

Original Research

Liposomal Glutathione Supplementation Mitigates Extrapulmonary Tuberculosis in the Liver and Spleen

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Abstract

Background: Extrapulmonary tuberculosis (EPTB) accounts for a fifth of all *Mycobacterium tuberculosis* (*M. tb*) infections worldwide. The rise of multidrug resistance in *M. tb* alongside the hepatotoxicity associated with antibiotics presents challenges in managing and treating tuberculosis (TB), thereby prompting a need for new therapeutic approaches. Administration of liposomal glutathione (L-GSH) has previously been shown to lower oxidative stress, enhance a granulomatous response, and reduce the burden of *M. tb* in the lungs of *M. tb*-infected mice. However, the effects of L-GSH supplementation during active EPTB in the liver and spleen have yet to be explored. **Methods:** In this study, we evaluated hepatic glutathione (GSH) and malondialdehyde (MDA) levels, and the cytokine profiles of untreated and L-GSH-treated *M. tb*-infected wild type (WT) mice. Additionally, the hepatic and splenic *M. tb* burdens and tissue pathologies were also assessed. **Results:** L-GSH supplementation increased total hepatic levels and reduced GSH. A decrease in the levels of MDA, oxidized GSH, and interleukin (IL)-6 was also detected following L-GSH treatment. Furthermore, L-GSH supplementation was observed to increase interferon-gamma (IFN- γ) and tumor necrosis factor (TNF)- α production and decrease IL-10 levels. *M. tb* survival was significantly reduced in the liver and spleen following L-GSH supplementation. L-GSH treatment also provided a host-protective effect in the liver and spleen of *M. tb*-infected mice. **Conclusions:** Overall, L-GSH supplementation elevated the levels of total and reduced forms of GSH in the liver and reduced the burden of *M. tb* by decreasing oxidative stress, enhancing the production of immunosupportive cytokines, and reducing the levels of immunosuppressive cytokines. These observed benefits highlight the potential of L-GSH supplementation during active EPTB and provide insight into novel therapeutic interventions against *M. tb* infections.

Keywords: tuberculosis; *Mycobacterium tuberculosis*; glutathione; oxidative stress; extrapulmonary tuberculosis; liver; spleen

1. Introduction

Mycobacterium tuberculosis (*M. tb*), the causative agent of tuberculosis (TB), is responsible for over 10 million infections worldwide and continues to be a global health concern [1]. *M. tb* infection is primarily caused by the inhalation of *M. tb*-containing aerosol droplets into the lungs, known as pulmonary TB [2]. *M. tb* contact with alveolar macrophages initiates a type 1 T helper (Th1) cytokine response that recruits immune cells to form a complex aggregate, ultimately, leading to the formation of granuloma in the lung [3]. Granulomas create a protective barrier to contain *M. tb* and prevent systemic dissemination, leading to latent pulmonary *M. tb* infection [4]. However, necrosis, caseation, and cavitation of granulomas during advanced stages of active *M. tb* infection or during immunocompromised states can potentially lead to systemic dissemination, also known as extrapulmonary TB (EPTB) [5].

EPTB occurs primarily through lymphatic and hematogenous dissemination of *M. tb* and can affect

extrapulmonary sites, such as the central nervous system, gastrointestinal system, liver, and spleen [5]. Approximately 20% of all *M. tb* infections are EPTB, which are highly prevalent in immunocompromised patients, such as those with Human Immunodeficiency Virus (HIV) or type 2 diabetes mellitus [6]. The systemic *M. tb* burden resulting from EPTB is associated with higher mortality rates and worse clinical outcomes [7]. EPTB treatment consists of a 4 to 6-month regimen of first-line antibiotics such as Rifampicin and Isoniazid; however, treatment duration can lead to poor compliance and is associated with hepatotoxicity [8]. Additionally, EPTB has been associated with a higher prevalence of multidrug-resistant (MDR) *M. tb* strains, prompting a need for additional therapeutic strategies against *M. tb* infection [9].

Recent attention has been given to host-directed therapies, which augment host-immune responses to control infectious diseases [10]. Glutathione (GSH) is a tripeptide, which consists of L-gamma-glutamyl-L-cysteinyl-glycine and is produced ubiquitously across most cells possess-



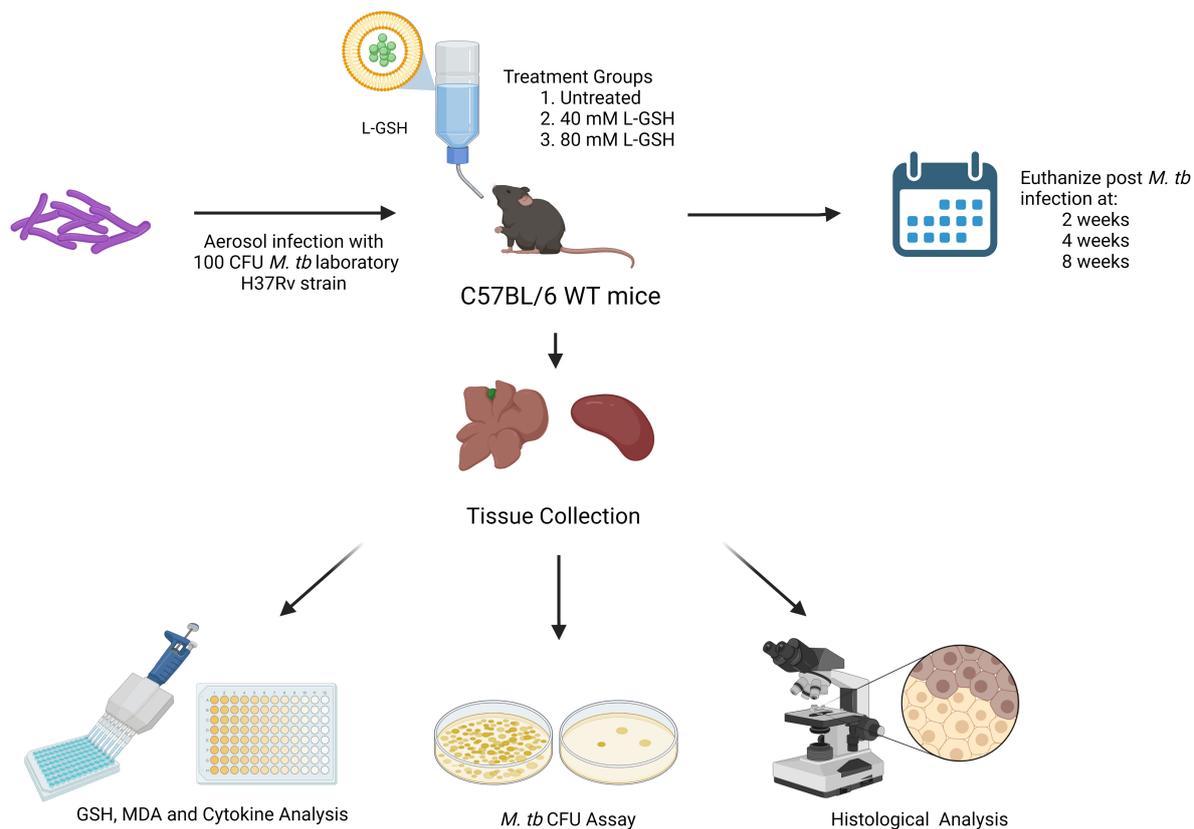


Fig. 1. Study Design. C57BL/6 male and female mice were infected with 100 colony forming units (CFUs) of *M. tb* H37Rv using aerosolization apparatus. Mice were untreated or treated with 40 mM or 80 mM L-GSH in their drinking water until sacrificed at 2, 4, and 8 weeks post-*M. tb* infection. The livers and spleens were collected and homogenized. A GSH colorimetric assay, Thiobarbituric Acid Reactive Substances (TBARS) assay, and cytokine Enzyme-linked Immunosorbent (ELISA) assays were performed using the liver homogenates. Portions of the livers and spleens were sectioned and stained for histological analysis. The *M. tb* loads in the livers and spleens were measured as CFU counts by plating liver and spleen homogenates from *M. tb*-infected mice. The sample size (n) included 3 mice in each of the 2-, 4-, and 8-week groups. CFU, colony-forming unit.

ing both antioxidant and immunomodulatory properties [11,12]. Emerging evidence has shown the role of GSH in enhancing the granulomatous response to *M. tb* infection by restoring redox homeostasis [13–18]. Previous animal models have demonstrated that GSH depletion exacerbates *M. tb* infection [19]. Furthermore, oral supplementation of liposomal glutathione (L-GSH) reduced oxidative stress and enhanced the production of Th1-promoting cytokines, thereby reducing *M. tb* survival in WT mice during an active pulmonary infection [20]. However, a study into the consequences of L-GSH supplementation to the liver or spleen during an active EPTB has yet to be conducted.

