Supplementary Information for

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

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Fig. S8. Δ*mceG* exhibits no difference in MXF susceptibility compared to wild-type and complemented strains *in vitro.*

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

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

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

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

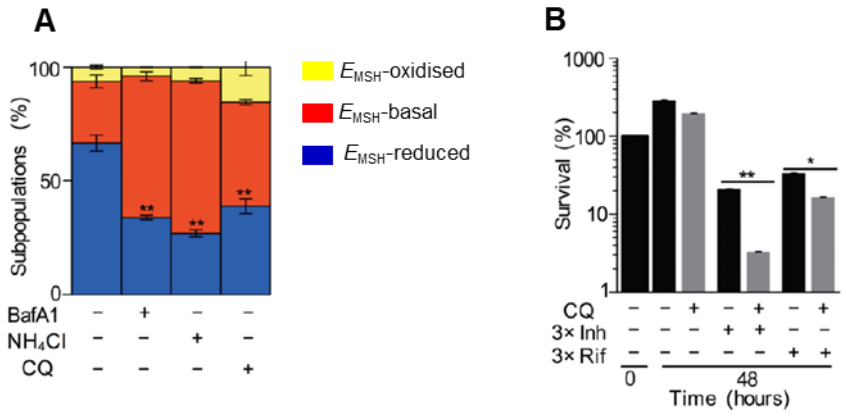
Other Supplementary Material for this manuscript includes the following:

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

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

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

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



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48 **Fig. S1. CQ-driven phagosomal deacidification reduces *E*MSH -reduced *Mtb* and drug**
49 **tolerance in THP-1 macrophages.** (**A**) THP-1 macrophages, either untreated or treated with
50 10 nM BafA1, 10 mM NH_4Cl, or 10 μM CQ, were infected with *Mtb*-roGFP2, and the
51 percentage distribution of redox-heterogeneous populations was assessed at 24 hours post-
52 infection. Statistical significance (**$P < 0.01$) was determined using the Mann–Whitney test
53 by comparing the E_{MSH}-reduced fraction of CQ-treated conditions to that of the untreated
54 control. (**B**) THP-1 macrophages, either untreated or treated with 10 μM CQ, were infected
55 with *Mtb* H37Rv for 24 hours, followed by exposure to INH (2.18 μM), RIF (1 μM), or no drug
56 for an additional 48 hours. Bacterial survival was expressed as per cent survival by normalising
57 CFU counts from drug-treated samples at 72 h p.i. (48 hours) to untreated controls at 24 h p.i.
58 (0 hours). Statistical significance (*$P < 0.05$, **$P < 0.01$) was determined using the Mann–
59 Whitney test. From (25), reprinted with permission from AAAS.

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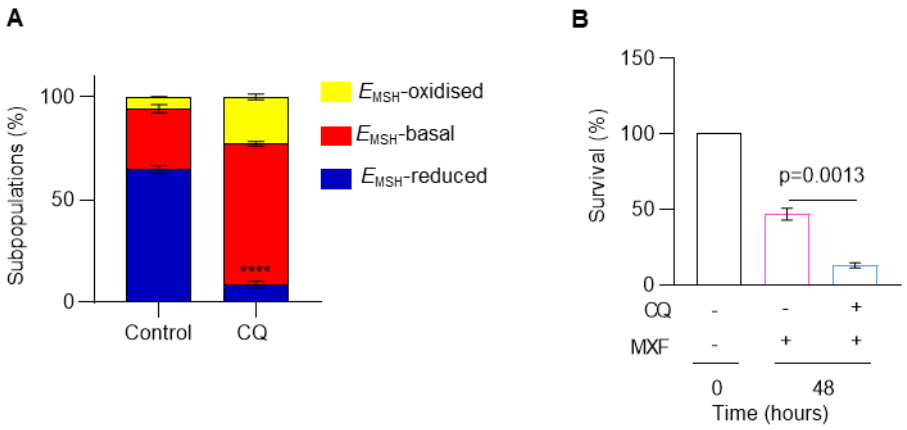


Fig. S2. CQ-driven phagosomal deacidification decreases E_{MSH}-reduced *Mtb* and mitigates drug tolerance in BMDMs. Untreated (Control) or CQ-treated BMDMs were infected with *Mtb*-roGFP2 (MOI 10, 24 h) followed by quantification of redox-diverse *Mtb* subpopulation by flow cytometry, comparing the E_{MSH}-reduced population in Control and CQ-treated BMDMs. ****$P < 0.0001$ was determined using the Mann–Whitney test. (**B**) Percent survival of *Mtb* H37Rv exposed to 3X MIC of MXF in untreated or CQ-treated BMDMs. The *p*-value was determined using a Student's two-tailed *t*-test with Welch's correction. Data shown are expressed as mean ± S.D., representative of three independent experiments.

