Published in final edited form as:

Sci Transl Med. 2019 November 13; 11(518): . doi:10.1126/scitranslmed.aaw6635.

Targeting redox heterogeneity to counteract drug tolerance in replicating *Mycobacterium tuberculosis*

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Abstract

The capacity of *Mycobacterium tuberculosis* (*Mtb*) to tolerate multiple antibiotics represents a major problem in tuberculosis (TB) management. Heterogeneity in *Mtb* populations is one of the factors that drives antibiotic tolerance during infection. However, the mechanisms underpinning this variation in bacterial population remain poorly understood. Here, we show that phagosomal acidification alters the redox physiology of *Mtb* to generate a population of replicating bacteria that display drug tolerance during infection. RNA sequencing of this redox-altered population revealed the involvement of iron-sulfur (Fe-S) cluster biogenesis, hydrogen sulfide (H₂S) gas, and drug efflux pumps in antibiotic tolerance. The fraction of the pH- and redox-dependent tolerant population increased when *Mtb* infected macrophages with actively replicating HIV-1, suggesting that redox heterogeneity could contribute to high rates of TB therapy failure during HIV-TB coinfection. Pharmacological inhibition of phagosomal acidification by the antimalarial drug chloroquine (CQ) eradicated drug-tolerant *Mtb*, ameliorated lung pathology, and reduced postchemotherapeutic relapse in in vivo models. The pharmacological profile of CQ ($C_{\max}$ and AUC_{last}) exhibited no major drug-drug interaction when coadministered with first line anti-TB drugs in mice. Our data establish a link between phagosomal pH, redox metabolism, and drug

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Author contributions: R.M. and A.S. conceptualized the research and prepared the manuscript. R.M. and S.K. performed the experiments and analyzed data. N.M. and A.S.N.S. conducted the RNA-seq analysis. P.B., M. Mehta, and M. Munshi constructed the knockout and complemented strains and conducted the HIV-TB coinfection and H₂S measurement assays. V.A. performed the flow sorting. V.K.A. and R.K.S. conducted the pharmacokinetic drug-drug interaction studies. R.S.R. conducted animal experiments.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. RNA-seq data generated and analyzed in this study have been uploaded to the NCBI Gene Expression Omnibus under accession number GSE123267.

tolerance in replicating *Mtb* and suggest repositioning of CQ to shorten TB therapy and achieve a relapse-free cure.

Introduction

An unusually long-term (6 months) therapy involving multiple antibiotics is required to cure tuberculosis (TB) in humans. This protracted treatment is necessary to prevent relapses due to genetically drug-sensitive bacteria that become transiently resistant inside host cells and tissues, a phenomenon called phenotypic drug tolerance. Thus, the mechanistic basis of phenotypic drug tolerance needs to be studied to develop new drugs with treatment-shortening properties. Recent studies indicate that heterogeneity in both the host environment and the bacterial population can promote phenotypic drug tolerance. For example, variability in the activation status of macrophages distinctly modulates drug tolerance in *Mycobacterium tuberculosis* (*Mtb*) (1). Immune activation of macrophages leads to release of antibacterial effectors such as reactive nitrogen species and reactive oxygen species (ROS) (2, 3), leading to a quiescent drug-tolerant state of *Mtb* (4). In support of this theme, drug tolerance is diminished in mice and macrophages deficient in producing nitric oxide (NO) (1). Moreover, extracellular *Mtb* present in the cavity caseum derived from *Mtb*-infected rabbits show slow replication and extreme tolerance to several first- and second-line anti-TB drugs (5). Single-cell measurements have revealed that stress conditions (for example, starvation) in vitro and host immune pressures (interferon- γ , a cytokine critical for anti-TB host immunity) in vivo create phenotypic heterogeneity within the *Mtb* population, which allows for the selection of nongrowing metabolically active bacteria responsible for postchemotherapeutic relapse (4).

However, recent studies suggest that adoption of a nongrowing state is not a prerequisite for drug tolerance (6–10). A fraction of both replicating and nonreplicating bacteria shows regrowth after drug withdrawal (4, 7), emphasizing that growth-arrested bacteria do not solely mediate tolerance. Alternate mechanisms—such as induction of drug efflux pumps, asymmetric cell division, and increased mistranslation rates—can contribute to substantial drug tolerance in actively multiplying cells (6, 8, 9, 11). Induction of efflux pumps is, so far, the only mechanism known to confer drug tolerance in replicating *Mtb* inside macrophages (6). Despite their importance, we lack understanding of macrophage-specific cue(s) and associated changes in the physiology of replicating *Mtb* that drive drug tolerance. Filling this knowledge gap will help in developing strategies to target both bacterial and host determinants crucial for mobilizing a drug-tolerant phenotype in vivo. A detailed summary of our current understanding of phenotypic drug tolerance in *Mtb* is described in fig. S1.

Using a ratiometric fluorescence biosensor (Mrx1-roGFP2) of the major mycobacterial antioxidant mycothiol (MSH), we previously showed that the environment inside macrophages rapidly generates heterogeneity in the MSH redox potential (E_{MSH}) of the *Mtb* population (12). Confocal and flow cytometry measurements categorized infected macrophages into two distinct populations, one predominantly harboring E_{MSH} -reduced bacteria (-300 ± 6 mV) and the other predominantly harboring E_{MSH} -basal bacteria (-275 ± 5 mV) (12). In addition, a minor fraction of infected macrophages was enriched for *Mtb* in

the E_{MSH} -oxidized state (-242 ± 6 mV) (12). These results are consistent with the heterogeneous and dynamic nature of both host and pathogen (13–15), suggesting that their interaction is likely to result in bacterial populations with diverse phenotypes. The E_{MSH} -reduced population was found to be refractory to anti-TB drugs compared to other populations (E_{MSH} -oxidized and E_{MSH} -basal) (12). Therefore, understanding the basis of redox heterogeneity could inform strategies that result in better targeting of drug-tolerant *Mtb*. In this study, we performed RNA sequencing (RNA-seq) of redox-altered intraphagosomal *Mtb* populations and identified bacterial factors and host cues associated with drug tolerance.

Results

Transcriptional profiling of redox-diverse populations identifies determinants of drug tolerance

We followed our previously developed flow cytometry protocol that averages median fluorescence ratio (405 nm/488 nm) of the Mrx1-roGFP2 biosensor expressed by intraphagosomal *Mtb* to gate macrophages into fractions enriched with either E_{MSH} -reduced ($E_{\text{MSH}} = -300 \pm 6$ mV) or E_{MSH} -basal ($E_{\text{MSH}} = -275 \pm 5$ mV) bacteria (fig. S2) (12). Using this gating strategy, we sorted THP-1 macrophages infected with *Mtb*/Mrx1-roGFP2 at 24 hours postinfection (p.i.), treated them with isoniazid [Inh; threefold the in vitro minimal inhibitory concentration (MIC)] for 48 hours, and confirmed that the E_{MSH} -reduced fraction is more tolerant to Inh than E_{MSH} -basal fraction (fig. S3). As reduced susceptibility to Inh originated from a population of intraphagosomal *Mtb*, our findings align with a recent consensus statement defining tolerance as the general ability of a population to survive longer antibiotic exposure (16). To investigate the physiological basis of the differential tolerance of redox-altered *Mtb*, we performed global transcriptional profiling by RNA-seq of E_{MSH} -reduced and E_{MSH} -basal populations derived from THP-1 macrophages flow-sorted 24 hours after infection with *Mtb*/Mrx1-roGFP2 (Fig. 1).

