested within phagocytes.

Iron—Iron is essential for *Mtb* growth, although the labile pool of free ferrous form (Fe^{2+}) is low given that most Fe is complexed with transferrin, ferritin, lactoferrin, and other hemoproteins inside host cells (Schaible and Kaufmann 2004). Macrophage iron uptake proceeds via hemoglobin scavenger receptors (CD163) as well as capture via transferrin receptors (TFs) that internalize two Fe^{3+} moieties as part of the holoTF complex. Release of Fe^{3+} occurs in the endosome with which the *Mtb* phagosome intersects, thus allowing bacterial access to iron (Sturgill-Koszycki et al. 1996). IFN-γ-activated macrophages limit this availability by down-regulating TF expression and preventing accumulation of Fe^{2+} in its saturated storage form (as Fe^{3+} -holoferritin complex) within the cytosol (Byrd and Horwitz 1993; Schaible et al. 1998).

Phagosomal Fe^{2+} (as well as Mn^{2+}, Mg^{2+}, and Zn^{2+}) is concomittently lowered by approximately two- to sixfold in IFN-γ-activated macrophages infected with *Mtb* (Wagner et al. 2005), a reduction due in part to the proton-dependent Mn^{2+} and Fe^{2+} efflux pump, NRAMP1 (encoded by *SLC11A1*; Jabado et al. 2000). *Nramp1* was originally identified via positional cloning in inbred mouse strains harboring G169A variants of the *Bcg/Ity/Lsh* allele (denoting *Mycobacterium bovis* BCG, *Salmonella typhimurium*, and *Leishmania donovani* susceptibility), and *NRAMP1* polymorphisms are thought to predispose to human TB as well (see Fortin et al. 2007). NRAMP1 is a 12-transmembrane spanning protein found

on lysosomes, tertiary granules (neutrophils), and phagosomes, where it aids mycobacterial killing (Jabado et al. 2000; Fortin et al. 2007). Genetic studies infer cationic competition between NRAMP1 and an *Mtb* homolog, Mramp, because Mn^{2+} and Fe^{2+} concentrations are elevated in phagosomes harboring Mramp-deficient *Mtb* (Wagner et al. 2005). Phagosomal recruitment of the Fe export protein ferriportin-1 also reduces intravacuolar iron after IFN-γ activation (Van Zandt et al. 2008), whereas lipocalin-2 directly sequesters iron to inhibit *Mtb* in macrophages, neutrophils, and airway epithelium (Martineau et al. 2007; Saiga et al. 2008; Johnson et al. 2010).

Other Cations—Magnesium, zinc, and copper are likewise sensed or used by *Mtb*; however, as with iron, high concentrations of these transitional metals are directly toxic or promote tuberculocidal ROS (Russell 2011; Samanovic et al. 2012). A requirement for Mg^{2+} in *Mtb* replication was identified under low cation and pH conditions akin to those found in the phagolysosome where bacterial growth was impaired (Piddington et al. 2000). This finding was reinforced by *Mtb* mutants lacking the mgtC (Mg^{2+} transporter) gene that perform poorly under the same conditions and are highly attenuated inhuman macrophages and mice (Buchmeier et al. 2000). For Zn^{2+}, intracellular stores are pumped into *Mtb* phagosomes via a ROS-dependent pathway to promote killing in TLR-activated macrophages (Botella et al. 2011). Here *Mtb* attempts to counter Zn^{2+} toxicity by expressing a P-type ATPase efflux transporter encoded in the ctpC locus (Botella et al. 2011). For Cu^{2+}, a host P-type ATPase pump ATP7A imports Cu^{2+} into the phagosome for generating intraluminal OH. radicals, which is countered to some extent by *Mtb* transport proteins CtpV and MctB (Ward et al. 2010; Wolschendorf et al. 2011; Samanovic et al. 2012). Divalent cations are thus selectively furnished or withheld to limit *Mtb* growth.

Nutritional Immunity: Carbon and Amino Acid Metabolism

ICLs and IRG1—Carbon catabolism is critical for *Mtb* survival and persistence (Muñoz-Elías and McKinney 2006). Conserving C_2 carbon during periods of nutrient restriction via anaplerosis helps maintain bacterial metabolism fueled by fatty acids. One metabolic target for cell-autonomous immunity is the *Mtb* glyoxylate shunt pathway that replenishes tricarboxylic acid (TCA) cycle intermediates normally diverted for biosynthetic purposes via successive carboxylation reactions (McKinney et al. 2000). Early work discovered a glycoxylate shunt enzyme, isocitrate lyase 1 (ICL1), was essential for *Mtb* persistence in IFN-γ-activated macrophages and mice (McKinney et al. 2000). Subsequent discovery of a second functional ICL isoform (ICL2) corroborates the strong metabolic pressure placed by cell-autonomous immunity on *Mtb* to use anaplerotic pathways (Muñoz-Elías and McKinney 2005).