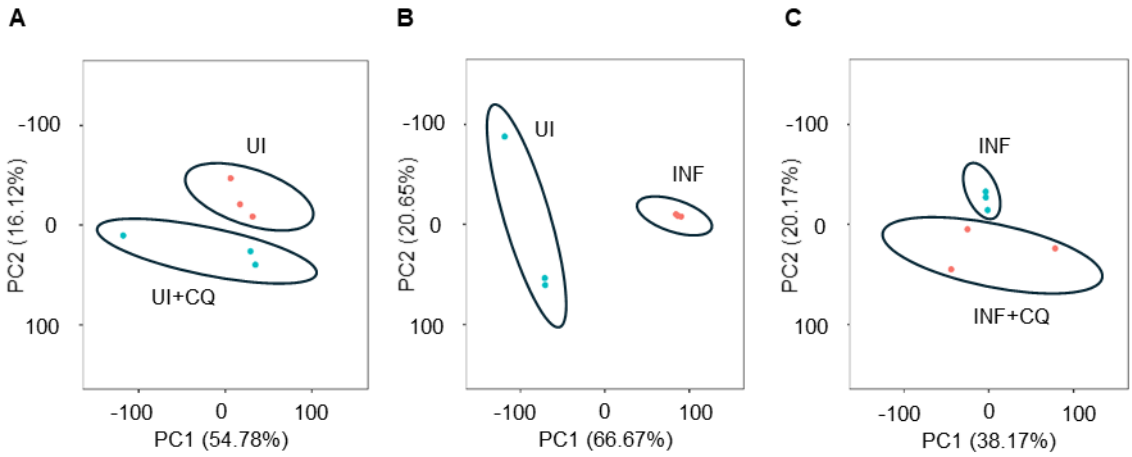


Fig. S3. RNA-Seq samples cluster distinctly with their biological replicates. RNA-Seq was performed on samples as mentioned in the legend of Figure 1. (**A**–**B**) PCA plots comparing UI and UI+CQ **(A)**, UI and INF **(B)**, and INF and INF+CQ **(C)** conditions.

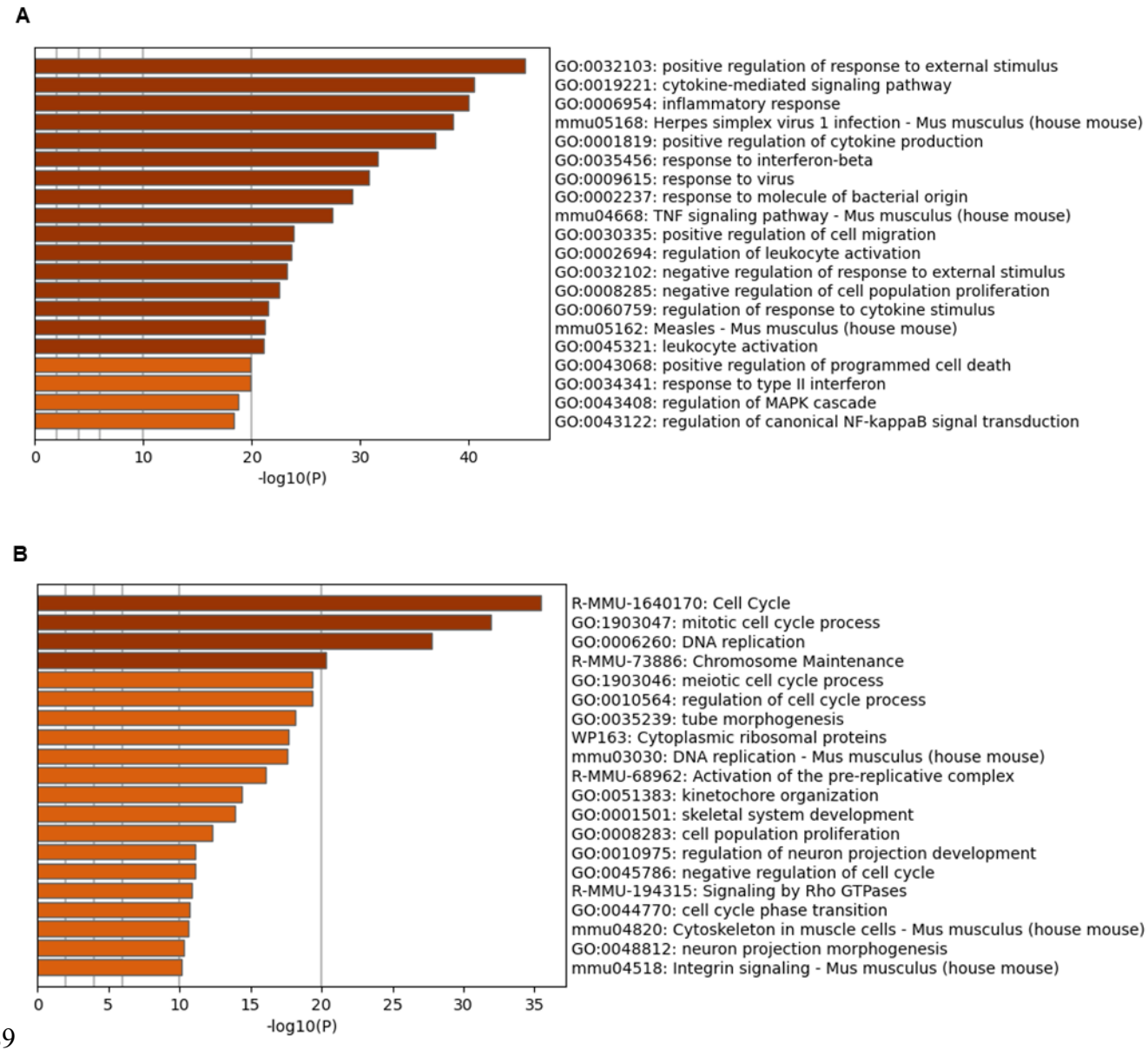


Fig. S4. *Mtb* infection induces pro-inflammatory pathways and arrests cell cycle in BMDMs. RNA-Seq was performed on samples as mentioned in the legend of Figure 1. (**A**) Metascape pathway analysis of the upregulated genes in INF BMDMs relative to UI BMDMs (FDR<0.05 and Log2FC >=1). (**B**) Metascape pathway analysis of the downregulated genes in INF BMDMs relative to UI BMDMs (FDR<0.05 and Log2FC=1).

