Mycobacterial Subversion of Chemotherapeutic Reagents and Host Defense Tactics: Challenges in Tuberculosis Drug Development

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mycobacteria, antibiotic resistance, macrophage, phagosome maturation, phagosome-lysosome fusion, tuberculosis

Abstract

Recent worldwide emergence of multidrug-resistant and extensively drug-resistant tuberculosis is threatening to destabilize tuberculosis control programs and urging global attention to the development of alternative tuberculosis therapies. Major roadblocks limiting the development and effectiveness of new drugs to combat tuberculosis are the profound innate resistance of *Mycobacterium tuberculosis* to host defense mechanisms as well as its intrinsic tolerance to chemotherapeutic reagents. The triangle of interactions among the pathogen, the host responses, and the drugs used to cure the disease are critical for the outcome of tuberculosis. We must better understand this three-way interaction in order to develop drugs that are able to kill the bacillus in the most effective way and minimize the emergence of drug resistance. Here we review our recent understanding of the molecular basis underlying intrinsic antibiotic resistance and survival tactics of *M. tuberculosis*. This knowledge may help to reveal current targets for the development of novel antituberculosis drugs.

INTRODUCTION

"*To point out that beginning immediately with the most intense tuberculosis eradication movement would require a hundred or more years for the eradication of tubercle bacilli may seem discouraging to those who lack courage to gird for such an encounter for the remainder of their lives and then pass it on to other generations for completion. However, 100 years is an exceedingly brief period for such an accomplishment when one considers that the lifetime of the disease extends far back into antiquity*."

J. Arthur Myers (*1*)

In the 1960s, with the exciting introduction of effective antibiotics such as streptomycin and isoniazid into tuberculosis (TB) treatment, it was optimistically predicted that tuberculosis would be eradicated from the world in the next 100 years (1). Yet, after more than 40 years of continuous attempts, no one can say if we will not see tuberculosis in another 100 years. *Mycobacterium tuberculosis* has evolved together with us since the dawn of civilization. A continuous struggle with our immune system and the recent exposure to chemotherapeutic reagents have forced the bacterium to evolve molecular mechanisms effective in neutralizing host defense tactics as well as antibacterial activities of antibiotics.

The World Health Organization (WHO) has estimated that approximately 2 billion people worldwide are latently infected with *M. tuberculosis* and ~10% of these people will develop active disease during their lifetimes. Together with the Human Immunodeficiency Virus (HIV) pandemic and the emergence of Multi-Drug Resistant (MDR) and Extensively-Drug Resistant (XDR) forms, tuberculosis is becoming one of the most significant threats to global health. Chemotherapeutic options to treat tuberculosis are severely restricted by the intrinsic tolerance of *M. tuberculosis* to most available drugs. Among hundreds of antibiotics commercially available, antituberculosis treatment is limited to a handful of drugs that were developed almost half a century ago. These narrow antibiotic choices, lengthy treatment regimens, and patient noncompliance have provided conditions for the accumulation of antibiotic resistance mutations (acquired antibiotic resistance) that have led to worldwide emergence of strains resistant to virtually all available drugs (2, 3). The mycobacterial intrinsic resistance to chemotherapeutic reagents should also be considered a challenge for the development of new drugs that target pathways operating inside the mycobacterial cytoplasm. Another key to the success of *M. tuberculosis* lies in its ability to evade the defense mechanisms of our immune system. Unlike other microbes, which may avoid being internalized by phagocytic cells such as dendritic cells or macrophages, *M. tuberculosis* allows its capture, but remains within a safe vacuole, the mycobacterial phagosome (4). *M. tuberculosis* living within phagosomes is capable of manipulating complex membrane trafficking events such that these vacuoles are excluded from fusion to lysosomes; the latter is a compartment characterized by a hostile environment, including low pH and degrading enzymes. By remaining within nondestructive phagosomes, and simultaneously reducing its own metabolism and increasing resistance to toxic host-derived reagents such as oxidants and antibacterial peptides, *M. tuberculosis* can survive within the macrophage—the cell designed to destroy invaders. To develop a next generation of effective antituberculosis drugs that have the desired characteristics (shortened treatment, minimized resistance, and greater effectiveness against MDR/XDR and latent tuberculosis), the multi-directional interactions among host responses, the tubercle bacillus, and the drugs used to treat the disease (**Figure 1**) must be further studied. Only then may we regain the winning position in the battle against tuberculosis and continue the hope that one day this disease will be eradicated.

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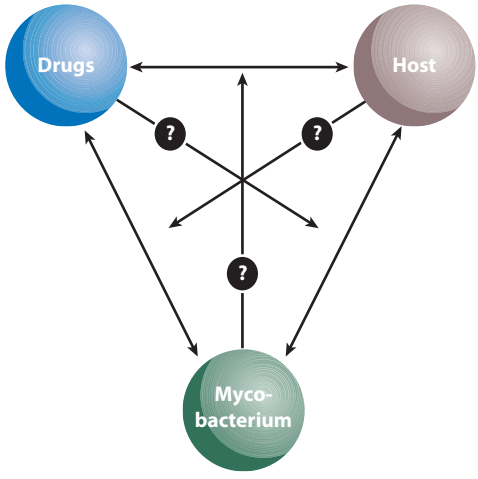

Figure 1

The triangle of interactions among pathogenic mycobacteria, host responses, and drugs that are used to treat the diseases. Although the pairwise interactions between mycobacteria, host defenses, and antimicrobial reagents have been studied at some levels, other more complex interactions [e.g., effects of antibiotics on host-mycobacterial interactions, effects of host responses on antibiotic activity and resistance (question marks)] are still elusive and need further investigation.

INTRINSIC ANTIBIOTIC TOLERANCE OF MYCOBACTERIA AS A HINDRANCE FOR CHEMOTHERAPY AND DEVELOPMENT OF NEW ANTBIOTICS

The antibacterial activity of an antibiotic is a function of its inhibitory activity on cellular targets and the total activity loss caused by resistance mechanisms that the affected bacterium operates. Mycobacterial species possess an array of molecular mechanisms capable of neutralizing the activity of many classes of antibiotics. Drugs that target molecules functioning within the mycobacterial cytoplasm or located within the inner peptidoglycan layer lose most of their activities before reaching their targets. One of the classical examples is the activity of β-lactam antibiotics. Although these drugs are commonly used against bacterial infections, β-lactam antibiotics have virtually no effect on mycobacteria. Because these antibiotics inhibit penicillin-binding proteins present in proximity to the inner peptidoglycan layer, in mycobacteria they have to travel through thick layers of mycolic acids and arabinogalactan before reaching these targets. In addition to this permeability barrier, another firewall for β-lactam drugs residing in the mycobacterial cell wall is the presence of β-lactamase enzymes that effectively inactivate β-lactam antibiotics. This concept is supported by the fact that β-lactamase-resistant β-lactam antibiotics such as imipenem have greater activities against mycobacteria because they overcome the obstacle of degradation by β-lactamase enzymes (5), and the fact that the bactericidal activity of these antibiotics on mycobacteria is extended on mycobacterial mutants having defects in cell wall integrity (**Figure 2**).

An Impermeable Cell Wall Limiting Penetration of Antibiotics

The intrinsic resistance of mycobacteria to multiple antibiotics has been ascribed to their unique cell wall structure, which functions as an effective barrier for the penetration of antibiotics. This cell envelope consists of a layer of hydrophilic arabinogalactan covering the peptidoglycan layer, which prevents the entry of hydrophobic chemicals (6). The arabinogalactan layer is wrapped by a network of long-chain hydrophobic mycolic acids, which limits the penetration of hydrophilic

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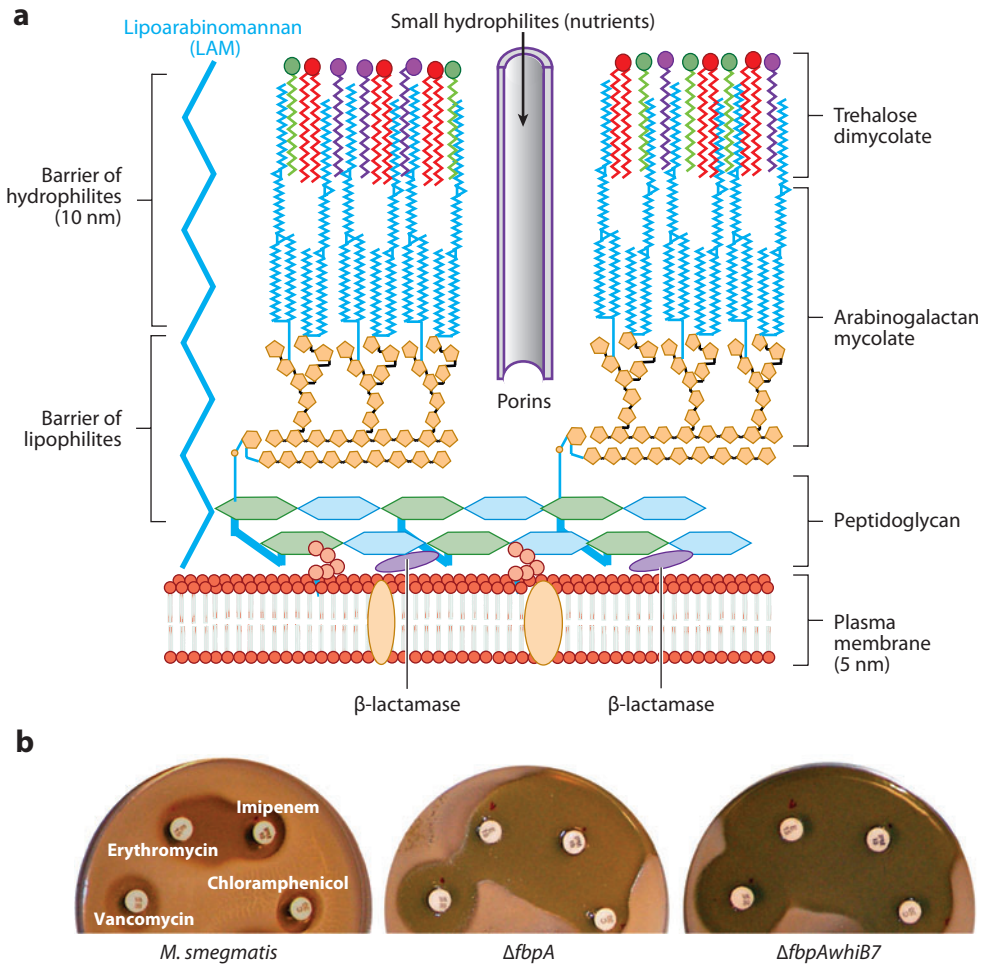