In this study, *M. tb*-infected WT C57BL/6 mice were treated with 40 mM or 80 mM L-GSH. We examined hepatic GSH levels, malondialdehyde (MDA) levels, and cytokine profiles of untreated and L-GSH-treated *M. tb*-infected WT mice. Additionally, hepatic and splenic *M. tb* burdens and tissue pathologies were also assessed. We hypothesize that L-GSH supplementation may confer a therapeutic effect during EPTB.

2. Materials and Methods

2.1 Preparation of *M. tb* for Infection

Virulent *M. tb* laboratory H37Rv strain (ATCC, Manassas, VA, USA) was cultivated in Middlebrook 7H9 media with OADC supplement and 0.05% Tween-80 (Thermo Fisher Scientific, Waltham, MA, USA) [21]. *M. tb* H37Rv culture was propagated until reaching the logarithmic phase at an absorbance between 0.6 and 0.8 OD₆₀₀. H37Rv was harvested by centrifugation at 3000 × g, washed with phosphate-buffered saline (PBS), and gently vortexed with sterile glass beads (3 mm) to disaggregate bacterial clumps. Then, the residual bacterial clumps were eliminated by filtration at 5 μm. Bacterial concentrations of the processed *M. tb* cultures were determined by serially diluting stock processed *M. tb* cultures, plating onto Middlebrook 7H10 agar plates, and incubating in 5% CO₂ at 37 °C for 3–4 weeks. Processed *M. tb* cultures were aliquoted into 1 mL cryotubes and stored at –80 °C until further use.

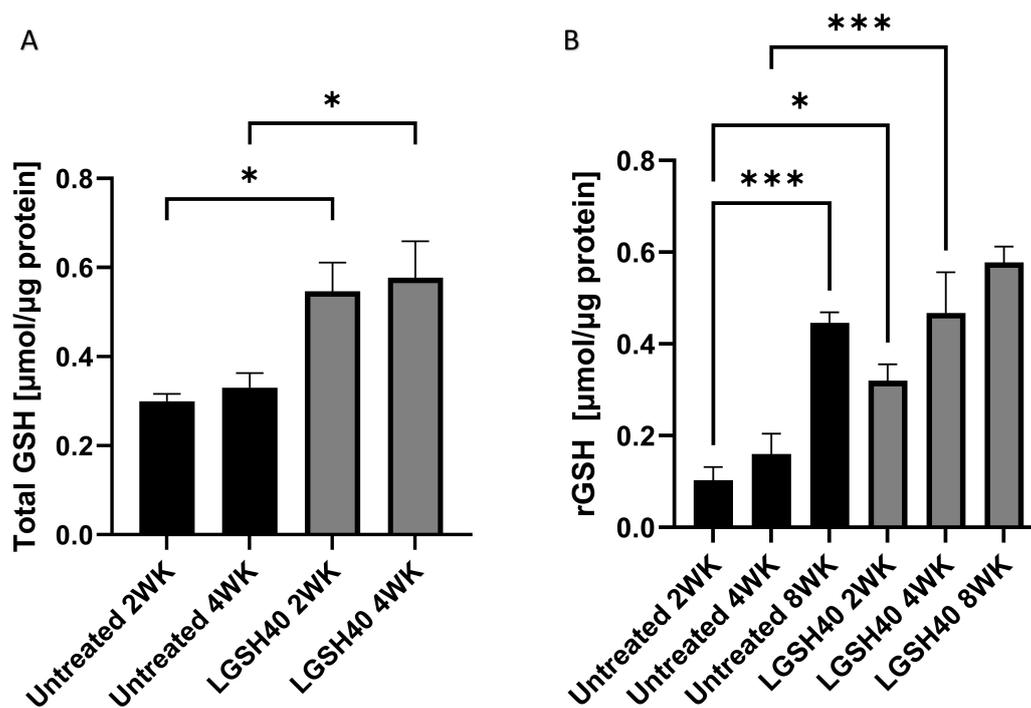


Fig. 2. Measurement of total and reduced forms of GSH in livers of *M. tb*-infected female C57BL/6 mice treated orally with 40 mM L-GSH. (A) Total GSH levels in the livers of untreated and 40 mM L-GSH-treated *M. tb*-infected WT mice 2 and 4 weeks post-infection. (B) Reduced GSH (rGSH) levels in the livers of untreated and 40 mM L-GSH-treated mice 2, 4, and 8 weeks post-*M. tb* infection. Total and reduced GSH levels were normalized against total protein levels in the liver and expressed as the mean value \pm standard error of the mean. Untreated and treated groups ($n = 3$) were compared by one-way ANOVA and statistically significant p -values are indicated as asterisks above the comparison bars. Single asterisk (*) indicates a p -value < 0.05 . Triple asterisk (***) indicates a p -value < 0.0005 .

2.2 Aerosol Infection of Mice, Treatment, and Bacterial Colony-Forming Unit Assay

C57BL/6 specific pathogen-free (SPF) male and female wildtype (WT) mice of 8–10 weeks old were purchased from Envigo (Envigo, Indianapolis, IN, USA) and infected with around 100 CFUs of H37Rv via the Glas-Col aerosolization apparatus (Glas-Col, Terre Haute, IN, USA), as previously described [22]. *M. tb*-infected mice were supplemented with either 40 mM or 80 mM L-GSH in their drinking water, as previously described [20]. Our collaborator, Dr. Guilford, provided us with L-GSH, from Your Energy Systems, Palo Alto, CA, US. A total of 3 mice comprised the experimental groups, which were euthanized at 2 weeks, 4 weeks, and 8 weeks post-infection, and liver and spleen samples were collected at the time of necropsy. The timepoints were based on the growth curve of *M. tb* infection in the mice [23]. Liver and spleen portions were homogenized, serially diluted, plated onto Middlebrook 7H11 agar plates, and incubated in 5% CO₂ at 37 °C for 3–4 weeks, to calculate the bacterial load. Liver tissue homogenates from female mice were purified through 0.2-micron filters and used to assess the levels of relevant biomarkers, including GSH, MDA, and cytokine profiles. Parts of the liver and spleen tissues, from the male mice, were used for the histological analysis (Fig. 1).

2.3 Ethics Statement

All animals in the study were humanely handled in accordance with National Institute of Health (NIH) guidance procedures. The animal protocols were performed in biosafety level 3 facilities as per the approved procedures of the Rutgers University Institutional Animal Care and Use Committee (IACUC). This study was approved by the IACUC (Protocol#R19 IACUC008).

2.4 Total Protein Quantification

Total protein levels from liver homogenates were assessed using the Pierce™ BCA Protein Assay Kit (catalog #23227) procured from Thermo Fisher Scientific, following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA).