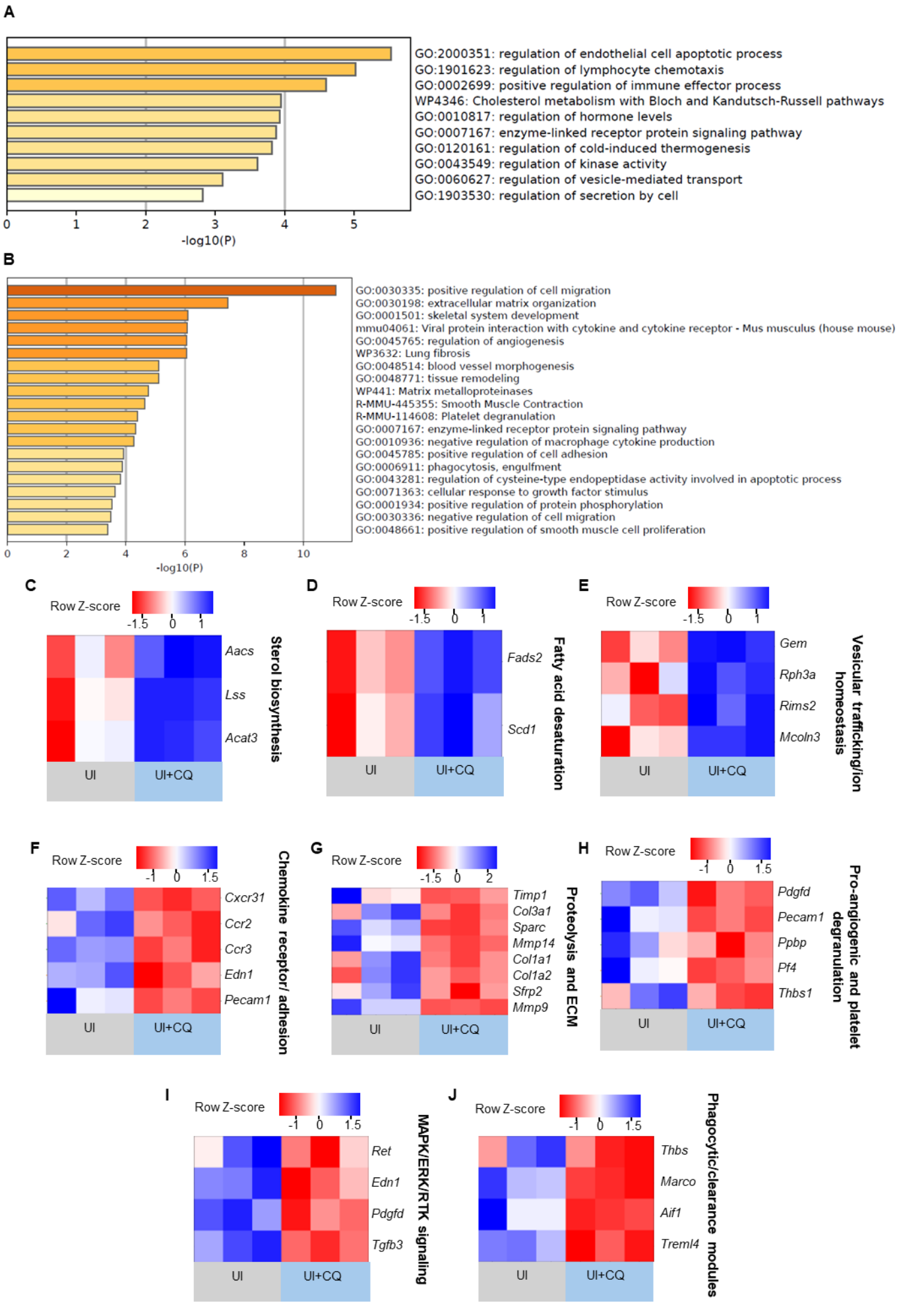

A
GO:2000351: regulation of endothelial cell apoptotic process
GO:1901623: regulation of lymphocyte chemotaxis
GO:0002699: positive regulation of immune effector process
WP4346: Cholesterol metabolism with Bloch and Kandutsch-Russell pathways
GO:0010817: regulation of hormone levels
GO:0007167: enzyme-linked receptor protein signaling pathway
GO:0120161: regulation of cold-induced thermogenesis
GO:0043549: regulation of kinase activity
GO:0060627: regulation of vesicle-mediated transport
GO:1903530: regulation of secretion by cell
0 1 2 3 4 5
-log10(P)
B
GO:0030335: positive regulation of cell migration
GO:0030198: extracellular matrix organization
GO:0001501: skeletal system development
mmu04061: Viral protein interaction with cytokine and cytokine receptor - Mus musculus (house mouse)
GO:0045765: regulation of angiogenesis
WP3632: Lung fibrosis
GO:0048514: blood vessel morphogenesis
GO:0048771: tissue remodeling
WP441: Matrix metalloproteinases
R-MMU-445355: Smooth Muscle Contraction
R-MMU-114608: Platelet degranulation
GO:0007167: enzyme-linked receptor protein signaling pathway
GO:0010936: negative regulation of macrophage cytokine production
GO:0045785: positive regulation of cell adhesion
GO:0006911: phagocytosis, engulfment
GO:0043281: regulation of cysteine-type endopeptidase activity involved in apoptotic process
GO:0071363: cellular response to growth factor stimulus
GO:0001934: positive regulation of protein phosphorylation
GO:0030336: negative regulation of cell migration
GO:0048661: positive regulation of smooth muscle cell proliferation
0 2 4 6 8 10
-log10(P)
C
Row Z-score
-1.5 0 1
Aacs
Lss
Acat3
UI
UI+CQ
Sterol biosynthesis
D
Row Z-score
-1.5 0 1
Fads2
Scd1
UI
UI+CQ
Fatty acid desaturation
E
Row Z-score
-1.5 0 1
Gem
Rph3a
Rims2
Mcoln3
UI
UI+CQ
Vesicular trafficking/ion homeostasis
F
Row Z-score
-1 0 1.5
Cxcr31
Ccr2
Ccr3
Edn1
Pecam1
UI
UI+CQ
Chemokine receptor/ adhesion
G
Row Z-score
-1.5 0 2
Timp1
Col3a1
Sparc
Mmp14
Col1a1
Col1a2
Sfrp2
Mmp9
UI
UI+CQ
Proteolysis and ECM
H
Row Z-score
-1 0 1.5
Pdgfd
Pecam1
Ppbp
Pf4
Thbs1
UI
UI+CQ
Pro-angiogenic and platelet degranulation
I
Row Z-score
-1 0 1.5
Ret
Edn1
Pdgfd
Tgfb3
UI
UI+CQ
MAPK/ERK/RTK signaling
J
Row Z-score
-1 0 1.5
Thbs
Marco
Aif1
Treml4
UI
UI+CQ
Phagocytic/clearance modules