The immune-responsive gene 1 (IRG1) has recently been identified as exerting part of that metabolic pressure in activated human and mouse macrophages (Michelucci et al. 2013) (Fig. 5). IRG1 is transcriptionally induced by autocrine IFN/STAT1 signaling in response to both virulent and environmental mycobacteria (Shi et al. 2005; Basler et al. 2006; Michelucci et al. 2013). Thereafter it localizes to mitochondria (DeGrandi et al. 2009), where it decarboxylates the TCA intermediate cis-aconitate needed for isocitrate synthesis, thus depriving ICL1 and ICL2 of their substrate to run the glyoxylate shunt (Michelucci et

al. 2013) (Fig. 5). Decarboxylation of *cis*-aconitate also produces itaconic acid (alternatively known as methylenesuccinic acid) that may be directly toxic to bacilli (Michelucci et al. 2013). IRG1 could additionally promote b-oxidation-dependent mitochondrial ROS production for some of its bactericidal activities (Hall et al. 2013; Roca and Ramakrishnan 2013).

IDO—Other effector mechanisms target the production of amino acids catabolized or assimilated by *Mtb* for its growth (Zhang and Rubin 2013). Tryptophan degradation is an effective defense against obligate bacterial pathogens like Chlamydia and protozoan parasites including *Toxoplasma* and *Leishmania* spp. (MacMicking 2012). IFN-γ induces indoleamine-2,3-dioxygenase (IDO), a heme-containing oxidoreductase responsible for the initial rate-limiting step of the kynurenine pathway in which it degrades L-tryptophan to generate N-formyl-kynurenine (MacMicking 2012) (Fig. 5).

Although most laboratory *Mtb* strains are not tryptophan auxotrophs and IDO inhibition does not impact their growth within IFN-γ-activated macrophages (MacMicking et al. 2003), recent use of *Mtb Trp*$^{-}$ mutants has revealed tryptophan degradation by the host does exert metabolic pressure on *Mtb* in macrophages and infected mice (Zhang et al. 2013). *Mtb* synthesizes L-tryptophan via an operon containing *TrpE*, encoding an enzyme that converts chorismate to anthranilate, and *TrpD*, which encodes a ribosylating enzyme for the substrate (Zhang et al. 2013) (Fig. 5). Deletion of *TrpE* greatly attenuates *Mtb* growth in IFN-γ-activated macrophages that is restored in IDO-1-deficient cells; a similar outcome occurs in vivo. Moreover, fluorinated anthranilate (5-FABA and 6-FABA) inhibitors that block *Mtb* tryptophan synthesis render the bacillus susceptible to IDO-mediated killing. Thus drugs targeting bacterial counterimmune mechanisms allow the corresponding host effector to regain functionality inside phagocytic cells.

Concluding Remarks

This perspective has outlined the major cell-autonomous effectors deployed against Mtb. Because apoptosis and autophagy are covered extensively elsewhere in this collection, they have been given limited coverage or omitted altogether in this particular article. Furthermore, the list of effectors is by no means complete. Cell-autonomous immunity typically uses all the tools at its disposal—proteins, peptides, nucleotides, cations, metabolites, and physiological gases—to defend the host against infection. Hence, it can be anticipated that new pathways and effector molecules will emerge with antitubercular defense functions. IFN-γ- or PRR-independent pathways like GM-CSF-mediated killing (Rothchild et al. 2014) and unconventional activities such as mitochondrial immunity (via ROS, IRGM1, and IRG1) or autophagic engulfment of cytosolically exposed bacilli will be fertile areas of future investigation. Once understood, these novel defense factors may join established mechanisms as a platform from which to design host-directed therapies or adjunctive treatments that more fully expose *Mtb* to killing by the human immune system (Nathan 2012; Hawn et al. 2013; Wilkinson 2014). Both offer hope for shortening treatment as well as for tackling the problem of bacterial multidrug resistance (Singh et al. 2008; Zhang et al. 2013; Stanley et al. 2014).

