Efflux-Mediated Multidrug Tolerance in Replicating Mycobacteria

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Abstract

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A major roadblock in the global eradication of tuberculosis lies in the long duration of treatment required for cure. This well documented need for long-term therapy is historically attributed to the infecting mycobacteria becoming dormant within the host so that they are no longer killed by currently used antitubercular drugs that target actively growing bacteria. The dormancy model of tolerance has been the center of antitubercular drug research with the pursuit of drugs to target bacterial determinants that are expressed in the nonreplicating phase of infection. My PhD dissertation research determined that replicating bacteria become tolerant to multiple antitubercular agents upon macrophage residence through the induction of distinct efflux pumps that are also required for intracellular growth. My findings provide a mechanistic explanation for the clinical tuberculosis treatment data gathered over the last 50 years where the length of treatment required for cure correlates with bacterial burdens. Moreover, the addition of the calcium channel blocker, verapamil reverses this tolerance to the front-line drugs, isoniazid and rifampicin. If the tolerance mechanism revealed by my work is indeed a substantial contributor to the tolerance of active tuberculosis, then the addition of efflux pump inhibitors such as verapamil to the current antitubercular regimen should shorten the course of curative therapy.

Efflux-mediated multidrug tolerance in replicating mycobacteria

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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), is a global health problem resulting in two million deaths annually (WHO, 2011). With an estimated 9 million incident cases in 2010, there has been a concerted effort to reduce the number of TB cases through the Stop TB Partnership with the goal of less than one TB case per million by 2050 (WHO, 2011). TB typically presents in the lungs (pulmonary TB) but can also affect other sites (extrapulmonary TB). The disease is spread by inhaling aerosolized droplets from an individual with pulmonary disease. After TB exposure, multiple outcomes are possible such as clearance, progression to active disease, or establishment of an asymptomatic state (latency) which may be complicated by later reactivation of disease (Flynn and Chan, 2001; Gomez and McKinney, 2004). The lack of an efficacious vaccine and the need for lengthy multidrug therapy are the major obstacles for eradication.

Due to the intrinsic resistance of MTB to many antibiotics, TB chemotherapy is restricted to a limited number of drugs (WHO, 2009). At minimum, six months of combination antibiotic treatment is required for cure (Connolly et al., 2007; WHO, 2009). Since the discovery of pyrazinamide over sixty years ago (Ryan, 1992), drug discovery efforts have yet to produce a new front-line TB drug, indicating that a better understanding of MTB's resistance mechanisms to antibiotics is needed to identify new drugs and decrease the duration of therapy.

MTB initially infects and replicates within single alveolar macrophages that then migrate into deeper tissues and aggregate to form the hallmark lesion of TB, the granuloma (Adams, 1976; Russell et al., 2010). The granuloma is an organized structure made up of macrophages and

lymphocytes (Adams, 1976; Russell, 2007) and in human granulomas, a central acellular core of necrotic material called caseum develops and in some lesions, the caseum contains large numbers of mycobacteria (Connolly et al., 2007). Despite being a concentrated focus of host immune cells, granulomas often fail to clear MTB, which can persist for long periods of time in the granuloma (Cosma et al., 2003; Russell, 2007).

Studies show mycobacterial growth to be restricted in Th1 cytokine-stimulated cultured macrophages or upon coincubation with lymphocytes, which suggests that an adaptive immune response has an important role in bacterial killing (Flesch and Kaufmann, 1990; Russell, 1995). However, innate immune mechanisms are increasingly appreciated to have bacteriostatic or bactericidal effects during the initial stages of mycobacterial infection. Using the zebrafish embryo model of *Mycobacterium marinum* infection (Davis et al., 2002), Clay et al demonstrated that naïve macrophages can limit mycobacterial numbers and that a substantial portion of this restriction is dependent on tumor necrosis factor (TNF) (Clay et al., 2007b; Clay et al., 2008). Conversely, the ability of these bacteria to survive and cause disease is dependent upon specific virulence determinants. Whole genome screens looking at bacterial genes required for growth in vitro, in macrophages and in the mouse has lead to the discovery of a huge number of newly-recognized bacterial factors involved in pathogenesis and survival (Fontan et al., 2008a; Sassetti et al., 2001; Sassetti and Rubin, 2003; Schnappinger et al., 2003b).

Current models for drug tolerance and drug discovery efforts are focused on MTB persistence. Studies dating back to the early twentieth century have clearly documented this. Prior to the discovery of antibiotics, only some patients were able to clear TB while the majority either succumbed rapidly to infection or were able to control the disease temporarily. The persistence of MTB was highlighted in a study by Corper and Cohn, who in 1920 left several hundred sealed

cultures of human and bovine isolates in an incubator for twelve years and found that they could isolate culturable bacteria (Corper and Cohn, 1933) reviewed in (Gomez and McKinney, 2004). Of the viable bacteria, those known to be virulent at the beginning remained so after the twelve year incubation period (Corper and Cohn, 1933).

Another study in the 1920's by Opie and Aronson also demonstrated the persistence of MTB by inoculating guinea pigs with specimens collected from deceased TB patients (Opie and Aronson, 1927). They found that even grossly normal-looking lung tissue was able to cause disease. Additionally, they also observed that infectivity varied depending on the lesion type. Fibrotic lesions were most likely to cause disease while calcified or encapsulated lesions rarely did (Opie and Aronson, 1927). Later studies also demonstrated that primary calcified lesions and the tissue surrounding was not likely to be infectious (Feldman and Baggenstoss, 1938, 1939).

The discovery of streptomycin by Schatz and Waksman and para-amino salicylate by Lehmann in the 1940's signified a cure for the "Great White Plague." Upon antibiotic treatment, relapse rates decreased to 20% and once long term multi-drug therapy was introduced, post-treatment relapse rates fell to 1-2% (Gomez and McKinney, 2004; van Rie et al., 1999). However, it was soon realized the elimination of TB would be much more difficult than originally anticipated, with studies demonstrating that drug-sensitive MTB was persisting in the face of multidrug therapy (McCune and Tompsett, 1956). A better understanding of MTB antibiotic resistance and tolerance mechanisms was needed to address this problem.

Antibiotic resistance mechanisms in MTB:

There are a number of mechanisms and pathways that limit drug efficacy in MTB, which contribute to the need for multidrug and long term antibiotic therapy. These include intrinsic, genetic and phenotypic antibiotic resistance mechanisms.

MTB is intrinsically resistant to many of the antibiotics that are currently in use. A major determinant of intrinsic resistance of MTB is its unique cell wall structure, which serves as an effective permeability barrier to both hydrophobic and hydrophilic solutes (Minnikin et al., 2002; Warner and Mizrahi, 2006b). The various classes of mycolic acids that make up the outer membrane have been shown to change depending on growth phase, oxygen tension and intraphagosomal growth (Minnikin et al., 2002).

Mycobacteria also contain a number of efflux systems that are capable of expelling antibiotics and other toxic compounds from the cell. For example, exposure of MTB to isoniazid (INH) and ethambutol (EMB) results in the upregulation of *iniA*, a transmembrane protein that has been shown to confer tolerance to cell wall inhibitors through a multi-drug resistant (MDR) pump mechanism (Alland et al., 2000a; Colangeli et al., 2005b). Overexpression of the histone-like protein Lsr2 was shown to repress *iniA* when exposed to INH and EMB making it an interesting candidate to study how MTB responds to certain antibiotics (Colangeli et al., 2007). Transcription of *whiB7* regulon, which confers low-level resistance to multiple antibiotics, has been shown to be induced in response to subinhibitory antibiotic concentrations as well as to exposure to fatty acids that are likely present in the *in vivo* environment from early timepoints of infection (Burian et al., 2012; Geiman et al., 2006; Morris et al., 2005). Recently, it was demonstrated that activation of WhiB7 in response to certain antimicrobials conferred cross resistance to other classes of anti-tubercular drugs (Burian et al., 2012).

Genetic antibiotic resistance refers to susceptible bacteria becoming resistant to antibiotics as a result of mutations (2005). Multi-drug treatment is designed to counter this type of resistance (Connolly et al., 2007). Identifying mutations that confer antibiotic resistance also gives insight into the mechanisms of antibiotic resistance and drug action (2005). MTB is unique among bacteria in that three of the antibiotics used to treat infection require activation, hence resistance to these drugs can be mediated by mutations that inhibit prodrug conversion or modification of the drug target (2005). The catalase-peroxidase KatG oxidizes INH resulting in an INH-NADH adduct that is an inhibitor of InhA, a protein involved in MTB cell wall biosynthesis (Ghiladi et al., 2005; Pym et al., 2001). Mutations in *katG*, *inhA* and *ndh* (NADH dehydrogenase II) all confer resistance to INH in MTB (Ghiladi et al., 2005). In contrast, the mechanism of genetic resistance to antibiotics such as RIF and streptomycin parallels that seen in other bacteria with mutation of the antibiotic targets (*rpoB* for RIF and *rpsL* and *rrs* for streptomycin)(2005).

Phenotypic resistance is another major problem for antibiotic therapy. This mechanism of resistance is thought to underlie early relapses with genetically susceptible bacteria in TB patients who have completed a full course of multidrug therapy (Connolly et al., 2007). Additionally, phenotypic resistance can contribute to genetic resistance. Phenotypic antibiotic resistance has been proposed to arise late in infection by exposure of the bacteria to growth limiting factors (hypoxia, nutrient limitation, host defenses) that essentially render the bacteria non-replicating within the granuloma and thus resistant to antibiotics that are effective only against replicating bacteria (Connolly et al., 2007; Gomez and McKinney, 2004; Russell, 2007).

There are a number of potential mechanisms that have been proposed to lead to non-replication of MTB during infection that are common in other bacteria. Bacterial persisters are a stochastically determined subset of non-replicating bacteria that arise in an otherwise growing

bacterial population (Lewis, 2007). These persisters are characterized by their slow or non-growth state making them resistant to antibiotics (Lewis, 2007; Shah et al., 2006). The mechanisms controlling the switch to a persistent state are beginning to be understood and include activation of the stringent response and toxin-antitoxin (TA) modules that can lead to growth arrest (Lewis, 2007). Interestingly, a recent study by Nguyen et al. demonstrates that the active stringent response mediates antibiotic tolerance in *Pseudomonsa aeruginosa* biofilms (Nguyen et al., 2011). These investigators found that inactivation of the stringent response increased levels of oxidant stress and also increased sensitivity to antibiotics (Nguyen et al., 2011). This increased sensitivity was independent of passive growth arrest, indicating that antibiotic tolerance, even during nutrient deprivation, is an active process (Nguyen et al., 2011).

Most models looking at phenotypic resistance in MTB operate under the assumption that the bacteria are non-replicating or metabolically inactive. While slow and non-replicating bacteria are likely playing a role in bacterial persistence and tolerance, other mechanisms are possible. New methods looking at mycobacterial growth *in vitro* and *in vivo* are being developed (Aldridge et al., 2012; Gill et al., 2009), which can be used to study the linkage between replication and antibiotic tolerance. Gill et al developed a method using an unstable plasmid that is lost at a steady quantifiable rate in replicating, but not non-replicating bacteria (Gill et al., 2009). Using the unstable plasmid in a mouse model of MTB infection, they were able to calculate bacterial growth and death rates in vivo (Gill et al., 2009). They found that even when bacterial numbers remain unchanged late during infection, there is in fact a substantial amount of bacterial growth occurring but is countered by bacterial death mediated by the host immune response (Gill et al., 2009). Recently, Aldridge et al were able image a single mycobacterium in a microfluidic chamber to examine bacterial growth and responses to various stresses, including antibiotics (Aldridge et al.,

2012). They found that due to the unipolar and asymmetric nature of mycobacterial cell division, there are subpopulations of bacteria within the clonal population that are differentially susceptible to antibiotics (Aldridge et al., 2012).

The hypothesis that phenotypic resistance is not just an attribute of non-replicating bacteria may have interesting implications for interpreting clinical data collected from pulmonary TB patients. When evaluating the relapse rates of short-course anti-tubercular drug treatment in pulmonary TB patients, a theme emerges indicating that rate of relapse is proportional to bacterial burden rather than lesion type (Connolly et al., 2007). These observations indicate that even during active disease, when a large proportion of the bacteria are replicating, phenotypic resistance is still playing a major role.

Phenotypic drug tolerance to INH has been extensively studied in human early bactericidal activity (EBA) studies (Donald and Diacon, 2008b; Jindani et al., 2003b). During INH monotherapy a distinctive biphasic curve in bacterial killing is apparent, featuring an initial quick drop in bacterial numbers within the first couple days of therapy followed by a slower decrease in bacterial burden (Jindani et al., 2003b).

Combining these clinical observations, along with recent developments looking at bacterial growth during chronic infection, there are likely mechanisms other than non-replication to explain MTB's tolerance to anti-tubercular drugs. One area of increasing interest is the role and regulation of efflux pumps in MTB. While efflux pumps have been extensively studied in both Gram-negative and Gram-positive bacterial pathogens, interest in mycobacterial efflux pumps has more recently gained momentum.

Efflux pumps in MTB

Efflux pumps, also known as transporters, have been classified into five major superfamilies: the ATP-binding cassette (ABC), major facilitator superfamily (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) and multidrug and toxic-compound extrusion (MATE). Transporters from all these superfamilies are represented in mycobacteria except for the MATE family, which have not yet been reported on (da Silva et al., 2011).

The ABC family of efflux pump transporters is conserved from bacteria to humans and consists of 52 subfamilies (Li and Nikaido, 2009). They are able to expel a wide assortment of substrates driven by ATP hydrolysis, including drugs, sugars, amino acids, caboxylates, metal ions and peptides (da Silva et al., 2011; Paulsen and Lewis, 2001). There are at least 26 putative ABC drug transporters in MTB, making up 2.5% of the genome (Braibant et al., 2000; Li and Nikaido, 2009). Three ABC transporters have been described in detail to be involved in anti-tubercular drug efflux: DrrABC, Rv2686c-Rv2687c-Rv2688c and Rv0194 (Choudhuri et al., 2002; Danilchanka et al., 2008; Pasca et al., 2004) reviewed in (da Silva et al., 2011). The *drrABC* operon has been shown to be involved in efflux of ethambutol, fluoroquinolones and streptomycin (Choudhuri et al., 2002). Rv2686c-Rv2687c-Rv2688c has been shown to be involved in fluoroquinolone efflux (Pasca et al., 2004) and Rv0194 is involved in STR efflux (Danilchanka et al., 2008). However, clinically relevant resistance has not yet been linked to any of the ABC-transporters (Piddock, 2006).

The MFS family of efflux pumps is the largest group of secondary transporters (Saier, 2003). These transporters are antiporters that are thought to function as monomers. Compounds known to be exported by MFS transporters include sugars, amino acids, nucleosides, oligosaccharides, Kreb cycle intermediates, drugs and inorganic ions and cations (De Rossi et al.,

2002). MFS (along with RND, SMR and MATE) are typically energized by proton motive force (da Silva et al., 2011; Li and Nikaido, 2009). There have been at least 20 potential MFS pumps identified in the MTB genome so far (da Silva et al., 2011; Saier et al., 2009).

There are a number of MFS efflux pumps involved in the efflux of anti-tubercular drugs. Rv1258c (also known as the Tap-like efflux pump) is one of the earliest described MFS efflux pump and was originally found to be involved in resistance to tetracycline and aminoglycosides (Ainsa et al., 1998). A RIF resistant clinical isolate was identified to overexpress this pump (Siddiqi et al., 2004). Exposure to ofloxacin has been shown to induce Rv1258c (Siddiqi et al., 2004), as did RIF and INH in studies of another MDR TB isolate (Jiang et al., 2008). Additionally, Rv1258c was also responsible for RIF-tolerance early during macrophage infection (Adams et al., 2011). Rv1410c, also known as P55, is also involved in efflux of anti-tubercular drugs. A MDR TB isolate was shown to increase transcription of Rv1410c when exposed to RIF and INH (Jiang et al., 2008). Correspondingly, deletion of this pump increases sensitivity to RIF, INH and ethambutol (Bianco et al., 2011; Ramon-Garcia et al., 2009b). Rv1634 was the first MTB pump shown to confer resistance to several fluoroquinolones when expressed on a plasmid in *M. smegmatis* (De Rossi et al., 2002). JefA (Rv2459) was identified in MDR MTB isolates as being involved in INH and ethambutol resistance since overexpression of this pump was observed following drug exposure (Gupta et al., 2010b; Gupta et al., 2010c).

Although RND efflux pumps are most commonly found in Gram-negative bacteria, MTB contains 13-putative RND transporters designated MmpL (mycobacterial membrane proteins large). However, inactivation of 11 or 13 MmpL genes (including MmpL7) did not alter drug susceptibility (Domenech et al., 2005). When MmpL7 was over expressed in *Mycobacterium*

smegmatis, it was able to confer high-level resistance to INH, which was reversed in the presence of the efflux pump inhibitors (Pasca et al., 2005).

The Mmr protein of MTB has been identified as an SMR family pump that confers resistance to acriflavine, ethidium bromide and erythromycin when overexpressed (De Rossi et al., 1998).

Another MDR pump of interest is the *iniBAC* operon. IniBAC does not belong to any characterized class of pump. When overexpressed in *Mycobacterium bovis* BCG and MTB, IniA confers resistance to INH and ethambutol (Colangeli et al., 2005b). These two anti-tubercular drugs also induce expression of the *iniBAC* operon. Deletion of *iniA* (not *iniB* and *iniC*) restored sensitivity to INH, suggesting that IniA is a pump component (Colangeli et al., 2005b). Additionally, mutations in this operon have been found in both INH and EMB resistant strains of MTB (Ramaswamy et al., 2000; Zhang et al., 2005).

According to long-standing dogma in the TB field, the long-term antibiotic therapy is necessitated by the bacteria adopting a non-replicating state during chronic infection (Connolly et al., 2007). While this is likely the case for those harboring latent MTB, long-term therapy is still needed for those with active disease and high bacterial burdens (Connolly et al., 2007). It has been well documented with MTB and other bacterial pathogens that in vitro killing by antibiotics occurs much more rapidly than what is observed clinically (Connolly et al., 2007). Studies of another intracellular pathogen, *Legionella pneumophila,* have shown that bacterial growth in cultured macrophages rapidly induces phenotypic antibiotic resistance to multiple antibiotic classes very early during infection (Barker et al., 1995). Likewise, *Mycobacterium abscessus* was shown to have decreased susceptibility to antibiotic killing while in human macrophages (Greendyke and Byrd, 2008). Additionally, it has been shown that intracellular MTB has a higher minimum

inhibitory concentration for several anti-tubercular drugs compared to culture grown bacteria and that the difference in susceptibility was not due to lack of penetration of the host cell (Dhillon and Mitchison, 1989).

These findings raise the possibility that phenotypic antibiotic resistance arises as a result of intracellular growth during the earliest stages of infection rather than as a result of growth restricting conditions that arise only after adaptive immunity is operant. The focus of my thesis research has been to determine when pathogenic mycobacteria develop antibiotic tolerance and the mechanisms by which this occurs.

This research began using *M. marinum* as a model pathogen for TB infection. *M. marinum* is the closest genetic relative of the MTB complex in both its genetics and in its pathogenesis (Tobin and Ramakrishnan, 2008). *M. marinum* grows optimally at 25-35˚C and is a natural pathogen of fish and amphibians causing tuberculosis like disease (Ramakrishnan et al., 1997). It is also a human pathogen causing chronic skin infections in the extremities referred to as swimming pool granuloma or aquarium tank granuloma (Ramakrishnan et al., 1997). The zebrafish is a genetically tractable, natural host for *M. marinum* and also has the added benefit of being optically transparent for the first two week post-fertilization. Using this model, we were able monitor in real-time bacterial responses to multiple anti-tubercular drugs, translate our findings into a tissue-culture model system and subsequently test both *M. marinum* and MTB for early antibiotic tolerance. I have found that both *M. marinum* and MTB become tolerant to the front-line antitubercular drugs, rifampicin and INH within days of macrophage infection and that this tolerance is due to the induction of bacterial efflux pumps. The addition of efflux pump inhibitors, such as the calcium channel blocker verapamil, is effective at reversing this tolerance and may be useful as an adjunct to current antitubercular therapy.

SECTION I: Macrophage-Induced Efflux-Mediated Drug Tolerance in Replicating Mycobacteria

SUMMARY

Treatment of tuberculosis, a complex granulomatous disease, requires long-term multidrug therapy to overcome tolerance, an epigenetic drug resistance that is widely attributed to nonreplicating bacterial subpopulations. Here, we deploy *Mycobacterium marinum*-infected zebrafish larvae for in vivo characterization of antitubercular drug activity and tolerance. We describe the existence of multi-drug tolerant organisms that arise within days of infection, are enriched in the replicating intracellular population, and are amplified and disseminated by the tuberculous granuloma. Bacterial efflux pumps that are required for intracellular growth mediate this macrophage-induced tolerance. This newly discovered tolerant population also develops when *Mycobacterium tuberculosis* infects cultured human macrophages, suggesting that it contributes to the burden of drug tolerance in human tuberculosis. Efflux pump inhibitors like verapamil reduce this tolerance. Thus, the addition of this currently approved drug, or more specific inhibitors, to standard antitubercular therapy may shorten the duration of curative treatment.

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INTRODUCTION

Despite more than 50 years of effective antitubercular drugs, TB eradication remains elusive (Sacchettini et al., 2008) due to the complexity of curative treatment regimens (Connolly et al., 2007; Donald and McIlleron, 2009). Long-term therapy is required to prevent relapses with genetically drug-sensitive bacilli that become transiently resistant in the host, a phenomenon called tolerance. The best-case regimen of six months was made possible by drug combinations that presumably reduce the tolerant bacterial population (Donald and McIlleron, 2009). This so-called "short course therapy" represented a major advance as prior regimens lasted 12-18 months. However, adherence to six months of multidrug treatment is difficult, leading to relapses that perpetuate the epidemic and fuel the development of genetic resistance (Sacchettini et al., 2008). Thus, an urgent goal of antitubercular drug discovery is to overcome tolerance (Connolly et al., 2007; Sacchettini et al., 2008). Several new drugs are in development (Sacchettini et al., 2008), yet most lack this key treatment-shortening property. This failure underscores a poor understanding of TB tolerance mechanisms (Barry et al., 2009; Connolly et al., 2007; Sacchettini et al., 2008; Warner and Mizrahi, 2006a).

Drug tolerance in TB is best contextualized to the in vivo lifestyle of Mtb. Mtb resides within complex immunological structures called granulomas, either within macrophages or extracellularly, in its necrotic core (caseum) (**Figure S1**) (Barry et al., 2009; Connolly et al., 2007). Multiple granuloma types, reflecting different levels of local disease activity, may co-exist in a given patient (**Figure S1**) (Barry et al., 2009; Canetti, 1955;

Connolly et al., 2007; Rich, 1946). A longstanding model is that tolerant bacteria are sequestered in a subset of granulomas wherein their replication and metabolism are uniformly slowed (**Figure S1**) (Barry et al., 2009; Connolly et al., 2007). Newer models posit that quiescent, drug tolerant bacteria are present in all lesion types and may be induced by deterministic mechanisms responsive to stress (e.g. hypoxia) and/or the stochastic formation of persister cells, some of which may be nonculturable under standard laboratory conditions (**Figure S1**) (Barry et al., 2009; Connolly et al., 2007; Garton et al., 2008; Mukamolova et al., 2010; Sacchettini et al., 2008; Warner and Mizrahi, 2006a). However, all tolerance models and consequently drug discovery efforts are centered on the assumption that slowed growth and/or metabolic quiescence is the primary mediator of tolerance.

In this study we demonstrate that growing mycobacteria develop multi-drug tolerance soon after they infect macrophages. This understanding was gained by a two-pronged approach: first we used zebrafish larvae infected with *Mycobacterium marinum* (Mm) to spatially and temporally characterize the responses of individual animals to frontline antitubercular drugs. Like Mtb infections, Mm infections feature drug tolerance and require long-term treatment in both humans and fish (Aubry et al., 2002; Decostere et al., 2004). Using the larval model, we discovered that tolerant bacteria arise within individual macrophages soon after infection and are then expanded and disseminated by tuberculous granulomas. Guided by these findings, we used cultured human and mouse macrophage infection models combined with bacterial efflux pump mutants and pharmacological inhibitors to identify both a mechanism and a therapy for macrophage-induced tolerance in TB.

RESULTS

A zebrafish larval model for in vivo characterization of antitubercular drug activity

To assess antitubercular drug activity in the model, we infected larvae intravenously with GFP-expressing Mm and maintained them in the presence of antitubercular drugs. Concentrations that represented multiples of the in vitro minimum inhibitory concentration (MIC) for Mm were used, and the drug-supplemented water was changed daily (**Table S1.1, Figure S1.2A** and **Experimental Procedures**). We established minimal isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and moxifloxacin (MOX) concentrations that were nontoxic and normalized host survival when administered within one day post infection (dpi), which we term the minimum effective concentration (MEC) (**Figure 1.1A** and **Table S1.1**). Bacterial burdens of infected larvae can be assessed visually by fluorescence microscopy, or quantitatively by larval lysis and enumeration of bacterial colony forming units (CFU). To facilitate rapid, serial quantification of bacterial burden, we developed software that enumerates fluorescent pixels in images of infected larvae, or fluorescent pixel count (FPC), and showed it to be an accurate predictor of bacterial burdens (**Figure S1.3** and **Experimental Procedures)**. Increased survival was associated with lower bacterial burdens than in untreated controls at four days post treatment (dpt) as judged by fluorescence microscopy, and confirmed quantitatively by FPC and CFU analyses (**Figure 1.1A-1.1F, Table S1.2, Figure S1.3** and **Figure S1.4**). Findings consistent with human TB data were also obtained with streptomycin (STM), which has resurged as a cornerstone of treatment for extremely drug resistant (XDR) TB (Donald and McIlleron, 2009) (**Figure S1.2C-E**). In summary, clinically relevant anti-tubercular drugs were efficacious in the zebrafish-Mm

infection model with the expected exception of pyrazinamide to which Mm is innately resistant (Rastogi et al., 1992)(**Figure S1.2F** and **S1.2G**).