We isolated total bacterial RNA, performed RNA-seq, and analyzed data using DESeq2 (Fig. 1A). Control RNA was isolated from logarithmically grown *Mtb* resuspended in complete RPMI for 24 hours (in vitro control). We compared the transcription profiles of macrophage-derived populations to in vitro control and to one another to identify responses that were induced in both populations and responses that were significantly induced in E_{MSH} -reduced bacteria [false discovery rate (FDR), 0.05]. Principal components analysis and clustering of heat map plots showed that the three samples clustered by their biological replicates (fig. S4 and tables S1, A to C). As compared to the in vitro control, the expression of 560 and 617 genes was affected in the E_{MSH} -reduced and E_{MSH} -basal populations, respectively (FDR, 0.05; fold change, 1.5) (table S1, B and C). Of 295 genes showing overlap, 151 were more induced in the drug-tolerant E_{MSH} -reduced population (Fig. 1B and table S1D). The transcriptome of both populations considerably overlapped with that of a previously reported transcriptional signature of intraphagosomal *Mtb* ($P < 0.05$, Fisher's exact test; Fig. 1C) (17). Consistent with studies showing phagosomal acidification as the earliest cue that alters the transcriptome of *Mtb* inside unstimulated macrophages (18, 19), RNA-seq data of the E_{MSH} -reduced fraction overlapped significantly with the transcriptome

of *Mtb* grown in 7H9 broth at pH 5.5 ($P = 1.05 \times 10^{-2}$) and pH 4.5 ($P = 1.5 \times 10^{-15}$) (Fig. 1C) (17, 20). The E_{MSH} -basal transcriptome showed little similarity to the genes down-regulated at pH 5.5 (Fig. 1C) (17).

Using *Mtb*/Mrx1-roGFP2 conjugated with pHrodo dye, which emits fluorescence only in acidic pH (21), we infected THP-1 macrophages and confirmed phagosomal pH to be 6.25 ± 0.14 , as previously reported for unstimulated macrophages (fig. S5) (22). Likewise, we examined the pH of flow-sorted macrophages enriched with E_{MSH} -basal or E_{MSH} -reduced bacteria. Macrophages enriched in E_{MSH} -reduced bacteria are more acidic ($\text{pH } 5.79 \pm 0.2$) than E_{MSH} -basal bacteria ($\text{pH } 6.67 \pm 0.08$) (fig. S5D), indicating that subtle variations in phagosomal pH underlie heterogeneity in E_{MSH} of *Mtb* during infection. We also compared the transcriptional profiles of E_{MSH} -altered *Mtb* and a mutant of the redox-sensitive transcription factor WhiB3 (*Mtb whiB3*), which has been linked to *Mtb*'s transcriptional response to low pH (20). We isolated RNA from wild-type (WT) *Mtb*, *Mtb whiB3*, and *whiB3*-complement (*whiB3*-Comp) cultured in 7H9 broth at neutral pH (6.6) and acidic pH (4.5) and performed RNA-seq. The WhiB3-specific, low pH-induced gene set showed comparable expression in the E_{MSH} -reduced population. In contrast, only a fraction of the WhiB3 regulon coincided with the transcriptome of E_{MSH} -basal bacteria, with lesser induction of the regulon than that seen in the case of the E_{MSH} -reduced *Mtb* (fig. S6 and table S2D). These findings link the transcriptome of E_{MSH} -reduced bacteria with *Mtb*'s response to low pH.

Low pH increases the solubility of transition metals including iron and copper (23), thereby allowing these metals to cross biological membranes and participate in metal-catalyzed ROS generation via the Fenton reaction (24). Consistent with this phenomenon, transcriptional sensors of metal toxicity (*furA*, *csor*, and *cmtR*), exporters of toxic metals (*ctpV* and *ctpG*), redox sensors (*whiBs* and *sufR*), and antioxidant systems (*rubA/B*, *ahpC*, and *trxB2*) were induced in the E_{MSH} -reduced fraction (Fig. 1D and table S3). Because ROS damages DNA, proteins, and lipids, we observed that several genes implicated in DNA repair, protein quality control, and envelope stress were induced in E_{MSH} -reduced fraction (Fig. 1D and table S3). A previous study linked the stochastic expression of catalase (*katG*) in mediating Inh tolerance (7). However, *katG* expression was not differentially regulated in the E_{MSH} -reduced fraction, indicating that redox-mediated Inh tolerance is unrelated to *katG* expression. Genes coordinating glyoxylate and methyl citrate cycles (*icl1* and *prpD*) and alternate respiration (*cydAB*) were up-regulated in the E_{MSH} -reduced fraction (Fig. 1D and table S3); both *icl1* and *cydAB* are vital for mitigating oxidative stress and promoting multidrug tolerance in *Mtb* (25–27). The overlap between acidic pH and oxidative stress responses has been reported in several bacteria (28–30), indicating a link between pH- and oxidative stress-driven adaptations.

A reductive shift in the E_{MSH} of *Mtb* indicates an increase in the cytoplasmic pool of reduced MSH. Supporting this, genes associated with the biogenesis of cysteine (CySH), a component of MSH (31), were up-regulated in the E_{MSH} -reduced fraction. For example, *lat*, *metA*, and *metC* involved in methionine (Met) biogenesis (32), and *metB* encoding a bifunctional enzyme (cystathionine- γ -lyase/cystathionine- γ -synthase) that incorporates sulfur from Met to CySH (reverse transsulfuration pathway) (33) were induced in the E_{MSH} -

reduced fraction (Fig. 1D). The enzyme MetB generates H₂S gas as a by-product (33), which protects several bacterial species from antibiotics and oxidative stress (34–36). We detected that *Mtb* cultured in 7H9 broth at pH 6.2 and pH 4.5 generated more H₂S than at neutral pH (fig. S7), indicating a link between H₂S biogenesis, acid stress, and reductive shift in E_{MSH} . CySH also serves as a source of sulfide for the biogenesis of Fe-S clusters, which modulate bacterial response to antibiotics (37). Accordingly, genes involved in Fe-S cluster biogenesis [Rv1460 (*sufR*) and Rv1461 (*sufB*)] (38, 39) were up-regulated in the E_{MSH} -reduced fraction (Fig. 1D and table S3). Other transcriptional changes in E_{MSH} -reduced cells involving genes that are known to promote drug refractoriness include genes associated with *S*-adenosyl methionine (SAM) biosynthesis (*metK*) (32), methyl transferases (*menH*, Rv0560c, Rv1403c, and Rv1405c) (40–42), and drug efflux pumps (*mmr*, Rv1258c, and Rv1250) (6, 43, 44) (Fig. 1D and table S3). In conclusion, the RNA-seq data suggest a major role of host acidification and bacterial mechanisms involved in alleviating metal toxicity, ROS remediation, and realignment of sulfur metabolism in the emergence of drug-tolerant E_{MSH} -reduced population during infection.

CySH-disposal pathways coordinate redox-mediated drug tolerance in *Mtb*

CySH-dependent pathways such as biogenesis of H₂S, low molecular-weight thiols, and Fe-S clusters protect bacteria against antibiotics and oxidative stress (37, 45). In *Mtb*, supplementation with H₂S donor [sodium hydrosulfide NaHS]) restored the imbalance in the E_{MSH} of MSH recycling mutants (46), and Fe-S cluster-dependent regulators (for example, WhiB3 and WhiB7) mediate a reductive shift in E_{MSH} of *Mtb* in response to acidic pH and antibiotics (20, 47). On this basis, we reasoned that the induction of *metB* (H₂S biogenesis) and *sufR* (regulator of Fe-S cluster biogenesis) could contribute to the emergence of a drug-tolerant E_{MSH} -reduced population (Fig. 2A). To test this idea, we independently disrupted *metB* (*Mtb metB*) and *sufR* (*Mtb sufR*) in *Mtb* H37Rv (fig. S8, A to E). As expected, *Mtb metB* displayed a reduced capability to produce H₂S compared to WT *Mtb* (fig. S8F). Similarly, disruption of *sufR* abrogated the induction of the *suf* operon (Rv1461 to Rv1466) involved in Fe-S cluster biogenesis (fig. S8G). Because WhiB3 promotes the emergence of E_{MSH} -reduced population inside macrophages (20), we used the *Mtb whiB3* strain as a control. THP-1 macrophages infected with *Mtb metB* or *Mtb sufR* expressing Mrx1-roGFP2 showed a significant decrease in the reductive- E_{MSH} fraction compared to WT *Mtb* ($P < 0.01$) (Fig. 2B). We next examined the influence of these pathways on Inh tolerance during infection. THP-1 macrophages infected for 24 hours with *Mtb* strains were exposed to 3× in vitro MIC of Inh, and survival was determined at 48 hours of Inh treatment. Intramacrophage growth of the mutants was marginally reduced compared to WT *Mtb* (Fig. 2C). However, upon Inh treatment, *Mtb metB*, *Mtb sufR*, and *Mtb whiB3* displayed 8.75-, 7-, and 9-fold reductions in survival compared to WT *Mtb*, respectively. Decreased tolerance displayed by these mutants was restored in the complemented strains to a degree similar to WT (Fig. 2C).