Fig. S5. Phagosomal alkalinization remodels macrophage inflammatory, metabolic, and vesicular trafficking programs in uninfected BMDMs. RNA-Seq was performed on samples as mentioned in the legend of Figure 1. (**A**) Metascape pathway analysis of the upregulated genes in UI+CQ BMDMs relative to UI BMDMs (FDR<0.05 and Log2FC>=1). (**B**) Metascape pathway analysis of the downregulated genes in UI+CQ BMDMs relative to UI BMDMs, FDR<0.05 and Log2FC>=1. (**C-J**) Heatmaps showing the genes of sterol biosynthesis (**C**), fatty acid desaturation (**D**), vesicular trafficking/ion homeostasis (**E**), chemokine receptor/adhesion (**F**), proteolysis and ECM (**G**), pro-angiogenic and platelet degranulation (**H**), MAPK/ERK/RTK signaling (**I**), phagocytic/clearance modules (**J**), between UI and UI+CQ BMDMs respectively. The heatmap was generated from three independent experiments, with FDR < 0.05 and Log2FC = 1. *Abbreviations: ECM – Extracellular matrix, UI – Uninfected, UI+CQ – Uninfected plus CQ-treated.*

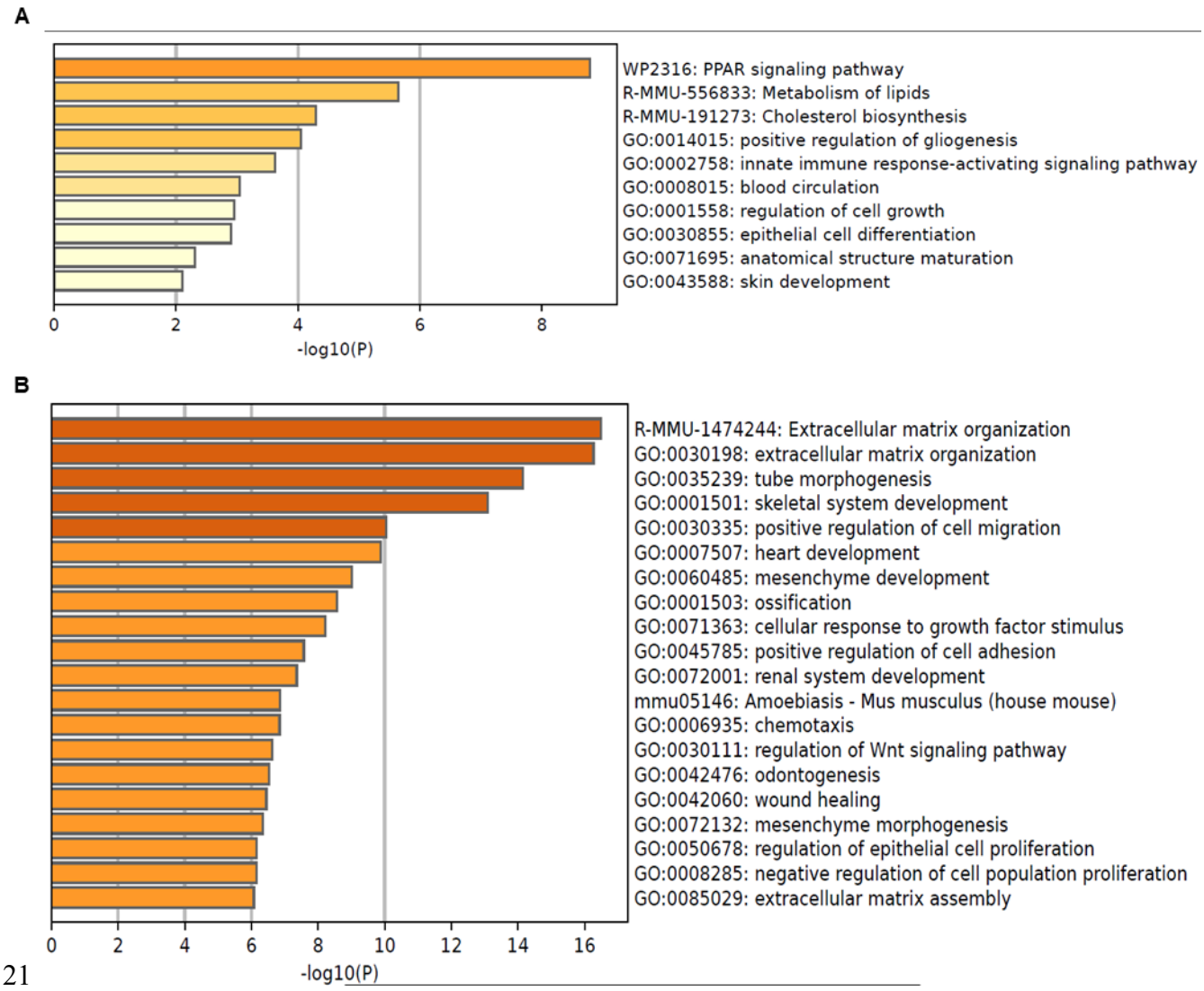


