

Divergent downstream biosynthetic pathways are supported by L-cysteine synthases of *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis's (*Mtb*) autarkic lifestyle within the host involves rewiring its transcriptional networks to combat host-induced stresses. With the help of RNA-seq performed under various stress conditions, we identified that genes belonging to *Mtb* sulfur metabolism pathways are significantly upregulated during oxidative stress. Using an integrated approach of microbial genetics, transcriptomics, metabolomics, animal experiments, chemical inhibition, and rescue studies, we investigated the biological role of non-canonical L-cysteine synthases, CysM and CysK2. While transcriptome signatures of *RvΔcysM* and *RvΔcysK2* appear similar under regular growth conditions, we observed unique transcriptional signatures when subjected to oxidative stress. We followed pool size and labelling (^{34}S) of key downstream metabolites, viz. mycothiol and ergothioneine, to monitor L-cysteine biosynthesis and utilization. This revealed the significant role of distinct L-cysteine biosynthetic routes on redox stress and homeostasis. CysM and CysK2 independently facilitate *Mtb* survival by alleviating host-induced redox stress, suggesting they are not fully redundant during infection. With the help of genetic mutants and chemical inhibitors, we show that CysM and CysK2 serve as unique, attractive targets for adjunct therapy to combat mycobacterial infection.

eLife assessment

Sulphur atoms derived from cysteine are thought to play significant roles in maintaining redox homeostasis in *Mycobacterium tuberculosis*, which encounters stresses associated with immune cell interactions. In this **valuable** manuscript, the authors provide **solid** evidence that the genes encoding cysteine biosynthetic enzymes (cysM and cysK2) are required to maintain full viability of *M. tuberculosis* under in vitro stress conditions, macrophage infections, and within the lung tissues of mice. The manuscript presents transcriptomic and metabolomic evidence to support the hypothesis that CysM and CysK2 play distinct roles in maintaining cysteine-derived metabolite pools under stress conditions. The work will be of interest to microbiologists in general.

Introduction

Mycobacterium tuberculosis (*Mtb*) continues to stride as the number one killer among all infectious diseases, accounting for nearly 1.5 million deaths yearly. The aggravating situation is despite the clinical use of over 20 antibiotics and a century-old vaccine, BCG. The gradual rise in the emergence of increasingly drug-resistant strains and HIV-TB co-infection further highlights the urgency to identify newer attractive drug targets. Throughout the course of infection, *Mtb* is exposed to a continuum of dynamic host-induced stresses such as severe nutrient deprivation, acidified compartments, and toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by its resident phagosomes. In turn, *Mtb* produces copious amounts of actinomycetes-specific mycothiol, the major antioxidant in actinomycetes that act as the functional equivalent of glutathione, to combat ROS and RNS. In addition to mycothiol, *Mtb* also produces ergothioneine, a low molecular weight thiol, and several enzymes that act concertedly to subvert host-induced redox stress. The redox-active group of both mycothiol and ergothioneine is derived from L-cysteine. Hence, genes involved in the biosynthesis of L-cysteine are upregulated in the host and *in vitro* upon oxidative and nutritional stress [1–5]. Notably, an increased expression of these genes is functionally crucial, as suggested by the attenuated survival of transposon mutants of many sulfur and L-cysteine biosynthesis genes within the host [6].

In mycobacteria, sulfur assimilation begins with the import of sulfate through a sulfate transporter composed of SufI.CysT.W.A. Intracellular sulfate is a substrate for APS synthase CysD.N.C, which adenylates and phosphorylates sulfate to form adenosine 5'-phosphosulfate (APS). APS sits at a metabolic branch point; it can either be converted into sulfolipids by consequent actions of multiple Stfs enzymes or reduced via SirA and SirH to sulfide (Figure S1). This pathway encompasses sulfide formation from sulfate, called the sulfur assimilation pathway. *Mtb* genome encodes three L-cysteine synthases – the canonical CysK1 and non-canonical CysM and CysK2 enzymes. Interestingly, humans do not possess L-cysteine synthases, raising the possibility of developing antibiotics without a homologous target in the host. CysK1 utilizes sulfide produced via the sulfur assimilation pathway and O-acetyl-L-serine produced from glycolytic intermediate 3-phosphoglycerate to produce L-cysteine. CysM, on the other hand, uses O-phospho-L-serine and a small sulfur carrier protein CysO as substrates. Like CysK1, CysK2 utilizes O-phospho-L-serine and sulfide as substrates (Figure S1). In addition, *Mtb* can also synthesize L-cysteine through a reverse transsulfuration pathway from L-methionine. This example of convergent metabolic redundancy raises several interesting questions: (1) Why would *Mtb* rely on multiple enzymes and pathways to produce the same biomolecule? (2) Are these “functionally redundant” enzymes

dispensable, or are they required at a distinct cellular space, time, and condition? (3) Is the L-cysteine pool produced through a particular pathway functionally compartmentalized? That is, is it metabolized into a specific kind of downstream thiol?

To define these unsolved aspects of *Mtb* L-cysteine metabolism, we sought to investigate the interplay of non-canonical L-cysteine synthases of *Mtb* and elucidate their roles in abetting virulence. We aimed to decipher the relative contribution of CysM and CysK2 enzymes in alleviating host-induced stresses and promoting the survival of *Mtb* within the host. We also investigated their role in secondary metabolism, synthesizing low molecular weight thiols, such as mycothiol and ergothioneine, and understanding the consequential effects of their deletion on the global transcriptome of *Mtb*. Lastly, with the help of specific inhibitors, we evaluated their potential to serve as attractive drug targets for adjunct antibiotic therapy.

Results

Non-canonical L-cysteine synthases facilitate *Mtb* in combating host-induced stresses

Mtb is a generalist, a prototroph organism that can produce all 20 proteinogenic amino acids. In agreement with this notion, numerous microarray studies depict the upregulation of multiple amino acid pathways within the host [3, 4, 7], indicating a higher dependency of *Mtb* survival and virulence on amino acid biosynthesis and regulation. Beyond their role in protein synthesis, amino acids such as tryptophan, lysine, and histidine interact with the host immune cells to facilitate mycobacterial survival within the host [8, 9]. We sought to identify distinct host stresses that result in the transcriptional modulation of specific amino acid biosynthetic pathways with the help of RNA sequencing (RNA-Seq). We compared the transcriptional profile of *H37Rv* (*Rv*) grown in 7H9-ADC with the profiles obtained when bacilli were subjected to oxidative, nitrosative, starvation, and acidic stresses (Table S1). The volcano plot illustrates differentially expressed genes (DEGs) that were significantly upregulated (blue) and downregulated (red) under indicated stress conditions (absolute $\log_2$ Fold change > 1 and P_{adj} < 0.05) (Figure S2a-e). Exposure to starvation conditions resulted in drastic transcription modulation compared with other stresses, suggesting that nutrient deficiency is the primary driver of transcriptome remodeling. In contrast, we observed the lowest number of DEGs when the bacteria were subjected to mildly acidic conditions (pH 5.5). Heat maps of normalized DEGs depict that DEG changes were comparable across biological replicates within each sample set (Figure S2f-j).

To further refine our understanding of the DEGs, we grouped them into various functional categories and found that genes belonging to intermediary metabolism & respiration remained the most affected in all conditions highlighting their role of metabolic rewiring (Figure S3a). Pathway enrichment analysis of the most enriched Gene Ontology (GO) further revealed that, while, expectedly, metabolic pathways were found to be downregulated during starvation, we observed enrichment of nitrogen metabolism (Figure S3b & Table S2). Surfactant stress resulted in the upregulation of branched-chain amino acids/keto acids degradation pathways (Figure S3c), and nitrosative stress ensued up-regulation of fatty acid and lipid biosynthetic processes (Figure S3d). There were no changes observed in mildly acidic conditions (Figure S3e). Importantly, oxidative stress resulted in significant up-regulation of genes involved in sulfur metabolism (Figure S3f). We further analyzed the DEGs involved in sulfur and L-cysteine metabolism across sample sets and discerned an overlap of the genes affected under two or more conditions. Interestingly, we observed upregulation of sulfate transporters genes (*subI*, *cysT*, *cysW*, *cysA1*) across multiple stresses and sulfur assimilation (*cysH*, *cysA2*, *cysD*, *cysNC*) during starvation, oxidative and SDS stress. CysK2, a non-canonical L-cysteine synthase, was found to be up-regulated during all stresses except SDS stress (Figure 1a).