It can be argued that the loss of MetB and SufR functions can profoundly affect the normal growth and metabolism of *Mtb*, complicating any association with redox heterogeneity and drug tolerance. However, we found that the growth of *Mtb metB* and *Mtb sufR* in 7H9 broth was not different from WT *Mtb* (fig. S9, A and B). Furthermore, E_{MSH} of *Mtb metB*

(-280 ± 3 mV) and *Mtb sufR* (-276 ± 4 mV) remained comparable to WT *Mtb* (-275 ± 2 mV) in vitro. In addition, *metB* or *sufR* disruption did not perturb the oxygen consumption rate or extracellular acidification rate (fig. S9, C and D), quantifiable readouts of oxidative phosphorylation and glycolysis, respectively (48). Last, we examined whether *metB* and *sufR* influenced tolerance to Inh under acidic pH. The MIC of Inh remained comparable (0.06 to 0.1 $\mu\text{g/ml}$) for *Mtb metB* and *Mtb sufR* at neutral pH. However, at pH 4.5, WT *Mtb* showed $79 \pm 3.97\%$ survival to $10\times$ MIC of Inh as compared to $19.79 \pm 0.58\%$ and $18.8 \pm 1.85\%$ in the case of *Mtb metB* and *Mtb sufR*, respectively (Fig. 2, D and E). These data indicate that *metB* and *sufR* influence drug tolerance in the context of acidic pH and the intramacrophage milieu. In sum, the genetic data support our RNA-seq findings indicating CySH flux as an important mechanism underlying redox diversity and drug tolerance in *Mtb* during infection.

Phagosomal acidification is required for the redox-dependent multidrug tolerance of *Mtb*

To clarify the link between phagosomal pH, redox heterogeneity, and drug tolerance during infection, we pretreated THP-1 macrophages with nontoxic doses of well-established inhibitors of phagosomal acidification [bafilomycin A1 (BafA1), ammonium chloride (NH_4Cl), and chloroquine (CQ)], infected them with *Mtb/Mrx1-roGFP2*, and measured E_{MSH} (12, 20, 49–51). Pretreatment with BafA1/ NH_4Cl /CQ uniformly diminished the fraction of *Mtb* displaying reductive E_{MSH} at 24 hours p.i. (Fig. 3A). Next, we examined whether phagosomal pH enhanced drug tolerance during infection. THP-1 macrophages with or without BafA1 pretreatment were infected with *Mtb* for 24 hours and exposed to $3\times$ MIC of Inh for an additional 48 hours before lysis and enumeration of viable counts. The addition of BafA1 further increased Inh-mediated killing of *Mtb* by fivefold (Fig. 3B). A similar increase in killing was observed upon substitution of Inh with rifampicin (Rif) or BafA1 with CQ (Fig. 3, B and C). We noted that although CQ and BafA1 uniformly increased killing efficacy of Inh and Rif, the effect was more pronounced in the case of Inh (Fig. 3, B and C). Consistent with findings in THP-1 cells, infection of peritoneal macrophages from BALB/c mice also led to a pH-dependent increase in the E_{MSH} -reduced fraction and Inh tolerance in *Mtb* (fig. S10, A and B). These results suggest that phagosomal pH is a potent enhancer of multidrug tolerance in *Mtb*.

We next examined whether the drug tolerance and redox heterogeneity displayed by intraphagosomal *Mtb* were a reversible phenotypic change or a stable genetic variation. We flow-sorted THP-1 macrophages infected with *Mtb/Mrx1-roGFP2* for 24 hours into E_{MSH} -reduced and E_{MSH} -basal fractions, lysed the macrophages in 7H9 broth, and measured E_{MSH} of the released *Mtb*. Incubation in 7H9 broth resulted in the loss of redox heterogeneity within 2 hours, indicating that the macrophage environment supports the emergence of drug-tolerant E_{MSH} -reduced population (Fig. 3D). When these redox-homogeneous bacteria were used to reinfect THP-1 macrophages with or without CQ pretreatment, both the heterogeneity in E_{MSH} and tolerance to Inh returned in untreated macrophages but not in CQ-treated macrophages (Fig. 3, D and E). Last, the bacteria that survived Inh treatment inside THP-1 macrophages showed an MIC comparable to the parental strain in 7H9 broth (fig. S10C). We conclude that pH- and redox-dependent tolerance of *Mtb* inside macrophages was due to reversible phenotypic changes rather than stable genetic mutations.

Phagosomal pH and redox heterogeneity drive drug tolerance during HIV-TB coinfection

Limited acidification is one of the hallmarks of *Mtb*-containing alveolar macrophages derived from HIV-TB-coinfecting patients (52). We reasoned that pH- and redox-driven tolerance to anti-TB drugs could contribute to the lower TB treatment success rates commonly observed in HIV-TB-coinfecting patients (53). To test this idea, we used the U1 monocytic cell line model of HIV-TB coinfection (54). U1 cells are derived from U937 monocytes wherein two copies of the HIV-1 genome are integrated and viral replication can be induced by phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor- α (55, 56). We confirmed viral replication by monitoring the expression of the HIV-1 *gag* transcript by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Treatment with PMA (5 ng/ml) induced HIV-1 replication in a time-dependent manner in U1 cells (Fig. 4A). Next, we infected PMA-treated U1 and U937 (uninfected HIV-1 control) with *Mtb*/Mrx1-roGFP2 and measured heterogeneity in E_{MSH} . Both U1 and U937 macrophages showed the emergence of redox-diverse fractions upon infection. However, a marked increase in the E_{MSH} -reduced fraction was clearly evident in U1 compared to U937 macrophages (Fig. 4, B and C), indicating that HIV-1 replication is accompanied with the rise of E_{MSH} -reduced population. Furthermore, treatment with BafA1, NH_4Cl , and CQ uniformly decreased the E_{MSH} -reduced fraction in U1 (Fig. 4D), confirming the role of phagosomal pH in the emergence of redox heterogeneity. Last, we tested Inh tolerance in U1 and U937 cells, as described earlier. Consistent with the increased E_{MSH} -reduced fraction, a significantly higher proportion of *Mtb* tolerates exposure to $3\times$ MIC of Inh in U1 ($65.9 \pm 11.67\%$) versus U937 ($21.93 \pm 0.42\%$) ($P = 0.0015$) (Fig. 4, E and F). As expected, pretreatment with CQ/BafA1 increased Inh-mediated killing of *Mtb* in U1 and U937 (Fig. 4, E and F). These results suggest that the phagosomal acidification encountered by *Mtb* inside HIV-*Mtb*-coinfecting macrophages facilitates the development of a redox-altered drug-tolerant population.

The drug-tolerant E_{MSH} -reduced population is replicative inside macrophages

We next examined whether drug tolerance exhibited by the E_{MSH} -reduced population is associated with slow replication as shown in several bacteria, including *Mtb* (1, 4, 57). We used an unstable replication clock plasmid, pBP10, which is uniformly lost from replicating but not from nonreplicating *Mtb* (58). Because the plasmid is resistant to kanamycin (Kan), its retention or loss can be easily estimated by determining colony-forming units (CFUs) on Kan-containing medium. We infected THP-1 macrophages with pBP10-containing *Mtb*/Mrx1-roGFP2. At 0, 24, and 72 hours p.i., 0.5×10^6 macrophages harboring an E_{MSH} -reduced or E_{MSH} -basal population were flow-sorted, after which, the bacteria were released and differences in replication were measured by enumerating Kan^r (Kan-resistant) and Kan^s (Kan-sensitive) colonies. Expression of the pBP10 plasmid in *Mtb*/Mrx1-roGFP2 did not influence redox heterogeneity during infection (fig. S11A). The pattern of pBP10 plasmid loss indicated that both populations were replicative; however, the plasmid loss was faster over time in the E_{MSH} -reduced population than the E_{MSH} -basal population (Fig. 5, B and C). For example, at 72 hours p.i., only $17.8 \pm 0.2\%$ of cells retained pBP10 in the E_{MSH} -reduced population as opposed to $61.19 \pm 0.02\%$ in the E_{MSH} -basal population (Fig. 5, B and C). The cumulative bacterial burden, which provides the total number of living, dead, or damaged *Mtb* based on a mathematical model established for the clock plasmid (58), also

confirmed the comparatively higher replication rate in the E_{MSH} -reduced population (Fig. 5, B and C).