Figure 2

Schematic structure of mycobacterial cell wall, and the synergy of cell wall exclusion and internal defense mechanisms in controlling intrinsic antibiotic resistance in mycobacteria. (*a*) Schematic structure of mycobacterial cell wall depicted according to Minnikin's model (132). Intercalation of hydrophilic arabinogalactan and hydrophobic mycolate containing layers creates an extremely impermeable envelope for antibiotic penetration. Small molecules and nutrients are transported through porin channels that are deposited through these layers. Modified from (133). (*b*) Deletion of *fbpA* involved in cell wall integrity (assembly of trehalose dimycolate into the outermost layer of the cell wall) and *whiB7* responsible for resistance to drugs that have penetrated into the cytoplasm leads to increased sensitivity to two distinct groups of antibiotics. *fbpA* mutants become most sensitive to drugs that target the inner layer of the cell wall, whereas *whiB7* mutants are more sensitive to ribosome-targeted antibiotics. A double mutant carrying both *fbpA* and *whiB7* deletion shows a combined sensitivity to both groups of antibiotics. Drug sensitivity is estimated by an antibiotic diffusion test. Disks containing the same amount of vancomycin, erythromycin, imipenem, and chloramphenicol are placed on the surface of medium plates cultivated with wild-type *M. smegmatis*, the Δ*fbpA* mutant, and the double mutant Δ*fbpAwhiB7*. Sensitivity is proportional to the clear zone (inhibition zone) around the antibiotic disks in which the bacterial growth is inhibited.

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molecules. These three layers are covalently connected; thus, defects in one layer could consequently affect the integrity of the whole cell wall. In addition, on the exterior of these layers is a mobile layer consisting of trehalose dimycolates and glycopeptidolipids (**Figure 2**). Mycolic acids are initially synthesized as two alkyl chains by enzymes similar to those that assemble fatty acids. These alkyl chains are then fused and subject to numerous modifications to generate a complex family of lipids that are assembled into the cell envelope (6). Analyses of mycobacterial mutants indicate a strong link between cell wall mycolate content and antibiotic resistance. A *Mycobacterium smegmatis* mutant defective for mycolate biosynthesis displays increased rates of uptake and sensitivity to erythromycin, chloramphenicol, novobiocin, and rifampicin (7); and an analysis of a transposon library identifies many antibiotic-sensitive mutants that have disruptions in polyketide synthase genes (8) potentially involved in mycolate biosynthesis. Also, a transposon mutant in *kasB*, a defined mycolic acid biosynthetic gene, is more permeable and sensitive to various antibiotics as well as to lysozyme and defensin, two host-defensive antimicrobial compounds (9). In addition, deletion of the *M. tuberculosis mymA* operon (*rv3083* to *rv3089*) and its transcriptional regulator gene *virS* (*rv3082c*), both of which are important for the mycolate content of the cell envelope (10), leads to an increased sensitivity to antituberculosis antibiotics such as rifampicin, ciprofloxacin, isoniazid, and pyrazinamide (11). Mycolic acids are incorporated into the cell wall arabinogalactan (to generate arabinogalactan mycolates) and trehalose (to generate trehalose dimycolates or TDM or cord factor) by a family of redundant mycolyltransferase enzymes initially known as the antigen 85 complex (12). Disruption of the gene encoding FbpA, one of the mycolyltransferases of this complex, reduces the trehalose dimycolates on the cell wall to almost half their numbers (13). The *fbpA* mutant displays increased sensitivity to broad-spectrum antibiotics widely used for antibacterial chemotherapy (13) (**Figure 2*b***). These observations have convincingly supported the model that the mycobacterial cell wall plays an essential role in mycobacterial intrinsic tolerance against antibiotics.

If this cell wall permeability barrier is so effective in preventing chemical passage, how do mycobacteria take up nutrients and molecules required for their growth? Transport of low-molecular-weight nutrients and metabolites through this impermeable cell wall is facilitated by porins that are similar to those found in the outer membranes of gram-negative bacteria (14) (**Figure 2**). Porins also play a role in the import of antibiotics; expression of the major porin MspA from *M. smegmatis* increases susceptibility of *M. tuberculosis* and *M. bovis* to β-lactam antibiotics as well as front-line antituberculosis drugs isoniazid, ethambutol, and streptomycin (15). In contrast, disruption of *mspA* in *M. smegmatis* leads to increased resistance to not only hydrophilic but also hydrophobic and large antibiotics such as vancomycin, erythromycin, and rifampicin (15). Whether MspA plays a direct role in the penetration of hydrophobic and large-molecular-weight compounds is still largely unknown; understanding the nature of porins could aid in the design of new anti-tuberculosis drugs that circumvent the inaccessible cell wall.

Finally, recent work has also highlighted the importance of efflux pumps that are located within the mycobacterial cell wall in the export of a number of antibacterial compounds, thereby allowing the microbes to resist several antibiotics (3, 16, 17).

Internal Defense Systems Neutralizing Activities of Antibiotics

Studies of mycobacterial cell wall permeability, however, suggest that this cell wall barrier provides only part of the extremely high level of antibiotic tolerance and that synergistic mechanisms are required for full protection of mycobacterial species against antibiotics (18) (see also **Figure 2*b***). In fact, the cell envelope allows passage of lethal amounts of hydrophilic antibiotics within a small fraction of the generation time of the cell. Without additional protection, this

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antibiotic accumulation in the cytoplasm would be enough to kill the bacteria (18). As discussed above, the cell wall obviously slows the passage of antibiotics, which may help to delay the accumulation of drugs such that internal resistance systems can effectively detoxify resident antibiotics (**Figure 2*b***).

Drug degradation and modification. β-lactam antibiotics kill bacteria by inhibiting cell wall biosynthesis. These drugs bind and inhibit the activities of penicillin binding proteins involved in peptidoglycan assembly. Besides the cell wall–mediated permeability barrier, which limits the influx of these antibiotics, mycobacteria possess effective β-lactamase enzymes that bind and degrade the β-lactam drugs (**Figure 2*a***) (19). β-lactamase degradation appears to be the major mechanism conferring resistance to β-lactam antibiotics. Both in vitro experiments and clinical evidence indicate that β-lactamase-resistant β-lactams or the use of β-lactams in combination with β-lactamase inhibitors are effective in killing *M. tuberculosis* (19, 20). Two β-lactamase activities are produced by *M. tuberculosis:* The predominant enzyme is a class A penicillinase similar to the β-lactamases present in common enteric bacilli and the other is a cephalosporinase. Genetic analysis revealed that β-lactamase activity in *M. tuberculosis* is encoded by *blaC* and *blaS* (20). A combinatory chemotherapy using β-lactamase-resistant β-lactams (19) with cell wall–destabilizing drugs is a potentially effective treatment for tuberculosis.

Mycobacteria are also able to inactivate antibiotics by chemical modifications. Aminoglycosides constitute a large group of broad-spectrum antibiotics such as streptomycin that are used to treat a variety of bacterial infections, including tuberculosis. There are three known chemical modifications that can inactivate aminoglycoside antibiotics: (*a*) ATP-dependent O-phosphorylation catalyzed by phosphotransferases (APH), (*b*) ATP-dependent O-adenylation by nucleotidyltransferases (ANT), and (*c*) acetylCoA-dependent N-acetylation by acetyltransferases (AAC). The aminoglycoside 2′-N-acetyltransferase (*aac*) gene from *M. fortuitum* and *M. smegmatis* provides resistance to gentamicin, tobramycin, dibekacin, and netilmicin (21). Homologs of AAC are also present in the genomes of pathogenic mycobacteria such as *M. tuberculosis* and *M. leprae*. Interestingly, structural comparison suggested that *M. tuberculosis* AAC could have a role in the biosynthesis of mycothiol (which is also an antibiotic resistance determinant, see below) where it functions as a regulator controlling the flux of mycothiol precursors via acetylation and deacetylation (22).

Rifampicin has been one of the front-line drugs in the treatment of infections by mycobacteria and related Actinobacteria, although many of these bacteria are able to inactivate rifampicin via several chemical modifications such as glycosylation, phosphorylation, and ribosylation. At least two separate ADP ribosyltransferases were cloned from *M. smegmatis*, which catalyze ribosylation and provide resistance to rifampicin (23, 24). Curiously, one of the encoded genes is located adjacent to an insertion sequence (IS1623). Although acquisition of drug resistance via mobile genetic elements has not been described for slow-growing mycobacteria, it was speculated that such factors may catalyze the spread of the antibiotic resistance gene to pathogenic mycobacteria (24).

