nature portfolio

Peer Review File

Mycobacterium bovis frd operon phase variation hijacks succinate signaling to drive immunometabolic rewiring and pathogenicity

Corresponding Author: Professor Xiangmei Zhou

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript presented by Dong et al. elucidates the molecular mechanism by which HT insertion mutations in the frd operon of M. bovis regulate the host succinate/HIF-1α/IL-1β immunometabolic axis, providing groundbreaking insights into host adaptation strategies of MTBC pathogens. The authors identified polymorphisms in the frd 7G HT of clinical M. bovis isolates and demonstrated through murine infection models that these mutations enhance pathogenicity by suppressing Th1 immunity and promoting pathogenic Th17 responses. Genetic knockout and complementation experiments confirmed that frd insertion mutations disrupt FRD functionality, thereby inhibiting succinate secretion, reducing HIF-1α accumulation, and diminishing IL-1β production. IL-1R blockade experiments further validated that loss of IL-1 signaling recapitulated the Th17 polarization phenotype observed in mutant infections, exacerbating pulmonary bacterial replication and tissue pathology.

Overall, by integrating in vitro macrophage assays, in vivo infection dynamics, and clinical strain profiling, this study rigorously delineates a phase variation-driven strategy wherein metabolic reprogramming subverts host immunity. This is an interesting study, well-organized, with clearly articulated data, and conclusions are fully supported by the data. Below are minor considerations that could further enhance the study:

1) This study identifies frd insertion mutations in both M. bovis and Mtb, yet notably reveals a stark disparity in mutation frequencies between them. If frd phase variation serves as an immune evasion strategy, the significantly lower incidence of such mutations in Mtb raises an intriguing question. This discrepancy warrants further investigation or discussion to clarify its evolutionary or functional implications.

2) The authors analyzed genomes of 74 clinical M. bovis isolates in China. However, it remains unclear whether the frd 7G HT insertion mutation exists in M. bovis isolates from other countries or regions. A critical question arises: Does this mutation represent a conserved evolutionary strategy across M. bovis populations, or is it restricted to specific lineages? Addressing this geographical and phylogenetic variability would clarify its role in host adaptation and global pathogenicity.

3) Line 80: The abbreviation "WT" (wild-type) should be defined upon its first occurrence in the text.

4) Line 112: The species name Escherichia coli should be italicized to adhere to microbiological nomenclature conventions.

5) Fig 2d: The color-coded subunits in the structural model require explicit annotation. Please specify in the figure legend or caption which subunit each color represents to ensure interpretability.

6) Lines 377-381: The discussion regarding the differential experimental outcomes observed between high-dose and low-dose infection models would benefit from a more in-depth analysis. Specifically, it is critical to address whether variations in host immune responses (e.g., cytokine profiles, immune cell recruitment, or metabolic reprogramming) under distinct infectious doses contribute to the divergent pathological and bacteriological outcomes.

Reviewer #2

(Remarks to the Author)
The authors are thanked for these studies advancing the investigation into a single genetic change in M. bovis that impacts the host response in C57BL/6 inbred mice, infected with a high dose of M.bovis (5000 CFU, intranasally). The mutation altered the lung lesions from non-necrotic to neutrophilic and necrotizing in 2-3 weeks after high dose infection. The authors show that the Δfrd mutation disrupts the fumarate reductase operon, leading to reduced succinate production during infection. This drop prevents stabilization of HIF-1α in macrophages (bone marrow derived), resulting in decreased IL-1β expression. In the mouse infection experiments, M. bovis strains lacking the frd operon (Δfrd) caused less severe disease compared to wild-type (WT) or complemented strains with reduced lung and spleen inflammatory responses. When Δfrd mutants were complemented with wild-type frd alleles (either 7G or 8G variants), the pathogenic features—including tissue damage and inflammatory responses—were largely restored.

I have 2 considerations the authors may wish to address:

1) In the discussion Watanabe et al 2011 is cited as a reference that infection with a physiological low dose (100 CFU) Mtb H37Rv with the same mutation does not have the same phenotype (bacterial burden increase). Have the authors performed studies with low and mid-dose aerosol infections of the M bovis mutants and observed similar phenomena and mechanisms? What mechanisms or interactions may explain the differences attributed to dose and not genotype? Could the host responses be driven by additional initiating events, or influenced by other genes? How do the authors envision the translatability of the high infection - what patient population is represented?

2) What is the evidence that the mechanism identified using bone marrow derived macrophages is the same mechanism as in the lungs and lungs macrophages, or other lung cell types?

I have 2 minor recommendations:

1) Suggest to change from in the abstract line 20 from host "adaptation" to host "infection."
2) Suggest to present the organ weight data as absolute weights or percentage of body weight.

Reviewer #3

(Remarks to the Author)
Dong and colleagues present a compelling study revealing a novel mechanism during Mycobacterium bovis infection, where bacterial genomic homopolymeric tracts phase variation in the fumarate reductase-encoding frd operon modulates host immunometabolism. This leads to suppression of the succinate/HIF-1α/IL-1β axis and a consequential shift from protective Th1 to pathogenic Th17 immunity. The work meaningfully contributes to our understanding of host-pathogen interactions and adds valuable insights into the pathogenesis of Mycobacterium tuberculosis complex organisms. The manuscript is well-structured and the data are generally convincing. Nonetheless, addressing the following suggestions could further enhance the clarity and impact of the manuscript:
L35: It would be helpful to clarify whether the phrase "targeting metabolic checkpoints" refers to interventions on the bacterial or host side to avoid potential ambiguity.
L46: Reference 4 could be updated to reflect the most recent Global Tuberculosis Report from the WHO (2023), ensuring readers have access to the most current data.
L109: Please specify the name of the source data file to improve transparency and reproducibility.
L112: The species name Escherichia coli should be italicized. A thorough review of the manuscript for similar formatting issues would be beneficial.
L152–153: While the observed differences are visually apparent, authors should describe the method used to identify lymphocytes and neutrophils, or alternatively, cite appropriate references.
L286: Consider specifying what accumulates in this context to ensure the meaning is clear.
L320: It is generally advisable to avoid starting sentences with “and”; a rephrasing may improve the flow.
L326 / Figure 8: There appears to be a misplaced letter “e,” possibly intended for panel h. Clarification or correction would be appreciated.
Methods section: For consistency and completeness, please include the country of origin for all reagents and kits used.
Discussion: The discussion provides strong insights into host immune responses. It could be further enriched by discussing the broader implications of the altered metabolites induced by the M. bovis mutant. Additionally, the claim that this study “provides a roadmap for novel host-directed therapies…” could be elaborated upon, especially considering that the initiating factor for the altered host response is a microbial genetic variation.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript presented by Dong et al. elucidates the molecular mechanism by which HT insertion mutations in the frd operon of M. bovis regulate the host succinate/HIF-1α/IL-1β immunometabolic axis, providing groundbreaking insights into