To further probe the model's relevance to human TB, we used the MECs as starting points from which to examine drug potency and dose-dependent activity. In human pulmonary TB studies, the early bactericidal activity (EBA) of antitubercular drugs, defined as their ability to reduce sputum bacterial counts in pulmonary TB patients over the first several days, has been useful to guide drug dosing (Donald and Diacon, 2008a; Jindani et al., 2003a). Each drug has distinctive EBA characteristics. For RIF, doses above the currently used therapeutic dose increase the EBA, raising the question of whether higher doses would have greater efficacy (Donald and Diacon, 2008a). In the larvae, treatment with the RIF MEC caused a 1.2 $\log_{10}$ reduction in bacterial burden, while treatment with twice the MEC caused an additional 1.2 $\log_{10}$ reduction despite increased toxicity (**Figure 1.1E-G).** We similarly observed dose-dependent activity for MOX: stepwise increases in concentration produced greater diminutions in bacterial burdens (**Figure S1.2H**).

In contrast to RIF, INH EBA does not increase when the conventional dose is doubled. We too observed no further reduction in bacterial counts at double the INH MEC (data not shown). Moreover, stepwise two-fold reductions from the conventional dose continue to show activity, with a graded EBA decrease down to $1/16^{th}$ the therapeutic dose (Donald and Diacon, 2008a). Similarly, we found a graded concentration-dependent bactericidal activity down to $1/24^{th}$ the MEC (**Figure 1.1H** and **1.1I**) and a therapeutic benefit at $1/5^{th}$ the MEC (**Figure 1.1J**).

Finally, since drug-resistant strains are increasingly associated with human TB (Sacchettini et al., 2008), we asked if the model could differentiate Mm strains with altered drug susceptibility. INH resistance is usually the first to occur in human TB and often the first step in the progression to XDR TB (Donald and McIlleron, 2009; Vilcheze and Jacobs, 2007). INH is a pro-drug activated by the bacterial catalase KatG and the vast majority of INH-resistant clinical Mtb isolates have *katG* mutations (Heym et al., 1999; Vilcheze and Jacobs, 2007). We identified a spontaneous Mm INH-resistant *katG* mutant with an vitro INH MIC of 464 μM, 8-fold higher than for wildtype (**Figure 1.1K; Experimental Procedures**). The relative INH resistance of the Mm *katG* mutant was detectable in vivo: in larvae infected with wildtype bacteria, maximal bacterial killing was observed at 290 μM whereas even an eight-fold higher concentration was ineffective against the *katG* mutant (**Figure 1.1L**). INH resistance was specific: EMB, which has a distinct target (Belanger et al., 1996), showed similar efficacy against both strains (**Figure 1.1L**).

In summary, the zebrafish larval model reliably replicates the activity of antitubercular compounds and their dosing characteristics in humans.

Drug tolerance occurs early in infection prior to granuloma formation

We observed that residual cultivatable bacteria persisted four dpt, despite initiation of treatment within a day of infection, when the bacteria were within individual macrophages (**Figures 1.1B-1.1D** and **S1.4**). This observation suggested the early presence of drug-tolerant bacteria, prior to granuloma formation. We pursued these observations in detail for INH because INH tolerance in human TB has been studied extensively (Donald and Diacon, 2008a; Donald and McIlleron, 2009; Jindani et al., 2003a). We confirmed that the residual

bacteria were tolerant, rather than genetically resistant to INH: the INH susceptibility of individual bacilli recovered from six INH-treated larvae (10.3 $\pm$1.2 CFU per larva) was unchanged from the parental strain.

INH tolerance is seen in human EBA studies where monotherapy produces biphasic killing. The majority of the bacteria are killed with two days of treatment, after which the rate of killing drops leaving a population of drug tolerant bacteria 14 dpt (**Figure 1.2A**)(Donald and Diacon, 2008a; Jindani et al., 2003a). We conducted an EBA-like study in the larvae, starting treatment after granulomas had formed, at three dpi. Serial quantitative tracking of infection by fluorescence microscopy revealed that INH killing kinetics mirror those observed in human EBA studies (**Figure 1.2A-C)**.

By tracking individual animals, we determined the location of the persistent bacteria (**Figure 1.2B**). Several infected macrophages had very little diminution in fluorescence, suggesting they contained tolerant bacteria, and their varying positions on consecutive days suggested their continued movement during treatment. There were also residual infected macrophages (**Figure 1.2B**, arrowhead) from granulomas that had dissolved with treatment (**Figure 1.2B**, arrow). Thus, tolerant bacteria were present both in individual macrophages and in granuloma macrophages.

In human TB, the addition of RIF to INH-containing regimens has shortened time to sterilization and thus treatment length. This is reflected in EBA studies where RIF is not rapidly bactericidal on its own and the additional bactericidal activity of the INH-RIF combination is seen only in the slower phase of killing, when INH-tolerant bacteria are implicated (**Figure 1.2A**)(Donald and Diacon, 2008a; Jindani et al., 2003a). Therefore, RIF's treatment-shortening effect is attributed to its capacity to reduce INH tolerant bacteria

(Donald and McIlleron, 2009). Reminiscent of the human data, RIF in the larvae was not rapidly bactericidal on its own (**Figure 1.2D)**, yet increased INH efficacy in the slow phase of its EBA curve (**Figure 1.2E**). Residual bacteria persisted after combination therapy, albeit fewer than with INH monotherapy (**Figure 1.2F**). In sum, the model demonstrates drug tolerance akin to that seen in human TB and the well-known synergistic effect of RIF in reducing tolerance. Furthermore, it reveals tolerant bacteria within individual macrophages at the earliest stages of infection.

Drug tolerant bacteria are expanded and disseminated by granuloma formation

All models of TB tolerance invoke metabolically quiescent bacteria (Barry et al., 2009; Connolly et al., 2007; Sacchettini et al., 2008; Warner and Mizrahi, 2006a). Yet during human TB treatment individual lesions can expand, or new lesions appear at distant sites, despite overall clinical improvement and reduction in lesion size and numbers (Akira et al., 2000; Barry et al., 2009; Bobrowitz, 1980; Canetti, 1955). Importantly, bacteria from new lesions were documented to be drug sensitive and the patients were ultimately cured with no change in therapy (reviewed in (Akira et al., 2000)). These observations are consistent with an uneven distribution of drug tolerant bacteria that can expand either locally or after dissemination. Even before the advent of effective TB therapy, it was appreciated that individual lesions within a given patient can progress differently: some resolve whilst others worsen (Canetti, 1955; Rich, 1946). These observations suggest distinct local responses that can be revealed by drug treatment. Since our data suggested that persistent bacteria were present within a few scattered macrophages, we wondered if these could then be amplified

and disseminated by mechanisms of granuloma formation described previously (Davis and Ramakrishnan, 2009).

To test this idea, we determined whether differential granuloma progression occurs in the context of untreated disease. Indeed, we found that in the first week following infection, some granulomas progressed while others resolved (**Figure 1.3A**). Lesions in the head region (containing the developing organs) were more likely to progress whereas those in the tail region (primarily muscle) were more likely to resolve (**Figure 1.3B** and 1.**3C**). The effect was even more striking at later developmental stages (days 7 to 11) when organogenesis is more advanced (**Figure 1.3C**). By one month, the muscles were devoid of infectious foci with granulomas found entirely within organs (**Figure 1.3D** and **1.3E**). This last finding is reminiscent of the observation that human TB seldom involves muscle (Rich, 1946).

To study the effect of drug treatment on individual lesions, we infected larvae with a range of inocula to achieve varying infection burdens (**Figure 1.3F**) and then began INH treatment for half the larvae. We quantified and spatially localized infection in each animal at the start of treatment and three days later by fluorescence microscopy. As expected, untreated larvae demonstrated an increase in bacterial burden (+0.2176 $\log_{10}$ FPC, P=0.0008, Student's t-test) whereas treatment reduced the bacterial burden (-1.001 $\log_{10}$ FPC).

Spatial monitoring of the untreated larvae confirmed differential progression of lesions found previously. Ten of the 12 treated larvae responded with reduction in bacterial burdens ranging from 32.3% to 99.7% (**Figure 1.3F** and **1.3G)**. We found local "nonresponsiveness" amongst the responding larvae, analogous to that observed in humans (**Figure 1.3G-3I**). For example, fish 11 (**Figure 1.3G** and **1.3H**) had an overall 32.3%

reduction in bacterial burden, yet two individual infected macrophages expanded into two infected cells and a granuloma and multiple new foci appeared. New foci developed even in animals with an overall >90% reduction in bacterial burdens (e.g. Fish 5, **Figure 1.3G** and 1.**3I**).

Finally, we determined whether new infection foci resulted from the egress of macrophages (containing tolerant bacteria) from existing granulomas, a phenomenon we have documented during untreated infection (Davis and Ramakrishnan, 2009). We infected larvae with bacteria expressing the fluorescent reporter Kaede that changes from green to red upon photoactivation (Davis and Ramakrishnan, 2009). We selectively photoactivated the largest granuloma and immediately began INH treatment (**Figure 1.3J,** left panel). We then assessed if macrophages infected with photoactivated bacteria left granulomas after initiation of treatment. Within 24 hours, three infected macrophages had left the granuloma and dispersed far from the initial site (**Figure 1.3J,** right panel). Importantly, the original granuloma had shrunk substantially showing its overall response to therapy, consistent with the human data (Bobrowitz, 1980). The bacteria within the departed macrophages were both red and green fluorescent, suggesting that they were synthesizing new Kaede protein and thus metabolically active, again consistent with the human data where the new foci had viable bacteria that were then able to expand.

In summary, as is the case in human TB, individual lesions in Mm-infected zebrafish larvae respond variably to treatment and dissemination of infection occurs during effective therapy. The detailed temporal monitoring possible in this model suggests a mechanism for these long-standing observations in humans: drug treatment favors growth of tolerant

bacteria within individual macrophages, which can then expand into granulomas by recruiting new macrophages, and then egress from the granulomas to disseminate infection.

Macrophage Residence Induces Drug Tolerance in Mm and Mtb

The drug tolerant bacteria observed within larval macrophages could result from three non-mutually exclusive mechanisms: 1) pre-existing drug tolerant persisters, 2) induction of tolerance upon exposure to the macrophage environment, and 3) induction of tolerance upon exposure to drug. Subtherapeutic concentrations of multiple drugs produce tolerance in actively growing Mtb cultures (de Steenwinkel et al., 2010; Morris et al., 2005; Viveiros et al., 2002b) and we found this to also be the case for Mm (**Figure S1.5A**). To probe the mechanism(s) of tolerance in the context of host infection, we examined the development of INH tolerance in larvae depleted of macrophages by anti-sense knock down of the PU.1 myeloid transcription factor - in the absence of macrophages the bacteria grow extracellularly (Clay et al., 2007a). Wildtype and macrophage-depleted (PU.1) larvae were infected at one dpf and INH treatment begun the following day. By two dpt, when INH tolerance is first apparent (**Figure 1.2C**), wildtype larvae had 10.4% $\pm$ 2.9% of the bacteria in the untreated controls, whereas PU.1 larvae had 3.4% $\pm$ 0.7%, a 3.1-fold reduction (**Figure 1.4A**). The improved relative efficacy of INH in the PU.1 larvae was even greater at 4 dpt with a 5.7-fold relative reduction (**Figure 1.4A** and **Table S1.3**). These data suggested that the tolerant bacterial population was enriched by macrophage residence.

To determine the mechanism of this macrophage-dependent tolerance, we turned to a cell culture infection model using J774 mouse or THP1 human macrophage cell lines (Volkman et al., 2004). Cells infected with Mm for either two or 96 hours were treated with

174 μM INH (three-fold the in vitro MIC) for an additional 48 hours and then lysed to obtain intracellular bacterial counts (**Figure 1.4B** and **1.4C**). After two hours of macrophage infection, 7.6% ± 0.8% Mm survived the subsequent 48 hours of INH treatment whereas after a 96-hour infection period, 49.5% ± 8.1% survived treatment, representing a 6.5-fold increase in persisting bacteria (**Figure 1.4B** and **1.4C**). There was no additional killing with 10-fold increased INH (1740 μM) (**Figure 1.4C**). The INH MIC for the bacteria recovered at 96 hours was unchanged from the parental strain, confirming that they had not acquired genetic resistance.

Our findings suggested that intramacrophage residence induced drug tolerance. Alternatively, drug activity could be limited intracellularly due to the residence of a bacterial subpopulation in a protected niche or to intracellular drug metabolism or modification. To differentiate between these possibilities, bacteria were grown in macrophages for two or 96 hours, released from the infected macrophages and then incubated in bacterial growth medium with and without drugs for an additional 48 hours prior to plating. Even with direct exposure to 174 μM INH, the proportion of tolerant bacteria was > 200-fold higher at 96 hours post infection than after two hours (**Figure 1.4D** and **Table S1.3**). Macrophage-conditioned Mm also developed tolerance to RIF with a 20.6-fold increase in survivors and to MOX with a 4.8-fold increase in survivors (**Figure 1.4D** and **Table S1.3**). Dilution of the macrophage lysates prior to exposure to antibiotics did not affect the proportion of tolerant organisms (**Figure S1.5B)**, excluding the possibility that tolerance was simply due to increased bacterial density at 96-hours (**Figure 1.4B**). Finally, we found that Mtb also develops macrophage-induced drug tolerance: 96 hour vs. two hour infection yielded > 2.3-fold increase in INH survival and > 2.8-fold increase in RIF survival (**Figure 1.4F** and **Table**

S1.3). Thus, macrophage residence rapidly induces Mm and Mtb to become tolerant to multiple drug classes.

Drug Tolerance is Associated with a Replicating Intracellular Population

Current models invoke host-induced bacteriostasis as a mechanism for drug tolerance during infection. Indeed, both Mtb and Mm exhibit drug tolerance under conditions of slowed growth in vitro **(Figure S1.5C**)(Paramasivan et al., 2005). Moreover, the net growth of Mm in macrophages appeared to decline slightly between 48 and 96 hours (Figure 4B), raising the possibility that tolerance might result from macrophage-induced bacteriostasis. However, both our zebrafish studies and prior human clinical data showed that bacteria expand and disseminate in vivo in the face of drug treatment. Thus, even if tolerance is initially associated with nongrowing persisters, they must somehow then retain the tolerance phenotype upon resuming growth.

To probe the replicative state of the tolerant bacteria, we manipulated intracellular growth by treating infected macrophages with dexamethasone (DEX), a broad-spectrum anti-inflammatory agent that increases net intracellular bacteria (Rook et al., 1987). DEX treatment resulted in a 2.1-fold increase in intracellular Mm at 96 hours that was accompanied by a 1.4-fold and 2.1-fold increase in the proportion of bacteria tolerant to INH and RIF, respectively (**Figure 1.5A, 1.5B** and **Table S1.4**). These experiments revealed that tolerance is not diminished by increased bacterial growth, and suggested rather that it is enhanced in growing intracellular bacteria.

We then directly compared the numbers of drug tolerant bacteria in non-growing and growing bacterial populations. We transformed Mm with the unstable plasmid pBP10 that is

lost at a constant rate from dividing, but not non-dividing mycobacteria (**Figure S1.6**) (Gill et al., 2009). Using Mm/pBP10, we showed the rate of plasmid loss per generation to be unchanged under different growth conditions, as was first shown with Mtb (**Figure S1.6** and **Supplemental Experimental Procedures**). We next used Mm/pB10 to examine bacterial growth in macrophages. The generation time of the intracellular population was not decreased over the 96-hour assay period (**Figure 1.5C** and **Figure S1.6**). Furthermore, the apparent slowed growth that we previously observed between 48 and 96 hours (**Figure 1.4B**) was in fact due to a large increase in bacterial death, which overshadowed a more modest increase in growth rate in this time period (**Figure S1.6E**).

We then compared the proportion of drug tolerant bacteria in the population that had retained the plasmid (Kan^R) (i.e. bacteria that had not yet replicated and daughter cells that had retained the plasmid upon replication) to that in the population that had lost the plasmid (Kan^S) (i.e. completed at least one round of replication). Drug tolerant bacteria were enriched in the Kan^S population: 3.5-fold for INH, 7.6-fold for RIF, and 7.6-fold for MOX (**Figure 1.5D** and **Table S1.4**). Together, these results suggested that drug tolerance was not associated with macrophage-induced bacteriostasis but rather that it was enhanced in the growing intracellular population.

Macrophage-induced bacterial efflux pumps mediate drug tolerance

One explanation for the multiclass drug tolerance we observed is a reduction in steady state intrabacterial drug concentration, which could be achieved by decreased drug entry and/or increased efflux. The induction of efflux pumps causes single or multidrug resistance in many bacteria, including Mtb (De Rossi et al., 2006; Li and Nikaido, 2009;

Louw et al., 2009b), so we investigated their potential role in macrophage-induced tolerance. Mtb and Mm encode numerous efflux pumps that can mediate resistance to antitubercular drugs when overexpressed (Li and Nikaido, 2009). Efflux pump activity has been invoked to explain multiple aspects of drug resistance in mycobacteria: 1) intrinsic resistance, 2) acquired multi-drug resistance, and 3) tolerance induced by antimicrobial exposure (De Rossi et al., 2006; Gupta et al., 2010a; Louw et al., 2009b; Morris et al., 2005; Viveiros et al., 2002b). Moreover, several mycobacterial efflux pumps and their regulators are induced during macrophage infection (Fontan et al., 2008b; Morris et al., 2005; Nguyen and Thompson, 2006; Ramon-Garcia et al., 2009a; Rohde et al., 2007b; Schnappinger et al., 2003a; Zahner et al., 2010). Finally, efflux pump inhibitors enhance the activity of certain drugs on drug-resistant Mtb; these include the currently approved pump inhibitors verapamil (VER), reserpine (RES) and thioridazine (Li and Nikaido, 2009).

To determine if bacterial efflux pumps mediated macrophage-induced tolerance, we added subinhibitory VER and RES concentrations to macrophage-released Mm and found that tolerance was reduced (**Figure 1.6A, 1.6B** and **Table S1.5**). VER produced a 15.6-fold reduction in INH survival and a 9.2-fold reduction in RIF survival; RES produced a 4.8-fold survival reduction in INH survival and a 7.9-fold reduction in RIF survival. These effects were specific to macrophage-induced tolerance: VER did not reduce stationary phase tolerance to INH or RIF (**Figure S1.7A**).

In Mtb, VER reduced tolerance to RIF but not INH (**Figure 1.6C** and **Table S1.5**). RIF survival was reduced by 1.4-fold and 1.9-fold, respectively, in bacteria grown intracellularly for 96 and 144 hours prior to challenge. This result suggests that Mtb possesses VER-resistant pumps distinct from those used by Mm. Consistent with this idea,

INH exposure induces transcription of more efflux pumps than does RIF in MDR-TB (Gupta et al., 2010a). Moreover, drug-induced INH tolerance in Mtb is sensitive to RES but not to VER (Colangeli et al., 2005b; Viveiros et al., 2002b).

Our data suggested that distinct efflux pumps mediate INH and RIF tolerance in Mtb. The Mtb Rv1258c efflux pump is transcriptionally induced upon: 1) macrophage infection (Morris et al., 2005; Schnappinger et al., 2003a) and 2) exposure to subinhibitory concentrations of RIF, but not INH, in an Mtb isolate resistant to both drugs (Siddiqi et al., 2004). Hypothesizing that Rv1258c mediates macrophage-induced RIF tolerance, we tested two Mtb strains with distinct transposon insertions in Rv1258c. After 96 hours of macrophage residence, both mutants retained INH, but lost RIF, tolerance (**Figure 1.6D** and **Table S1.5**). Indeed, they became hypersusceptible to RIF: while the wild-type had a 3.0-fold increase in tolerance, the mutants had 2.5- and 2.0-fold reductions in tolerance. Moreover, the mutants were compromised for intra-macrophage growth (**Figure 1.6E**), suggesting that this efflux pump is required for both intracellular growth and RIF tolerance.

To confirm that the loss of tolerance in the Rv1258c mutants was not simply a function of intracellular growth attenuation, we tested two additional mutants with macrophage growth defects: Mm mutants lacking the RD1/ESX-1 secretion system or the cell surface/secreted Erp protein retained antibiotic tolerance **(Figure S1.7B** and **S1.7C).** The Erp mutant developed macrophage-induced RIF tolerance despite being RIF-hypersusceptible at baseline (Cosma et al., 2006b; Gao et al., 2003). This would suggest that efflux pump induction due to intracellular residence allowed the bacteria to extrude sufficient RIF to become more resistant than at baseline, despite growth attenuation. In contrast, the

Rv1258c-deficient bacteria become hypersusceptible to RIF because their intracellular damage is coupled to an inability to expel RIF.

Finally, we showed that the Mtb Rv1258c mutants retained stationary phase-induced RIF tolerance, again showing specificity of this efflux pump in mediating macrophage-induced tolerance (**Figure S1.7D**). In sum, these results suggest that bacterial efflux pumps are induced upon macrophage infection to promote bacterial growth but also mediate drug tolerance. This mechanistic understanding explains the enrichment of tolerant bacteria in the growing intracellular population.

VER reduces intracellular Mm growth and tolerance

If VER reduces tolerance via its effects on intracellularly induced efflux pumps, it should, similar to the Rv1258c mutation, lead to growth attenuation and abrogation of tolerance when administered to infected macrophages. Indeed, efflux-pump and potassium-transport inhibitors have been found to promote intracellular killing of multi-drug resistant Mtb (Amaral et al., 2007b). We similarly found that incubation of Mm-infected macrophages with VER specifically reduced intracellular bacterial growth at concentrations that did not impact bacteria in axenic culture (**Figure 1.6F** and **S1.7A**). Incubation of infected macrophages with VER also inhibited tolerance (**Figure 1.6G**). 96 hour Mm-infected macrophages were treated with INH alone or INH and VER for an additional 48 hours before lysis and bacterial enumeration; the addition of VER reduced tolerance by 2.0 fold (**Figure 1.6G** and **Table S1.5**). Finally, when added to a synergistic combination of INH and RIF, VER further reduced tolerance by 2.2-fold (**Figure 1.6G** and **Table S1.5**), suggesting the potential of VER to increase the efficacy of existing treatment regimens. Taken together, the

finding that mutations in Rv1258c and treatment with the efflux pump inhibitor VER lead to the same two phenotypes (growth attenuation and loss of tolerance) indicates that efflux pumps are required for both processes. Moreover, the observation that VER treatment phenocopies the Rv1258c mutation argues that it is the loss of Rv1258c function in the mutants, rather than polar effects on downstream genes, that is responsible for the observed phenotypes.

Macrophage-induced tolerance persists after bacteria are rendered extracellular

Both the zebrafish larval model and the human EBA studies show that tolerant bacteria arise within a few days of treatment. However, in contrast to the intracellular bacteria present in the larvae, the bacteria assessed in the human studies are thought to be predominantly extracellular, residing within the necrotic cores of open cavitary lesions (**Figure S1.1**). Necrosis results from death of the granuloma's macrophage core, and is sustained by continued influx and lysis of infected and uninfected macrophages (Cosma et al., 2004; Dannenberg, 2003). For macrophage-induced tolerance to play a significant role in human cavitary TB, it must persist for some time after the bacteria are rendered extracellular by macrophage lysis. To test this model, we lysed Mm out of macrophages and monitored the proportion of tolerant organisms with time. Mm retained tolerance for at least 120 hours after macrophage release despite continued replication in the lysate (**Figure S1.8A** and **S1.8B**). Whether tolerance is retained due to slow turn over of the efflux pump or persistence of the original macrophage stimulus in the lysates, remains to be determined. Regardless of the mechanism, these findings argue that extracellular bacteria in cavitary TB may be rendered tolerant by prior growth in macrophages.

DISCUSSION

Drug tolerance presents a significant challenge to the eradication of TB. Here, we exploit two complementary TB infection models to demonstrate that macrophages and granulomas both play a role in the induction and expansion of drug tolerant bacteria in the face of treatment.

The zebrafish larval model has enabled a reassessment of fundamental dogmas about the role of macrophages and granulomas in TB pathogenesis (Davis and Ramakrishnan, 2009; Tobin and Ramakrishnan, 2008; Tobin et al., 2010; Volkman et al., 2010). These studies suggested that rather than the granuloma being a host-protective structure to contain infection, mycobacteria co-opt granulomas for their expansion and dissemination. We now show that tolerant bacteria arising within individual macrophages similarly exploit granulomas for their amplification and dissemination during the course of drug treatment. This finding helps to explain decades-old observations that human TB lesions can expand, and new ones appear, in the face of overall clinical and radiological response (Akira et al., 2000; Bobrowitz, 1980; Canetti, 1955).

The core finding of this study are that drug tolerant bacteria originate in the macrophage dependent on the activity of bacterial efflux pumps (**Figure 1.7**). Within a few days of macrophage infection, a bacterial subpopulation arises that manifests tolerance to multiple drugs independent of drug exposure. The association between intramacrophage growth and drug tolerance suggests that a common mechanism promotes both. This is borne out by our finding that Rv1258c is required for both macrophage growth and intracellular RIF tolerance in Mtb. The activation of bacterial efflux pumps in response to membrane and

oxidative stress and antimicrobial peptides in vitro (Morris et al., 2005; Nguyen and Thompson, 2006; Ramon-Garcia et al., 2009a; Zahner et al., 2010) suggests that their induction during intracellular residence may protect the bacteria from these same conditions in vivo. For example, efflux pumps mediate intrinsic resistance of *Neisseria meningitidis* to human antimicrobial peptides (Tzeng et al., 2005a). The finding that DEX-treated macrophages induce tolerance may shed some light on the intracellular stimuli involved. DEX-treatment suppresses macrophage oxidative bursts while preserving antimicrobial peptide expression (Duits et al., 2001a; Ehrchen et al., 2007). Antimicrobial peptides, which are induced in Mtb-infected human macrophages and are required for macrophage antimycobacterial activity (Liu et al., 2007a; Rivas-Santiago et al., 2008a), may mediate the induction of bacterial efflux pumps and thereby tolerance.