2.5 Glutathione Level Quantification

We measured total, reduced, and oxidized GSH levels in liver homogenates of untreated, 40 mM, and 80 mM L-GSH-treated *M. tb*-infected WT mice. We measured total and oxidized GSH levels using a Glutathione Colorimetric Detection Kit, procured from Thermo Fisher Scientific (catalog #EIAGSHC), following the manufacturer's instructions (Thermo Fisher Scientific Waltham, MA, USA). Reduced GSH levels were measured by subtracting oxi-

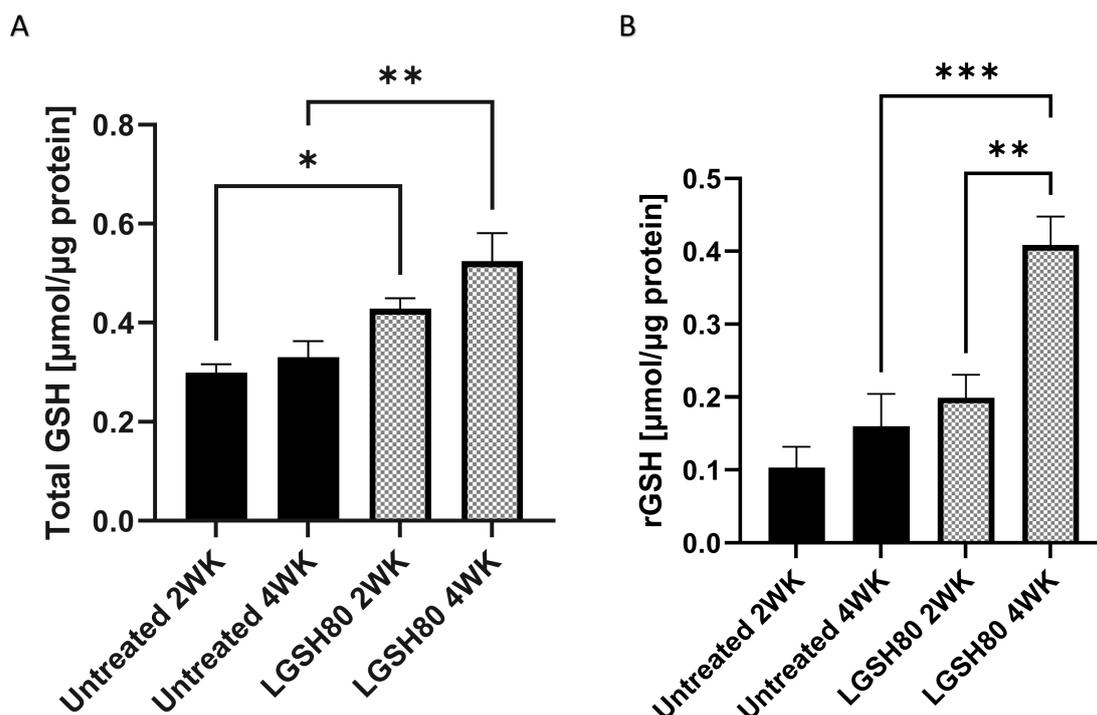


Fig. 3. Measurement of total and reduced forms of GSH in livers of *M. tb*-infected female C57BL/6 mice treated orally with 80 mM L-GSH. (A,B) Total GSH (A) and reduced GSH (rGSH) (B) levels in the livers of untreated and 80 mM L-GSH-treated *M. tb*-infected mice 2 and 4 weeks post-infection. Total and reduced GSH levels were normalized against total protein levels in the liver and expressed as the mean value \pm standard error of the mean. Untreated and treated groups ($n = 3$) were compared by one-way ANOVA and statistically significant p -values are indicated as asterisks above the comparison bars. A single asterisk (*) indicates a p -value < 0.05 . A double asterisk (**) indicates a p -value < 0.005 . A triple asterisk (***) indicates a p -value < 0.0005 .

dized GSH levels from total GSH levels. GSH levels were normalized to total protein levels for the samples and measured as micromoles of GSH per microgram of protein.

2.6 Malondialdehyde Level Quantification

MDA levels in the livers of untreated and L-GSH-treated mice were measured spectrophotometrically using a Cayman Chemicals TBARS Kit (catalog #10009055), following the manufacturer's protocols (Cayman Chemicals, Ann Arbor, MI, USA). MDA measurements were normalized to total sample protein levels and reported as micromoles of MDA per microgram protein.

2.7 Assessment of Cytokine Levels

Cytokine levels of interferon-gamma (IFN- γ), tumor necrosis factor (TNF)- α , and interleukin (IL)-6 and IL-10 were measured in the liver homogenates of untreated, 40 mM GSH-treated, and 80 mM GSH-treated *M. tb*-infected WT mice using a sandwich ELISA assay: IFN- γ Uncoated ELISA kit (catalog #88-7314-88), TNF- α uncoated ELISA kit (catalog #88-7324-88), IL-6 uncoated ELISA (catalog #88-7064-88), and IL-10 uncoated ELISA kit (catalog #88-7105-88). All ELISA kits were obtained from Thermo Fisher Scientific, and assays were performed using the pro-

cedure supplied by the manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). Cytokine concentrations were normalized to total sample protein levels and reported as picograms of cytokine per microgram protein.

2.8 Tissue Sectioning and Histology Staining of Liver and Spleen Samples

Portions of liver and spleen collected from *M. tb*-infected mice were fixed in 10% neutral formalin prior to embedding in paraffin. Embedded samples were cut into 5 μ m sections using a microtome blade and fixed onto a glass slide. We performed hematoxylin and eosin (H&E) (Poly Scientific, Bay Shore, NY, USA) staining to visualize the cellular organization and distribution of host-infected tissues. Briefly, tissue sections fixed on glass slides were deparaffinized with xylene and rehydrated by serially treating with absolute and 95% ethanol prior to soaking in distilled water. Rehydrated tissue sections were first stained with hematoxylin and rinsed with distilled water before eosin staining. Stained sections were serially treated with absolute and 95% ethanol and xylene before the coverslips were mounted onto glass slides. Stained liver and spleen sections were visualized using an Olympus Model BX41TF microscope and imaging was performed with an Olympus DP controller (East Lyme, CT, USA).