Fig. S6. Phagosomal deacification reprograms lipid metabolism, inflammatory signalling, and ECM organisation in *Mtb*-infected BMDMs. RNA-Seq was performed on samples as mentioned in the legend of Figure 1. (**A**) Metascape pathway analysis of the upregulated genes in INF+CQ BMDMs relative to INF BMDMs (FDR<0.05 and Log2FC>1). (**B**) Metascape pathway analysis of the downregulated genes in IFN+CQ BMDMs relative to INF BMDMs (FDR<0.05 and Log2FC>1).

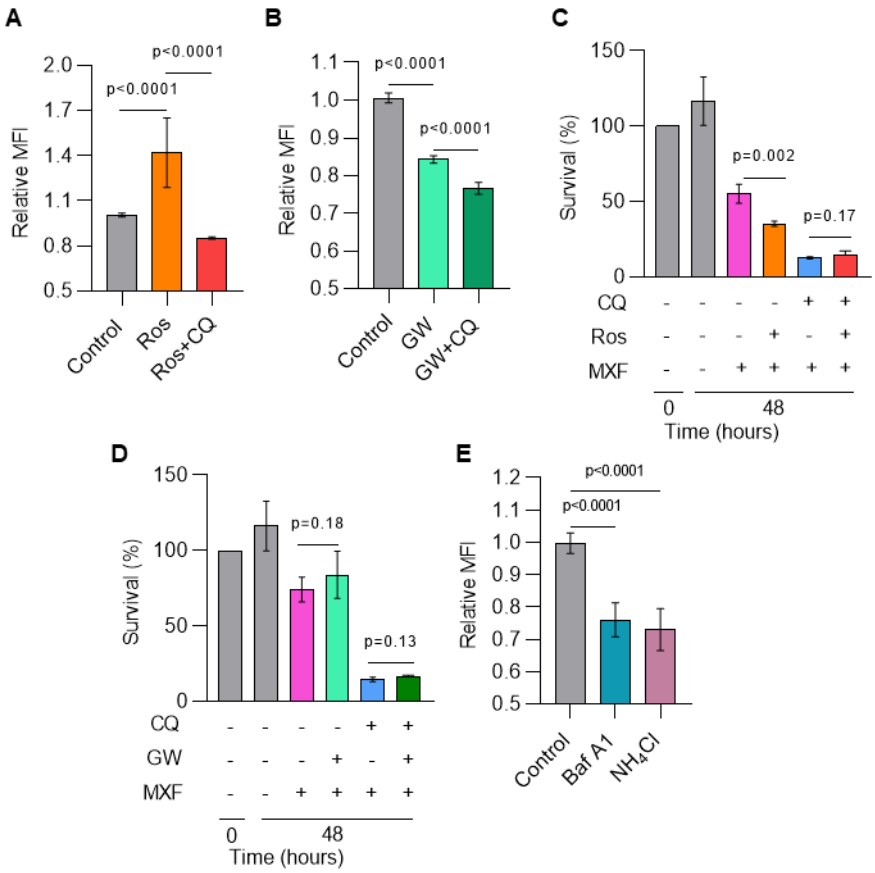


Fig S7. Phagosomal alkalinization uniformly reduces LDs independently of PPARγ. (A, B, E) Flow cytometry quantification of relative LipidTOX MFI of *Mtb*-roGFP2-infected BMDMs, either untreated (Control) or treated with Ros, GW, CQ, Baf A1, or NH4Cl. **(C, D)** Percent survival of *Mtb* H37Rv exposed to 3X MIC of MXF in untreated, CQ, Ros, GW or their combination-treated BMDMs. Data shown are the results of three independent experiments performed in triplicate (means ± SD). The *p*-values were determined using the Mann-Whitney test.