Our in vivo results show that tolerant bacteria arise within a subset of infected macrophages during treatment. This may be due to macrophage heterogeneity, again underscoring the complexity of the macrophage-*Mycobacterium* interface. Individual macrophages may cause distinct and/or differential induction of bacterial efflux pumps, and thus, variable tolerance within the bacterial population. Additionally, antibiotics have long been thought to act in co-operation with cellular host defenses, an idea further supported by the increasing recognition of the complex, multidimensional mechanisms of antibiotic activity (Kohanski et al., 2010).

How do our findings relate to human clinical studies? Among the readily cultivable bacilli in the sputum of humans with active cavitary TB, two distinct populations have been described: drug-susceptible and drug-tolerant (Jindani et al., 2003a). The metabolic status of the tolerant population has been the source of much debate. Spurred by our discovery of

early tolerance in zebrafish larvae, we revisited human EBA and radiological studies, and realized that growing, tolerant populations must mediate the progression and expansion of tubercular lesions that occur in the face of treatment (Bobrowitz, 1980; Jindani et al., 2003a). The association between tolerance and intracellular growth in the zebrafish larval and macrophage models provides a mechanism for the earlier human studies. Further strengthening this link is our finding that tolerance persists for days after the bacteria are released from macrophages suggesting that the bacteria present in the necrotic core of macrophage-lined cavities may utilize similar tolerance mechanisms.

Slowly growing bacteria that are not cultivable under standard conditions have also been observed in human sputum (Barry et al., 2009; Connolly et al., 2007; Garton et al., 2008; Mukamolova et al., 2010; Sacchettini et al., 2008; Warner and Mizrahi, 2006a). These too appear to be drug tolerant (Barry et al., 2009; Connolly et al., 2007; Garton et al., 2008; Mukamolova et al., 2010; Sacchettini et al., 2008; Warner and Mizrahi, 2006a), but their relative impact on treatment duration and clinical relapse is unclear. Importantly, tolerance models centered on nonreplicating bacteria do not account for the recent finding that diarylquinolones, which are equally bactericidal in culture for exponentially-growing and dormant bacteria (Koul et al., 2008), only shorten the time to cure from six months down to four in the mouse mode of TB (Ibrahim et al., 2009). While this shortening may be extremely important clinically, it does not support the notion that tolerance, and thus the long duration required for therapy, is mediated solely by dormant bacterial populations (Gill et al., 2009; Munoz-Elias et al., 2005). One possibility is that the growing, tolerant bacteria revealed in this work are responsible for the substantial residual tolerance observed after diarylquinolone-containing therapies.

In summary, we have identified a population of drug tolerant bacteria that are likely to be substantially present in TB patients as they are induced by macrophages, which host dynamic mycobacterial populations throughout infection. Current drug discovery efforts, with their emphasis on quiescent tolerant bacteria, fail to take into account the role of this population in tolerance. Our identification of pharmacological measures to reduce the numbers of growing tolerant bacteria suggests an approach to further shorten TB chemotherapy. Our finding that the same pump mediates both growth and tolerance suggests that the identification of more potent specific inhibitors with a dual bacterial killing mechanism is possible. Indeed, some are already being tested against Rv1258c (Sangwan et al., 2008; Sharma et al., 2010b). Ultimately, the best assessment of whether targeting these newly discovered tolerant bacteria is the key to truly short course chemotherapy will come from clinical trials using efflux pump inhibitors like VER.

Finally, the tolerance mechanisms and counter strategies that we describe for mycobacteria may be relevant to other intracellular pathogens. Indeed, macrophage-induced tolerance to multiple drug classes is described for *Legionella pneumophila,* an agent of pneumonia (Barker et al., 1995). Thus, our findings may have relevance for *Legionella* as well as other recalcitrant intracellular pathogens that produce serious and often relapsing infections where tolerance is a barrier to therapy.

EXPERIMENTAL PROCEDURES

Bacterial strains and Methods

M. marinum strain M (ATCC BAA-535) and its fluorescent derivatives have been described (Cosma et al., 2006a; Davis and Ramakrishnan, 2009). A spontaneous INH resistant mutant identified in our laboratory (KA1), was found to have increased resistance to INH (MIC 64 μg/ml) and the *katG* locus was subsequently sequenced. Plasmid pBP10 (gift of D. Sherman, Seattle Biomed) was transformed into strain M to yield strain KA2, referred to in the text as Mm/pBP10. Mm were grown in standard media (see **Supplemental Experimental Procedures)** and prepared for experimental manipulations by growth to mid-log phase, followed by passage through a sterile 5μM filter to yield a single cell suspension. INH susceptibility of bacteria isolated from INH-treated larvae was verified by patching all outgrown colonies onto 7H10 agar containing 10, 20, and 40 μg/ml INH, and comparing growth to that of the parental strain. The INH MIC of three colonies was confirmed to be identical to strain M.

Mtb strain H37Rv was from D. Sherman (Seattle Biomed). Mtb strains JHU1258c-715, and JHU1258c-833 (harboring transposon insertions at positions 715 and 833, respectively, in the Rv1258c ORF), and the wild-type parent strain, CDC1551, were a gift of W. R. Bishai and G. Lamichhane (Johns Hopkins University) (Lamichhane et al., 2003). Mtb were grown to mid log phase in standard medium prior to infection.

Zebrafish infections

Wild-type AB zebrafish were from our laboratory stock, and PU.1 morphant embryos have been described (Clay et al., 2007a).. Larvae were infected via caudal vein injections at 36-48 hours post-fertilization as described (Davis and Ramakrishnan, 2009).

Microscopy

Wide field fluorescence microscopy was performed using a Nikon E600 equipped with a Nikon D-FL-E fluorescence unit with a 100W Mercury lamp. Wide field fluorescence images were captured using a CoolSnap HQ CCD camera (Photometrics) with MetaMorph 7.1 (Molecular Devices).

Fluorescent Pixel Count (FPC)

Quantification of infection with fluorescent Mm using images of individual embryos was performed using custom-made MATLAB software developed in-house. Briefly, in each image, the number of pixels with a fluorescence intensity greater than the brightest pixel observed in images of control uninfected embryos is counted. This count represents the total fluorescent area (in pixels) for each infected larva.

Calculation of Early Bactericidal Activity

FPC counts were $\log_{10}$ transformed and for each larva the difference between each day's measurement and the initial burden was calculated. EBAs over defined intervals were calculated by taking the mean and SD of the $\Delta \log_{10}$ FPC values calculated for individual larvae.

Macrophage Growth and Infection

J774A.1 and THP-1 macrophages were grown in DMEM and RPMI, respectively, supplemented with 10% FBS and 1% L-glutamine. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate for 48 hours prior to infection. $1x10^5$ J774A.1 or $5x10^5$ THP-1 macrophages were infected at an MOI of 3 for 3 hours at 33°C (for Mm) or 37°C (for Mtb). Cells were washed with medium and then 20 (for Mm) or 6 (for Mtb) μg/ml STM was added for the duration of the intracellular growth. Medium was changed daily. Macrophages were lysed with 0.1% Triton X-100 for intracellular growth quantization. To lyse macrophages and release bacteria for subsequent tolerance assessment, each well was first washed once with 1x PBS, and once with diH_2O, with the latter wash being removed immediately. Then, 200μl of diH_20 was added and the cells incubated for 15 minutes to lyse macrophages. Finally, 800μl of 1.25x concentrated 7H9 medium (see **Supplemental Experimental Procedures**) was added and the wells scraped gently with a pipette tip to release all release all macrophages. CFU were enumerated from triplicate wells on supplemented 7H10 agar. For determination of antibiotic killing, the percent survival was calculated by dividing the CFU for each well by the mean CFU present prior to treatment.

Statistics

Statistical analyses were performed using Prism 5.01 (GraphPad). For data sets requiring $\log_{10}$ transformation prior to ANOVA, embryos with no detectable fluorescence above background, or with no detectable CFU were assigned a value of 0.9, with 1 being the limit of detection, prior to $\log_{10}$ transformation. A Mann Whitney rank test was used when values

in one group were all below the limit of detection. Post test *P* values are as follows: *, $P<0.05$; **, $P<0.01$; ***$P<0.001$.

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FIGURES

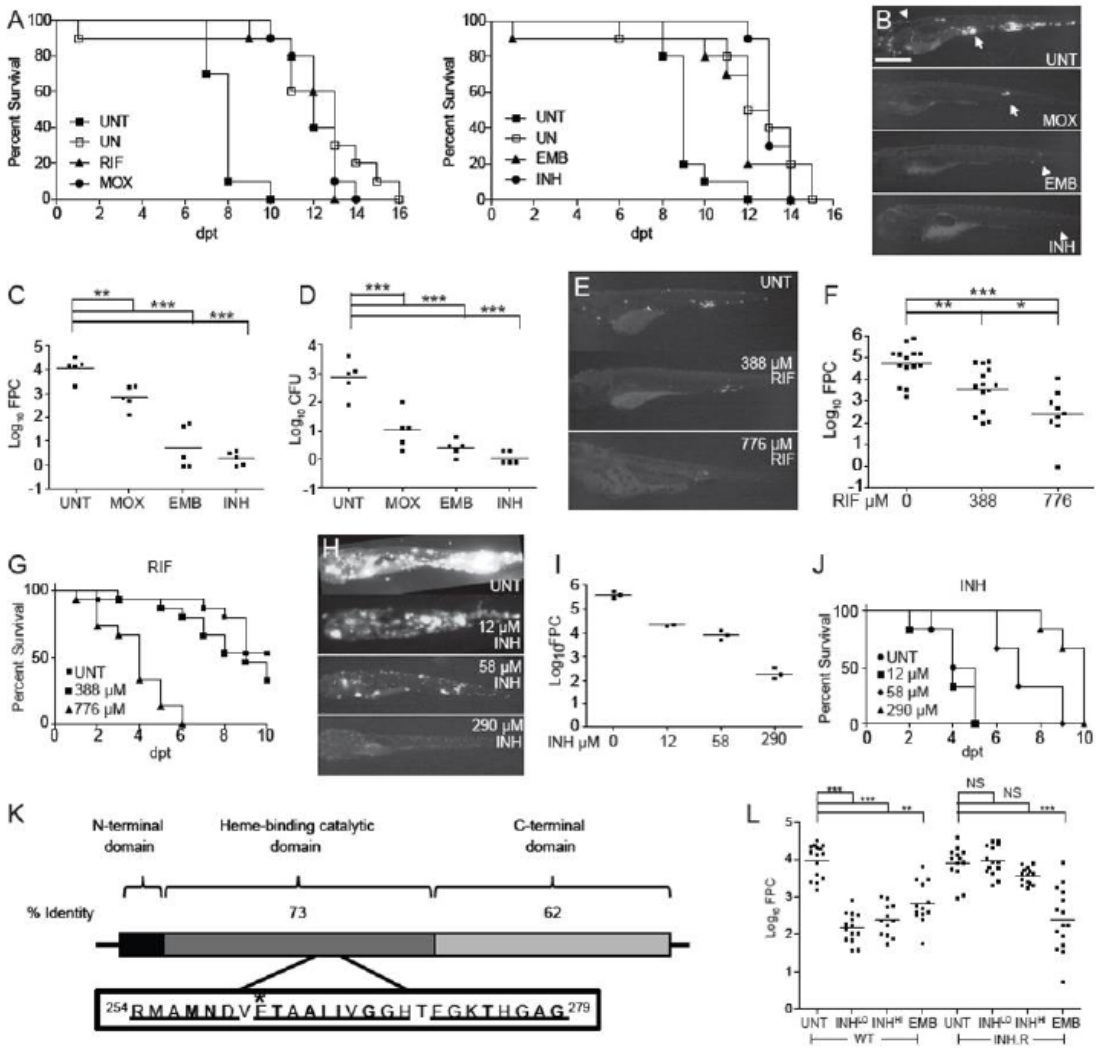


Figure 1.1. The Mm-larval zebrafish infection model replicates the specificity and activity of clinically relevant antitubercular drugs.

(A-D) Larvae were soaked in the MEC of RIF (388 µM), MOX (62.3 µM), EMB (1442 µM) or INH (290 µM) (**Table S1**). (A) Survival of uninfected (UN) larvae vs. those infected with 800 Mm and immediately treated with RIF, MOX, EMB, INH or left untreated (UNT). Left panel in the presence of 1% DMSO (see **Figure S2A**). Survival of treated infected larvae was significantly different from UNT larvae for all drugs (**Table S2**). Results are representative of at least two independent experiments.

(B-D) Larvae were infected with 155 Mm and left untreated (UNT) or treated with MOX, EMB, or INH for 4 days, prior to assessment of bacterial burdens by fluorescence microscopy (B, representative larvae are shown), FPC (C) or CFU enumeration of the lysed larvae immediately after imaging (D). Arrow, granuloma; arrowhead, single infected macrophage. Scale bar 500 µm. For C and D, individual larvae (points) and means (bars) are shown. Significance testing by one way ANOVA with Dunnett's post test.

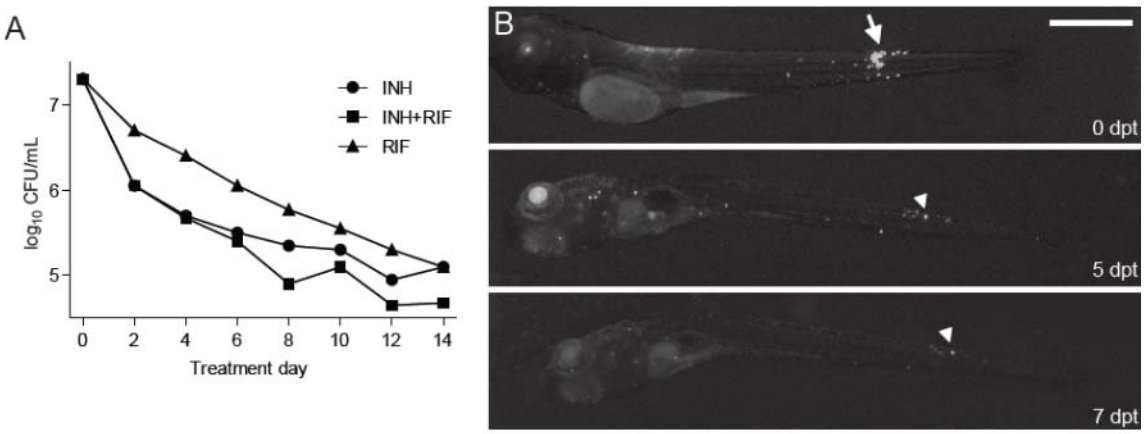
(E and F) Larvae infected with 46 Mm were soaked for 3 days in 388 or 776 μM RIF or left untreated. Representative fluorescence images (E) and bacterial burdens (F) of survivors are shown. Significance testing by one-way ANOVA with Tukey's post-test.

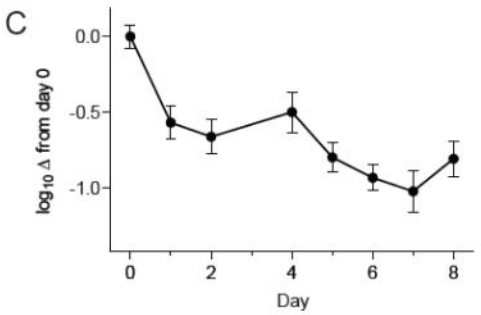
(G) Survival of uninfected larvae upon treatment with 0, 388 or 776 μM RIF added 2 dpf . P=0.0010 for 0 vs. 776; 0 vs. 388 μM, NS by Log-rank test. N=15 per group.

(H-J) Larvae infected with 1800 Mm were left untreated or immediately soaked in 12, 58 or 290 μM INH. Representative fluorescence images (H) and bacterial burdens (I) of survivors at 4 dpt are shown. Mean bacterial burdens (bars) compared by one-way ANOVA with Tukey's post-test resulted in $P < 0.001$ for all comparisons, with the exception of 12 vs. 58 μM INH, which was not significant ($P > 0.05$). (J) Survival curve, N= 6 larvae per group. P=0.0018, Log-rank test for trend comparing all curves and P=0.0010 for comparison of untreated vs. 58 μM or untreated vs. 290 μM.

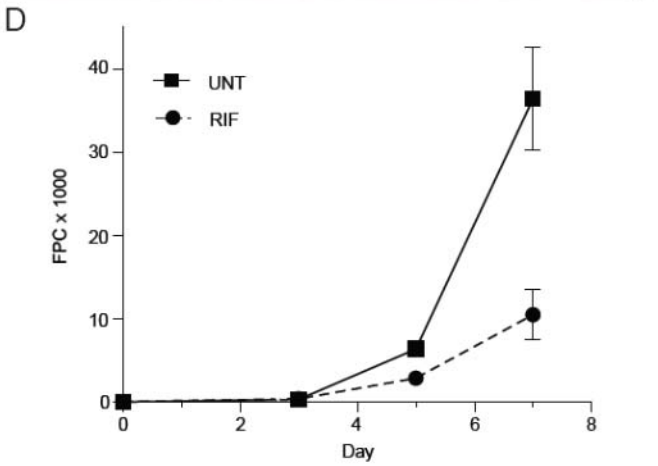
(K) Functional domains of KatG. Mtb KatG is 740aa and Mm KatG is 743aa. Boxed inset of Mtb catalytic domain shows regions of identity with Mm (underlined) and point mutations (in bold) that confer INH resistance in Mtb (Sandgren et al., 2009). *Position of the single amino acid substitution (E265V) in the INH-resistant Mm strain corresponds to the E261 position of Mtb KatG.

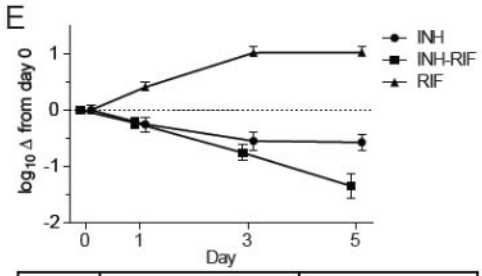
(L) Bacterial burdens of 3 dpt larvae infected with 300 WT or INH-resistant (INH.R) Mm and soaked in 290 (LO) or 2320 μM (HI) INH, 1442 μM EMB, or left untreated (UNT) beginning 1 dpi. Median $\log_{10}$FPC (bars) compared using Kruskal-Wallis test with Dunn's post-test. For all panels, *, P<0.05; **, P<0.01; ***, P<0.001, NS, not significant.





	Δ/d	sd	N
EBA_{0-2}	0.33	0.24	19
EBA_{2-7}	0.06	0.14	11
EBA_{0-7}	0.14	0.07	11





	INH			INH-RIF		
	Δ/d	sd	N	Δ/d	sd	N
EBA_{0-5}	-0.12	0.079	10	-0.27	0.14	8
EBA_{1-5}	-0.08	0.095	10	-0.28	0.14	8

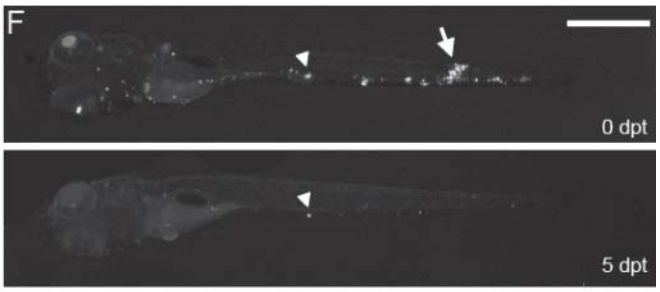


Figure 1.2. INH treatment of Mm-infected larvae results in a biphasic EBA with persistence of tolerant organisms.

(A) Authors' rendition of human clinical EBA data (see Figure 1 of (Jindani et al., 2003a)) showing the rate of clearance of Mtb from sputum in patients with previously untreated, smear-positive pulmonary tuberculosis, upon treatment with INH and/or RIF. (B and C) Twenty larvae were infected with 300 Mm and treated with 290 μM INH beginning 3 dpi. Each larva was imaged daily for 8 dpt, and bacterial burdens quantified by FPC. (B) Representative larva imaged at the beginning of treatment (0 dpt), and again at five and seven dpt. Arrow, granuloma, arrowhead, macrophage containing persistent bacteria. (C) EBA curve for INH-treated larvae, showing the mean $\log_{10}$ FPC change from day 0. Error bars represent SEM. EBA was calculated as described in the **Experimental Procedures**.

(D-F) Ten larvae per group were infected with 300 Mm and were serially imaged for enumeration of bacterial burden by FPC. (D) Larvae were treated with 388 μM RIF or left untreated, beginning 1 dpi. Mean FPC and SEM are shown. (E) Larvae were treated with 290 μM INH, 388 μM RIF, or a combination of both drugs beginning at 3 dpi. Data analyzed as in (C). (F) Representative INH-RIF treated larvae annotated as in (B).

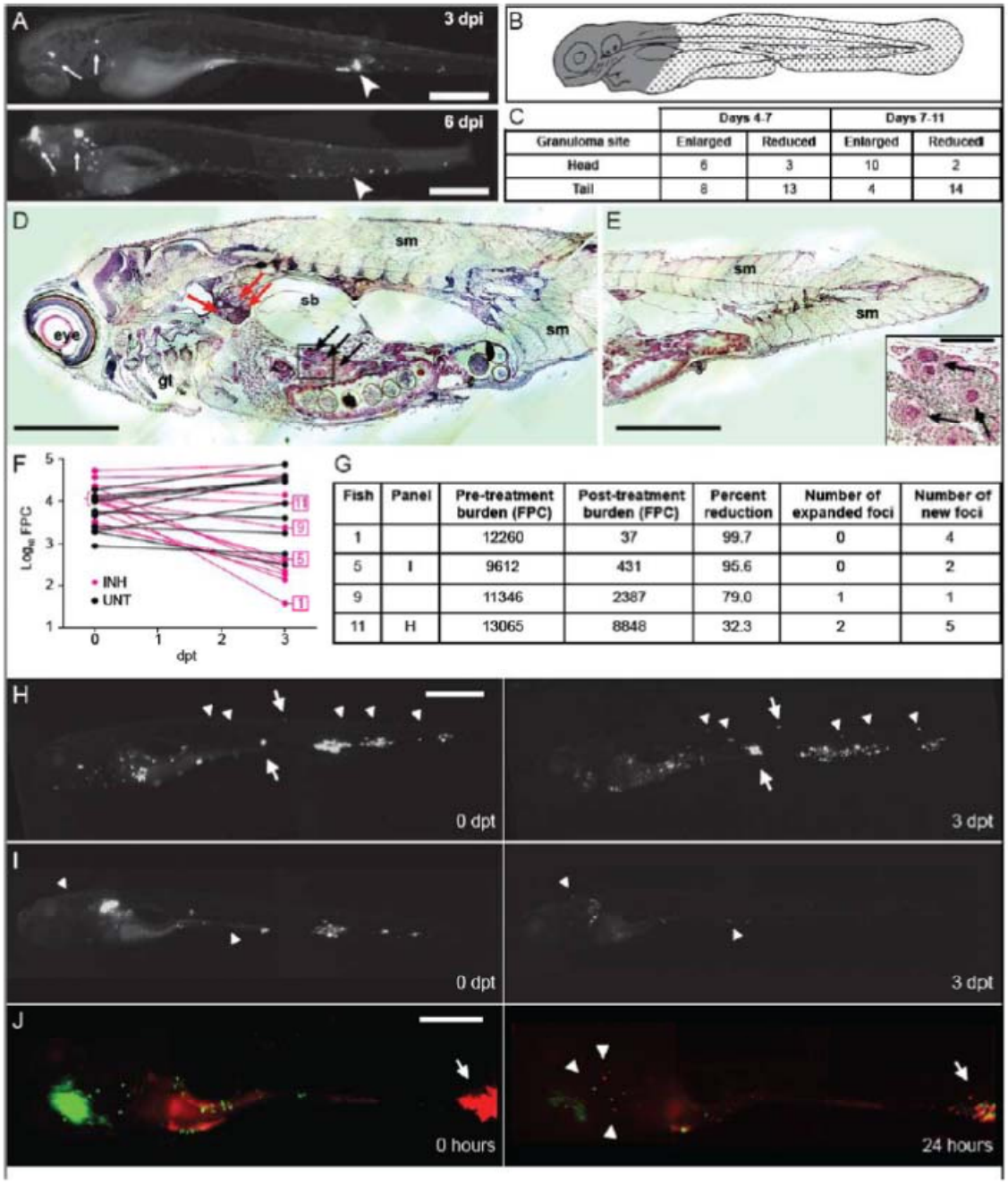


Figure1.3. Mm infections are dynamic during antibiotic treatment.
(A-D) Tracking of untreated Mm infections in individual larvae. (A) Fluorescence images of a representative larva at 3 and 6 dpi. Arrows, enlarging granulomas, arrowheads, shrinking granulomas. Scale bar, 400 µm. (B) Cartoon indicating "head" region (gray) primarily containing organs, and "tail" region (stippled) comprised mostly of muscle. (C) Enumeration of expanding and contracting granulomas over time. Differential region-specific outcomes of granulomas were statistically significant for changes occurring between days 7 and 11 (P=0.0022), but not for changes occurring between days 4 and 7 (P=0.2360, Fisher's exact test).
(D and E) Hematoxylin and eosin staining showing Mm in caseating granulomas in a 33 day-old fish that was infected at 1 dpf. Red and black arrows indicate granulomas in pronephros and liver, respectively. gl, gill; sb, swim bladder; sm, somite. Scale bar 300 µm. Higher magnification of granulomas in boxed inset of (D) is shown on bottom right of (E). Scale bar 50 µm.

