

Phagosomal acidification coordinates a lipid–redox program underlying antibiotic tolerance in *Mycobacterium tuberculosis*

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Phagosomal acidification coordinates a lipid–redox program underlying antibiotic tolerance in *Mycobacterium tuberculosis*

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One sentence summary: Phagosomal acidification drives lipid-mediated reductive shift and antibiotic tolerance in intracellular *Mycobacterium tuberculosis*.

ABSTRACT

Phenotypically drug-tolerant *Mycobacterium tuberculosis* (*Mtb*) subpopulations within macrophages delay bacterial clearance, contributing to treatment failure and resistance. Here, we provide evidence that phagosomal acidification functions as a metabolic checkpoint linking host lipid metabolism, bacterial redox balance, and antibiotic tolerance. Acidic phagosomes promote lipid droplet (LD) biogenesis in M-CSF-differentiated bone marrow-derived macrophages (BMDMs) and facilitate lipid access by intracellular *Mtb*. Access to these lipids enables intracellular *Mtb* to maintain a reductive cytoplasmic redox state that supports drug tolerance. Consistent with this, chloroquine (CQ)-mediated alkalinization disrupts this pH-LD axis, leading to reduced LD accumulation, diminished bacterial lipid access, and attenuation of redox-associated tolerance mechanisms in a drug-sensitive (H37Rv) and a multi-drug-resistant clinical strain (NHN1664). Transcriptomic profiling of intraphagosomal *Mtb* identifies the Fe-S transcription factor WhiB6 as a potential contributor linking phagosomal pH, host lipid availability, and reductive stress. In C3HeB/FeJ mice, which develop necrotic lesions and fibrotic lungs with limited MXF efficacy, CQ monotherapy was sufficient to attenuate lung fibrosis and improve pulmonary function without reducing bacterial burden in the lungs infected with NHN1664. Importantly, MXF treatment alone had little effect on bacterial burden in these mice, whereas combination treatment with CQ and MXF significantly reduced bacterial loads. Collectively, these findings identify phagosomal acidification as an organiser of a lipid-redox program that underlies antibiotic tolerance in *Mtb* and establish CQ as a promising host-directed adjunct therapy for multidrug-resistant tuberculosis.

51 INTRODUCTION

52 Standard therapy for drug-sensitive tuberculosis (DS-TB) requires at least six months
53 of multidrug treatment (isoniazid, rifampicin, pyrazinamide, and ethambutol) (1,2), whereas
54 multidrug-resistant TB (MDR-TB) historically required 18–24 months of therapy (3,4),
55 although newer regimens have shortened treatment to 6–9 months in selected patients (5).
56 Despite these advances, prolonged therapy remains the norm globally. This extended duration
57 largely reflects the presence of phenotypically drug-tolerant *Mycobacterium tuberculosis* (*Mtb*)
58 subpopulations (6,7). These bacteria remain genetically drug-susceptible but enter
59 metabolically rewired states in response to the host environment, allowing them to withstand
60 antibiotic pressure during infection. Drug tolerance in intracellular *Mtb*, therefore, is a
61 consequence of a spectrum of host-imposed physiological states rather than a single bacterial
62 mechanism. Host immune context, including macrophage activation state (8), ontogeny
63 (alveolar versus interstitial macrophages) (9–11) and immune-metabolic programs such as
64 oxidative phosphorylation (OXPHOS) or glycolysis can profoundly reshape metabolism, redox
65 balance, and antibiotic susceptibility of intracellular *Mtb* (9,12,13). Heterogeneous
66 macrophages expose *Mtb* to diverse and overlapping stresses, including nitric oxide, acidic pH,
67 hypoxia, and iron limitation, that reshape bacterial physiology and influence antibiotic
68 susceptibility (8,14–18).

69 *In vivo* evidence implicates foamy macrophages and caseous necrotic granulomas as
70 key drivers of drug tolerance in TB. Caseous granulomas restrict vascular drug delivery,
71 sequestering lipophilic antibiotics within lipid-rich debris and imposing hypoxic conditions
72 that slow bacterial replication (19). Consistently, necrotic caseum in rabbit lung cavities
73 harbours highly drug-tolerant, non-replicating *Mtb* populations (20). Lipid-laden foamy
74 macrophages, the cellular hallmark of caseum and necrotising TB lesions, further promote

bacterial persistence by supplying fatty acids that fuel mycobacterial metabolism and triacylglycerol synthesis (21,22). In addition to a lipid-rich diet, *Mtb* is exposed to heterogeneous acidic pH and variable oxygen tension in foamy macrophages and caseum (19,23), consistent with the role of these stresses in mobilising drug tolerance in *Mtb* (19,24). However, the underlying mechanism of how host microenvironmental cues and metabolic states, particularly the availability of host-derived fatty acids and acidic pH, mobilise bacterial adaptation programs that promote drug tolerance in *Mtb* remains poorly understood.

Using a genetically encoded redox biosensor, we previously demonstrated that *Mtb* residing within acidified phagosomes remains replicative and undergoes a reductive shift in its mycothiol redox potential (E_{MSH}) that promotes tolerance to frontline antibiotics (25). Reductive shift was also documented in *Mtb* grown in 7H9 medium containing specific carbon sources at acidic pH (26). In macrophages, the pH-inducible E_{MSH} -reduced fraction of *Mtb* counteracts anti-TB drugs by activating efflux pumps and cysteine disposal pathways, including biogenesis of iron-sulfur (Fe-S) clusters, hydrogen sulfide (H_2S), and mycothiol (MSH) (25). Importantly, pharmacological alkalinization of phagosomes using the antimalarial drug chloroquine (CQ) suppresses this reductive shift, accelerates bacterial clearance, and prevents relapse *in vivo* when combined with isoniazid (INH) and rifampicin (RIF) (25). Based on these encouraging pre-clinical data, a Phase I human clinical trial evaluating adjunctive CQ with standard anti-TB therapy is currently underway (27). Despite these advances, several fundamental questions remain unresolved. For example, (a) How does acidic phagosomal pH reprogram macrophage metabolism to drive redox-dependent drug tolerance in *Mtb*? (b) Which aspects of macrophage physiology and bacterial metabolism are disrupted by phagosomal deacidification? (c) Is there a bacterial regulator that links phagosomal pH, host physiology and redox-dependent antibiotic tolerance? (d) Can phagosomal deacidification be exploited to suppress drug tolerance associated with necrotizing TB lesions while ameliorating lung fibrosis

and restoring pulmonary function? Elucidating these mechanisms is imperative not only for dissecting the molecular basis of pH- and redox-dependent drug tolerance, but also for validating if the pharmacological alkalization agents such as CQ can be leveraged as immunometabolic modulators and adjuncts to anti-tuberculosis therapy.

Here, we address these questions by integrating biochemical, genetic, transcriptomic, and *in vivo* analyses. We show that phagosomal acidification drives macrophage lipid droplet (LD) biogenesis and promotes the acquisition of host fatty acids by *Mtb*. This process enables intracellular *Mtb* to maintain a reductive redox state that drives antibiotic tolerance. Conversely, phagosomal alkalization by CQ, BafA1, and NH₄Cl disrupts this pH–lipid–redox axis by limiting LD formation, thereby suppressing drug tolerance in both laboratory and clinical multidrug-resistant (MDR) strains. RNA-sequencing of macrophage-derived *Mtb* revealed that phagosomal deacidification by CQ starves the bacteria of iron and diminishes the expression of WhiB6, a major regulator of the ESX-1 secretion system involved in LD biogenesis (28–30). Genetic ablation of *whiB6* impaired macrophage LD pool and suppressed the reductive shift in the *EMSH* of *Mtb*, suggesting its role in coordinating host-pathogen metabolism and drug tolerance. Finally, in a murine model of necrotising TB, adjunct CQ therapy enhances antibiotic efficacy while simultaneously controlling lung fibrosis and improving respiratory function.

RESULTS

Transcriptomic signatures associate phagosomal deacidification with lipid remodelling, inflammatory response, and fibrotic rewiring.

Using *Mtb* expressing the redox-sensitive biosensor (*Mtb-roGFP2*), we previously demonstrated that E_{MSH} -reduced bacilli localise within acidified phagosomes (pH ~ 5.7) of THP-1 macrophages (25). These bacilli exhibit a replicative phenotype and display pronounced tolerance to frontline anti-TB agents such as INH and RIF (25). In contrast, E_{MSH} -basal and oxidised *Mtb* reside in phagosomes with near-neutral pH (~ 6.7), where they remain drug-susceptible (25). Notably, phagosomal deacidification by the antimalarial agent CQ (25) diminished the proportion of E_{MSH} -reduced *Mtb* fraction (fig. S1A) and attenuated drug tolerance inside macrophages (fig. S1B) and in animals (25). CQ alone had a minimal effect on intracellular bacterial viability (25) (Fig. S1B).

To assess whether these observations extend to primary macrophages and second-line anti-TB drugs, we employed M-CSF differentiated murine bone marrow-derived macrophages (BMDMs) and moxifloxacin (MXF), a fluoroquinolone integral to both drug-sensitive and drug-resistant TB regimens (31,32). Also, given the limited efficacy of MXF in chronic necrotic lesions (33), where drug penetration and bacterial metabolic state constrain activity (34), we asked if phagosomal deacidification by CQ could enhance its efficacy. CQ treatment (10 μ M) uniformly diminished the frequency of E_{MSH} -reduced *Mtb* at 24 hours post-infection (p.i.) (Fig. S2A). Consistent with this, *Mtb* growing in CQ-treated BMDMs were significantly more sensitive to MXF than those in untreated BMDMs (percentage survival in MXF alone: 47% $\pm$ 3.2% vs CQ+MXF: 12.9% $\pm$ 1.4%) (Fig. S2B).

To delineate the mechanism of how phagosomal deacidification by CQ alters redox physiology and drug susceptibility of *Mtb* during infection, we performed RNA sequencing (RNA-seq) of BMDMs (i) uninfected, (ii) uninfected; CQ-treated, (iii) *Mtb*-roGFP2-infected, and (iv) *Mtb*-roGFP2-infected; CQ-treated. At 24 h post-infection (p.i.), we flow-sorted GFP-positive (infected) macrophages to minimise the contribution of bystander cells that often undergo paracrine activation (35) and influence bulk RNA-seq signature (Fig. 1, A). Principal component analysis (PCA) showed that samples distinctly clustered with their biological replicates, with clear segregation between uninfected and *Mtb*-infected BMDMs and CQ-treated or untreated BMDMs (Fig S3, A-C). As expected, *Mtb* infection induces wide-ranging changes in the BMDM transcriptome with over 5,000 differentially expressed genes (DEGs; 2,275 upregulated and 3,469 downregulated; fold change >2, false discovery rate (FDR) < 0.05) (Supplementary file 1). Consistent with published findings, *Mtb* infection upregulated the expression of genes associated with TNF α signalling, interferon signalling, general cytokine response and programmed cell death (Fig. S4A) (12). *Mtb* infection downregulated cell cycle and DNA replication pathways, cell adhesion and collagen fibril organisation genes (Fig. S4B). In contrast to the broad transcriptional changes induced by *Mtb* infection, CQ treatment of uninfected BMDMs altered a distinct transcriptional program comprising 182 DEGs (54 upregulated, 128 downregulated; fold-change >2, FDR < 0.05) (Supplementary file 1). Gene ontology analysis indicated that CQ deregulates genes associated with angiogenesis and vascular remodelling, apoptotic signalling, immune synapse formation, lymphocyte chemotaxis, macrophage differentiation, and extracellular matrix (ECM)/fibrotic remodelling (Fig. S5, A-B).

