

Dipeptidyl peptidase 3 sets the threshold for immune activation and survival during experimental bacterial infection

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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)

In this manuscript Facoetti and Lambroia et al. aim to decipher the role of dipeptidyl peptidase (Dpp3) in antimicrobial immunity. Authors performed in-depth characterization of the immune response and assessed vascular homeostasis involving the angiotensin II-AT1R axis. Whereas extensive analyses have been performed, there are points which require attention.

- Introduction:

Information about the ubiquitous expression of Dpp3 is lacking. This should be addressed and linked to the chosen experimental model: ubiquitous Dpp3 knockout mice.

- Results: p 9, lines 231-247: The authors evaluate the expression of Dpp3 in splenocytes stimulated with LPS and found an upregulation following LPS stimulation. Further deciphering in which immune cells the upregulation in Dpp3 occurred is required.

- Results: p 14, lines 416-420. In which Dpp3^{-/-} splenocytes is the AT1R expression increased?

- Methods: The sex of the animals is not reported (as requested by the ARRIVE guidelines). Since sex is an important determinant of the immune response, the sex of the animals should be stated. Are all mice male? Or are the mice a pool of male and female mice, if so, in which ratio?

- Methods: The strain background of Dpp3 knockout mice is not reported. Whereas reference 15 is added, in which the generation of the Dpp3 knockout mice is explained in the C57BL6/J background, this information should be directly present in the manuscript, for reasons of clarity.

- Figure legends: The N number of mice differs between the analyzed groups and between the different methods analyzed. The base for these differences in N size is not to deduct and is not described. This needs to be elaborated for reasons of reproducibility and clarity.

- Authors address as part of the limitations of the study that the precise involvement of vascular versus immune mechanisms warrants further dissection. How could pharmacological modulation of the renin-angiotensin system in Dpp3-deficient models hereby help?

Minor:

- Discussion: p 16, line 482: add reference to "These observations extend previous reports linking elevated circulating Dpp3 with poor prognosis."

Reviewer #2

(Remarks to the Author)

The study is very interesting as it proposes new roles for DPP3 in defending against bacteria and possibly other pathogens. It is also highly complex, involving various technologies and experimental setups. The authors primarily used WT and Dpp3 KO mice to evaluate the role of DPP3 in the host response to infection. Guided by clinical observations, the majority of infection studies target the lungs or investigate host responses in bacterial sepsis. The resistance of the KO animals in all models is clear, and better protection has also been observed upon adoptive transfer of splenocytes in Rag KO animals. In order to evaluate the underlying mechanisms of this resistance, the authors profiled the animals at time points where CFUs were already divergent. However, it is difficult to determine what triggered the resistance, as the bacterial loads confound many follow-up measurements. Mechanistic insights are largely derived from the analysis of naïve mice (e.g. metabolism and immunometabolism, transcriptomics) or ex vivo stimulation (e.g. phagocytosis and ROS production following PMA stimulation). Results or hypotheses should be validated in infection models, and extrapolations from the naïve state to the infected state should be made with caution.

Additional concerns are presented below:

Fig. 1: Do the CFUs differ after just a few hours (<20 hours) of infection? Is there no bacteremia KO mouse at 20 hpi? Panels d and f should calculate the difference of differences more accurately, as the abundances of cytokines and chemokines differ even at steady state. How is the granulomatous lesion defined in panel g? Panel h shows patches/clusters of MPO-positive cells, likely neutrophils, but not real granulomas. Are the differences in blood frequencies of immune cells statistically significant at steady state (panel i)?

Fig. 2: Publicly available scRNA-seq data were used and computed to evaluate changes in frequencies. Does the differential abundance testing take in the consideration potential sample errors inherent to scRNA-seq samples, e.g. variability due to loading, etc (panels b and c)? Does it compute credible changes? An alternative could be a compositional analysis with scCODA, a tool designed for scRNA-seq to identify credible changes in cell type composition (doi: 10.1038/s41467-021-27150-6). It remains unclear what statistics have been used. Panel f is not labeled as such.

Fig. 3: The figure has the title “Dpp3 deficiency remodels the transcriptional programs of immune cells to enhance antibacterial responses”. It is unclear why this title. Indeed, some immune functions are enhanced, but why antibacterial? The data comes from naïve animals only. It is unclear why the authors conclude on tolerance and regulatory signaling based on hallmark analysis in panel b.

Fig. 4: Since CD11b+ cells are hardly engrafted in the lung upon transfer, it is likely that lymphocytes cause the resistance. What about CD3 or CD19 adoptive transfer? Or serum transfer?

Fig. 5: Again, all data comes from 48h pi, when CFU are already very different. Cell frequencies (e-h) may differ due to chemotaxis, proliferation or cell death – all due to variable infection loads. An unpaired t-test is unlikely to be suitable for n = 4 or 5.

Fig. 6: All analyses are done in absence of any infection (PKC or TLR activators). It is difficult to extend findings to the infectious state. Are basal ROS levels already different (panel a)? OXPHOS is enriched in immune populations (Fig. 2b). What is the origin of ROS? MitoSox measurements may be helpful. What about ROS in infection? How does the data in panel i fit with the upregulation of the hallmark shown in Fig. 2b? Bioenergetic flux measurements may be helpful to evaluate mitochondrial metabolism.

Data visualization: it is unclear why various plots are used for similar assays. For example, sometimes individual animals are used, sometimes violin plots and sometimes bar plots.

The legends are incomplete with regard to the statistics used for each measurement. These should be carefully checked and amended.