Thus RNA-Seq data suggest that sulfur assimilation and L-cysteine biosynthetic pathway genes are the most commonly upregulated pathways in *Mtb* exposed to multiple host-like stresses (**Figure S2**). To address the functional relevance of this observation, we deleted two non-canonical L-cysteine synthases-CysM [5] and CysK2, from the *Mtb* chromosome. CysK2 mutant, *RvΔcysK2*, was generated using the recombineering method, and the recombination at the native loci was confirmed with the help of multiple PCRs (**Figure 1b**). While deletion of *cysM* or *cysK2* did not affect mycobacterial growth under *in vitro* nutrient-rich 7H9-ADC or 7H9-ADS (**Figure S4a** and **S4b**), it significantly compromised *Mtb* growth in nutrient-limiting Sauton's media ($\sim 2 \log_{10}$; **Figure 1c**), suggesting the importance of *cysM*- and *cysK2*-derived L-cysteine. Restoring *cysM* or *cysK2* expression in the complementation strains, *RvΔcysM::M* and *RvΔcysK2::K2*, rescued the growth defects (**Figure S4** & **Figure 1c**). *Mtb* is a metabolically versatile organism capable of utilizing a large variety of carbon and nitrogen sources [10]. Unlike 7H9, wherein glucose, glycerol, glutamate, and ammonia act as carbon and nitrogen sources, Sauton's media contains glycerol and asparagine as the sole carbon and nitrogen sources, suggesting metabolic reprogramming aided by CysM and CysK2 enable *Mtb* to grow optimally in a limited nutritional environment. This observation was further recapitulated under nutrient starvation (PBS), wherein the survival of *RvΔcysM* and *RvΔcysK2* was lower than parental *Rv* or *RvΔcysM::M* or *RvΔcysK2::K2* strains (**Figure 1d**). Interestingly, when exposed to acidic conditions (pH 4.5), *RvΔcysM* and *RvΔcysK2* survival were observed to be ~ 0.85 and $\sim 0.24 \log_{10}$ lower, respectively, compared with *Rv*, suggesting CysM is relatively more important for bacillary survival under acidic stress (**Figure 1e**). The highest attenuation of *RvΔcysM* and *RvΔcysK2* was observed upon the addition of cumene hydroperoxide (CHP), an organic hydroperoxide that, upon decomposition, generates free radicals. The relative survival of *RvΔcysM* and *RvΔcysK2* was ~ 2.04 and $\sim 1.31 \log_{10}$ lower, respectively, compared to *Rv* (**Figure 1f**). The addition of diamide, which results in thiol oxidation, also attenuated the survival of *RvΔcysM* and *RvΔcysK2* by ~ 1.33 and $\sim 1.26 \log_{10}$ compared with *Rv* (**Figure S4c**). When the mutant strains were subjected to nitrosative stress, the survival of *RvΔcysM* and *RvΔcysK2* was ~ 0.93 and $\sim 0.86 \log_{10}$ lower than *Rv* (**Figure 1g**). However, no significant attenuation was found during reductive and surfactant stress (**Figure 1h-i**). Collectively, *RvΔcysM* and *RvΔcysK2* displayed increased susceptibility towards oxidative, nitrosative, mild acidification, and PBS starvation to varying degrees compared with *Rv*, *RvΔcysM::M* and *RvΔcysK2::K2*. The data suggests that L-cysteine, produced via CysM and CysK2, and its downstream products help mycobacteria thwart specific stresses that *Mtb* encounters within the host.

Distinct roles of CysM and CysK2.C

To decipher the mechanism through which CysM and CysK2 combat oxidative stress, we performed a global transcriptomic analysis of *Rv*, *RvΔcysM*, and *RvΔcysK2* in the presence and absence of oxidative stress (CHP) (Table S3). Principal Component Analysis demonstrated clear separation of strains under different conditions. Intriguingly, while *RvΔcysM* and *RvΔcysK2* were closely located on a PCA plot in the absence of any stress, oxidative stress resulted in a significant divergence between the two groups (**Figure 2a**). Deletion of *cysM* resulted in differential expression of 322 genes (159 downregulated and 163 upregulated) (**Figure S5a-b**), while deletion of *cysK2* impacted 278 genes (155 downregulated and 123 upregulated) under regular growth conditions (**Figure S5c-d**) (absolute $\log_2$ Fold change > 1 and $\text{Padj} < 0.05$). In contrast, upon treatment with CHP, nearly $\sim 33\%$ and $\sim 53\%$ of *Mtb* genes were differentially expressed in *RvΔcysM* and *RvΔcysK2*, respectively, compared with *Rv* (**Figure 2b-c**). To understand the individual contribution of CysM and CysK2 in combating oxidative stress, we compared the DEGs of *RvΔcysK2* to *RvΔcysM* under regular growth and oxidative stress conditions. While DEGs between *RvΔcysK2* and *RvΔcysM* were limited to 13 in untreated conditions (**Figure 2d**), CHP treatment resulted in differential expression of 1372 genes (**Figure 2e** and **Figure S5e**), highlighting unique transcriptional signatures associated with each L-cysteine synthase under oxidative stress. Subsequently, we compared the DEGs of *Rv*, *RvΔcysM*, and *RvΔcysK2* obtained under oxidative stress with the help of a Venn diagram. This analysis revealed that CysM and CysK2 influence a shared repertoire of 1023 genes (529 upregulated and 494 downregulated) compared to *Rv* upon

CHP treatment (**Figure 2f**). Importantly, CysM and CysK2 distinctively modulate 324 and 1104 genes, respectively, during oxidative stress (**Figure 2f-g**). Assortment of DEGs into functional classes revealed intermediary metabolism & respiration and cell wall pathways among the most impacted categories (**Figure 2h**). These results corroborate the recent finding that CysK2 alters the phospholipid profile of the *Mtb* cell envelope [11]. Pathway enrichment analysis of the most enriched Gene ontology biological process indicated that while most pathways are commonly affected by CysM and CysK2, these L-cysteine synthases also have a unique transcriptional footprint (Table S4). Pathways such as DNA binding, homologous recombination, translation, ribosome, etc., were commonly affected in *RvΔcysM* and *RvΔcysK2* compared to *Rv* during oxidative stress (**Figure S5f, g**). Genes belonging to oxidative phosphorylation and quinone binding were upregulated, while DNA binding and cholesterol catabolism gene categories were found to be downregulated in *RvΔcysM* compared to *RvΔcysK2* during oxidative conditions (**Figure 2i**). Together, our data point to the unique roles of CysM and CysK2, as their deletion differentially affects various cellular pathways under oxidative stress.

Key metabolites are differentially affected upon CysM and CysK2 deletion

L-Cysteine concentration in *Mtb* is notoriously low, usually 10-fold lower than most other amino acids [12]. As expected, L-cysteine levels were below the limit of detection/quantification (not shown). Therefore, we used key downstream metabolites to monitor L-cysteine biosynthesis and utilization. We followed pool size and labelling (^{34}S) of L-methionine, mycothiol/mycothione, and ergothioneine, made from L-cysteine, via three distinct and dedicated metabolic pathways (**Figure S6**). First, L-methionine, mycothiol, and ergothioneine concentrations vary from zero to 24 h, indicating that their pool sizes are not as stable as those of other core metabolites. This is consistent with their function, susceptibility to redox homeostasis, and L-methionine's role in protein synthesis. Second, upon challenge with CHP for 24 h, L-methionine levels decrease in *Rv*, remain constant in *RvΔcysK2*, and increase in *RvΔcysM* (**Figure 3a**). Ergothioneine levels are identical in the three strains, and upon treatment with CHP, they are decreased in the parent strain but remain unchanged in the *RvΔcysK2* and *RvΔcysM* mutant (**Figure 3b**). Mycothiol levels increase in the *Rv*, *RvΔcysK2* strains and remain nearly constant in *RvΔcysM* (**Figure 3c**). Finally, mycothione levels increased slightly in the parent strain, decreased in the *RvΔcysK2*, and remained stable in the *RvΔcysM* strain (**Figure 3d**). These changes revealed a significant role of distinct L-cysteine biosynthetic routes on redox stress and homeostasis.

Employing ^{34}S labelling, we observed a reduced rate of synthesis of mycothiol, mycothione and ergothioneine in *RvΔcysM* compared to *Rv* and *RvΔcysK2* (**Figure 3e-g**). Interestingly, ergothioneine levels were found to be reduced in both *RvΔcysM* and *RvΔcysK2* compared to *Rv*, possibly underlying one of the reasons for their enhanced sensitivity to oxidative stress.

This result further indicates that these two routes are partially redundant; that is, they generate the same end product, L-cysteine. Yet, pool size measurements demonstrate significant changes, highlighting that while these pathways produce the same metabolite, their complex biological roles and requirements (co-substrates, pathways, regulation, etc.) are not fully redundant. This interpretation is in accordance with transcriptomics and phenotypic results described above. These non-overlapping metabolic requirements (e.g., O-acetyl-L-serine vs. O-phospho-L-serine vs. CysM) are likely the source of the different metabolic phenotypes observed. Therefore, even producing the same end-product, genetic disruption of different L-cysteine synthases differently affects bacterium metabolism and fitness, leading to the distinct phenotypes observed between *RvΔcysK2* and *RvΔcysM* *in vitro*, *in cellulo*, and *in vivo*.