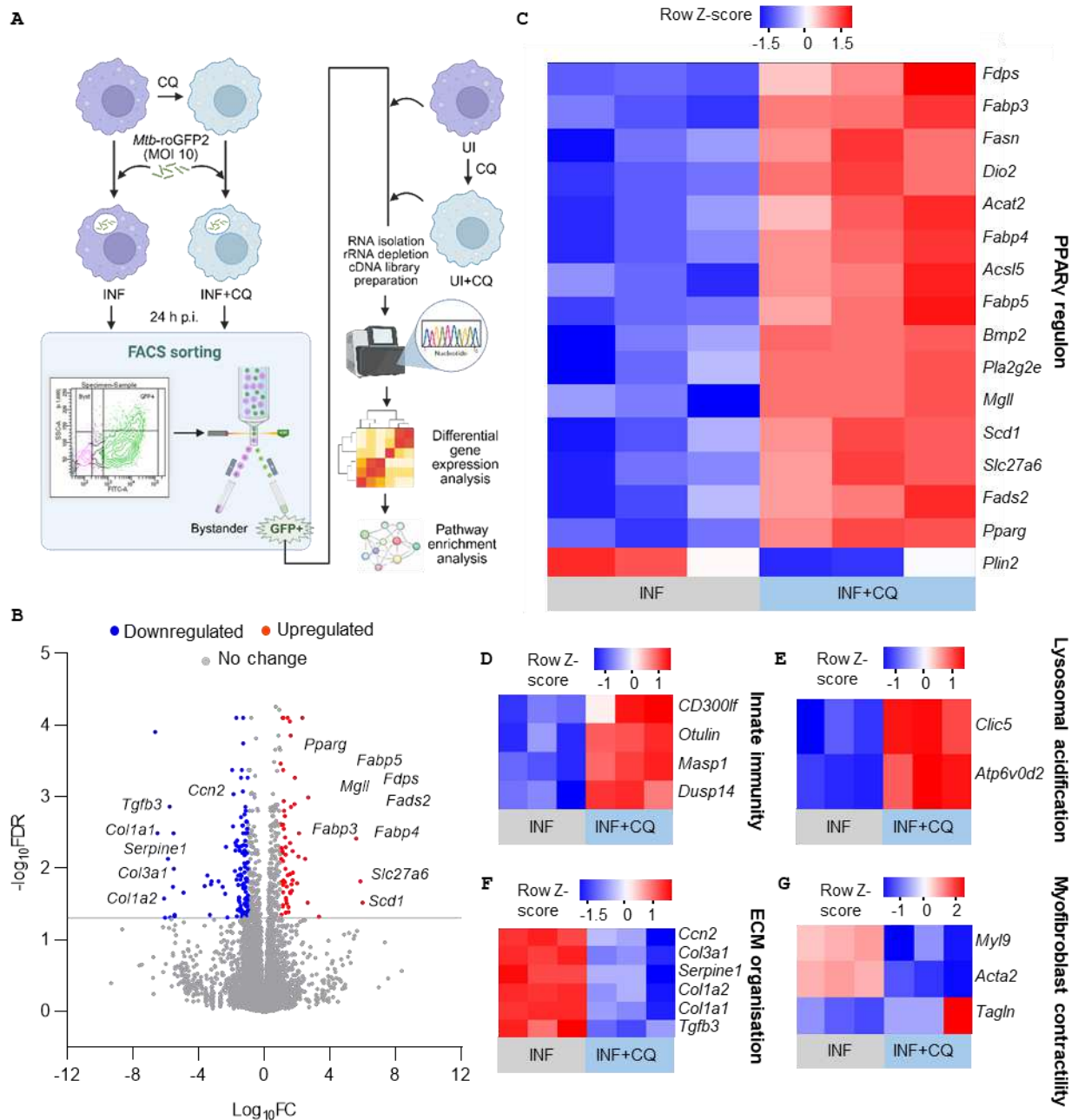


Fig. 1. Phagosomal deacidifier, CQ, induces lipid metabolism, inflammatory, and fibrotic reprogramming in *Mtb*-infected macrophages. (A) Schematic showing flow-sorting coupled RNA-seq of *Mtb*-roGFP2-infected BMDMs. Briefly, uninfected BMDMs were treated with CQ or left untreated for 24 h. For infection conditions, untreated or CQ-treated BMDMs were infected with *Mtb*-roGFP2 (MOI 10, 24 h), followed by flow sorting of GFP-positive cells from both conditions. (B) Volcano plot showing 2-fold upregulated (red) and downregulated (blue) genes in CQ-treated *Mtb*-roGFP2-infected BMDMs relative to untreated infected BMDMs. (C–G) Heatmaps showing expression

of genes associated with the PPAR γ regulon (C), innate immune signalling (D), lysosomal acidification (E), ECM organisation (F), and myofibroblast contractility (G) in CQ-treated infected BMDMs relative to untreated infected BMDMs. Heatmaps were generated from three independent experiments with $P < 0.05$ and Log2FC = 1. Abbreviations: FC, fold change; UI, Uninfected; UI+CQ, Uninfected plus CQ-treated; INF, infected; INF+CQ, infected plus CQ-treated.

Although modest in magnitude, CQ upregulated components of Peroxisome Proliferator-Activated Receptor- γ (PPAR γ)–driven lipid anabolic pathways, including sterol biosynthesis (Lss, Aacs, Acat3), fatty acid desaturation (Scd1, Fads2), lipid transport (Slco4a1), and lipid-sensing GPCR signalling (Hcar2), along with genes involved in cell-cycle progression (Fig. S5, C-D and Supplementary file 1). Consistent with its lysosomal alkalinizing activity, CQ also induced genes linked to vesicular trafficking and ion homeostasis (Mcoln3, Rph3a, Rims2, Gem) (Fig. S5E). Analysis of downregulated transcripts suggests that CQ shifts uninfected macrophages toward a less migratory and less inflammatory phenotype by reducing the expression of chemokine receptors and adhesion molecules, ECM assembly and proteolytic enzymes, pro-angiogenic and platelet degranulation–associated factors, MAPK/ERK and TNF- α signalling components, and genes associated with endosomal stress and phagocytic/clearance modules (Fig S5, F-J).

CQ treatment of *Mtb*-infected BMDMs resulted in differential expression of 189 genes (fold change > 2; FDR < 0.05) relative to untreated control (Fig. 1B and Supplementary file 1). Gene ontology enrichment analysis indicated that, in the context of infection, CQ broadens its regulatory footprint across pathways linked to lipid catabolism and remodelling, inflammatory signalling, and ECM organisation and fibrosis (Fig. S6). The most significantly enriched cluster corresponded to PPAR γ -driven immunometabolic reprogramming (Fig S6A). CQ enhanced

the expression of PPAR γ , and genes involved in fatty acid uptake and trafficking (Fabp4, Fabp5, Fabp3, Slc27a6), desaturation and lipid remodelling (Scd1, Fads2, Pla2g2e), lipolysis (Mgl1), and the cholesterol/mevalonate pathway (Fdps, Cyp51, Idi1-ps1) (Fig. 1C). In contrast, the LD-associated protein (Plin2) was down-regulated upon treatment of *Mtb*-infected BMDMs with CQ (Fig. 1C). Concomitantly, RNA-seq analysis revealed active attenuation of inflammatory signalling. CQ treatment of infected macrophages induced genes associated with negative regulation of NF- κ B, MAPK, and TLR pathways and lipid-sensing circuits (e.g., OTULIN, DUSP14, CD300lf), along with activation of the lectin complement pathway (MASP1), consistent with dampening of general inflammatory stress (Fig. 1D). Genes encoding lysosomal acidification components (Atp6v0d2, Clic5) were also upregulated, likely reflecting compensatory responses to CQ-induced lysosomal alkalinization (Fig. 1E). In parallel with activation of lipid remodelling programs, CQ repressed transcripts involved in ECM organization, collagen biosynthesis, and TGF- β -dependent fibrotic pathways, including Colla1, Colla2, Col3a1, Ccn2, Serpine1, and Tgfb3 (Fig. 1F). Genes associated with myofibroblast contractility (Acta2, Tagln, Myl9) (Fig. 1G), integrin-mediated adhesion, angiogenic signalling, and developmental transcriptional programs were similarly downregulated (Supplementary file 1).

These findings are consistent with prior reports demonstrating CQ-mediated modulation of TGF- β /CTGF signalling, α -SMA expression, and PPAR γ -linked lipid reprogramming in attenuating fibrosis and collagen deposition in lung injury models (36,37). Given that collagen-rich fibrotic niches are hypoxic and enriched in host-derived lipids, both of which influence *Mtb* redox physiology, drug permeation, and drug tolerance (19,38), our transcriptional data support a model in which phagosomal deacidification by CQ likely suppresses drug tolerance by recalibrating host lipid metabolism and restraining profibrotic programs characteristic of chronic granulomatous inflammation.

Phagosomal acidification enhances, whereas deacidification reduces, lipid droplet accumulation in *Mtb*-infected macrophages

Our transcriptomic analysis implicates PPAR γ and lipid metabolism as mediators of pH-dependent modulation of redox heterogeneity and drug tolerance in intracellular *Mtb*. Because PPAR γ is a master regulator of LD biogenesis and lipid handling (39–41), we next examined the impact of deacidification by CQ on LD. LD consist predominantly of triacylglycerols (TAGs) and cholesteryl esters surrounded by a phospholipid monolayer (42,43). Neutral lipids were detected using LipidTOX Deep Red staining and quantified by flow cytometry and confocal microscopy (Fig. 2A). In uninfected BMDMs, CQ treatment significantly increased LD accumulation, consistent with RNA-seq data showing induction of lipogenic genes (*Fasn*, *Fads2*, *Scd1*), the LD-associated protein *Plin2*, and the cholesterol biosynthesis enzyme *Fdps* (Fig. 2, B and E). *Mtb* infection similarly increased LD content in BMDMs, accompanied by elevated *Plin2* expression and reduced expression of lipid catabolic genes, including *Fabp5*, and *Mgll* (Fig. 2, C and E). Notably, CQ treatment significantly reversed these infection-induced metabolic alterations. LD accumulation was reduced approximately twofold, together with repression of *Plin2* (Fig. 2, D-E). In parallel, CQ derepressed *Ppar γ* and lipogenic gene expression while also upregulating lipid catabolic genes, *Fabp5* and *Mgll*, relative to untreated *Mtb*-infected BMDMs (Fig. 2, D and E). Confocal microscopy corroborated that CQ treatment had an opposite effect on LD accumulation in *Mtb*-infected macrophages relative to untreated *Mtb*-infected macrophages (Fig. 2, F-H).

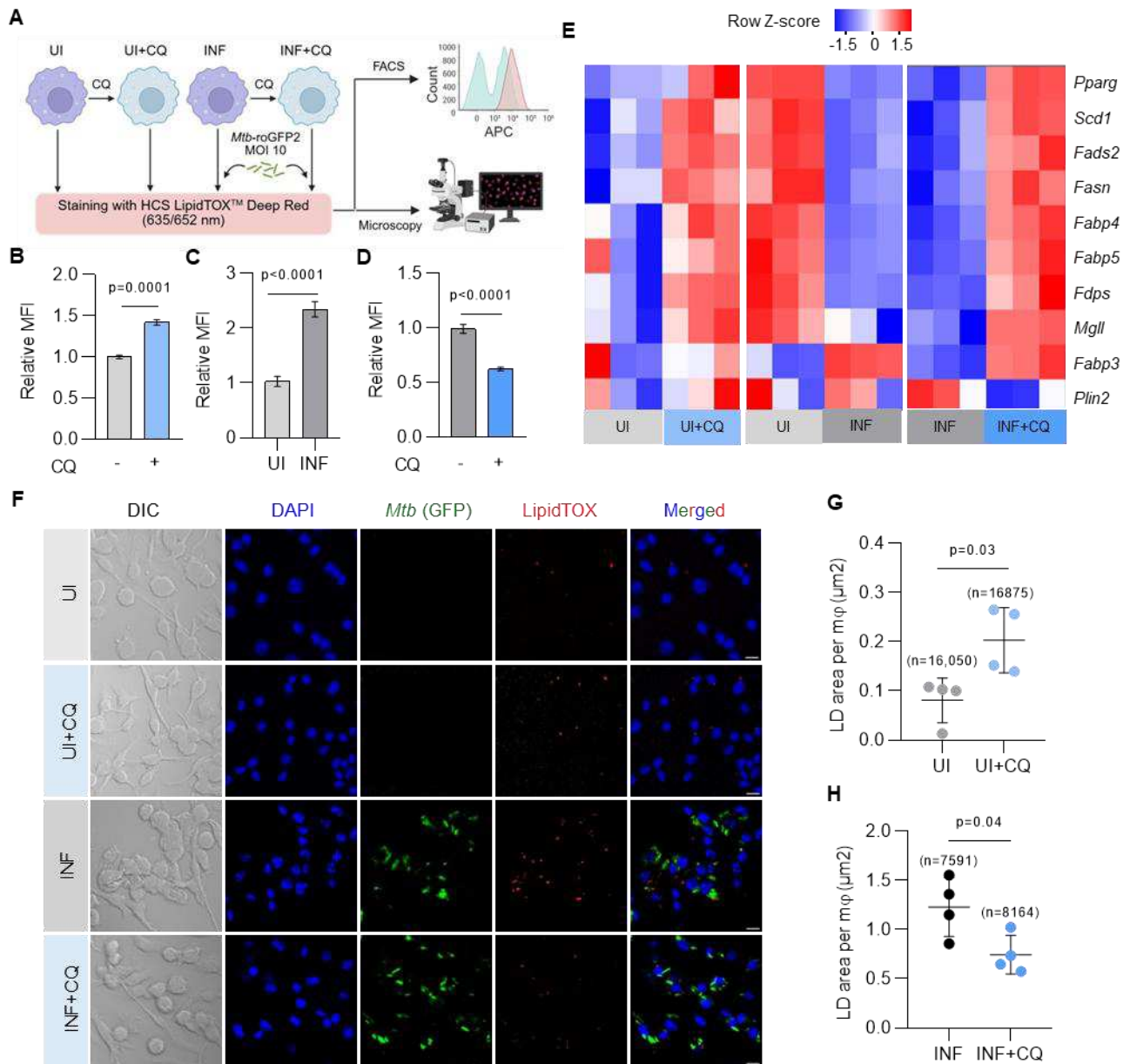


Fig. 2. *Mtb* infection induces and phagosomal deacidification reduces LD accumulation in BMDMs. (A) Schematic of LipidTOX staining of BMDMs under various conditions for flow cytometry and confocal microscopic analysis. (B–D) Flow cytometry quantification of relative LipidTOX mean fluorescence intensity (MFI) comparing uninfected (UI) and uninfected treated with chloroquine (UI+CQ). (B), UI and *Mtb*-infected (INF) (C), and INF and *Mtb*-infected treated with chloroquine (INF+CQ) (D) at 48 h pi. (E) Heatmaps comparing the DEGs belonging to the PPAR γ regulon in UI and UI+CQ; UI and INF; and INF and INF+CQ groups. (F) Representative confocal images of LipidTOX-stained UI, UI+CQ, INF, and INF+CQ BMDMs at 24 h.p.i. Scale bars represent 10 μ m. (G–

H) Quantification of LD area per macrophage comparing **(G)** UI and UI+CQ and **(H)** INF and INF+CQ. Each data point represents the average LD area/macrophage from one experiment, and “n” indicates the total number of cells examined in all four experiments. The *p*-values were obtained using the Mann–Whitney test. Data shown are the results of three independent experiments performed in triplicate (means $\pm$ SD). *Abbreviations: UI, uninfected BMDMs; UI+CQ, CQ-treated uninfected BMDMs; INF, Mtb-roGFP2-infected BMDMs; INF+CQ, CQ-treated infected BMDMs; LTOX, LipidTOX Deep Red; mφ, macrophage.*

