Ornithine-A urea cycle metabolite enhances autophagy and controls *Mycobacterium tuberculosis* infection

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Supporting Information
Supplementary Fig. 1: The yield of alveolar, peritoneal, liver macrophages and bone marrow-derived monocytes. Representative dot plots depicting the purification of AMs, PMs, KCs and BDMs. The expression of two markers commonly expressed on AMs (F4/80 and CD11c), KCs (F4/80 and CD68) and BDMs (CD11b and Ly6C), was analyzed by flow cytometry, resulting in (A) 95% pure AMs. (B) approximately 94% pure PMs (C) 90% pure KCs and (D) 94% pure BDMs. The experiments were performed five times and they all yielded similar results. **Gating strategies used for all FACS figures.** (A) Alveolar macrophages (B) Peritoneal macrophages (C) Kupffer cells. The same gating strategy used for Fig. 2E, F and J.
Supplementary Fig. 2: ROS production, NO levels and arginase activity in *Mtb*-infected site-specific macrophages. AMs, PMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv at an MOI of 1:2.5. After 24 h, macrophages were labeled with 20 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) and their ROS levels were evaluated by flow cytometry. (A) A representative flow cytometry plot for DCFDA staining is shown. (B) Mean fluorescence intensity (MFI) of ROS production is shown. p<0.0025, p<0.0028. n=5 experiments. (C-D) AMs, PMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv at an MOI of 1:2.5. After 72 h, (C) The NO levels (in the form of nitrate and nitrite) in the culture supernatants were measured. The NO concentrations were determined by spectrophotometric analysis at 540 nm with extrapolation from the standard curve and expressed as µM (mean ± SD). Data are representative of five independent experiments. p<0.0042, p<0.0072, p<0.0049. (D) Arginase activity measured by the conversion of L-arginine into L-ornithine in the cell lysates and determined by spectrophotometric analysis at 570 nm with extrapolation from the standard curve and expressed as nmol/mg of protein (mean ± SD). p<0.0029. Statistical analysis was performed using paired two-tailed t test. Data are representative of five independent experiments.
Supplementary Fig. 3: *Mtb* H37Rv-infected alveolar, peritoneal and liver macrophages are equally apoptotic. AMs, PMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv at an MOI of 1:2.5. After 72 h, apoptotic cells were determined by the Phosphatidyl-serine exposure assay. A representative flow cytometry plot for Annexin-V/PI staining is shown.
Supplementary Fig. 4: *Mtb*-infected liver macrophages have increased levels of autophagy compared to *Mtb*-infected alveolar and peritoneal macrophages. AMs, PMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv at an MOI of 1:2.5. After 72 h, (A) total protein was extracted from the above cells and western blotting was performed. A representative blot depicting the levels of LC-3, ATG-5, ATG-7 and Beclin-1 in the control and *Mtb* H37Rv-infected macrophages is shown. (B) Normalized fold intensities of LC-3B, ATG-5, ATG-7 and Beclin-1 levels in *Mtb* H37Rv-infected macrophages relative to the levels in the uninfected control. p<0.0051. (C) LC-3B, ATG-5, p<0.0022, p<0.0074, p<0.0039; ATG-7, p<0.0026 and Beclin-1, p<0.0044 gene expression was determined by quantitative real-time PCR. n=5 experiments. Data are presented as the mean ± SD and analyzed using paired two-tailed t test.
Supplementary Figure. 5: *Mtb*-infected liver macrophages have increased levels of autophagy. AMs, PMs, and KCs from C57BL/6 mice were infected with *Mtb* H37Rv. After 72 h, the immunofluorescence intensities (A.U.) of LC-3B, *p*<0.0028, 0.0015; ATG-5, *p*<0.0028; ATG-7, *p*<0.0073, *p*<0.0039; and Beclin-1, *p*<0.0039, *p*<0.0048 calculated for each group. n=5 experiments. Data are presented as the mean ± SD and analyzed using paired two-tailed t test.
Supplementary Fig. 6: siRNA efficacy on autophagy molecules. KCs from C57BL/6 mice were isolated and transfected with siRNAs targeting LC-3B, ATG-5, ATG-7 and Beclin-1 or with control siRNA and then infected with *Mtb* H37Rv at an MOI of 1:2.5. Total protein was extracted, and western blotting was performed. A representative blot depicting the efficiencies of the siRNAs for LC-3B, ATG-5, ATG-7 and Beclin-1 in *Mtb* H37Rv-infected KCs is shown.