host adaptation strategies of MTBC pathogens. The authors identified polymorphisms in the frd 7G HT of clinical M. bovis isolates and demonstrated through murine infection models that these mutations enhance pathogenicity by suppressing Th1 immunity and promoting pathogenic Th17 responses. Genetic knockout and complementation experiments confirmed that frd insertion mutations disrupt FRD functionality, thereby inhibiting succinate secretion, reducing HIF-1α accumulation, and diminishing IL-1β production. IL-1R blockade experiments further validated that loss of IL-1 signaling recapitulated the Th17 polarization phenotype observed in mutant infections, exacerbating pulmonary bacterial replication and tissue pathology.

Overall, this is an interesting study, well-organized, with clearly articulated data, and conclusions are fully supported by the data. And the authors have completed the questions which needed to supplemented.

Point-by-point response to the reviewers' comments

We are deeply grateful to the reviewers for their rigorous evaluation of our manuscript and their valuable suggestions, which have significantly enhanced the academic quality and clarity of presentation in this paper. We have conscientiously addressed all comments and provided point-by-point responses as follows.

Reviewer #1 (Remarks to the Author):

The manuscript presented by Dong *et al*. elucidates the molecular mechanism by which HT insertion mutations in the *frd* operon of *M. bovis* regulate the host succinate/HIF-1α/IL-1β immunometabolic axis, providing groundbreaking insights into host adaptation strategies of MTBC pathogens. The authors identified polymorphisms in the *frd* 7G HT of clinical *M. bovis* isolates and demonstrated through murine infection models that these mutations enhance pathogenicity by suppressing Th1 immunity and promoting pathogenic Th17 responses. Genetic knockout and complementation experiments confirmed that *frd* insertion mutations disrupt FRD functionality, thereby inhibiting succinate secretion, reducing HIF-1α accumulation, and diminishing IL-1β production. IL-1R blockade experiments further validated that loss of IL-1 signaling recapitulated the Th17 polarization phenotype observed in mutant infections, exacerbating pulmonary bacterial replication and tissue pathology.

Overall, by integrating in vitro macrophage assays, in vivo infection dynamics, and clinical strain profiling, this study rigorously delineates a phase variation-driven strategy wherein metabolic reprogramming subverts host immunity. This is an interesting study, well-organized, with clearly articulated data, and conclusions are fully supported by the data. Below are minor considerations that could further enhance the study:

We thank the reviewer for recognizing the novelty and significance of our work. We sincerely appreciate the reviewer's insightful comments and constructive feedback, which have significantly enhanced the rigor and depth of our study. Below, we address each point raised:

1) This study identifies *frd* insertion mutations in both *M. bovis* and *Mtb*, yet notably reveals a stark disparity in mutation frequencies between them. If *frd* phase variation serves as an immune evasion strategy, the significantly lower incidence of such mutations

in *Mtb* raises an intriguing question. This discrepancy warrants further investigation or discussion to clarify its evolutionary or functional implications.

We thank the reviewer for raising this insightful question. We fully agree that the biological significance of this mutation frequency discrepancy warrants further exploration and have expanded our analysis accordingly.

Our extended analysis of clinical *M. bovis* isolates from five countries confirmed the conserved high-frequency occurrence of *frd* 7G HT insertions (Supplementary Fig. 1). Combined with our prior observation of elevated *glpK* HT mutation rates in *M. bovis* compared to *M. tb* (*Infect Drug Resist*, **2022**, 15, 1469), these data suggest *M. bovis* may have evolved unique mechanisms to tolerate or promote replication errors.

HT frameshift mutations arise from replication-associated slipped-strand mispairing, a phenomenon widely observed across bacterial species (*BMC Genomics*, **2010**, 11, 102). These indel mutations are hypothesized to stem from DNA strand slippage events, where HT regions misalign with complementary sequences due to secondary structure formation in the DNA template (*Mutat Res*, **2006**, 598, 148). Recent structural studies revealed that *M. tb* DNA polymerase III (Pol III) subunits DnaQ (3'-5' exonuclease) and DnaE1 (replicase) physically interact with the β-clamp (DnaN) during replication, suggesting a potential coproofreading mechanism to maintain replication fidelity (*Proc Natl Acad Sci U S A*, **2024**, 121, e2322938121; *Sci Rep*, **2016**, 6, 18418).

However, in *M. bovis*, we observed a conserved D99G mutation in DnaN (β-clamp), while wild-type sequences for DnaQ and DnaE1. The β-clamp, a homodimeric ring-shaped protein encircling duplex DNA, serves as the critical processivity factor for Pol III (*Cell*, **2008**, 132, 46). By tethering the polymerase to DNA and sliding along the template during replication, it ensures efficient DNA synthesis while also recruiting accessory proteins involved in translesion synthesis and DNA mismatch repair (*Cell Mol Life Sci*, **2024**, 81, 245).