(F-I) Larvae infected with varying Mm inocula for 4 days were then treated with 290 μM INH or left untreated for an additional 3 days. Larvae were imaged at 4 and 7 dpi (0 and 3 dpt). (F) Pre- and post-treatment $\log_{10}$ FPC values for individual larvae are plotted with data points from the same individual connected. (G) Raw FPC values before and after treatment, percent change, and the presence of expanding and new foci are reported for representative fish indicated in (F). (H and I) Fluorescence images of fish 11 (H) and fish 5 (I) as reported in (F and G), shown before and after treatment. Arrows, enlarging granulomas, and arrowheads, new foci. Scale bars 500 μm.

(J) A single larva was infected with 500 Mm constitutively expressing the Kaede photoactivatible GFP for 4 days. Composite red and green fluorescence images immediately after photoactivation of a granuloma (**left** panel) and 24 hours later (**right** panel). Arrows, photoactivated granuloma, arrowheads, single macrophages containing red fluorescent bacteria. Scale bar 250 μm.

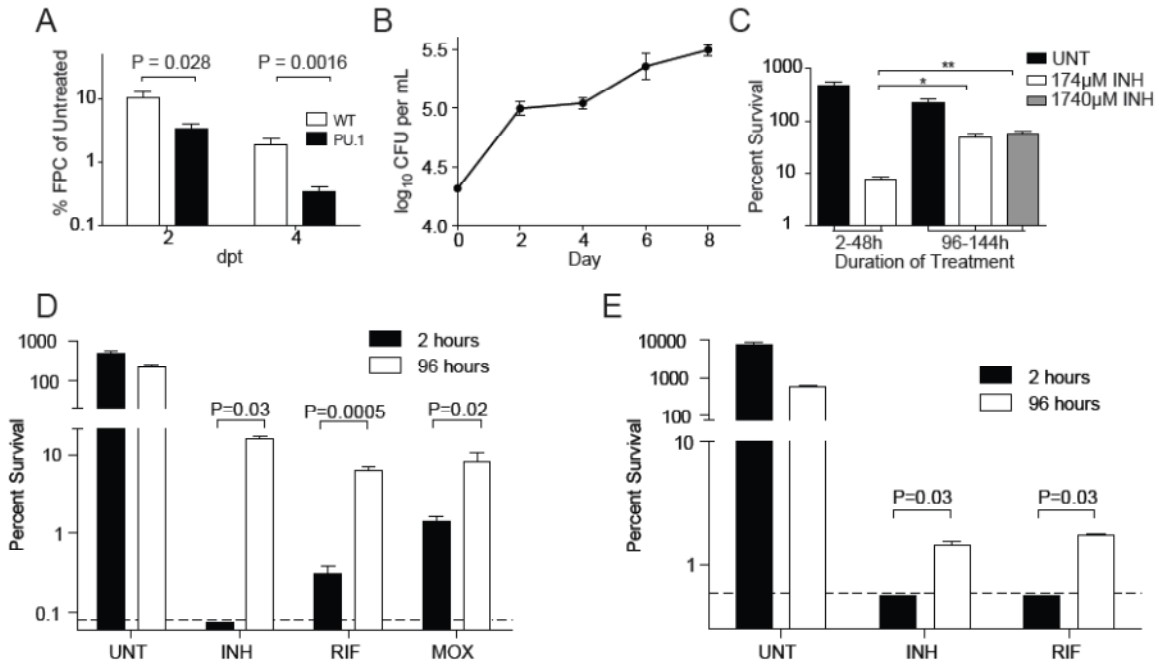


Figure1. 4. Antibiotic tolerance is induced by macrophage residence

A) Wild-type and PU.1-morphant larvae were infected with 300 Mm. One dpi, larvae were treated with 290 μM INH, or left untreated. Larvae were imaged at two and four dpt and bacterial burdens determined by FPC. For each timepoint, FPC of each treated larva was normalized to the mean FPC of the untreated control group. N=20 wild-type or 12 PU.1 morphant larvae per group. *P* values determined using Student's *t*-test.

(B and C) J774A.1 macrophages were infected with Mm and were left untreated, or were treated with INH prior to lysis and enumeration of CFU. (B) Growth of Mm in the untreated control wells. (C) Survival of intracellular Mm upon exposure to 174 or 1740 μM INH during the time periods indicated (two-48 hours, or 96-144 hours) prior to macrophage lysis and enumeration of CFU. Percent survival was compared using one-way ANOVA with Dunnett's post-test.

(D) Mm were used to infect THP-1 macrophages for 2 or 96 hours prior to being released by macrophage lysis. CFU were enumerated at the time of release, and again following 48 hours exposure to 174 μM INH, 1.21 μM RIF, 7.48 μM MOX, or left untreated. For the purpose of display, values below the limit of detection (0.08%, dashed line) were arbitrarily set to 0.074%. *P* values were determined using Student's *t*-test (RIF and MOX), or the Mann-Whitney rank test (INH).

(E) Mtb strain H37Rv was used to infect J774A.1 macrophages at an MOI of 1, and were grown and treated as described for (D), except that the concentration of INH was 4.4 μM, reflecting the greater inherent susceptibility of this organism to INH. For the purpose of display, values below the limit of detection (0.6%, dashed line) were arbitrarily set to 0.57%. *P* values were determined using the Mann-Whitney rank test. In all panels, error bars represent SEM.

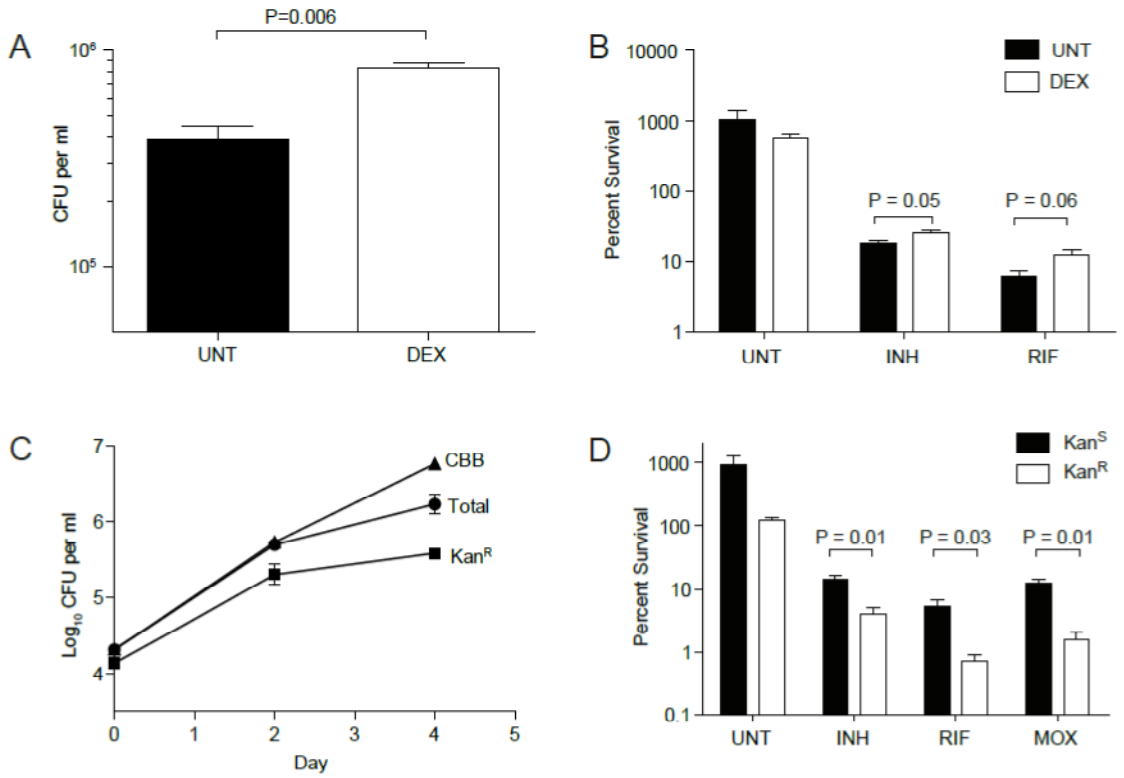


Figure 1.5. Growing bacteria are enriched for antibiotic tolerance
(A and B) J774A.1 macrophages were infected with Mm for three hours, prior to treatment with 100 nM dexamethasone. Macrophages were lysed at 96 hpi to release bacteria. (A) Total CFU at time of release. (B) Percent survival of released bacteria upon 48 hours exposure to 174 μM INH, 1.21 μM RIF, or left untreated. (C) J774A.1 macrophages were infected with Mm/pBP10 and total and Kan^R CFU enumerated at 48 and 96 hpi. The cumulative bacterial burden (CBB) was calculated as described in the **Supplemental Experimental Procedures**. (D) Mm/pBP10 grown intracellularly for 96 hours (described in (C)) were released by macrophage lysis and then treated for an additional 48 hours with 174 μM INH, 1.21 μM RIF, 7.48 μM MOX, or left untreated, prior to enumeration of total and Kan^R CFU. Kan^S CFU were calculated as the total CFU minus the mean Kan^R CFU. In all panels, error bars represent SEM, and *P* values were determined using Student's *t*-test.

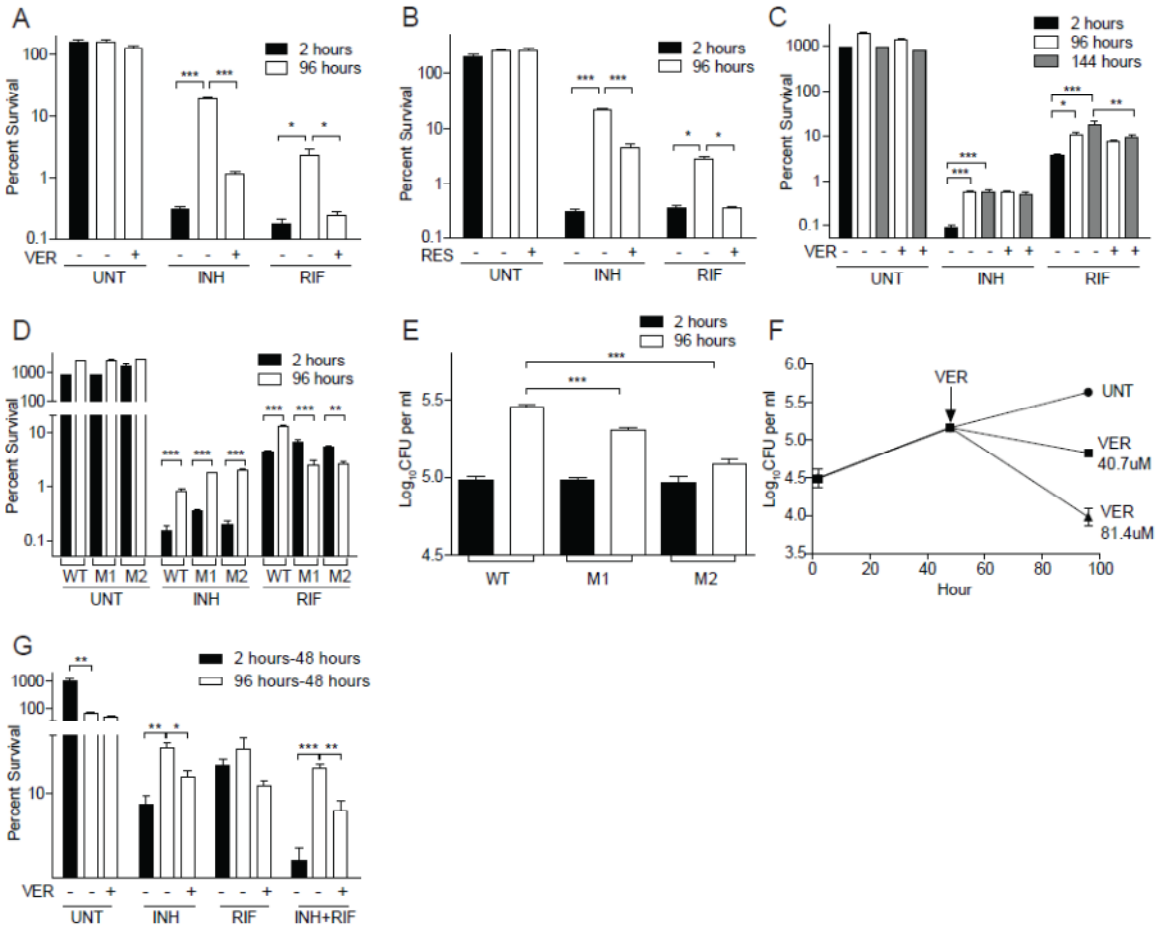


Figure1.6. Bacterial efflux pumps confer tolerance within macrophages

(A and B) THP-1 macrophages were infected with Mm and lysed at two or 96 hpi. The released bacteria were treated for an additional 48 hours with 174 μM INH, 1.21 μM RIF or left untreated, in the presence or absence of 81.4 μM verapamil (A) or 65.7 μM reserpine (B), prior to enumeration of CFU.

(C) THP-1 macrophages were infected with Mtb strain CDC1551 and lysed at two, 96 or 144 hpi. The released bacteria were then treated with antibiotics for 48 hours as described in panel (A), except that the concentration of INH was 4.4 μM, as described for **Figure 4E**.

(D and E) THP-1 macrophages were infected with Mtb strains JHU1258c-715 ("M1"), JHU1258c-833 ("M2") and the isogenic wild-type control, CDC 1551, for two or 96 hours prior to lysis and enumeration of CFU. (D) Released bacteria were treated as described in panel (C).

(F) THP1 cells were infected with Mm for 48 hours, prior to addition of 0 (UNT), 40.7 or 81.4 μM VER for an additional 48 hours. $P<0.001$ using one-way ANOVA, with Dunnett's post-test comparing each treatment group to the untreated control after 48 hours of VER treatment.

(G) THP1 cells infected with Mm for two hours or 96 hours were incubated for an additional 48 hours with 174 μM INH, 1.21 μM RIF or both, and in the presence or absence of 40.7μM VER. Cells were lysed and CFU were enumerated at the end of the 48 hour treatment. Percent survival was calculated relative to the mean intracellular counts present at the start of antibiotic exposure. For all panels, error bars represent SEM. Significance testing was performed using one-way ANOVA with Dunnett's (A,B, D and E) or Bonferroni (C and D) post-tests.

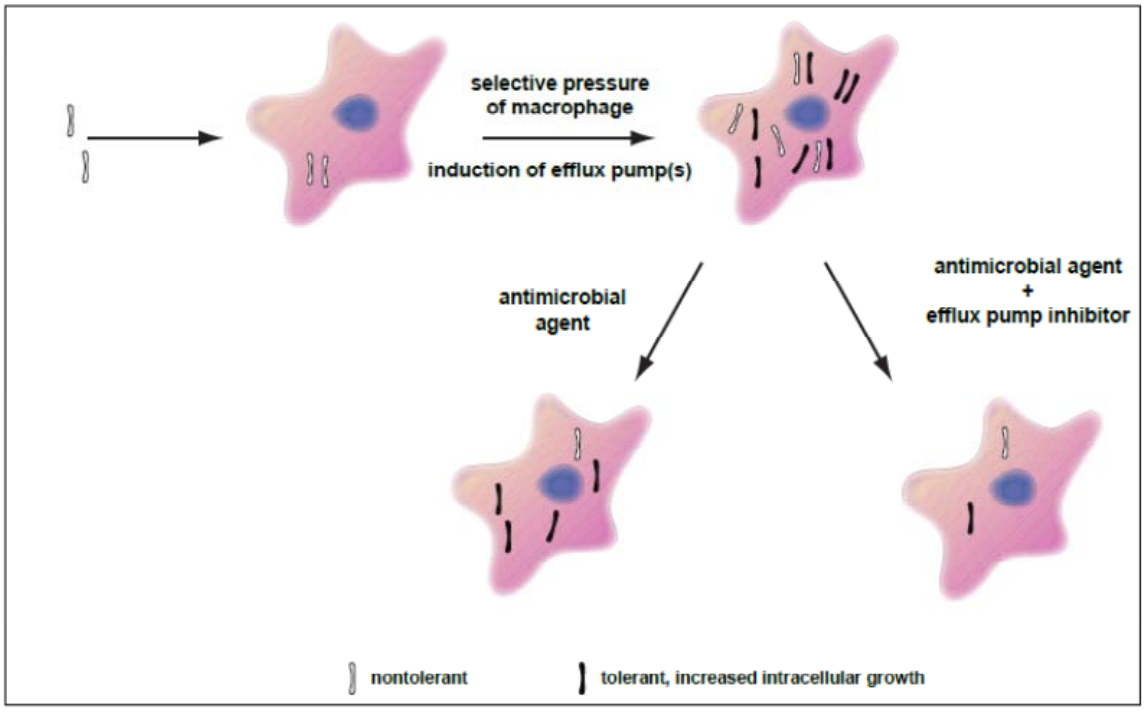


Figure 1.7. Model for the mechanism of antibiotic tolerance in TB and its treatment.
Nontolerant bacteria are phagocytosed by macrophages soon after infection wherein they induce efflux pumps to counter macrophage defenses. These efflux pumps render bacteria tolerant to multiple antitubercular drugs. The tolerant bacteria are associated with the growing population because of their enhanced ability to counter macrophage defenses. Antitubercular drug treatment spares tolerant bacteria and the addition of efflux pump inhibitors reduces their numbers.

SUPPLEMENTAL INFORMATION

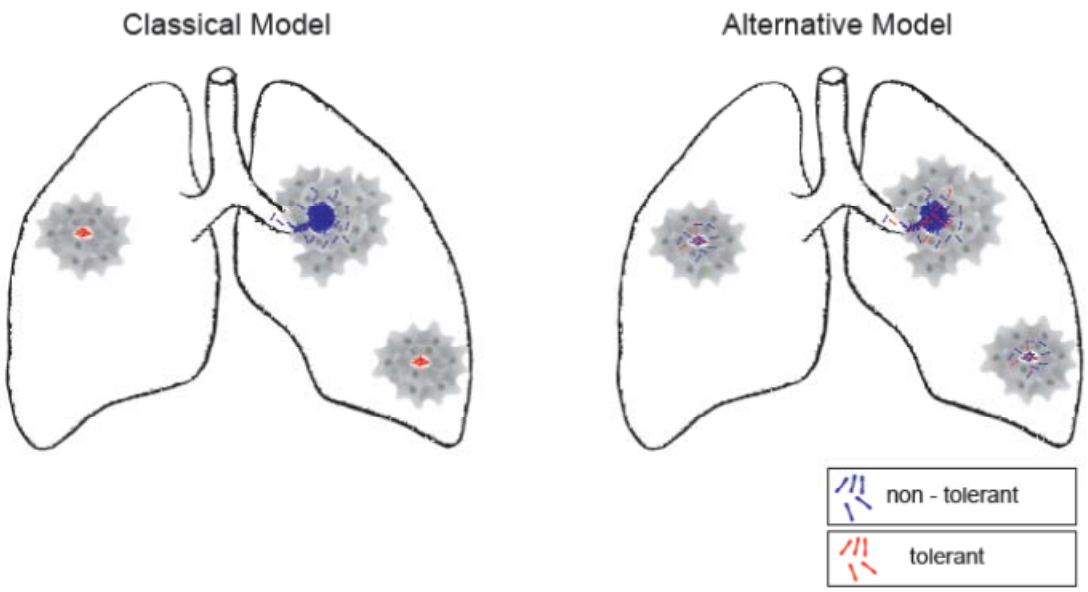


Figure S1.1. Current models for tolerance in TB. Related to Figure 1.7. The figure depicts two models for the localization of tolerant bacteria in the context of necrotic (caseous) tubercular granulomas. The upper granuloma in the left lung (right side of drawing) opens into the bronchial tree, allowing bacteria to escape the lung and be transmitted via aerosol. It is this type of lesion from which bacteria can be cultured from sputum. The other two granulomas, while still necrotic, do not connect with the bronchial passages (i.e. they are "closed") and do not contribute to sputum burdens. In the classical model of tolerance, closed lesions are presumed to be hypoxic and it is this condition that is thought to induce a uniformly tolerant dormancy state (Barry et al., 2009). Alternative models of tolerance implicate a variety of stresses including nutrient limitation, DNA damage, nitric oxide, as well as the existence of a stochastic population of persister cells that contribute to tolerance. Such models are more agnostic about the placement of the tolerant bacteria, which might reside in all different granuloma types, both within macrophages and in the caseum (Burgos et al., 2008; Connolly et al., 2007; Dhar and McKinney, 2007; Sacchettini et al., 2008; Warner and Mizrahi, 2006a). Both models implicate slowly growing and/or metabolically inactive cells in the generation of tolerance.

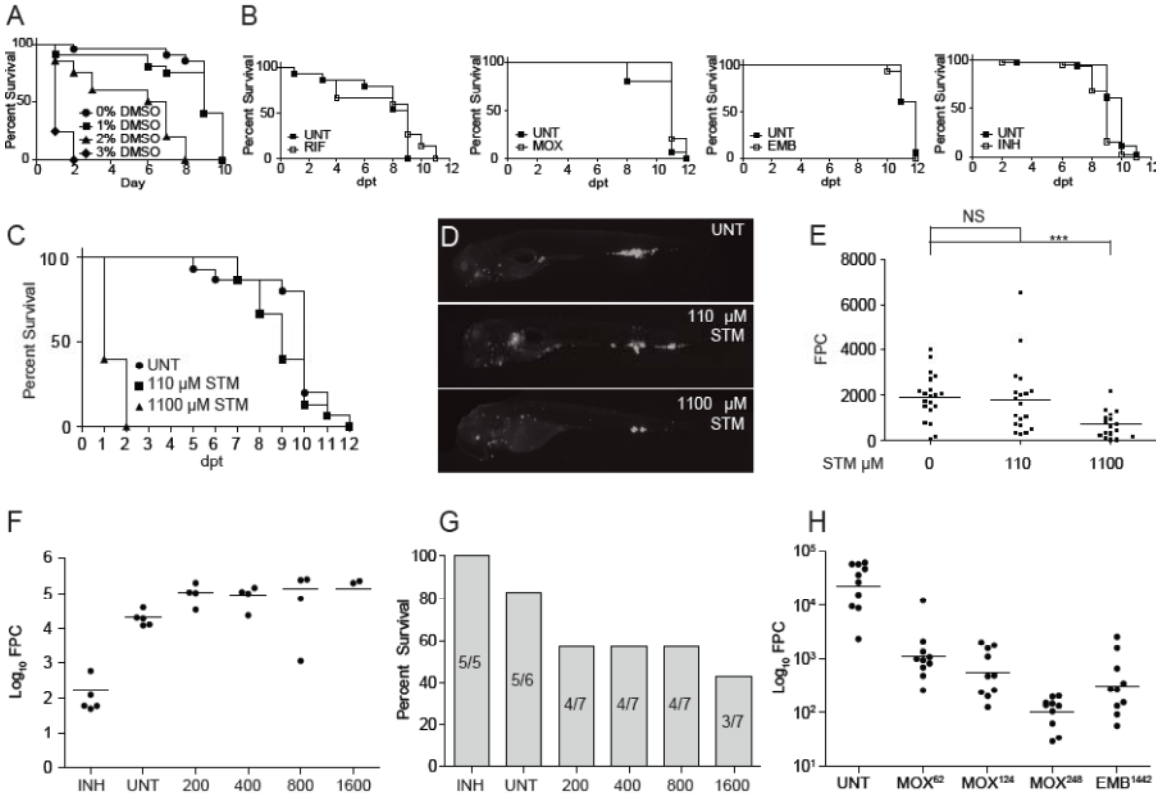


Figure S1.2. Toxicity and/or efficacy of DMSO, streptomycin (STM), pyrazinamide (PZA), and MOX in the larval model. Related to Figure 1.1. (A) To determine the maximum amount of DMSO that could be used to facilitate dissolution of drugs for addition to fish water, the effects of DMSO alone on survival of uninfected larvae was tested. Twenty larvae were placed in each of four concentrations of DMSO at one dpf and monitored daily for survival thereafter. 1% DMSO did not significantly impact survival when compared to 0% DMSO (P=0.73, Log-rank test), while 2% and 3% DMSO reduced survival (P<0.0001, Log-rank test).

(B) Survival curves of uninfected larvae soaked in fish water alone (UNT) or in the presence of 388 µM RIF, 62.3 µM MOX, 1442 µM EMB, or 290 µM INH (see also **Table S2)**.

(C-E) STM treatment of larvae. (C) Reminiscent of its toxicity in humans, we could not establish a STM MEC due to toxicity in the larvae. Survival of uninfected larvae left untreated (UNT) or in the presence of 110 or 1100 µM STM added starting 2dpf. P<0.0001, UNT vs. 1100 µM STM. Log-rank test. N=15 per group. (D and E) Larvae infected with 250 Mm were left untreated for 24 hours prior to adding STM (0, 110 or 1100 µM) for an additional 42 hours. Representative fluorescence images (D) and bacterial burdens as determined by FPC (E) are shown. Bars represent median FPC. Significance testing by Kruskal-Wallis test with Dunn's post test comparing each treatment group to the untreated control. ***, P <0.001. The minimum concentration that reduced bacterial burdens at 42 hours post treatment (1100 µM) was associated with 100% mortality only a few hours later (Panel C).