Non-parametric tests compare medians, not means; therefore, the data should be plotted accordingly, i.e. median +/- IQR.

Reviewer #3

(Remarks to the Author)

This manuscript presents evidence that dipeptidyl peptidase 3 (Dpp3) acts as a negative regulator of host defence against extracellular bacteria, including *Klebsiella pneumoniae*. Dpp3-deficient mice exhibit reduced bacterial burden and attenuated tissue inflammation. The study suggests Mechanistically that Dpp3 deficiency lowers Nrf2 levels upon stimulation and enhances NF- κ B signaling. While the findings are intriguing and contribute to our understanding of immune regulation during infection, the manuscript lacks focus and does not convincingly establish a causal link between Dpp3 and Nrf2 expression.

Major Comments

1. Mechanistic clarity. The manuscript proposes that Dpp3 modulates inflammation via regulation of Nrf2, yet the causal relationship remains unclear. It is possible that Dpp3 influences NF- κ B signaling or metabolic pathways independently of Nrf2, or through intermediary factors. This ambiguity should be addressed with additional mechanistic data. Furthermore, in Figure 6d, Nrf2 appears predominantly cytosolic in LPS-stimulated cells. The authors should include images showing basal expression and subcellular localization of Nrf2 in unstimulated cells.

2. Immune profiling. While IFN- γ production has been assessed, the role of Dpp3 in regulating IL-17 and neutrophil responses is not investigated in the context of extracellular bacterial infections. These parameters should be evaluated to provide a more comprehensive picture of the immune landscape.

3. Data presentation and interpretation. The manuscript includes extensive data across multiple tissues and experimental approaches. However, key findings (eg, pulmonary response to *Klebsiella pneumoniae*) are obscured by less relevant details. A clearer narrative structure and reorganization of results are needed to highlight the central discoveries. Importantly, extrapolating mechanisms from LPS-stimulated splenic cultures to in vivo lung responses is not sufficiently justified and should be reconsidered or better supported. In the current form, almost half of the data were generated using spleen cells, eg in Fig. 4 entire data were from spleens, it's unclear why spleens should be used.

Minor comments

1. Fig. 1b, no error bars included.
2. Fig. 1d and f, please clarify what the scale bars represent.
3. Fig. 3a, rationale is not unclearly stated why naïve mice were used to understand host defence mechanisms.
4. Fig. 4, panels j and k are missing, panel organization is confusing.
5. Fig. 5i, statistical analysis not included.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have mainly addressed my comments satisfactorily. Though 2 points still require attention.

- 1) For reasons of consistency, the background of the Rag 1 ^{-/-} mice should still be given.
- 2) The formulation: "All in vivo experiments were performed using both male and female mice, with comparable representation of sexes within each genotype." is still vague. Specific distribution of male and female mice in the groups should be given.

Reviewer #2

(Remarks to the Author)

The comments have been adequately addressed.

Reviewer #3

(Remarks to the Author)

The authors have made considerable efforts to address the reviewers' comments, and the revised manuscript reflects meaningful revisions. However, while some concerns have been addressed, several key issues raised by this reviewer remain unresolved.

1. I remain unconvinced that the mechanisms / pathways identified using LPS-stimulated splenic cultures adequately explain resistance mechanisms operating in the lungs. The cellular composition of naïve spleens differs substantially from that of infected lungs, with B cells representing the predominant population in the former setting. In contrast, infected lungs exhibit a markedly distinct immune landscape. To strengthen the physiological relevance of the findings, T and B cell responses should be examined directly in infected lungs or lung-draining lymph nodes rather than in spleens (Fig. 3). Similarly, Figures 6 and 7 are difficult to interpret, as the composition of myeloid populations in naïve spleens and inflamed lungs differs significantly. For macrophage-focused experiments, stimulation with live *Klebsiella pneumoniae* rather than LPS would be more appropriate and biologically relevant.
2. I also do not agree with the authors' assertion that "adoptive transfer experiments demonstrate that Dpp3-deficient immune cells are sufficient to confer protection in vivo, thereby directly linking splenic phenotypes to pulmonary infection outcomes." Unless it can be demonstrated that T cells from lymph nodes do not transfer protection, the proposed link between splenic phenotypes and pulmonary immunity remains insufficiently supported. It is well established that adoptively transferred immunity in RAG models can be mediated by T lymphocytes independently of their tissue of origin, which weakens the authors' interpretation.
3. Key methodological details should be included in the figure legends. In several instances, it is difficult to discern how the experiments were performed based on the figures alone, which hampers interpretation of the data.
4. Related to the point above, it is unclear how the mean fluorescence intensity (MFI) data shown in the revised Figure 4F were quantified. Please clarify whether MFI was determined by flow cytometry or by image-based analysis. If flow cytometry was used, this should be explicitly stated in both the figure legend and the Methods section. If image-based quantification was performed, the authors should comment on the accuracy and limitations of this approach, particularly given that DPP3 signals in many images appear saturated. The same concern applies to the quantification of NRF2 expression. In addition, representative staining for both WT and Dpp3^{-/-} cells should be shown. Inclusion of Dpp3^{-/-} cells as negative controls in immunofluorescence experiments throughout the manuscript would further strengthen the rigor of the study.
5. Finally, the illustration and legend in Figure 3A do not correspond and should be corrected for accuracy.

Reviewer #4

(Remarks to the Author)

The reviewers made nice adjustments to the manuscript and were thorough in addressing our critiques.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

My comments have been adequately addressed.