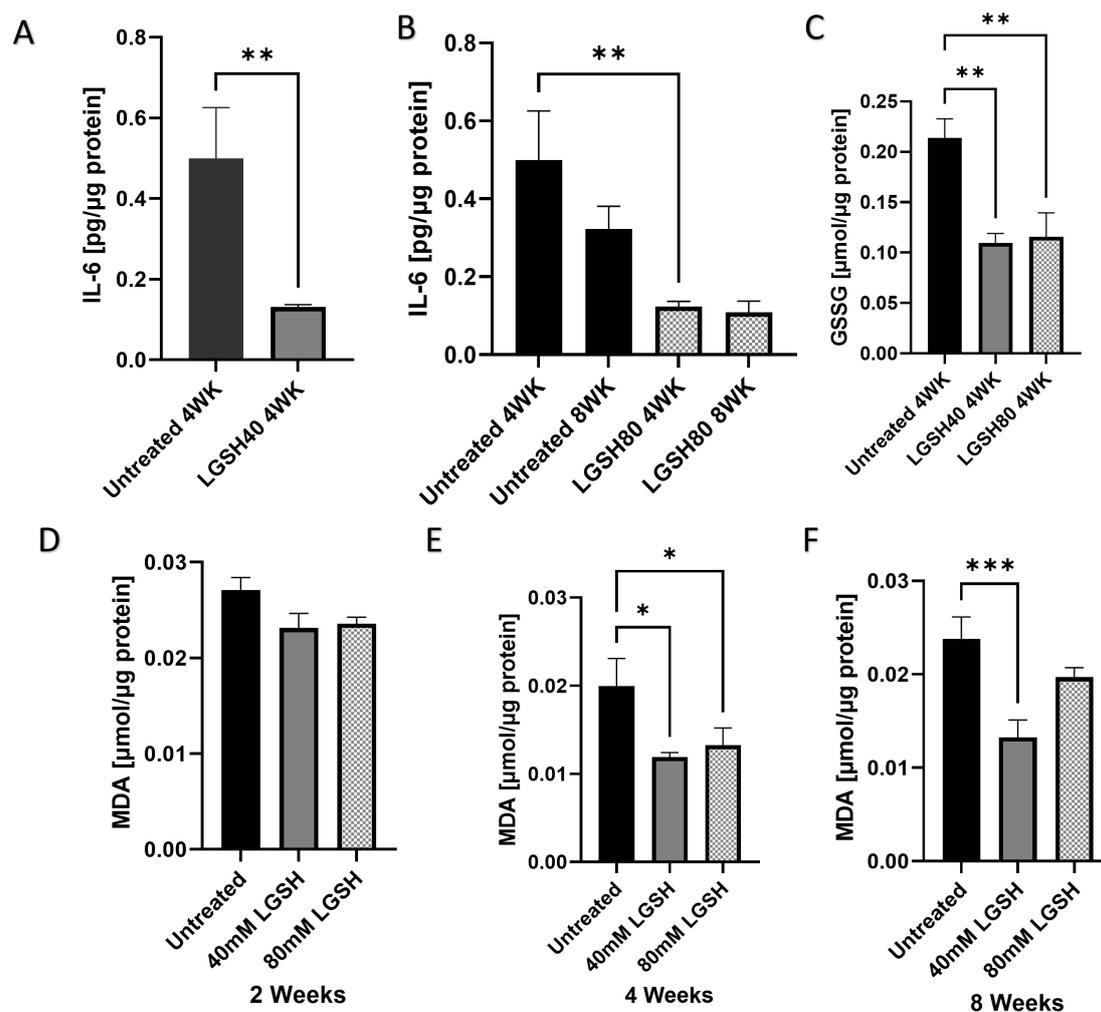


Fig. 4. Measurement of IL-6 and oxidized GSH in the livers of *M. tb*-infected female C57BL/6 mice treated orally with 40 and 80 mM L-GSH. (A) IL-6 levels in the livers of untreated and 40 mM L-GSH-treated *M. tb*-infected mice 4 weeks post-infection. (B) IL-6 levels in the livers of untreated and 80 mM L-GSH-treated *M. tb*-infected 4 and 8 weeks post-infection. (C) Oxidized glutathione (GSSG) levels in the livers of untreated, 40 mM L-GSH, and 80 mM LGSH-treated *M. tb*-infected mice 4 weeks post-infection. MDA levels in the livers of untreated and L-GSH-treated mice. (D-F) Malondialdehyde (MDA) levels in the livers of untreated, 40 mM, and 80 mM L-GSH-treated *M. tb*-infected mice 2 weeks (D), 4 weeks (E), and 8 weeks (F) post-infection. IL-6, GSSG, and MDA levels were normalized against total protein levels in the liver and are expressed as mean value \pm standard error of the mean. Graphs consisting of two comparison groups were compared by unpaired *t*-test with Welch's correction; three or more comparison groups were compared by one-way ANOVA. Statistically significant *p*-values are indicated by asterisks above the comparison bars. A single asterisk (*) indicates a *p*-value < 0.05 . A double asterisk (**) indicates a *p*-value < 0.005 . A triple asterisk (***) indicates a *p*-value < 0.0005 .

2.9 Statistical Analysis

GraphPad Prism Software 8 (GraphPad Prism 8.0.2, Boston, MA, USA) was used for statistical analysis. Statistical significance between the two groups was determined by an unpaired *t*-test with Welch's correction or Mann-Whitney test. Multiple groups were compared by one-way Analysis of variance (ANOVA). All values reported are representative of the mean and standard error of the mean for each respective category, while *p*-values of < 0.05 were considered statistically significant.

3. Results

3.1 Total and Reduced Glutathione are Elevated after Liposomal Glutathione Supplementation in the Livers of *M. tb*-Infected Mice

Hepatic total and reduced GSH (rGSH) levels were assessed between untreated and L-GSH-treated *M. tb* infected mice by one-way ANOVA. Untreated mice showed a non-significant difference in the total and rGSH levels, 2 and 4 weeks post-*M. tb* infection, although rGSH levels were significantly elevated 8 weeks ($p = 0.0001$) post-*M. tb* infection (Fig. 2A,B). A L-GSH treatment of 40 mM caused

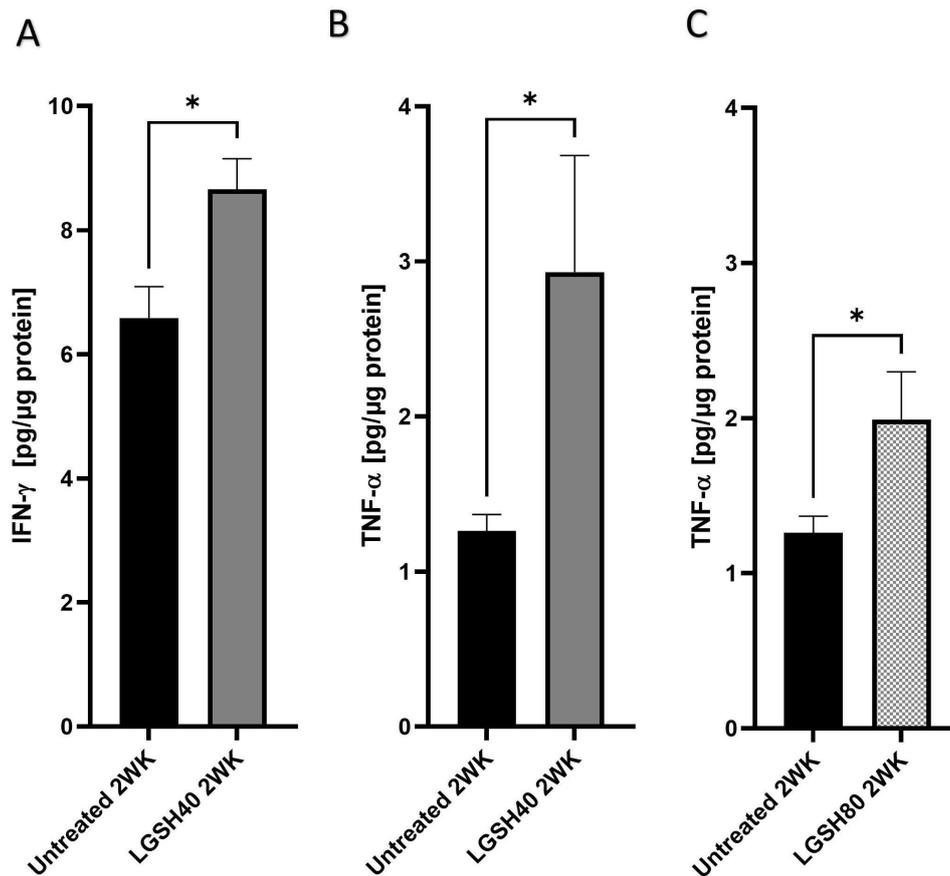


Fig. 5. IFN- γ and TNF- α levels in the livers of untreated and L-GSH-treated mice. (A) IFN- γ levels in the livers of untreated and 40 mM L-GSH-treated *M. tb*-infected mice 2 weeks post-infection. (B,C) TNF- α levels in the livers of untreated, 40 mM (B), and 80 mM (C) L-GSH-treated *M. tb*-infected mice 2 weeks post-infection. IFN- γ and TNF- α levels were normalized against total protein levels in the liver and are expressed as mean value \pm standard error of the mean. IFN- γ levels in untreated and treated groups ($n = 3$) were compared by unpaired *t*-test with Welch's correction. TNF- α levels in untreated and treated groups were compared by Mann-Whitney test. Statistically significant *p*-values are indicated by an asterisk above the comparison bars. A single asterisk (*) indicates a *p*-value < 0.05 .

a significant increase in the total GSH levels in the liver at 2 weeks ($p = 0.0355$) and 4 weeks ($p = 0.0170$) post-infection (Fig. 2A). Additionally, hepatic rGSH levels were significantly elevated after 40 mM L-GSH treatment at 2 weeks ($p = 0.0351$), and 4 weeks ($p = 0.0005$) post-*M. tb* infection (Fig. 2B). An increase in rGSH levels was also observed after 40 mM L-GSH treatment at 8 weeks post-infection, although was not statistically significant (Fig. 2B). A L-GSH treatment of 80 mM significantly increased the total GSH levels in the liver at 2 weeks ($p = 0.0381$) and 4 weeks ($p = 0.0020$) post-infection (Fig. 3A). Furthermore, an 80 mM L-GSH treatment significantly increased rGSH levels 4 weeks ($p = 0.0006$) post-infection, whereby 4 weeks of 80 mM L-GSH treatment yielded significantly higher levels of hepatic rGSH than 2 weeks of 80 mM L-GSH treatment ($p = 0.0031$) (Fig. 3B).