(F and G) PZA, to which Mm is innately resistant (Rastogi et al., 1992, see also **Table S1**), fails to reduce bacterial burdens even at toxic doses. (F) Larvae infected with 500 Mm were immediately soaked in INH (290 µM), left untreated (UNT), or treated with PZA (200, 400, 800, or 1600 µM). Bacterial burdens were assessed by FPC at 4 dpt. Bars represent mean $\log_{10}$FPC. Bacterial burdens of PZA treated embryos were not significantly different from untreated (One-way ANOVA with Dunnett's post-test comparing treated groups to untreated control). (G) Percent survival of larvae in (F) at the time of imaging at 4 dpt. Numbers within each bar represents $n_{final}/n_{initial}$. Survival of

embryos treated with 1600 μM PZA was not significantly different from that of untreated embryos (Fischer's exact test).
(H) Dose-dependent efficacy of MOX, and its superior activity compared to EMB. Three-dpf larvae (N=10 per group) were infected with 300 CFU Mm and treatment started the following day with varying concentrations of MOX or a single high dose of EMB (shown in μM). Bacterial burdens were assessed five dpt. Bars represent mean $\log_{10}$FPC. 248 μM MOX had greater activity than 1442 μM EMB (P=0.0213, Student's t-test), consistent with its greater antitubercular potency in humans (Conde et al., 2009).

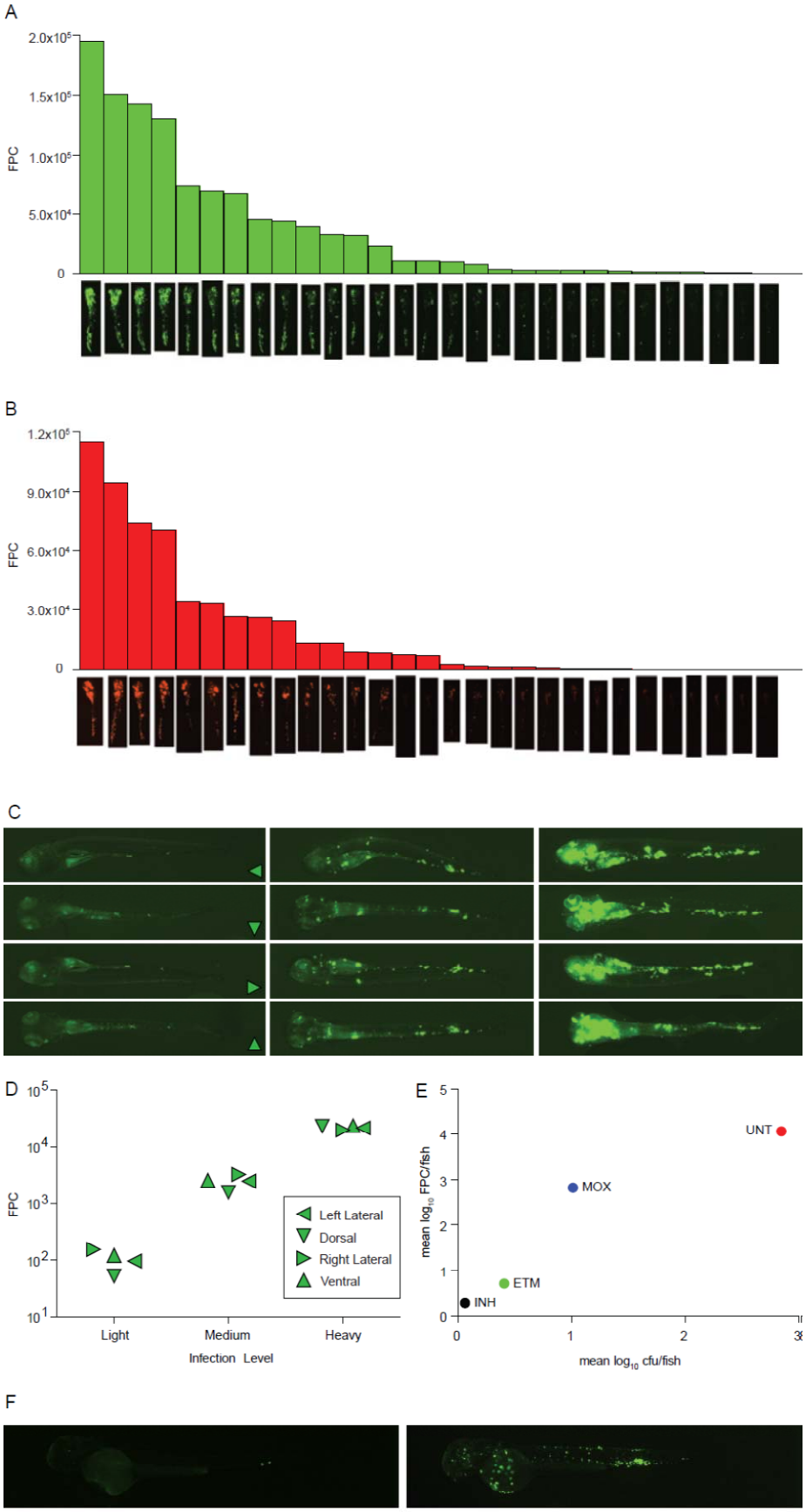

A
2.0x10⁵
1.5x10⁵
1.0x10⁵
5.0x10⁴
0
FPC
B
1.2x10⁵
9.0x10⁴
6.0x10⁴
3.0x10⁴
0
FPC
C
D
10⁵
10⁴
10³
10²
10¹
FPC
Light
Medium
Heavy
Infection Level
Left Lateral
Dorsal
Right Lateral
Ventral
E
5
4
3
2
1
0
mean log10 FPC/fish
UNT
MOX
ETM
INH
0
1
2
mean log10 cfu/fish
F

Figure S1.3. Fluorescent Pixel Count (FPC) as an accurate measure of relative infection burden in logarithmic and stationary phase bacteria. Related to Figure 1.1. (A and B) Larvae infected with varying doses of (A) green-fluorescent or (B) red-fluorescent Mm were imaged and bacterial burdens quantified by FPC. Data arranged in order of decreasing FPC (bar) with corresponding embryo image shown underneath. (C and D) Larvae were infected with variable doses of Mm and the infection allowed to progress for five days. Three individual larvae with variable infection burdens (light, medium and heavy) were then mounted in 1% agarose and imaged in dorsal, ventral, left and right lateral orientations. (C) Fluorescence images: top row = left lateral view; second row = dorsal view; third row = right lateral view, and bottom row = ventral view. (D) FPC measurements of the images shown in (C). (E) Correlation between mean $\log_{10}$ FPC and mean $\log_{10}$ CFU counts from populations shown in Figure 1C and 1D. Pearson's Coefficient of Correlation (r) = 0.9341, P= 0.0659. (F) To determine the detection sensitivity of stationary phase Mm, light (left panel) and heavy (right panel) inocula of Mm (OD_{600} of 1.8) were injected into 30 hpf larvae, which were imaged immediately at the same settings used in (C). By fluorimetry, stationary phase Mm/pMSP12::GFP expresses ~ 20% of the fluorescence of logarithmic phase bacteria, yet are still readily visualized by microscopy. Furthermore, FPC analysis counts fluorescent pixels above a user defined threshold, regardless of intensity. Therefore, all bacteria will count equally, independent of their level of GFP expression and thereby of their growth phase.

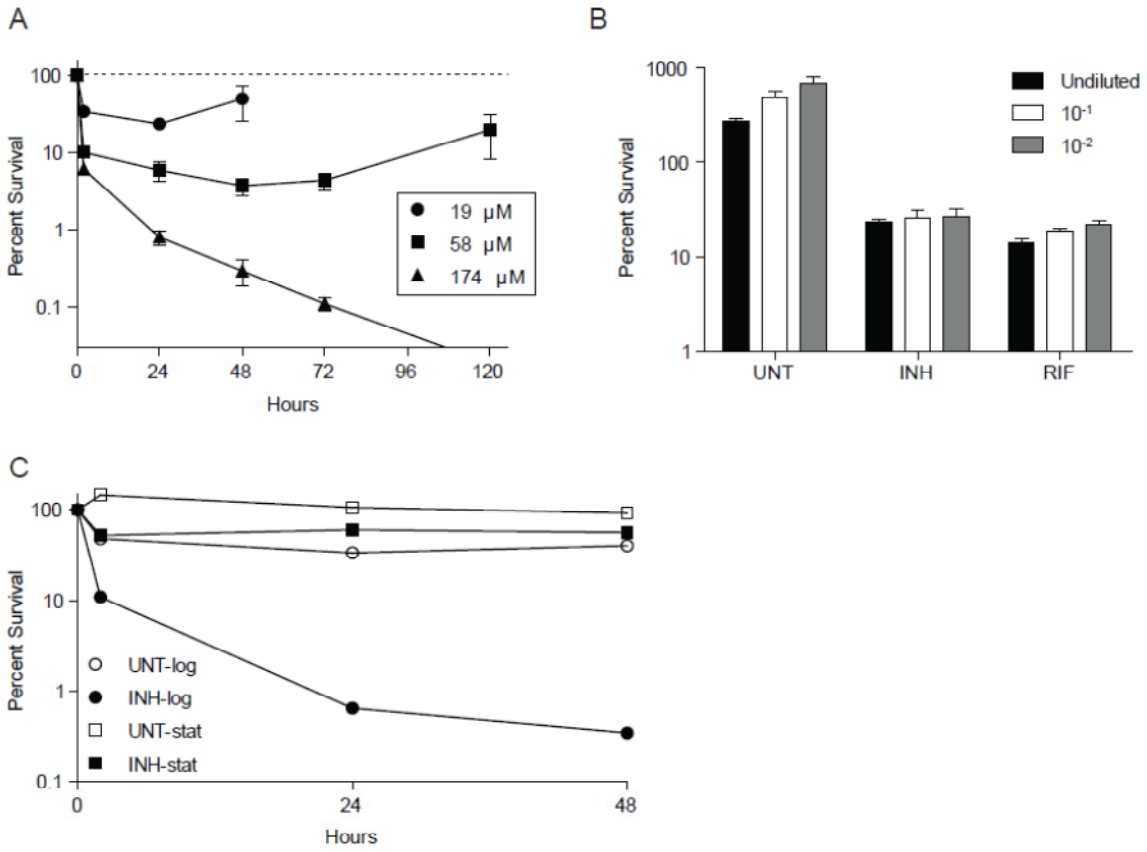
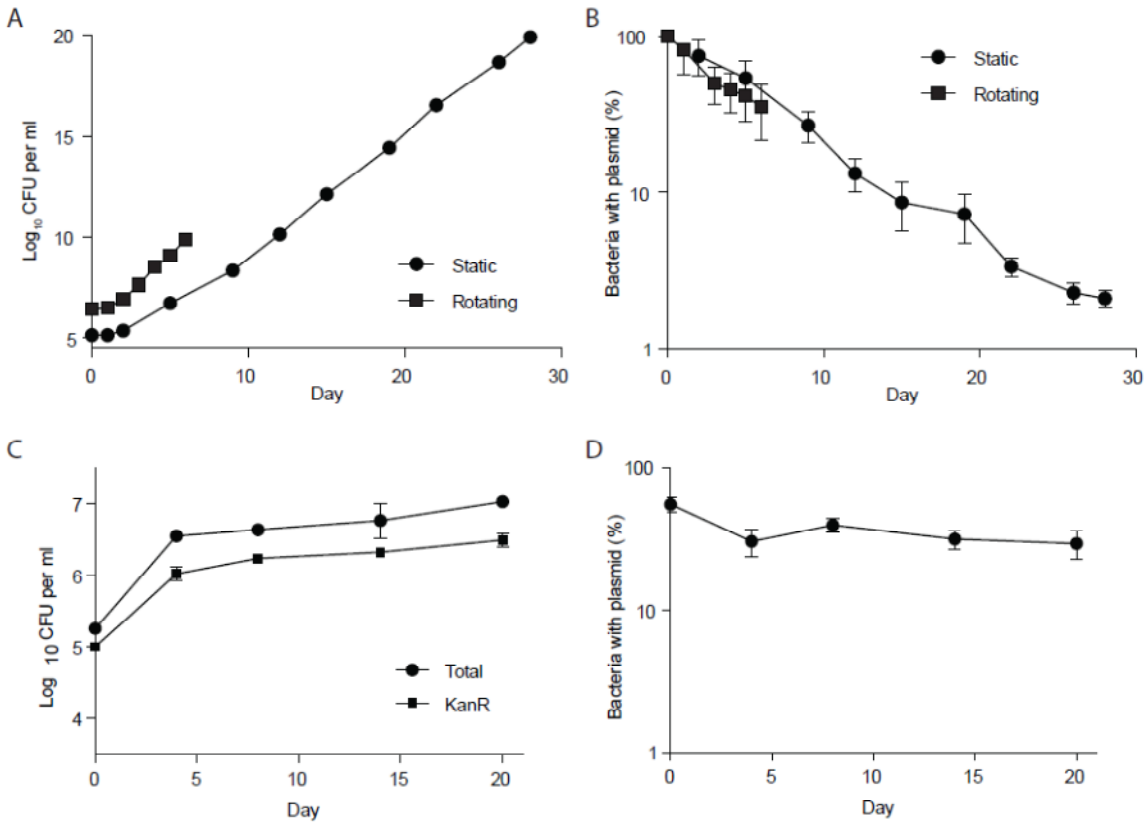


Figure S1.4. INH tolerance in axenic culture. **Related to Figure 1.4 and Figure 1.5.** A) INH-tolerance is induced by exposure to INH. Approximately 3000 *M. marinum* were incubated in 19, 58, or 174 µM INH (representing 0.33-, 1- and 3-fold the in vitro MIC) and survival assessed at times shown. Mean percent survival +/- SEM of triplicate samples are plotted. Stippled line and lower limit of y-axis indicate upper and lower limits of detection, respectively. At 174 µM two of three samples were sterilized by 120 hours, and thus are below the limit of detection of 1 CFU. By 72 hours cultures containing 19 µM INH had grown above the limit of detection (3000 CFU). Representative colonies arising from all cultures were verified to be INH-sensitive. B) Bacterial density does not alter tolerance. J774 cells infected with Mm for 96 hours were lysed and the lysates diluted 10 and 100-fold in 7H9 medium. Undiluted and diluted lysates were treated with INH and RIF for 48 hours. C) INH tolerance of stationary phase Mm. Single cell suspensions (see **Experimental Procedures**) of Mm were isolated from broth-grown log and stationary phase cultures and were treated with 174 µM INH or left untreated prior to enumeration of CFU at the time points shown.



E

Cell line	Day	Standard plating CFU/ml	Plasmid frequency %	r	δ	Generation Time h	Cumulative Bacterial Burden (CBB) CFU/ml
THP1	0	1.28 x 10^4	100.3				1.28 x 10^4
	2	6.63 x 10^4	65.3	1.9	1.1	8.8	1.37 x 10^5
	4	2.70 x 10^5	34.9	2.8	2.2	5.9	9.46 x 10^5
J774	0	2.08 x 10^4	65.7				2.08 x 10^4
	2	4.97 x 10^5	45.1	1.7	0.12	9.8	5.32 x 10^5
	4	1.83 x 10^6	20.2	3.4	2.7	4.9	5.68 x 10^6

Figure S1.5. Assessment of intramacrophage growth rates using the unstable plasmid, pBP10. Related to Figure 1.5.

A) In vitro stability of pBP10 in Mm in the absence of antibiotic selection. The total number of Mm, as determined by CFU enumeration, accounting for serial dilutions in continuous log-phase cultures. Bacteria were grown in 25 ml flasks, standing (static) or in 50 mL conical tubes in a roller drum (rotating). B) The frequency of plasmid bearing Mm/pBP10 as determined by plating on 7H10 agar with and without 20 µg/mL kanamycin. C) The absolute numbers of all bacteria (Total, circles) and those retaining pBP10 (KanR, squares) in static culture. D) Frequency of plasmid bearing Mm/pBP10. Mean of triplicate cultures shown with error bars representing SEM. E) Estimates of Mm generation times during intracellular growth in THP-1 and J774A.1 cell lines. The growth rate (r), death rate (δ) and Cumulative Bacterial Burden (CBB) were calculated as described in **Supplementary Experimental Procedures**. The generation time was calculated by ln(2)/*r*.

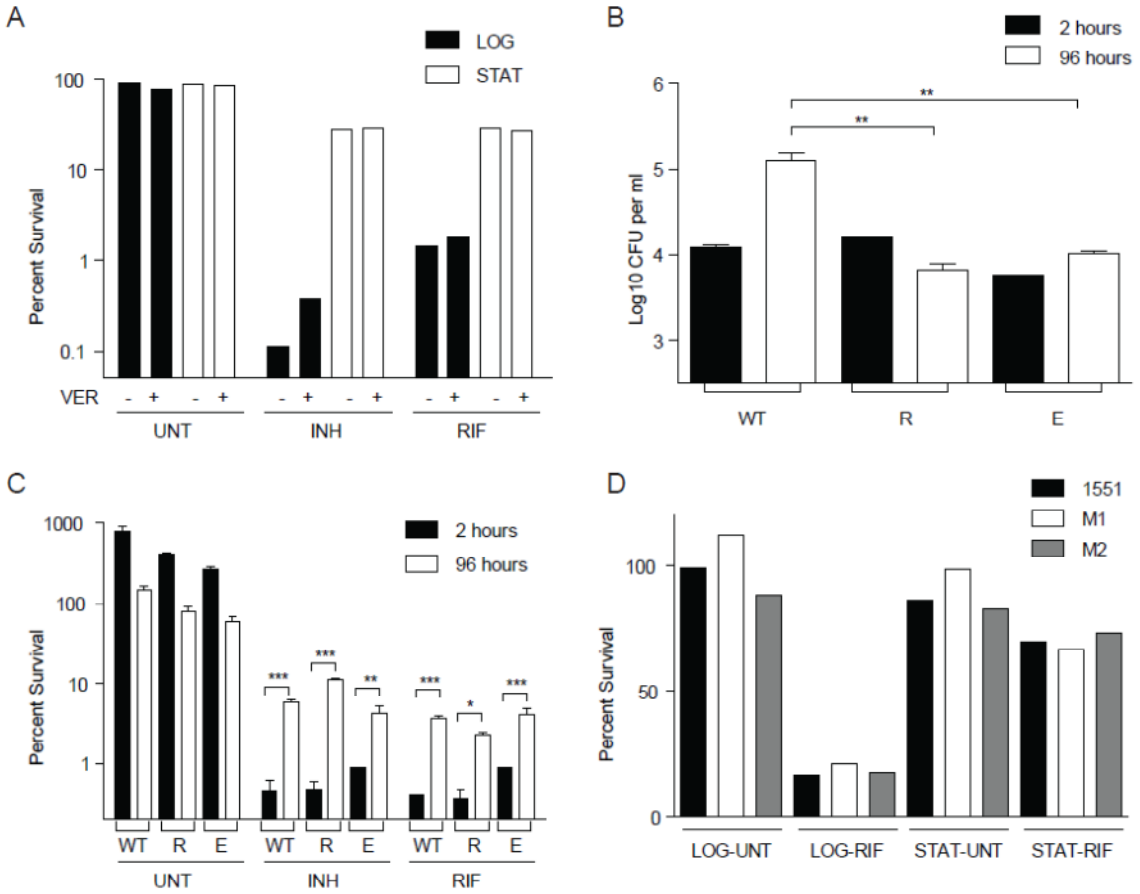


Figure S1.6. Specificity of VER and Mtb Rv1258c in regulating intramacrophage tolerance. Related to Figure 1.6.

(A) VER does not reduce stationary phase Mm tolerance to INH or RIF. Logarithmic (LOG) and stationary phase (STAT) Mm cultures were treated for 48 hours with 174 µM INH, 1.21 µM RIF, or neither drug (UNT), and in the presence or absence of 81.4 µM VER. (B and C) Attenuated mutants develop tolerance. J774 macrophages were infected with Mm strains M ("WT"), RD1-6 ("R") and KK33 ("E"), which are wild-type, or defective for the ESX-1 and Erp virulence determinants, respectively for two or 96 hours. (B) Intracellular growth attenuation of mutant bacteria. Statistical testing performed using one way ANOVA with Dunnett's post test: **, $P<0.01$. (C) Percent Survival of macrophage-released bacteria after an additional 48 hours in 174 µM INH, 1.21 µM RIF, or left untreated (UNT). Statistical testing was performed for INH and RIF cohorts separately, in each case using one way ANOVA with Bonferroni's post test for selected multiple comparisons. Error bars represent SEM. *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$. (D) Antibiotic susceptibility of Rv1258c mutants in logarithmic and stationary phase culture. Logarithmic (LOG) or 6-week old stationary (STAT) phase cultures of CDC1551 ("1551"), JHU1258c-715 ("M1") and JHU1258c-833 ("M2") were incubated in the presence or absence of 1.21 µM RIF for 48 hours prior to enumeration on 7H10 agar.

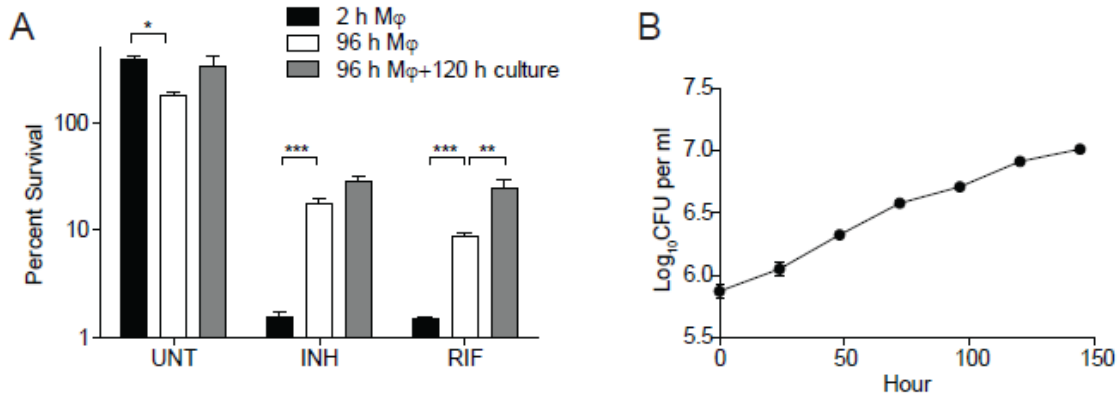


Figure S1.7. Macrophage-induced antibiotic tolerance persists after bacteria resume extracellular growth. Related to Figure 1.6. Mm was used to infect J774 macrophages for 2 hours (black bars) or 96 hours (white and gray bars) prior to being released by macrophage lysis. The lysates were then immediately subjected to 48 hours treatment with 174 μM INH, 1.21 μM RIF, or left untreated (black and white bars), or were allowed to resume growth in the lysate for 120 hours prior to the 48 hour antibiotic treatment (gray bars). (A) Percent survival of bacteria after 48 hours antibiotic treatment relative to pretreatment. Error bars represent SEM. Significance testing was performed using one-way ANOVA for each treatment separately (UNT, INH or RIF). Multiple comparisons were made with Dunnett's post-test using the 96 hour macrophage group (white bars) as the reference group; *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$. (B) Graph shows growth of released bacteria (after 96 hours growth in macrophages) in the lysate, with t=0 representing the CFU count at time of macrophage lysis.

SUPPLEMENTAL TABLES

Antibiotic	Molecular Weight	Mtb MIC (μg/ml)	Mm MIC (μg/ml)	Minimum Effective Concentration (MEC)[1]			
				Fold MIC	(μg/ml)	(μM)	Solvent
Isoniazid	137.14	<0.025-0.2[a]	8.0[d]	5x	40	290	H_2O
Rifampicin	822.95	<0.0625-0.5[a]	0.32[d]	1000x	320	388	DMSO[1%]
Ethambutol	277.23	1-4[a]	8.0[d]	50x	400	1442	H_2O
Moxifloxacin	401.43	0.06-1[b]	1.0[e]	25x	25	62.3	DMSO[1%]
Streptomycin	728.69	<0.5-2.0[a]	8.0[d]	100x	800	1098[2]	H_2O
Pyrazinamide	123.11	6-60[a]	R^+	NA	>200	>1600	H_2O

Table S1.1. In vitro MIC of antituberculous drugs and their MEC in zebrafish larvae. Related to Figure1.1. [1]MEC was defined as the lowest concentration tested that normalized host survival, as shown for INH, EMB, MOX, and RIF (**Figure 1A**). [2]STM was highly toxic **(Figure S2F)** and so no MEC was determined, however bacterial burdens were shown to be reduced **(Figure S2G-H)** at this concentration. MICs reported in [a](Heifets, 1991), [b](Gillespie and Billington, 1999), [c](Kremer et al., 2000), and [d](Cosma et al., 2006b). [e]MIC was determined by the agar dilution method as described in the **Supplemental Experimental Procedures**. R^+, innate resistance (Rastogi et al., 1992), although contradictory results have been reported using a non-standard pH (Silcox and David, 1971).

	Mock infected (see Figure S2B-E)				*M. marinum*-infected (see Figure 1A)			
	-Drug[a]	+Drug[a]	HR[b]	*P*[c]	-Drug[a]	+Drug[a]	HR[b]	*P*[c]
Rifampicin	9 (15)	9 (15)	0.57 [0.21-1.6]	0.2781	8 (10)	13 (10)	0.042 [0.010-0.18]	<0.0001
Moxifloxacin	11 (15)	11 (15)	0.23 [0.049-1.1]	0.0693	8 (10)	12 (10)	0.040 [0.009-0.17]	<0.0001
Ethambutol	12 (15)	12 (15)	1.4 [0.37-5.0]	0.6468	9 (10)	12 (10)	0.15 [0.041-0.53]	0.0037
Isoniazid	10 (45)	9 (45)	4.6 [2.4-8.7]	<0.0001	9 (10)	13 (10)	0.036 [0.008-0.16]	<0.0001

Table S1.2. Summary statistics and significance testing for survival of treated and untreated larvae. Related to Figure 1 and Figure S2B-E. [a]Data reported are median survival days (number of larvae) in the absence and presence of drug. [b]Hazard Ratio [95% confidence interval] of treated larvae relative to untreated. [c]Results of Log-rank test comparing survival of treated vs. untreated larvae. For survival of uninfected larvae, results shown are from four independent experiments, and thus the *P* values can be interpreted relative to $\alpha = 0.05$. For survival of infected larvae, RIF and MOX groups were compared to a single UNT control, which contained 1% DMSO, while the EMB and INH groups were compared to a single UNT control lacking DMSO. Thus the *P* values can be interpreted relative to $\alpha = 0.025$ to account for multiple comparisons. All statistical analyses performed using GraphPad Prism v. 5.01.