Recent work also reveals a novel drug-resistance determinant in mycobacteria that is related to their unique system for coping with oxidative stresses. In most bacteria, protection against oxygen toxicity is provided by a reducing thiol called glutathione. Interestingly, Actinobacteria do not make glutathione. Instead, they produce mycothiol, an alternative thiol-containing compound that has thus far been found present only in this order of bacteria (25, 26). Mycothiol provides resistance to several antibiotics such as vancomycin, rifamycin, macrolides, and β-lactams, probably through direct interactions (25, 26). Inhibition of mycothiol-mediated antibiotic resistance may render mycobacterial pathogens more susceptible to antibiotics as well as facilitate destruction by host macrophages.

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Target modification. Macrolide and lincosamide antibiotics inhibit bacterial protein synthesis by binding reversibly to a specific site of the ribosomal RNA in the subunit 50S of bacterial ribosomes, thereby inhibiting translocation of peptidyl tRNA. This group of antibiotics usually has little or no effect on *M. tuberculosis* complex (MTC), which includes *Mycobacterium bovis* (27). Interestingly, the Pasteur vaccine strain derived from *M. bovis*, *Bacillus* of Calmette and Guérin (BCG), is highly susceptible to many macrolide and lincosamide antibiotics. It was later found that this sensitivity of BCG is because of its lack of the *erm37* gene encoding a ribosomal RNA methyltransferase (27). Erm37 methylates the 23S ribosomal RNA, thereby altering ribosomal structure such that it lowers the affinity of the antibiotics to ribosomes. The *erm37* gene is located within a chromosomal location of *M. bovis* called Region of Difference 2 (RD2), which was deleted in BCG during culture passage. In *trans* expression of the *M. tuberculosis erm37* gene in BCG restores the macrolide and lincosamide resistance to the susceptible bacterium. In vitro analyses of macrolide affinity to ribosomes isolated from these bacteria confirmed that Erm37 protects the ribosomes from the binding of the antibiotics and thus may reduce the inhibitory activity of macrolides on protein synthesis (27).

Another *erm* gene was identified in both *M. smegmatis* and *M. fortuitum*, whose expression is also induced by exposure to macrolides and confers resistance to macrolide and lincosamide antibiotics (28, 29). Importantly, analyses of *M. fortuitum* clinical isolates reveal that the majority of clarithromycin-susceptible isolates carry a mutation in the putative start codon of this *erm* gene (GTG to CTG), which may have inactivated its expression (29).

The chromosomal conservation of *erm* genes and their similar functions in many mycobacterial species suggest an ancestral origin. Although the impact of *erm* genes on the clinical usage of macrolides and lincosamides to treat mycobacterial infections needs further investigation, their contribution to mycobacterial resistance to macrolide and lincosamide antibiotics is well established.

Target mimicry. Fluoroquinolones (fluoridated quinolones) are entirely synthetic antibiotics and have recently emerged as important antibiotics to shorten the tuberculosis treatment regimens (30). The first fluoroquinolone antibiotic was norfloxacin, which was synthesized from nalidixic acid by Kiorin in 1978. In a short time, chemical modifications led to the birth of many second- and third-generation fluoroquinolones such as pefloxacin, ofloxacin, ciprofloxacin, and levofloxacin, which gradually improved the treatment efficacy of Gram-negative infections. Since then, many generations of fluoroquinolones have been developed (moxifloxacin, gatifloxacin, etc.) to improve lethal effects on Gram-positive, anaerobic microbes and mycobacteria. Because fluoroquinolones have a broad antimicrobial spectrum and relatively fast bactericidal effects, they have been recommended to treat multidrug-resistant tuberculosis (30, 31).

Fluoroquinolones prevent bacterial reproduction by blocking replication, repair, and transcription of DNA through interactions with DNA gyrase (or topoisomerase II) and/or topoisomerase IV (32). By binding to the complex of these enzymes with DNA, fluoroquinolones stabilize the DNA breaks but inhibit resealing of DNA strands, leading to degradation of DNA and eventual bacterial death (32).

In a screen for fluoroquinolone-resistant mutations in *M. smegmatis*, a chromosomal gene was identified (*mfpA)* whose expression from a multicopy plasmid results in low-level resistance to ciprofloxacin and sparfloxacin in *M. smegmatis* and *M. bovis* (33). Conversely, disruption of *mfpA* leads to increased susceptibility of wild-type *M. smegmatis* to the antibiotics, suggesting that resistance to the fluoroquinolones is dependent on *mfpA* expression level. The amino acid sequence of MfpA has highest homology to the pentapeptide repeat protein family, in which every fifth amino acid is either a leucine or phenylalanine; each pentapeptide repeat is expected to form a sheet that

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is important for protein-protein interaction (34). Elucidation of the *M. tuberculosis* MfpA structure reveals a fascinating secret of mycobacterial defense tactics to fluoroquinolones: the protein has a three-dimensional structure resembling that of DNA double helix (34, 35) (**Figure 3*a***). The tandem of five amino acid repeats coiling around in a rod-shaped, right-handed helix has the same width as DNA (34, 36). MfpA could therefore mimic DNA to sequester fluoroquinolones

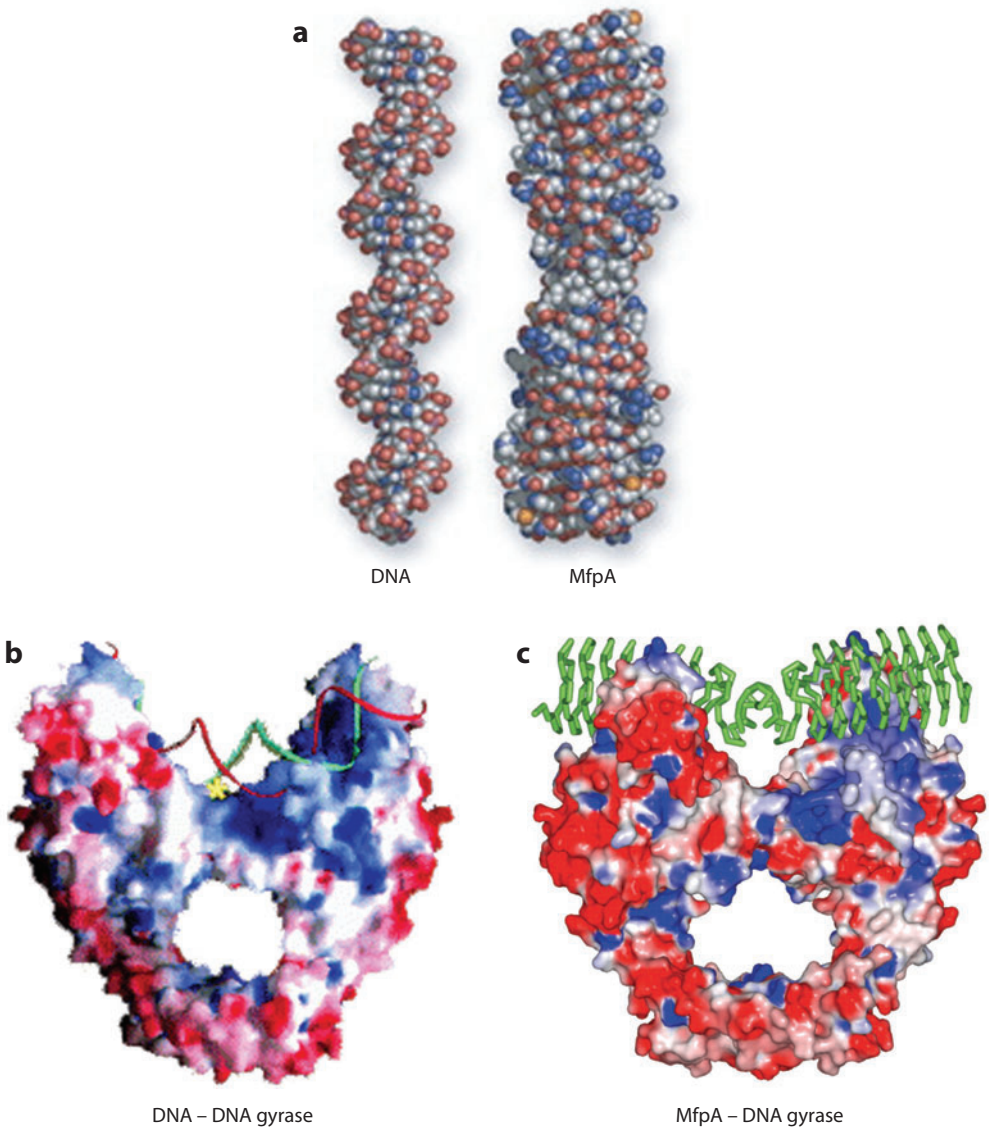

Figure 3

MfpA mimicry of DNA structure and interaction of MfpA with DNA gyrase. (*a*) MfpA structure (*right*) mimics that of DNA (*left*). Figure reproduced with permission from (35). (*b*) Molecular model of DNA bound to the dimeric form of the N-terminal domain of the *E. coli* gyrase A subunit. The DNA backbone is shown as a green and red ribbon; the active-site tyrosines as yellow stars. Reproduced with permission from (36). (*c*) Molecular model of dimeric MfpA (*green trace*) bound to the dimeric form of the N-terminal domain of the *E. coli* gyrase A subunit (surface). Negatively charged surfaces are in red, positively charged surfaces in blue. Reproduced with permission from (34).