We examined the health of *Mtb* in the E_{MSH} -reduced and E_{MSH} -basal fractions using a fluorogenic cell-permeable dye calcein violet–acetoxymethyl ester (CV-AM), an established metabolic indicator (59, 60). THP-1 macrophages infected for 24 hours with *Mtb*/Mrx1-roGFP2 were flow-sorted, and then, bacteria were released from the E_{MSH} -reduced and E_{MSH} -basal fractions and stained with CV-AM. Bacilli in the E_{MSH} -reduced ($91.5 \pm 0.07\%$) and E_{MSH} -basal fractions ($99.1 \pm 0.14\%$) showed strong CV-AM fluorescence, indicating healthy metabolic activity (Fig. 5D). As expected, metabolically active *Mtb* cultured in 7H9 broth at 37°C showed $91.3 \pm 0.99\%$ CV-AM staining as compared to negligible staining in bacteriostatic cells incubated at 4°C (Fig. 5D). Treatment of *Mtb*/Mrx1-roGFP2 with 2 mM cell-permeable thiol-reductant dithiothreitol (DTT) induces a reductive shift in E_{MSH} (–320 mV) without any influence on metabolism and viability in vitro (20). Consistent with this, $83.9 \pm 2.97\%$ of DTT-treated *Mtb* cells scored positive for CV-AM staining (Fig. 5D). Together, these results suggest that the drug-tolerant E_{MSH} -reduced population is replicative and metabolically active inside macrophages.

Redox-diverse populations of *Mtb* show differential activation of efflux pumps

Induction of efflux pumps is associated with drug tolerance in replicating *Mtb* during infection (6). We investigated whether the drug-tolerant E_{MSH} -reduced population exhibited variation in efflux pump activity relative to the E_{MSH} -basal population. THP-1 macrophages infected for 24 hours with *Mtb*/Mrx1-roGFP2 were flow-sorted into E_{MSH} -reduced and E_{MSH} -basal populations, and bacterial RNA was isolated for qRT-PCR of efflux pump transcripts. As a control, we performed qRT-PCR of efflux pumps on *Mtb* grown in 7H9 broth. We selected efflux pumps (Rv0194, Rv1348, Rv1250, *ctpV*, *mmr*, and Rv1819c) that are induced in intraphagosomal *Mtb* upon exposure to anti-TB drugs (44, 61–64). The transcripts of *ctpV*, *mmr*, Rv1348, and Rv1250c were enriched in the E_{MSH} -reduced fraction (Fig. 5E). We tested pH- and redox-dependent expression of efflux pumps by examining transcripts in response to pH 6.2, pH 4.5, and 2 mM DTT in vitro. Each of these conditions uniformly induces reductive shift in E_{MSH} of *Mtb* in vitro (12, 20). All of these treatments increased expression of the efflux pumps (fig. S11, B and C). As a control, we analyzed efflux pump expression in an *Mtb* strain lacking the antioxidant buffer MSH (*Mtb mshA*); this strain maintains oxidative E_{MSH} (>–240 mV) at both neutral and acidic pH (20, 65). The expression of pH-inducible efflux pumps was significantly down-regulated in *Mtb mshA* relative to WT *Mtb* ($P < 0.05$) (fig. S11D), suggesting redox-dependent regulation of efflux pump expression in *Mtb*.

To clarify the association between efflux pump activity and E_{MSH} of *Mtb*, we assessed the steady-state distribution of Inh in E_{MSH} -reduced and E_{MSH} -basal populations inside THP-1 macrophages using [^{14}C]-labeled Inh. We infected THP-1 macrophages with *Mtb*/Mrx1-roGFP2 cells preloaded with [^{14}C]-Inh (0.5 $\mu\text{Ci/ml}$ for 2 hours). At 24 hours p.i., equal numbers of macrophages harboring either E_{MSH} -reduced or E_{MSH} -basal populations were sorted using flow cytometry. We chose the 24-hour time point because bacterial load was comparable in both populations (10^6 CFU/ml). Infected macrophages were lysed, the

bacterial (pellet) and macrophage (supernatant) fractions were separated, and [^{14}C]-labeled Inh radioactivity was measured. The distribution of [^{14}C]-Inh was different in macrophages containing E_{MSH} -reduced *Mtb* versus E_{MSH} -basal *Mtb* and in the bacteria themselves. Whereas macrophages harboring E_{MSH} -reduced *Mtb* showed high counts for [^{14}C]-Inh and corresponding lower counts remained in the bacteria, the E_{MSH} -basal population showed an inverse drug distribution. These data indicate higher efflux from the E_{MSH} -reduced population into macrophages (Fig. 5F). Direct comparison of [^{14}C]-Inh counts in E_{MSH} -reduced and E_{MSH} -basal bacterial pellets confirmed lower accumulation of the drug in the former (Fig. 5F). In summary, our data indicate that variations in efflux pump activity can be one of the factors managing drug tolerance in the E_{MSH} -reduced population during infection.

CQ counteracts drug tolerance and relapse in vivo

Given our findings that acidic pH promotes redox heterogeneity and enhances drug tolerance in vitro, we sought to determine the impact of pharmacological inhibition of phagosomal acidification on drug tolerance in vivo. We used the antimalarial drug CQ, which deacidifies endosomes and lysosomes (66, 67), to test *Mtb*'s response to Inh in a chronic murine model of infection (Fig. 6A) (68).

We treated chronically infected BALB/c mice (4 weeks p.i.) with Inh (25 mg/kg body weight), CQ (10 mg/kg body weight), or Inh along with CQ. After 2 and 8 weeks of therapy, we harvested the lungs and quantified the recovered bacteria. As reported (68), Inh monotherapy reduced the bacterial load from 10^6 to 10^4 at 2 weeks ($P = 0.00012$) and 10^3 per lung at 8 weeks ($P = 0.0022$) of treatment (Fig. 6B). CQ treatment alone showed no effect on bacterial viability over time (Fig. 6B). Relative to the control regimen (Inh alone), the addition of CQ did not alter lung CFUs after 2 weeks of treatment (Fig. 6B). However, 8 weeks of treatment with a combination of CQ with Inh (CQ plus Inh) completely sterilized the lungs of mice compared to 10^3 CFUs in the animals treated with Inh alone (Fig. 6B). The gross and histopathological changes observed in the lungs after 8 weeks of therapy were proportionate with the bacillary load observed (Fig. 6, D and E, and fig. S12A). After 8 weeks of treatment, the extent of pulmonary tissue destruction was highest in the untreated (score, 4) and CQ-treated animals (score, 3), intermediate in the case of the Inh-treated animals (score, 2), and negligible in CQ plus Inh-treated animals (score, 1 or 0). We also examined whether adjunct therapy with CQ for 8 weeks increased the efficacy of Rif (10 mg/kg body weight). The addition of CQ substantially reduced the fraction of Rif-tolerant *Mtb* in animal lungs ($P = 0.021$ for Rif alone versus a combination of CQ with Rif) (Fig. 6, C to E, and fig. S12A). However, the influence of CQ in reducing tolerance was more notable in the case of Inh as compared to Rif.