Figure	Strain	Time point	UNT	INH	RIF	MOX
4D	WT-Mm M	2 hr	490 ± 54	0.07	0.3 ± 0.1	1.4 ± 0.2
		96 hr	222 ± 27	16 ± 0.8	6.2 ± 0.6	6.7 ± 1.4
4E	WT-Mtb H37Rv	2 hr	7500 ± 1180	0.57	0.57	-
		96 hr	595 ± 49	1.4 ± 0.1	1.7 ± 0.04	-

Table S1.3. Summary of the percent survival data shown in Figure 1.4. Related to Figure 1.4. The percent survival was calculated relative to the starting number of bacteria at the beginning of treatment. Numbers are the means of triplicate wells +/- SEM.

Figure	Strain	Condition	UNT	INH	RIF	MOX
5B	WT	UNT	1050 ± 380	18 ± 1.0	6.0 ± 1.5	-
		DEX	510 ± 67	25 ± 2.3	12 ± 2.0	-
5D	Mm/pBP10	Kan^S	928 ± 364	14 ± 2.1	5.3 ± 1.3	12 ± 2.3
		Kan^R	122 ± 16	4.0 ± 1.1	0.7 ± 0.2	1.6 ± 0.5

Table S4. Summary of the percent survival data shown in Figure 1.5. Related to Figure 1.5. The percent survival was calculated relative to the starting number of bacteria at the beginning of treatment. Numbers are the means of triplicate wells +/- SEM.

Figure	Strain	Condition	Timepoint	UNT	INH	RIF	INH+RIF
6A	WT Mm	UNT	2 hr	157 ± 15	0.3 ± 0.03	0.2 ± 0.03	
			96 hr	155 ± 18	19 ± 1.2	2.3 ± 0.6	
		VER	96 hr	127 ± 9.7	1.2 ± 0.07	0.3 ± 0.03	
6B	WT Mm	UNT	2 hr	199 ± 22	0.3 ± 0.04	0.3 ± 0.04	
			96 hr	254 ± 7.4	21 ± 1.0	2.7 ± 0.2	
		RES	96 hr	251 ± 30	4.4 ± 0.7	0.3 ± 0.02	
6C	CDC1551	UNT	2 hr	940 ± 26	0.09 ± 0.008	3.7 ± 0.2	
			96 hr	1990 ± 45	0.5 ± 0.05	10.9 ± 1.2	
			144 hr	958 ± 38	0.5 ± 0.07	18.4 ± 2.9	
		VER	96 hr	1360 ± 150	0.6 ± 0.03	7.6 ± 0.4	
			144 hr	774 ± 27	0.5 ± 0.07	9.6 ± 1.0	
6D	CDC1551		2 hr	796 ± 45	0.2 ± 0.03	4.2 ± 0.4	
			96 hr	2690 ± 130	0.8 ± 0.1	13 ± 0.5	
	M1		2 hr	859 ± 10	0.3 ± 0.04	6.5 ± 0.9	
			96 hr	2810 ± 215	1.8 ± 0.05	2.5 ± 0.6	
	M2		2 hr	1630 ± 435	0.2 ± 0.03	5.3 ± 0.3	
			96 hr	2980 ± 219	1.9 ± 0.2	2.6 ± 0.2	
6G	WT Mm	UNT	2 hr	1030 ± 221	8.2 ± 1.4	17 ± 1.9	2.8 ± 0.8
			96 hr	67 ± 4.6	24 ± 2.5	23 ± 5.4	16 ± 1.4
		VER	96 hr	50 ± 5.3	12 ± 1.8	12 ± 1.1	7.3 ± 1.5

Table S1.5. Summary of the percent survival data shown in Figure 1.6. Related to Figure 1.6. The percent survival was calculated relative to the starting number of bacteria at the beginning of treatment. Numbers are the means of triplicate wells +/- SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Bacterial Growth Media

Mm was cultured in Middlebrook 7H9 broth (Difco) supplemented with 0.5% BSA, 0.005% Oleic Acid, 0.2% glucose, 0.2% glycerol, 0.085% sodium chloride, and 0.05% Tween-80, or on Middlebrook 7H10 agar (Difco) supplemented with 0.5% BSA, 0.005% Oleic Acid, 0.2% glucose, 0.2% glycerol, and 0.085% sodium chloride. Mtb was cultured in Middlebrook 7H9 broth, or on 7H10 agar supplemented with enriched OADC enrichment (BBL). Media were supplemented with 20 μg/ml kanamycin and 50 μg/ml of hygromycin when appropriate to select for plasmids.

MIC assays

MICs were determined as follows: Individual plates of 7H10 agar (supplemented as described above) were prepared with two-fold dilutions of the antibiotic being tested. Approximately 10^4 Mm CFU were spotted in triplicate onto each plate and incubated at 33°C. The MIC was defined as the lowest concentration that prevented growth after 8-11 days. Alternately, MICs were determined by inoculating round bottom Costar 96-well plates with 100 μl drug-supplemented medium with ~10^4 CFU Mm and incubating for 6-8 days, prior to reading OD_{590} using a Tecan GENios Pro microplate reader. The MIC was defined as the lowest concentration that prevented growth.

Determination of bacterial growth rates using the unstable plasmid, pBP10

Mm strain M was transformed with pBP10, and colonies were selected by growth on 7H10 medium with 20 μg/ml kanamycin, yielding strain Mm/pBP10. For in vitro log-phase

cultures, Mm/pBP10 was grown in the absence of kanamycin under both static and rotating conditions to obtain different doubling times. The cultures were diluted daily to maintain continuous log-phase growth. For stationary-phase cultures, Mm/pBP10 was grown in the absence of kanamycin for 20 days. Plasmid loss was determined by CFU by plating on 7H10 with and without kanamycin. The segregation constant (*s*) was determined as previously described (Gill et al., 2009). Briefly, the growth rate and segregation constant of Mm was determined by using the following equations:

$$N(t)=N(0)e^{rt}$$

$$f(t)=f(0)e^{-rst}$$

Where *N*=the total number of bacteria, *r*=population growth rate, *t*=time in days and *f*=the frequency of plasmid-bearing bacteria at time *t*. *r* and *rs* was estimated by fitting regression lines through plots of *ln[N(t)]* and *ln[f(t)]* versus *t* (referred to as *SlopeN* and *Slopef*). *N* and *f* were estimated by CFU on plates with and without kanamycin. The value for *s* was calculated by dividing the estimate for *rs* by the estimate for *r*. The segregation constant *s* for Mm was 0.112 ± 0.001 (SEM). The in vitro determined segregation constant was then used to calculate the growth (*r*) and death rate (δ) of Mm during intracellular growth in THP1 and J774A.1 cell lines. The growth rate and death rate of Mm in THP1 and J774A.1 cell lines were determined by using the following equations:

$$r(t)=[SlopeN - SlopeP] / s$$

$$\delta(t)=r(t) - SlopeN$$

SlopeN and *SlopeP* were estimated from plots of *ln[N(t)]* and *ln[P(t)]* versus *t* (*P* is the number of bacteria carrying the plasmid). The number of dead bacteria for each time interval was determined using the equation:

$$D(t_f)=D(t_s) + \delta N(t_s)[e^{(r-\delta)(tf-ts)} - 1] / (r - \delta)$$

where $D(t)$ is the total number of bacteria killed at that interval. The total cumulative bacterial burden (CBB) was determined by adding $N(t)+D(t)$.

Notes to Section 1:

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SECTION 2: The *Mycobacterium tuberculosis* IniA membrane transporter confers macrophage-induced isoniazid tolerance that is inhibited by verapamil

SUMMARY

Drug tolerance has been largely attributed to bacterial non-replication in the host. However, we recently discovered that replicating mycobacteria quickly become tolerant to the front-line antitubercular drugs, rifampicin and isoniazid. We found that tolerance to rifampicin but not isoniazid was due to the induction of the Tap-like efflux pump Rv1258c that was also required for macrophage growth. These results linked efflux pump induction to rifampicin tolerance and intracellular growth. Here, we identify IniA, a component of an MDR-like efflux pump, as the mediator of macrophage-induced tolerance to isoniazid. An *iniA* mutant specifically fails to develop isoniazid tolerance while becoming tolerant to rifampicin. Like the *Rv1258c* mutant, it is attenuated for intracellular growth. These results and their fundamental and clinical implications will be described in this section.

INTRODUCTION

It has long been thought that drug tolerance is largely due to MTB becoming non-replicating in the host. Recently we found that rapidly-growing MTB becomes tolerant to the front-line anti-tubercular drugs rifampicin (RIF) and isoniazid (INH) upon macrophage infection (Adams et al., 2011). We showed that macrophage-induced RIF tolerance (but not INH tolerance) was mediated by the bacterial efflux pump Rv1258c, a macrophage-induced member of the major facilitator superfamily (MFS) (Adams et al., 2011). The Rv1258c mutant was also attenuated for macrophage growth (Adams et al., 2011). This result allowed us to formulate the model where mycobacteria induce efflux pumps upon entry into macrophages to facilitate their intracellular growth. These pumps then also pump out antibiotics. Furthermore, we showed that macrophage-induced RIF tolerance was reduced by the addition of verapamil, a calcium channel blocking drug that is known to also block efflux pumps (Amaral et al., 2007a; Nobili et al., 2006).

Because INH tolerance was also induced in rapidly growing bacteria upon macrophage residence, we reasoned that it must also be mediated by a different efflux pump. Consistent with this hypothesis, we found that verapamil also reduces macrophage-induced INH tolerance as it does RIF tolerance. To identify candidate INH pumps, we made a comprehensive literature search for MTB efflux pumps that were first associated with INH resistance and also transcriptionally induced during macrophage growth. *iniA* was the only one that met both criteria and we show here that IniA specifically confers macrophage-induced INH (but not RIF tolerance). Like Rv1258c, it too enhances macrophage growth in the absence of antimicrobial agents. Together these results support the model that both INH

and RIF tolerance are mediated by specific efflux pumps that are induced within macrophages as virulence determinants to promote intracellular bacterial growth.

RESULTS

Verapamil reduces macrophage-induced INH tolerance of MTB

Previously, we had found that in *M. marinum* the addition of 81.4 μM verapamil inhibited macrophage-induced RIF tolerance completely; in contrast, INH tolerance was inhibited substantially but not completely (**Figure 1.6A**) (Adams et al., 2011). For MTB, this same concentration of verapamil had a smaller effect on RIF and especially on INH tolerance (**Figure 1.6C**)(Adams et al., 2011). These data suggested that for both *M. marinum* and MTB, INH tolerance is more resistant to verapamil than RIF tolerance and that both RIF and INH tolerance are more resistant to verapamil in MTB than in *M. marinum*. This later result could be explained by the finding that the minimum inhibitory concentration (MIC) of verapamil for MTB is 2.5-fold higher than it is for *M. marinum* (407 μM for MTB vs 162.8 μM for *M. marinum*) (Balganesh et al., 2010)(KNA unplublished data).

Therefore, we tested in our assay a 2-fold higher concentration of verapamil (162.8 μM) than we had for *M. marinum* that was still sub inhibitory to broth-grown MTB. This verapamil concentration resulted in a significant reduction in macrophage-induced INH tolerance while completely inhibiting RIF tolerance (**Fig 2.1**). This conclusive demonstration that INH tolerance in MTB was inhibited by verapamil lent further support to the model that macrophage-induced INH tolerance of MTB is also efflux pump mediated. The relative resistance of INH tolerance to even this higher verapamil dose suggested two possibilities:

either multiple INH pumps are induced, some of which were verapamil insensitive or the single INH pump induced that is less sensitive to verapamil.

Discovery of MTB efflux pump mediating intramacrophage induced INH tolerance

We searched the literature for pumps associated with INH resistance and are also induced early during macrophage growth and found that only *iniA* matched both criteria (**Table 2.1**)(Colangeli et al., 2005a; da Silva et al., 2011; Louw et al., 2009a; Schnappinger et al., 2003b). IniA is a component of a MDR-like efflux pump known to be involved in drug-induced resistance to INH and ethambutol (Colangeli et al., 2005a).

We found that the *iniA* mutant failed to develop macrophage-induced tolerance to INH while developing tolerance to RIF that was comparable to wildtype (WT) (**Fig 2.2A and 2.2B**) distinct from the Rv1258c mutant that failed to develop RIF tolerance while developing INH tolerance (**Fig 2.2D and E**). Like the *Rv1258c* mutant, the *iniA* mutant is also attenuated for intracellular growth (**Fig 2.2C and F**), indicating a dual role for this efflux pump in also promoting intracellular growth and INH tolerance.

DISCUSSION

These results identify the IniA efflux pump as the major mediator of macrophage-induced INH tolerance and show that this tolerance is also sensitive to verapmail inhibition. Two lines of evidence support the clinical relevance of our finding: mutations in *iniA* have been found in both INH and ethambutol resistant strains of MTB (Ramaswamy et al., 2000; Zhang et al., 2005) and *iniA* is induced upon macrophage infection in clinical MTB strains from around the world (Homolka et al., 2010).

Expression of *iniA* is induced by a broad range of cell-wall inhibitors including INH and ethambutol, but not other biological stresses including low pH, hydrogen peroxide, lysozyme and other antibiotics that do not directly inhibit cell-wall biosynthesis (Alland et al., 2000b; Colangeli et al., 2005a). Its induction during macrophage infection would suggest that *in vivo* cell wall modifications occur early within the macrophage environment. Indeed, multiple genes thought to be involved in cell wall remodeling are induced within several hours of infection (Rohde et al., 2007a; Schnappinger et al., 2003b), including those like *fadD26* and *umaA,* which are involved either in the synthesis (FadD26) or modification (UmaA) of cell wall lipids (Laval et al., 2008; Simeone et al., 2010). Further analysis of genes involved in cell wall remodeling during infection may give insight to the natural substrate(s) of IniA and how it functions to promote intracellular growth and macrophage-induced tolerance to INH.

Further structural analysis by Colangeli et al has shown that IniA forms a transmembrane pore, similar to other transporters. Interestingly, they find that IniA is unlikely directly exporting INH itself as WT and *iniA* mutant MTB strains accumulated similar amounts of intracellular INH (Colangeli et al., 2005a). The authors postulate that IniA may be functioning to remove toxic cell wall components that accumulate as a result of INH's (or other cell wall inhibitors) inhibition of cell wall synthesis (Colangeli et al., 2005a). Consistent with their distinct structural features and postulated mechanisms of actions, we find that IniA and Rv1258c play a nonredundant role in promoting intracellular bacterial growth. Therefore the finding that verapamil can target both pumps suggest its potential not only to reduce both INH and RIF tolerance but also suggests that it will produce greater reduction in intracellular bacterial numbers than inhibition of either pump alone.

Identifying an efflux pump inhibitor that can target these macrophage-induced pumps may be useful for increasing the effectiveness of the current anti-tubercular drug treatment regimen and have the potential to decrease the time needed for treatment by reducing antibiotic tolerance. Such an inhibitor might also be useful in combating some forms of genetic resistance, as is seen with overexpression of *iniA* and *Rv1258c* (Colangeli et al., 2005a; Jiang et al., 2008; Siddiqi et al., 2004). Ultimately, understanding how IniA and Rv1258c are functioning *in vivo* and identifying their substrate(s) may lead to even better approaches to reduce INH and RIF tolerance and subsequently shorten TB treatment time.

As predicted, the *iniA* mutant failed to develop tolerance to INH in our assay (Figure 2.2A), and the finding that it is also attenuated for intracellular growth (Figure 2.2C) is consistent with these efflux pumps being required to expel other growth limiting substrate(s) during intracellular residence in the absence of antibiotics.

EXPERIMENTAL PROCEDURES

Bacterial strains, methods and chemicals

MTB strain JHU0342-1574 (harboring a transposon insertion at position 1574 in *iniA*), JHU1258c-833 (harboring transposon insertions at positions 833, in Rv1258c) and the wild-type parent strain, CDC1551, were a gift of W. R. Bishai and G. Lamichhane (Johns Hopkins University) (Lamichhane et al., 2003). MTB were grown to mid log phase in Middlebrook 7H9 medium (Becton Dickinson) with 0.05% Tween-80 and albumin, dextrose, catalase (Middlebrook ADC Enrichment, BBL Microbiology) prior to infection. When required kanamycin was added at a final concentration of 20 μg/ml. Streptomycin (STR), rifampicin (RIF), isoniazid (INH) and verapamil (VER) were purchased from Sigma.

Macrophage Growth and Infection

THP-1 macrophages were grown in RPMI, supplemented with 10% FBS and 1% L-glutamine. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate for 48 hours and allowed to recover for 48 hours prior to infection. $5x10^5$ THP-1 macrophages were infected at an MOI of 1.5 for 3 hours at 37°C. Cells were washed with medium and then 6 μg/ml STR was added for the duration of the intracellular growth. Medium was changed daily. Macrophages were lysed with 0.1% Triton X-100 for intracellular growth quantization. To lyse macrophages and release bacteria for subsequent tolerance assessment, each well was first washed once with 1x PBS, and once with diH_2O, with the latter wash being removed immediately. Then, 100μl of diH_20 was added and the cells incubated for 15 minutes to lyse macrophages. Finally, 900μl of 1.1x concentrated 7H9 medium was added and the wells scraped gently with a pipette tip to release all release all macrophages. CFU were enumerated from triplicate wells on supplemented 7H10 agar. For determination of antibiotic killing, the percent survival was calculated by dividing the CFU for each well by the mean CFU present prior to treatment.

Statistics

Statistical analyses were performed using Prism 5.01 (GraphPad). Post test *P* values are as follows: *, $P<0.05$; **, $P<0.01$; ***$P<0.001$.

FIGURES

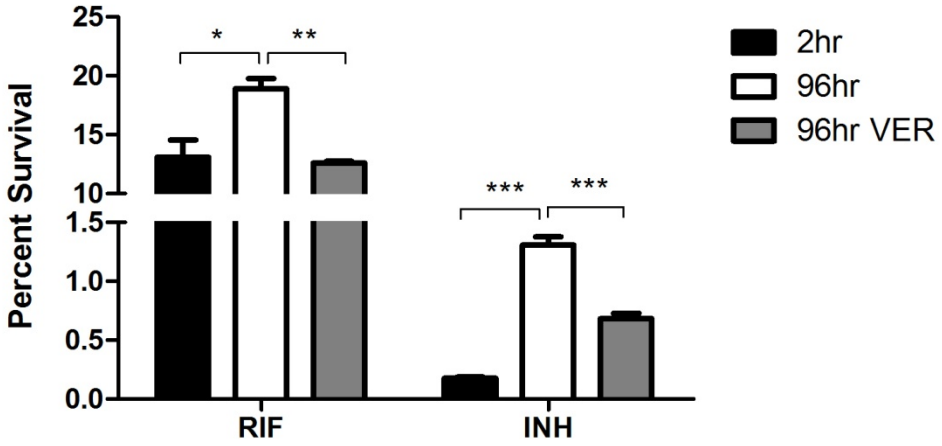

Figure 2.1. Verapamil reduces INH tolerance in MTB.
THP-1 macrophages were infected with wild-type CDC 1551 MTB and lysed at 2 or 96 hours post infection (hpi). The released bacteria were treated for an additional 48 hr with 4.4 μM INH or 1.2 μM RIF or left untreated in the presence or absence of 162.8 μM verapamil prior to enumeration of CFU.
Error bars represent SEM. Significance testing was performed using one-way ANOVA with Dunnett's post-test.

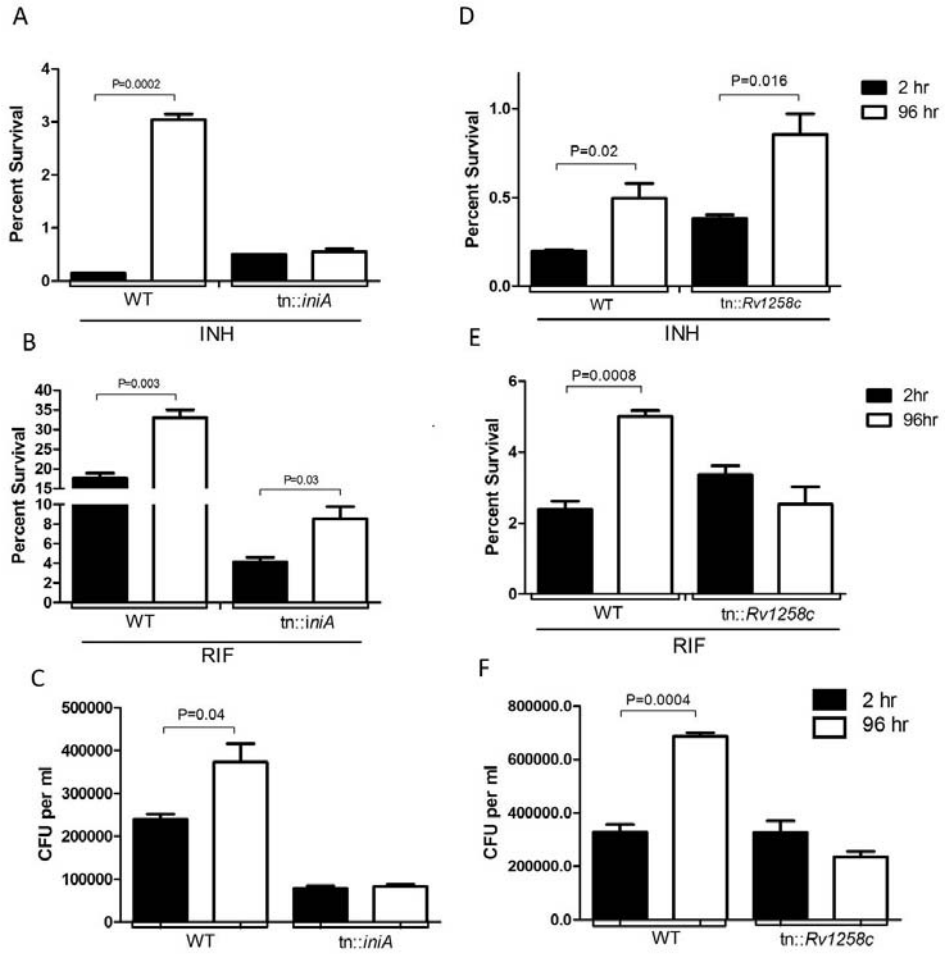

Figure 2.2. IniA mediates macrophage-induced INH tolerance and intracellular growth.
(A and B) THP-1 macrophages were infected with Mtb strains JHU0342 and the isogenic wild-type control, CDC 1551, for two or 96 hours prior to lysis and enumeration of CFU. Released bacteria were treated with 4.4 μM INH or 1.2 μM RIF left untreated for 48 hr prior to enumeration of CFU.
(C) Released bacteria were treated as described in panel (A).
(D and E) THP-1 macrophages were infected with Mtb strains JHU1258c-833 and the isogenic wild-type control, CDC 1551, and treated as described in (A).
(F) Released bacteria were treated as described in panel (A).
Error bars represent SEM. *P* values were determined using Student's *t*-test.

Drug Efflux Pump	Transporter Family	Drug(s)	Induction in macrophages*	References
pstB	ABC	INH, RIF, EMB, CIP	0.7	(Gupta et al., 2006)
Rv1747	ABC	INH	0.9	(Louw et al., 2009a)
Rv1258c	MFS	INH, RIF, OFX	1.7	(Jiang et al., 2008; Rodrigues et al., 2011; Siddiqi et al., 2004; Zhang et al., 2005)
Rv1410c	MFS	INH, RIF, STR	0.8	(Jiang et al., 2008)
Rv2459 (jefA)	MFS	INH, EMB	0.8	(Gupta et al., 2010c)
Rv2846c (efpA)	MFS	INH, ETH	ND	(Rodrigues et al., 2011; Zhang et al., 2005)
mmpL7	RND	INH	ND	(Pasca et al., 2005; Rodrigues et al., 2011)
iniA	Membrane Protein	INH, EMB	1.4 and 2.0**	(Alland et al., 2000b; Colangeli et al., 2005a; Ramaswamy et al., 2000; Ramaswamy et al., 2003)

Isoniazid (INH); rifampicin (RIF); ofloxacin (OFX); streptomycin (STR); ethambutol (EMB); ethionamide (ETH); ciprofloxacin (CIP)

Table 2.1. Identified transporters to be involved in INH resistance.
*Microarray data from 48 hr naïve macrophages (Schnappinger et al., 2003b).
** Two values listed

Notes to Section 2:

This work was done in collaboration with Dr. John D. Szumowski in the laboratory of Dr. Lalita Ramakrishnan. Transposon mutants were a gift from Dr. Gyanu Lamichhane. Experiments involving IniA were performed by K.N. Adams and J.D. Szumowski. All other experiments were done by K.N. Adams.

SECTION 3: Practical applications of an efflux pump inhibitor during tuberculosis infection

SUMMARY

A major goal of antitubercular drug discovery is to overcome tolerance thereby reducing treatment time. We previously found that macrophage-induced drug tolerance is sensitive to the calcium channel blocker verapamil with rifampicin tolerance being more sensitive than isoniazid tolerance. An examination of two additional efflux pump inhibitors currently marketed as drugs, reserpine and piperine, again revealed they had better activity against rifampicin than isoniazid. Of the three tested inhibitors, verapamil had the best activity against both isoniazid and rifampicin tolerance and is a well-tolerated drug to treat hypertension and arrhythmias. Therefore, verapamil is the best candidate drug amongst currently used efflux pump inhibitors for a clinical trial to test the treatment-shortening effects of these inhibitors. However, verapamil is metabolized in vivo by CYP3A4 whose expression is induced by rifampicin, a critical drug for TB treatment. Here, we find that the major metabolite of verapamil, norverapamil, is similarly capable of reducing efflux-mediated drug tolerance. These results suggest that it will be practical to use verapamil as adjunctive therapy.