3.2 Liposomal Glutathione Supplementation Reduces Hepatic Interleukin-6, Oxidized Glutathione, and Malondialdehyde Levels

Markers of oxidative stress in the livers of *M. tb* infected mice were assessed between L-GSH treated and untreated mice. IL-6 levels in untreated and L-GSH-treated groups were compared by unpaired *t*-test with Welch's correction. A treatment of 40 mM L-GSH to *M. tb*-infected mice was associated with a significant decrease in IL-6 levels, 4 weeks ($p = 0.0062$) post-infection (Fig. 4A). A treatment of 80 mM L-GSH to *M. tb*-infected mice had a significant decrease in IL-6 levels 4 weeks ($p = 0.0020$) post-*M. tb* infection and an observable, yet nonsignificant, decrease at 8 weeks post-infection (Fig. 4B). Oxidized GSH (GSSG) levels were compared between the untreated and L-GSH-treated groups by one-way ANOVA. A significant decrease in GSSG levels was observed following 40 mM L-GSH treatment 4 weeks ($p = 0.0044$) post-infection (Fig. 4C). Similarly, GSSG was also signif-

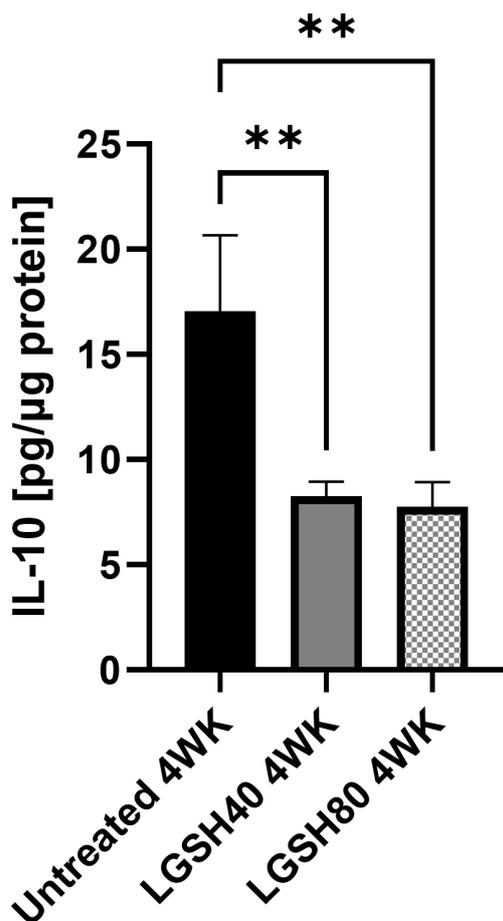


Fig. 6. IL-10 levels in the livers of untreated and L-GSH-treated mice. IL-10 levels in the livers of untreated, 40 mM, and 80 mM L-GSH-treated mice at 4 weeks post-infection. IL-10 levels were normalized against total protein levels in the liver and are expressed as the mean value \pm standard error of the mean. Untreated and treated groups ($n = 3$) were compared by one-way ANOVA and statistically significant p -values are indicated by asterisks above the comparison bars. Double asterisks (**) indicate a p -value < 0.005 .

icantly decreased after 80 mM L-GSH treatment 4 weeks post-infection ($p = 0.0065$) (Fig. 4C). MDA levels between untreated and treated groups were compared by one-way ANOVA (Fig. 4D–F). A 40 mM L-GSH treatment to *M. tb*-infected mice caused a significant decrease in the MDA levels in the liver at 4 weeks ($p = 0.0107$) and 8 weeks ($p = 0.0005$) post-infection (Fig. 4E,F). An 80 mM L-GSH treatment to *M. tb*-infected mice caused a significant decrease in the MDA levels in the liver at 4 weeks ($p = 0.0419$) post-infection, which was reduced at 8 weeks post-infection but not to levels of significance (Fig. 4E,F).

3.3 Liposomal Glutathione Supplementation Increases Th1 Cytokines Interferon Gamma and Tumor Necrosis Factor- α in the Liver

We assessed whether L-GSH treatment stimulated the production of Th1 cytokines. IFN- γ levels in the livers of the untreated and L-GSH-treated groups were compared by unpaired t -test with Welch's correction. A treatment of 40 mM L-GSH significantly increased hepatic IFN- γ 2 weeks ($p = 0.0194$) post-infection in *M. tb*-infected mice (Fig. 5A). The TNF- α levels in the untreated and L-GSH-treated groups were compared using the Mann–Whitney test. Hepatic TNF- α was significantly elevated at 2 weeks ($p = 0.0190$) post-infection following the 40 mM L-GSH treatment (Fig. 5B). Similarly, 80 mM L-GSH significantly increased hepatic TNF- α levels at 2 weeks ($p = 0.0260$) post-infection (Fig. 5C).

3.4 Liposomal Glutathione Supplementation Reduced Immunosuppressive Interleukin-10 Levels in the Liver

A comparison of the IL-10 levels in the livers between untreated and L-GSH-treated groups was performed by one-way ANOVA. A 40 mM L-GSH treatment to the *M. tb*-infected mice significantly decreased IL-10 in the liver at 4 weeks ($p = 0.0086$) post-infection (Fig. 6). Treatment with 80 mM L-GSH caused a significant decrease in hepatic IL-10 at 4 weeks ($p = 0.0060$) post-infection (Fig. 6).

3.5 Hepatic and Splenic *M. tb* Survival Decreased after Liposomal Glutathione Treatment

Hepatic *M. tb* burden between L-GSH-treated and untreated groups was compared by one-way ANOVA. A L-GSH treatment of 40 mM administered to *M. tb*-infected mice significantly decreased the hepatic *M. tb* burden at 4 weeks ($p = 0.0130$) and 8 weeks ($p = 0.0389$) post-infection (Fig. 7A,B). However, an 80 mM L-GSH treatment significantly reduced the *M. tb* viability at 4 weeks ($p = 0.0046$) post-infection (Fig. 7A). The splenic *M. tb* burden between L-GSH-treated and untreated groups was compared by the Mann–Whitney Test. Treatment with 40 mM L-GSH resulted in a significant reduction in the splenic *M. tb* burden at 2 weeks ($p = 0.0496$) and 8 weeks ($p = 0.0023$) post-infection (Fig. 7C,D).