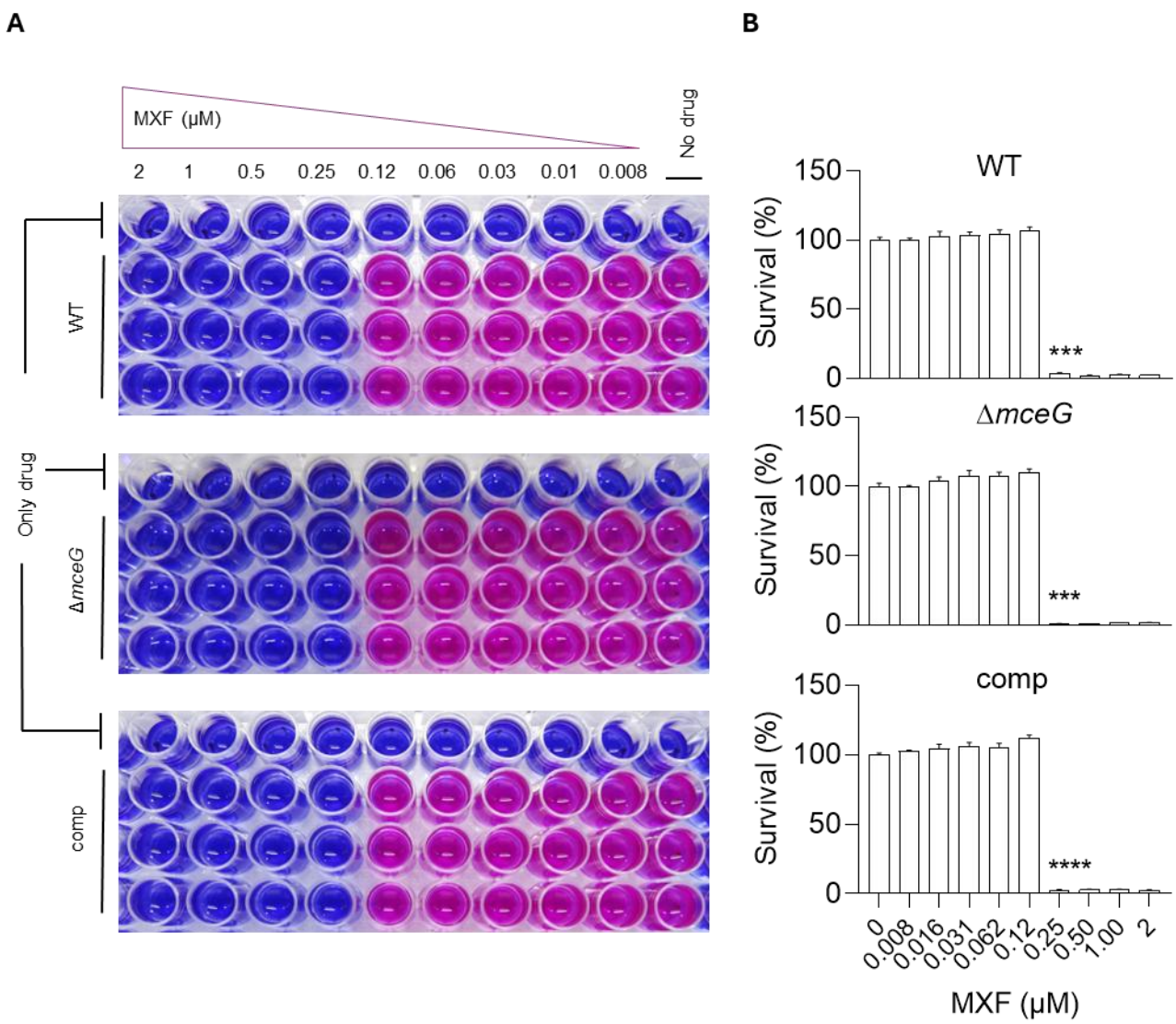
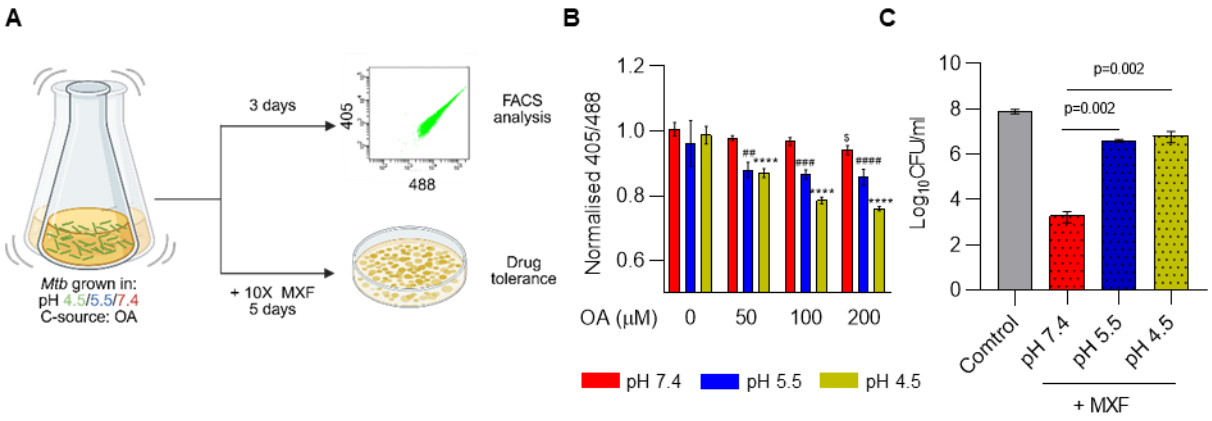


Fig. S8. **Δ*mceG* exhibits no difference in MXF susceptibility compared to wild-type and complemented strains *in vitro.*** (**A**) Representative Alamar blue assay plate pictures of the indicated *Mtb* Erdman strains treated with the indicated concentrations of MXF and the **(B)** associated percent survival of the *Mtb* Erdman strains in the indicated concentrations of MXF. Percent survival was calculated by normalising the fluorescence intensities of the MXF-added samples with that of the no MXF-added (0 μM MXF) sample. For detailed protocol, check the *Materials and Methods* section. ***$P < 0.001$, ****$P < 0.0001$ by Mann-Whitney test comparing percentage survival at 0.25 μM MXF with 0 μM MXF for all three strains.