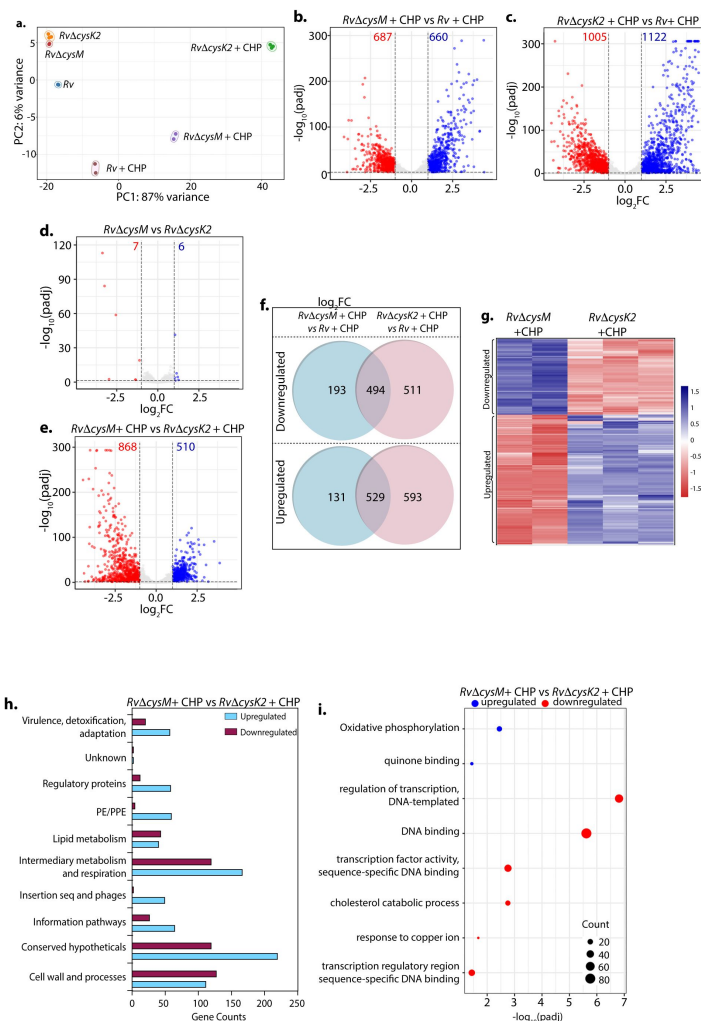


Fig 2

Distinct roles of CysM and CysK2 in attuning cellular processes.

(a) PCA plot demonstrating separation of various bacterial strains under different conditions (b-e) Volcano plots illustrating significantly upregulated (blue) and downregulated (red) genes in indicated strains and conditions with absolute $\log_2$ Fold change ≥ 1 and P_{adj} value < 0.05 . Numbers in the top quadrant highlight the number of significantly upregulated (blue) and downregulated (red) genes in each condition (f) Venn diagram showing the number of significantly downregulated and upregulated DEGs that overlap between indicated strains (g) Heat Maps depicting normalized gene count of differentially expressed genes (DEGs) in independent replicates of *RvΔcysM* and *RvΔcysK2* grown under oxidative conditions with absolute $\log_2$ Fold change ≥ 1 and P_{adj} value < 0.05 . The colour intensity indicates relative upregulated (blue) and downregulated (red) genes compared to the control. (h) Horizontal bar graph depicting the number of DEGs belonging to a particular functional category upon oxidative stress in *RvΔcysM* compared to *RvΔcysK2* under oxidative conditions. (i) Pathway enrichment by DAVID depicting significantly enriched Gene Ontology (GO) biological processes based on DEGs upon oxidative stress in *RvΔcysM* compared to *RvΔcysK2* (absolute $\log_2$ Fold change > 1 and $P_{adj} < 0.05$).

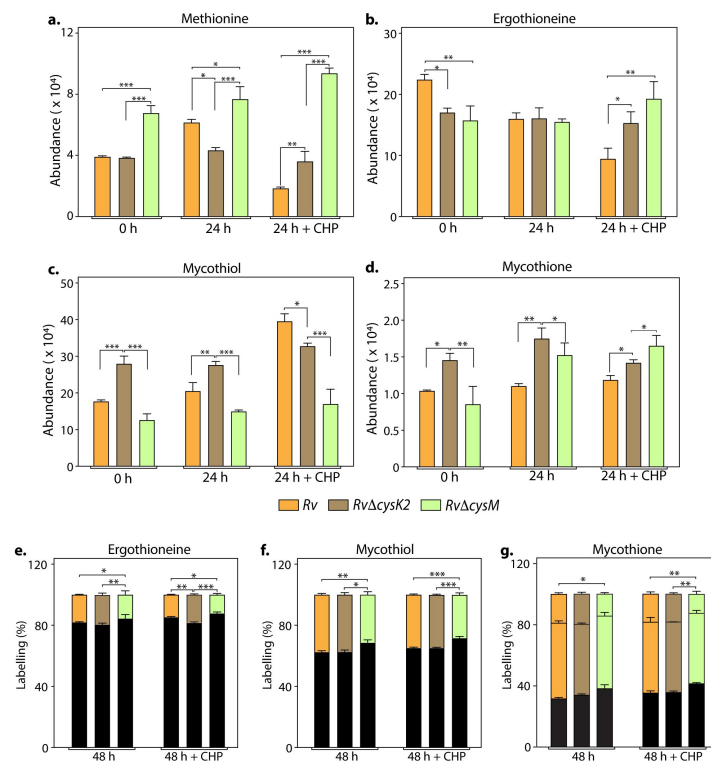


Fig 3

Key metabolites are differentially affected upon CysM and CysK2 deletion

(a-d) Total abundance of L-methionine (a), ergothioneine (b), mycothiol (c), and mycothione (d) in *Rv* with or without CHP treatment. **(e-g)** The percentage of labelled (colored) and unlabeled (black) ergothioneine (e), mycothiol (f), and mycothione (g) in *Rv* with or without CHP treatment at 48 h. Percentage was calculated with respect to 0 h abundance for each replicate. Bars depict the mean of biological replicates (n=3), and error bars represent the standard deviation. The same samples were used for analysis (a-d) and (e-g). Statistical significance was calculated between *Rv* and *RvΔcysM*/*RvΔcysK2*; and between *RvΔcysM* and *RvΔcysK2* using non-parametric t-test. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

CysM and CysK2 alleviate the toxicity of host-produced ROS and RNS

To define whether the attenuation of mutant strains observed during defined *in vitro* host-like conditions can be recapitulated within the host cells, we compared the survival of parental, mutants, and complementation strains in murine peritoneal macrophages (**Figure 4a**). Survival of *RvΔcysM* and *RvΔcysK2* was ~ 1.22 and $\sim 0.85 \log_{10}$ lower, respectively, compared with *Rv* at 96 h post-infection (p.i) (**Figure 4b**). Notably, the addition of L-cysteine alleviated survival differences (**Figure 4c**), suggesting that reduced L-cysteine levels in mutant strains are responsible for their attenuated survival. To further validate that CysM and CysK2 support *Mtb* survival by detoxifying peroxides generated by the host, we compared the survival of strains in the peritoneal macrophages extracted from wild-type C57BL/6 (WT) mice that are proficient in eliciting oxidative and nitrosative stress or murine strains that are deficient in their ability to produce hydrogen peroxide (H_2O_2) (*phox^{-/-}*) or both oxidative and nitrosative stress (*IFN γ ^{-/-}*). As anticipated, regardless of the mice genotype, there was no difference in the survival of *Rv* and complementation strains (**Figure 4d-e**). On the other hand, attenuation of *RvΔcysM* and *RvΔcysK2* observed in wild-type macrophages is partially reversed in the macrophages obtained from *phox^{-/-}* mice (**Figure 4d**) and completely nullified in macrophages isolated from *IFN γ ^{-/-}* mice (**Figure 4e**). To further dissect the relative contribution of CysM and CysK2 in combating ROS and RNS independently or collectively, the survival of *Mtb* strains was examined in peritoneal macrophages isolated from WT and *phox^{-/-}* in the presence or absence of iNOS inhibitor. While the inhibition of ROS or RNS independently only partially alleviated the attenuated survival *RvΔcysM* and *RvΔcysK2*, inhibition of both ROS and RNS by treating peritoneal macrophages isolated from *phox^{-/-}* with iNOS inhibitor completely nullified survival defects of the mutants (**Figure 4f**). These results suggest that non-canonical L-cysteine synthases, CysM and CysK2, play an essential role in combating host-induced redox stress.

CysM and CysK2-derived L-cysteine support mycobacterial survival in vivo

Given the importance of non-canonical L-cysteine synthases in mitigating host-induced redox stress, we next sought to examine whether CysM and CysK2 independently facilitate mycobacterial survival in a murine infection model. We followed disease progression by enumerating colony forming units (CFU) of the lung and spleen of the infected mice at day 1, 4, and 8 weeks p.i (**Figure 5a** & d). CFUs enumerated on day 1 showed equal bacillary deposition across strains in the lungs (**Figure 5b** & e). Compared with *Rv*, survival of *RvΔcysM* was $\sim 1.25 \log_{10}$ lower at 4 weeks p.i, which further attenuated to $\sim 1.71 \log_{10}$ at 8 weeks p.i in the lungs (**Figure 5b**). Similarly, dissemination of *RvΔcysM* in the spleen was $\sim 1.63 \log_{10}$ lower at 4 weeks p.i and $\sim 1.72 \log_{10}$ lower at 8 weeks p.i compared with *Rv* (**Figure 5c**). Similarly, *RvΔcysK2* was $\sim 1.67 \log_{10}$ and $\sim 1.87 \log_{10}$ lower at 4 and 8 weeks p.i, respectively, compared with *Rv* in the lungs (**Figure 5e**) and $\sim 1.63 \log_{10}$ and $\sim 1.72 \log_{10}$ lower at 4 and 8 weeks p.i, respectively in the spleens of infected mice (**Figure 5f**).