Because the pathophysiology of human TB is more closely recapitulated in guinea pigs (69), we aerosol-infected outbred Hartley guinea pigs with *Mtb*, followed 4 weeks later by treatment with Inh, CQ, or CQ plus Inh for an additional 8 weeks, and then estimated the lung bacillary load. The bacterial burden in the lungs of guinea pigs was 10^3 CFUs in Inh-treated animals and 10^5 CFUs in CQ-treated animals, compared to 100 CFUs in CQ plus Inh-treated animals (Fig. 6F). The effectiveness of CQ plus Inh was also reflected in the lung histopathology of guinea pigs (Fig. 6G and fig. S12B). Studying relapse can be another

predictor of therapeutic efficacy in TB. Therefore, we aerosol-infected mice with WT *Mtb*, followed 4 weeks later by treatment of infected animals with Inh or CQ plus Inh for 8 weeks. As shown earlier, 8 weeks of CQ plus Inh treatment completely sterilized mouse lungs. At 20 weeks p.i., which was 8 weeks after completion of therapy, mice received immunosuppressant dexamethasone (10 mg/kg body weight) for 2 weeks, and the lung bacillary load was determined at 22 weeks p.i. Relapse of disease was observed in five of five Inh-treated mice with bacterial loads of 2×10^4 CFUs in the lungs. Only three of five CQ plus Inh-treated mice relapsed, with only 30 CFUs in the lungs ($P = 0.0069$) (Fig. 6H).

Although in vitro studies indicate that CQ mainly exerts its influence on intracellular *Mtb* by raising the vacuolar pH (70), CQ can interfere with other cellular processes such as DNA synthesis, generation of ROS, and necrosis (71, 72). Therefore, we questioned whether the effect of CQ in reducing tolerance was associated with pH alkalization in vivo. Using Magic Red cathepsin B substrate that fluoresces only upon cleavage by cathepsin B protease inside acidic lysosomes (73), we confirmed that 6 weeks of CQ treatment raised the vacuolar pH of macrophages isolated from the lungs of mice chronically infected with *Mtb* (fig. S13, A and B). Other antibacterial mechanisms such as ROS production and necrosis were not stimulated in macrophages derived from the lungs of mice chronically infected with *Mtb* after 6 weeks of treatment with CQ plus Inh as compared to Inh or CQ alone (fig. S13, A, C, and D). Together, these results confirm that adjunct therapy with CQ counteracts drug tolerance and reduces disease relapse.

CQ exhibits no adverse interaction with anti-TB drugs

Excellent oral bioavailability, oral human pharmacokinetics (half-life of 10 to 15 days), high tissue penetration, and years of clinical use in humans (74) make CQ a good candidate for developing new therapeutic combinations for the treatment of TB. We investigated the pharmacological compatibility of CQ by measuring its potential drug-drug interactions with first-line anti-TB drugs (Inh or H, Rif or R, Emb or E, and Pza or Z) given as a combination. A single-dose pharmacokinetic interaction test was performed by administering anti-TB drugs with and without CQ [10 mg/kg body weight, intraperitoneally (i.p.)] in mice. Another group of mice was dosed with CQ (10 mg/kg body weight, i.p.) to compare the pharmacokinetic behavior of CQ in the presence of combination therapy (Fig. 7A). Plasma samples were analyzed for individual drugs using liquid chromatography–mass spectrometry, and key pharmacokinetic parameters such as maximum plasma concentration ($C_{\max}$) and area under the plasma concentration–time curve (AUC_{last}) were calculated as a ratio for combination versus single-treatment groups. Pharmacokinetic profiles revealed no adverse drug-drug interactions when CQ was coadministered with HREZ (Fig. 7, B and G). $C_{\max}$ and AUC_{last} for CQ alone was 228.5 ng/ml and 1358.0 ng·hour/ml and 297.2 ng/ml and 1358.0 ng·hour/ml for HREZ, respectively (Fig. 7, B and G).

The plasma pharmacokinetic profiles of anti-TB drugs remained largely unchanged in the presence of CQ. We observed no major interaction for Rif, Emb, or Pza in the presence or absence of CQ (Fig. 7, D to G) because $C_{\max}$ and AUC_{last} were within 80 to 125% criteria for equivalence (Fig. 7G) (75). Comparative ratios of $C_{\max}$ and AUC_{last} for Rif, Emb, and Pza, with and without CQ, were close to one except for Inh, which showed a minor

interaction ($C_{\max}$ ratio, 0.685), although AUC_{last} was not affected (Fig. 7, C and G). This minor influence of CQ on the pharmacokinetics of Inh may be due to the effect of CQ in reducing Inh influx in the intestines (76). Overall, the pharmacokinetic results suggested no adverse drug-drug interactions between the HREZ combination regimen versus CQ and vice versa. In summary, our study shows an effect of CQ on drug susceptibility, no major drug-drug interaction with HREZ, and enhanced in vivo efficacy of CQ-based combinations. With years of safe clinical history for CQ, these findings suggest that CQ could be repurposed for developing new curative combinations for TB.

Discussion

Generation of phenotypic heterogeneity and metabolic quiescence in response to stresses induced by immune activation is the most commonly invoked mechanism of antibiotic tolerance in *Mtb* (1, 4). However, clinical evidence in humans indicates that tolerance can also be associated with growing bacterial populations (77, 78). Consistent with this idea, early tolerance was documented in actively multiplying *Mycobacterium marinum* and *Mtb* in zebrafish larvae and in macrophages, respectively (6). In the present study, we showed a dominant role of phagosomal acidification in facilitating heterogeneity in the redox physiology of *Mtb* to generate an actively replicating, drug-tolerant population exhibiting higher antioxidant capacity (E_{MSH} -reduced) during infection. Our study constitutes an important foundation linking the macrophage environment with the core redox physiology of *Mtb* to promote drug tolerance in replicating *Mtb*, a population not typically associated with tolerance.

Host-induced oxidative stress is a major environmental stress encountered by *Mtb* during infection and is further exacerbated by anti-TB drugs (12, 79). Because a modest decrease in vacuolar pH is the earliest cue *Mtb* encounters (18), a pH-dependent reductive shift in E_{MSH} might offer cross-protection to oxidative stress generated by immune activation and drugs. This is supported by the identification of a small molecule (AC2P36) that interferes with thiol homeostasis at acidic pH and increases vulnerability to antibiotics in vitro (80). We also found that the expression of drug efflux pumps and consequent accumulation of antibiotics in *Mtb* populations are also dependent on the pH-induced remodeling of intramycobacterial E_{MSH} . Because efflux pumps have recently been shown to export oxidatively damaged proteins in *Mtb* (81), the pH-responsive induction of efflux pumps may be an elegant adaptation strategy to maintain redox homeostasis and tolerance in the face of antibiotics and host immune pressures. Consistent with this idea, the *Mtb* Rv1258c efflux pump is required for both survival and drug efflux during infection (6).

Because acidic pH encountered inside macrophages does not perturb intramycobacterial pH homeostasis (82), it is more likely that the phagosomal pH-dependent selection of an E_{MSH} -reduced population is part of a bacterial adaptation program during infection. In support of this notion, we have recently found that a redox-sensitive transcription factor, WhiB3, is required to generate an E_{MSH} -reduced population in response to phagosomal acidification (20). As a consequence, a WhiB3-deficient strain showed a growth defect in macrophages and guinea pigs (34) and also exhibited increased susceptibility to Inh. Acidic pH-mediated changes in gene expression and redox potential of *Mtb* were also reported to be dependent

on the PhoPR two-component system (83), indicating overlapping roles of WhiB3/PhoPR in modulating *Mtb* adaptation to pH stress. Data from our transcriptional profiling also align with the adaptation model wherein the drug-tolerant population ($E_{\text{MSH-reduced}}$) showed enhanced expression of stress-responsive regulons relative to the drug-sensitive fraction ($E_{\text{MSH-basal}}$) inside macrophages. However, although heterogeneity in *Mtb* populations has been reported during infection (4, 78), it was unclear whether there were variations in the expression of stress regulons or virulence factors in *Mtb* populations as seen with other pathogens including *Salmonella typhimurium* and *Yersinia pseudotuberculosis* (84, 85). Our data demonstrate heterogeneity in the expression of several regulators involved in sensing toxic metals and oxidative stress in redox-altered populations. We also observed a major realignment of sulfur metabolism and proposed that the flux of reduced sulfur metabolites such as CySH into Fe-S cluster assembly, reverse transsulfuration, SAM biosynthesis, and MSH biosynthesis is likely to coordinate *Mtb*'s defense against antibiotics. Deviations in CySH flux contribute to potentiation of the mycobactericidal efficacy of anti-TB drugs (86). Furthermore, increased expression of SAM-dependent methyl transferases in $E_{\text{MSH-reduced}}$ bacteria can promote drug tolerance by *N*-methylation of antibiotics (40). These transcriptional changes—along with the reduced tolerance shown by *Mtb metB*, *Mtb sufR*, and *Mtb whiB3*—led us to propose a model of how phagosomal acidification and bacterial pathways integrate to reset *Mtb*'s redox physiology for successfully counteracting anti-TB drugs (fig. S14).