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and thereby rescue the mycobacterial DNA from the attack of the drugs (35) (**Figure 3*b***). Although the precise physiological role of MfpA is not known and its relevance to clinical isolates of fluoroquinolone-resistant mycobacteria has not been tested, this discovery provides a striking example of how evolution has provided bacteria with a perfect adaptability to environmental pressures, including human interventions.

Transcriptional control of multidrug resistance. The ability to monitor and simultaneously respond to multiple harmful conditions in their surroundings is important for microbes living in complex environments such as soil or infected hosts. There is growing evidence suggesting that pathogenic mycobacteria utilize comprehensive machineries to recognize and protect themselves against dangerous compounds, including antibiotics. These systems might indeed help *M. tuberculosis* to survive attacks from the host immune system as well as chemotherapeutic treatment, which often includes several drugs targeting different molecules. These machineries are usually composed of several structural proteins that protect the bacteria from one or more toxic compounds and whose expression or activity is controlled by a master regulator, which is directly or indirectly able to recognize the presence of the toxic compounds.

One of such systems discovered recently is the transcription control exerted by the histone-like protein Lsr2. This protein is a 12-kDa basic protein present exclusively in Actinobacteria, including mycobacteria. Biochemical and atomic force microscopy recently showed that Lsr2 functions as a DNA-bridging protein able to connect distant DNA segments, suggesting that this protein plays a role in the overall organization and packaging of mycobacterial chromosomes (37). Indeed, it has been demonstrated previously that bacteria may use chromatin packaging as a common strategy to respond to stress-related conditions such as antibiotic treatment (38). This protein was recently identified as a suppressor of the ethambutol-induced transcription of the *iniABC* operon, which encodes a multidrug efflux pump in *M. tuberculosis* (39, 40). Comparative gene expression analyses suggested that Lsr2 controls the transcription of hundreds of genes potentially involved in many cellular processes, including cell wall biosynthesis and metabolic functions (40). Disruption of *lsr2* leads to increased transcription of the *iniABC* operon as well as *efpA* gene (encoding another multidrug efflux pump), which results in increased resistance of mycobacteria to multiple drugs.

Antibiotic tolerance may also reflect physiological adaptations that occur within the host, perhaps including an undefined developmental state that underlies persistent infection. As an example, the early sporulation gene *whiB* is essential for the assembly of septa needed to initiate cell division and sporulation in *Streptomyces* (41). The presence of *whiB*-like genes in *M. tuberculosis* suggests the existence of a developmental program mechanistically and functionally analogous to sporulation in *Streptomyces*. Indeed, the mycobacterial *whiB* gene (*whmD*) is essential for septum formation and cell division in *M. smegmatis* (42). In addition, the retention in the downsized *Mycobacterium leprae* genome and absence in all other taxa suggests that *whiB*-like genes encode important and Actinomycete-specific functions (3). A *whiB*-like gene (*whiB3*) affects mycobacterial virulence in mouse and guinea pig models. WhiB3 protein binds to the wild-type allele of SigA, the principal sigma factor, but does not bind to a mutant allele that is associated with virulence attenuation in a BCG vaccine strain (43). Another *whiB*-like gene (*whiB7*) initially identified in *Streptomyces lividans* is a multidrug resistance determinant (44). The *Streptomyces lividans whiB7* mutant grows and differentiates normally but becomes hypersensitive to a broad spectrum of chemically and functionally unrelated clinical antibiotics (chloramphenicol, macrolides, lincomycins, fusidic acid, imipenem, pristinamycin, rifampicin, and tetracycline) (44). Deletion of *whiB7* in many mycobacterial species (*M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis*; **Figure 2**) leads to a multidrug-sensitive phenotype, suggesting that *whiB7* is an ancestral multidrug resistance determinant (44).

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whiB7 encodes for a 122–amino acid protein carrying conserved motifs of the WhiB protein family (44).

How can such a small protein as WhiB7 provide resistance to multiple antibiotics with such chemical and target variety? Gene expression analysis using cDNA microarrays indicates that WhiB7 controls expression of a regulon of at least eight genetic loci (44), including at least four genes whose functions relate to intrinsic antibiotic resistance (8; L. Nguyen, unpublished data). These include a transporter Tap (45), which provides resistance to tetracycline and aminoglycosides, and a ribosome modifying enzyme, Erm37 (27) (see above), which provides resistance to macrolides and lincosamides. This observation suggests that WhiB7 functions as a master regulator of internal defense mechanisms that provide resistance to drugs penetrated into the cytoplasm (**Figures 2** and **4**). Interestingly, *whiB7* expression and subsequently expression of its regulon are induced by exposure to subinhibitory concentrations of antibiotics in a dose- and time-dependent manner (44). Expression of *whiB7* is also inducible in the presence of fatty acids, suggesting that WhiB7 functions are needed for mycobacterial survival/persistence in macrophages (44). A role

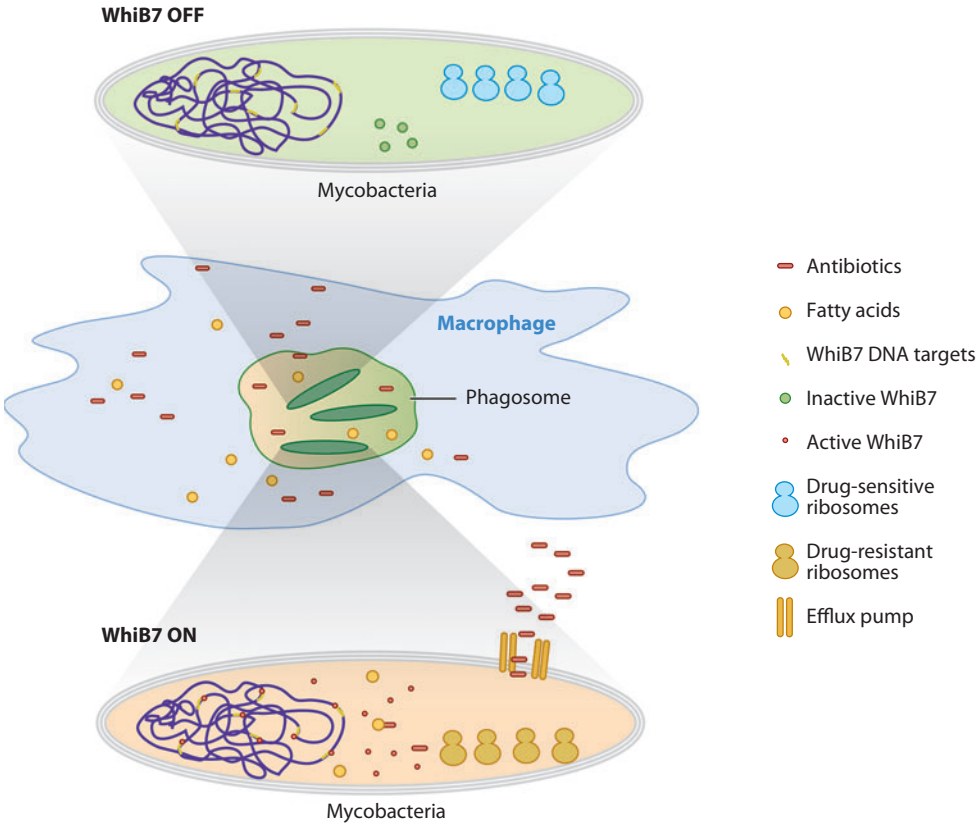

Figure 4

Model of WhiB7-mediated recognition and phenotypic resistance to antibiotics. The presence of antibiotics, fatty acids, or other unknown stimuli in macrophages may signal hostile environment clues, triggering metabolic shifts that result in alterations of redox balance in mycobacterial cells. This turns on the WhiB7 activity, which results in transcription activation of the WhiB7 regulon and thus leads to increased antibiotic tolerance and other unknown responses.

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for WhiB7 in mycobacterial survival and/or interactions with the host cells is supported by recent observations that (*a*) *whiB7* transcription is induced during mycobacterial infection in macrophages (46) and (*b*) the *eis* gene (enhanced intracellular survival), whose transcription is controlled by WhiB7 (44), encodes a secreted protein able to modulate mycobacterial survival in macrophages (47, 48) and affect cytokine secretion and T cell function (48, 49). The fact that antibiotic treatment induces mycobacterial defense systems such as those controlled by WhiB7, which leads to enhanced mycobacterial tolerance to both chemotherapy and host immunity, should be viewed as a caution for the use of antibiotics in treating bacterial infections (**Figure 4**). However, targeting those bipartite systems may be effective in both eradicating pathogenic bacteria and minimizing the emergence of drug resistance.

The observation that *whiB7* expression is exclusively induced by ribosome-targeting antibiotics and fatty acids suggests that the real substrates of the WhiB7 system may be naturally occurring or immune-generated toxins that affect ribosomal functions and might derive from fatty acid biosynthetic pathways. Under anaerobic and reduced condition, WhiB7 forms an iron-sulfur cluster that is sensitive to oxygen exposure (L. Nguyen, P.R. Jensen, R.P. Morris, M. Folcher, S. Durr, S. Grzesiek, & C.J. Thompson, unpublished data; 3), suggesting that WhiB7 and other WhiB proteins might function in vivo as redox-responsive transcription regulators. However, it is important to note that there is no experimental evidence thus far that WhiB proteins actually bind DNA. The upstream DNA region of *whiB7* contains the consensus motif recognized by the iron-responsive transcription regulator IdeR (50). This suggests that WhiB7 function may be controlled and related to iron metabolism, which is essential for mycobacterial survival in macrophages (51). This observation is further supported by recent work showing that, in addition to responses to several ribosome-targeted antibiotics, transcription of *whiB7* also occurs under iron-depleted conditions (52).