To understand whether the ability of CysM to mitigate oxidative and nitrosative stress is linked to its role in facilitating mycobacterial survival *in vivo*, we infected WT, *phox^{-/-}* and *IFN γ ^{-/-}* mice with *Rv*, *RvΔcysM* and *RvΔcysM::M* and analyzed bacillary load at 4 weeks p.i (**Figure 5g**). As shown in **Figures 5h** & 5i, the survival of *RvΔcysM* is impaired in WT mice that produce both ROS and RNS, compared with *phox^{-/-}* and *IFN γ ^{-/-}* mice. The survival defect observed due to the absence of CysM in the lungs was partially rescued in *phox^{-/-}* mice. Due to the lack of ROS and RNS in *IFN γ ^{-/-}* mice, *RvΔcysM* showed higher bacillary load than in *phox^{-/-}* mice (**Figure 5h**). *RvΔcysM* displayed better survival in *phox^{-/-}* and *IFN γ ^{-/-}* mice in the spleen than lungs, which could either be because of relatively lesser ROS and RNS stress or imperiled response in clearing *Mtb* infection (**Figure 5i**). In contrast to the peritoneal macrophage infection experiment (**Figure 4**), attenuated survival of *RvΔcysM* was not completely salvaged in *IFN γ ^{-/-}* mice, suggesting that CysM

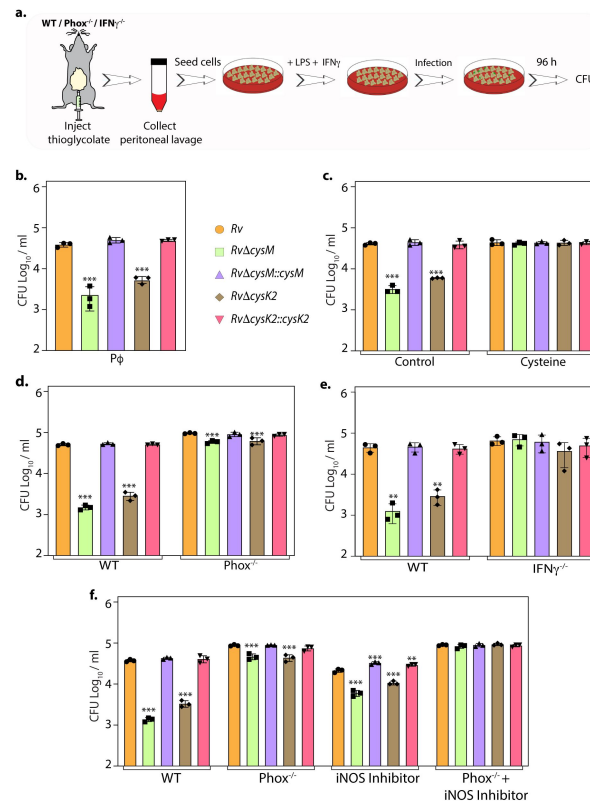


Fig 4

L-cysteine synthases ameliorate mycobacterial survival in response to host-induced oxidative and nitrosative stress

(a) Pictorial representation of peritoneal macrophage infection experiments. Thioglycolate was injected into the peritoneum cavity of C57BL/6, *phox*^{-/-}, *IFN γ* ^{-/-} mice. Four days post-injection, peritoneal macrophages were extracted and activated by treatment with IFN γ overnight and LPS 2 h prior to the infection. In specific cases, iNOS inhibitor 1400 W was added along with IFN γ /LPS. The intracellular bacillary survival was calculated 96 h p.i. **(b)** Peritoneal macrophages from C57BL/6 mice were infected, and intracellular bacillary survival was enumerated 96 h p.i. **(c)** Peritoneal macrophages from C57BL/6 mice were infected, left untreated (control) or treated with 0.2mM L-cysteine at the time of infection and intracellular bacillary survival was assessed 96 h p.i. **(d-f)** Peritoneal macrophages isolated from mice of indicated genotypes were either left untreated or treated with iNOS inhibitor, 1400W. Intracellular bacillary survival was enumerated 96 h p.i. **(b-f)** Data points are presented as CFU log₁₀/ml $\pm$ SD of each replicate (n=3). Statistical significance was drawn in comparison with *Rv* using one-way ANOVA followed by a *post hoc* test (Tukey test; GraphPad prism). ***P* < 0.005; ****P* < 0.0005.

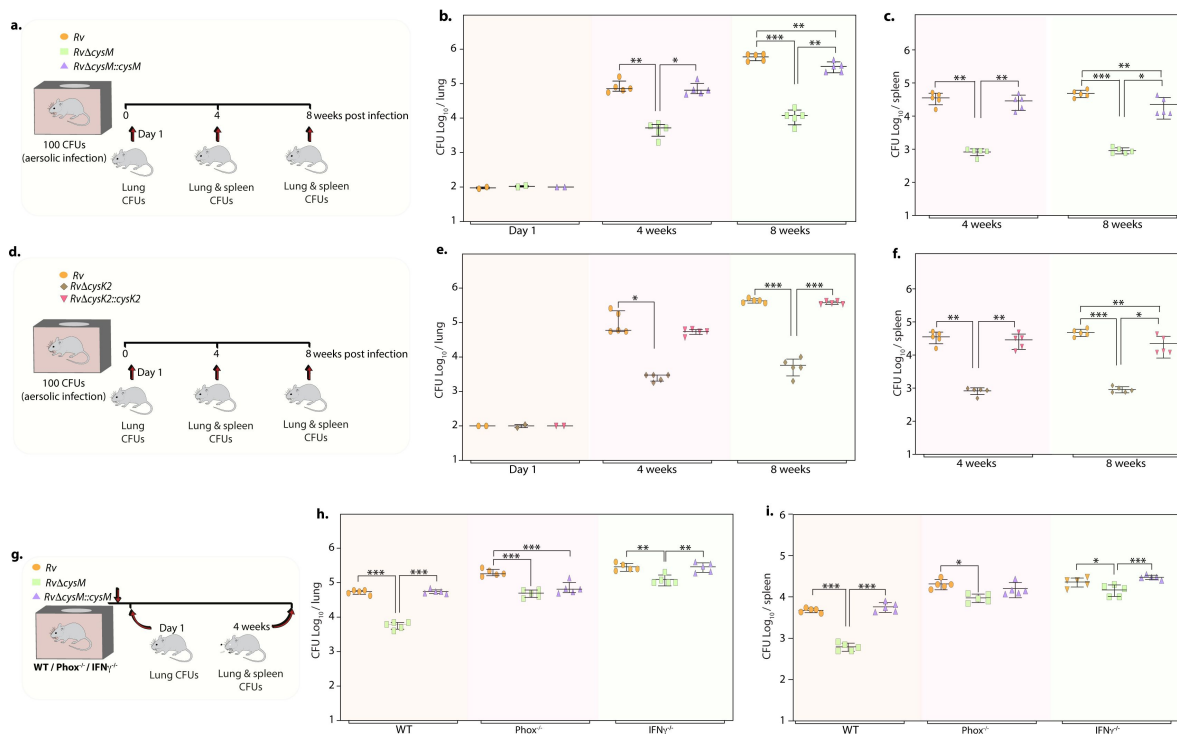


Fig 5

Deletion of non-canonical L-cysteine synthases attenuates mycobacterial survival in murine lungs and spleen

(a) Schematic outline of murine infection experiment. C57BL/6 ($n = 12$ per group) were infected with *Rv*, *RvΔcysM*, or *RvΔcysM::cysM* strains via an aerosol route. CFU was enumerated at day 1 ($n=2$), week 4 ($n=5$) and week 8 ($n=5$). **(b-c)** Each data point represents Log₁₀ CFU in lung (b) and spleen (c) of an infected animal, and the error bar indicates the median with interquartile range for each group. **(d)** Schematic outline of murine infection experiment. C57BL/6 ($n = 12$ per group) were infected with *Rv*, *RvΔcysK2*, or *RvΔcysK2::cysK2* strains via an aerosol route. CFU was enumerated at day 1 ($n=2$), week 4 ($n=5$) and week 8 ($n=5$). **(e-f)** Each data point represents Log₁₀ CFU in the lung (e) and spleen (f) of an infected animal, and the error bar indicates the median with interquartile range for each group. **(g)** Schematic outline of murine infection experiment. C57BL/6 ($n = 7$ per group), *phox^{-/-}* ($n = 7$ per group), *IFNγ^{-/-}* ($n = 7$ per group) were infected with *Rv*, *RvΔcysM*, or *RvΔcysM::cysM* strains via an aerosol route. CFU was enumerated at day 1 ($n=2$) and week 4 ($n=5$). **(h-i)** Each data point represents Log₁₀ CFU in the lung (h) and spleen (i) of an infected animal, and the error bar indicates the median with interquartile range for each group. **(a-i)** Statistical significance was drawn in comparison with *Rv* using one-way ANOVA followed by a *post hoc* test (Tukey test; GraphPad prism). *** $P < 0.0005$.

may also be involved in alleviating additional stresses such as nutrient deprivation and/or IFN γ independent ROS/RNS produced by the host. Data presented suggests that L-cysteine produced through non-canonical pathways are independently important for mycobacterial survival *in vivo*.