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149 **Fig. S9. OA induces reductive *E*$_{MSH}$ in *Mtb* and drives drug tolerance *in vitro*. (A)**
150 Experimental scheme for panels (**B**–**C**). **(B)** *Mtb*-roGFP2 was cultured in 7H9 medium at pH
151 7.4, 5.5, or 4.5 with the indicated OA concentrations for 72 h, and ratiometric sensor response
152 (405/488) was measured by flow cytometry. Values were normalised to no-carbon controls at
153 the respective pH. Statistical analysis was performed using 2-way ANOVA with Tukey's
154 multiple comparisons test. **(C)** Quantification of *Mtb* H37Rv CFU grown at pH 7.4, 5.5 or 4.5
155 with OA 200 μM as the sole carbon source and exposed to 10X MIC MXF for 5 days. Control
156 denotes CFU of *Mtb* grown in OA+0.2% glycerol containing media set to pH 6.6. The *p*-values
157 were determined using the Mann-Whitney test. Data shown are expressed as mean ± S.D.,
158 representative of two independent experiments performed in triplicate.

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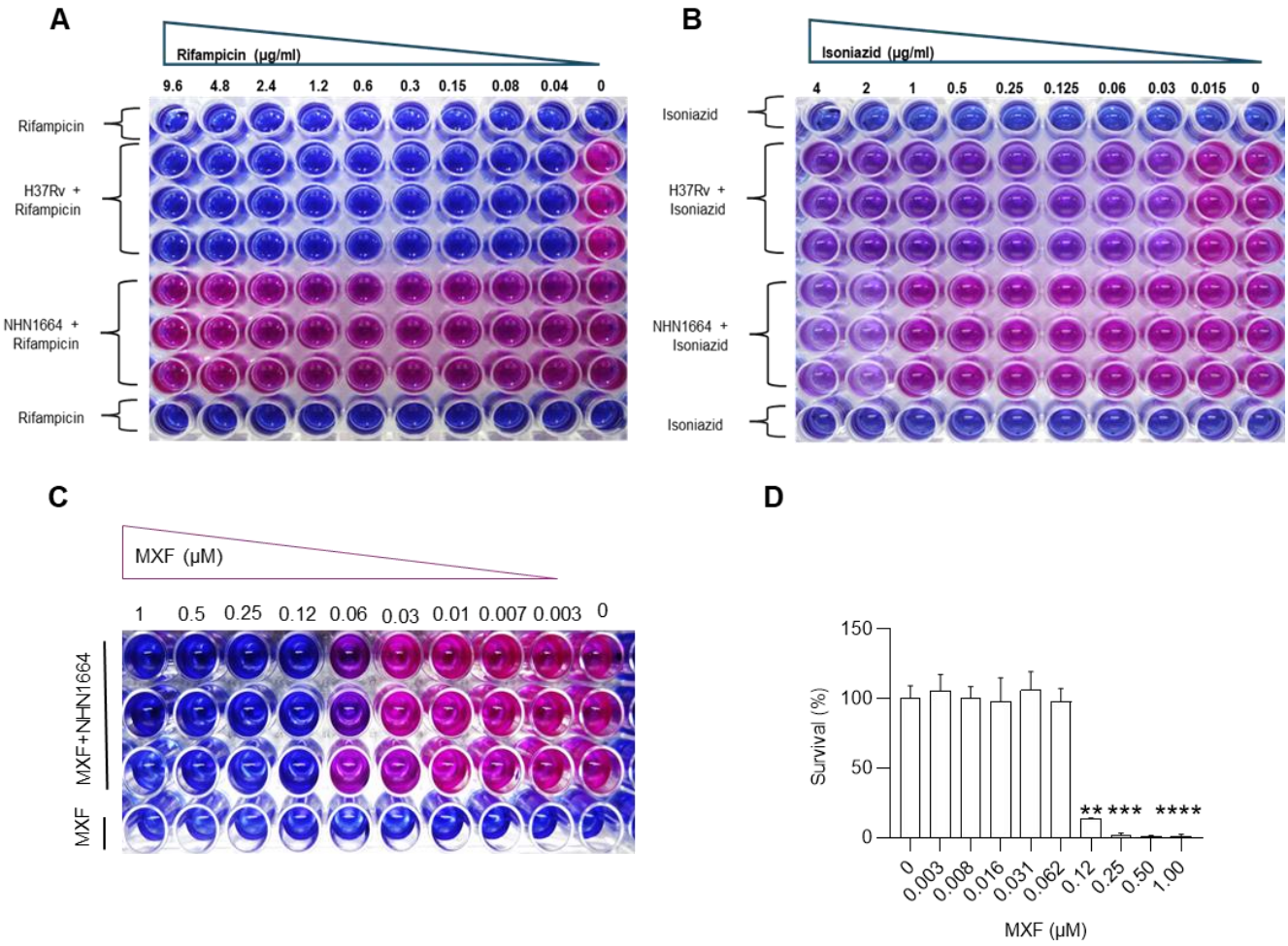

Fig. S10. ***Mtb*** **NHN1664 is genetically resistant to frontline anti-TB drugs but susceptible to MXF.** (**A-B**) Representative Alamar blue assay plate pictures of *Mtb* H37Rv and *Mtb* NHN1664 strains treated with different concentrations of Rifampicin (**A**) and Isoniazid (**B**). (**C**) Representative Alamar blue assay plate picture of *Mtb* NHN1664 exposed to different concentrations of MXF and (D) associated quantification of percent survival of the *Mtb* NHN1664 in the indicated concentrations of MXF. Percent survival was calculated by normalising the fluorescence intensities of the MXF-added samples with that of the no MXF-added (0 µM MXF) sample. For detailed protocol, check the *Materials and Methods* section. ***P*=0.002, ****P*=0.0001, ****P*<0.0001, by Kruskal-Wallis test with Dunn's Multiple Comparisons test. Data shown are expressed as mean ± S.D., representative of two independent experiments.