Our preliminary bioinformatics analysis suggests the DnaN D99G substitution increases surface positive charge density, which may enhance electrostatic interactions with negatively charged DNA, increase replication fork friction, and alter β-clamp-DNA binding dynamics, potentially disrupting its coordination with replication/repair machinery.

These biophysical changes could theoretically promote DNA strand slippage events, thereby increasing HT mutation rates. This hypothesis aligns with our empirical observations of elevated HT instability in *M. bovis* compared to *M. tb* and provides a mechanistic framework for future experimental validation. Future work will functionally validate this mutation's molecular consequences and explore its role in the ecological adaptation of the MTBC.

We have incorporated this discussion in the revised manuscript (Lines 443-452):

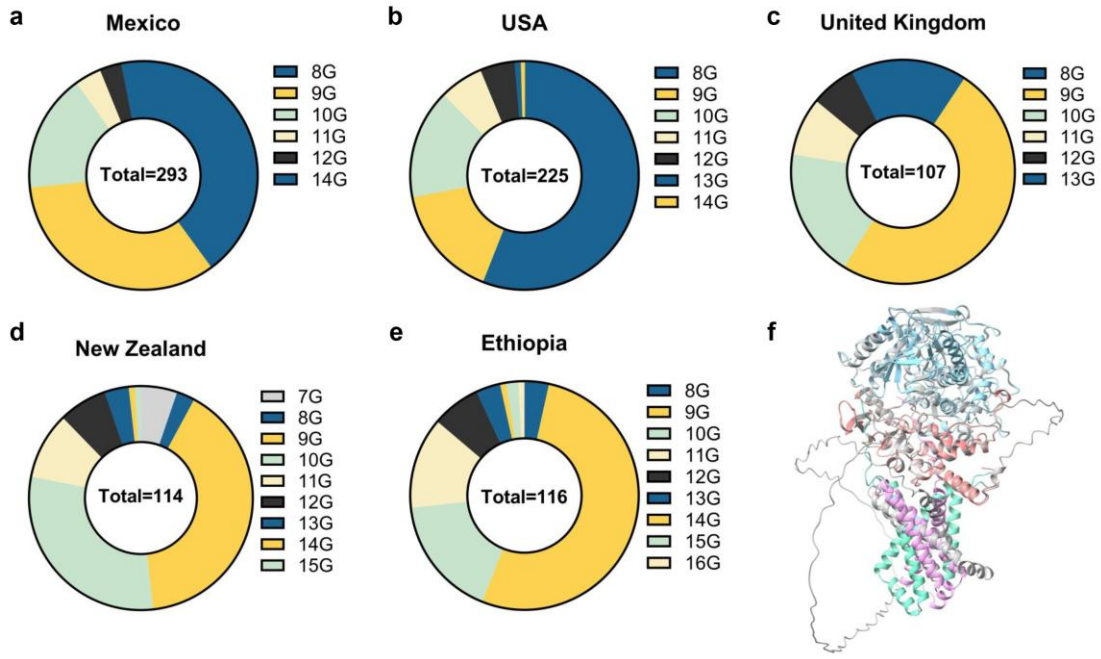
"Our analysis revealed frequent insertion mutations within the frd 7G HT of M. bovis, consistent with our previous finding of significantly higher glpK 7C HT mutation rates in M. bovis compared to M. tb[64]. These combined observations suggest that M. bovis may have evolved unique mechanisms to tolerate or promote replication errors. Frameshift mutations in HTs typically originate from replication-associated slipped-strand mispairing[65]. In M. tb, the DNA polymerase III subunits DnaQ (3'-5' exonuclease) and DnaE1 (replicase) physically interact with the β-clamp (DnaN), suggesting a potential coproofreading mechanism to maintain replication fidelity[66,67]. Strikingly, M. bovis harbors a conserved D99G mutation in DnaN, which could promote strand slippage during replication, thereby elevating HT mutation rates, although this finding requires additional investigation."

2) The authors analyzed genomes of 74 clinical *M. bovis* isolates in China. However, it remains unclear whether the *frd* 7G HT insertion mutation exists in *M. bovis* isolates from other countries or regions. A critical question arises: Does this mutation represent a conserved evolutionary strategy across *M. bovis* populations, or is it restricted to specific lineages? Addressing this geographical and phylogenetic variability would clarify its role in host adaptation and global pathogenicity.

We sincerely appreciate the reviewer's insightful comments regarding the evolutionary and geographical implications of the *frd* 7G HT insertion mutations. As suggested, we supplemented and analyzed the data of *M. bovis* isolates in five countries spanning five biogeographical regions (Mexico, USA, UK, New Zealand, and Ethiopia). We added the following statement and Supplementary Fig. 1 (Lines 111-122 in the revised manuscript):

"To elucidate the phylogeographic distribution and evolutionary conservation of the frd 7G HT insertion mutation in M. bovis, we analyzed genomes from Mexico, the USA, the

United Kingdom, New Zealand, and Ethiopia. Notably, isolates from Mexico and the USA predominantly exhibited the 8G HT mutation pattern observed in Chinese strains, while those from New Zealand, the United Kingdom, and Ethiopia exhibited a distinct 9G HT mutation as the dominant genotype (Supplementary Fig. 1a-e). Structural modeling revealed that the 9G HT insertion introduces a frameshift that fundamentally alters the FRD architecture by inducing complete translational fusion of FrdB, FrdC, and FrdD subunits into a single contiguous polypeptide chain (Supplementary Fig. 1f). This aberrant fusion results in marked structural divergence from the wild-type FRD, exceeding the conformational changes caused by the 8G HT mutation. These findings suggest that high-frequency 7G HT insertion mutations represent a conserved evolutionary strategy across geographically distinct M. bovis populations."



Supplementary Fig. 1 Insertion mutations in the *frd* 7G HT of *M. bovis* clinical isolates.

a–e Mutation frequency of *M. bovis* isolates from Mexico (a, *n*=293), USA (b, *n*=225), United Kingdom (c, *n*=107), New Zealand (d, *n*=114), and Ethiopia (e, *n*=116). **f** 3D structural models of WT FRD (colored subunits: FrdA in blue, FrdB in red, FrdC in green, FrdD in purple) and the 9G HT mutant (gray). Source data are provided as a Source Data file.