(Remarks on code availability)

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POINT-BY-POINT RESPONSE TO REVIEWER COMMENTS

We thank the Reviewers for their careful and constructive evaluation of our manuscript. We greatly appreciate the insightful comments and suggestions, which have helped us improve the clarity, focus, and mechanistic depth of the study. In the revised version, we have strengthened the manuscript by:

(i) clarifying the conceptual framework linking Dpp3 to immune activation thresholds; (ii) adding new biochemical and functional data supporting the Dpp3–Keap1–Nrf2 axis; (iii) integrating quantitative proteomic network analyses to provide systems-level mechanistic insight; and (iv) improving figure clarity and organization as requested.

Importantly, we have revised the text to avoid overinterpretation and to clearly distinguish correlative observations from mechanistic conclusions. We believe these changes substantially enhance the robustness and conceptual coherence of the manuscript.

Below, we respond point-by-point to each comment.

Point-by-point response to Reviewer #1

Reviewer: *In this manuscript Facchetti and Lambroia et al. aim to decipher the role of dipeptidyl peptidase (Dpp3) in antimicrobial immunity. Authors performed in-depth characterization of the immune response and assessed vascular homeostasis involving the angiotensin II-AT1R axis. Whereas extensive analyses have been performed, there are points which require attention.*

We thank the reviewer for the careful evaluation of our manuscript and for the constructive comments, which have helped us improve the clarity and rigor of the study. Below we address each point in detail.

Reviewer: *“Information about the ubiquitous expression of Dpp3 is lacking. This should be addressed and linked to the chosen experimental model: ubiquitous Dpp3 knockout mice.”*

Response: We thank the reviewer for this constructive suggestion. In the revised Introduction, we have added a paragraph explicitly describing the broad tissue distribution of Dpp3, including its expression in metabolic, cardiovascular, and hematopoietic compartments. This clarification strengthens the rationale for employing a ubiquitous Dpp3 knockout model to investigate both immune-intrinsic and systemic aspects of Dpp3 function during infection.

We have also clarified this point in the Discussion. While the global Dpp3-deficient model enables assessment of integrated immune and vascular responses, it does not allow discrimination between cell-intrinsic and systemic effects. We now explicitly acknowledge this limitation and outline the need for future studies using cell-type-specific Dpp3 deletion models.

Revisions made: Introduction page 4, lines 108; Discussion page 16, lines 507-510.

Reviewer: *“Results: p 9, lines 231-247: The authors evaluate the expression of Dpp3 in splenocytes stimulated with LPS and found an upregulation following LPS stimulation. Further deciphering in which immune cells the upregulation in Dpp3 occurred is required”.*

Response: We thank the Reviewer for this important comment. In the revised manuscript, we have directly addressed this point by performing subset-specific analysis of Dpp3 expression during in vivo infection.

We sorted splenic CD11b⁺, CD19⁺, and CD3⁺ immune populations from WT mice following intraperitoneal *Klebsiella pneumoniae* infection and quantified Dpp3 protein levels in positive cells from each subset by immunofluorescence. Dpp3 expression increased significantly across all examined immune populations compared to naïve controls, with the strongest induction observed in CD3⁺ T cells (**new Fig. 4F**).

These data demonstrate that Dpp3 upregulation during bacterial infection is not restricted to a single lineage but occurs broadly across immune compartments, with preferential enhancement in T cells. The Results section has been revised accordingly to incorporate these findings and clarify the cellular context of Dpp3 regulation in vivo.

Reviewer: “Results: p 14, lines 416-420. In which Dpp3^{-/-} splenocytes is the AT1R expression increased?”.

Response: We thank the Reviewer for raising this point. In the revised manuscript, we have specifically assessed AT1R expression in distinct splenocyte subsets from WT and Dpp3^{-/-} mice. qRT-PCR analysis revealed that the increase in AT1R expression was predominantly observed in lymphocytes. These findings are now presented in **new Supplementary Figure 7D**.

Reviewer: “Methods: The sex of the animals is not reported (as requested by the ARRIVE guidelines). Since sex is an important determinant of the immune response, the sex of the animals should be stated. Are all mice male? Or are the mice a pool of male and female mice, if so, in which ratio?”

Response: We thank the reviewer for highlighting this methodological point. All in vivo experiments were performed using both male and female mice, with comparable representation of sexes within each genotype and experimental group. No sex-specific effects were observed in preliminary analyses; therefore, data from both sexes were pooled for statistical evaluation.

We have now explicitly included this information in the Methods section to ensure full compliance with ARRIVE guidelines.

Revisions made: Methods, page 17, lines 538–540.

Reviewer: “Methods: The strain background of Dpp3 knockout mice is not reported. Whereas reference 15 is added, in which the generation of the Dpp3 knockout mice is explained in the C57BL/6J background, this information should be directly present in the manuscript, for reasons of clarity”.

Response: As required, we now explicitly indicate in the Methods section that the Dpp3 knockout mice were generated and maintained on a C57BL/6J background. Although this information is included in Reference 15, we agree that stating it directly increases clarity and transparency.

Revisions made: Methods, page 18, line 529.

Reviewer: “Figure legends: The N number of mice differs between the analyzed groups and between the different methods analyzed. The base for these differences in N size is

not to deduct and is not described. This needs to be elaborated for reasons of reproducibility and clarity”.

Response: We thank the Reviewer for raising this important point. We agree that differences in sample size (N) across experimental groups and assays should be clearly justified. The variation in N reflects differences in experimental endpoints, tissue availability, and the number of animals required for specific downstream analyses (e.g., histology, flow cytometry, RNA-seq, or proteomics). In some instances, limited cell yield from infected lungs or predefined technical exclusion criteria contributed to variation in final sample numbers.