Our data reveal an inverse relationship between LD abundance and PPAR γ -associated transcriptional changes in lipid metabolic genes. Despite the established role of PPAR γ in lipid storage, *Mtb* infection increased LD accumulation while repressing PPAR γ and its downstream targets, indicating that infection-induced LD formation could be driven by pH and infection-dependent mechanisms rather than a canonical PPAR γ program. This is consistent with CQ-mediated reduction in LD content along with the derepression of PPAR γ -dependent genes in our RNA-seq data. To understand the relationship between PPAR γ activity, CQ, and drug tolerance, we pharmacologically modulated PPAR γ using an antagonist, GW9662 and an agonist, rosiglitazone (44,45). As expected, rosiglitazone increased, whereas GW9662 reduced LD accumulation in *Mtb*-infected BMDMs (Fig. S7, A-B) (46,47). However, these changes in LD did not correlate with drug tolerance as rosiglitazone marginally decreased MXF tolerance, whereas GW9662 had no effect (Fig. S7, C-D). Importantly, CQ uniformly suppressed both LD build-up and MXF tolerance irrespective of PPAR γ activation or inhibition (Fig. S7, C-D), suggesting that phagosomal deacidification acts upstream of, or in parallel to, PPAR γ -dependent lipid metabolic pathways. These findings indicate that phagosomal pH-dependent regulation of host LD biogenesis, rather than PPAR γ activity alone, might be a dominant determinant of intracellular drug tolerance. Agreeing to this, maturation and acidification of

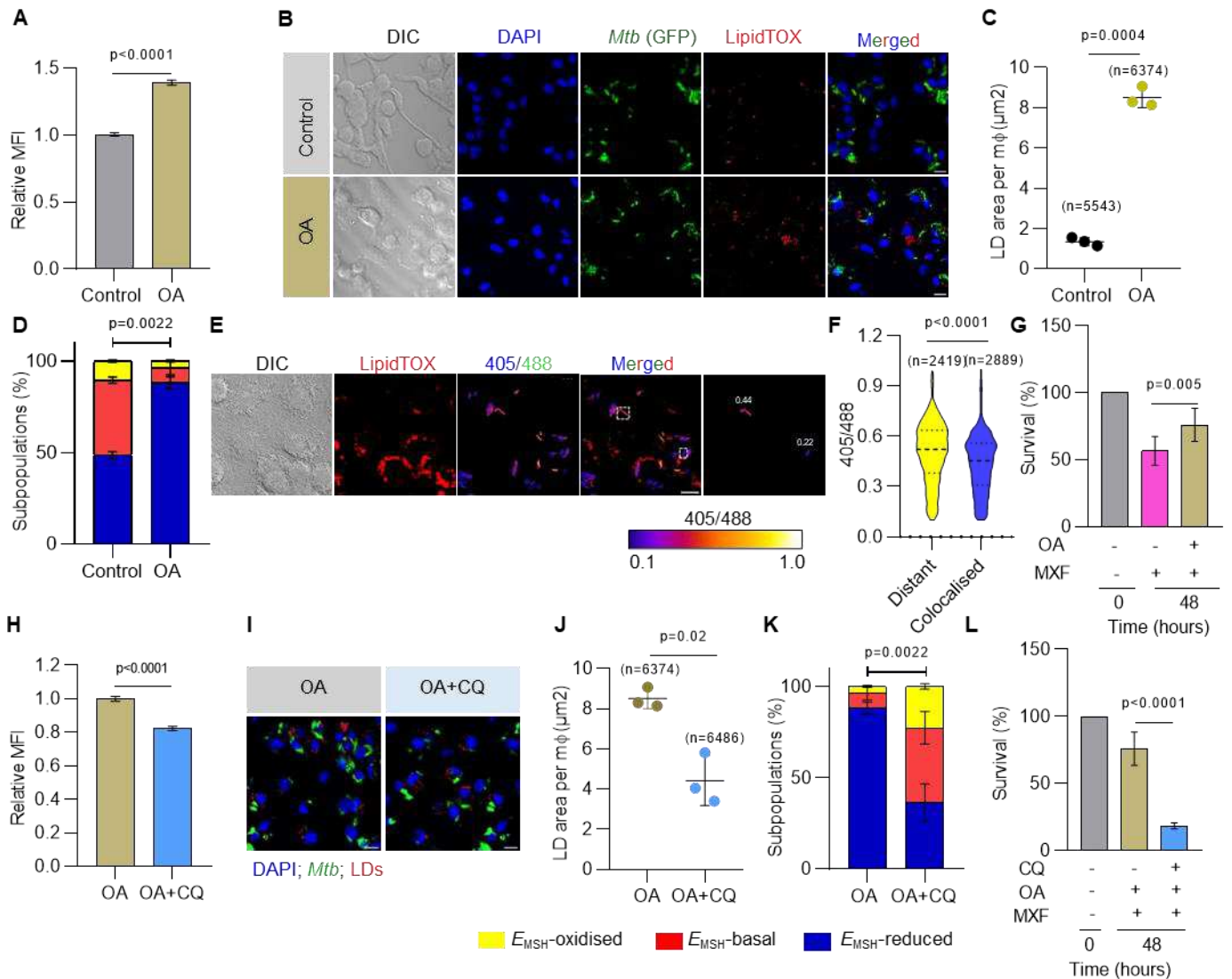
pathogen-containing phagosomes occur in parallel with formation of lipid bodies (LBs), and LDs are promptly recruited to mycobacterial phagosomes (22,47–51). We next examined whether phagosomal maturation influences LD biogenesis during *Mtb* infection. Consistent with this, inhibition of phagosomal maturation via deacidification using bafilomycin A1 (BafA1; an H⁺-ATPase inhibitor) or ammonium chloride (NH₄Cl; a lysosomotropic weak base) significantly reduced LD accumulation in *Mtb*-infected BMDMs (Fig. S7E). Both BafA1 and NH₄Cl are known to diminish *E*_{MSH}-reduced subpopulation and drug tolerance in intracellular *Mtb* (25). The data establish that phagosomal pH itself, not a drug (CQ)-specific effect, as the key determinant of LD accumulation and downstream redox-dependent antibiotic tolerance.

Phagosomal alkalinization impairs redox-dependent drug tolerance by disrupting lipid acquisition by intracellular *Mtb*

Acidic pH imposes metabolic and redox stress on *Mtb*, driving adaptive remodelling of bacterial metabolism, including increased reliance on host-derived fatty acids (26,38,52,53). Based on this framework, we hypothesised that CQ-mediated phagosomal alkalinization and the associated reduction in LD accumulation would impair lipid acquisition by intracellular *Mtb*, thereby limiting reductive metabolism and suppressing drug tolerance. We systematically tested this model.

We first tested whether increasing host lipid availability alters intrabacterial redox state and drug tolerance. Exogenous oleic acid (OA) is well established to induce LD accumulation in infected macrophages, thereby providing a nutrient reservoir for intracellular *Mtb* (54,55). Supplementation of *Mtb*-infected BMDMs with 50 μM OA robustly increased LD formation, as quantified by LipidTOX staining, followed by flow cytometry (Fig. 3A), or confocal microscopy (Fig. 3, B-C). Importantly, OA supplementation increased the proportion of macrophages harbouring *E*_{MSH}-reduced *Mtb* from ~50% to ~90% (Fig. 3D). To strengthen the

association between OA-induced LD accumulation and reductive E_{MSH} , we examined the spatial relationship between intracellular Mtb-roGFP2 and host LD. *Mtb* located in close proximity to LD exhibited a lower 405/488 biosensor ratio, indicative of a more reduced E_{MSH} , whereas bacteria positioned away from LD displayed comparatively higher ratios consistent with a more oxidised E_{MSH} state (Fig. 3, E-F). OA-driven enrichment of LD and close association with reductive- E_{MSH} bacteria were accompanied by a ~50% increase in MXF tolerance ($3\times$ MIC), with survival increasing from ~50% in OA-untreated BMDMs to ~75% in OA-treated BMDMs (Fig. 3G). CQ pre-treatment significantly reversed OA-induced LD accumulation, reductive shift, and MXF tolerance (Fig. 3, H-L).



312

313 **Fig. 3. OA-induced LD accumulation resulted in reductive shift and MFX tolerance in**

314 **intracellular *Mtb*, reversed by CQ. (A-D) Untreated (Control) or OA-treated BMDMs were infected**

315 **with *Mtb*-roGFP2 (MOI 10, 24 h) and stained with LipidTOX. (A) Flow cytometry quantification of**

316 **relative LipidTOX MFI of Control and OA-treated BMDMs. (B) Representative confocal images of**

317 **Control and OA-treated BMDMs and (C) associated quantification of LD area per macrophage. Each**

318 **data point represents the average LD area/macrophage from one experiment, and “n” = total number of**

319 **cells analysed in all three experiments. (D) Quantification of redox-diverse *Mtb* subpopulation by flow**

320 **cytometry, comparing the E_{MSH} -reduced population in Control and OA-treated BMDMs. (E-F) OA-**

321 **treated *Mtb*-roGFP2-infected BMDMs were analysed by confocal microscopy to assess the ratiometric**

322 **response of bacilli distant or colocalised to LD. Representative false-coloured images (E). For clarity,**

the final panel shows two representative *Mtb* cells: an LD-associated bacterium with a lower 405/488 ratio and a distal bacterium with a higher ratio. **(F)** Quantification of confocal images of bacillary 405/488 ratios is shown (n= total number of *Mtb*-roGFP2 cells analysed). **(G)** Percent survival of *Mtb* H37Rv exposed to 3X MIC of MXF for 48 h in Control or OA-treated BMDMs. **(H-K)** OA-treated BMDMs were further left untreated or treated with CQ, infected with *Mtb*-roGFP2 (MOI 10, 24 h) and stained with LipidTOX. **(H)** Flow cytometry quantification of relative LipidTOX MFI of OA and OA+CQ-treated BMDMs. **(I)** Representative confocal images of OA and OA+CQ-treated BMDMs and **(J)** associated quantification of LD area per macrophage. Each data point represents the average LD area/macrophage from one experiment, and “n” = total number of cells analysed in all three experiments. **(K)** Quantification of redox-diverse *Mtb* subpopulation by flow cytometry in OA and OA+CQ-treated BMDMs. **(L)** Percent survival of *Mtb* H37Rv exposed to 3X MIC of MXF for 48 h in OA and OA+CQ-treated BMDMs. Scale bars represent 10 μ m. Data shown are the results of three independent experiments performed in triplicate (means $\pm$ SD). The *p*-values were obtained by the Mann-Whitney test.

Since acidic phagosomal conditions and the macrophage milieu are known to facilitate *Mtb* adaptation to host lipids, including the uptake and catabolism of OA (38,56,57), we reasoned that CQ-mediated alkalization might disrupt fatty acid acquisition by *Mtb*, thereby altering intrabacterial redox homeostasis and suppressing drug tolerance. We next directly examined fatty acid import by intracellular *Mtb*. BMDMs were infected with tdTomato-expressing *Mtb* and subsequently pulsed with the fluorescent fatty acid analogue BODIPY FL-C16 in the presence or absence of CQ. Uptake of BODIPY FL-C16 by intracellular bacilli was quantified by flow cytometry (Fig. 4A) (58). Compared with untreated -infected BMDMs, CQ treatment reduced BODIPY FL-C16 uptake by approximately 40% (Fig. 4B), indicating that CQ impairs the acquisition of fatty acids by intracellular *Mtb*.

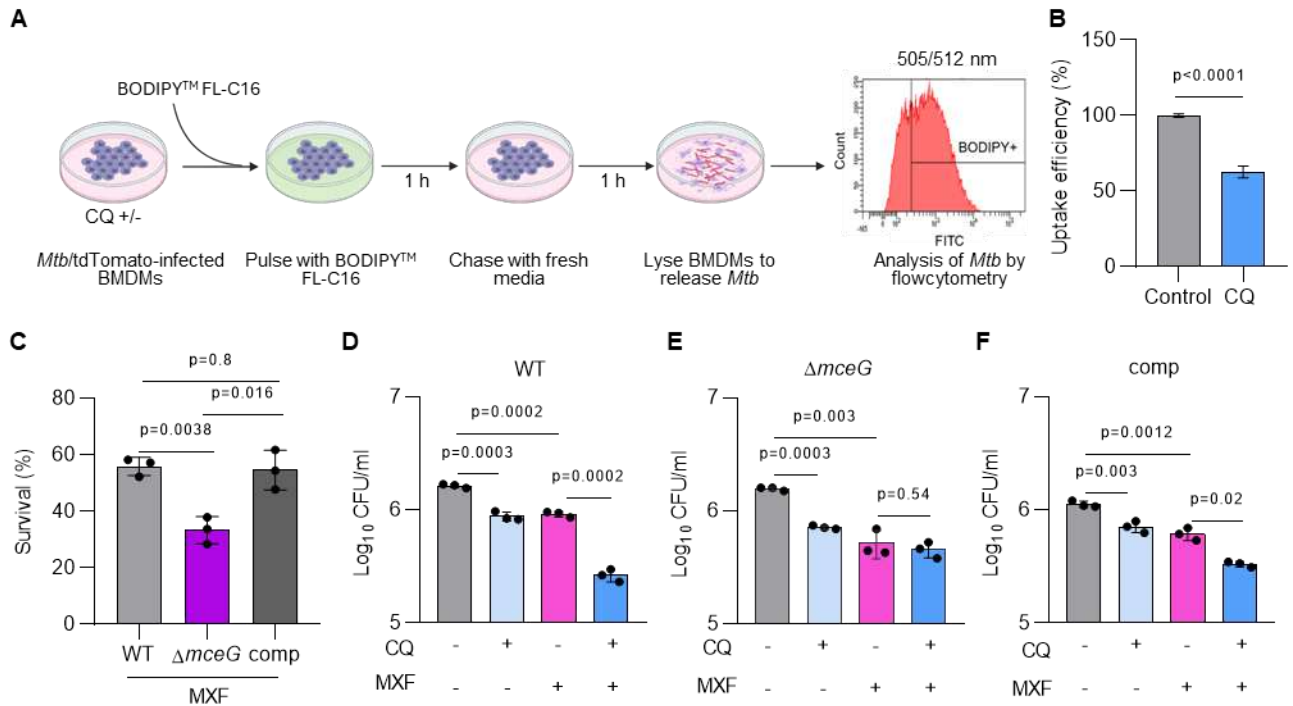


Fig. 4. Redox- and pH-dependent drug tolerance is mediated by increased lipid acquisition by intracellular *Mtb*. (A) Schematic of the BODIPYTM FL-C16 uptake assay in *Mtb* H37Rv/tdTomato-infected BMDMs. (B) Quantification of BODIPY FL-C16 uptake by intracellular *Mtb* H37Rv/tdTomato. MFI from *Mtb* recovered from untreated BMDMs (Control) was set to 100%, and uptake in CQ-treated samples was normalised to Control and expressed as uptake efficiency. (C) Percent survival of *Mtb* Erdman (WT), $\Delta mceG$, and complemented strains (comp) exposed to 3X MIC of MXF for 48 h in BMDMs. (D–E) CFU of WT, $\Delta mceG$, and comp strains in CQ-treated or untreated BMDMs following 3X MIC MXF exposure for 48 h. Data are expressed as mean $\pm$ S.D. of three biological replicates done in triplicate. The *p*-value was determined using a Student's two-tailed *t*-test with Welch's correction.