3) Line 80: The abbreviation "WT" (wild-type) should be defined upon its first occurrence in the text.

We thank the reviewer for highlighting this oversight. We have revised the manuscript to explicitly define "WT" (wild-type) upon its first occurrence in line 80 of the original text (now line 81 in the revised manuscript). Additionally, we conducted a full-text audit to ensure proper definition of all abbreviations at their first mention throughout the article.

4) Line 112: The species name Escherichia coli should be italicized to adhere to microbiological nomenclature conventions.

We sincerely thank the reviewer for highlighting this formatting oversight. We have corrected the species name *Escherichia coli* to italicized format (line 125 in the revised manuscript) and conducted a comprehensive review of the entire manuscript to ensure consistent adherence to scientific nomenclature standards.

5) Fig 2d: The color-coded subunits in the structural model require explicit annotation. Please specify in the figure legend or caption which subunit each color represents to ensure interpretability.

We sincerely appreciate the reviewer's constructive feedback on improving the clarity of our structural analysis. As suggested, we have revised Figure 2d's legend as follows (lines 145-147 in the revised manuscript):

*"**d** The 8G HT mutation-induced FrdB-FrdC in-frame fusion (gray) sterically obstructs the MQ9-binding pocket, with structural occlusion mediated by an orange-highlighted peptide segment."*

6) Lines 377-381: The discussion regarding the differential experimental outcomes observed between high-dose and low-dose infection models would benefit from a more in-depth analysis. Specifically, it is critical to address whether variations in host immune responses (e.g., cytokine profiles, immune cell recruitment, or metabolic reprogramming) under distinct infectious doses contribute to the divergent pathological and bacteriological outcomes.

We sincerely appreciate the reviewer's insightful suggestion. We fully agree that the observed dose-dependent pathological divergence likely reflects complex interactions between bacterial burden and host immune modulation. While low-dose (100 CFU)

murine models remain prevalent in TB research, emerging evidence demonstrates that higher bacterial challenges better recapitulate the immune mechanism of active TB, which aligns with our experimental design: high-dose *M. tb* infection in C57BL/6 mice replicates the blood transcriptomic signatures observed in active TB patients-a critical feature unattainable with conventional low-dose models (*Nat Immunol*, **2020**, 21, 466). This experimental approach aligns with recent non-human primate studies where aerosolized high-dose *M. tb* induced progressive active TB through macrophage interferon responses, mirroring the elevated IFN signatures characteristic of human active TB (*Cell Host Microbe*, **2021**, 29, 168). Notably, the high-dose infection paradigm uncovers protective roles of multiple autophagy genes in the lung, where autophagy promotes effective T cell response to control infection (*Nat Microbiol*, **2024**, 9, 692). Our selection of high-dose infection specifically targets to better reflect the immune response during active TB. While we appreciate the reviewer's valid concerns about dose effects, our focus on acute-stage host-pathogen interactions makes the high-dose model optimal for capturing critical pathophysiological events. Future studies will employ single-cell sequencing to systematically dissect dose-dependent immunomodulation.

As suggested by the reviewers, we have expanded our discussion of these concepts in lines 396–408 of the revised manuscript to further elucidate the immunological and metabolic mechanisms underlying dose-dependent pathological outcomes:

"This striking contrast underscores the pivotal role of infection dose in TB pathogenesis modeling. While the low-dose (100 CFU) murine model remains prevalent in TB research, emerging evidence demonstrates that higher bacterial challenges better recapitulate the immune mechanisms of active TB[43-45]. High-dose M. tb infection in C57BL/6 mice replicates the blood transcriptomic signatures observed in active TB patients—a critical feature unattainable with conventional low-dose models[45]. This paradigm is further supported by non-human primate studies where aerosolized high-dose M. tb induced progressive active TB through macrophage interferon responses, mirroring the elevated IFN signatures characteristic of human active TB[44]. Furthermore, recent work demonstrates that high-dose challenges unveil otherwise cryptic host defense mechanisms: autophagy activation in lung macrophages suppresses myeloid-derived suppressor cell accumulation, thereby potentiating T cell-mediated bacterial control[43]. Our selection of high-dose infection specifically targets to better reflect the immune response during active TB."

Reviewer #2 (Remarks to the Author):

The authors are thanked for these studies advancing the investigation into a single genetic change in *M. bovis* that impacts the host response in C57BL/6 inbred mice, infected with a high dose of *M. bovis* (5000 CFU, intranasally). The mutation altered the lung lesions from non-necrotic to neutrophilic and necrotizing in 2-3 weeks after high dose infection. The authors show that the Δ*frd* mutation disrupts the fumarate reductase operon, leading to reduced succinate production during infection. This drop prevents stabilization of HIF-1α in macrophages (bone marrow derived), resulting in decreased IL-1β expression. In the mouse infection experiments, *M. bovis* strains lacking the *frd* operon (Δ*frd*) caused less severe disease compared to wild-type (WT) or complemented strains with reduced lung and spleen inflammatory responses. When Δ*frd* mutants were complemented with wild-type *frd* alleles (either 7G or 8G variants), the pathogenic features—including tissue damage and inflammatory responses—were largely restored.

We sincerely appreciate the reviewer's thoughtful and comprehensive summary of our work, as well as their time and expertise in evaluating our manuscript. Below, we have carefully addressed each of the specific comments and suggestions, with detailed responses and revisions incorporated into the revised manuscript.