INTRODUCTION

The use of efflux pump inhibitors such as verapamil has immediate clinical relevance; indeed, based on our finding, clinical trials are now being designed to determine if the addition of verapamil to standard antitubercular regimens will shorten the time to cure. In this section I will describe experiments I performed as a prelude to clinical trials to address the feasibility of using verapamil in the context of tuberculosis (TB) treatment.

There are two issues with the use of verapamil, the first is host toxicity and the second is bioavailability. Fortunately, verapamil has been well studied in cardiac patients and has historically been fairly well tolerated (Leone et al., 2000; Pfizer, 2010). Of course, it is not clear if verapamil will be equally well tolerated in TB patients who may be sicker and have lower body weights than cardiac patients. Regarding its bioavailability, a potential problem arises with verapamil in combination with RIF. The major drug-metabolizing enzyme for verapamil is CYP3A4, whose gene expression is induced by RIF (Niemi et al., 2003). In the presence of RIF, verapamil levels are rapidly decreased (Aristoff et al., 2010; Barbarash et al., 1988), potentially hindering its efficacy *in vivo*. RIF has a smaller effect on norverapamil the major metabolite of verapamil (Barbarash et al., 1988). NOR has only 20% of the activity of verapamil (Frishman et al., 1982), suggesting greatly reduced calcium channel blocking activity.

Additional human approved drugs have been shown to inhibit antibiotic efflux in MTB including Piperine, an inhibitor of CYP3A4 and reserpine, an inhibitor of vesicular catecholamine transporter, VMAT2 (Bhardwaj et al., 2002; Henry et al., 1994). Both piperine and reserpine have been shown to inhibit RIF efflux (Adams et al., 2011; Piddock et al., 2000; Sharma et al., 2010a), however, in addition to inhibiting RIF efflux, reserpine has also

been shown to inhibit the efflux of INH (Adams et al., 2011; Bianco et al., 2011; Colangeli et al., 2005a; Pasca et al., 2005; Viveiros et al., 2002a).

Here, we found the efflux pump inhibitors piperine and reserpine reduce macrophage-induced RIF tolerance, but are less effective against INH tolerance. Given verapamil's effectiveness at reducing both, we further pursued verapamil as a potential drug to reduce macrophage-induced RIF and INH tolerance. We show that verapamil's efflux pump activity is independent of its calcium channel blocking activity and then show that norverapamil has equivalent inhibitory activity as verapamil for both INH and RIF tolerance.

RESULTS

Additional efflux pump inhibitors reduce macrophage-induced RIF tolerance

We searched for additional efflux pump inhibitors for two reasons; first, Verapamil reduces macrophage-induced RIF tolerance completely but its effect on INH tolerance, though substantial is incomplete (Figure 2.1) and second, verapamil use presents a potential clinical problem as its levels are profoundly decreased by concomitant RIF treatment, a key component of modern TB therapy (Aristoff et al., 2010; Barbarash et al., 1988). Reserpine has been shown to inhibit a number of mycobacterial efflux pumps involved in INH resistance (Choudhuri et al., 2002; Pasca et al., 2005; Viveiros et al., 2002a).We previously reported that the pump mediating INH tolerance is IniA (**Figure 2.2A**). The data on the sensitivity of this pump to reserpine are ambiguous. Reserpine can inhibit the INH resistance caused by overexpression of the *iniA* gene in *Mycobacterium bovis* BCG but not MTB (Colangeli et al., 2005a). We have previously shown that in *M. marinum*, reserpine is effective at reducing macrophage-induced INH tolerance (**Figure 1.6B**) (Adams et al., 2011).

However, we found that reserpine had no effect on INH tolerance in MTB, whereas it did reduce RIF tolerance (**Fig 3.1A**). Taken together with the results from Colangeli et al, these data suggest that MTB IniA is more resistant to reserpine than in BCG and *M. marinum*. Importantly, they indicate that verapamil is the better option to reduce both RIF and INH tolerance in MTB.

We next evaluated piperine, a major alkaloid from the black pepper (*Piper nigrum*) plant, that has been previously identified as an efflux pump inhibitor specifically targeting Rv1258c (Khan et al., 2006; Sharma et al., 2010a). The combination of RIF and piperine has been shown to sustain RIF levels in humans for a longer periods versus RIF alone (Zutshi et al., 1985). According to the makers of Risorine (a combination drug made up of RIF, INH and piperine), piperine acts as a RIF bioavailabity enhancer so they are using it to reduce the levels of RIF to be administered during TB treatment (Cadila). We considered the possibility that the mechanism of piperine's activity *in vivo* might be a direct result of mycobacterial pump inhibition. We found that PIP was effective at reducing RIF tolerance (1.4-fold for verapamil and 1.7-fold for PIP) but did not decrease in INH tolerance significantly (**Fig 3.1B**). These results suggest that in addition to sustaining RIF levels *in vivo* (Zutshi et al., 1985), PIP may also be acting on bacterial efflux pumps. However, again verapamil is revealed to produce the best overall reduction of both INH and RIF tolerance.

Inhibition of bacterial efflux pumps by verapamil is independent of its calcium channel blockade

Verapamil is a calcium channel blocker, and calcium supplementation is used to treat verapamil toxicity (DeWitt and Waksman, 2004). We felt it was important to determine if

verapamil's action on efflux pumps was mediated directly or indirectly via its calcium channel activity. If so then calcium supplementation should interfere with verapamil's efflux pump inhibitory properties. We first tested two different concentrations of calcium in untreated, macrophage-released *Mycobacterium marinum* to determine a concentration that is not toxic to the bacteria. We found that 10mM $CaCl_2$ to be toxic on its own, whereas 1mM CaCl2 did not impede bacterial growth (**Fig 3.2A**), so we chose to supplement 1mM $CaCl_2$ in our RIF-tolerance assay. We found that in the presence of 1mM $CaCl_2$, verapamil was still effective at reducing macrophage-induced RIF tolerance in *M. marinum* (**Fig 3.2B**) indicating that even the very high calcium concentration of 1mM did not reduce verapamil's activity.

These results indicate that verapamil's inhibition of bacterial efflux pumps is not mediated via its calcium channel activity. This has two important implications: first that co-administration of calcium to treat or prevent verapamil's calcium blocking effects will not have an effect on bacterial efflux pumps, secondly, and perhaps more importantly it suggests that verapamil metabolites resulting from induction of CYP3A4 that are structurally similar, but lack calcium channel blocking activity may also inhibit bacterial efflux pumps.

Norverapamil reduces macrophage-induced RIF and INH tolerance

The amount of verapamil needed to block MTB efflux pumps *in vivo* is not known. Verapamil is metabolized *in vivo* by CYP3A4, a major drug-metabolizing enzyme present in both the liver and intestine whose expression is induced by RIF (Niemi et al., 2003). Verapamil levels are profoundly decreased by concomitant RIF treatment, presenting a significant hurdle to the clinical use of verapamil as a TB drug therapy shortening agent

(Aristoff et al., 2010; Barbarash et al., 1988). The major metabolite of verapamil *in vivo* is norverapamil (Kates, 1983) as levels of norverapamil are comparable to verapamil in humans. In addition, RIF has much less of an effect on norverapamil levels (Barbarash et al., 1988). Therefore, we asked if norverapamil inhibits macrophage-induced INH and RIF tolerance for MTB. We found that norverapamil caused a similar reduction in tolerance for both drugs compared to verapamil (for RIF, 1.5-fold for verapamil and 1.4-fold for norverapamil and for INH, 1.9-fold for verapamil and 1.7-fold for norverapamil) (**Fig 3.3**). These results suggest that even in the face of rapid verapamil metabolism, norverapamil can reduce efflux-mediated drug tolerance.

DISCUSSION

While identifying reserpine and piperine as additional inhibitors of RIF tolerance, these findings show that verapamil is the best agent to overcome both INH and RIF tolerance. We find that verapamil's efflux pump activity is independent of its calcium channel blockade and show that its major metabolite norverapamil is equally active against drug efflux. In sum, these findings support the use of verapamil as an adjunct to current antitubercular chemotherapy to shorten the duration of treatment.

In MTB, a number of efflux pumps have been identified that are capable of exporting a diverse array of antibiotics and are thought to contribute to MTB's intrinsic resistance as well as to the development of genetic resistance during TB chemotherapy (da Silva et al., 2011; Louw et al., 2009a; Warner and Mizrahi, 2006b). Approximately 20 to 30 percent of isoniazid (INH)-resistant MTB isolates lack mutations in genes known to be associated with

INH resistance (*katG* and *inhA*) and five percent of rifampicin (RIF) resistant isolates do not harbor mutations in *rpoB* (Louw et al., 2009a; Ramaswamy et al., 2003; Telenti et al., 1993). Overexpression of efflux pumps has been identified in some of these resistant MTB isolates (Ramaswamy et al., 2000; Ramaswamy et al., 2003; Siddiqi et al., 2004; Zhang et al., 2005), implicating drug efflux as an additional mechanism for the acquisition of genetic resistance. Given the role of efflux pumps in both drug tolerance and genetic resistance, it stands to reason that inhibition of bacterial efflux pumps has the potential to greatly enhance antibiotic efficacy. Multiple MTB efflux pumps, from different classes, have been shown to be sensitive to verapamil (Choudhuri et al., 2002; Gupta et al., 2010c; Louw et al., 2011; Pasca et al., 2004; Silva et al., 2001), including those pumps induced during intramacrophage growth (Schnappinger et al., 2003b).

We also examined the mycobacterial efflux pump inhibitor activity of piperine, which was originally identified as an inhibitor of the *Staphlococcus aureus* NorA efflux pump and was later reported to inhibit RIF-resistance mediated by Rv1258c in MTB (Khan et al., 2006; Sharma et al., 2010a). Piperine is already being used in combination with RIF and INH (Risorine ®, Cadila Pharmaceuticals) as a bioavailability enhancer (Bhardwaj et al., 2002; Zutshi et al., 1985). The combination of RIF and piperine was shown to sustain RIF levels in humans for longer periods versus RIF alone (Zutshi et al., 1985). The precise mechanisms of how piperine is acting remains poorly understood but may in part result from the inhibition of gut P-glycoprotein (Cadila), which is thought to expel RIF from host cells (Magnarin et al., 2004). We found that piperine was able to reverse macrophage-induced RIF tolerance (**Figure 3.1B**). Along with studies from Calida, these results suggest that piperine may be another efflux pump inhibitor that will be effective at shortening drug treatment.

piperine is a known inhibitor of CYP3A4 and has been shown to inhibit the catalysis of verapamil to norverapamil via CYP3A4 inhibition (Bhardwaj et al., 2002). The combination of verapamil and piperine given as adjuncts to standard TB drugs may be a productive approach, as piperine may help sustain RIF and verapamil levels *in vivo*. However, pharmacokinetic studies of piperine in humans would need to be done to assess toxicity and possible drug interactions.

A potential pitfall for the use of verapamil in combination with RIF is that verapamil is rapidly metabolized by CYP3A4, which is induced by RIF (Barbarash et al., 1988). Although verapamil levels in plasma may be lowered significantly by concomitant RIF treatment, verapamil is concentrated in the lung, so local concentrations might still be adequate (Schwartz et al., 1986). Indeed, Louw et al found that adding verapamil to INH, RIF and pyrazinamide lead to a significant reduction in pulmonary bacterial burdens in mice infected with MDR MTB (Louw et al., 2011). Additionally, even in the face of rapid metabolism, our macrophage model of drug tolerance demonstrates that norverapamil is just as effective at reducing RIF and INH tolerance (**Figure 3.3**), and its levels are reduced to a lesser extent than verapamil by concomitant RIF treatment (Barbarash et al., 1988).

EXPERIMENTAL PROCEDURES

Bacterial strains, methods and chemicals

M. marinum strain M (ATCC BAA-535) has been described (Cosma et al., 2006a). MTB strain CDC1551, was a gift of W. R. Bishai and G. Lamichhane (Johns Hopkins University) (Lamichhane et al., 2003). MTB were grown to mid log phase in Middlebrook

7H9 medium (Becton Dickinson) with 0.05% Tween-80 and albumin, dextrose, catalase (Middlebrook ADC Enrichment, BBL Microbiology) prior to infection. Streptomycin (STR), rifampicin (RIF), isoniazid (INH), verapamil (VER) and piperine (PIP) were purchased from Sigma. Norverapamil (NOR) was purchased from Santa Cruz Biotechnology.

Macrophage Growth and Infection

THP-1 macrophages were grown in RPMI, supplemented with 10% FBS and 1% L-glutamine. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate for 48 hours and allowed to recover for 48 hours prior to infection. $5x10^5$ THP-1 macrophages were infected at an MOI of 1.5 for 3 hours at 37°C. Cells were washed with medium and then 6 μg/ml STR was added for the duration of the intracellular growth. Medium was changed daily. Macrophages were lysed with 0.1% Triton X-100 for intracellular growth quantization. To lyse macrophages and release bacteria for subsequent tolerance assessment, each well was first washed once with 1x PBS, and once with diH_2O, with the latter wash being removed immediately. Then, 100μl of diH_20 was added and the cells incubated for 15 minutes to lyse macrophages. Finally, 900μl of 1.1x concentrated 7H9 medium was added and the wells scraped gently with a pipette tip to release all release all macrophages. CFU were enumerated from triplicate wells on supplemented 7H10 agar. For determination of antibiotic killing, the percent survival was calculated by dividing the CFU for each well by the mean CFU present prior to treatment.

Statistics

Statistical analyses were performed using Prism 5.01 (GraphPad). Post test *P* values are as follows: *, $P<0.05$; **, $P<0.01$; ***$P<0.001$.

FIGURES

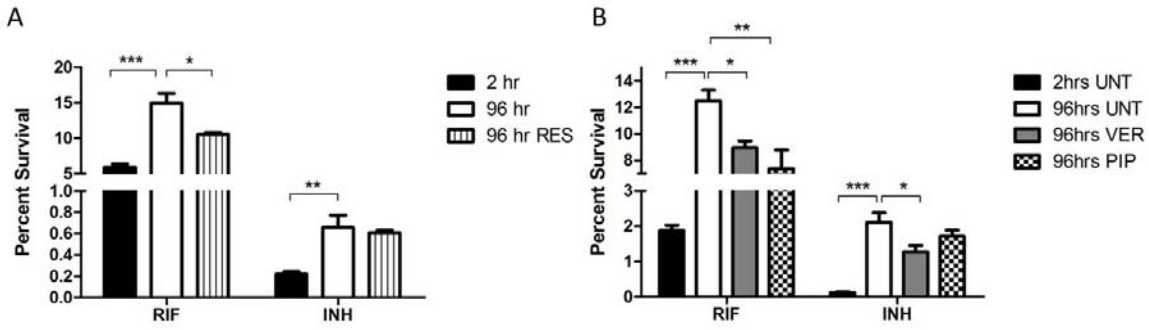

Figure 3.1. Piperine reduces RIF tolerance in MTB.
(A) THP-1 macrophages were infected with MTB strain CDC 1551 and lysed at 2 or 96 hours post infection (hpi). The released bacteria were treated for an additional 48 hr with 1.2 μM RIF, 4.4 μM INH or left untreated in the presence or absence of 65.7 μM reserpine prior to enumeration of CFU.
(B) THP-1 macrophages were infected with MTB strain CDC 1551 treated as in (A). The released bacteria were treated for an additional 48 hr with 1.2 μM RIF, 4.4 μM INH or left untreated in the presence or absence of 162.8 μM verapamil or 350 μM piperine prior to enumeration of CFU.
Error bars represent SEM. Significance testing was performed using one-way ANOVA with Dunnett's post-test.

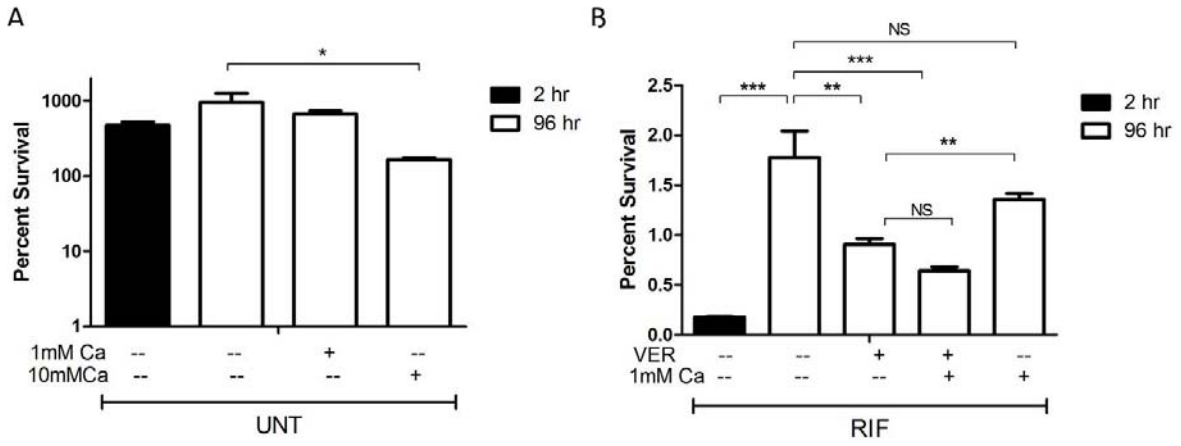

Figure 3.2. Reduction of RIF tolerance by verapamil in the presence of calcium.

(A) THP-1 macrophages were infected with *M. marinum* strain M lysed at 2 or 96 hours post infection (hpi). Bacteria released at 96 hours were treated for an additional 48 hours with 1mM or 10mM $CaCl_2$ or left untreated prior to enumeration of CFU.

(B) The released bacteria were treated as in (A) except for an additional 48 hr with 1.2 μM RIF, or left untreated in the presence or absence of 81.4 μM verapamil and/or 1mM $CaCl_2$ prior to enumeration of CFU.

Error bars represent SEM. Significance testing was performed using one-way ANOVA with Bonferroni's multiple comparisons post-test.

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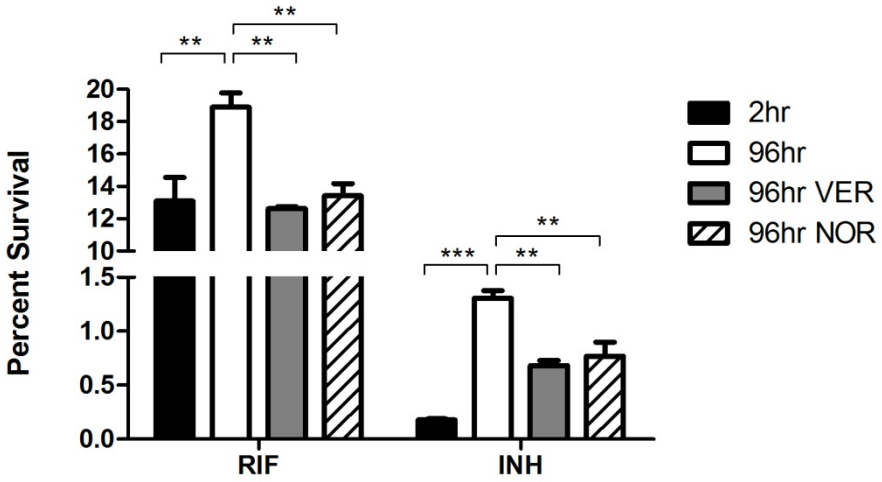

Figure 3.3. Norverapamil reduces RIF and INH tolerance in MTB.
THP-1 macrophages were infected with MTB strain CDC 1551and lysed at 2 or 96 hours post infection (hpi). The released bacteria were treated for an additional 48 hr with 1.2 μM RIF, 4.4 μM INH or left untreated in the presence or absence of 162.8 μM verapamil or 167.7 μM norverapamil prior to enumeration of CFU.
Error bars represent SEM. Significance testing was performed using one-way ANOVA with Dunnett's post-test.

SECTION 4: Macrophage-induced tolerance to secondary antitubercular drugs moxifloxacin and streptomycin is not inhibited by verapamil and piperine

SUMMARY

Cure of multi-drug resistant (MDR) tuberculosis requires a longer treatment course than does drug susceptible TB and relies on more toxic and less efficacious drugs. We have previously found that *Mycobacterium tuberculosis* develops drug tolerance to first-line antitubercular drugs following macrophage residence. Here, we report that macrophage-induced tolerance to moxifloxacin and streptomycin, which may have significant clinical implications as fluoroquinolones such as moxifloxacin are an important component of treatment for MDR tuberculosis and streptomycin is still frequently used in retreatment regimens. Tolerance to moxifloxacin and streptomycin was not sensitive to verapamil that reduces isoniazid and rifampicin tolerance. We have identified two efflux pumps, Rv1634 and ArsB2, mediating macrophage-induced moxifloxacin tolerance. Identifying effective efflux pump inhibitors to reduce macrophage-induced tolerance may permit shortening of treatment for drug-resistant tuberculosis.

INTRODUCTION

As of 2008, 3.6 percent of tuberculosis (TB) cases worldwide are caused by multidrug resistant (MDR) TB defined as being resistant to both isoniazid (INH) and rifampicin (RIF) (WHO, 2010). MDR TB is particularly common amoung person previously treated for TB: 15% of relapsed TB patients have MDR, five times higher than the average for treatment-naive patients (Mitnick et al., 2003). Treatment of MDR TB requires a minimum of 18 months with regimens containing drugs that are not only less efficacious than INH and RIF but also not as well tolerated (2008a; 2008b; 2008c; WHO, 2011). Even with these drugs, cure rates of MDR TB are much lower than those with drug sensitive TB (WHO, 2011). Current WHO MDR treatment guidelines call for four drugs that include a fluoroquinolone, an aminoglycoside, ethionamide, and cycloserine (WHO, 2009).

Since the fluoroquinolones and aminoglycosides comprise the bactericidal drugs in the MDR TB regimen, we asked if macrophage-induced tolerance develops to these drugs also. Here we show that tolerance does indeed develop and find that neither moxifloxacin (MOX) nor streptomycin (STR) tolerance is sensitive to verapamil, suggesting that it is mediated by efflux pumps other than Rv1258c and IniA. We identified two additional efflux pumps mediating MOX tolerance, Rv1634 and ArsB2, which also contributes to RIF tolerance. These results further support a model whereby diverse macrophage-induced efflux pumps mediate multidrug tolerance during intracellular growth of MTB.

RESULTS

Macrophage-induced tolerance to moxifloxacin and streptomycin of MTB is insentive to verapamil

Given the importance of fluoroquinolones in treatment of MDR TB and continued reliance on STR-based regimens for retreatment cases, we wanted to determine if MTB also develops macrophage-induced tolerance to MOX and STR. We found that this is indeed the case (**Fig 4.1A and B**). Next, we asked if macrophage-induced MOX and STR tolerance was sensitive to verapamil and piperine. Verapamil, its metabolite norverapamil and piperine all failed to inhibit MOX and STR tolerance (**Fig 4.1A and B**). This result suggests that MOX and STR tolerance are mediated by distinct pumps from Rv1258c and IniA as the RIF and INH tolerance they mediate is inhibited by verapamil (**Figure 2.1**).

Rv1634 and ArsB2 mediate macrophage-induced moxifloxacin tolerance

We searched the literature to create a list of MTB efflux pumps, finding representatives from four of the five major efflux pump families (ABC, MFS, RND and SMR) (**Table 4.1**). Among these, we first looked for pumps associated with fluoroquinolone resistance and then determined which pumps were induced in macrophages (Schnappinger et al., 2003b). We identified three additional pumps, two of which are ABC transporters and one which is a MFS transporter. Since clinically relevant resistance has yet to be linked to ABC transporters (Piddock, 2006), we choose to test the MFS pump Rv1634. Like Rv1258c, Rv1634 is a member of the MFS family of efflux pumps and has been shown to be involved in fluoroquinolone efflux (De Rossi et al., 2002). *Rv1634* is variably induced in

macrophages in different studies, ranging from 1.2-fold (**Table 4.1**) (Schnappinger et al., 2003b) to 2-fold (www.tbdb.org) (Fontan et al., 2008a).

We found that the *Rv1634* mutant failed to develop macrophage-induced tolerance to MOX while developing tolerance to STR, RIF and INH comparable to wildtype (**Fig 4.2A-D**). However, unlike the *Rv1258c* and *iniA* mutants, the *Rv1634* mutant did not show evidence of reduced growth in macrophages (**Fig 4.2E**), indicating that it is not a MTB virulence factor. An additional *Rv1634* transposon mutant was also tested and also failed to develop tolerance to MOX and grew like WT in macrophages (data not shown).

In our ongoing search for efflux pumps mediating tolerance to commonly-used TB drugs, we also tested an additional macrophage-induced pump *arsB2*, the membrane component of a predicted arsenic pump (Borges-Walmsley and Walmsley, 2001). We found that the *arsB2* mutant failed to develop macrophage-induced tolerance to MOX and RIF while developing tolerance to INH and STR (**Fig 4.3A-D**). Additionally, the *arsB2* mutant was attenuated for intracellular growth (**Fig 4.3E**), as we observed for the *Rv1258c* mutant (**Figure 2.2F**).

DISCUSSION

These results demonstrate that while MTB develops macrophage-induced tolerance to MOX and STR, this tolerance is not sensitive to verapamil and piperine, indicating that tolerance to these drugs is not mediated by Rv1258c or IniA. Whereas verapamil is effective at reducing macrophage induced tolerance against the first-line drugs RIF and INH, other efflux pump inhibitors need to be identified that can reduce tolerance to STR, a component of

some retreatment regimens (WHO, 2009), as well as MOX, an agent used in treatment of MDR TB.