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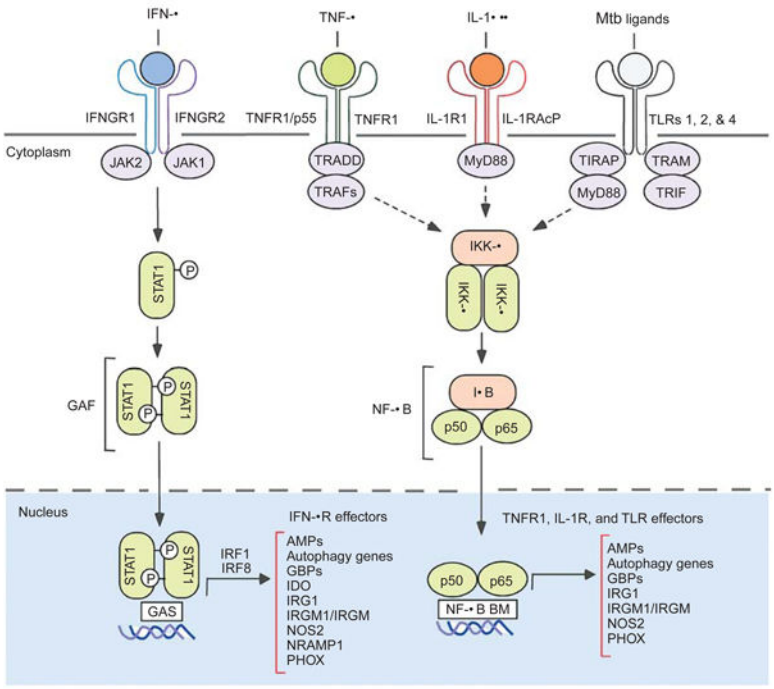

Figure 1.

Immunoreceptor signaling for mobilizing the major cell-autonomous effector programs against Mtb. In addition, new NLR, VDR-, and P2XR-induced activities are emerging (see text). *Mtb* ligands that elicit TLR signaling include mannosylated lipoarabinomann, phosphatidylinositol mannosides (PIMs), and the 19-kDa lipoprotein. These pathways synergize to induce distinct as well as overlapping signatures of effector proteins for bacterial restriction as shown. For schematic simplicity, receptors are depicted as single chains instead of their trimeric, tetrameric, and heteromeric forms (especially in the case of the TLRs). Adaptor and transcription factor abbreviations: IRF, interferon regulatory factor; JAK, Janus kinase; NF-κB BM, NF-κB binding motif; STAT1, signal transducer and activator of transcription 1; TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TRADD, tumor necrosis factor receptor type 1–associated DEATH domain; TRAF, TNF receptor–associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β.

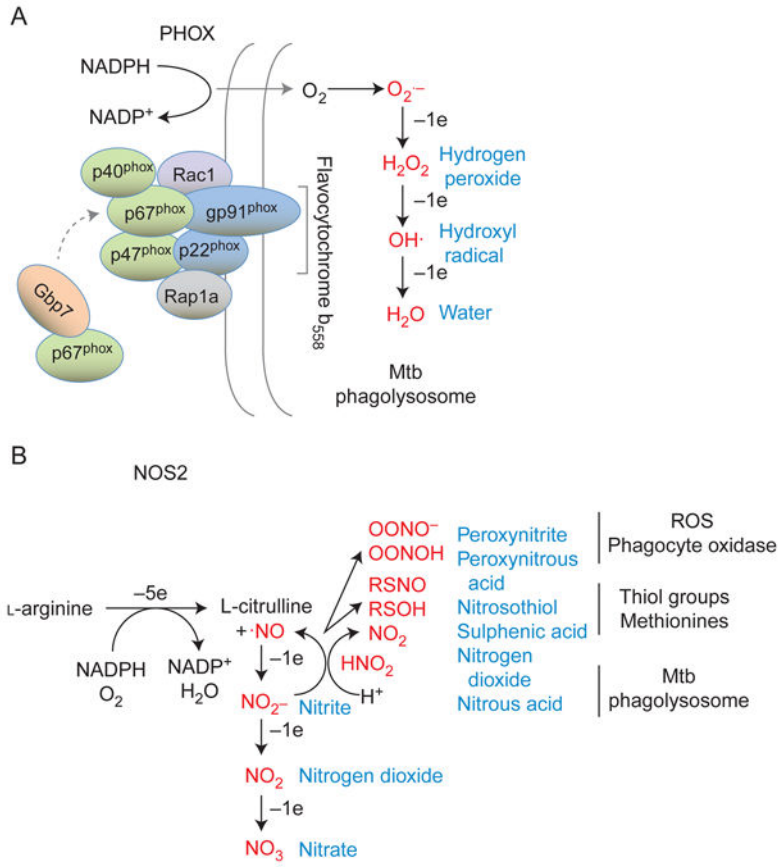

Figure 2.