Additionally, several experiments were performed in independent biological replicates conducted at different time points. While the overall findings were highly consistent across these independent experiments, variability in experimental conditions and timing did not allow all datasets to be pooled into a single figure. For clarity, representative datasets are shown in the main figures, and the corresponding N values are now explicitly reported for each condition.

To improve transparency and reproducibility, all figure legends have been revised to state the exact N for each experiment and condition.

Revisions made: Figure legends throughout.

Reviewer: *“Authors address as part of the limitations of the study that the precise involvement of vascular versus immune mechanisms warrants further dissection. How could pharmacological modulation of the renin-angiotensin system in Dpp3-deficient models hereby help?”*

Response: We thank the Reviewer for this important question. Pharmacological modulation of the renin–angiotensin system (RAS) provides a functional approach to dissect the relative contribution of vascular and immune mechanisms in Dpp3-deficient models.

To address this, we treated infected mice with the AT1R antagonist losartan. AT1R blockade selectively worsened infection outcomes in *Dpp3*^{-/-} mice, whereas it had no significant effect in WT animals. This genotype-specific sensitivity indicates that intact Ang II–AT1R signaling contributes to the protective phenotype observed in Dpp3-deficient mice. In contrast, ACE inhibition with ramipril increased susceptibility in both *Dpp3*^{-/-} and WT animals, consistent with prior reports (DOI: 10.1126/scitranslmed.abj2138).

[editorial note: unpublished data redacted]

This broader effect likely reflects upstream suppression of Ang II production and systemic hemodynamic alterations that are not genotype-restricted.

Together, these pharmacological data suggest that Dpp3-dependent immune activation occurs within a physiological context requiring preserved Ang II–AT1R signaling. Given the established links between AT1R activation, redox regulation, and NF-κB signaling, RAS modulation likely influences both vascular tone and immune effector pathways in this setting.

Because increased AT1R expression was detected in *Dpp3*^{-/-} immune cells, we have included the losartan experiments in the **new Supplementary Figure 10** and clarified this interpretation in the revised Discussion.

Revisions made: Results, page 8, lines 245-258; Discussion, page 16, lines 486-493.

Reviewer: “Minor comment: Discussion: p 16, line 482: add reference to ‘These observations extend previous reports linking elevated circulating Dpp3 with poor prognosis.’”

Response: We thank the Reviewer for this suggestion. We have now added the appropriate reference supporting the statement that elevated circulating Dpp3 levels are associated with poor prognosis in septic patients.

Revisions made: Discussion, page 15, line 450.

Point-by-point response to Reviewer #2

Reviewer: *The study is very interesting as it proposes new roles for DPP3 in defending against bacteria and possibly other pathogens. It is also highly complex, involving various technologies and experimental setups. The authors primarily used WT and Dpp3 KO mice to evaluate the role of DPP3 in the host response to infection. Guided by clinical observations, the majority of infection studies target the lungs or investigate host responses in bacterial sepsis. The resistance of the KO animals in all models is clear, and better protection has also been observed upon adoptive transfer of splenocytes in Rag KO animals. In order to evaluate the underlying mechanisms of this resistance, the authors profiled the animals at time points where CFUs were already divergent. However, it is difficult to determine what triggered the resistance, as the bacterial loads confound many follow-up measurements. Mechanistic insights are largely derived from the analysis of naïve mice (e.g. metabolism and immunometabolism, transcriptomics) or ex vivo stimulation (e.g. phagocytosis and ROS production following PMA stimulation). Results or hypotheses should be validated in*

infection models, and extrapolations from the naïve state to the infected state should be made with caution.

We thank the Reviewer for the thoughtful and balanced evaluation of our work and for recognizing the complexity of the experimental approach. We agree that distinguishing cause from consequence in infection models is essential, particularly when immune profiling is performed at time points where bacterial burdens have already diverged. To address this concern, we have revised and strengthened the manuscript in several ways. First, we now include early time-point analyses (8 h post-infection), demonstrating that bacterial clearance in *Dpp3*^{-/-} mice diverges prior to extensive immune remodeling. These data support the notion that the protective phenotype is not solely a secondary consequence of altered inflammatory responses at later stages. Second, we have reorganized the Results section to more clearly separate in vivo phenotypic observations from mechanistic analyses performed under naïve or ex vivo conditions. We now explicitly frame transcriptomic, metabolic, and functional assays in naïve cells as cell-intrinsic mechanistic investigations that complement, but do not substitute for, the infection models. Third, we have moderated the language throughout the manuscript to avoid over-extrapolation from baseline or ex vivo findings to the infected lung environment. We believe that these revisions clarify the causal framework and better integrate the complementary experimental systems used in this study. Below, we address each specific point in detail.

Reviewer: *Fig. 1: Do the CFUs differ after just a few hours (<20 hours) of infection? Is there no bacteremia KO mouse at 20 hpi? Panels d and f should calculate the difference of differences more accurately, as the abundances of cytokines and chemokines differ even at steady state. How is the granulomatous lesion defined in panel g? Panel h shows patches/clusters of MPO-positive cells, likely neutrophils, but not real granulomas. Are the differences in blood frequencies of immune cells statistically significant at steady state (panel i)?*

Response: We thank the Reviewer for the detailed and constructive comments regarding Fig. 1. We address each point below.