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PHAGOCYTOSIS OF MYCOBACTERIA BY MACROPHAGES

Phagocytosis is a fundamental defense mechanism of vertebrate innate immunity which allows phagocytic cells such as macrophages to engulf and eventually digest invading pathogens. For most microbes, capture usually means a dead end as the phagosome quickly fuses with lysosomes, thereby destroying the bacilli inside. However, *M. tuberculosis* and related pathogenic mycobacteria have evolved mechanisms to subvert the host's killing machinery.

Mycobacteria are phagocytosed by macrophages through several host cell receptors present at the cell surface to mediate mycobacterial entry into macrophages without altering the subsequent mycobacterial survival. These include the mannose receptor, complement receptors, Fc receptors, and scavenger receptors (53, 54). These receptors may also work together to mediate host cell signaling pathways, thereby leading to early host responses. Although it remains unknown how mycobacterial phagocytosis occurs in vivo, the uptake via complement receptor type 3 (CR3) is one of the most predominant entry routes in vitro (55–57). CR3 receptor-mediated uptake of mycobacteria requires the accumulation of cholesterol at the phagocytic cup (58). Treatment of macrophages with the cholesterol-reducing drug lovastatin and cholesterol chelator methyl-β-cyclodextrin results in an abrogation of mycobacterial entry into macrophages. The precise function of cholesterol, however, remains unknown. Cholesterol might be involved in the signal transduction reactions following engagement of CR3 (55, 58). Alternatively, cholesterol might help to increase the microviscosity of the membrane domains that are in contact with the hydrophobic mycobacterial cell wall and permit efficient formation of the actin network, thereby facilitating phagocytic uptake (59).

PHAGOSOME MATURATION PATHWAYS IN RESTING MACROPHAGES

Coronin 1 Pathway

Cholesterol-mediated phagocytosis is required for the retention of the host membrane protein coronin 1 [also known as Tryptophan Asparte Containing Coat protein (TACO) or P57] on mycobacterial phagosomes (58, 60), which is essential for the prevention of phagosome maturation (60–62). Coronin 1 is a 51-kDa protein with a C-terminal coiled-coil sequence of 30 amino acids and a tryptophan-aspartate 40 (WD40) repeat domain at the N-terminus. It belongs to a conserved family of WD-repeat actin-binding proteins predominantly found in eukaryotes (63). The first member of this family, termed coronin, was isolated from *Dictyostelium discoideum* using actin/myosin affinity chromatography (64). Deletion of coronin results in *Dictyostelium* mutants that have defects in several F-actin-mediated processes, such as macropinocytosis, cell motility, and phagocytosis, hence it was designated an actin-interacting protein. Whereas coronin 1 is the closest homologue to *Dictyostelium* coronin, there are up to six additional coronin isoforms expressed in mammalian cells, some more ubiquitously, and others in a tissue-specific fashion (60, 64). For most mammalian coronin isoforms, the biological role remains enigmatic.

The mechanism underlying coronin 1–mediated mycobacterial blockage of phagosome maturation and intracellular survival remained a puzzle until recently. The generation of macrophages as well as mice lacking coronin 1 expression showed that coronin 1 is fully dispensable for any of the F-actin processes analyzed (65–67). However, coronin 1 was shown to be required for the activation of the Ca^{2+}-dependent phosphatase calcineurin that is essential for inhibiting phagolysosome biosynthesis. Strikingly, chemical inhibition of calcineurin activation by cycloporin A or FK506 results in increased lysosomal delivery and reduced intracellular mycobacterial survival (66) (**Figure 5**). Calcineurin is not activated when macrophages isolated from coronin 1–deficient mice are infected with mycobacteria, resulting in rapid lysosomal delivery and intracellular mycobacterial killing (**Figure 5**).

Calcineurin is also known as protein phosphatase 2B and is responsible for activating T cell responses by dephosphorylation of different transcription factors (NF-AT) that in turn activate transcription of genes involved in the biosynthesis of IL-2 and other cytokines essential for T cell survival. Calcineurin inhibitors such as cyclosporin and FK506 have been used in immunosuppressive therapy. Interaction of a T cell receptor with antigens presented in the context of MHC molecules triggers the rise of intracellular concentration of calcium, which in turn activates calcineurin by binding a regulatory subunit and activating calmodulin. In macrophages, calcineurin negatively regulates the activation of NF-κB, MAPKs, and IFN response factors by inhibiting the Toll-like receptor (TLR)-mediated signaling pathways (68). Inhibition of calcineurin by cyclosporin A, FK506, or by small interfering RNAs leads to activation of these signaling pathways. In contrast, overexpression of a constitutively active form of calcineurin results in inhibiting the activation of these TLR-mediated pathways (68). Whether coronin 1–mediated inhibition of phagosome-lysosome fusion occurs through modulation of TLR signaling pathways remains to be established. However, both Ca^{2+} signaling as well as calcineurin activation can result in a pleithora of downstream signaling reactions. Therefore, further investigations into the coronin 1–mediated signaling pathway will allow a full understanding of its mechanisms of action.

The observation that only live mycobacteria can cause retention of coronin 1 on phagosomal membranes suggests that this is an active process and mycobacteria possess a mechanism to retain this host protein for their own sake. In a screen for mycobacterial factors responsible for coronin 1 retention, a mycobacterial protein termed LpdC was identified that specifically binds to coronin 1 in a cholesterol-dependent manner (69). Although the molecular mechanism mediating this

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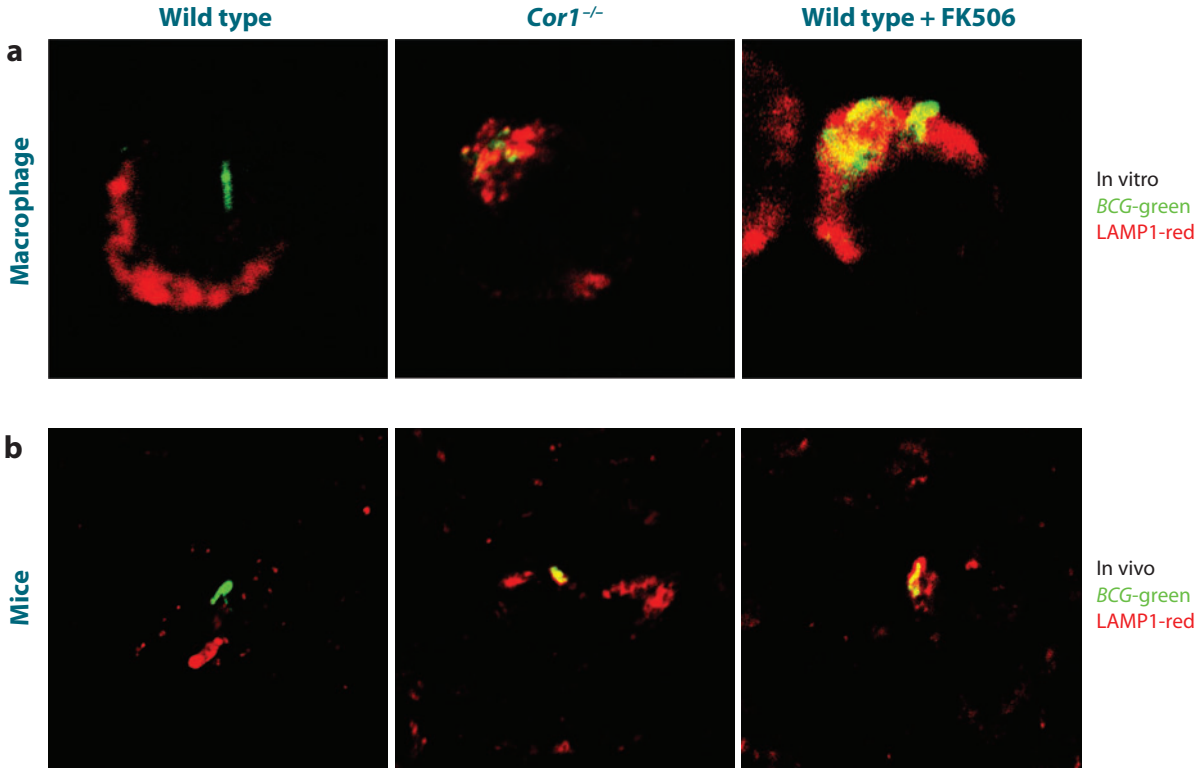

Figure 5

Genetic and chemical depletion of coronin 1 affects intracellular trafficking of pathogenic mycobacteria both in vitro and in vivo. (*a*) Wild-type and coronin 1–deficient (Cor $1^{-/-}$) macrophages were infected with *M. bovis Bacillus* of Calmette and Guérin (BCG) and chased for 3 h, followed by methanol fixation and staining for mycobacteria (*green*) and LAMP-1 (*red*). In wild-type macrophages, mycobacteria were retained in LAMP-1 negative compartments. In contrast, in Cor $1^{-/-}$ macrophages, mycobacteria were rapidly transported to lysosomes. Chemical inhibition of calcineurin activation by the inhibitor FK506 (0.5 μM) resembled the effect of coronin 1 genetic disruption. Figure reproduced with permission from (66). (*b*) Wild-type and coronin $1^{-/-}$ mice were infected with 10^6 CFU of *M. tuberculosis* H37Rv for one week. The mice were sacrificed and cryosections (8 μm) were prepared from the liver and immunostained for mycobacteria (*green*) and LAMP-1 (*red*). In wild-type mice, *M. tuberculosis* arrested phagolysosome formation. In contrast, the tubercle bacillus is predominantly located within lysosomes in coronin $1^{-/-}$ mice. In wild-type mice treated with a daily dose of FK506 (5 mg/kg body weight), *M. tuberculosis* also localized in lysosomal compartments. Figure reproduced with permission from (66).

process needs further investigation, the tactic of using a host protein for mycobacterial survival in macrophages is a perfect example of how pathogenic microorganisms have coevolved to parasitize the host cells that are designed to eliminate them.