S. No.	Gene name	Nucleotide change	Amino acid change	Resistant to:
1	*katG*	c.944G>C	Ser315Thr	Isoniazid
2	*rpoB*	c.1334A>C	His445Pro	Rifampicin
3	*rpsl*	c.128A>G	Lys43Arg	Streptomycin
4	*embB*	c.916A>G	Met306Val	Ethambutol

Table S1. Whole-genome sequence analysis identified SNPs that confirmed the MDR status of *Mtb* NHN1664. The whole-genome sequence of *Mtb* NHN1664 was compared with the *Mtb* H37Rv reference genome to identify antibiotic resistance-conferring mutations.

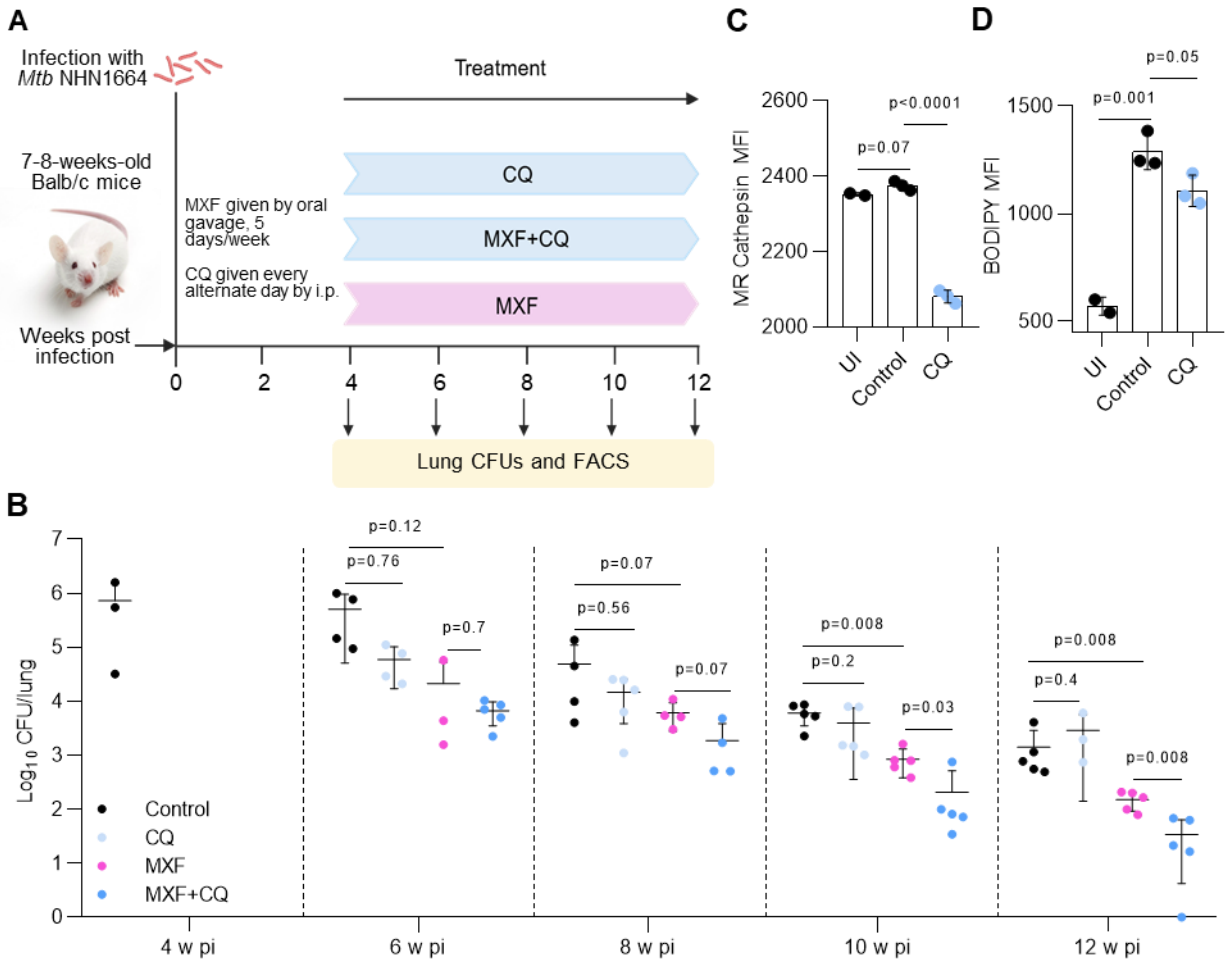

Fig. S11. CQ marginally increases activity of MXF against *Mtb* NHN1664 in BALB/c mice. (**A**) Strategy to investigate the efficacy of CQ in reducing tolerance against MXF *in vivo* and measure acidity and LDs of murine lung cells across experimental groups. BALB/c mice ($n \geq 3$) were given an aerosol challenge with *Mtb* NHN1664. From 4 weeks p.i. onward, groups of mice were left untreated or treated with 25 mg/kg MXF alone or in combination with 10 mg/kg CQ. (**B**) Bacterial CFUs were measured in the lungs at the indicated time points, and the statistical analysis was done using the Mann-Whitney test. (**C-D**) At 12 weeks p.i., a single cell suspension of the lungs of BALB/c mice ($n \geq 2$) of the indicated treatment groups was stained with either MR Cathepsin (**C**) or BODIPY (**D**) and analysed by flow cytometry. The *p*-values were determined using a Student's two-tailed *t*-test with Welch's correction.