I have 2 considerations the authors may wish to address:

1) In the discussion Watanabe et al 2011 is cited as a reference that infection with a physiological low dose (100 CFU) Mtb H37Rv with the same mutation does not have the same phenotype (bacterial burden increase). Have the authors performed studies with low and mid-dose aerosol infections of the M bovis mutants and observed similar phenomena and mechanisms? What mechanisms or interactions may explain the differences attributed to dose and not genotype? Could the host responses be driven by additional initiating events, or influenced by other genes? How do the authors envision the translatability of the high infection - what patient population is represented?

We fully concur with the reviewer's emphasis on infection dosage in shaping immunological outcomes. While we have not performed low/mid-dose aerosol infections with *M. bovis* mutants in this study, we respectfully submit that our experimental design remains scientifically justified and ethically responsible for the following reasons:

Experimental Design Rationale: While the low-dose (100 CFU) murine model remains prevalent in TB research, emerging evidence demonstrates that higher bacterial challenges better recapitulate the immune mechanism of active TB, which aligns with our experimental design: High-dose *M. tb* infection in C57BL/6 mice replicates the blood transcriptomic signatures observed in active TB patients-a critical feature unattainable with conventional low-dose models (*Nat Immunol*, **2020**, 21, 466). This experimental approach aligns with recent non-human primate studies where aerosolized high-dose *M. tb* induced progressive active TB through macrophage interferon responses, mirroring the elevated IFN signatures characteristic of human active TB (*Cell Host Microbe*, **2021**, 29, 168). Notably, the high-dose infection paradigm uncovers protective roles of multiple autophagy genes in the lung, where autophagy promotes effective T cell response to control infection (*Nat Microbiol*, **2024**, 9, 692). Our selection of high-dose infection specifically targets to better reflect the immune response during active TB.

Ethical Considerations: Our study strictly adheres to the principles of Replacement, Reduction, and Refinement in animal research. The high-dose infection in mice already demonstrates clear phenotypic differences between wild-type *M. bovis* and mutant, fulfilling the primary objective of this work. Conducting low/mid-dose experiments would necessitate additional animals without providing mechanistically novel insights, as

(a) If low/mid-dose infections fail to replicate the high-dose phenotype (as in Watanabe et al. 2011), this would suggest that high-dose infections better recapitulate human active TB immunopathology—a conclusion supported by Feng et al. (*Nat Microbiol*, **2024**, 9, 685), who demonstrated that high-dose infection uniquely uncovers protective autophagy pathways. This outcome would reinforce, rather than negate, the relevance of our current model.

(b) If low/mid-dose infections do reveal phenotypic differences, this would redundantly confirm the role of *frd* in virulence, which is already established at high doses.

Neither scenario would alter our core conclusions about the genotype-specific impact of the *frd* mutant.

While we appreciate the reviewer's valid concerns about dose effects, our focus on acute-stage host-pathogen interactions makes the high-dose model optimal for capturing critical pathophysiological events. We share the reviewer's interest in dose-dependent immunomodulation; future studies will employ single-cell RNA sequencing to systematically dissect transcriptional heterogeneity across immune cell subsets.

We have expanded our discussion of these concepts in lines 396–408 of the revised manuscript:

"This striking contrast underscores the pivotal role of infection dose in TB pathogenesis modeling. While the low-dose (100 CFU) murine model remains prevalent in TB research, emerging evidence demonstrates that higher bacterial challenges better recapitulate the immune mechanisms of active TB[43-45]. High-dose M. tb infection in C57BL/6 mice replicates the blood transcriptomic signatures observed in active TB patients—a critical feature unattainable with conventional low-dose models[45]. This paradigm is further supported by non-human primate studies where aerosolized high-dose M. tb induced progressive active TB through macrophage interferon responses, mirroring the elevated IFN signatures characteristic of human active TB[44]. Furthermore, recent work demonstrates that high-dose challenges unveil otherwise cryptic host defense mechanisms: autophagy activation in lung macrophages suppresses myeloid-derived suppressor cell accumulation, thereby potentiating T cell-mediated bacterial control[43]. Our selection of high-dose infection specifically targets to better reflect the immune response during active TB."

2) What is the evidence that the mechanism identified using bone marrow derived macrophages is the same mechanism as in the lungs and lungs macrophages, or other lung cell types?

We appreciate the reviewer's insightful question regarding the translational relevance of our findings from bone marrow-derived macrophages (BMDMs) to lung alveolar macrophages and other pulmonary cell types. While we fully acknowledge the cellular heterogeneity of lung tissue (encompassing alveolar macrophages, neutrophils, etc.) and potential discrepancies between in vitro BMDM models and pulmonary microenvironments, multiple lines of evidence support the functional conservation of the succinate-HIF-1α-IL-1β axis across respiratory immune cells. This evolutionarily

conserved immunometabolic pathway has been extensively documented in various pulmonary cell types, including alveolar macrophages (*Immunity*, **2021**, 54, 1206; *Elife*. **2022**, 11, e77457), airway epithelial cells (*JCI Insight*, **2023**, 8, e166860), and neutrophils (*Am J Respir Crit Care Med*, **2019**, 200, 1385).

Our selection of BMDMs for in vitro modeling was based on two key considerations: First, BMDMs have been widely adopted in tuberculosis pathogenesis research due to their experimental tractability and established translational validity-mechanistic insights obtained from this model system have been successfully replicated in animal studies (*Science*, **2022**, 378, eabq0132; *Nat Microbiol*, **2024**, 9, 1859; *Nat Immunol*, **2016**, 17, 683). Second, while AMs indeed constitute the first phagocytes encountering inhaled *M. tb*, their limited antigen-presenting capacity to T cells and dynamic migration patterns within alveoli lead to subsequent bacterial transfer to recruited monocyte-derived cells or neutrophils (*Cell Host Microbe*, **2018**, 24, 440; *Cell*, **2020**, 183, 114). Crucially, Mtb becomes predominantly sequestered within monocyte-derived populations during later infection stages (*Cell Host Microbe*, **2018**, 24, 440), thereby establishing BMDMs-which share functional and developmental homology with these recruited cells-as a biologically relevant model for studying sustained intracellular pathogenesis.