OA is a highly reduced carbon source; its β -oxidation and subsequent entry into the TCA cycle generate 35 NADH equivalents per molecule of OA (59). The OA-induced expansion of the *EMSH*-reduced population and increased drug tolerance, therefore, likely reflect

efficient fatty acid uptake and catabolism within macrophages. We predicted that genetic impairment of lipid import would reduce drug tolerance inside macrophages. To test this, we employed an *Mtb* Erdman $\Delta mceG$ mutant. MceG is an ATPase required for stabilisation of the Mce1 and Mce4 lipid transport complexes that mediate fatty acid and cholesterol uptake (60). The $\Delta mceG$ mutant exhibited ~40% lower MXF tolerance inside BMDMs compared to wild-type and complemented strains (Fig. 4C), without any difference in MXF MIC in the axenic 7H9 medium (Fig. S8). Importantly, CQ reduced MXF tolerance in MceG-sufficient strains but had no additional effect on the $\Delta mceG$ mutant (Fig. 4, D-F). The inability of CQ to further reduce tolerance in the $\Delta mceG$ mutant suggests that CQ suppresses tolerance primarily by limiting bacterial lipid acquisition.

To isolate the effect of acidic pH on lipid-driven redox remodelling, we examined *Mtb* metabolism in axenic 7H9 medium (Fig. S9A). *Mtb* preferentially catabolizes OA under acidic conditions (38). Using the Mrx1-roGFP2 biosensor, OA supplementation induced a concentration-dependent decrease in the biosensor ratio at pH 4.5 and 5.5 compared to neutral pH (7.4), consistent with enhanced reductive stress (Fig. S9B). Correspondingly, *Mtb* cultured in OA-supplemented medium at pH 5.5 and pH 4.5 displayed nearly 2000- and 3000- fold more survival than OA-supplemented pH 7.4, respectively, after exposure to 10 $\times$ MIC of MXF for 5 days (Fig. S9C). Collectively, these findings support a model in which phagosomal pH–lipid import–redox axis governs intracellular drug tolerance in *Mtb*. Acidic phagosomes promote LD accumulation, enabling fatty acid-driven reductive metabolism that supports antibiotic tolerance, whereas CQ disrupts this pathway by alkalinising phagosomes and restricting lipid access to intracellular *Mtb*.

***Mtb* WhiB6 is linked to pH-dependent LD accumulation and changes in E_{MSH} redox state.**

387 To define the transcriptional consequences of CQ-induced lipid deprivation on *Mtb*
388 within macrophages, we performed RNA-seq on *Mtb* isolated from CQ-treated and PBS-
389 treated BMDMs at 24 h post-infection (Fig. 5A). Differential expression analysis ($\text{FDR} \leq 0.05$,
390 fold change > 1.5) identified a relatively restricted transcriptional response comprising 21
391 significantly altered genes (18 upregulated, 3 downregulated), consistent with a host-directed
392 effect of CQ resulting in a targeted adaptive calibration of *Mtb* (Fig. 5, B and C). Among the
393 upregulated genes were those associated with cholesterol metabolism and fatty acid oxidation
394 (Rv3160c, Rv3161c, PPE37, acg) and fatty acid transport (Mce3 operon components,
395 Rv1773c) (61–66) (Fig. 5C). The induction of these loci suggests a compensatory response
396 aimed at enhancing lipid scavenging and catabolism in the context of CQ-mediated lipid
397 restriction. However, the limited breadth of transcriptional remodelling indicates that this
398 response is constrained, consistent with lipid deprivation.

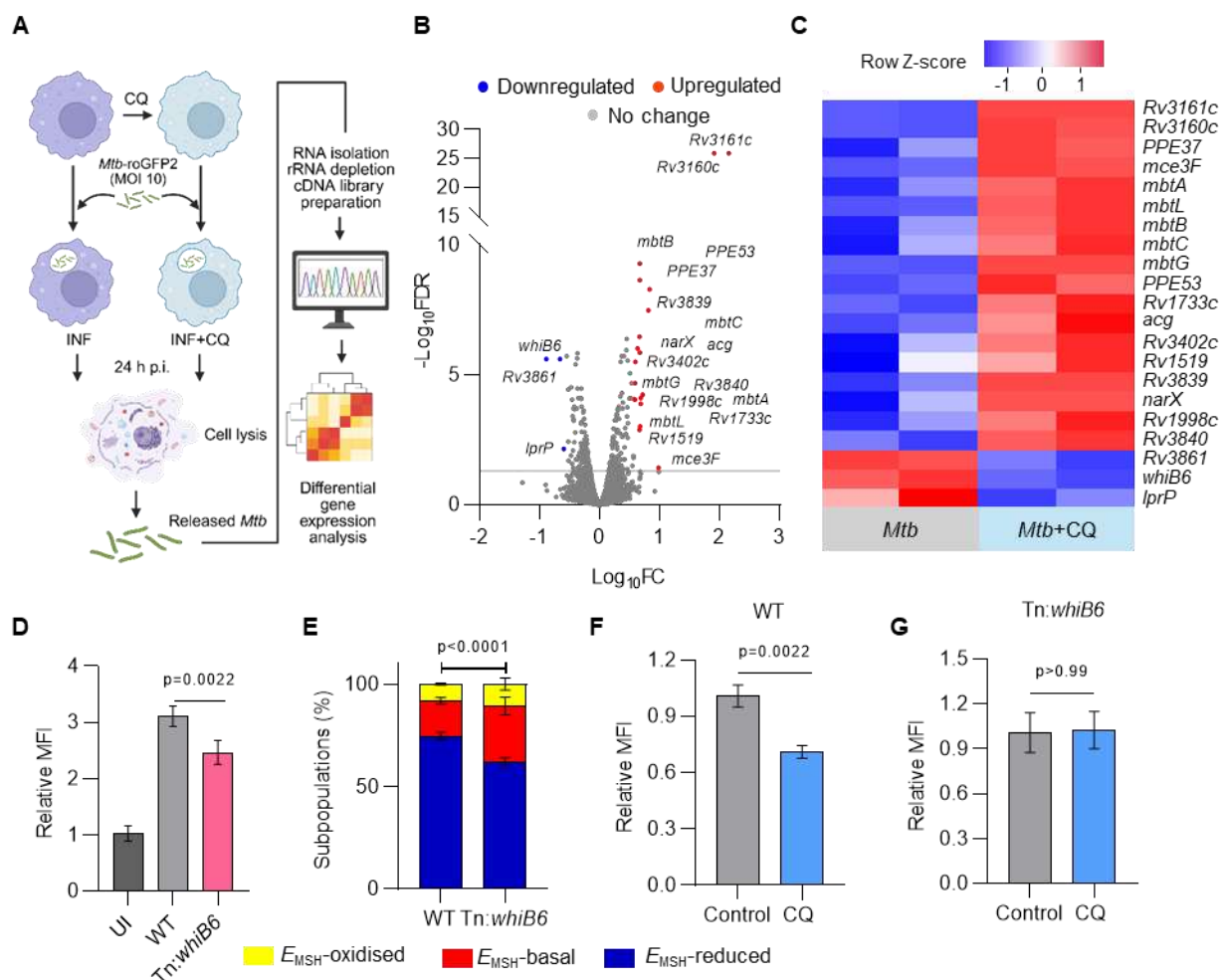


Fig. 5. RNA-seq of intracellular *Mtb* identifies *WhiB6* as a potential regulator of redox and LDs.

(A) Experimental scheme for RNA-seq of intracellular *Mtb* from BMDMs with and without CQ treatment. (B) Volcano plot showing 1.5-fold upregulated (red) and downregulated (blue) genes in intracellular *Mtb* from CQ-treated BMDMs compared to untreated BMDMs. (C) Heatmap of DEGs in *Mtb* isolated from untreated (*Mtb*) or CQ-treated (*Mtb*+CQ) BMDMs (FDR <0.05, Log₂FC >0.6). (D) Flow cytometry quantification of relative LipidTOX MFI of uninfected BMDMs (UI) and BMDMs infected with *Mtb* CDC1551 wild-type (WT) or transposon mutant of *whiB6* (Tn:*whiB6*) strains (MOI 10, 24 h). (E) BMDMs were infected with Mrx1-roGFP2 strains of WT or Tn:*whiB6* (MOI 10, 24 h) and the redox-diverse *Mtb* subpopulation was quantified by flow cytometry, comparing the *E*_{MSH}-reduced population of WT and Tn:*whiB6*. (F-G) Flow-cytometry quantification of relative LipidTOX MFI of untreated (Control) and CQ-treated BMDMs infected with either WT (F) or Tn:*whiB6* (G) *Mtb*

strains (MOI 10, 24 h). Data shown are the results of two independent experiments performed in triplicate (means $\pm$ SD). The *p*-values were obtained using the Mann-Whitney test.

We also observed consistent induction of genes involved in mycobactin biosynthesis (Fig. 5C), indicative of iron limitation. CQ-mediated alkalinization of the phagosome is known to impair iron release from host iron-binding proteins such as transferrin, thereby restricting iron availability to intracellular pathogens (67). CQ-induced iron dysregulation has been reported across multiple biological systems, including bacteria, fungi, endothelial cells, and macrophages (67–71). Importantly, mycobactin biosynthesis requires long-chain fatty acyl precursors (C16–C22) (72). Thus, upregulation of mycobactin synthesis genes under CQ treatment likely reflects dual stress: iron deprivation and reduced availability of host-derived lipid substrates required for siderophore maturation. This reinforces the notion that CQ perturbs both lipid and iron homeostasis within the phagosomal niche.

Notably, two of the three downregulated genes, *whiB6* (Rv3862c) and Rv3861 (Fig 5C), are positioned upstream of the ESX-1 secretion locus and positively regulate secretion of key virulence effectors such as EsxA (ESAT-6) and CFP-10 (28,29,73). The ESX-1 secretion system has been implicated in modulating host lipid metabolism and promoting LD biogenesis within infected macrophages (30,74). Downregulation of the *whiB6–Rv3861* locus therefore suggests attenuation of ESX-1–dependent host lipid remodelling, potentially contributing to the reduced LD accumulation observed in CQ-treated macrophages. WhiB6, in particular, functions as a pH-inducible Fe-S cluster-containing redox regulator integrating environmental cues with virulence gene expression (28,75,76). WhiB6 repression under CQ treatment may limit both LD accumulation and lipid acquisition, thereby impairing the ability of *Mtb* to sustain a reductive *E_{MSH}* state conducive to drug tolerance.

Consistent with this model, BMDMs infected with a transposon mutant of *whiB6* (Tn:*whiB6* *Mtb* CDC1551) (77) showed partial reduction LD accumulation and *E*_{MSH}-reduced fraction relative to WT *Mtb* CDC1551-infected macrophages (Fig.5D) (Fig. 5E). Notably, CQ treatment failed to further reduce LD levels in Tn:*whiB6*-infected BMDMs (Fig. 5, F and G). Collectively, the transcriptional data indicate that CQ disrupts phagosomal acidification, thereby depriving *Mtb* of host-derived lipids and iron, two critical determinants of intracellular redox balance and antibiotic tolerance. Through coordinated effects on LD availability and the WhiB6 pathway, phagosomal alkalization constrains redox adaptation and suppresses drug tolerance during infection.

Phagosomal deacidification reduces redox-dependent MXF tolerance in a clinical MDR-TB strain by recalibrating LD formation

Genetically diverse clinical isolates of *Mtb* differ substantially from the laboratory strain H37Rv in their ability to modulate host immunity and respond to antibiotics (78,79). MXF is a core component of both drug-sensitive and drug-resistant TB regimens and retains activity against many MDR-TB strains (31,32). To determine whether phagosomal pH-driven MXF tolerance extends beyond H37Rv, we evaluated a clinically isolated MDR strain, NHN1664.

NHN1664 is resistant to first-line drugs: isoniazid (Inh), rifampicin (Rif), ethambutol (Emb), and streptomycin (Str) (80), but susceptible to MXF (34) which was confirmed by phenotypic drug susceptibility testing (Fig. S10). Whole-genome sequencing (WGS) of NHN1664 revealed a genome size of approximately 4.2 Mbp (Fig. 6A). Phylogenetic analysis classified NHN1664 as lineage 2, sublineage L2.2.1 (East Asian/Beijing genotype), distinct from H37Rv (lineage 4; Euro-American) (Fig. 6B). Variant analysis identified canonical

resistance-conferring mutations corresponding to the observed drug resistance phenotype (Table S1).