3.6 Liposomal Glutathione Treatment Provides Host-Protective Effects on Liver and Spleen

Histopathological analysis of the livers from untreated *M. tb*-infected mice at 4 weeks post-infection showed remarkable immune cell infiltration, particularly in perivascular mononuclear cells, including lymphocytes. However, 40 mM or 80 mM L-GSH treatments administered to *M. tb*-infected mice reduced the immune cell infiltration and inflammation. At 8 weeks post-infection, the sizes of the lesion and immune cell infiltration were reduced in the *M. tb*-infected untreated mice. Compared to the untreated mice, the L-GSH-supplemented mice exhibited reduced lesion sizes and pathological manifestations, which were pro-

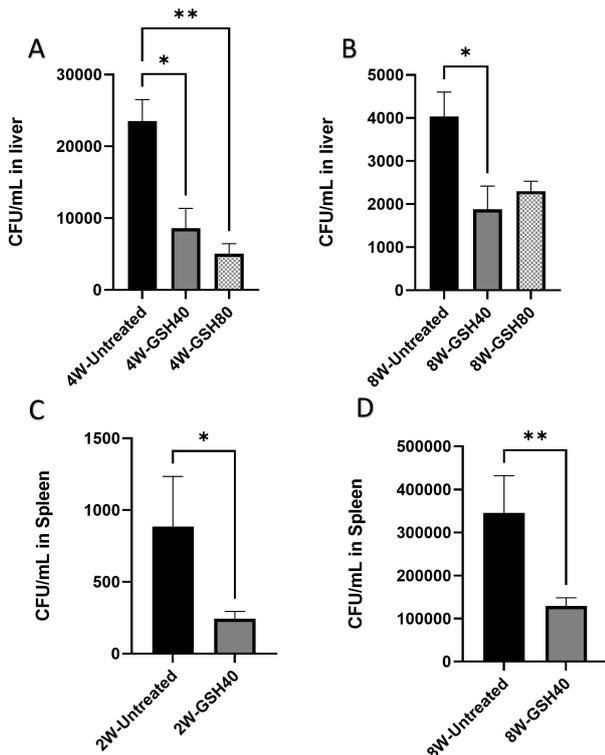


Fig. 7. Survival of *M. tb* in the livers of untreated and L-GSH-treated mice. (A,B) CFU counts of *M. tb* H37Rv strain per milliliter of liver homogenate of untreated, 40 mM, and 80 mM L-GSH-treated *M. tb*-infected mice at 4 weeks (A) and 8 weeks (B) post-infection. Untreated and treated groups ($n = 3$) are expressed as mean value \pm standard error of the mean and compared via one-way ANOVA. Survival of *M. tb* in the spleens of untreated and L-GSH-treated mice. (C,D) *M. tb* CFU counts per milliliter spleen homogenate from untreated and 40 mM L-GSH-treated *M. tb*-infected mice at 2 weeks (C) and 8 weeks (D) post-infection. Untreated and treated groups ($n = 3$) are expressed as mean value \pm standard error of the mean and compared via Mann–Whitney test. Statistically significant p -values are indicated as asterisks above the comparison bars. A single asterisk (*) indicates a p -value < 0.05 . Double asterisks (**) indicate a p -value < 0.005 .

portional to the dosing (i.e., 80 mM showed a more prominent effect than 40 mM L-GSH), particularly at 8 weeks post-infection.

Histopathological analysis of the spleens from untreated *M. tb*-infected mice at 4 weeks post-infection showed abundant immune cell infiltration, particularly by macrophages, lymphocytes, and a few polymorphonuclear (PMN) cells, in the red pulp and in the associated inflammation throughout the red and white pulps. However, neither 40 mM nor 80 mM L-GSH treatments reduced immune cell infiltration and inflammation in the spleens of the *M. tb*-infected mice at 4 weeks post-infection. Comparatively, more PMN infiltration and associated inflammation were

noted in the spleens of the *M. tb*-infected untreated mice at 8 weeks post-infection. At this time point, 40 mM L-GSH supplementation also presented a similar pathological manifestation as the one observed in the untreated mouse spleen. In contrast, 80 mM L-GSH supplementation showed a significant improvement in disease pathology in the infected mice with the sporadic presence of PMNs and inflammation.

4. Discussion

Manifestation of *M. tb* infection outside of the lungs leads to extrapulmonary tuberculosis (EPTB). This study focuses on EPTB, specifically in the liver and spleen. Hepatosplenic TB occurs hematogenously through the hepatic artery but can also be spread by gastrointestinal TB through the portal vein [5]. GSH is a tripeptide antioxidant that is highly synthesized in the liver and is responsible for maintaining redox homeostasis [24]. A preliminary infection study in untreated *M. tb*-infected mice showed that CFUs in the liver significantly increased at 4 weeks post-infection before decreasing drastically at 8 weeks post-infection (Supplementary Fig. 1). We also observed significantly elevated hepatic reduced GSH (rGSH) levels in the untreated *M. tb*-infected mice at 8 weeks post-infection (Fig. 2B). Prolonged *M. tb* infections have been known to generate reactive oxygen species (ROS), which activate the redox-sensitive transcription factor (Nrf2) that binds to the antioxidant response element (ARE) and activates the expression of ROS-cytoprotective proteins as a host response [25]. Enzymes regulated by ARE include GSH synthesizing and regulating enzymes, such as glutamate cysteine ligase, glutathione-S-transferase (GST), and glutathione synthetase (GS) [26,27]. We hypothesized that the GSH content in the liver may play a role in mitigating *M. tb* infection.

A previous research model demonstrated that supplementing mice with 40 and 80 mM of L-GSH can reduce oxidative stress, enhance Th1-promoting cytokines, reduce lung-tissue pathology, and the overall *M. tb* burden during active *M. tb* infection in the lungs of WT C57BL/6 mice [20]. Conversely, diethyl maleate (DEM) induced the depletion of GSH in the *M. tb*-infected mice, which led to an exacerbation of the *M. tb* lung infection [19]. Furthermore, L-GSH supplementation in clinical trials, involving healthy, HIV-infected, and type 2 diabetic patients, enhanced the granuloma formation of patient-blood-derived peripheral blood mononuclear cells (PBMCs) *in vitro* against *M. tb* infection [14–18]. Here, we aimed to assess whether L-GSH supplementation could elevate GSH levels, thereby reducing oxidative stress, enhancing the host's immune response, and *M. tb* clearance in extrapulmonary sites, such as the liver and spleen, in *M. tb*-infected mice.

The supplementation of L-GSH at doses of 40 mM and 80 mM, both, significantly increased total hepatic GSH levels at 4 weeks post-infection (Figs. 2A and 3A). Addition-

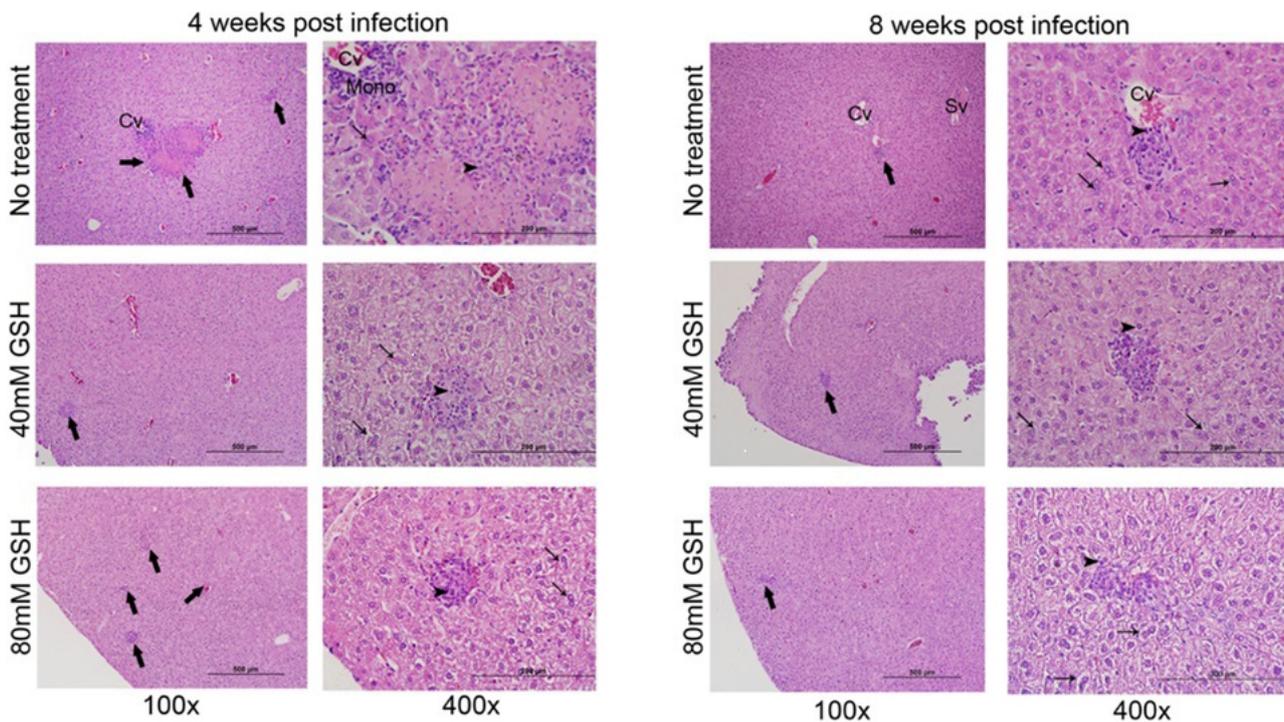


Fig. 8. Representative images of liver sections from *M. tb*-infected mice with or without 40 mM or 80 mM GSH supplementation for 4 weeks or 8 weeks stained with H&E. Mono, mononuclear cells; Cv, central vein; Sv, sublobular vein. Block arrows indicate immune cell accumulation into a granulomatous lesion; thin arrows indicate binuclear hepatocytes and arrowheads indicate lymphocytes. Images were photographed at 100× or 400× the original magnification. The scale bar for 100× panels refers to 500 μm and 400× panels refers to 200 μm.