Oxidative and nitrosative defense against *Mtb*. (*A*) Multisubunit complex of the phagocyte oxidase (NADPH oxidase) depicting the core membrane (gp91^{phox}, p22^{phox}; known as flavocytochrome b_{558}) and cytosolic (p67^{phox}, p47^{phox}, p40^{phox}) components. Also shown are immune (Gbp7) and nonimmune GTPases (Rac1, Rap1a) that regulate trafficking to the *Mtb* phagosome for membrane anchorage as well as complex assembly. The product superoxide (O_2^-) and oxidant derivatives are shown in red font. (*B*) Enzymatic production of nitric oxide (·NO) via 5-electron oxidation of the guanidino nitrogen of L-arginine by inducible nitric oxide synthase 2. Downstream intermediates or congeners are shown in red font.

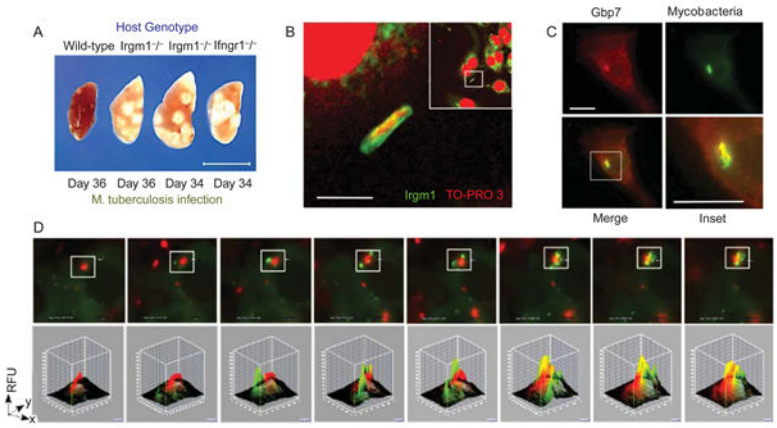

Figure 3.

IFN-γ- and TLR-induced immune GTPases involved in autophagic trafficking of *Mtb* and antimicrobial cargo to lysosomes. (*A*) Deletion of the Irgm1 gene leads to profound TB susceptibility after aerogenic challenge (MacMicking et al. 2003). Scale bar, 1.0 cm. (*B*) Targeting of endogenous Irgm1 to mycobacterial phagosomes in IFN-γ-activated macrophages. TO-PRO-3 detects nuclear and bacterial DNA. Original inset for higher magnification shown. Scale bar, 2 mm. (*C*) Recruitment of the 65-kDa guanylate binding protein, Gbp7, to mycobacterial phagosomes in IFN-γ-activated macrophages. Scale bar, 5 mm. Gbp7 helps recruit $p67^{phox}$ to assemble the phagocyte oxidase complex in this location (Kim et al. 2011). (*D*) Live imaging of Gbp7 vesicle recruitment (green) to internalized mycobacteria (red) over a 30.8-min data collection period. Image J software reveals fluorescence convergence within the chosen inset area (Kim et al. 2011).

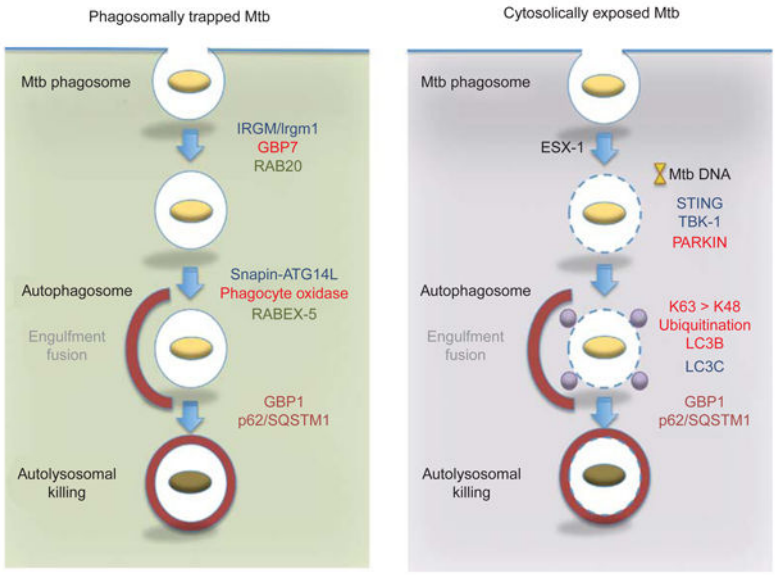

Figure 4.