Membrane Trafficking Regulatory Lipids Pathway

The fusion of phagosomes with lysosomes is also dependent on the synthesis of membrane lipids, in particular phosphatidylinositol 3-phosphate (PI3P), that function as a docking site for several proteins involved in trafficking within the lysosomal pathway (70, 71). Interestingly, analyses of the *M. tuberculosis* cell wall composition identified a PI3P analog, glycosylated phosphatidylinositol lipoarabinomannan (ManLAM), that is able to block phagosomal maturation (72). ManLAM most likely blocks a PI3P-dependent pathway involved in the transport of cargo between the *trans*-Golgi network and phagosomes, a transport step required for phagolysosome biogenesis

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(72, 73). Another mycobacterial factor which interferes with the PI3P pathway is the acid phosphatase SapM, a lipid phosphatase removing PI3P from phagosomes and thereby preventing phagosomal maturation (74).

Host and Mycobacterial Protein Kinases

Many host kinases have been implicated in controlling phagosomal maturation and survival of intracellular bacteria. Classically, isozymes of the protein kinase C family have been shown to play an essential role in these processes (75, 76). A recent screen of the human kinome using an RNA interference approach identified a network of several host kinases involved in phagolysosome biosynthesis and capable of inhibiting intracellular growth of *Salmonella* and *Mycobacterium* (77). The identified kinome involved in intracellular bacterial killing includes at least ten interactive kinases around the protein kinase B (PKB/AKT-1) enzyme. Chemical inhibition of these kinases suppresses the growth of *Salmonella typhimurium* and *M. tuberculosis* strains. This pathway is targeted by *S. typhimurium* through an effector of the type III secretion system, SopB, a phosphoinositide phosphatase, which may activate the host signaling cascade leading to inhibition of phagosome maturation (77). The mycobacterial factors involved in this pathway are currently unknown. By functional analogy, mycobacterial phosphoinositide phosphatase enzymes such as SapM (74) may function in the same way as SopB in activating the PKB pathway to prevent phagosome maturation.

The mycobacterial protein kinases make up another class of candidates that potentially participate in host signaling pathways. Indeed, the mycobacterial genome encodes eleven different eukaryotic-like protein kinases (78). One of these, protein kinase G (PknG), was recently shown to play an essential role in blocking phagosome maturation and intracellular survival of mycobacteria (79). After phagocytosis, *M. bovis* BCG *pknG*-deleted mutant is rapidly transported to lysosomes and the mutant mycobacterium is unable to survive inside macrophages (79) (**Figure 6**). Subcellular localization studies suggest that PknG is present in the phagosomal lumen as well as in the cytosol of macrophages infected with live bacteria but not in macrophages infected with dead bacteria, which suggests that pathogenic mycobacteria actively secrete PknG (79, 80). These data also suggest that PknG is a mycobacterial virulence factor that is delivered into the cytosol of host cells and may interfere with the regulation of phagosome-lysosome transfer (**Figure 6**).

The molecular mechanism responsible for PknG secretion is currently not known. PknG lacks the signal sequence required for its translocation via the classical Sec secretion system. PknG also does not have the sequence motif at the C-terminus required for secretion via the recently characterized specialized secretion machinery RD1 in *M. tuberculosis* (81). In addition to the characterized RD1 secretion machinery, there are five RD1-homologous regions distributed at different loci in the genome of *M. tuberculosis* (82). Whether PknG is secreted via an RD1-like secretion machinery

Figure 6

PknG-dependent trafficking of pathogenic mycobacteria and crystal structure of PknG with its inhibitor AX20017. (*a*) Model of PknG-mediated phagosome-lysosome fusion blockage by pathogenic mycobacteria. PknG (*purple*) is secreted by pathogenic mycobacteria into the phagosomal lumen and cytosol of macrophages to block the fusion of phagosomes to lysosomes. Deletion of the *pknG* gene leads to rapid lysosomal transfer and destruction of the resulting mutant. (*b*) X-ray crystal structure of the PknG-AX20017 complex. Ribbon representation of the dimeric PknG-AX20017 complex as seen in the asymmetric unit of the crystal. The three PknG domains of the left subunit are labeled and depicted in different colors. Rubredoxin domain (RD) with the bound metal, cadmium in the present structure, depicted as a sphere. KD, kinase domain; TPRD, tetratricopeptide repeat-containing domain. Inserted panel shows picture of PknG crystal. Figure reproduced with permission from (84).

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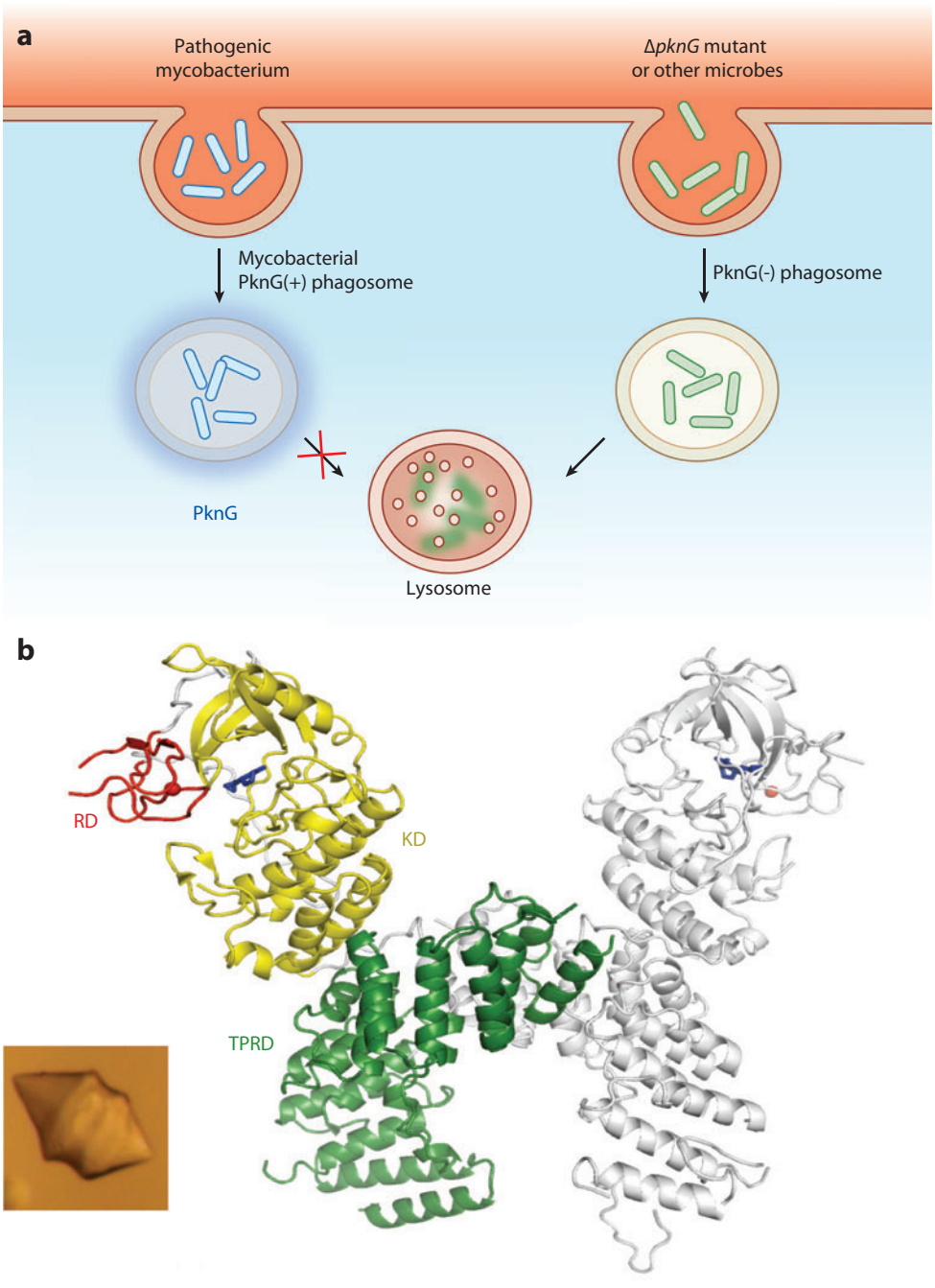

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remains to be investigated. Another question is how PknG, after secretion from mycobacteria, translocate to the outer surface of phagosomes and cytosol. Interestingly, mycobacterial infection results in permeability of phagosomal membranes, allowing accessibility of molecules of up to 70 kDa through the membrane (83). This ability may enable antigen presentation processes as well as facilitating protein translocation, thus allowing mycobacterial communication with signaling pathways in the host cytoplasm.