Supplementary Fig. 7: Autophagy is involved in the restriction of *Mtb* growth in alveolar and peritoneal macrophages. AMs and PMs from C57BL/6 mice were isolated and transfected with siRNAs targeting LC-3B, ATG-5, ATG-7 and Beclin-1 or with control siRNA and then infected with *Mtb* H37Rv at an MOI of 1:2.5. After 5 days, CFUs were counted. AMs (p<0.015, p<0.0232, p<0.0277) and KCs (p<0.0323, p<0.0015, p<0.0051) transfected with control siRNA shown p values compared to LC-3B, ATG-5, ATG-7 and Beclin-1 transfected siRNAs. Data are presented as mean ± SD using paired two-tailed t test from five independent experiments.
Supplementary Fig. 8: Differentially generated metabolites in alveolar and liver macrophages up on *Mtb* H37Rv infection. AMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv. After 72 h, cell lysates were analyzed using LC-MS. A representative score plot of the partial least squares discriminant analysis (PLS-DA) was generated using MetaboAnalyst. PLS-DA models were validated using R$^2$ and Q$^2$ based on LOOCV (leave one out cross-validation); the four-component model was selected as the optimized model with R$^2$=0.95 and Q$^2$=0.58. The significance of the model was demonstrated by a permutation test with 100 testing iterations using a separation distance of p<0.01. (A) AMs control (red); AMs *Mtb* H37Rv (green); KC control (dark blue); KC *Mtb* H37Rv (light blue). (B) Heat map representation of the metabolic changes in AMs and KCs up on *Mtb* infection compared with the control. Each row represents a single metabolite detected in the study. Color differences demonstrate the relative concentrations of metabolites across the different groups. Individual samples (horizontal axis) and metabolites (vertical axis) are separated using hierarchical clustering (Ward’s algorithm), with the dendrogram scaled to represent the distance between each branch (Pearson’s correlation). From each group, five biological samples were analyzed. Dark-orange color in the tile indicates high levels, and dark blue indicates low levels.
Supplementary Fig. 9: Quantification of ornithine and imidazole levels in various macrophages and monocytes up on *Mtb* infection. AMs, PMs, BDMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv. After 72 h, the cell lysates were quantified for ornithine (p<0.0093, p<0.0093, p<0.0034) and imidazole (p<0.0124, p<0.0133, p<0.0243) levels using LC-MS and expressed as µM. IL-4 treated macrophages and monocytes were used as positive control for ornithine levels. Statistical analysis performed using paired two-tailed t test and expressed as mean ± SD. Data are representative of three independent experiments.
Supplementary Figure. 10: Effect of ornithine and imidazole on autophagy. AMs were infected with Mtb H37Rv. At 2 h post-infection, ornithine or imidazole were added. After 72 h, the immunofluorescence intensities (A.U.) of LC-3B, ATG-5, ATG-7 and Beclin-1 are shown. ns: not significant. Data shown are representative of five independent experiments. Data presented as the mean ± SD and analyzed using paired two-tailed t test.
Supplementary Figure. 11: Ornithine reduces ammonia levels and enhances AMPK mediated autophagy. AMs and KCs from C57BL/6 mice were isolated and infected with *Mtb* H37Rv at an MOI of 1:2.5. After 72 h, the immunofluorescence intensities (A.U.) of LC-3B, p<0.0163, p<0.0011; ATG-5, p<0.0415; ATG-7, p<0.0273 and Beclin-1, p<0.0065 in AMs and KCs (p<0.0155, p<0.0122, p<0.0214, p<0.0147) are shown. n=5 experiments. Data presented as the mean ± SD and analyzed using paired two-tailed t test.
Supplementary Fig. 12: Arginase inhibitor reduces autophagy-relevant genes expression in liver macrophages.

KCs from C57BL/6 mice were isolated and infected with Mtb H37Rv at an MOI of 1:2.5. At 2 h post-infection, ornithine and compound C were added at indicated concentrations. After 72 h, LC-3B, ATG-5, ATG-7 and Beclin-1 gene expression was determined by quantitative real-time PCR. Statistical analysis performed using paired two-tailed t test and expressed as mean ± SD. Data are representatives of five independent experiments.