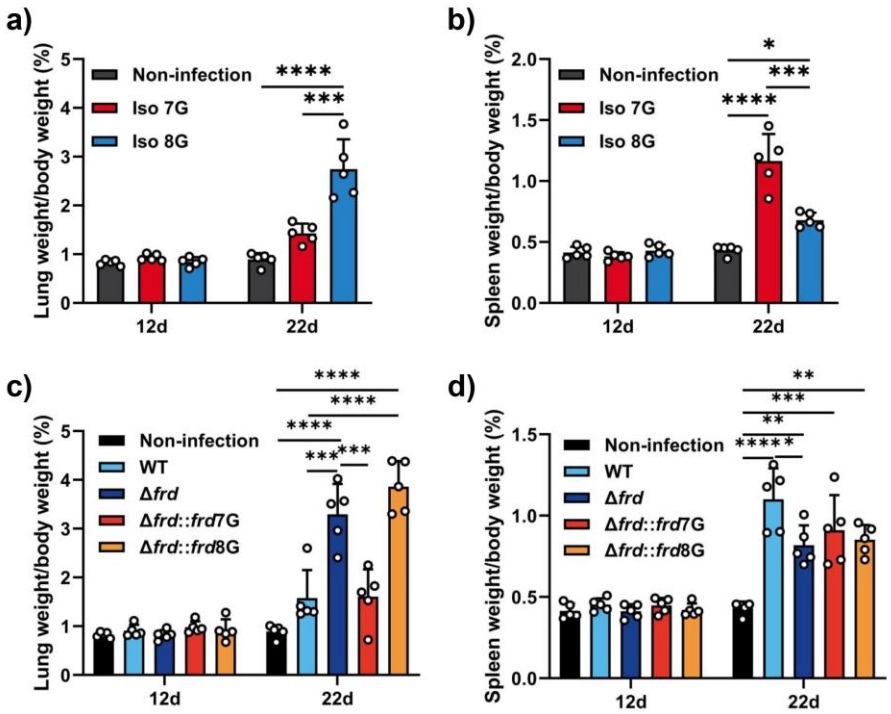
I have 2 minor recommendations:

1) Suggest to change from in the abstract line 20 from host "adaptation" to host "infection."

We thank the reviewer for pointing this out. We have corrected this mistake in the revised manuscript line 20.

2) Suggest to present the organ weight data as absolute weights or percentage of body weight.

We sincerely appreciate the reviewer's constructive suggestion regarding data presentation. In accordance with this constructive feedback, we have revised Figs 3b, 3c, 4b, and 4c to present organ weight data as percentages of body weight (%) in the revised manuscript (figure below).



a) Fig. 3b

b) Fig. 3c

c) Fig. 4b

d) Fig. 4c

Reviewer #3 (Remarks to the Author):

Dong and colleagues present a compelling study revealing a novel mechanism during *Mycobacterium bovis* infection, where bacterial genomic homopolymeric tracts phase variation in the fumarate reductase-encoding *frd* operon modulates host immunometabolism. This leads to suppression of the succinate/HIF-1α/IL-1β axis and a consequential shift from protective Th1 to pathogenic Th17 immunity. The work meaningfully contributes to our understanding of host-pathogen interactions and adds valuable insights into the pathogenesis of *Mycobacterium tuberculosis* complex organisms. The manuscript is well-structured and the data are generally convincing. Nonetheless, addressing the following suggestions could further enhance the clarity and impact of the manuscript:

We thank the reviewer for recognizing the novelty and significance of the work. We are grateful for the reviewer's insightful comments/suggestions to improve the manuscript. In light of these comments, we respond to the specific comments and suggestions below.

L35: It would be helpful to clarify whether the phrase "targeting metabolic checkpoints" refers to interventions on the bacterial or host side to avoid potential ambiguity.

We thank the reviewer for highlighting this critical semantic distinction. In the revised manuscript (L35 in the revised manuscript), we have explicitly specified that the phrase "targeting metabolic checkpoints" refers to host-directed therapeutic strategies modulating immunometabolic pathways, rather than bacterial metabolic targets. The concluding sentence now reads:

"*...providing a framework for targeting **host** metabolic checkpoints in TB therapy.*"

L46: Reference 4 could be updated to reflect the most recent Global Tuberculosis Report from the WHO (2023), ensuring readers have access to the most current data.

We appreciate the reviewer's suggestion to update our reference to the most recent WHO Global Tuberculosis Report. After thorough examination of the 2024 report, we found that while it provides comprehensive updates on global TB burden, treatment outcomes, and emerging strategies, it does not specifically report disaggregated data for *M. bovis*-induced TB cases or deaths in humans. The 2024 report focuses primarily on aggregate TB statistics (e.g., 10.8 million new cases and 1.25 million deaths globally in 2023) without specifying *M. bovis* contributions.

Given this limitation, we have retained the original data (140,000 new cases and 11,400 deaths annually) from the 2020 WHO report, as these remain the most recent species-specific estimates for *M. bovis*. Should species-specific *M. bovis* data emerge in future WHO reports, we will prioritize updating these estimates to reflect the latest epidemiology. However, to ensure readers have access to the latest global TB context, we have updated the reference to the Global Tuberculosis Report 2024 in the introduction (L43, 44 in the revised manuscript):

"This ancient pathogen caused 10.8 million incident human TB cases and 1.25 million deaths worldwide in 2023[3]."

3 World Health Organization. Global Tuberculosis Report 2024. Geneva, Switzerland: https://iris.who.int/bitstream/handle/10665/379339/9789240101531-eng.pdf?sequence=1 (2024).

L109: Please specify the name of the source data file to improve transparency and reproducibility.