Our data also suggest that redox-dependent drug tolerance in replicating *Mtb* is multifactorial. Although the deletion of single redox-responsive pathways (WhiB3, SufR, and MetB) exhibited substantial influence on drug tolerance, complete clearance of *Mtb* was not achieved. Similar to our findings, drug tolerance in the growth-arrested *Mtb* population possibly requires activation of multiple stress regulons and toxin-antitoxin modules (DosR, PhoP, MprA, and MazEF) (1). These studies imply that targeting few bacterial genes or pathways is unlikely to severely affect the ability of *Mtb* to mobilize drug tolerance in response to host-induced pressures. Conversely, targeting host cues that alter the physiology of *Mtb* could be an effective mechanism to diminish phenotypic heterogeneity-driven drug tolerance. Our animal data showing the nearly sterilizing effect of CQ in combination with anti-TB drugs provide a compelling argument for targeting vacuolar pH to subvert early emergence of drug tolerance in vivo. Although CQ reinstates redox homogeneity and drug susceptibility mainly by increasing phagosomal pH, other immunomodulatory properties of CQ such as iron depletion (70), blocking phagosomal maturation and autophagy (66), and reversing inflammation-dependent efflux pumps (51) could also contribute to the effect of CQ on multidrug tolerance in vivo. It has been recently shown that CQ potentiates the antimycobacterial activity of Inh and Pza in immune-activated macrophages (51). Because shortening TB chemotherapy requires rapid sterilization of *Mtb*, our study provides empirical evidence that targeting phagosomal acidification by small molecules has the potential to provide relapse-free control by subverting redox heterogeneity. Because CQ is clinically used, stable, cost effective, and highly tolerable with few side effects, it can be conveniently repurposed to formulate new combinations with the current anti-TB regimen to reduce therapy duration. CQ also reduced redox heterogeneity and Inh tolerance in a HIV-TB coinfection background. Along with clinical evidence showing anti-HIV properties of

CQ (87), this raises the possibility of potentiating current anti-TB and anti-HIV therapies by CQ. Last, redox-mediated multidrug tolerance may be relevant to other chronic pathogens. For example, heightened antioxidant capacity is linked to the acquisition of phenotypic antibiotic resistance in the human pathogen *Pseudomonas aeruginosa* (88). Thus, our findings may have broad relevance to several human pathogens where a sterilizing cure is therapeutically challenging.

Although CQ therapy reduced drug tolerance in vivo, several issues remain to be addressed before it can be combined with the standard anti-TB therapy. First, it needs to be investigated whether a combination of CQ with current therapeutic regimens reduces therapy duration and facilitates the development of immunological memory to prevent relapse. Second, the efficacy of CQ in shortening regimens for drug-resistant *Mtb* infections remains to be evaluated. Third, despite treatment with anti-TB drugs, *Mtb* cells persist in animal tissues and the sputum of patients with TB in a metabolically altered state and remain undetectable by viable counts (89, 90). It will be interesting to investigate the impact of CQ on these heterogeneous subpopulations of *Mtb* that are difficult to detect and retain persister phenotypes in animal models. All these issues require further experimentation using animal models and prospective clinical trials to properly test the efficacy of CQ as adjunct anti-TB therapy.

Materials and Methods

Study design

The overall objective of this study was to evaluate host and bacterial mechanisms underlying the emergence of redox heterogeneity and drug tolerance in *Mtb* during infection. First, we characterized the transcriptome of redox-diverse *Mtb* fractions (E_{MSH} -reduced and E_{MSH} -basal), which led to the identification of CySH metabolism of *Mtb* and phagosomal acidification as important factors coordinating redox-mediated drug tolerance inside macrophages. Next, we performed detailed mechanistic studies on the role of phagosomal acidification in generating drug-tolerant E_{MSH} -reduced bacteria in macrophages infected with *Mtb* alone or coinfecting with HIV-1. We applied multiple approaches to studying replication dynamics, metabolic status, and efflux pump activity in E_{MSH} -reduced and E_{MSH} -basal *Mtb* inside macrophages. We then evaluated the licensed antimalarial drug CQ, which deacidifies phagosomes, in reducing tolerance to standard anti-TB drugs and relapse in animal models of *Mtb* infection. We assessed the pharmacological compatibility of CQ with first-line anti-TB drugs in mice. Animals were randomly allocated into groups and were identifiable with respect to their treatment. All studies were carried out as per guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, with approval from the Institutional Animal Ethical Committee. Drug treatment and euthanasia were carried out in humane ways to minimize suffering for animals. All experiments were carried out in a biosafety level 3 containment facility and approved by the Institutional Biosafety Committee.

In vivo experiments

For the chronic model of infection (68), 4- to 6-week-old female BALB/c mice ($n = 6$ per group) were infected by the aerosol route with 100 *Mtb* H37Rv bacilli using a Madison chamber aerosol generation instrument, housed for 4 weeks for progression of infection, and then left untreated or started under various treatment conditions: (i) 10 mg/kg body weight intraperitoneal doses of CQ on alternate days, (ii) 25 mg/kg body weight of Inh in drinking water daily, (iii) 10 mg/kg body weight of Rif in drinking water daily, (iv) a combination of CQ and Inh (CQ plus Inh) at earlier mentioned doses, and (v) a combination of CQ and Rif (CQ plus Rif) at the mentioned doses (68). At indicated time points of treatment, mice were euthanized, and the lungs were harvested for bacterial burden, gross pathology, and tissue histopathology analysis. The upper right lobe of the lungs of animals from each group was fixed in 10% neutral-buffered formalin. Fixed tissues were prepared as 5- μ m-thick sections, embedded in paraffin, and stained with hematoxylin and eosin. Tissue sections were coded, and coded sections were analyzed by a certified pathologist to assess for granuloma formation and lung damage (91). Remaining tissue samples were homogenized in 2 ml of sterile 1 $\times$ phosphate-buffered saline (PBS), serially diluted, and plated on 7H11-OADC agar plates supplemented with lyophilized BBL MGIT PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, as supplied by BD). Plates were incubated at 37°C for 3 weeks before colonies were enumerated.

For mice receiving treatment with Inh alone or a combination of CQ and Inh, all treatments were stopped at 12 weeks p.i. (when animals were found to be culture negative for *Mtb*) for remaining animals ($n = 5$ per group). Animals were further housed for 8 weeks without treatment, after which four intraperitoneal doses of dexamethasone at 10 mg/kg body weight were administered over 2 weeks for pan-immunosuppression. In the 22nd week p.i., animals in both groups were euthanized, and lung burden of reactivated *Mtb* was determined by plating lung homogenates for CFUs, as mentioned earlier.