Differential recruitment of effectors to “trapped” and “escaped” *Mtb* in immunologically activated macrophages. (*Left*) Delivery of phagosomal bacilli to autolysosomes after autophagic capture requires both immune and nonimmune GTPases. Their position in the pathway and corresponding interacting partners are color matched. (*Right*) Cytosolically exposed bacilli following permeabilization of the phagosomal membrane by ESX-1 releases DNA for detection by STING. This recruits TBK-1 and probably LC3C for *Mtb* capture. The E3 ligase PARKIN also ubiquitinates *Mtb* directly or its surrounding membrane remnants for detection by the autophagic pathway. A preferential requirement for K63 over K48 ubiquitin linkage is reported (Manzanillo et al. 2013).

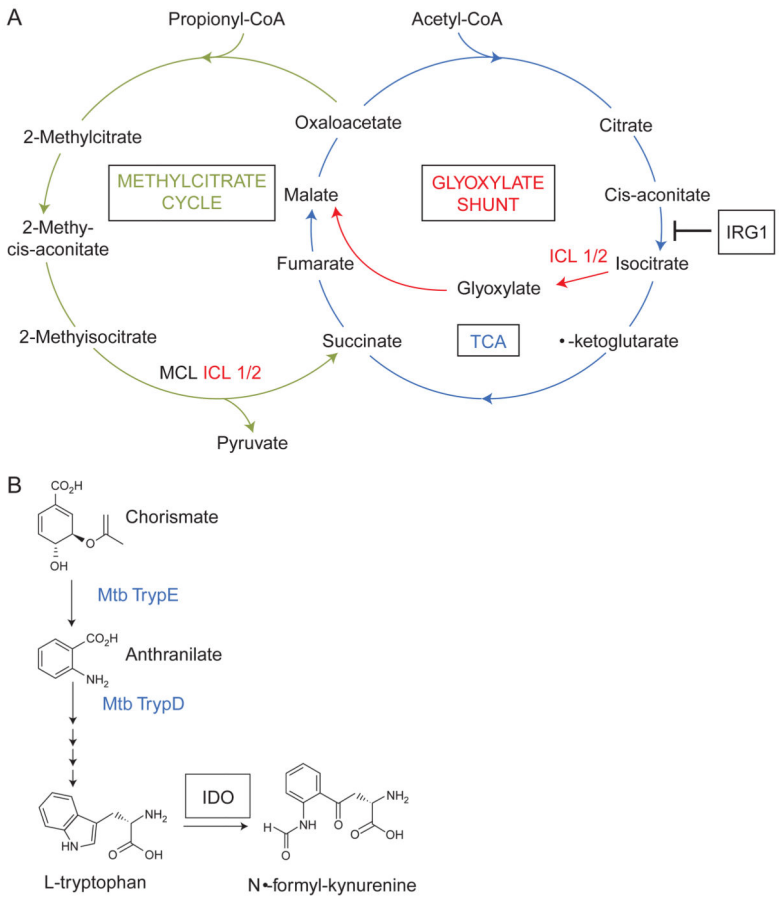

Figure 5.

Nutritional immunity to *Mtb*. (*A*) Depletion of tricarboxylic acid (TCA) cycle and potential glyoxylate shunt substrates via a *cis*-aconitate decarboxylase known as immunoresponsive gene-1 (IRG1) that resides in mitochondria. Position at which IRG1 blocks anaplerotic substrate production for *Mtb* is shown. Isocitrate lyases 1 and 2 (ICL1/2) that serve as important *Mtb* anaplerotic enzymes in the glycoxylate shunt and that are the functional *Mtb* paralogs of methylcitrate lyases (MCLs) in the methylcitrate pathway (Muñoz-Elias et al. 2006) are also depicted. (Adapted from data in Michelucci et al. 2013.) (*B*) Tryptophan depletion by the IFN-γ-induced enzyme, indolamine-2,3-dioxygenase (IDO), in activated macrophages. *Mtb* genes encoding the enzymes known to be involved in generating precursors of tryptophan biosynthesis are shown in blue font.