Early CFU divergence. To determine whether enhanced bacterial control occurs at early stages of infection, we quantified pulmonary CFUs at 8 hours post-infection (hpi). At this early time point, *Dpp3*^{-/-} mice already exhibit significantly reduced bacterial burden compared to WT animals. These data demonstrate that divergence in bacterial clearance precedes the immune remodeling observed at 20–48 hpi and support the conclusion that *Dpp3* deficiency impacts early host defense mechanisms. **Revisions made:** Figure 1A-B, Results page 5, lines 139-146.

Bacteremia at 20 hpi. We have clarified the bacteremia data at 20 hpi. Bacteremia is detectable in both WT and *Dpp3*^{-/-} mice; however, the proportion of bacteremic animals is lower in the *Dpp3*^{-/-} group. This is now explicitly illustrated in the **revised Figure 1B**.

Cytokine normalization (panels d and f). We agree that baseline cytokine and Ig levels differ between genotypes and that this should be accounted for. The heatmaps in **revised panels 1E and 1J** are based on z-score normalization calculated relative to the median expression value across samples, enabling visualization of relative changes independent of baseline offsets. To enhance transparency, we have added a **new Supplementary Table 1** reporting raw cytokine concentrations (mean $\pm$ SD) for each group and condition. Figure legends have been updated to clarify the normalization method and statistical representation. **Revisions made:** Legend Figure 1.

Granulomatous terminology. We agree that the term “granulomatous lesion” was imprecise. The structures observed represent inflammatory foci characterized by clusters of MPO⁺ myeloid cells rather than classical organized granulomas. We have revised the terminology accordingly and clarified that MPO-positive aggregates most likely correspond to neutrophilic infiltrates. **Revisions made:** Results, page 6, lines 158-164.

Baseline immune cell frequencies. We have now explicitly indicated the statistical analysis for baseline immune cell frequencies. Minor differences observed under steady-state conditions did not reach statistical significance unless specified. This clarification has been added to the figure legend. We believe these revisions improve clarity, accuracy, and transparency of the data presentation.

Reviewer: “Fig. 2: Publicly available scRNA-seq data were used and computed to evaluate changes in frequencies. Does the differential abundance testing take in the consideration potential sample errors inherent to scRNA-seq samples, e.g. variability due to loading, etc (panels b and c)? Does it compute credible changes? An alternative could be a compositional analysis with scCODA, a tool designed for scRNA-seq to identify credible changes in cell type composition (doi: 10.1038/s41467-021-27150-6). It remains unclear what statistics have been used. Panel f is not labeled as such”.

Response: We thank the Reviewer for this technically insightful comment.

In the revised manuscript, we have complemented the original feature plot (**Fig. 4B**) with a quantitative assessment of Dpp3⁺ cell frequencies across cell types and experimental groups (**revised Fig. 4C**). Percentages of Dpp3⁺ cells were calculated at the individual sample level and normalized to the total number of cells captured per sample to account for variability in sequencing depth and cell loading. These normalized proportions were then compared across conditions.

To statistically evaluate differences in Dpp3⁺ cell proportions between groups within each cell type, we applied Fisher’s exact test. We have now explicitly described this statistical approach in the Methods and clarified it in the figure legend. The analysis is therefore descriptive but statistically supported, and consistent with the distribution shown in the original feature plots.

We appreciate the suggestion to perform compositional analysis using scCODA. However, scCODA requires multiple biological replicates per condition to model sample-level variability within a Bayesian framework. The publicly available dataset used in our study includes one naïve WT sample and two infected samples, which precludes robust compositional modeling. Given these structural limitations, frequency-based comparison represents the most appropriate and statistically

transparent approach for this dataset. Finally, we have corrected the panel labeling to ensure consistency and clarity in the revised figure. **Revisions made:** Methods, page 21, lines 645-650; Legend Figure 4.

Reviewer: “Fig. 3: The figure has the title “Dpp3 deficiency remodels the transcriptional programs of immune cells to enhance antibacterial responses”. It is unclear why this title. Indeed, some immune functions are enhanced, but why antibacterial? The data comes from naïve animals only. It is unclear why the authors conclude on tolerance and regulatory signaling based on hallmark analysis in panel b.”

Response: We agree with the Reviewer that the original title of Fig. 3 (**now Fig. 5** in the revised version) overstated the functional interpretation of the data. Because the transcriptional analyses were performed on naïve immune subsets, the figure reflects baseline immune programming rather than direct antibacterial activity.

In the revised manuscript, we have modified the figure title to more accurately reflect the experimental context. The new title emphasizes baseline transcriptional priming in Dpp3-deficient immune cells, avoiding any implication of infection-driven antibacterial responses.

Regarding the interpretation of tolerance and regulatory signaling pathways in panel B, our conclusions were based on gene set enrichment analysis (GSEA) indicating relative downregulation of hallmark pathways associated with immune tolerance and regulatory programs. We acknowledge that the original wording could be interpreted as implying functional suppression. We have therefore revised the text to clarify that these findings represent transcriptional signatures consistent with reduced regulatory tone at baseline, rather than direct functional impairment of tolerance mechanisms.

Importantly, we now clearly distinguish baseline transcriptional priming from infection-induced antibacterial responses, which are demonstrated separately in the in vivo infection models (Fig. 1 and **revised Fig. 2**) and in adoptive transfer experiments (**revised Fig. 3**). We believe these revisions improve conceptual clarity and ensure that interpretations are appropriately aligned with the experimental conditions.

Reviewer: “Fig. 4: Since CD11b⁺ cells are hardly engrafted in the lung upon transfer, it is likely that lymphocytes cause the resistance. What about CD3 or CD19 adoptive transfer? Or serum transfer?”