We identify Rv1634 and ArsB2 as pumps mediating macrophage-induced MOX tolerance (**Figures 4.2A and 4.3A)**, with ArsB2 but not Rv1634 additionally promoting growth (**Figures 4.2E and 4.3E**). Since MOX tolerance, like RIF and INH tolerance is also enriched in replicating bacteria (**Figure 1.5D**) (Adams et al., 2011), we propose that ArsB2 is the principle efflux pump involved in macrophage-induced MOX tolerance. In addition, the discovery of a pump that mediates tolerance but not bacterial growth highlights our lack of understanding of the natural substrates transported by these pumps. It is possible that the Rv1634 pump functions during normal infection under circumstances that are not in play during early infection of cultured macrophages. For instance, it may be involved for bacterial survival only in activated macrophages, as it was found to be induced in interferon-γ treated macrophages (Schnappinger et al., 2003b). In support of this, mycobacterial genes have been previously identified that are not induced in cultured macrophages *in vitro*, but are in macrophages and granulomas *in vivo* (Davis et al., 2002).

Given that MTB develops macrophage-induce STR tolerance, we still need to identify the responsible pump(s). Several candidate STR pumps belong to the MFS and ABC transporter families. The MFS transporter Rv1410c has been shown to be involved in STR resistance and though not induced in macrophages (Schnappinger et al., 2003b), it has been found to been essential for growth within macrophages (Rengarajan et al., 2005) and survival in vivo in a murine TB model (Sassetti and Rubin, 2003). Rv0194 is an ABC transporter shown to be invovled in STR resistance (Danilchanka et al., 2008) and is also both induced in

macrophages and important for intramacrophage growth (Rengarajan et al., 2005; Schnappinger et al., 2003b).

Even in the absence of the identification of the exact pumps, a targeted search for inhibitors of macrophage-induced STR and MOX tolerance should provide candidate compounds for adjunctive treatment to shorten the duration of treatment time needed for MDR TB. Potential compounds to be screened include phenothiazine derivatives, reserpine, or P-glycoprotein inhibitors such as tariquidar for the ability to block the MTB efflux pumps mediating MOX and STR tolerance (Amaral and Molnar, 2012; Leitner et al., 2011).

Finally, we note that the ArsB pump also mediates RIF tolerance and the fact that MOX tolerance is completely resistant to verapamil and piperine suggests that this pump is resistant to these compounds. What is the relative importance of the ArsB2 and Rv1258c pumps in mediating RIF tolerance? We note that RIF tolerance is completely suppressed by verapamil, suggesting that the ArsB2 pump is in play in the absence of the Rv1258c pump. This could be because the relative kinetics of pumping rifampicin could be higher for Rv1258c pump vs the ArsB2 pump. Nevertheless, this finding of a second pump that mediates RIF tolerance suggests that a comprehensive search for additional efflux pump inhibitors is in order to provide the best adjunctive treatments for drug sensitive and MDR TB.

EXPERIMENTAL PROCEDURES

Bacterial strains, methods and chemicals

MTB strain B0150 (transposon insertion at position 187 in *Rv1634*), STN1121 (transposon insertion at 1334 in *Rv1634*), J00145 (transposon insertion at 1078 in *arsB2*) and

the wild-type parent strain, CDC1551, were a gift of W. R. Bishai and G. Lamichhane (Johns Hopkins University) (Lamichhane et al., 2003). MTB were grown to mid log phase in Middlebrook 7H9 medium (Becton Dickinson) with 0.05% Tween-80 and albumin, dextrose, catalase (Middlebrook ADC Enrichment, BBL Microbiology) prior to infection. When required kanamycin was added at a final concentration of 20 μg/ml. Streptomycin (STR), rifampicin (RIF), isoniazid (INH) and verapamil (VER) were purchased from Sigma.

Macrophage Growth and Infection

THP-1 macrophages were grown in RPMI, supplemented with 10% FBS and 1% L-glutamine. THP-1 cells were differentiated with phorbol 12-myristate 13-acetate for 48 hours and allowed to recover for 48 hours prior to infection. $5x10^5$ THP-1 macrophages were infected at an MOI of 1.5 for 3 hours at 37°C. Cells were washed with medium and then 6 μg/ml STR was added for the duration of the intracellular growth. Medium was changed daily. Macrophages were lysed with 0.1% Triton X-100 for intracellular growth quantization. To lyse macrophages and release bacteria for subsequent tolerance assessment, each well was first washed once with 1x PBS, and once with diH_2O, with the latter wash being removed immediately. Then, 100μl of diH_20 was added and the cells incubated for 15 minutes to lyse macrophages. Finally, 900μl of 1.1x concentrated 7H9 medium was added and the wells scraped gently with a pipette tip to release all release all macrophages. CFU were enumerated from triplicate wells on supplemented 7H10 agar. For determination of antibiotic killing, the percent survival was calculated by dividing the CFU for each well by the mean CFU present prior to treatment.

Statistics

Statistical analyses were performed using Prism 5.01 (GraphPad). Post test *P* values are as follows: *, $P<0.05$; **, $P<0.01$; ***$P<0.001$.

FIGURES

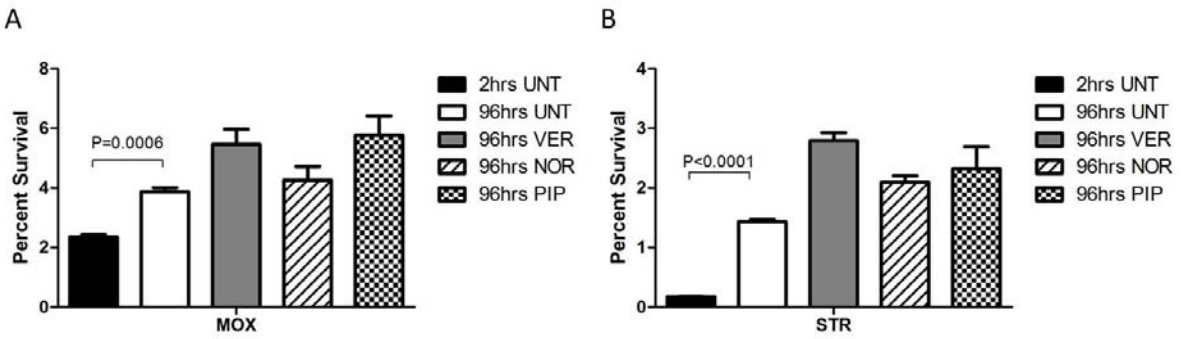

Figure 4.1. Macrophage-induced moxifloxacin and streptomycin tolerance not sensitive to efflux pump inhibitors.

(A) THP-1 macrophages were infected with MTB strain CDC 1551 and lysed at 2 or 96 hours post infection (hpi). The released bacteria were treated for an additional 48 hr with 3.75 μM MOX in the presence or absence of 162.8 μM verapamil or 167.7 μM norverapamil or 350 μM piperine prior to enumeration of CFU.
(B) THP-1 macrophages were infected with MTB strain CDC 1551 treated as in (A). The released bacteria were treated for an additional 48 hr with 8.23 μM STR in the presence or absence of 162.8 μM verapamil or 167.7 μM norverapamil or 350 μM piperine prior to enumeration of CFU. Error bars represent SEM. Significance testing was performed using one-way ANOVA with Dunnett's post-test. *P* values were determined using Student's *t*-test.

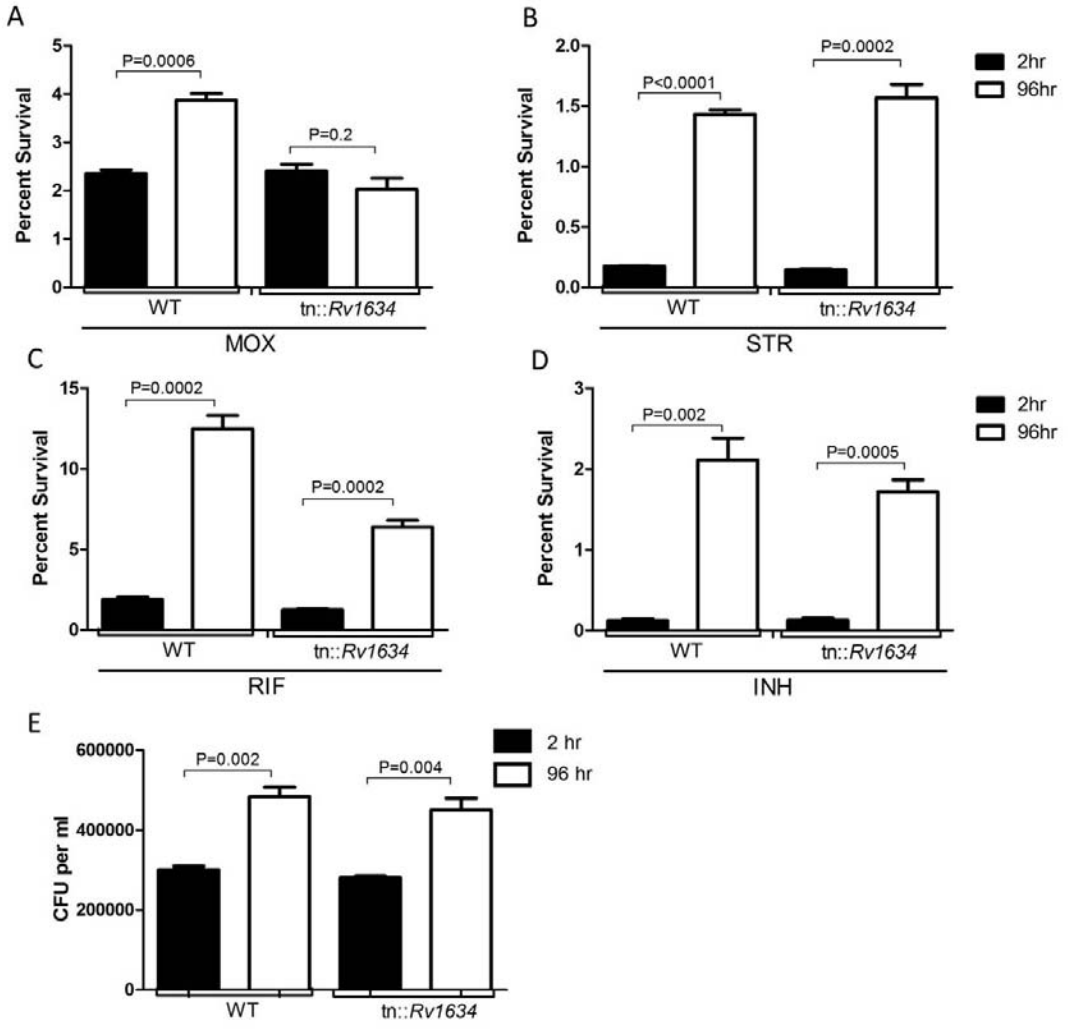

Figure 4.2. Rv1634 mediates macrophage-induced moxifloxacin tolerance but does not promote intracellular growth.

(A-D) THP-1 macrophages were infected with MTB strains JHU1634 and the isogenic wild-type control, CDC 1551, for two or 96 hours prior to lysis and enumeration of CFU. Released bacteria were treated with 3.75 μM MOX, 8.23 μM STR, 4.4 μM INH, 1.2 μM RIF or left untreated for 48 hr prior to enumeration of CFU.
(E) Released bacteria were treated as described in panel (A).
Error bars represent SEM. *P* values were determined using Student's *t*-test.

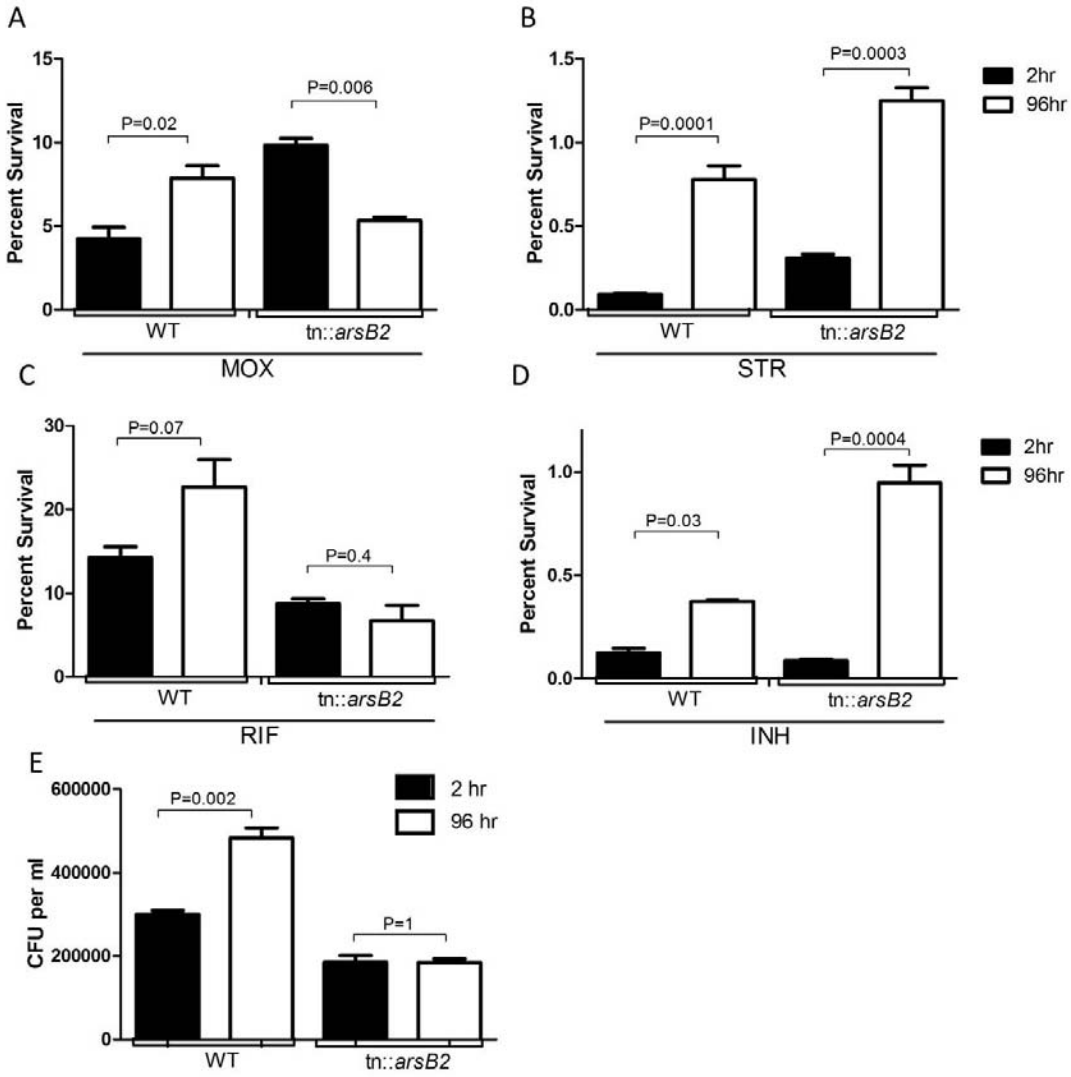

Figure 4.3. ArsB2 mediates macrophage-induced moxifloxacin and rifampicin tolerance.

(A-D) THP-1 macrophages were infected with MTB strains JHU3578 and the isogenic wild-type control, CDC 1551, for two or 96 hours prior to lysis and enumeration of CFU. Released bacteria were treated with 3.75 μM MOX, 8.23 μM STR, 4.4 μM INH, 1.2 μM RIF or left untreated for 48 hr prior to enumeration of CFU.
(E) Released bacteria were treated as described in panel (A).
Error bars represent SEM. *P* values were determined using Student's *t*-test.

Drug Efflux Pump	Transporter Family	Drug(s)	Induction in macrophages*	TARGET transposon mutant available	References
drrABC	ABC	TET,STR, EMB	1	Yes	(Choudhuri et al., 2002)
pstB	ABC	INH, RIF, EMB, CIP	0.7	No	(Gupta et al., 2006)
Rv0194	ABC	STR	1.6	Yes	(Danilchanka et al., 2008)
Rv1218c	ABC		1.3	No	
Rv1348	ABC	multiple drugs	2	Yes	
Rv1456c	ABC	undetermined	1	Yes	
Rv1463	ABC	undetermined	1.8	No	
Rv1747	ABC	INH	0.9	Yes	(Louw et al., 2009)
Rv1819c	ABC		1	Yes	
Rv2477c	ABC		0.8	No	
Rv2686c-87c-88c	ABC	CIP	2.1	Yes	(Pasca et al., 2004)
Rv0037c	MFS		1.1	No	
Rv0191	MFS		1.2	Yes	
Rv0783c (emrB)	MFS		ND	Yes	
Rv0849	MFS		1	No	
Rv1250	MFS		1.2	Yes	
Rv1258c	MFS	RIF, OFX, INH	1.7	Yes	(Jiang et al., 2008; Rodrigues et al., 2011; Siddiqi et al., 2004; Zhang et al., 2005)
Rv1410c	MFS	STR, INH, RIF	0.8	Yes	(Jiang et al., 2008)
Rv1634	MFS	FQ	1.2	Yes	(De Rossi et al., 2002)
Rv1877	MFS	TET, KAN, erythromycin	1.2	Yes	(Li et al., 2004)
Rv2333c	MFS	TET	1.2	Yes	(Ramon-Garcia et al., 2007)
Rv2456c	MFS		1	Yes	
Rv2459 (jefA)	MFS	INH, EMB	0.8	Yes	(Gupta et al., 2010b)
Rv2846c (efpA)	MFS	INH, ETH	ND	No	(Rodrigues et al., 2011; Zhang et al., 2005)
Rv2994	MFS	undetermined	0.9	No	
Rv3239c	MFS	undetermined	1.8	Yes	
Rv3728	MFS	undetermined	1.6	Yes	

Drug Efflux Pump	Transporter Family	Drug(s)	Induction in macrophages*	TARGET transposon mutant available	References
mmpl1	RND		1	Yes	
mmpl2	RND		1	Yes	
mmpl3	RND		0.8	No	
mmpL4	RND		1	Yes	
mmpL5	RND		1.1	Yes	
mmpL6	RND		1.1	Yes	
mmpL7	RND	INH	ND	Yes	(Pasca et al., 2005; Rodrigues et al., 2011)
mmpL8	RND		1.2	Yes	
mmpL9	RND		0.9	No	
mmpL10	RND		1.3	Yes	
mmpL11	RND		1.1	Yes	
mmpL12	RND		1.2	Yes	
mmpL13a	RND		0.8	No	
mmpL13b	RND		1.3	No	
mmr (Rv3065)	SMR	erythromycin	1.8	Yes	(Gupta et al., 2010a)
iniA	Membrane Protein	INH, EMB	1.4 and 2.0**	Yes	(Alland et al., 2000; Colangeli et al., 2005; Ramaswamy et al., 2000; Ramaswamy et al., 2003)
arsB2	Arsenic Pump		1.5	Yes	

Isoniazid (INH); rifampicin (RIF); ofloxacin (OFX); streptomycin (STR); ethambutol (EMB); ethionamide (ETH); ciprofloxacin (CIP)

Table 4.1. List of identified efflux pumps in *Mycobacterium tuberculosis*.
Efflux pumps listed came from (da Silva et al., 2011; De Rossi et al., 2002; Gupta et al., 2010a; Louw et al., 2009; Saier et al., 2009)
Transposon mutants from (Lamichhane et al., 2003)
*Microarray data from 48 hr naïve macrophages (Schnappinger et al., 2003).
** Two values listed

CONCLUSION

Overcoming drug tolerance may allow the treatment time for tuberculosis to be shortened, providing significant benefits to both individual patients and to health systems. My thesis research has focused on when pathogenic mycobacteria develop drug tolerance and the mechanism by which this occurs. These investigations led to the discovery of both new bacterial virulence determinants that mediate drug tolerance and to the identification of drugs that may be able to counter this process.

The central finding of this work is that drug tolerance to both front-line antitubercular drugs RIF and INH and second-line drugs MOX and STR occurs in MTB within days of macrophage infection and that this tolerance is dependent on the activity of distinct mycobacterial efflux pumps. *Rv1258c* (Adams et al., 2011) and *iniA* mediate tolerance to RIF and INH, while *Rv1634* and *arsB2* mediate tolerance to MOX. *arsB2* contributes to RIF tolerance as well. Identification and *in vivo* characterization of the natural substrates for these pumps are important next steps. Analyzing these efflux pump mutants *in vivo* will help elucidate their relative role for virulence and tolerance during a longer course of infection. Once the natural substrates for these pumps are identified, it will not only increase our understanding of how these pumps are enhancing intracellular growth, but potentially lead to strategies that may enhance host-mediated bacterial killing.

Given the large number of diverse efflux pumps present in MTB, further in vivo investigations will be helpful in determining which are most important for virulence and drug tolerance. In a screen identifying MTB genes essential for *in vivo* growth, only four efflux pumps were found to be essential (Rv1410c, MmpL7, Rv0194 and DrrABC) (Sassetti and Rubin, 2003), none of which overlapped with the pumps identified as being important for macrophage-induced tolerance. Our

observation that antibiotic tolerance is conferred by multiple pumps (both Rv1258c and ArsB2 to be involved in RIF tolerance and Rv1634 and ArsB2 to be involved in MOX tolerance) demonstrates redundancy in the induced bacterial efflux pumps. Although multiple pumps may handle a particular antibiotic, it is not yet known how the absence of one pump may affect the activity or expression of other pumps. However, the identification of the natural substrate(s) of these pumps should provide insight as what the role of each pump is during infection.

The induction of efflux systems either via physiological changes or mutations allows bacteria to survive in hostile environments by lowering the intracellular concentration of various toxins, including multiple classes of antibiotics. Identifying the host and/or bacterial factor(s) inducing these pumps will give further insight into how to disarm efflux pumps and enhance antibiotic efficacy.

Antimicrobial peptides (AMPs) in the macrophage may provide an important stimulus for MTB pump expression, as they do in several other bacterial pathogens (Koprivnjak and Peschel, 2011). In both *Neisseria gonorrhoeae* and *meningitidis*, the MtrCDE efflux pump, which expels antibiotics, also confers resistance to human AMPs (Shafer et al., 1998; Tzeng et al., 2005b). Expression of the MFS transporter QacA in *Staphlococcus aureus* causes resistance to AMPs, however, inhibition of QacA efflux fails to confer sensitivity to AMPs, suggesting that alterations in the membrane fluidity, rather than active transport confers AMP resistance in *S. aureus* (Bayer et al., 2006).

MTB infected macrophages induce AMPs (Rivas-Santiago et al., 2008b), which is important for host antimycobacterial activity (Liu et al., 2007b). We previously reported that macrophage-induced tolerance is preserved in dexamethasone-treated macrophages (Adams et al., 2011). Dexamethasone is a broadly acting anti-inflammatory agent, but AMP

expression is preserved (Duits et al., 2001b) unlike other macrophage denfenses (Newton, 2000). Together, these results suggest that AMPs may be involved in the induction of mycobacterial efflux pumps, which confer antibiotic tolerance, though this hypothesis requires further investigation.

Macrophage-induced multidrug tolerance occurs within a subset of bacteria during infection. This may be due to heterogeneity among macrophages and/or subcellular localization of bacteria, although escape into the cytoplasm is not required for development of tolerance (Adams et al., 2011). Mycobacteria are commonly found within nonacidified phagosomes and their genomes encode a number of bacterial effectors that inhibit phagosome maturation as well resisting acidic conditions in vivo (Deretic et al., 2006; Vandal et al., 2008). However, acidification of the phagosome appears to be associated with mycobacterial death under some but not all circumstances; for example, mycobacteria taken up by the Fc Receptor survive acidification; whereas treating infected macrophages with interferon gamma (IFN-γ) is associated with bacterial death (Cosma et al., 2003). Interestingly, a recent study reported that the release of IFN-γ induces phagosomal maturation and the induction of AMPs via a vitamin D-dependent pathway (Fabri et al., 2011). Therefore testing MTB infected in IFN-γ-treated macrophages may shed further light on what host factors (AMPs and/or acidification) are inducing MTB efflux pumps mediating macrophage-induced drug tolerance.

Our finding that verapamil reduces macrophage-induced tolerance to the front-line antitubercular drugs RIF and INH, warrants further pursuit using *in vivo* models. A recent study found that adding verapamil to a RIF, INH and pyrazinamide regimen in mice infected with a RIF resistant strain of MTB lead to a significant reduction in pulmonary bacteria

burdens (Louw et al., 2011), suggesting *in vivo* efficacy for verapamil. Additional investigation of verapamil as adjunctive therapy for drug-suseptible TB using the murine TB model are underway (William Bishai, personal communication).

Because verapamil does not inhibit pumps that mediate tolerance to other clinically-important drugs such as moxifloxacin and streptomycin, further efforts to identify and test additional potential efflux pump inhibitors are warranted. For example, piperine also blocks macrophage-induced RIF tolerance. Other agents such as phenothiazine derivatives and tariquidar may also be effective at reducing macrophage-induced tolerance (Amaral et al., 2001; Amaral and Molnar, 2012; Leitner et al., 2011). Ultimately combinations of efflux pump inhibitors may show greater efficacy given the wide spectrum of efflux pumps mediating mycobacterial antibiotic tolerance, though this strategy may be difficult to implement in clinical care of TB patients due to additive toxicities and additional drug-drug interactions.

In summary, this research provides a new mechanistic explanation for why length of treatment correlates with bacterial burdens in active tuberculosis (Connolly et al., 2007). Growing bacteria express bacterial efflux pumps upon macrophage residence which render them tolerant to TB drugs, so longer courses of treatment are required to eliminate this tolerant population. If macrophage-induced efflux pumps substantially contribute to antitubercular drug tolerance, then the addition of efflux pump inhibitors such as verapamil to the current antitubercular regimen may accelerate bacterial killing and allow for shorter TB treatment courses.

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Publications:

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