Supplementary Fig. 13: Imidazole directly inhibits *Mtb* growth in a concentration-dependent manner by reducing cytochrome P450 monooxygenase (CYP). (A) Growth curves of *Mtb* H37Rv cultures with or without ornithine and imidazole over the course of 5 days generated by OD_{600} measurements. Each data point corresponds to the average of five measurements ± standard deviation. (B) Total RNA was extracted from *Mtb* H37Rv cultures with or without imidazole on the 3rd and 4th days and 20 mRNA expression of CY P450 genes were determined by quantitative real-time PCR. Heat map representation of CYP gene expression in imidazole treated *Mtb* H37Rv cultures on the 3rd (D3) and 4th days (D4) compared with the levels in the absence of imidazole and normalized with 16S rRNA. Each row represents a single CYP gene detected. Color differences demonstrate the relative expression of the CYP genes. Row Z-score scaled from −1 to +1 and is illustrated in green, black, and red colors. Red color indicates higher expression and green color indicates lower expression of CYP genes. Data are representatives of three independent experiments using two-way ANOVA and mean value of log fold change is shown.
Supplementary Fig. 14: Quantification of ornithine or imidazole levels in the lungs of *Mtb*-infected mice. C57BL/6 mice were infected with 100 CFU of aerosolized *Mtb* H37Rv and then treated with ornithine and imidazole at varying concentrations. (A) Schematic representation of *Mtb*-infected and PBS or ornithine or imidazole treated mice euthanized for respective metabolite quantification at different time intervals. (B) Concentrations of ornithine or imidazole in lung homogenates were evaluated after the administration by using LC-MS and are expressed as µM. Statistical analysis was performed using paired two-tailed t test and shown as mean ± SD. Data are representative of three independent experiments. Five mice per group were used for each independent experiment.
Supplementary Fig. 15: Schematic representation of urea cycle metabolite ornithine in *Mtb* growth restriction. Red color indicates inhibitory line and green arrows indicates activation.
| Pathway name                                                                 | Hits/Total | P        | Holm adjust | FDR  |
|-----------------------------------------------------------------------------|------------|----------|-------------|------|
| Purine Metabolism (Adenosine, Adenosine mono phosphate, guanine, Hypoxanthine, inosinic acid, L-aspartic acid, Inosine, xanthine, Guanosine mono phosphate) | 9/74       | 2.65E-5  | 0.00259     | 0.00259 |
| Aspartate Metabolism (Adenosine mono phosphate, inosinic acid, L-aspartic acid, N-Acetyl-L-aspartic acid) | 4/35       | 0.00869  | 0.843       | 0.426 |
| Methionine Metabolism (Betaine; Adenosine monophosphate, Adenosine, L-serine) | 4/43       | 0.0179   | 1           | 0.49 |
| Glutamate Metabolism (Adenosine monophosphate, Glutathione, L-Aspartic acid, Guanosine monophosphate) | 4/49       | 0.0278   | 1           | 0.49 |
| Selenoamino Acid Metabolism (Adenosine monophosphate; Adenosine; L-Serine) | 3/28       | 0.028    | 1           | 0.49 |
| Urea cycle (L-Aspartic acid; Ornithine)                                     | 2/29       | 0.0309   | 1           | 0.49 |
| Arginine and Proline Metabolism (Adenosine monophosphate; L-Proline; L-Aspartic acid; Ornithine) | 4/63       | 0.036    | 1           | 0.49 |