Outbred Hartley guinea pigs ($n = 5$ per group) were given an aerosol challenge of 100 *Mtb* H37Rv (92) using a Madison chamber aerosol generation instrument, housed for 4 weeks for progression of infection, and then left untreated or started on treatment in one of three groups: (i) 5 mg/kg body weight intraperitoneal doses of CQ on alternate days, (ii) 30 mg/kg body weight of Inh in drinking water daily, and (iii) a combination of CQ and Inh (CQ plus Inh) at earlier mentioned doses. At 8 weeks after commencement of treatment, guinea pigs were euthanized, and lung burden of *Mtb* was determined by homogenizing organs in 5 ml of sterile 1 $\times$ PBS, serial dilution, and plating on 7H11-OADC agar plates supplemented with PANTA. Upper right lobes of the lungs from different treatment groups were fixed in neutral-buffered formalin and prepared, as mentioned earlier, for histopathological analysis (91).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 6.0). All data indicated are means $\pm$ SD except figs. S12 and S13, where median $\pm$ interquartile range was plotted for animal groups. The Mann-Whitney rank sum test was used for comparison of nonparametric data between two experimental groups. Nonparametric multiple group

comparisons were analyzed using the Kruskal-Wallis test with Dunn's post hoc correction. For overlap analysis of differentially expressed (DE) genes with other microarray studies, the significance of gene number overlap was determined by Fisher's exact test on a two-by-two contingency table (93). Differences with $P < 0.05$ were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to C. Grundner at the Seattle Children's Research Institute for critical reading of the manuscript and valuable input. We acknowledge W. R. Jacobs Jr. at the Albert Einstein College of Medicine for the *Mtb mshA* mutant and D. J. V. Beste at the University of Surrey for providing the zeocin-marked vector pANEE001. C. N. Naveen at the Foundation of Neglected Disease Research (FNDR), India is acknowledged for supporting pharmacokinetic and drug-drug interaction studies in mice. We thank S. Nayak for valuable discussions about the key findings of the study and designing of schematics and models. We acknowledge the CIDR BSL-3 facility, Indian Institute of Science (IISc) for carrying out experiments with *Mtb* and A. Pandit and the NGS facility, NCBS for RNA-seq. The *mshA* mutant of *Mtb* was obtained from W. R. Jacobs Jr. under a material transfer agreement (MTA) between the Albert Einstein College of Medicine of Yeshiva University and the International Centre for Genetic Engineering and Biotechnology.

Funding

This work was supported by Wellcome Trust-DBT India Alliance grant IA/S/16/2/502700 (to A.S.); in part, by Department of Biotechnology (DBT) grants BT/PR11911/BRB/10/1327/2014 and BT/PR13522/COE/34/27/2015 (to A.S.); and by the DBT-IISc Partnership Program (22-0905-0006-05-987436). A.S. is a senior fellow of Wellcome Trust-DBT India Alliance. A.S.N.S. is supported by the Wellcome Trust-DBT India Alliance Grant IA/I/16/2/5-2711. R.M. is supported by the IISc, Bengaluru. S.K. and M. Mehta are national postdoctoral fellows supported by the Department of Science and Technology (DST), India. N.M. is supported by the National Centre for Biological Sciences—Tata Institute of Fundamental Research (NCBS-TIFR), Bengaluru. P.B. is supported by a fellowship provided by the Council of Scientific and Industrial Research (CSIR), India.

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Fig. 1. RNA-seq of intraphagosomal *Mtb* derived from *E*_{MSH}-reduced and *E*_{MSH}-basal fractions.

(A) Schematic depiction of flow sorting–coupled RNA-seq of intraphagosomal bacteria present in *E*_{MSH}-basal and *E*_{MSH}-reduced fractions of THP-1 macrophages infected with *Mtb*/Mrx1-roGFP2. *Mtb* cells (optical density at 600 nm, 0.4) harvested and resuspended in RPMI for 24 hours were used as an in vitro control. FACS, fluorescence-activated cell sorting; GTC, guanidinium thiocyanate. (B) Scatter plot indicates relative distribution of differentially expressed genes (DEGs) from the *E*_{MSH}-reduced and *E*_{MSH}-basal fractions on the basis of log₂ fold changes (FC) (blue, DEGs specific to *E*_{MSH}-reduced; red, DEGs unique to *E*_{MSH}-basal; black, DEGs common to both; gray, nonsignificant genes). (C) The table summarizes the transcriptional overlap between this study and the response of *Mtb* under intramacrophage and pH stress conditions. Fisher's exact test with $P < 0.05$ as a cutoff

for significance. ns, no significant difference. **(D)** Heat maps indicate $\log_2$ fold changes of DEGs belonging to various functional categories (obtained from Mycobrowser, École polytechnique fédérale de Lausanne) in E_{MSH} -reduced and E_{MSH} -basal fractions. Genes were considered differentially expressed on the basis of the false discovery rate (FDR) of 0.05 and absolute fold change of ≥ 1.5 (tables S1 and S3).

Fig. 2. Cysteine utilization pathways promote redox heterogeneity and drug tolerance in *Mtb*.

(A) Various cysteine (CySH) utilization pathways in *Mtb*. Expression of genes (blue) coordinating CySH flux into pathways for mycothiol (MSH), Fe-S cluster, and H₂S biogenesis is induced in the *E*_{MSH}-reduced fraction. (B) THP-1 macrophages were infected for 24 hours with the indicated strains of *Mtb* expressing Mrx1-roGFP2, and the percent distribution of redox-diverse fractions was measured at 24 hours p.i. and depicted as a stacked bar plot. ***P* < 0.01, by Mann-Whitney test compares *E*_{MSH}-reduced fraction in various strains of *Mtb* with WT *Mtb*. (C) THP-1 macrophages infected for 24 hours with the

indicated strains of *Mtb* were exposed for an additional 48 hours to Inh (2.18 μ M, 3 $\times$ of in vitro MIC) or left untreated. Bacillary load was determined by CFU enumeration, and percent survival was quantified by normalizing the CFU in Inh-treated samples at 48 hours against untreated samples (UT) at 0 hours. * P < 0.05, ** P < 0.01, # P < 0.05, and ## P < 0.01, by Mann-Whitney test. Number signs (#) and asterisks (*) compare survival between WT *Mtb* and other strains under UT and Inh-treated conditions, respectively. (D and E) Indicated strains of *Mtb* grown in 7H9-tyloxapol broth acidified to pH 6.2 or pH 4.5 were exposed to Inh (7.25 μ M, 10 $\times$ of in vitro MIC) or kept unexposed. Bacterial load was quantified after 5 days of treatment by CFU enumeration, and percent survival was quantified strain-wise by normalizing the bacterial load in Inh-treated samples at day 5 against untreated samples. * P < 0.05, ** P < 0.001, by Mann-Whitney test. Asterisks compare survival between WT *Mtb* and other strains after 5 days of Inh treatment. Data shown in each panel are the results of three independent experiments performed in triplicate (means $\pm$ SD). ns, no significant difference (P > 0.05).

Fig. 3. Phagosomal pH is required for the redox-dependent multidrug tolerance of *Mtb*.

(A) THP-1 macrophages—untreated or pretreated with 10 nM BafA1, 10 mM NH₄Cl, or 10 μM CQ—were infected with *Mtb*/Mrx1-roGFP2, and percent distribution of redox-diverse fractions was measured at 24 hours p.i. ***P* < 0.01, by Mann-Whitney test to compare the *E*_{MSH}-reduced fraction with untreated sample. (B and C) THP-1 macrophages, untreated or pretreated with 10 nM BafA1 or 10 μM CQ, were infected with WT *Mtb* for 24 hours and exposed to Inh (2.18 μM) or Rif (1 μM) or left unexposed for an additional 48 hours. Percent survival was quantified by normalizing the CFU in drug-treated samples at 48 hours against

untreated samples at 0 hours. $*P < 0.05$, $**P < 0.01$, by Mann-Whitney test. **(D)** THP-1 macrophages were infected with *Mtb*/Mrx1-roGFP2, and E_{MSH} was measured at 24 hours p.i. After this, intraphagosomal bacteria were released and incubated in 7H9-albumen-dextrose-sodium chloride for 2 hours, and E_{MSH} was determined. The 7H9-ADS-adapted *Mtb* was used to reinfect fresh THP-1 macrophages, with or without pretreatment with 10 μM CQ, and E_{MSH} was measured at 24 hours p.i. $**P < 0.01$, $###P < 0.001$, by Mann-Whitney test. Number signs (#) compare E_{MSH} -reduced fractions between intramacrophage and 7H9-ADS-adapted *Mtb*. Asterisks (*) compare E_{MSH} -reduced fractions between untreated and CQ-treated samples. **(E)** THP-1 macrophages harboring E_{MSH} -reduced and E_{MSH} -basal bacteria were flow-sorted at 24 hours p.i. (cycle 1 infection), and bacteria were released into 7H9-ADS. At 24-hour incubation, 7H9-ADS-adapted *Mtb* were used to infect THP-1 macrophages, with or without pretreatment with 10 μM CQ, for 24 hours (cycle 2 infection), and Inh tolerance was determined as mentioned earlier. $*P < 0.05$, $**P < 0.01$, by Mann-Whitney test. Data shown in each panel are the results of three independent experiments performed in triplicate (means $\pm$ SD). ns, no significant difference ($P > 0.05$).