ally, hepatic rGSH levels were also significantly elevated following L-GSH supplementation and enhanced to similar levels in untreated mice infected with *M. tb* for 8 weeks following treatment with 40 mM L-GSH (Figs. 2B and 3B). While an observable increase in the hepatic rGSH was noted at 8 weeks post-infection following a L-GSH treatment of 40 mM, the increase was not statistically significant, possibly indicating that the rGSH levels are reaching the physiological limit at 8 weeks post-infection, when mice are supplemented with L-GSH.

Then, we assessed whether the increase in total and rGSH levels was associated with a decrease in oxidative stress, which refers to a condition resulting from excessive ROS and deficient antioxidant defenses that also play a role in impairing the immune response to *M. tb* infections [28]. Oxidative stress levels were measured by evaluating known markers of oxidative stress, such as oxidized GSH levels, malondialdehyde (MDA) levels, and IL-6 levels. GSH protects cells from ROS by reducing free radicals and peroxides to form oxidized GSH (GSSG) [29]. GSSG levels decreased with an increase in rGSH levels after the supplementation of both 40 mM and 80 mM L-GSH, indicating a reduction in rGSH expenditure from ROS (Fig. 4C). Furthermore, MDA is the stable product of lipid peroxidation and is used to assess cellular injury by ROS [30]. Interest-

ingly, hepatic MDA levels were significantly reduced in the *M. tb*-infected mice at 4 and 8 weeks post-infection following the 40 mM L-GSH treatment (Fig. 4D–F). Similarly, a reduction in MDA levels was observed at 2, 4, and 8 weeks post-*M. tb* infection following 80 mM L-GSH supplementation, although a significant reduction was only observed at 4 weeks post-*M. tb* infection (Fig. 4D–F). We also measured IL-6 levels, which serve as an indicator of the host-immune responses against oxidative stress. We observed a significant reduction in IL-6 production at 4 weeks post-*M. tb* infection with 40 mM L-GSH supplementation, and at both 4 and 8 weeks post-*M. tb* infection with an 80 mM L-GSH treatment, thereby signifying a reduction in the ROS-induced responses (Fig. 3A). Altogether, supplementation of L-GSH was observed to cause reductions in oxidative stress in the livers of *M. tb*-infected mice.

Th1 cytokines, IFN- γ , and TNF- α play pivotal roles in the immune response of the host against intracellular *M. tb* infections [31]. IFN- γ is a type II interferon produced predominantly by natural killer (NK) cells and T-lymphocytes, which serve to activate macrophage function and induce major histocompatibility complex class II expression [32]. IFN- γ deficiencies were associated with increased susceptibility to *M. tb* [33]. TNF- α , produced by activated macrophages and lymphocytes, works synergis-

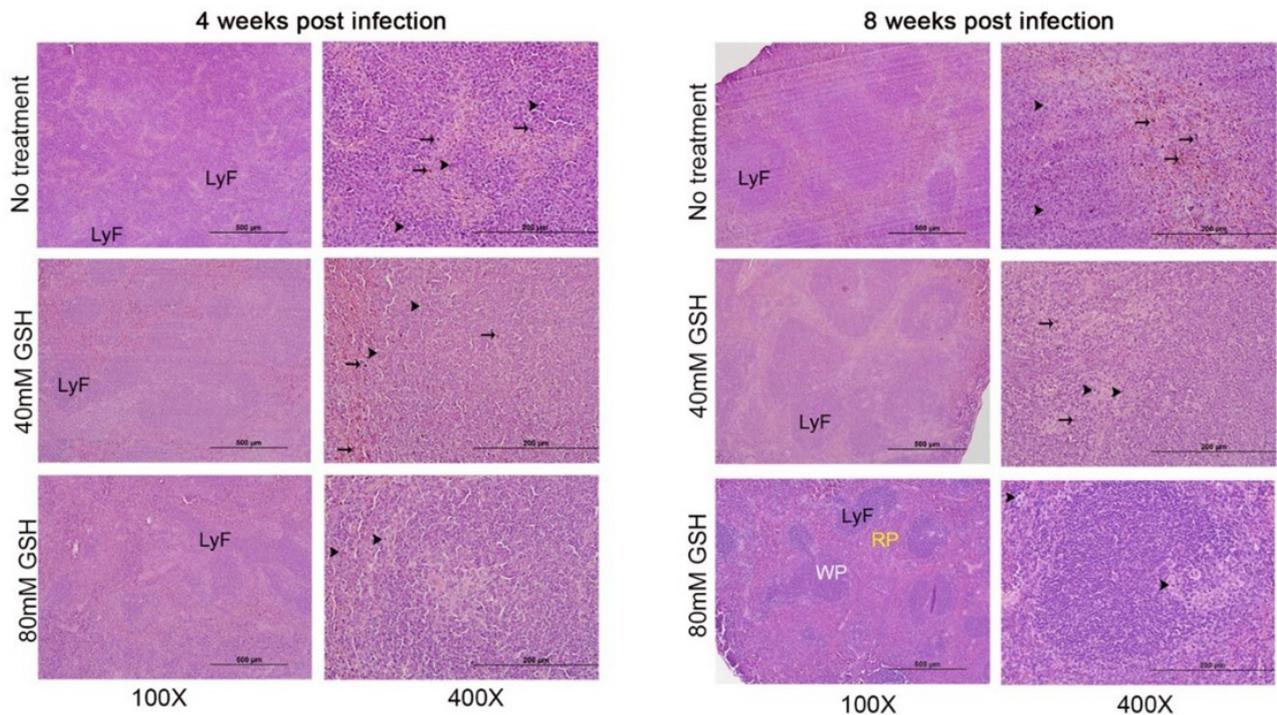


Fig. 9. Representative images of spleen sections from *M. tb*-infected mice with or without 40 mM or 80 mM GSH supplementation for 4 weeks or 8 weeks stained with H&E. LyF, lymphoid follicle; WP, white pulp; RP, red pulp. Arrows indicate PMNs and arrowheads indicate macrophages. Images were photographed at 100 \times or 400 \times the original magnification. The scale bar for 100 \times panels refers to 500 μ m and 400 \times panels refers to 200 μ m.

tically with IFN- γ to stimulate immune cell migration and granuloma formation at the site of infection [34]. *M. tb* contains virulence factors, such as phthiocerol dimycocerosate (PDIM), and ESX-1, which enable mycobacterium to resist phagocytic degradation by macrophages [35]. An increase in TNF- α induces phagocytic maturation of *M. tb* infected macrophages and induces apoptosis of infected tissues [36]. Neutralization of TNF- α in a humanized mouse model was demonstrated to resuscitate latent *M. tb* infections [37]. Furthermore, TNF- α inhibitors increased the risk of nontuberculous mycobacteria infections in patients with rheumatoid arthritis [38]. Thus, TNF- α provides a critical role in the granulomatous response against mycobacterium infection. An increase in both IFN- γ and TNF- α was detected at 2 weeks post-infection after 40 mM L-GSH treatment, indicating the augmentation of an early *M. tb* response in the liver (Fig. 5A–C). IL-10 is an inhibitory cytokine that is produced by macrophages to maintain a balance between pro- and anti-inflammatory actions; however, high levels of IL-10 have been shown to support increased *M. tb* survival in the host [34]. L-GSH supplementation significantly reduced IL-10 levels in the liver at 4 weeks post-infection (Fig. 6A). L-GSH supplementation resulted in the production of granuloma-promoting cytokines and diminished immunosuppressive cytokines.