Response: We thank the Reviewer for this suggestion. As correctly noted, CD11b⁺ donor cells exhibit limited engraftment in the lung following adoptive transfer, suggesting that adaptive lymphocytes are likely major contributors to the protective phenotype observed in *Rag1*^{-/-} recipients.

To directly address this point, we performed additional adoptive transfer experiments using purified CD19⁺ B cells. B cells were selected based on their abundance within splenocyte populations and their relatively high Dpp3 expression at the transcriptomic level. Transfer of Dpp3^{-/-} B cells reduced pulmonary bacterial burden compared to WT B-cell transfer, indicating a significant contribution of B cells to host protection. However, IL-6 levels were only partially normalized, suggesting that B cells alone do not fully account for the attenuated inflammatory phenotype observed with total splenocyte transfer. Together with the coordinated immune rewiring observed across lineages, these data support a model of integrated immune modulation rather than

single-subset dominance. Our primary objective was therefore to demonstrate that Dpp3 deficiency lowers immune activation thresholds broadly across immune populations.

To maintain focus on the central mechanistic framework, the subset-specific transfer data were not included in the main figures but can be provided as supplementary material for the Reviewer's evaluation.

[editorial note: unpublished data redacted]

Reviewer: *"Fig. 5: Again, all data comes from 48h pi, when CFU are already very different. Cell frequencies (e-h) may differ due to chemotaxis, proliferation or cell death – all due to variable infection loads. An unpaired t-test is unlikely to be suitable for n = 4 or 5."*

Response: We thank the Reviewer for this important comment. We agree that analyses performed at 48 h post-infection require careful interpretation, as bacterial burdens already diverge between WT and *Dpp3*^{-/-} mice at this stage.

The immune cell frequency changes observed at 48 hpi may therefore reflect both primary Dpp3-dependent effects and secondary consequences of differential bacterial loads. Importantly, we now include early time-point data (8 hpi) (**revised Fig. 1A-B**), demonstrating that bacterial clearance diverges prior to extensive immune remodeling, thereby supporting an early, Dpp3-dependent component of the phenotype.

In addition, analysis of selected chemokines (CCL3, CXCL10, CCL4, CXCL1) at baseline (**new Fig. 1E**) revealed modest, non-significant differences between genotypes (**new Supplementary Table 1**). Although these differences did not reach statistical significance, they are consistent with a pre-existing primed immune state in *Dpp3*^{-/-} mice. These observations suggest that part of the immune divergence observed at 48 hpi may reflect baseline immune conditioning rather than solely secondary effects of differential bacterial burden.

Moreover, to evaluate whether altered immune cell frequencies might result from differences in survival rather than recruitment or activation, we directly assessed cell viability within the relevant immune subsets at 48 hpi. No significant differences in apoptosis or necrosis were detected between WT and *Dpp3*^{-/-} mice (data not shown), indicating that altered cell death is unlikely to account for the observed changes in cell composition. These data are now included in the revised manuscript.

Regarding statistical analysis, we agree that the use of an unpaired Student's t-test may not be optimal for small sample sizes ($n = 4-5$). We have therefore reanalyzed these datasets using the non-parametric Mann-Whitney U test, which does not assume normal distribution. Figures and legends have been updated accordingly to ensure consistency between statistical testing and graphical representation.

We believe these revisions clarify the causal framework and improve the rigor of the data interpretation.

Reviewer: *"Fig. 6: All analyses are done in absence of any infection (PKC or TLR activators). It is difficult to extend findings to the infectious state. Are basal ROS levels already different (panel a)? OXPHOS is enriched in immune populations (Fig. 2b). What is the origin of ROS? MitoSox measurements may be helpful. What about ROS in infection? How does the data in panel i fit with the upregulation of the hallmark shown in Fig. 2b? Bioenergetic flux measurements may be helpful to evaluate mitochondrial metabolism".*

Response: We thank the Reviewer for these comments and technical suggestions.

We agree that mechanistic analyses should be interpreted cautiously when performed outside the infectious context. In the revised manuscript, **Fig. 6** has been substantially reorganized and now includes analyses performed following LPS stimulation to more closely mimic infection-associated inflammatory signaling. Importantly, we have also confirmed altered ROS production *ex vivo* in immune cells isolated from infected mice (**revised Fig. 2**), demonstrating that the enhanced redox phenotype is not restricted to artificial stimulation but is recapitulated during infection.

Regarding basal ROS levels, we now report that *Dpp3*^{-/-} immune cells exhibit modestly elevated ROS production under steady-state conditions, with a more pronounced increase following LPS stimulation. These findings indicate that *Dpp3* deficiency alters both baseline redox tone and inducible oxidative responses.

To address the origin of ROS and the relationship with oxidative phosphorylation (OXPHOS) signatures observed in transcriptomic analyses, we performed extracellular flux (Seahorse) assays under basal and LPS-stimulated conditions. *Dpp3*^{-/-} splenocytes displayed increased basal and maximal mitochondrial respiration, as well as elevated ATP-linked respiration. Following LPS stimulation, WT cells exhibited reduction in oxidative phosphorylation, whereas *Dpp3*-deficient cells maintained higher respiratory capacity (**new panels E-F of revised Figure 6**). These data indicate mitochondrial remodeling with enhanced oxidative flux rather than impaired respiration.

The enrichment of OXPHOS-related gene signatures in the transcriptomic dataset is therefore consistent with enhanced mitochondrial capacity, rather than dysfunctional oxidative metabolism. We have revised the Results to clarify this distinction.