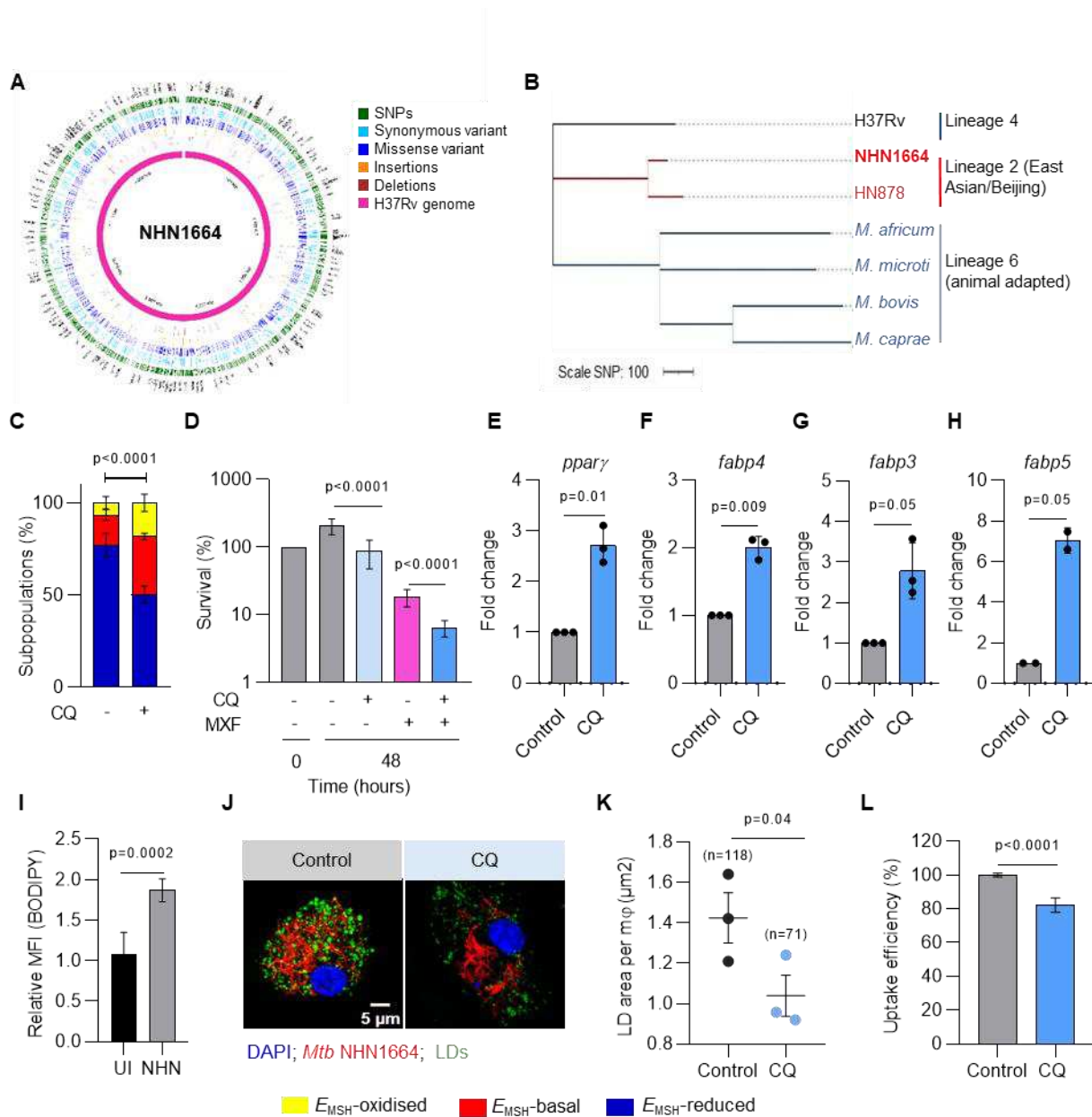


Fig. 6. Phagosomal alkalization reduces LD accumulation and MXF tolerance in a clinical MDR-TB strain. (A–B) Circos plot (A) and phylogenetic analyses (B) of the *Mtb* NHN1664 genome relative to the genome of *Mtb* H37Rv. (C) Quantification of redox-diverse *Mtb* subpopulation by flow cytometry in untreated or CQ-treated BMDMs infected with *Mtb* NHN1664-roGFP2 (MOI 10, 24 h), comparing the E_{MSH} -reduced population in untreated and CQ-treated BMDMs. (D) Percent survival of

Mtb NHN1664 exposed to 3X MIC of MXF for 48 h in Control and CQ-treated BMDMs. **(E–H)** qRT-PCR analysis of indicated host genes in untreated (Control) and CQ-treated BMDMs infected with *Mtb* NHN1664-roGFP2 (MOI 10, 24 h) and flow-sorted. Fold change was normalised to β -actin. **(I)** Flow cytometry quantification of relative BODIPY 493/503 MFI of BMDMs that were uninfected or infected with *Mtb* NHN1664/tdTomato (MOI 10, 48 h). **(J)** Representative confocal images of untreated (Control) or CQ-treated BMDMs, infected with NHN1664/tdTomato (MOI 4, 48 h) and stained with BODIPY 493/503 and **(K)** associated quantification of LD area per macrophage. Each data point represents the average LD area/macrophage from one experiment, and “n” = total number of cells analysed in all three experiments. **(L)** Quantification of BODIPY FL-C16 import by intracellular *Mtb* NHN1664/tdTomato isolated from CQ-treated BMDMs. Uptake efficiency was measured as described in the legend for Figure 4. Data shown are the results of three independent experiments performed in triplicate (means $\pm$ SD). The *p*-values were obtained by the Mann-Whitney test.

Functionally, NHN1664 displayed redox heterogeneity and MXF tolerance within BMDMs (Fig. 6, C and D). Approximately 20% of intracellular NHN1664 bacilli survived 24 h exposure to MXF at 3 $\times$ MIC (Fig. 6D). Importantly, CQ treatment of infected BMDMs significantly reduced the *E*_{MSH}-reduced subpopulation and abolished MXF tolerance in a concentration-dependent manner (Fig. 6, C and D), demonstrating that CQ effectively disrupts redox-dependent tolerance in a clinically relevant MDR background.

We next assessed whether phagosomal deacidification by CQ similarly modulates host lipid metabolism during NHN1664 infection. As observed with H37Rv, CQ induced expression of genes involved in fatty acid metabolism in infected macrophages (Fig. 6, E-H). Flow cytometric analysis and confocal microscopy of BODIPY-stained cells confirmed that NHN1664 infection promoted robust LD accumulation in BMDMs (Fig. 6, I-K), whereas CQ treatment markedly reduced LD content (Fig. 6, J-K). Furthermore, CQ impaired uptake of the

fluorescent fatty acid analogue BODIPY FL-C16 by intracellular NHN1664 (Fig. 6L), indicating reduced lipid access to intracellular bacteria. Collectively, these findings demonstrate that alkalization by CQ uniformly disrupts host LD biogenesis and fatty acid availability, thereby limiting the ability of genetically distinct *Mtb* strains to adopt a reductive redox state required for MXF tolerance.

CQ marginally improved MXF efficacy in the BALB/c mouse model

To determine whether CQ-mediated enhancement of MXF activity extends *in vivo*, we employed a chronic aerosol infection model using BALB/c mice. Mice were infected with ~100 CFU of the clinical MDR strain NHN1664 and, at 4 weeks post-infection (p.i.), were treated with MXF (25 mg/kg), CQ (10 mg/kg), or the combination of MXF and CQ. Lung bacterial burdens were quantified at 4, 6, 8, 10, and 12 weeks of treatment (Fig. S11A).

Unlike H37Rv, NHN1664 did not establish a stable persistent plateau by week 4 p.i. Instead, lung CFU declined progressively, approximately 10³-fold between weeks 4 and 12 p.i. (Fig. S11B), consistent with lineage-dependent differences in virulence and host immune modulation reported for clinical isolates (78,79). This dynamic was mirrored in treatment response. MXF monotherapy reduced lung CFU from ~10⁶ to ~10⁴ after 4 weeks of treatment (8 weeks p.i.) and further to ~10² after 8 weeks of treatment (12 weeks p.i.) (Fig. S11B). CQ monotherapy did not significantly affect bacterial burden compared to untreated controls (Fig. S11B). The MXF+CQ combination therapy showed a modest additional ~5-fold reduction in lung CFU at 6 and 8 weeks of treatment compared with MXF alone (Fig. S11B). Given our macrophage data showing that CQ increases vacuolar pH and reduces LD accumulation, we assessed these parameters directly in lung cells. Total lung cells isolated from CQ-treated BALB/c mice were stained with Magic Red cathepsin B substrate, which fluoresces upon cleavage in acidified lysosomes, and BODIPY 493/503 to quantify LDs. CQ treatment reduced

lysosomal acidification and decreased LD content after 8 weeks of therapy compared with untreated controls (Fig. S11, C-D).

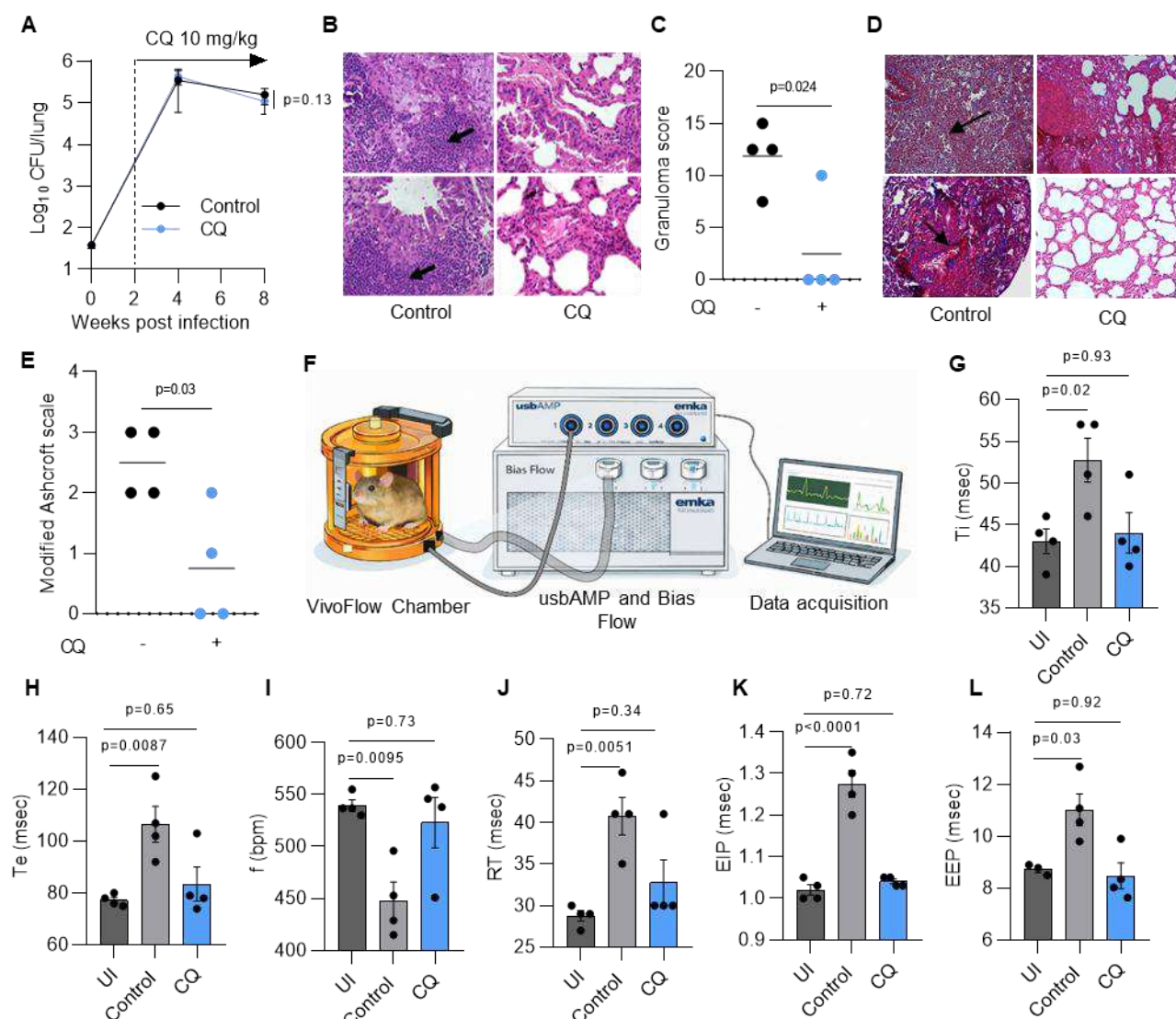
CQ attenuates lung fibrosis, improves respiratory function, and significantly potentiates MXF efficacy in the C3HeB/FeJ model

Because BALB/c mice develop predominantly cellular, non-necrotic granulomas that permit efficient drug penetration, we next evaluated CQ efficacy in the C3HeB/FeJ model, which develops caseating necrotic granulomas with severe pulmonary dysfunction (81,82). Our RNA-seq analysis demonstrated significant downregulation of pro-fibrotic pathways, including TGF- β signalling and extracellular matrix (ECM) programs, following CQ treatment in BMDMs. We therefore investigated whether CQ treatment is sufficient to mitigate lung fibrosis and restore pulmonary function in NHN1664-infected mice. C3HeB/FeJ mice were aerosol-infected with NHN1664, and CQ treatment was initiated at 2 weeks p.i. and continued until week 8. In contrast to BALB/c, NHN1664 established a persistent infection in C3HeB/FeJ animals by 4 weeks p.i. (Fig. 7A). CQ alone had minimal impact on lung bacillary burden (~1.4-fold; $p = 0.32$) (Fig. 7A). After 4 weeks of treatment, H&E staining of lung sections of untreated mice exhibited extensive pulmonary damage (histopathology score ~12), while CQ produced striking attenuation (score ~2) (Fig. 7, B-C). Fibrotic remodelling was assessed using Masson's Trichrome staining and quantified by the modified Ashcroft scoring system (83). Infected lungs exhibited prominent collagen deposition and well-defined fibrotic lesions (Ashcroft score ~3) at 8 weeks post-infection (p.i.) (Fig 7, D-E). Strikingly, CQ treatment decreased collagen accumulation, reducing fibrosis to near-baseline levels (Ashcroft score = 0.75) (Fig. 7, D-E).