In addition to genetic evidence, chemical inhibition of PknG also confirms the role of this kinase in blocking the phagosome-lysosome fusion process. A screen of more than 10,000 compounds for PknG inhibitors identified a tetrahydrobenzothiophene, named AX20017, which specifically inhibits the kinase activity of PknG. When added to macrophages infected with *M. tuberculosis* and *M. bovis* BCG, AX20017 induces the maturation of phagosomes and promotes destruction of the intracellular mycobacteria (79) (**Figure 6**). The crystal structure of PknG in complex with its inhibitor AX20017 reveals the binding site of the inhibitor, which is located deep within the adenosine-binding site, targeting an active conformation of the kinase domain (84) (**Figure 6**). This kinase domain is flanked by an N-terminal rubredoxin domain and a C-terminal tetratricopeptide repeat-containing domain. Interestingly, AX20017 also targets a unique set of amino acid residues adjacent to the ATP site that are not found in any human kinases (84). This unique interaction provides a high specificity of AX20017 to PknG. Indeed, in vitro phosphorylation assays showed that AX20017 has no significant activity on 28 archetypical human kinases classified in 6 major kinase groups (84). This work raises the possibility that rational design could improve AX20017 activity to develop it into drugs that target persistent *M. tuberculosis* and thus cure latent tuberculosis.

The activity of PknG in blocking phagosome-lysosome fusion suggests that this kinase might be an excellent target for the development of drugs that induce mycobacterial death inside macrophages. An advantage of targeting PknG is that it does not kill the bacteria per se but instead facilitates the macrophage to carry out its natural antibacterial activity, delivering intracellularly surviving mycobacteria to lysosomes for destruction. Another possible advantage of targeting a secreted molecule such as PknG is that its inhibitors are not required to transport through the extremely impermeable mycobacterial cell wall. This may greatly improve the bactericidal activity of the compounds.

HOST-MYCOBACTERIAL INTERACTIONS IN ACTIVATED MACROPHAGES

Lrg-47

The phagosome-lysosome fusion blockage by pathogenic mycobacteria is bypassed when macrophages are activated by interferon-γ (IFN-γ) (85). IFN-γ is an essential component of immunological defense against infections (86). In fact, several immune mechanisms, such as leukocyte-endothelium cell interactions, antigen presentation, reactive nitrogen and oxygen intermediates, cell growth and apoptosis, as well as phagosome-lysosome fusion, can be modulated through the activity of IFN-γ (87). Both mice and humans with genetic defects in IFN-γ signaling are highly susceptible to mycobacterial diseases (88). How IFN-γ-induced signaling may overcome the mycobacterial blockage of phagosome-lysosome fusion process and eliminate mycobacteria persisting within phagosomes is still not completely known. Recently, an IFN-γ effector required for restriction of intracellular growth of pathogenic mycobacteria was identified. Lrg-47, a guanosine triphosphatase protein, is a member of the large family of the IFN-γ-inducible P47 GTPases, which are regulators of immunity to a variety of intracellular pathogens (85, 89). In cultured macrophages, Lrg-47 also appears to be involved in the regulation of phagosome maturation.

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Lrg-47 is normally associated with Golgi membranes, but upon phagocytosis of cargo it becomes translocated to the plasma membrane and is cointernalized with phagosomes (90). Interestingly, in Lrg-47-depleted macrophages, activation by IFN-γ is no longer able to restore phagosome-lysosome fusion and eradicate intracellular mycobacteria. This observation suggests that Lrg-47 is a direct mediator of IFN-γ function in the restoration of phagosomal maturation blocked by pathogenic mycobacteria. As a consequence, mice lacking Lrg-47 are unable to control mycobacterial infection. This confirms the importance of phagosome maturation in the host control of *M. tuberculosis* proliferation (91, 92).

Autophagy and Ubiquitin

IFN-γ activation of macrophages also induces autophagy, a classical degradation process in eukaryotic cells to remove unwanted cytoplasmic proteins and organelles by sequestering them into a vacuole called an autophagosome that finally fuses with the lysosome (93). Besides ensuring degradation of autophagocytic cargo, autophagy is also implicated in innate immune mechanisms against a variety of intracellular pathogens, including *M. tuberculosis* (94–96). Starvation of macrophages, the conventional inducer of autophagy, (*a*) increases colocalization of internalized mycobacteria with autophagosomal and lysosomal markers, (*b*) induces the transfer of mycobacteria to acidic compartments, and (*c*) reduces intracellular mycobacterial survival, suggesting that autophagy can overcome the block in phagosome-lysosome fusion by mycobacteria.

Similar to its role in IFN-γ modulated phagosome maturation and mycobacterial killing (91), expression of Lrg-47 also induces the formation of autophagolysosomes and leads to reduced mycobacterial survival in macrophages (96). This observation confirms the role of Lrg-47 as the key mediator of IFN-γ-activated killing of intracellular mycobacteria. Because autophagy plays several important functions in both innate and adaptive immunity and its disorder leads to many diseases (97), how this process is specialized in vivo for the defense against mycobacterial and other infections remains to be established.

How pathogenic mycobacteria counter autophagy is not known. Because autophagy is dependent on PI3P (98), mycobacteria may prevent autophagy by inhibiting PI3P production. Alternatively, as *M. tuberculosis* is able to translocate from phagosomes to the macrophage cytoplasm using the secreted ESAT-6 protein (99) (see more details below), it is possible that pathogenic mycobacteria use the same strategy as *Shigella* to respond to autophagy (94).

Macrophage activation and autophagy, besides restoring the phagosome maturation process, also induces a mycobactericidal mechanism which depends on the ubiquitin-mediated protein degradation process (100). Ubiquitin (ubiquitous immunopoietic polypeptide) is a small linear polypeptide of 76 amino acids that is highly conserved and ubiquitously present in eukaryotes. Ubiquitination is a process in which proteins are marked for proteolytic destruction by the attachment of one or more ubiquitin molecules to a lysine residue of the target protein (101). A search for molecules present in macrophage lysosomes having mycobactericidal activity identified a cathepsin-digested ubiquitin derived peptide called Ub2, which effectively kills mycobacteria, whereas full-length ubiquitin does not (100). It was proposed that ubiquitinated proteins delivered to lysosomes for degradation are digested by the local proteinase cathepsin, which releases the Ub2 and other ubiquitin-derived peptides that have mycobactericidal activity (100). Induction of autophagy accelerates transport of ubiquitinated proteins to the lysosome, which coincides with increased killing of intracellular mycobacteria (100). This finding suggests that the increased fusion of phagosomes or autophagosomes to lysosomes in activated macrophages and the subsequent lysosomal destruction are not two separate mechanisms but are orchestrated to eliminate intracellular mycobacteria.

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Toll-Like Receptors and the Vitamin D Dependent Cathelicidin Response

Toll-like receptors (TLRs) mediate the activation of cells of the innate immune system, resulting in destruction of invading microbes through the activation of signaling cascades that also trigger the adaptive immune system (102). TLR stimulation induces the production of several cytokines that activate immune cells and directly upregulates phagocytosis of bacteria. Interestingly, TLRs appear to regulate fusion events between phagosomes and lysosomes, rather than accelerate the uptake per se, which could occur as a result of local signaling events at the phagosomal membrane (103–105).

A breakthrough has been made recently in understanding how TLR-mediated host cell eradication of intracellular mycobacteria takes place. TLR activation leads to upregulation in expression of the vitamin D receptor and the vitamin D-1-hydrolase genes, which in turn induce the production of the antimicrobial peptide cathelicidin that effectively kills intracellular *M. tuberculosis* (106). This work also established the role for vitamin D in tuberculosis and explained the differences in susceptibility of different ethnic populations to tuberculosis. African Americans, because of the high content of melanin in their skin, usually have a lower UV light-dependent vitamin D3 synthetic capacity. The lower serum level of provitamin D3 hormone may lead to a lower cathelicidin response when exposed to *M. tuberculosis*, thereby reducing the innate bactericidal activity of the immunity against the bacterium.

Methylglyoxal

Methylglyoxal, a highly reactive metabolic intermediate produced by several metabolic pathways in cells of eukaryotic and prokaryotic origins, was recently discovered to be an unusual host factor important for controlling intracellular mycobacteria (107). The physiological function of this compound is not known. However, it is clear that methylglyoxal is highly toxic to cells, and increased production of methylglyoxal may contribute to aging, diabetes, and other disorders. This electrophilic chemical interacts with macromolecules such as proteins and lipids and thus interferes with their functions. Recent work also indicated that methylglyoxal and its advanced glycation end products (AGEs) trigger generation of reactive oxygen species, apoptosis, and MAPK activation (108). Interestingly, intracellular concentrations of methylglyoxal and AGE are elevated during mycobacterial infection of macrophages (109). These high concentrations lead to apoptosis and activation of macrophages, resulting in eventual elimination of the intracellular bacilli. Gene expression profiling demonstrated that methylglyoxal induces diverse responses in the host cells, which include the induction of genes involved in apoptosis and immune responses (109). The role of methylglyoxal in tuberculosis pathology is further evidenced by the fact that local concentrations of methylglyoxal and AGEs in pulmonary lesions are significantly elevated in tuberculosis patients (109). Although controversial data suggest that pathogenic mycobacteria may inhibit or induce apoptosis as their tactic for survival, apoptosis may play a role in interactions between host immune systems and pathogenic mycobacteria within granulomatous lesions (110). Further work is required to understand how pathogenic mycobacteria are tolerant to this bactericide and whether they are able to modulate the mechanism of methylglyoxal-mediated apoptosis.