We sincerely appreciate the reviewer's meticulous attention to data accessibility. In full compliance with the journal's data deposition policy, we have uploaded all source data files through the submission portal with standardized nomenclature (e.g., "Source Data for Fig.1c" for Fig. 1c data). These files are systematically organized under the "Source Data" section of the submission system, corresponding precisely to each figure identifier. While the manuscript text maintains the standard statement "Source data are provided as a Source Data file" per editorial guidelines, we would be pleased to include explicit in-text file references (e.g., "Source Data Fig. 1") pending editorial approval.

L112: The species name *Escherichia coli* should be italicized. A thorough review of the manuscript for similar formatting issues would be beneficial.

We sincerely thank the reviewer for highlighting this formatting oversight. We have corrected the species name *Escherichia coli* to italicized format (L125 in the revised manuscript) and conducted a comprehensive review of the entire manuscript to ensure consistent adherence to scientific nomenclature standards.

L152–153: While the observed differences are visually apparent, authors should describe the method used to identify lymphocytes and neutrophils, or alternatively, cite appropriate references.

We sincerely appreciate the reviewer's insightful inquiry regarding cellular identification methodology. The differential identification of lymphocytes and neutrophils was conducted through standardized H&E morphological analysis by two board-certified veterinary pathologists, adhering to established histological criteria from internationally recognized guidelines:
Lymphocytes: Identified by their characteristic round nuclei with condensed chromatin, high nuclear-to-cytoplasmic ratio, and scant cytoplasm (Fig. 3g, arrows).
Neutrophils: Distinguished by their segmented nuclei and cytoplasmic granules showing neutral staining properties (Fig. 3g, arrowheads).

The complete diagnostic protocol has been supplemented in the Methods section (L552-554 in the revised manuscript) with explicit citations to the references:

"Standardized morphological identification of inflammatory cells was performed independently by two board-certified veterinary pathologists under double-blinded conditions[74,75]."

74 Renne, R. et al. Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. Toxicol. Pathol. 37, 5S-73S (2009).

75 Taylor, I. A Practical Guide to the Histology of the Mouse (John Wiley & Sons, Ltd, Oxford, 2014).

L286: Consider specifying what accumulates in this context to ensure the meaning is clear.

We appreciate the reviewer's suggestion to enhance clarity. In the revised manuscript (L300–302), we have explicitly specified the accumulating entity as follows:

*"HIF-1α dynamics in infected BMDMs revealed sustained accumulation of **HIF-1α protein** in M. bovis WT- and Δfrd::frd7G-infected cells at 12 and 24 hours post-infection, whereas M. bovis Δfrd and Δfrd::frd8G infections attenuated this response (Fig. 7a, b)."*

L320: It is generally advisable to avoid starting sentences with "and"; a rephrasing may improve the flow.

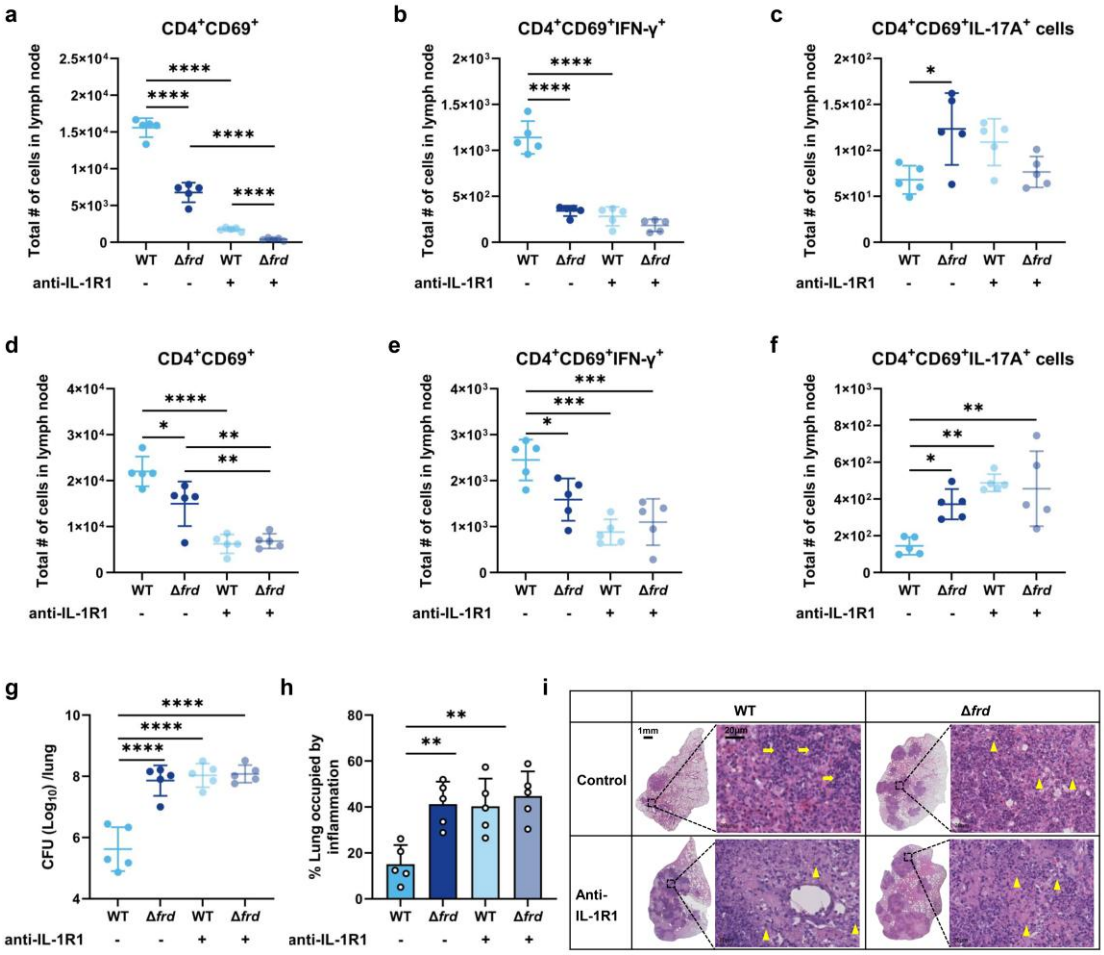
We sincerely appreciate the reviewer's constructive feedback regarding sentence structure. The sentence initially starting with "and" has been revised to employ the transitional phrase "Concurrently, this blockade…", ensuring logical coherence and alignment with academic writing standards. As shown in the revised manuscript (L333–335), the modified text now reads:

*"IL-1R blockade during M. bovis infection suppressed lymph node $CD4^+CD69^+$ T cells at 12 and 22 dpi (Fig. 8a, d). **Concurrently, this blockade** decreased $CD4^+CD69^+IFN\text{-}\gamma^+$ T cells while elevating $CD4^+CD69^+IL\text{-}17A^+$ T cells (Fig. 8b, c, e, f)."*

L326 / Figure 8: There appears to be a misplaced letter "e," possibly intended for panel h. Clarification or correction would be appreciated.