To determine the functional consequences of these structural changes, we evaluated respiratory mechanics using unrestrained whole-body plethysmography (WBP) at 8 weeks p.i.

(Fig. 7F). NHN1664-infected mice displayed severe respiratory dysfunction characterised by prolonged inspiratory (T_i) and expiratory times (T_e), reduced breathing frequency (f , breaths per minute), impaired elastic recoil, and extended inspiratory (EIP)/expiratory pauses (EEP), all consistent with restrictive fibrotic lung disease (Fig. 7, G-L). Consistent with the histopathology and fibrosis findings, CQ treatment significantly improved respiratory parameters to near-baseline levels (Fig. 7, G-L). Altogether, CQ treatment reduced pathological manifestations of TB without significantly altering bacterial burden, consistent with a host-directed mechanism that limits tissue damage independently of bacillary control.

547



548

549 **Fig. 7. CQ decreases fibrosis and improves lung function of *Mtb* NHN1664-infected C3HeB/FeJ**

550 **mice.** C3HeB/FeJ mice were aerosol-infected with *Mtb* NHN1664 and treated with CQ (10 mg/kg) from

551 2 weeks p.i. till 8 weeks p.i. **(A)** Lung bacterial burden of Control ($n=8$) and CQ ($n=8$) groups were

552 quantified by CFU enumeration at 8 weeks p.i. **(B)** Representative H&E-stained lung section (8 weeks

553 p.i.) images taken at 40x magnification from infected mice of Control and CQ-treated groups; arrows

554 indicate granulomas. **(C)** Quantification of granuloma scores from lung histopathology. **(D)**

555 Representative Masson's Trichrome-stained lung section (8 weeks p.i.) images taken at 20x

556 magnification from infected mice of Control and CQ-treated groups; arrows indicate fibrotic lesions

557 stained with aniline blue. **(E)** Quantification of fibrosis using the modified Ashcroft scale. **(F)**

Schematic of the vivoFlow whole-body plethysmograph for non-invasive respiratory assessment. (G–
L) Respiratory parameters of infected C3HeB/FeJ mice measured by whole-body plethysmography.
The *p*-values were determined using the Mann-Whitney test for comparison between two groups or
one-way ANOVA with Dunnett's T3 test for multiple comparisons test for multiple comparisons.
Abbreviations: Ti, inspiratory time; Te, expiratory time; f, breathing frequency; RT, relaxation time;
EIP, end inspiratory pause; EEP, end expiratory pause.

We next examined whether CQ-mediated mitigation of lung pathology enhances
antibiotic efficacy in C3HeB/FeJ mice. NHN1664-infected animals were treated with CQ at 2
weeks p.i., and MXF treatment was started at 4 weeks p.i. and continued till 8 weeks p.i. (Fig.
8A). Similar to CQ alone, MXF monotherapy achieved only a modest non-significant reduction
in bacterial burden (~1.7-fold; $p = 0.18$) (Fig. 8B), consistent with heterogeneous
fluoroquinolone penetration into necrotic lesions (33). Strikingly, MXF+CQ combination
treatment produced an approximately 25-fold reduction in lung CFU compared with untreated
controls ($p = 0.0043$) (Fig. 8B), indicating CQ-mediated potentiation of MXF efficacy in a
pathologically stringent model.

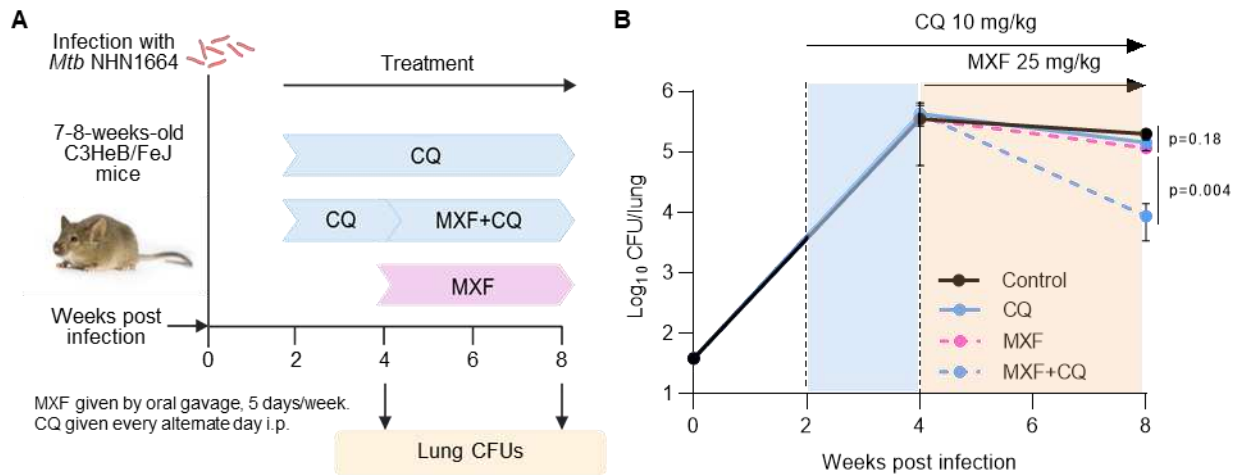


Fig. 8. CQ potentiates activity of MXF against *Mtb* NHN1664 in C3HeB/FeJ mice. (A) Experimental scheme to evaluate the efficacy of CQ in reducing MXF tolerance *in vivo*. C3HeB/FeJ mice were aerosol-infected with *Mtb* NHN1664 and treated with CQ (10 mg/kg) from 2 weeks p.i. and/or MXF (25 mg/kg) from 4 weeks p.i. Drug treatments were continued till 8 weeks p.i. **(B)** Lung bacterial burden of Control ($n=5$), CQ ($n=5$), MXF ($n=6$) and MXF+CQ ($n=6$) groups were quantified by CFU enumeration at the indicated time points. The p -values were obtained using the Mann–Whitney test.

590 DISCUSSION

591 This study identifies that phagosomal deacidification suppresses redox-dependent drug
592 tolerance in *Mtb* by rewiring macrophage lipid metabolism, inflammatory tone, and fibrotic
593 programs. By integrating redox biosensor analysis, host–pathogen dual transcriptomics, LD
594 quantification, genetic perturbation of bacterial lipid import, and validation in a necrotising TB
595 mouse model, we support a mechanistic model linking phagosomal pH, host lipid availability,
596 and antibiotic tolerance. Our previous work showed that *Mtb* localised to moderately acidified
597 phagosomes (pH 5.7) undergoes a reductive shift in E_{MSH} and exhibits tolerance to first-line
598 drugs. Here, we extend this paradigm to MXF and demonstrate that intraphagosomal pH
599 functions not simply as a stress signal but as a regulator of host lipid metabolism that shapes
600 bacterial redox state and drug susceptibility. Acidic phagosomes promote LD accumulation
601 and facilitate lipid access to intracellular *Mtb*, enabling a reductive metabolic program
602 associated with antibiotic tolerance. Phagosomal de-acidifiers such as CQ, BafA1, and NH_4Cl
603 disrupt this axis by limiting lipid availability and constraining the E_{MSH} -reduced bacterial
604 subpopulation.

605 *Mtb* infection is associated with the phagosomal acidification and LD accumulation,
606 accompanied by suppression of PPAR γ expression (25,30,84). Activation of macrophages by
607 IFN- γ further enhances phagosome–lysosome fusion and phagosomal acidification (pH ~4.5),
608 leading to further increased LD accumulation (8,84,85). The mechanisms underlying LD
609 biogenesis in activated macrophages remain incompletely understood but may involve
610 mycobacterial factors such as the terpene nucleoside 1-tuberculosinyladenosine (1-TbAd) and
611 keto mycolic acids (kMAs) (86,87). However, despite increased LD abundance, drug tolerance
612 in IFN- γ -activated macrophages arises primarily through nitric oxide (NO)-dependent
613 metabolic quiescence rather than lipid-driven reductive stress (8). Despite increased LD

abundance, *Mtb* residing within IFN- γ -activated macrophages exhibits limited access to host lipids (85) and maintains an oxidised E_{MSH} state (88). This contrasts with the reductive shift in E_{MSH} observed in naïve macrophages, where intracellular bacilli actively acquire host-derived lipids. Collectively, these findings suggest that phagosomal acidification can promote distinct tolerance states depending on the activation status of the host macrophage, resulting either in NO-dependent metabolic quiescence or lipid-dependent reductive buffering. Multiple bacterial and host factors cooperate to induce LD formation during infection (30,85–87). Our bacterial RNA-seq analysis identified WhiB6 as a potential molecular link between acidic pH, host lipid remodelling, and mycobacterial redox homeostasis during infection. WhiB6 is a major regulator of ESX-1 secretion and associated virulence programs controlled by the pH-responsive PhoP/R system (28–30,73,74). Notably, ESX-1-dependent host cell remodelling has been shown to promote LD biogenesis in *Mtb*-infected macrophages, increasing the availability of host lipids to intracellular bacilli (22,30). In parallel, WhiB6 regulates expression of the Dos dormancy regulon, a pathway implicated in reductive stress, persistence, and drug tolerance (28,89). We therefore propose that acidification-induced WhiB6 coordinates two complementary processes: ESX-1-driven LD formation that enhances host lipid availability and Dos regulon-mediated redox adaptation that promotes reductive buffering. Together, these pathways may contribute to the emergence of a lipid-fuelled, drug-tolerant state in intracellular *Mtb*. (28,30,81). Importantly, recent large-scale genomic and transcriptomic analyses of clinical *Mtb* strains identified variants in *whiB6*, *phoPR*, *dosR*, and *ESX-1* associated with transmission and drug resistance (90). In this context, our findings raise the possibility that acidic phagosomal pH may shape the activity of pH-responsive regulatory networks that coordinate host lipid remodelling and bacterial redox adaptation. Further studies will be required to determine whether such pathways contribute to the emergence of drug tolerance and influence the evolutionary trajectories associated with resistance and transmission.

639 Mycobacterial ManLAM engages TLR2 and Dectin-2 to promote LD biogenesis (91).
640 TLR2 signalling activates NF- κ B and MAPK pathways, which suppress PPAR γ expression
641 and activity through competition for coactivators (CBP/p300) (92–95) and inhibitory
642 phosphorylation via ERK/JNK (96). In parallel, TLR2 signalling induces COX-2 and
643 eicosanoid production, driving LD formation independently of PPAR γ levels (97). Thus,
644 inflammatory LD accumulation can occur despite suppressed PPAR γ transcription. CQ-
645 mediated alkalinization of endosomal compartments likely disrupts this axis at multiple levels.
646 By altering receptor trafficking and endosomal maturation, CQ could dampen sustained TLR2
647 signalling, thereby relieving inflammatory repression of PPAR γ . Additionally, lysosomal
648 acidification is required for lipophagy-mediated liberation of endogenous fatty acid-derived
649 PPAR γ ligands. CQ impairs this process, potentially reshaping the availability and oxidation
650 state of lipid mediators that activate PPAR γ . Importantly, lipid mediators such as arachidonic
651 acid (AA) and other polyunsaturated fatty acids (PUFAs) can serve both as precursors of
652 endogenous PPAR γ ligands (98) and as substrates for *Mtb* lipid catabolism (84). We propose
653 that infection redirects PUFA flux away from immunoregulatory signalling and toward
654 bacterial metabolism, promoting reductive shift and drug tolerance. This metabolic diversion,
655 coupled with the inflammatory suppression of PPAR γ transcription, may explain reduced
656 PPAR γ activity despite increased LD accumulation in infected macrophages. LD stability
657 during infection is further reinforced by the recently described magnesium transporter-
658 GPCR156 (MMGT1–GPR156) axis (99). Although no direct link has been established between
659 magnesium homeostasis and phagosomal acidification, Mg²⁺–ATP is essential for V-ATPase
660 activity (100), and GPCR signalling modulates endosomal maturation (101). These pathways
661 may intersect with the pH–lipid–PPAR γ network described here, necessitating further
662 investigation.

In addition to restricting lipid availability, deacidification by CQ imposes functional iron limitation. Endo-lysosomal alkalization impairs pH-dependent iron release from transferrin, reducing bioavailable iron and inducing siderophore biosynthesis in intracellular *Mtb*. Consistent with this, iron chelation by deferoxamine (DFO) similarly limits iron availability and can potentiate anti-TB drugs (102). However, and in contrast to CQ, DFO promotes LD accumulation through HIF-1 α stabilisation and suppression of mitochondrial respiration (98). Thus, iron deprivation cannot explain the LD-reducing effects of CQ in infected macrophages. CQ therefore acts pleiotropically through coordinated modulation of pH, lipid flux, and iron availability rather than through iron restriction alone. Consistent with its multifaceted mode of action, CQ has also been reported to potentiate the activity of INH and PZA by inhibiting the BCRP-1 efflux transporter in macrophages, thereby increasing intracellular drug accumulation (103).

How do these findings relate to human TB? *In vivo*, LD-laden foamy macrophages contribute to disease progression by promoting pro-inflammatory and pro-necrotic signalling, facilitating necrotising granuloma formation, and impairing pulmonary function (104). Necrotising granulomas drive tissue destruction and transmission (105). Because foamy macrophages contribute to pathology in diverse diseases, including TB and atherosclerosis (106), they represent attractive targets for host-directed interventions (107). By defining how LD accumulation, lipid import, and phagosomal acidification are integrated during *Mtb* infection and disrupted by CQ, our work highlights CQ as a promising host-directed strategy. Nonetheless, clinical translation will require attention to dose, safety window, and potential QT interactions with fluoroquinolones, and may be most relevant to MDR-TB, necrotising/cavitary disease, or prevention of post-TB lung impairment. Importantly, recent murine models of post-TB lung disease demonstrate that fibrosis and inflammatory macrophage signatures persist despite standard HREZ therapy, mirroring clinical post-

treatment lung impairment in humans (108). The ability of CQ to attenuate fibrosis and restore pulmonary function in a necrotising TB model suggests that CQ-based adjunctive therapy may improve long-term structural and functional outcomes beyond bacterial clearance. These findings are further supported by epidemiological observations indicating reduced TB risk in rheumatoid arthritis patients receiving hydroxychloroquine (109), reinforcing the translational relevance of targeting the pH–lipid–PPAR γ axis.