L-Cysteine synthase inhibitors can effectively kill *Mtb* within the host

Data presented above demonstrated that CysM and CysK2 might serve as clinically-important targets for adjunct TB therapy. Brunner *et al.* screened a compound library to identify inhibitors of mycobacterial L-cysteine synthases-CysK1, CysK2, and CysM. We selected three compounds - Compound 1 (C1) (named compound 2 in [13]), which inhibits all three synthases; Compound 2 (C2) (compound 6 in [13]), which inhibits both non-canonical L-cysteine synthases and Compound 3 (C3) (compound 31 in [13]), which selectively inhibits CysK1 (Figure 6a & b). To examine the therapeutic potential of these inhibitors, we first tested their effect on the survival of *Rv* within host peritoneal macrophages. Treatment with C1 resulted in ~ 1 log₁₀ attenuation compared with the untreated cells. Killing mediated by C2 or C3 was marginally lower compared with C1 (Figure 6c). Next, we examined the ability of these drugs to enhance the bactericidal activity of INH, a drug whose activity depends on redox state of the cell [14], and therefore could be affected by disruption of L-cysteine downstream pathways. Towards this goal, we first measured the MICs of INH (0.06 μ g/ml) and C1 (0.6 mg/ml), C2 (0.6 mg/ml), and C3 (0.15 mg/ml) independently. As expected, all the compounds were ineffective in killing *Mtb* because of redundant roles and relatively less requirement of L-cysteine synthases during regular growth conditions. To examine the combinatorial effect, we selected sub-MIC values of INH (0.03 μ g/mL, encircled orange) and increasingly two-fold diluted concentrations of C1, C2, or C3, all below MIC values (Figure 6c-e; blue encircled denotes the starting concentration) and assessed bacterial survival upon combinatorial treatment with INH and the three L-cysteine synthase inhibitors. The addition of either of the inhibitors below MIC rendered *Mtb* highly susceptible to INH, highlighting the potency of these compounds to serve as an adjunct therapy to combat mycobacterial infection.

Discussion

Throughout its course of infection, *Mtb* must persist in a hostile, oxidizing, and nutrient-deprived environment of host macrophage. Understanding the dynamic metabolic interactions between the pathogen and its host is imperative to identify its weaknesses which can be exploited to design new-age chemotherapy. Our study provides convincing evidence that the genes involved in the L-cysteine biosynthetic pathway are attractive targets for the design of anti-mycobacterial drugs. Genes involved in sulfur assimilation and L-cysteine biosynthesis were found to be upregulated in the transcriptome profile of *Rv* subjected to various host-like stresses. The sulfur metabolism pathway was particularly enriched upon the addition of oxidizing agent, CHP (Figure 1, S2 & S3). This observation has been consistently reported by multiple studies demonstrating the up-regulation of sulfur assimilation and L-cysteine biosynthetic genes in response to oxidative stress, nutrient deprivation, and macrophage infection [1-5]. Besides being involved in protein synthesis, L-cysteine is also important for the biosynthesis of L-methionine, S-adenosyl methionine, coenzyme A, and iron-sulfur clusters. As a thiol-containing molecule, L-cysteine contributes to the intracellular redox state directly and through the production of major antioxidants like mycothiol and ergothioneine. These numerous essential functions of L-cysteine prompted us to further examine its roles in the context of *Mtb* cellular metabolism and virulence.

Unlike *Mtb* Δ cysH, which was reported to be an L-cysteine auxotroph and thus required L-methionine or glutathione supplementation to grow *in vitro* (from which L-cysteine can be generated catabolically)[15], neither deletion of *cysK2* nor *cysM* impacted *Mtb* growth kinetics *in vitro* suggesting that these genes are functionally redundant during rich growth conditions [5] [11] (Figure S4). Interestingly, the induction of various host-like stresses attenuated the growth of these mutants compared to the parental strain, pointing towards the possibility of an

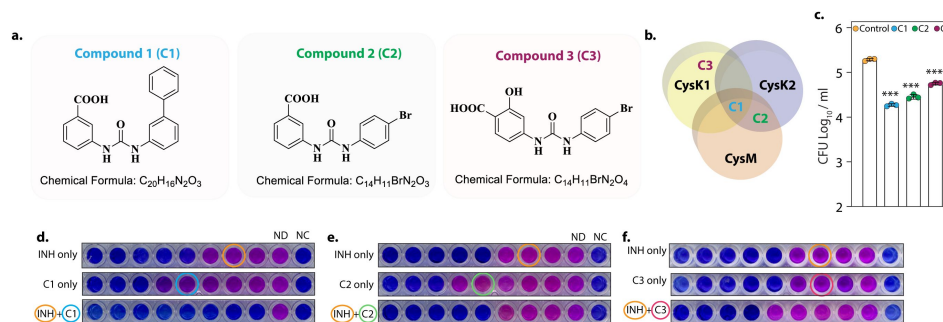


Fig 6

L-cysteine synthase inhibitors can effectively kill *Mtb* within the host.

(a) Chemical structure and formula of lead compounds. (b). Venn diagram illustrating the specificity of compounds on mycobacterial L-cysteine synthases. (c). Peritoneal macrophages were infected with *Rv*. 4 h post-infection, cells were either left untreated (control) or 5 mg/ml C1 (a), C2 (b) or C3 (c) was added to the infected cells. Bar graphs represent mean Log_{10} CFU/ml $\pm$ SD, and values from independent replicates are represented by individual data points ($n=3$). (d-f). Alamar blue assay to determine MIC values of C1 (d), C2 (e) and C3 (f) with and without Isoniazid in *Rv*.

enhanced requirement of L-cysteine-derived antioxidants and other biomolecules to subvert these stresses (**Figure 1**). In agreement with this hypothesis, we previously showed that the cellular thiol levels are higher during oxidative stress in *Mtb* [5]. Similarly, various MSH and ERG mutants display enhanced sensitivity to oxidative stress caused by treatment with H₂O₂, cumene hydroperoxide, or O^{2•-} [16–20].

While the transcriptomic profiles of mutants were highly similar to each other during regular growth conditions, the addition of CHP resulted in differential expression of >30% *Mtb* genes, indicating that cues and downstream effects of the two non-canonical L-cysteine synthases are partially non-overlapping, further underlying their importance for the bacillary growth inside the host (**Figure 2**). We also found that the steady-state levels (pool sizes) of key L-cysteine derived antioxidants, ergothioneine, and mycothiol, are significantly different at 24 h (**Figure 3**). The rate of synthesis of mycothiol, mycothione and ergothioneine were observed to be lower in *RvΔcysM* compared to *Rv* and *RvΔcysK2*.

The addition of L-cysteine in oxidatively stressed cells nullified the compromised survival of the mutants indicating that *Mtb* cells are able to uptake L-cysteine from the extracellular medium, as shown previously [5], and reduced levels of L-cysteine in the mutants are chiefly responsible for their attenuated growth in the presence of stresses (**Figure 4**). Various *Mtb* mutants in mycobacterial sulfur metabolism genes are severely compromised to persist within the host and cause disease [15, 21–26]. Inorganic sulfate is present at 300–500 μM in human plasma [26]. However, the inability of host-derived sulfur/L-cysteine to compensate for attenuated survival of mutants is suggestive of either inaccessibility of sufficient L-cysteine in *Mtb* niches or inefficient expression, function, or uptake by transporters *in vivo*. Importantly, compromised survival of *RvΔcysM* and *RvΔcysK2* *in vivo* was largely mitigated in IFNγ^{-/-} and Phox^{-/-}, indicating that the non-canonical L-cysteine synthases, CysM and CysK2, independently facilitate mycobacterial survival during immune-mediated redox stress (**Figure 5**).

Using network analysis of the *Mtb* protein interactome, a flux balance analysis of the reactome and randomized transposon mutagenesis data, along with sequence analyses and a structural assessment of targetability, Raman *et al.*, reported CysK2 and CysM as high confidence drug targets [27]. Similarly, inhibitors of CysM showed mycobactericidal activity in a nutrient-starvation model of dormancy [28]. Interestingly, humans do not reduce sulfur to produce L-cysteine; they rather synthesize L-cysteine through SAM-dependent transmethylation followed by transsulfuration of L-methionine. Owing to their complete absence in humans, mycobacterial L-cysteine biosynthetic genes and their regulators represent unique, attractive targets for therapeutic intervention [29]. We found that both *RvΔcysM* and *RvΔcysK2* displayed enhanced antibiotic sensitivity *in vitro* and within the host (data not shown). Importantly, a combination of L-cysteine synthase inhibitors with front-line TB drugs like INH, significantly reduced the bacterial load inside the host (**Figure 6**). Altogether, this study demonstrates for the first time that the two non-canonical L-cysteine synthases have non-redundant biological functions. Deletion or biochemical inhibition of CysM or CysK2 perturbs redox homeostasis of *Mtb* and allow for the maximal effect of host macrophages antibacterial response, and thus increased elimination of virulent *Mtb*.

Methods

Bacterial strains and culturing conditions

Mtb culturing conditions were performed as described previously [5, 30].