Nitric Oxide

Upon macrophage activation, several mechanisms are utilized by host immune systems to eradicate internalized microbes. Together with lysosomal delivery and increased production of antimicrobial peptides such as cathelicidin and ubiquitin, reducing pH and increasing the local concentrations of reactive oxygen and reactive nitrogen intermediates such as nitric oxide also aid in bacterial

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destruction. In macrophages, nitric oxide is generated by inducible nitric oxide synthase (iNOS). iNOS is a cytosolic enzyme that catalyzes the conversion of L-arginine to L-citrulline and nitric oxide. Nitric oxide is highly bactericidal (111) and plays an essential role in control of mycobacterial infection. Mice lacking iNOS succumb rapidly to *M. tuberculosis* (112). Similarly, inhibition of iNOS functions during acute *M. tuberculosis* infection leads to rapidly fatal disease progression and accelerated bacterial replication in host organs (113). Furthermore, treatment of mice latently infected with *M. tuberculosis* by the iNOS2 inhibitor aminoguanidine leads to reactivation of the persistent *M. tuberculosis* (114).

iNOS expression is under the control of IFN- γ (115); IFN-γ disrupted mice are unable to produce reactive nitrogen intermediates in early infection and are also unable to control *M. tuberculosis* infection (116). IFN-γ not only controls iNOS synthesis but also affects its cellular localization. In macrophages that have internalized latex beads or *Escherichia coli* and are activated by IFN-γ and lipopolysaccharides, iNOS is recruited from the cytosol to the phagosomal membrane, which may help to increase the local biosynthesis and concentration of NO within the phagosome vacuole. Interestingly, in activated macrophages infected with pathogenic mycobacteria, iNOS does not localize to the phagosome harboring the bacteria (117). Thus, pathogenic mycobacteria may counter the destructive effects of nitric oxide by disassembling iNOS from phagosomal membranes in order to lower the phagosomal concentration of NO.

How can pathogenic mycobacteria recognize and respond to this bactericidal chemical? Recent work suggests that the WhiB protein family (see above) may function as redox-responsive transcription regulators helping *M. tuberculosis* to respond to oxidative stress and reactive nitrogen intermediate. The iron-sulfur cluster in WhiB3 protein specifically interacts with O_2 and NO, which may lead to specific transcriptional control upon gene expression of metabolic or stress responsive genes (118). Signaling pathways leading to the NO response may also be mediated by the mycobacterial protein kinase PknE (119). Exposure of mycobacteria to NO leads to upregulation of promoter activity, and *M. tuberculosis* lacking the *pknE* gene is more resistant to NO donors and induces increased apoptosis when infecting macrophages (119).

Interestingly, bactericidal activity of NO can also be neutralized by the mycobacterial proteasome (120). The proteasome is a multisubunit molecular machinery, highly conserved from archaebacteria to humans and responsible for the proteolysis of cytoplasmic proteins (101). In eukaryotes, the proteasome is involved in multiple cellular events, including cell cycle, development and differentiation, immune and inflammatory responses, and signal transduction, but the function of the proteasome in prokaryotes is less clear (121). *M. tuberculosis* has adapted the proteasome machinery to protect itself from the killing effect of NO (120, 122). Deletion of genes encoding proteins involved in the formation of the proteasome causes hypersensitivity of the bacilli to NO (120) and results in attenuation of mycobacterial persistence in mice (120, 123). Although the mycobacterial proteasome clearly functions in protein degradation (124), it remains to be elucidated if the protease activity is involved in the resistance to NO. In addition, as the primary substrates of the proteasome are enzymes required for the synthesis of fatty acids and polyketides essential for *M. tuberculosis* virulence (124), further study is required to evaluate the role of NO resistance in the proteasome involvement in tuberculosis pathogenesis.

Mycobacterial Escape from Phagolysosome

If the innate immune mechanisms described above have such a powerful ability to overrule the mycobacterial phagosomal maturation blockage as well as enhance mycobacterial killing when macrophages are activated, how do pathogenic mycobacteria survive upon macrophage activation? A recent observation may provide an explanation (99). At later periods during in vitro infection

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when macrophages undergo gradual activation, *M. tuberculosis* progressively translocates from its resident phagosome (that is under attack by lysosomal fusion) into the cytosol, which leads to acceleration of the macrophage apoptosis process (99). Indeed, the ability of translocation from phagosomes to the cytosol was described earlier for *M. tuberculosis* (125) and *M. marinum* (126). Once in the cytosol, *M. marinum* can also recruit host cell cytoskeletal factors to induce actin polymerization, which leads to direct cell-to-cell spread (126). This ability depends on the secretion of the mycobacterial virulence factors ESAT-6 and CFP-10; dead *M. tuberculosis* or live *M. bovis* BCG (which cannot secrete ESAT-6 and CFP-10 because of the lack of the secretion machinery RD1) are unable to escape from phagolysosomes (99, 125). In *M. marinum*, secretion of ESAT-6 and CFP-10 promotes phagosomal maturation (127) and cytolysis of host cells, which is also required for cell-to-cell spread of infection (128). Translocation of *M. tuberculosis* from the residing phagosome under attack by lysosomal fusion to the cytosol may help the bacterium to escape the dead end once their blockage of phagosome-lysosome fusion fails. This strategy may also help to spread the initial infection to other cells in close contact. Interestingly, macrophages infected with *M. tuberculosis* isolated directly from tuberculosis patients showed that the bacillus residing within phagosomes arrests the progression of lysosomal delivery (129). Further studies are therefore required to identify the in vivo mechanisms leading to mycobacterial translocation to the cytosol (99) as well as to address the relevance of this phenomenon to human tuberculosis.

CONCLUSIONS AND PERSPECTIVES

The last 50 years have brought drastic changes in the relationship between humans and pathogenic bacteria such as *M. tuberculosis*, which have coexisted and coevolved with their human host for thousands of years. The long-established balance was broken by the addition of a new component: antibiotics. The use of antibiotics has undoubtedly revolutionized chemotherapeutic treatments of infectious diseases and saved billions of lives. However, it will probably take hundreds of years to see what this new relationship brings and who (we or the bacteria) will eventually win the battle. The exposure to a massive amount of antibiotics within a short period has unexpectedly accelerated the evolution of pathogenic bacteria toward adaptation and increased tolerance to the drugs. The innate ability of bacteria to survive and resist chemical eradication is probably beyond our expectation (130). Besides the ability to quickly acquire mutations in target genes conferring resistance to antimycobacterial drugs, the genetic backup of *M. tuberculosis* allows the bacterium to resist most available antibiotics, including those that are never used for antituberculosis treatment and synthetic drugs that the bacterium has never been exposed to before. This natural resistance is built on an impermeable cell wall limiting the access of antibiotics to their targets and an effective internal defense system composed of many antibiotic-resistant structural proteins whose expression is under the control of drug-responsive regulators. Pharmaceutical inhibition of these drug resistance systems might be an alternative to searching for brand new drugs, which may again quickly encounter (and thus also introduce) new resistance mechanisms. Currently ineffective antibiotics, used in combination with potentiators inhibiting resistance mechanisms (and thus rendering mycobacteria susceptible to the drugs), may help to broaden chemotherapeutic options for antituberculosis treatment. Besides the ability to tolerate antibiotics, *M. tuberculosis* has evolved multiple molecular mechanisms allowing it to resist destruction by the host. In spite of numerous bactericidal strategies utilized by the host immune system in both resting and activated macrophages, *M. tuberculosis* can survive and evade many of those mechanisms (**Table 1**).

Interesting questions remaining to be answered are whether a specialized developmental or virulence state of pathogenic bacteria determines changes in their antibiotic tolerance, and whether exposures to antibiotics influence transitions of developmental, metabolic, or virulence states.

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Table 1 Summary of factors involved in *Mycobacterium*-macrophage interactions and mycobacterial survival

Host factors		Mycobacterial factors	
Naive	Coronin 1-calcineurin pathway	LpdC	
	Regulatory lipid PI3P pathway	ManLAM, PIM, SapM	
	Host protein kinases (PKC, PKB, etc.)	SapM ?, ManLAM ?, Mycobacterial protein kinases (PknG, PknH,. . .) ?	
Activation	Interferon-γ Lrg-47 Autophagy Ubiquitin degradation Apoptosis	WhiB7 ?	Escape macrophages ?
	Toll-like receptor and Vitamin D-dependent Cathelicidin biosynthesis	TLR agonists (ESAT-6) ?	
	Methylglyoxal-mediated apoptosis	PknE ?	
	Nitric oxide toxicity	Proteasome, PknE, WhiB-like proteins Mycobacterial exclusion of iNOS	

Although intrinsic antibiotic resistance in mycobacteria has been viewed as an isolated characteristic of mycobacterial species, in vivo and in vitro models have demonstrated that *M. tuberculosis* surviving in a dormant state has a higher antibiotic tolerance compared with that of the bacterium during an active infection (131). In the lung, mycobacterial infection is normally contained by macrophages within granulomatous structures. *M. tuberculosis* may remain in a dormant state within granulomas for extended periods of time when they are phenotypically more resistant to antibiotics. Conversely, antibiotics may also trigger *M. tuberculosis* to transform from its vegetative growth to a dormant state. Thus, antibiotic tolerance relates both to phenotypic metabolic changes induced by conditions in the macrophage as well as to genotypic changes due to selective pressure imposed by chemotherapy and host immunity. Considering possible medical applications, the correlation of intrinsic antibiotic resistance with mycobacterial persistence suggests that these mechanisms might be coregulated, possibly allowing the inactivation of both processes by a single compound.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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