Finally, we assessed hepatic and splenic *M. tb* survival after L-GSH treatment. Treatments with L-GSH at both doses of 40 and 80 mM to the *M. tb*-infected mice caused a significant decrease in the *M. tb* burden in the liver, with the 40 mM L-GSH treatment promoting a sustained reduction in the *M. tb* burden at 4 and 8 weeks post-infection (Fig. 7A,B). A significant decrease in the *M. tb* survival in the spleen was also observed following L-GSH treatment (Fig. 7C,D). A reduction in *M. tb* burden was also associated with a reduction in lesion size and pathological manifestation, in the liver, at 4 and 8 weeks post-infection following 40 mM L-GSH treatment. Moreover, there was a significant improvement in the disease pathology in the spleens of the infected mice at 8 weeks post-infection following 80 mM L-GSH supplementation, as demonstrated by the sporadic presence of polymorphonuclear cells and inflammation (Figs. 8,9). Overall, L-GSH supplementation was able to reduce the duration needed to raise hepatic rGSH levels to a therapeutic range, thereby reducing oxidative stress and improving *M. tb* clearance, compared to the untreated controls.

A dose-dependent response was observed in livers and spleens of L-GSH-treated *M. tb*-infected male mice, with an 80 mM L-GSH treatment resulting in the highest decrease in granuloma lesion size in the liver and splenic inflammation compared to the untreated and 40 mM-treated groups.

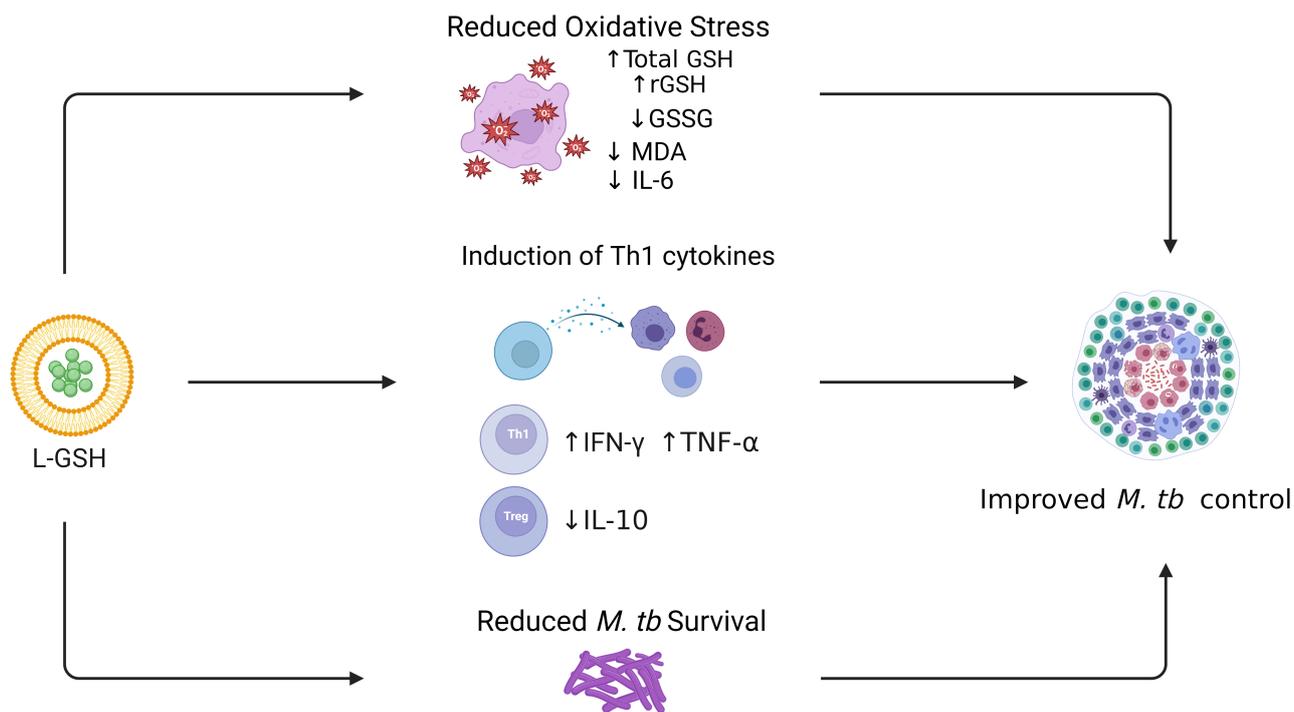


Fig. 10. Summary of study findings. L-GSH supplementation is associated with a reduction in oxidative stress, increased production of Th1-supporting cytokines in the liver, and reduced *M. tb* survival in the livers and spleens of infected mice. L-GSH supplementation increased hepatic total and reduced GSH (rGSH) levels and decreased oxidized GSH (GSSG), MDA, and IL-6 levels. An increase in IFN- γ and TNF- α levels was observed in the liver along with a decrease in the IL-10 immunosuppressive cytokine. A significant decrease in *M. tb* CFU counts was observed in the livers and spleens of *M. tb*-infected mice following L-GSH treatment. An upward arrow (\uparrow) indicates an increase in levels, whereas a downward arrow (\downarrow) indicates a decrease.

However, a dose-dependent response was not observed in the female mice, whereby 40 mM L-GSH provided more optimal antimycobacterial activity than the 80 mM L-GSH treatment. Additionally, female mice treated with 80 mM L-GSH demonstrated relatively higher MDA levels than female mice treated with 40 mM L-GSH. The mechanism behind this difference remains unclear. Therefore, further sex-dependent pharmacokinetic studies assessing optimal dose ranges and bioavailability are needed. Lastly, while L-GSH supplementation augmented the immune response of the host and reduced the *M. tb* survival, treatment did not result in complete *M. tb* clearance by the endpoint of the study. Thus, additional studies that assess L-GSH as an adjunctive therapy, in conjunction with first-line antimycobacterial treatments, in WT and immunocompromised animal models are necessary.

5. Conclusions

Our results indicate that GSH enhancement in the liver improves the control of the *M. tb* infection by restoring redox homeostasis and by modulating the levels of cytokines, thereby promoting a favorable immune response against *M. tb* infections. L-GSH supplementation increased the production of granuloma-promoting cytokines and diminished immunosuppressive cytokines, shifting the immune

response towards the production of Th1 cytokines. Specifically, 40 mM L-GSH treatment was efficacious in augmenting the production of IFN- γ and TNF- α at 2 weeks and significantly reducing *M. tb* viability at 4 and 8 weeks post-infection (Fig. 10). By elevating the total and reducing GSH levels, L-GSH supplementation can serve as an additional treatment modality against *M. tb* infections (Fig. 10).

Abbreviations

TB, tuberculosis; GSH, glutathione; L-GSH, liposomal glutathione; EPTB, extrapulmonary tuberculosis; rGSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; ROS, reactive oxygen species; Th1, type 1 T helper; IFN- γ , interferon-gamma; TNF, tumor necrosis factor; IL, interleukin; CFU, colony-forming unit.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

VV and SS designed the research study. KS, MK, AA, JO, SY, AB, NK, AY, AK, RK, and SR performed the re-

search. KS, MK, AA, JO, SY, AB, NK, AY, AK, RK, and SR contributed to data curation. VV and SS analyzed the data. VV obtained extramural funding. KS, VV, SS, MK, AA, AK, RK and SR, wrote and revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the IACUC (Protocol#R19 IACUC008).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbe1503015>.

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