Generation of *RvΔcysK2* mutant and complementation strain

We generated gene replacement mutants through the recombineering method [31] as previously described [5, 30]. Briefly, 671bp upstream of 176th nucleotide from 5' end (5' flank) and 643 bp downstream of 943rd nucleotide from 3' end (3' flank) of *cysK2* were PCR amplified from *Rv* genomic DNA using Phusion DNA polymerase (Thermo Scientific). The amplicons were digested with BstAPI and ligated with compatible hygromycin resistance (*hyg^r*) cassette and *oriE*+*cosλ* fragments [31], to generate the allelic exchange substrate (AES). AES was digested with SnaBI to release the LHS-*hyg^r*-RHS fragment, and the eluted fragment was electroporated into the recombineering proficient *Rv-ET* strain [5]. Multiple *hyg^r* colonies were examined by PCRs to screen for legitimate recombination at *cysK2* locus. *RvΔcysK2*, thus generated, was cured of pNit-ET through negative selection on LB Agar plates containing 2% sucrose. To generate the complementation strain, full length *cysK2* gene was PCR amplified from genomic DNA isolated from *Rv* as the template and Phusion DNA polymerase (ThermoFischer Scientific). The amplicon was digested with NdeI-HindIII (NEB), cloned into the corresponding sites in pNit-3F and the resultant plasmid was electroporated into *RvΔcysK2* to generate the *RvΔcysK2::cysK2* strain. *RvΔcysM* and *RvΔcysM::cysM* strains were generated in the lab previously using a similar method [2].

Ex vivo infection experiments

Balb/c, C57BL/6 (B6), *phox^{-/-}* (B6.129S6-Cybbtm1Din/J; JAX# 002365) or IFN- γ ^{-/-} (B6.129S7-Ifngtm1Ts/J; JAX#002287) mice were procured from The Jackson Laboratory. 4% thioglycolate (Hi-Media) was injected into the peritoneum cavity of 4- to 6-weeks old mice and peritoneal macrophages were extracted four days post-injection and seeded and processed as described [5]. In specific cases, the peritoneal macrophages were treated with 10 ng/ml IFN γ (BD Biosciences) overnight and 10 ng/ml lipopolysaccharide (LPS, Sigma) for 2 h for activation. Where indicated, cells were further pretreated with 100 μ M 1400 W (Sigma) overnight to inhibit iNOS or pretreated with 0.2 mM L-cysteine before infection. Single-cell *Mtb* suspensions were used for infection at 1:10 (host cells: bacteria) MOI. 4 h post-infection (p.i), cells were washed thrice and replenished with complete RPMI containing IFN γ + LPS, 1400 W, or L-cysteine, as required. The infected host cells were washed thrice with PBS, lysed using 0.05% Dodecyl sulfate sodium salt (SDS), and serial dilutions were plated on 7H11-OADC to enumerate bacillary survival.

In vivo infection experiments

Mice (4–6-weeks) housed in ventilated cages at the Tuberculosis Aerosol Challenge Facility at the International Centre for Genetic Engineering and Biotechnology (New Delhi, India) were infected via aerosol route with ~ 100 bacilli using the Madison Aerosol Chamber (University of Wisconsin, Madison, WI). At 24 h post-infection, mice (n=2; per group) were euthanized to determine the bacterial deposition. At 4/8 weeks p.i, lungs and spleen were homogenized and plated on 7H11+OADC containing PANTA.

RNA isolation and qRT-PCRs

Mtb strains were cultured in triplicates in 7H9-ADS till O.D. reached 0.3-0.4. One set was left untreated (control), the other was treated with 50 μ M of CHP for 6 h. A culture equivalent to 10 O.D.₆₀₀ was resuspended in TRIzol (Invitrogen). Zirconium beads were added to the cell-TRIzol mix to facilitate lysing *Mtb* cells with the help of a bead beater (MP FastPrep system, MP Biomedicals) and RNA was extracted and analyzed as described [5]. Data was plotted as 2^(-ΔΔCt) wherein the gene expression was normalized with respect to 16s rRNA (*rrs* gene), followed by normalization with control strain/condition/group.

RNA sequencing and analysis

Total RNA was isolated from two-three biological replicates of indicated *Mtb* strains, and their concentrations were checked by Qubit (ThermoFischer Scientific) followed by quality assessment through Agilent 2100 BioAnalyzer (Agilent RNA 6000 Nano Kit). Both sets of RNA seq represented in **Figures 1** and **5** were processed and run at the same time, the same set of control (*Rv*) triplicates were used for the analysis of both figures. Samples with RIN values ≥ 7 were processed for RNA sequencing using the Illumina NovaSeq 6000 Platform (CSIR-CCMB central facility; read length of 100 bp, 20 million paired-end reads / sample).

Illumina adapters and low-quality reads were discarded and those with quality scores < 20 and smaller than 36 bp were eliminated from raw sequencing reads using cutadapt [32]. Processed reads were mapped to the *Mtb* H37Rv, (https://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/Mycobacterium_tuberculosis/reference/GCF_000195955.2_ASM19595v2/), using hisat2 with default parameters [33]. Uniquely aligned reads were counted with the help of feature Counts of Subread package [34] and those with total read count < 10 across all the samples were removed, and the rest were used for further downstream analysis. Differentially expressed genes (DEGs) were identified using DESeq2 [35] and those with adjusted p-value < 0.05 and absolute $\log_2$ Fold change > 1 or 0.5 were considered. The raw read counts were rlog normalized the raw read counts for PCA plot and heat map with the DESeq2 package.

Functional enrichment analysis

Functional enrichment analysis was performed with DAVID web services [36]. We specifically used GO terms and KEGG pathways for this analysis. Only top 10 enriched hits based on gene counts were plotted.

Preparation of samples for metabolomics

Mtb strains were grown in 7H9 media until an OD 1 and then inoculated onto 0.22um nitrocellulose filters and grown on 7H10 plates containing 0.5g/L BSA Fraction V, 0.2% dextrose and 0.085% NaCl (ADS) for 5 days. The filters were then transferred to 7H10 plus ADS plates containing Sodium sulfate-34S (Merck 718882) containing either 50uM CHP or no CHP for 24 and 48 h. The metabolites were extracted by mechanical lysis in cold acetonitrile/methanol/water (2:2:1) containing 0.1mm acid washed Zirconia beads. The lysates were clarified by centrifugation and filtered through a 0.22um Spin-X column (Costar). The lysates were mixed 1:1 with acidified acetonitrile (0.1% formic acid). The samples were normalized to protein concentration using the BCA assay kit (Thermo).

Liquid chromatography – mass spectrometry metabolomics

LC–MS analysis was done in an Agilent 1290 Infinity II HPLC connected to a 6230B time-of-flight (ToF) mass spectrometer using a Dual AJS ESI ionization source. Compounds were separated in a Cogent Diamond Hydride Type C silica column (2.1 $\times$ 150 mm). Solvent A was LC– MS grade H₂O + 0.1% (v/v) formic acid and solvent B was acetonitrile + 0.1% (v/v) formic acid. The gradient was from 85% B to 5% B over 14 min. Flow rate was 0.4 mL/min. The ion source parameters were as follows: Gas temperature 250 °C, Drying gas flow rate 13 l/min, nebulizer pressure 35 psig, sheath gas temperature 350 °C, sheath gas flow 12 l/min, capillary voltage 3500 V and nozzle voltage 2000 V, The ion optic voltages were 110 V for the fragmentor, 65 V for the skimmer and 750 for the octopole radio frequency voltage. MS data were analyzed with the MassHunter suite version B0.7.0.00.

MIC analysis

MIC values were assessed using Alamar Blue assay, as described previously [37]. Briefly, 100 µl 7H9-ADS medium without Tween 80 was added to each well of a 96-well plate. First well of each column were filled with 100 µl of the test drug/antibiotic, which was then serially diluted across the column. *Mtb* cells corresponding to 0.01 A_{600} were diluted in 100 µl 7H9-ADS medium and were added to each well. Two rows one in which the drug was not added (no drug [ND]), and the other wherein *Mtb* cells were replaced with 7H9-ADS (no cells [NC]) acted as controls. The 96 well plate was sealed with parafilm and kept at 37°C. After 5 days, 20 µl of 0.25% filter-sterilized resazurin was added to each well, and color development was captured after 24 h.

Statistical analysis

Unless otherwise specified, experiments were performed in triplicates and repeated independently at least twice. CFU results were plotted, and significance of the datasets was calculated using one-way ANOVA followed by a *post hoc* test (Tukey test) on GraphPad Prism 5. Figures were customized using Adobe Illustrator version 26.3.1. Statistical significance was set at *P* values < 0.05 significant (*, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0005). Source datasets can be requested from the corresponding author.

Data availability

RNA Seq data is available at the NCBI Gene Expression Omnibus Database, accession no. GEO GSE225792, the link to the database is <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225792> and the reviewer token for private access is ipmnekailfmlbcj.