Taken together, the inclusion of LPS-stimulated Seahorse analyses and confirmation of altered ROS production in infected animals strengthen the link between redox remodeling, mitochondrial bioenergetics, and inflammatory amplification in *Dpp3*-deficient immune cells. These findings support the relevance of the mechanistic data to the infectious state. **Revisions made:** Results, page 13, lines 388-392; Discussion, page 15, lines 467-478.

Reviewer: *"Data visualization: it is unclear why various plots are used for similar assays. For example, sometimes individual animals are used, sometimes violin plots*

and sometimes bar plots. The legends are incomplete with regard to the statistics used for each measurement. These should be carefully checked and amended. Non-parametric tests compare medians, not means; therefore, the data should be plotted accordingly, i.e. median +/- IQR.”

Response: In the revised manuscript, figures have been adapted in accordance with the Nature Communications figure guidelines, taking into account sample size (n) and the most appropriate graphical representation for each dataset. Where the number of biological replicates was limited, individual data points are now consistently displayed to ensure transparency.

All statistical analyses have been carefully re-evaluated and are explicitly indicated in each figure legend. For comparisons analyzed using non-parametric tests (e.g., Mann–Whitney), data are now presented as median $\pm$ interquartile range (IQR). For datasets meeting assumptions of normality and analyzed using parametric tests, values are displayed as mean $\pm$ SEM. The graphical representation has been aligned accordingly to ensure consistency between statistical testing and data presentation. We believe these revisions improve clarity, transparency, and adherence to journal standards.

Point-by-point response to Reviewer #3

Reviewer: *This manuscript presents evidence that dipeptidyl peptidase 3 (Dpp3) acts as a negative regulator of host defence against extracellular bacteria, including Klebsiella pneumoniae. Dpp3-deficient mice exhibit reduced bacterial burden and attenuated tissue inflammation. The study suggests Mechanistically that Dpp3 deficiency lowers Nrf2 levels upon stimulation and enhances NF- κ B signaling. While the findings are intriguing and contribute to our understanding of immune regulation during infection, the manuscript lacks focus and does not convincingly establish a causal link between Dpp3 and Nrf2 expression.*

We thank the Reviewer for the thoughtful and balanced evaluation of our work. We appreciate the recognition that our findings contribute to a better understanding of immune regulation during pulmonary infection and that Dpp3 deficiency confers improved bacterial clearance with reduced tissue injury.

We acknowledge the Reviewer's concern regarding the mechanistic interpretation of the Dpp3–Nrf2 axis. In the original version of the manuscript, our intention was not to propose Nrf2 as the sole mediator of the Dpp3-deficient phenotype, but rather to identify altered Nrf2 dynamics as one component of a broader regulatory network integrating redox and inflammatory signaling. We agree that this distinction was not sufficiently emphasized.

In the revised manuscript, we have strengthened the mechanistic framework by incorporating additional biochemical and functional analyses, including direct assessment of inducible Nrf2 transcriptional activity, Dpp3–Keap1 interaction studies, and integrated proteomic network analysis. These data support a model in which Dpp3 modulates stimulus-dependent Nrf2 activation while simultaneously reshaping inflammatory and proteostasis pathways, rather than acting through a single linear mechanism.

We address the specific mechanistic concerns in detail below.

Reviewer: Mechanistic clarity. *The manuscript proposes that Dpp3 modulates inflammation via regulation of Nrf2, yet the causal relationship remains unclear. It is possible that Dpp3 influences NF- κ B signaling or metabolic pathways independently of Nrf2, or through intermediary factors. This ambiguity should be addressed with additional mechanistic data. Furthermore, in Figure 6d, Nrf2 appears predominantly cytosolic in LPS-stimulated cells. The authors should include images showing basal expression and subcellular localization of Nrf2 in unstimulated cells.*

Response: We thank the Reviewer for this important comment and agree that additional mechanistic evidence was necessary to clarify the relationship between Dpp3 and Nrf2. To directly address this point, we have incorporated complementary biochemical and functional analyses. First, co-immunoprecipitation experiments demonstrate a physical interaction between Dpp3 and Keap1 in primary splenocytes (**new Supplementary Figure 14**). This interaction is detectable under basal conditions and is enhanced following LPS stimulation, supporting a stimulus-dependent role for Dpp3 within the Keap1–Nrf2 regulatory axis.

Second, we assessed Nrf2 transcriptional activity using a DNA-binding ELISA assay. While basal Nrf2 activity was only modestly affected in *Dpp3*^{-/-} cells, LPS-induced Nrf2

transcriptional activity was significantly impaired compared to WT controls (**new Figure 7 C**). Consistently, intracellular Nrf2 protein accumulation was reduced in Dpp3-deficient cells following stimulation, whereas basal nuclear localization was detectable in both genotypes. These findings indicate that Dpp3 regulates inducible Nrf2 stabilization and activation rather than basal transcript expression.

Importantly, our novel quantitative proteomic analysis revealed coordinated remodeling of inflammatory and proteostasis networks in Dpp3-deficient cells, including upregulation of interferon- and NF- κ B-associated proteins alongside alterations in mitochondrial components (**new Figure 7 D-E**). Together with the revised metabolic and bioenergetic data (**new Figure 6 E-F**), these results indicate that Dpp3 deficiency reshapes a broader signaling landscape rather than acting exclusively through Nrf2. Thus, while impaired inducible Nrf2 activation represents a direct mechanistic consequence of Dpp3 loss, additional parallel pathways, including NF- κ B signaling and metabolic rewiring, contribute to the overall phenotype.

In response to the Reviewer's request, we have also included representative images showing basal Nrf2 expression and subcellular localization in unstimulated cells. These confirm detectable nuclear Nrf2 at baseline and reduced stimulus-dependent nuclear accumulation in *Dpp3*^{-/-} cells.