| Ammonia Recycling (Adenosine monophosphate; L-Serine; L-Aspartic acid)     | 3/32       | 0.04     | 1           | 0.49 |
| Glycine and Serine Metabolism (Betaine; adenosine monophosphate; L-Serine; Ornithine) | 4/69       | 0.0507   | 1           | 0.552 |
| Riboflavin Metabolism (Adenosine monophosphate; Riboflavin)                | 2/20       | 0.0831   | 1           | 0.807 |
| Betaine Metabolism (Betaine; Adenosine)                                    | 2/21       | 0.095    | 1           | 0.807 |
| Phenylalanine and Tyrosine Metabolism (Adenosine monophosphate; L-Phenyl Alanine) | 2/28       | 0.147    | 1           | 1    |
| Biotin Metabolism (L-lysine)                                               | 1/8        | 0.18     | 1           | 1    |
| Homocysteine Degradation (L-serine)                                        | 1/9        | 0.2      | 1           | 1    |
| Phenylacetate Metabolism (Adenosine Mono Phosphate)                        | 1/9        | 0.2      | 1           | 1    |
| Thiamine Metabolism (Adenosine mono Phosphate)                             | 1/9        | 0.2      | 1           | 1    |
| Malate-Aspartate Shuttle (L-Aspartic acid)                                 | 1/10       | 0.22     | 1           | 1    |
| Pyruvaldehyde Degradation (Glutathione)                                    | 1/10       | 0.22     | 1           | 1    |
| Phosphatidylethanolamine Biosynthesis (L-Serine)                            | 1/12       | 0.258    | 1           | 1    |
| Pyruvate Metabolism (Adenosine mono Phosphate; Glutathione)                | 2/48       | 0.329    | 1           | 1    |
Table 2. List of the *Mycobacterium tuberculosis* (Mtb) Cytochromes P450 primers used for real-time polymerase chain reaction

| Mtb Cyp gene | Sense                   | Anti-sense               |
|--------------|-------------------------|--------------------------|
| Cyp 51 (Rv0764c) | 5'-CTACGTCGACCGGTATCTGC-3' | 5'-CGGTTGATGTGTTCCCGAAC-3' |
| Cyp 138 (Rv0138)  | 5'-CACGTCATGCTATGTTTCG-3' | 5'-GGGATGCCATGTATCATGGG-3' |
| Cyp 136A1 (Rv0327c) | 5'-CAATAGCGACACGACAAG-3' | 5'-GGCCATGAGAACACAGGA-3' |
| Cyp 135B1 (Rv0568) | 5'-CACCATTAGAGGGTTGTCAC-3' | 5'-TCCAGAGCGATTGATCAGC-3' |
| Cyp 123 (Rv0766c)  | 5'-CGCTGTCAGCTAACGGGAA-3' | 5'-TCCAGGGATACCAGGCAAC-3' |
| Cyp 126 (Rv0778)  | 5'-CCGATTTGCTATGCAAGTG-3' | 5'-GCCGTTAGGTGATACCGCAG-3' |
| Cyp 130 (Rv1265c)  | 5'-CGACTAAGCTGCTGCTG-3' | 5'-CAATGATTCACAGCTGCGA-3' |
| Cyp 132 (Rv1384c)  | 5'-TTCTGCTGCGATGTAATTCG-3' | 5'-CGGTGATGTTGTGCACGAT-3' |
| Cyp 139 (Rv1666c)  | 5'-TGATCTTTGGGTGACATCC-3' | 5'-AATACGAGTCATCTAGGGG-3' |
| Cyp 144 (Rv1777)  | 5'-CGTTTATGTTGCGGATCC-3' | 5'-GGGCGATGTTGATCAGGCG-3' |
| Cyp 143 (Rv1785c)  | 5'-TTCTGCAATGCTTACATCC-3' | 5'-GCCGATCCAGAAGACGAAACA-3' |
| Cyp 140 (Rv1880c)  | 5'-TCACGGAAATTTGGGCAG-3' | 5'-GATATCCGATGATCCCCGC-3' |
| Cyp 124 (Rv2266)  | 5'-GTGACAGAGGCTCTTGCGG-3' | 5'-TCCAGGGATGCGTATGCAT-3' |
| Cyp 128 (Rv2268c) | 5'-AGGGATGACACTGTTCTCGG-3' | 5'-GATTGCAGTAGCGGACGC-3' |
| Cyp 121 (Rv2276)  | 5'-CGGAAGAGTCCCGCCTACT-3' | 5'-GAAATTTGCAGCGTGATAC-3' |
| Cyp 136 (Rv3059)  | 5'-CCTAGAGGCGCCGGATCG-3' | 5'-GAGTGGATGCTTCAAGATT-3' |
| Cyp 141 (Rv3121)  | 5'-GAGAAGACCTTGAGTCCGA-3' | 5'-GAAACTCATCTGGGACTACC-3' |
| Cyp 142 (Rv3518c) | 5'-GGATACCGAGCTCTCAGGCA-3' | 5'-AAGTGGATGCGGTCAGAAG-3' |
| Cyp 125 (Rv3545c) | 5'-TCTGGCGCATCAGGAAAC-3' | 5'-AGCCGGCCAGAGATCTTGTG-3' |
| Cyp 137 (Rv3665c)  | 5'-ATGCATAATCGGGATTTGGA-3' | 5'-CAAGATTCAACACCACCGCTCG-3' |
| 16S rRNA       | 5'-GTCAGTCTGGAGGAGTG-3' | 5'-CGGCGATTGTAGCATGTG-3' |