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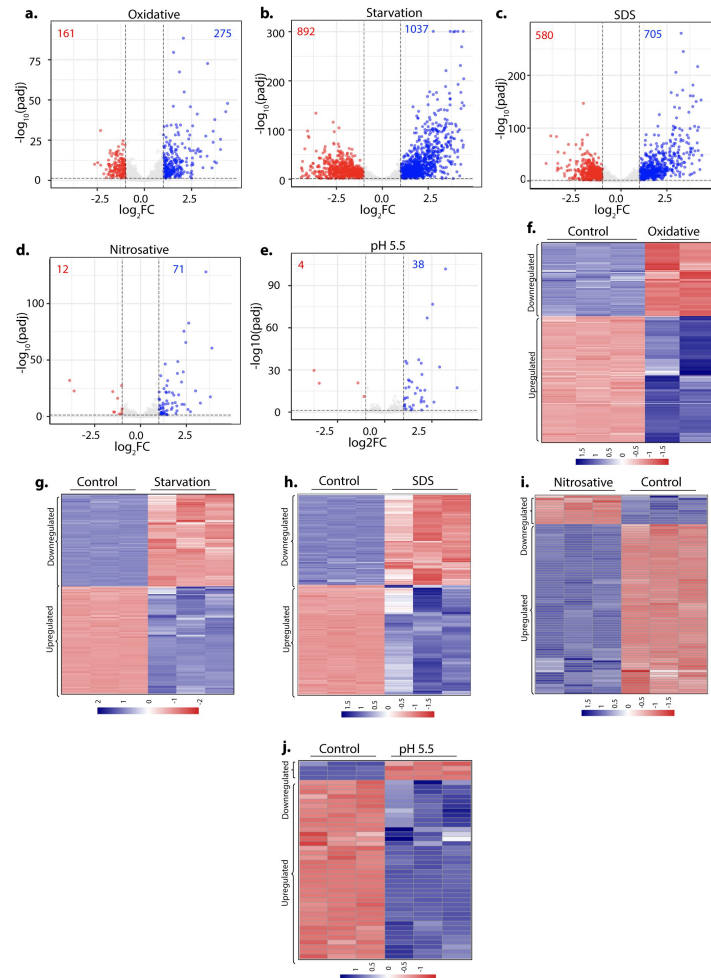
Author contributions

MZK, LPSC and VKN conceptualized the study; MZK contributed to generation of constructs and strains; BS and MZK were involved in the preparation of RNA sequencing samples, while NKS and DTS performed the analyses. MZK and BS performed animal studies at ICGB; MZK contributed to *in vitro* stress susceptibility assays, macrophage assays and inhibitor studies; DMH and LPSC performed the metabolomic studies; DVSP and DS synthesized chemical inhibitors; MZK curated the data. MZK, YK, LPSC and VKN wrote the manuscript; VKN and LPSC acquired funding for the study. All authors read and approved the manuscript.

Ethics statement

Protocols for animal experiments were preapproved by the Animal Ethics Committee of the National Institute of Immunology, New Delhi, India (IAEC numbers 409/16 and 462/18) as per standard institutional guidelines.

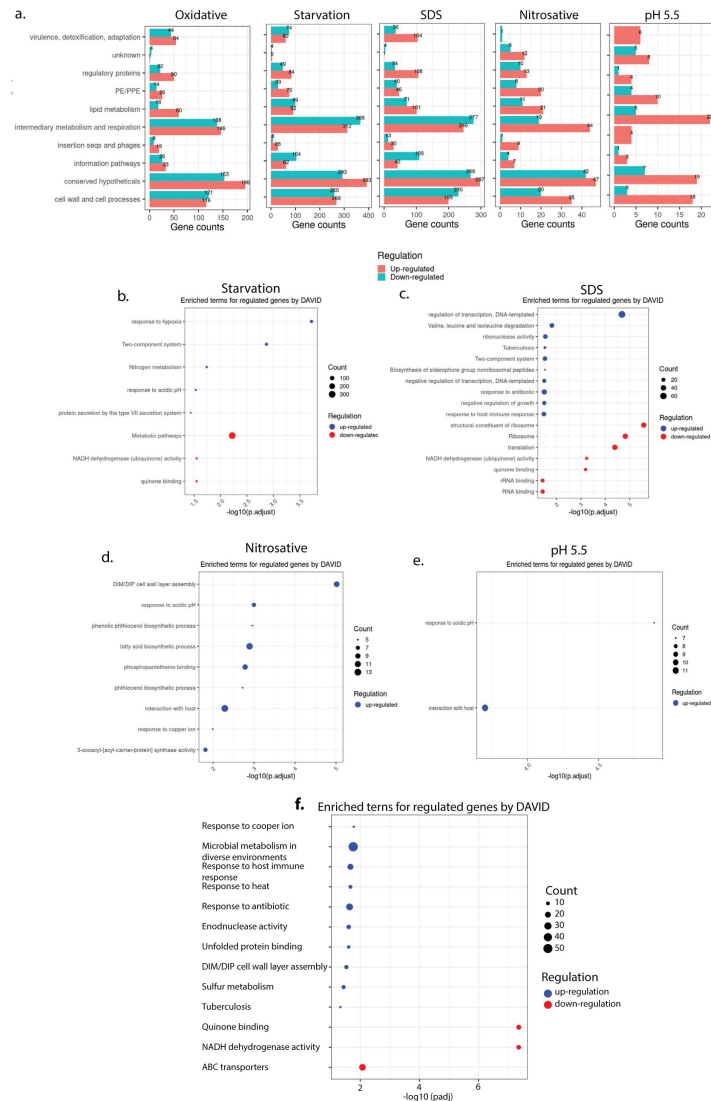
Table S1: Differentially Expressed Genes (DEG) analysis *Rv* under stress vs *Rv*.



Supplementary Figure 2

Sulfur assimilation and L-cysteine biosynthesis pathways are upregulated under stress conditions.

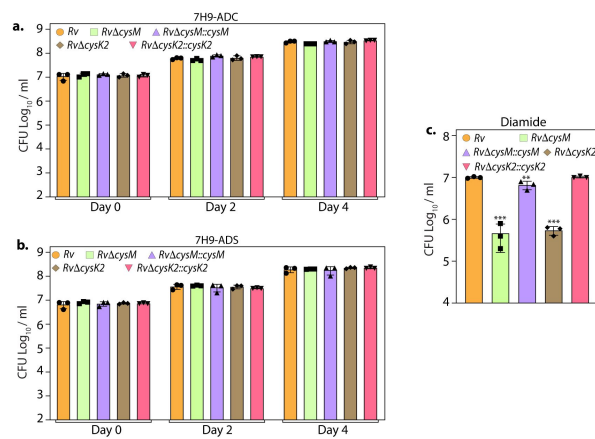
(a-e) Volcano plots illustrating significantly upregulated (blue) and downregulated (red) genes in *Rv* grown under indicated conditions with Log_2 Fold change ≥ 1 and P_{adj} value < 0.05 compared to control (untreated) *Rv*. Numbers in the top quadrant depict the number of significantly upregulated (blue) and downregulated (red) genes in each condition compared to the control. (f-j) Heat Maps depicting normalized gene count of differentially expressed genes (DEGs) in independent replicates of *Rv* grown under indicated conditions with Log_2 Fold change ≥ 1 and P_{adj} value < 0.05 . The colour intensity indicates relative upregulated (blue) and downregulated (red) genes compared to the control.



Supplementary Figure 3

Genes belonging to the Sulfur metabolism pathway are upregulated upon oxidative stress

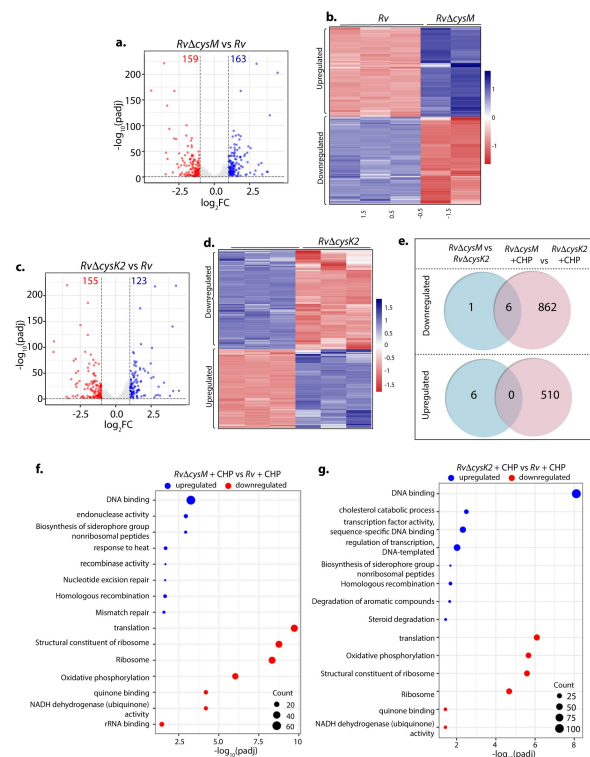
(a) Horizontal bar graph demonstrating the number of DEGs belonging to a particular functional category under indicated stress condition. (b-e) Pathway enrichment by DAVID depicting significantly enriched Gene Ontology (GO) biological processes based on DEGs in starvation (b), SDS (c), Nitrosative (d) and pH 5.5 (e). (f) Pathway enrichment by DAVID depicting significantly affected Gene Ontology (GO) biological processes based on DEGs (Log_2 Fold change ≥ 0.5 and P_{adj} value < 0.05).



Supplementary Figure 4

L-cysteine synthases facilitate mycobacterial survival upon oxidative stresses *in vitro*

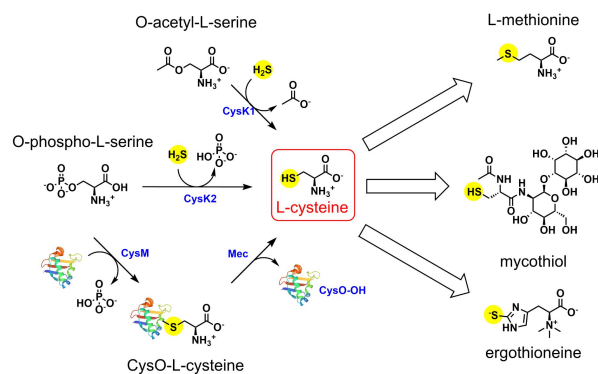
(a-c) *Rv*, *RvΔcysM*, *RvΔcysK2*, *RvΔcysM::cysM*, and *RvΔcysK2::cysK2* strains were inoculated in 7H9- ADC (a), 7H9-ADS (b), 7H9-ADS plus 10mM diamide (c) medium. Bar graphs represent the bacillary survival with data point indicating values CFU log₁₀/mL ± SD from individual replicate (n=3). Statistical significance was drawn in comparison with *Rv* using one-way ANOVA followed by a *post hoc* test (Tukey test; GraphPad prism). ***P* < 0.005; ****P* < 0.0005.