Collectively, our study establishes phagosomal acidification as a metabolic checkpoint controlling lipid access, redox balance, and antibiotic tolerance in *Mtb*. By disrupting this checkpoint, CQ recalibrates macrophage lipid metabolism, constrains reductive adaptation in *Mtb*, and enhances antibiotic efficacy while simultaneously mitigating fibrotic lung remodelling. These findings highlight a therapeutically actionable host pathway linking metabolic rewiring to drug tolerance and post-infectious lung pathology.

708 MATERIALS AND METHODS

709 Animal handling, maintenance and ethics statement

710 All experiments involving animals and virulent *Mtb* were carried out within the animal
711 handling facility of the Biosafety Laboratory Level 3 (BSL-3) in accordance with the guidelines
712 of the Committee for the Purpose of Control and Supervision on Experiments on Animals
713 (CPCSEA), Government of India. The animal experiment protocol was approved by the
714 Animal Ethics Committee on the Ethics of Animal Experiments, IISc, Bangalore, India
715 (approval number: CAF/Ethics/972/2023). All humane efforts were made to minimise
716 suffering.

717 *Mtb* culture conditions

718 H37Rv, Erdman, CDC1551 and NHN1664 strains of *Mtb* were used in the study. The
719 bacteria were grown to log phase in Middlebrook 7H9 broth supplemented with 10%
720 albumin/dextrose/saline (ADS) or 10% oleic acid-albumin-dextrose-catalase (OADC), 0.2%
721 glycerol, 0.05% Tween 80, and 50 µg/ml hygromycin B (for *Mtb*-roGFP2 variants). *Mtb*
722 Erdman strains (a kind gift from Prof Brian C. VanderVen) were grown in 7H9 supplemented
723 with OADC and 25 µg/ml Kanamycin since all three strains contained the pMV306 vector
724 (without mCherry). To create the $\Delta mceG$ mutant strain, the internal region of the rv0655 ORF
725 (111 bp to 499 bp) was replaced with a hygromycin cassette via allelic exchange; therefore, 50
726 µg/ml hygromycin B was added to the growth media (60). *Mtb* CDC1551 WT strain and
727 transposon mutant of *whiB6* (Rv3862c) were obtained from BEI resources and were cultured
728 as specified in (77).

729 Bone Marrow-derived macrophage (BMDM) culture and *ex vivo* assays

BMDMs were isolated from C57BL/6 mice and cultured in DMEM (Cell Clone) supplemented with 10% FBS (Cell Clone), 30 ng/ml macrophage colony-stimulating factor (M-CSF, BioLegend), 2 mM L-glutamine (Sigma Aldrich), 1 mM sodium pyruvate (Sigma Aldrich), 10mM HEPES buffer (Sigma Aldrich), and 1% penicillin/streptomycin (Sigma Aldrich) at 37°C for 6 days. BMDM purity was checked by staining with monoclonal antibodies against MerTK (Thermo Fischer, (DS5MMER) Alexa Fluor 700) and CD64(anti-mouse, BioLegend). BMDMs were infected with indicated strains of *Mtb* for 4h before extracellular bacteria were removed by washing thrice with Dulbecco's phosphate-buffered saline (DPBS). Fresh OptiMEM medium (Thermo-Fischer) supplemented with 5% FBS and 25 ng/ml M-CSF was added, and cells were incubated for varying time points as required by the experiment.

The following compounds were used: chloroquine diphosphate (CQ; 10 μ M for *Mtb* H37Rv and 15 μ M for all other strains), oleic acid (OA, 50 μ M), Rosiglitazone (Ros, 10 μ M), GW9662 (GW, 10 μ M), Bafilomycin A1 (Baf A1, 100 nM), and ammonium chloride (NH₄Cl, 10 mM). All compounds were purchased from Sigma-Aldrich. OA and Ros were added 24 h before infection. CQ, GW9662, Baf A1 and NH₄Cl were added 1-2 h before infection. All drugs were maintained in the medium throughout the experiment.

Redox profiling of intracellular *Mtb*-roGFP2

BMDMs were infected with *Mtb*-roGFP2 at an MOI of 10, and at different time points post-infection, cells were washed and scraped off in 1X PBS. The samples were acquired using a BD FACS Aria Fusion flow cytometer, and the data were analysed using BD FACS Diva software. At least 10,000 GFP-positive events were analysed by excitation at 405 and 488 nm with a constant emission (510 nm) to determine E_{MSH} under different treatment conditions. Events were gated based on a previously developed strategy (88), where infected BMDMs were

754 treated with 10 mM CHP for complete oxidation or 100 mM DTT for complete reduction of
755 the biosensor.

756 ***Ex vivo* drug tolerance assay**

757 For intracellular CFU determination, BMDMs were infected at an MOI of 2 with *Mtb*
758 for 4h, lysed with 0.06% SDS in 7H9, serially diluted and plated on 7H11 agar supplemented
759 with 10% OADC at different time points post-infection.

760 For antibiotic tolerance assays, MXF (0.75 μ M, 3X MIC) was added to the infected
761 BMDMs at 24 h pi. Per cent survival was calculated by normalising CFU of the different
762 samples at 72 h pi (48 hours of MXF treatment) with the CFU of the untreated (Control) sample
763 at 24 h pi (0 hours of MXF treatment). Concentrations of other drugs used are mentioned in the
764 Supplementary Materials.

765 **RNA isolation, cDNA library preparation, sequencing and data analysis for CQ-treated** 766 **BMDMs**

767 Differentiated BMDMs were either left uninfected or infected with H37Rv/Mrx1-
768 roGFP2 at an MOI of 10 for 4 hours, after which the extracellular bacteria were removed by
769 washing thrice with DPBS. Postinfection, cells were treated with 0.1% PBS (solvent control)
770 or 10 μ M chloroquine diphosphate (Sigma-Aldrich). At 24 h p.i., infected BMDMs from each
771 condition were flow-sorted using a BD FACS Aria Fusion flow cytometer. The FITC channel
772 was used to sort infected macrophages (i.e., GFP-positive macrophages). RNA sequencing was
773 done at the Next-Gen Genomics Facility at the National Centre for Biological Sciences
774 (NCBS), Bangalore. Total RNA was extracted from the uninfected macrophages and the
775 FACS-sorted macrophage sub-populations using the Qiagen RNeasy Mini Kit. All RNA
776 samples (three biological replicates in each group) passed quality control analysis on the
777 Agilent 4200 TapeStation system. rRNA depletion was done using NEBNext® rRNA

778 Depletion Kit (Human/Mouse/Rat) with RNA Sample Purification Beads (E6350X).
779 Directional poly-A+ RNA-sequencing libraries were generated with the NEBNext® Ultra™ II
780 Directional RNA Library Prep with Sample Purification Beads (E7765L). Libraries were
781 sequenced on a NovaSeq 6000 (Illumina) in paired-end mode with a read length of 100 bp.
782 Raw reads were obtained as fastq files. The reference genome sequence and annotation files
783 for mouse (*Mus musculus*, GCF_000001635.27_GRCm39) were downloaded from the NCBI
784 ftp ("ftp.ncbi.nlm.nih.gov"). The raw read quality was checked using the FastQC software
785 (version v0.11.5), and the differential gene expression analysis was done using the EdgeR
786 package in R (100).

787 **Pathway analysis using Metascape**

788 FDR<0.05 and Log2FC>1 or Log2FC>0.06 were applied to obtain the final lists of
789 upregulated and downregulated genes across the different comparison groups, unless otherwise
790 specified. The differentially expressed genes were analysed using Metascape (34), a freely
791 available online platform for pathway enrichment and functional annotation, to identify
792 significantly up- and downregulated pathways.

793 **LD quantification by flow cytometry**

794 BMDMs of the different conditions were stained with 5 µM BODIPY™ 493/503 dye
795 (Sigma-Aldrich) (Ex/Em = 493/503) or 1:200 HCS LipidTOX™ Deep Red Neutral Lipid Stain
796 (Invitrogen) (Ex/Em = 635/652). Briefly, BODIPY dissolved in DMSO was directly added to
797 the cell culture medium, incubated at 37°C and 5% CO₂ for 30 min. After incubation, the cell
798 culture was aspirated, the macrophage monolayers were washed 3 times in 1X PBS, and the
799 cells were scraped in 1X PBS and acquired by BD FACS Aria Fusion flow cytometer. For
800 LipidTOX staining, the BMDMs were infected with *Mtb-roGFP2* at an MOI of 10, fixed at the
801 indicated time points, stained with LipidTOX, scraped and analysed by flow cytometry.

802 **LD quantification by confocal microscopy**

803 For LD quantification by confocal imaging, BMDMs were seeded onto glass coverslips
804 in 24-well plates at a density of 3×10^5 BMDM per coverslip and infected with the indicated
805 strains of *Mtb*. At the specified time points, the samples were fixed and stained with LipidTOX
806 or BODIPY 493/503 at 1:200 or 2 μ M, respectively, and incubated at 37°C for 30 min. For
807 LipidTOX staining, the *Mtb*-roGFP2 variants were used to infect BMDMs. After incubation,
808 the media were aspirated, and the coverslips with macrophage monolayers were washed three
809 times with 1X PBS, stained with DAPI, and mounted on slides with an antifade mounting
810 medium. Imaging was performed on a Zeiss LSM880 or a Zeiss LSM710 confocal microscope.
811 Images shown were taken with a 63x objective. For quantification, larger fields were taken
812 with a 40x objective.

813 **405/488 ratio quantification by confocal microscopy.**

814 Images were acquired using sequential excitation at 405 and 488 nm, with emission
815 collected between 510 and 540 nm. Redox ratios were calculated for each bacterium:
816 Fluorescence acquisitions were analysed in grayscale, and pixel intensities were extracted for
817 each channel. The fluorescence ratio was then calculated pixel-by-pixel as the fluorescence
818 intensity at 405 nm divided by that at 488 nm. A false-colour ratio image was generated using
819 the "Fire" lookup table in ImageJ. The corresponding colour bar indicates fluorescence ratios
820 at 405/488 nm ranging from 0.1 (fully reduced sensor) to 1.0 (oxidised sensor).

821 **BODIPY FL-C16 uptake assay**

822 The protocol was taken from (58). Briefly, BMDMs untreated or treated with CQ were
823 infected with *Mtb* H37Rv/tdTomato or *Mtb* NHN1664/tdTomato at MOI 10 for 48 h, were
824 pulsed with BODIPY FL C16 (Invitrogen) for 1 h at 37°C and 5% CO₂, washed and chased

with fresh media for 1 h at 37°C and 5% CO₂. The BMDMs were lysed with 0.06% SDS, and the released bacteria were analysed by BD FACS Aria Fusion flow cytometer.

Redox profiling of *in vitro* grown *Mtb*

Primary cultures of *Mtb* H37Rv were grown in 7H9 supplemented with ADS, 0.2% glycerol and 0.05% Tween80. Secondary cultures were set at 0.05 OD₆₀₀ in 7H9 medium set to pH 4.5, 5.5 or 7.4, with OA as the sole carbon source, cultured in a shaker incubator for three days, after which the cultures were pelleted down, washed, resuspended in 1X PBS and analysed by BD FACS Aria Fusion flow cytometer.

Drug tolerance assay of *in vitro*-grown *Mtb*

Primary cultures of *Mtb* H37Rv were grown in 7H9 supplemented with ADS, 0.2% glycerol and 0.05% Tween80. Secondary cultures were set at 0.01 OD₆₀₀ in 7H9 medium and at pH 4.5, 5.5 or 7.4, with OA at 200 µM as the sole carbon source. 2.5 µM MXF (10X MIC) was added on the same day, and the cultures were allowed to grow in shaking conditions for 5 days. On days 0 and 5, dilutions were made from different conditions and plated on OADC-supplemented 7H11 agar to enumerate CFUs.

RNA isolation, cDNA library preparation, sequencing and data analysis for *Mtb* isolated from chloroquine-treated macrophages

Differentiated BMDMs were infected with H37Rv/Mrx1-roGFP2 (*Mtb*-roGFP2) at an MOI of 10 for 4 h, after which the extracellular bacteria were removed by washing thrice with DPBS. Post-infection, cells were treated with 0.1% PBS (solvent control) or CQ at 10 µM. At 24 h p.i., intracellular *Mtb* was collected in 4M GTC buffer containing 1% 2-mercaptoethanol for differential lysis of macrophages and fixation of bacterial RNA. Fixed bacteria were then centrifuged at 5000 rpm for 5 minutes. Total RNA was isolated as previously described (111). RNA sequencing was done at the Next-Gen Genomics Facility at the National Centre for

849 Biological Sciences (NCBS), Bangalore. All RNA samples (three biological replicates in each
850 group) passed quality control analysis on the Agilent 4200 TapeStation system. rRNA
851 depletion was done using NEBNext® rRNA Depletion Kit (Bacteria) with RNA Sample
852 Purification Beads (E7860L). Libraries were generated with the NEBNext® Ultra™ II
853 Directional RNA Library Prep with Sample Purification Beads (E7765L). Libraries were
854 sequenced on a NovaSeq 6000 (Illumina) in paired-end mode with a read length of 100 bp.
855 Raw reads were obtained as fastq files. The reference genome sequence and annotation files
856 for *Mtb* (NC_000962.3) were downloaded from the NCBI ftp ("ftp.ncbi.nlm.nih.gov"). The
857 raw read quality was assessed using FastQC (v0.11.5), and DEG analysis was performed using
858 the EdgeR package in R (110).