Fig. 4. Phagosomal pH and redox heterogeneity drive drug tolerance during HIV-TB coinfection.

(A) The course of HIV-1 replication upon stimulation of the U1 promonocytic cell line with PMA (5 ng/ml). Viral load was monitored by *gag* qRT-PCR. ** $P < 0.01$, by Mann-Whitney test comparing *gag* expression with 0 hours. U937 (uninfected HIV-1 control) (B) and U1 macrophages (C) were stimulated with PMA and infected with *Mtb*/Mrx1-roGFP2, and percent distribution of redox-diverse fractions was measured over time. * $P < 0.05$, by Mann-Whitney test. Asterisks (*) compare $E_{\text{MSH}}\text{-reduced}$ fraction at various time points with 0 hours. (D) U1 macrophages—untreated or pretreated with 10 nM BafA1, 10 mM NH₄Cl,

and 10 μ M CQ—were infected with *Mtb*/Mrx1-roGFP2, and percent distribution of redox-diverse fractions was measured at 12 hours p.i. * P < 0.05, ** P < 0.01, by Mann-Whitney test. Asterisks (*) compare E_{MSH} -reduced fractions between untreated and BafA1/ NH_4Cl /CQ-treated samples. U937 (**E**) and U1 macrophages (**F**), untreated or pretreated with 10 μ M CQ or 10 nM BafA1, were infected with WT *Mtb* for 12 hours and exposed to Inh (2.18 μ M) or left unexposed for an additional 48 hours. Bacillary load was determined by CFU enumeration, and percent survival was quantified by normalizing the CFU in drug-treated samples at 48 hours against untreated samples at 0 hours. * P < 0.05, ** P < 0.01, by Mann-Whitney test. Data shown in each panel are the results of three independent experiments performed in triplicate (means $\pm$ SD).

Fig. 5. The drug-tolerant *E*_{MSH}-reduced population is replicative and has high efflux pump activity.

(A) Graphical depiction of Mrx1-roGFP2-coupled flow-sorting strategy to determine replication dynamics, metabolic state, and drug efflux activity in intramacrophage *E*_{MSH}-reduced and *E*_{MSH}-basal populations. (B and C) THP-1 macrophages were infected with pBP10-containing *Mtb*/Mrx1-roGFP2. At indicated time points, macrophages harboring *E*_{MSH}-reduced and *E*_{MSH}-basal bacteria were flow-sorted, and bacteria were released and plated in the presence or absence of kanamycin (Kan). The frequency of pBP10 loss and increase in cumulative bacterial burden (CBB) were calculated. **P* < 0.05, ***P* < 0.01, #*P* < 0.05, ##*P* < 0.01, by Kruskal-Wallis test with Dunn's correction over time p.i. Asterisks (*) and number signs (#) compare CFU per milliliter and percentage of pBP10 + ve bacteria over time p.i., respectively. (D) THP-1 macrophages were infected with *Mtb*/Mrx1-roGFP2, and at 24 hours p.i., the redox state of intraphagosomal *Mtb*/Mrx1-roGFP2 thiols was fixed using *N*-ethylmaleimide. Bacteria were released from macrophages and stained with calcein violet-AM (CV-AM). The CV-AM staining and *E*_{MSH} status of *Mtb* cells were determined using multiparameter flow cytometric analysis. As a control, we performed CV-AM staining of *Mtb* grown in 7H9 broth for 24 hours at 4° and 37°C. **P* < 0.05, by Mann-Whitney test. (E) THP-1 macrophages harboring *E*_{MSH}-reduced and *E*_{MSH}-basal bacteria were flow-sorted at 24 hours p.i., bacterial RNA was isolated, and expression of efflux pumps was measured by qRT-PCR. Expression was compared with in vitro control *Mtb*, and fold change was quantified after normalizing by 16S ribosomal RNA. **P* < 0.05, ***P* < 0.01, by Mann-

Whitney test for comparison with in vitro control *Mtb*. (F) THP-1 macrophages were infected with *Mtb*/Mrx1-roGFP2 bacteria preloaded with [^{14}C]-Inh. At 24 hours p.i., macrophages harboring E_{MSH} -reduced (Red) and E_{MSH} -basal (Bas) bacteria were sorted and bacteria released. The relative distribution of radioactive [^{14}C]-Inh was measured in bacterial and macrophage (MΦ) fractions. * $P < 0.05$, ** $P < 0.01$, by Mann-Whitney test. Data shown in each panel are the results of two independent experiments performed in triplicate (means $\pm$ SD). ns, no significant difference ($P > 0.05$).

Fig. 6. CQ counteracts drug tolerance and reduces relapse in vivo.

(A) Strategy to investigate the efficacy of CQ in reducing tolerance against Inh and Rif and post-therapeutic relapse in vivo. BALB/c mice ($n = 6$) were given an aerosol challenge with WT *Mtb*. From 4 weeks p.i. onward, groups of mice were left untreated or treated with anti-TB drugs (Inh/Rif) alone or in combination with CQ (CQ + Inh/ CQ + Rif). (B and C) Bacterial CFUs were measured in the lungs at the indicated time points. $**P < 0.01$, $****P < 0.0001$, by Kruskal-Wallis test with Dunn's correction across experimental groups at 12 weeks p.i. (D) Gross pathology of the lungs of WT *Mtb*-infected mice at 8 weeks of

treatment across experimental groups. **(E)** Hematoxylin and eosin–stained lung sections (8 weeks of treatment) from mice infected with WT *Mtb* across experimental groups. The pathology sections show granuloma (G), alveolar space (AS), and bronchiole lumen (BL). All images were taken at $\times 40$ magnification. Scale bars, 200 μm . **(F)** Outbred Hartley guinea pigs ($n = 6$) were given aerosol challenge with WT *Mtb*, and efficacy of CQ in reducing Inh tolerance was assessed as described in (B) and (C). $**P < 0.01$, $****P < 0.0001$, by Kruskal-Wallis test with Dunn's correction across experimental groups at 12 weeks p.i. **(G)** Hematoxylin and eosin–stained lung sections (8 weeks of treatment) from guinea pigs infected with WT *Mtb* across experimental groups. The pathology sections show granuloma (G), alveolar space (AS), and necrotic core (N). All images were taken at $\times 40$ magnification. Scale bars, 200 μm . **(H)** Dexamethasone-induced reactivation of *Mtb* from the lungs of BALB/c mice ($n = 5$) after treatment with Inh alone or a combination of CQ plus Inh. Mann-Whitney test was used to compare the relapse frequency (Inh alone versus CQ + Inh combination) for effectiveness of CQ therapy ($P = 0.0069$). Data shown in each panel are the results of two independent experiments (means $\pm$ SD). ns, no significant difference ($P > 0.05$).

Fig. 7. CQ exhibits no adverse interactions with anti-TB drugs.

(A) The table indicates three groups of treatment in BALB/c mice used in the pharmacokinetic study: CQ alone, front line anti-TB combination therapy (HREZ), and combination (CQ + HREZ). (B to F) Line plots indicate pharmacokinetic profiles of CQ and individual drugs of the anti-TB therapy regimen analyzed individually and in the presence of each other in plasma of animals over 24 hours. No significant difference was observed between groups at each time point indicated in each panel by Mann-Whitney test ($P > 0.05$). (G) The table depicts ratios of C_{max} and AUC_{last} of individual drugs alone or in combination to analyze drug-drug interaction. Doses used are the following: CQ, 10 mg/kg body weight, i.p.; Inh/H, 25 mg/kg body weight, p.o.; Rif/R, 10 mg/kg body weight, p.o.; Emb/E, 200 mg/kg body weight, p.o.; Pza/Z, 150 mg/kg body weight, p.o. p.o., per os consumption; BDL, below detection limit. All data are means $\pm$ SD of concentrations at each time point of samples in triplicates ($n = 3$ animals per group).