We thank the reviewer for their meticulous evaluation. Upon reviewing the manuscript, we identified a typographical error in the labeling of Figure 8. Specifically, the panel originally labeled as “h” was mistakenly annotated with an extra letter “e” (likely due to a formatting error during figure assembly). This has been corrected in the revised manuscript (L341 and Figure 8), and all panel labels now align with the data presented. We apologize for any confusion caused by this oversight and appreciate the reviewer’s vigilance in ensuring accuracy.

Corrected Figure 8 (L341 in the revised manuscript):



Methods section: For consistency and completeness, please include the country of origin for all reagents and kits used.

We sincerely appreciate the reviewer’s careful evaluation and constructive feedback. In response to the suggestion regarding the Methods section, we have thoroughly revised

the manuscript to ensure consistency and completeness. Specifically, we have now explicitly included the country of origin for all reagents and kits used in the study, as detailed below:

In the revised Methods section, each reagent/kit is now accompanied by its manufacturer's name and country of origin (e.g., "BD, 271310, USA" or "Sigma-Aldrich, P1754, USA"). For clarity, we have also cross-checked the entire Materials subsection to confirm no reagents were inadvertently omitted. We believe these revisions enhance the reproducibility and transparency of our work. Please let us know if further adjustments are needed.

Discussion: The discussion provides strong insights into host immune responses. It could be further enriched by discussing the broader implications of the altered metabolites induced by the *M. bovis* mutant. Additionally, the claim that this study "provides a roadmap for novel host-directed therapies…" could be elaborated upon, especially considering that the initiating factor for the altered host response is a microbial genetic variation.

We sincerely appreciate the reviewer's insightful comments, which have significantly strengthened the manuscript. Below is our point-by-point response to the concerns raised:

"It could be further enriched by discussing the broader implications of the altered metabolites induced by the *M. bovis* mutant."

We thank the reviewer for highlighting this opportunity to expand the discussion. In the revised manuscript (L382-391), we now explicitly contextualize the metabolic and pathogenic implications of FRD-mediated succinate metabolism:

"Functionally, FRD catalyzes fumarate reduction to succinate, a critical reaction in anaerobic respiration across diverse organisms. However, under aerobic conditions, bacterial FRD enzymes paradoxically become major generators of reactive oxygen species, which may inflict oxidative damage on M. tb[39-41]. This dual role underscores the complexity of FRD in bacterial pathogenesis. Notably, frd operon expression may be dynamically regulated during host-pathogen interactions. Following internalization into macrophages, the M. tb H37Ra attenuated strain reveals elevated frd expression during

prolonged infections compared to the virulent H37Rv strain, yet displays reduced expression in acute phases[42]. These temporal expression differences suggest FRD-mediated metabolic adaptations may dynamically influence bacterial virulence."

"The claim about a roadmap for host-directed therapies should be elaborated, particularly given the microbial genetic variation as the initiating factor."

We have expanded this section (L469-472) to explicitly link microbial genetic variation to therapeutic design, providing mechanistic and translational rationale:

"Our study thus provides a roadmap for capitalizing on the pathogen's genetic footprint to guide novel host-directed therapies. By targeting the succinate-HIF-1α-IL-1β axis and related immunometabolic checkpoints, precise recalibration of host immunity could be implemented, thereby advancing the global fight against TB."

By targeting the succinate-HIF-1α-IL-1β axis—a nexus directly perturbed by the *M. bovis* mutant—we propose strategies to recalibrate host immunity in a pathogen-informed manner. For example, enhancing succinate-driven IL-1β production to boost antimicrobial Th1 immunity.

Once again, we sincerely extend our gratitude to the reviewers for their meticulous evaluation and constructive comments, which have substantially enhanced the rigor and clarity of our manuscript.

Point-by-point response to the reviewers' comments

Reviewer #1 (Remarks to the Author):

Reviewer #1 (Remarks to the Author):

The manuscript presented by Dong et al. elucidates the molecular mechanism by which HT insertion mutations in the frd operon of M. bovis regulate the host succinate/HIF-1α/IL-1β immunometabolic axis, providing groundbreaking insights into host adaptation strategies of MTBC pathogens. The authors identified polymorphisms in the frd 7G HT of clinical M. bovis isolates and demonstrated through murine infection models that these mutations enhance pathogenicity by suppressing Th1 immunity and promoting pathogenic Th17 responses. Genetic knockout and complementation experiments confirmed that frd insertion mutations disrupt FRD functionality, thereby inhibiting succinate secretion, reducing HIF-1α accumulation, and diminishing IL-1β production. IL-1R blockade experiments further validated that loss of IL-1 signaling recapitulated the Th17 polarization phenotype observed in mutant infections, exacerbating pulmonary bacterial replication and tissue pathology.

Overall, this is an interesting study, well-organized, with clearly articulated data, and conclusions are fully supported by the data. And the authors have completed the questions which needed to supplemented.

We extend our heartfelt gratitude to the reviewer for their meticulous and insightful evaluation of our manuscript, as well as for their constructive and encouraging feedback regarding our work.