Supplementary Figure 5

Unique transcriptional signatures of CysM and CysK2.

(a & c) Volcano plots illustrating significantly upregulated (blue) and downregulated (red) genes in indicated strains and conditions with Log_2 Fold change > 1 and P_{adj} value < 0.05 . Numbers in the top quadrant highlight the number of significantly upregulated (blue) and downregulated (red) genes in each condition (b & d) Heat Maps depicting normalized gene count of differentially expressed genes (DEGs) in independent replicates of *RvΔcysM* and *RvΔcysK2* compared to *Rv* with Log_2 Fold change > 1 and P_{adj} value < 0.05 . The colour intensity indicates relative upregulated (blue) and downregulated (red) genes compared to the control. (e) Venn diagram showing the number of significantly downregulated and upregulated DEGs that overlap between indicated strains (Log_2 Fold change > 1 and P_{adj} value < 0.05). (f & g) Pathway enrichment by DAVID depicting significantly enriched Gene Ontology (GO) biological processes based on DEGs upon oxidative stress in *RvΔcysM* (f) and *RvΔcysK2* (g) compared to *Rv* (Log_2 Fold change > 1 and P_{adj} value < 0.05).



Supplementary Figure 6

Overview of metabolites tracked through ^{35}S sulfur incorporation

Table S2: Pathway enrichment analysis of DEGs from Table S1.

Table S3: DEG analysis of RvΔcysM or RvΔcysK2 vs Rv with or without CHP stress & DEG analysis RvΔcysM +CHP vs RvΔcysK2 + CHP.

Table S4: Pathway enrichment analysis of DEGs from Table S3.

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Reviewer #1 (Public Review):

Summary:

Khan et. al., investigated the functional redundancy of the non-canonical L-cysteine synthases of *M. tuberculosis*, CysM and CysK2, focussing on their role in mitigating the effects of host-derived stress. They found that while deletion mutants of the two synthases (RvΔcysM, RvΔcysK2) have similar transcriptomes under standard conditions, their transcriptional response to oxidative stress is distinct. The impact of deleting the synthases also differentially affected the pools of L-cysteine-derived metabolites. They show that the mutants (RvΔcysM, RvΔcysK2) have impaired survival in peritoneal macrophages and in a mouse model of infection. Importantly, they show that the survival of the mutants increases when the host is defective in producing reactive oxygen and nitrogen species, linking the phenotype to a defect in combating host-derived stress. Finally, they show that compounds inhibiting L-cysteine synthases reduce the intracellular survival of *M. tuberculosis*.

Strengths:

1. The distinct transcriptome of the RvΔcysM and RvΔcysK2 mutants in the presence of oxidative stress provides solid evidence that these mutants are distinct in their response to oxidative stress, and suggests that they are not functionally redundant.
2. The use of macrophages from *phox*^{-/-} and *INF*^{-/-} mice and an iNOS inhibitor for the intracellular survival assays provides solid evidence that the survival defect seen for the RvΔcysM and RvΔcysK2 mutants is related to their reduced ability to combat host-derive

oxidative and nitrosative stress. This is further supported by the infection studies in *phox*^{-/-} and *INF*^{-/-} mice.

Weaknesses:

1. There are several previous studies looking at the transcriptional response of *M. tuberculosis* to host-derived stress, however, the authors do not discuss initial RNA-seq data in the context of these studies. Furthermore, while several of the genes in sulfur assimilation and L-cysteine biosynthetic pathway genes are upregulated by more than one stress condition, the data does not support the statement that it is the "most commonly upregulated pathway in *Mtb* exposed to multiple host-like stresses".
2. For the quantification of the metabolites, it isn't clear how the abundance was calculated (e.g., were standards for each metabolite used? How was abundance normalised between samples?), and this information should be included to strengthen the data. Furthermore, labelling with L-methionine was performed to determine the rate of synthesis of the L-cysteine-derived metabolites. L-cysteine is produced from L-methionine via the transsulfuration pathway, which is independent of CysM and CysK2. It is therefore difficult to interpret this experiment, as the impact of deleting CysM and CysK2 on the transsulfuration pathway is likely indirect.
3. The ability of L-cysteine to rescue the survival defect of the *RvΔcysM* and *RvΔcysK2* mutants in macrophages is interpreted as exogenous L-cysteine being able to compensate for reduced intracellular levels. However, there is no evidence that L-cysteine is being taken up by the mutants and an alternate explanation is that L-cysteine functions as an antioxidant within cells i.e., it reduces intracellular ROS.

The authors sought to investigate the functional redundancy of the non-canonical L-cysteine synthases CysM and CysK2. While their distinct transcriptional response to oxidative stress suggests distinct physiological roles, the study did not explore these differences and therefore provides only preliminary insight into the underlying reasons for this observation. In the context of drug development, this work suggests that while L-cysteine synthase inhibitors do not have high potency for killing intracellular *M. tuberculosis*, they have the potential to decrease the pathogen's survival in the presence of host-derived stress.

Reviewer #2 (Public Review):

Summary:

The paper examines the role L-cysteine metabolism plays in the biology of *Mycobacterium tuberculosis*. The authors have preliminary data showing that *Mycobacterium tuberculosis* has two unique pathways to synthesize cysteine. The data showing new compounds that act synergistically with INH is very interesting.

Strengths:

RNAseq data is interesting and important.

Weaknesses:

The paper would be strengthened if the authors were to add further detail to their genetic manipulations.

The authors provide evidence that they have successfully made a *cysK2* mutant by recombineering. This data looks promising, but I do not see evidence for the *cysM* deletion. It is also important to state what sort of complementation was done (multicopy plasmid, integration proficient vector, or repair of the deletion). Since these mutants are the basis for most of the additional studies, these details are essential. It is important to include complementation in mouse studies as unexpected loss of PDIM could have occurred.

Reviewer #3 (Public Review):

In this work, the authors conduct transcriptional profiling experiments with Mtb under various different stress conditions (oxidative, nitrosative, low pH, starvation, and SDS). The Mtb transcriptional responses to these stress conditions are not particularly new, having been reported extensively in the literature over the past ~20 years in various forms. A common theme from the current work is that L-cysteine synthesis genes are seemingly up-regulated by many stresses. Thus, the authors focused on deleting two of the three L-cysteine synthesis genes (*cysM* and *cysK2*) in Mtb to better understand the roles of these genes in Mtb physiology.

The *cysM* and *cysK2* mutants display fitness defects in various media (Sautons media, starvation, oxidative and nitrosative stress) noted by CFU reductions. Transcriptional profiling studies with the *cysM* and *cysK2* mutants revealed that divergent gene signatures are generated in each of these strains under oxidative stress, suggesting that *cysM* and *cysK2* have non-redundant roles in Mtb's oxidative stress response which likely reflects the different substrates used by these enzymes, CysO-L-cysteine and O-phospho-L-serine, respectively. Note that these studies lack genetic complementation and are thus not rigorously controlled for the engineered deletion mutations.

The authors quantify the levels of sulfur-containing metabolites (methionine, ergothioneine, mycothiol, mycothionine) produced by the mutants following exposure to oxidative stress. Both the *cysM* or *cysK2* mutants produce more methionine, ergothioneine, and mycothionine relative to WT under oxidative stress. Both mutants produce less mycothiol relative to WT under the same condition. These studies lack genetic complementation and thus, do not rigorously control for the engineered mutations.

Next, the mutants were evaluated in infection models to reveal fitness defects associated with oxidative and nitrosative stress in the *cysM* or *cysK2* mutants. In LPS/IFN γ activated peritoneal macrophages, the *cysM* or *cysK2* mutants display marked fitness defects which can be rescued with exogenous cysteine added to the cell culture media. Peritoneal macrophages lacking the NADPH oxidase (Phox) or IFN γ fail to produce fitness phenotypes in the *cysM* or *cysK2* mutants suggesting that oxidative stress is responsible for the phenotypes. Similarly, chemical inhibition of iNOS partly abrogated the fitness defect of the *cysM* or *cysK2* mutants. Similar studies were conducted in mice lacking IFN γ and Phox establishing that *cysM* or *cysK2* mutants have fitness defects in vivo that are dependent on oxidative and nitrosative stress.

Lastly, the authors use small molecule compounds to inhibit cysteine synthases. It is demonstrated that the compounds display inhibition of Mtb growth in 7H9 ADC media. No evidence is provided to demonstrate that these compounds are specifically inhibiting the cysteine synthases via "on-target inhibition" in the whole Mtb cells. Additionally, it is wrongly stated in the discussion that "combinations of L-cys synthase inhibitors with front-line TB drugs like INH, significantly reduced the bacterial load inside the host". This statement suggests that the INH + cysteine synthase inhibitor combinations reduce Mtb loads within a host in an infection assay. No data is presented to support this statement.