859 **Whole-genome sequencing and analysis of *Mtb***

860 Raw RNA-seq reads (paired-end, triplicate) were subjected to quality control using
861 FastQC and adaptor trimming with Trim Galore 0.6.7, applying a Phred score cutoff of 30 to
862 ensure >99.9% base-call accuracy. Low-quality reads and adaptors were removed using
863 cutadapt 3.10.12. Quality-filtered reads were aligned to the *Mtb* H37Rv reference genome
864 NCBI (RefSeq: GCF_000195955.2; GenBank: GCA_000195955.2, assembly ASM19595v2)
865 with Bowtie2 v2.4.4, yielding 91–93% alignment rates. The resulting SAM files were
866 converted to sorted BAM format using Samtools v1.13, and pileup files were generated for
867 genome-wide base call summaries. VarScan v2.4.2 was used to call SNPs and small indels for
868 variant identification, reporting genomic locations, allele frequencies, and coverage depths.
869 The resulting VCF files were annotated with snpEff.

870 **Lineage assignment and phylogenetic tree**

871 Lineage identification of *Mtb* isolates was performed using TB-Profiler v6.6.2 with the
872 Tbdb database (v82777), involving read trimming, mapping, variant calling, k-mer counting,

and depth calculation. Genomes from human-adapted MTBC lineages (1–7), animal-adapted clades (*M. bovis*, *M. caprae*, *M. microti*), and *M. canettii* were included for phylogenetic analysis. Genome assemblies were aligned using Parsnp with H37Rv as a reference.

qRT-PCR of flow-sorted *Mtb*-infected BMDMs

NHN1664-roGFP2-infected BMDMs were flow-sorted at 24 h p.i., GFP+ BMDMs were lysed in the RLT buffer (Qiagen), and total RNA was isolated according to the manufacturer's protocol using the Qiagen RNeasy Plus Mini Kit. Isolated RNA was further treated with DNase I (Thermo Scientific) to remove residual genomic DNA. 500ng of RNA was used to synthesise cDNA for downstream qRT-PCR analysis using the iScript Select cDNA Synthesis Kit (BioRad). (CFX96 RT-PCR System, BioRad) Furthermore, iQ SYBR Green Supermix (BioRad) were used to assess gene expression, and the primers used for qRT-PCR for different genes were as follows:

Ppar γ FP 5'-GTACTGTCGGTTTCAGAAAGTGCC-3'

Ppar γ RP 5'-ATCTCCGCCAACAGCTTCTCCT-3'

Fabp3 FP 5'-AGAGTTCGACGAGGTGACAGCA-3'

Fabp3 RP 5'-TTGTCTCCTGCCCCGTTCCACTT-3'

Fabp4 FP 5'-TGAAATCACCGCAGACGACAGG-3'

Fabp4 RP 5'-GCTTGTCACCATCTCGTTTTCTC-3'

Fabp5 FP 5'-GACGACTGTGTTCTCTTGTAACC-3'

Fabp5 RP 5'-TGTTATCGTGCTCTCCTTCCCG-3'

β -actin FP 5'-CATTGCTGACAGGATGCAGAAGG-3'

β -actin RP 5'-TGCTGGAAGGTGGACAGTGAGG-3'

895 **Alamar blue assay of *in vitro* grown *Mtb***

896 Antimicrobial susceptibility was assessed using the microplate Alamar Blue assay in
897 96-well flat-bottom plates. *Mtb* strains were cultured in Middlebrook 7H9 medium
898 supplemented with ADS or OADC to mid-exponential phase ($OD_{600} \approx 0.6$). Approximately 1
899 $\times 10^5$ bacilli were seeded per well in a final volume of 200 μ L medium under the indicated
900 treatment conditions. Following incubation at 37°C for 5 days, 30 μ L Alamar Blue reagent was
901 added to each well, and the plates were further incubated for 48 h. Fluorescence was measured
902 using a SpectraMax M3 microplate reader (Ex 530 nm/Em 590 nm). Percentage inhibition was
903 calculated from relative fluorescence units, and the minimum drug concentration producing
904 $\geq 90\%$ growth inhibition was defined as the minimum inhibitory concentration (MIC).

905 **Infection and drug treatment of BALB/c mice**

906 To establish the chronic infection model of *Mtb* infection, 4-6-week-old female
907 BALB/c mice were infected by aerosol with 30-40 *Mtb* NHN1664 bacilli using a Madison
908 chamber aerosol generation instrument, and the animals were then housed for 4 weeks to allow
909 progression of infection. Infected BALB/c mice were randomly distributed into four groups
910 and started on various treatments in the following doses: (i) 10 mg per kg body weight intra-
911 peritoneal (i.p.) doses of CQ on alternate days; (ii) 25 mg per kg body weight of MXF by oral
912 gavage five days a week; (iv) a combination of CQ and MXF (CQ + MXF), at the mentioned
913 doses. The animals were dosed for 8 weeks. Their lungs were harvested, homogenised, and
914 plated at the indicated time points for CFU determination.

915 **Single cell suspension preparation from lungs of BALB/c mice**

916 Chronically infected BALB/c mice from different treatment groups were sacrificed 12
917 weeks post-infection, and whole lungs were aseptically removed to prepare a single-cell
918 suspension for flow cytometry. Lungs were minced and digested in serum-free RPMI

containing 0.2 mg/mL Liberase DL (Roche) and 0.1 mg/mL DNase I (Roche) for 60 minutes at 37°C at 180 rpm. This was followed by mechanical disruption of the suspension using the GentleMACS dissociator (Miltenyi Biotech) and de-clumping by passage through 70 µM nylon cell strainers (BD Falcon). To remove all red blood cells, the single cell suspension was incubated in RBC lysis buffer for 15 minutes with gentle vortexing. Cell suspension was washed thrice with pre-warmed 1X PBS and re suspended in appropriate buffers for staining.

Analysis of acidification and LDs in murine lung cells by flow cytometry

The Magic Red Cathepsin B (Immunochemistry Technologies) fluorogenic substrate was resuspended in DMSO to assess macrophage acidity, as recommended by the manufacturer. A total lung cell suspension was seeded in a 96-well plate in RPMI-1640 supplemented with 10% FBS at a density of 50,000 cells per well and incubated for 1 h at 37°C in 5% CO₂ to allow cell adherence. This was followed by the removal of complete media and its replacement with RPMI-1640 containing 1X diluted MR Cathepsin B substrate for an additional 1h under the same conditions. After 1 h of incubation, the media was removed, cells were washed twice with 1X PBS, the cell pellet was resuspended in 1X PBS, and the cells were acquired on the BD FACS Aria Fusion flow cytometer. MR Cathepsin-positive cells were analysed by flow cytometry with excitation at 560 nm and emission at 585 nm.

To check LDs, the lung cell suspension was incubated with 5 µM BODIPY 493/503 at 37°C and 5% CO₂ for 30 min, washed three times with 1X PBS, the cell pellet was resuspended in 1X PBS, and acquired on a BD FACS Aria Fusion flow cytometer.

Infection and drug treatment of C3HeB/FeJ mice

For establishing the chronic infection model of *Mtb* infection, 6-8 weeks old mice (equal numbers of male and female) C3HeB/FeJ mice were infected by the aerosol route with 30-40 *Mtb* NHN1664 bacilli using a Madison chamber aerosol generation instrument, and the

animals were then housed for the progression of infection. At 2 weeks p.i. CQ treatment was started, and at 4 weeks p.i. MXF treatment was started. The drugs were given in the following doses: (i) 10 mg per kg body weight intra-peritoneal (i.p.) doses of CQ on alternate days; (ii) 25 mg per kg body weight of MXF by oral gavage five days a week; (iv) a combination of CQ and MXF (CQ + MXF), at the mentioned doses. Treatment was continued for 8 weeks p.i., after which their lungs were harvested, homogenised, and plated for CFU determination.

CFU estimation in *Mtb*-infected mice lungs

The *Mtb* NHN1664-infected BALB/c or C3HeB/FeJ mice from each group were sacrificed at different time points post-treatment. Lungs were harvested aseptically, weighed, and homogenised in 2 mL sterile PBS. Homogenates were serially diluted and plated on 7H11-OADC agar plates supplemented with lyophilised BBL MGIT PANTA antibiotic mixture (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin, as supplied by BD Biosciences). Plates were incubated at 37°C for 3 weeks before colonies were enumerated.

Histopathology analysis of formalin-fixed lung sections

At 8 weeks p.i., the *Mtb* NHN1664-infected C3HeB/FeJ mice were euthanised, and lungs were harvested to analyse gross pathology, histopathology and CFU load. 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-um-thick sections, embedded in paraffin, and stained with either haematoxylin, eosin, or Masson's Trichrome following established protocols (83,108,112). Tissue sections were coded, and a certified pathologist analysed them to assess granuloma formation and fibrotic lesions.

The table below illustrates the basis of a system for granuloma scoring in lung sections of mice infected with *Mtb*. The system ranked the granulomas on a scale of 0-5 (112). The

966 number of granulomas was multiplied by the rank of each granuloma to get the final granuloma
967 score.

Characteristics	Granuloma rank
Granulomas with necrosis	5
Granulomas with centrally placed epithelioid cell clusters but no necrosis	2.5
Granulomas with fibrous connective tissue surrounding lymphocytes and epithelioid cells	1

968

969 The table below shows the basis of scoring fibrosis in lung sections of *Mtb*-infected
970 mice. The Modified Ashcroft Scale was used for scoring (83).

Grade of fibrosis	Ashcroft Modified Scale
0	No fibrotic burden at the flimsiest small fibres in some alveolar walls
1	Isolated gentle fibrotic changes (septum $\leq 3\times$ thicker than normal) - Alveoli partly enlarged and rarefied, but no fibrotic masses present
2	Clearly fibrotic changes (septum $>3\times$ thicker than normal) with knot-like formation but not connected to each other - Alveoli partly enlarged and rarefied, but no fibrotic masses

3	Contiguous fibrotic walls (septum >3× thicker than normal) predominantly in the whole microscopic field - Alveoli partly enlarged and rarefied, but no fibrotic masses
4	Variable – Single fibrotic masses (≤10% of microscopic field)
5	Variable - Confluent fibrotic masses (>10% and ≤50% of microscopic field). Lung structure severely damaged but still preserved
6	Variable, mostly not existent - Large contiguous fibrotic masses (>50% of microscopic field). Lung architecture is mostly not preserved
7	Non-existent - Alveoli nearly obliterated with fibrous masses, but still up to five air bubbles
8	Non-existent - Microscopic field with complete obliteration by fibrotic masses

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972 **Whole body plethysmography of C3HeB/FeJ mice**

973 At the end of 8 weeks post-infection, respiratory parameters in *Mtb* NHN1664-infected
974 and uninfected C3HeB/FeJ mice were monitored in the vivoFlow-WBP (whole-body
975 plethysmography) system by SCIREQ and emka TECHNOLOGIES. Mice were acclimatised
976 in the WBP subject chamber before recording. Respiratory function was recorded for 20
977 minutes for each animal.

978 **Statistical Analysis**

979 All statistical analyses were performed using GraphPad Prism (version 8.0). Data are
980 presented as mean ± SD. Nonparametric datasets and normalised values were analysed using
981 the Mann–Whitney test (two groups) or the Kruskal–Wallis test followed by Dunn's multiple-

comparison correction (multiple groups). Parametric datasets were analysed using an unpaired Student's *t*-test with Welch's correction (two groups) or one-way ANOVA followed by Dunnett's T3 multiple-comparison test (multiple groups). A 2-way ANOVA with Tukey's multiple comparisons test was used for Figure S9B. A *P* value < 0.05 was considered statistically significant.

1000 **SUPPLEMENTARY INFORMATION**

1001 Fig. S1. CQ-driven phagosomal deacidification reduces E_{MSH} -reduced *Mtb* and drug tolerance
1002 in THP-1 macrophages.

1003 Fig. S2. CQ-driven phagosomal deacidification decreases E_{MSH} -reduced *Mtb* and mitigates
1004 drug tolerance in BMDMs.

1005 Fig. S3. RNA-Seq samples cluster distinctly with their biological replicates.

1006 Fig. S4. *Mtb* infection induces pro-inflammatory pathways and arrests cell cycle in BMDMs.

1007 Fig. S5. Phagosomal alkalization remodels macrophage inflammatory, metabolic, and
1008 vesicular trafficking programs in uninfected BMDMs.

1009 Fig. S6. Phagosomal deacidification reprograms lipid metabolism, inflammatory signalling,
1010 and ECM organisation in *Mtb*-infected BMDMs.

1011 Fig S7. Phagosomal alkalization uniformly reduces LDs independently of PPAR γ .

1012 Fig. S8. $\Delta mceG$ exhibits no difference in MXF susceptibility compared to wild-type and
1013 complemented strains *in vitro*.

1014 Fig. S9. OA induces reductive E_{MSH} in *Mtb* and drives drug tolerance *in vitro*.

1015 Fig. S10. *Mtb* NHN1664 is genetically resistant to frontline anti-TB drugs but susceptible to
1016 MXF.

1017 Table S1. Whole-genome sequence analysis identified SNPs that confirmed the MDR status of
1018 *Mtb* NHN1664.

1019 Fig. S11. CQ marginally increases activity of MXF against *Mtb* NHN1664 in BALB/c mice.

1020 **Other Supplementary Material for this manuscript includes the following:**

1021 Data file S1 (Microsoft Excel format). The file contains 3 sheets, with details given below.

1022 Sheet 1 (UI+CQ vs UI): List of differentially expressed genes in uninfected BMDMs treated
1023 with CQ compared to uninfected untreated BMDMs.

1024 Sheet 2 (INF vs UI): List of differentially expressed genes in *Mtb*-roGFP2-infected BMDMs
1025 compared to uninfected BMDMs.

1026 Sheet 3 (INF+CQ vs INF): List of differentially expressed genes in CQ-treated *Mtb*-roGFP2-
1027 infected BMDMs compared to *Mtb*-roGFP2-infected BMDMs.

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