We have revised the manuscript to clearly distinguish direct mechanistic evidence from correlative in vivo observations. We believe these additions substantially strengthen the mechanistic framework of the study while maintaining an appropriately cautious interpretation of causality. **Revisions made:** Results, page 13, lines 412-435; Discussion, page 15, lines 457-466.

Reviewer: Immune profiling. While IFN- γ production has been assessed, the role of Dpp3 in regulating IL-17 and neutrophil responses is not investigated in the context of extracellular bacterial infections. These parameters should be evaluated to provide a more comprehensive picture of the immune landscape.

Response: We thank the Reviewer for raising this point regarding IL-17 and neutrophil responses in extracellular bacterial infection.

These parameters were indeed assessed in our study. In the pulmonary *K. pneumoniae* model, IL-17 levels were quantified in lung homogenates at 48 h post-infection (**revised Fig. 1E**). While differences did not reach statistical significance, IL-17 was measured and is now more clearly highlighted in the revised Results section.

Neutrophil-associated responses were evaluated through multiple complementary approaches. MPO levels were quantified in lung homogenates (**revised Fig. 1G**), and histopathological analysis demonstrated reduced neutrophil-rich inflammatory aggregates in *Dpp3*^{-/-} lungs, indicating attenuated tissue-damaging neutrophilic infiltration.

Importantly, in the systemic (intraperitoneal) infection model, we observed increased LTB4 levels in *Dpp3*^{-/-} mice, a lipid mediator strongly associated with neutrophil activation and oxidative burst. This increase correlated with enhanced ROS production measured at 48 h post-infection. Together, these findings suggest that Dpp3 deficiency does not suppress neutrophil responses per se, but rather alters their activation state and effector profile, favoring enhanced antimicrobial activity while limiting excessive tissue injury. To improve clarity, we have revised the manuscript to explicitly emphasize these analyses and their relevance to neutrophil biology and IL-17-mediated responses.

Revisions made: Results, page 6, line 155; lines 158-161; pag 7, lines 217-220.

Reviewer: Data presentation and interpretation. *The manuscript includes extensive data across multiple tissues and experimental approaches. However, key findings (eg, pulmonary response to *Klebsiella pneumoniae*) are obscured by less relevant details. A clearer narrative structure and reorganization of results are needed to highlight the central discoveries. Importantly, extrapolating mechanisms from LPS-stimulated splenic cultures to in vivo lung responses is not sufficiently justified and should be reconsidered or better supported. In the current form, almost half of the data were generated using spleen cells, eg in Fig. 4 entire data were from spleens, it's unclear why spleens should be used.*

Response: We thank the Reviewer for this important and constructive comment. We agree that the original organization of the manuscript did not sufficiently foreground the central pulmonary phenotype and may have diluted the focus on the primary in vivo findings.

In the revised manuscript, we have substantially reorganized the Results section to emphasize the pulmonary *Klebsiella pneumoniae* infection model as the primary framework. All lung infection data are now presented first, followed by systemic validation experiments. Adoptive transfer studies are positioned as direct in vivo evidence of immune cell-intrinsic protection, and ex vivo splenocyte assays are explicitly framed as mechanistic dissection tools rather than as standalone phenotypic models.

Importantly, we have revised the text throughout to clearly distinguish between in vivo infection data and mechanistic analyses performed under controlled stimulation conditions. We no longer extrapolate directly from LPS-stimulated splenocyte assays to lung infection outcomes. Instead, splenic analyses are presented as complementary cell-intrinsic systems that help interpret the in vivo phenotype.

Regarding the use of splenocytes, these were selected for mechanistic studies due to their higher cell yield and suitability for functional, biochemical, metabolomic, and proteomic analyses that are technically not feasible with the limited number of cells obtainable from infected lung tissue. Furthermore, adoptive transfer experiments demonstrate that Dpp3-deficient immune cells are sufficient to confer protection in vivo, thereby directly linking splenic phenotypes to pulmonary infection outcomes. We believe that these structural and conceptual revisions substantially improve the clarity, coherence, and focus of the manuscript, while maintaining the mechanistic depth of the study.

Reviewer: Minor comments

1. Fig. 1b, no error bars included.
2. Fig. 1d and f, please clarify what the scale bars represent.
3. Fig. 3a, rationale is not unclearly stated why naïve mice were used to understand host defence mechanisms.
4. Fig. 4, panels j and k are missing, panel organization is confusing.
5. Fig. 5i, statistical analysis not included.

Response: We have carefully revised the figures and text to address each point:

1. **Fig. 1b:** Error bars have now been added to the graph as requested, and the legend has been updated accordingly.

2. **Fig. 1d and 1f:** In **revised Fig. 1D and 1J**, the color scale bars represent normalized cytokine and chemokine levels expressed as z-score values calculated relative to the median across samples. To facilitate visualization of relative differences, median-centered values were log-transformed and plotted as heatmaps using R (heatmap package). The corresponding figure legends have been updated to clearly describe the normalization and scaling procedure.
3. **Fig. 3a:** We thank the Reviewer for this comment. We agree that the rationale for using naïve mice in Fig. 3a was not sufficiently articulated in the original version. Naïve mice were used to assess whether Dpp3 deficiency establishes a baseline transcriptional or phenotypic immune state independent of infection-driven remodeling. Our intention was to distinguish pre-existing immune priming from secondary changes resulting from differential bacterial burden during infection. By analyzing sorted immune subsets under steady-state conditions, we aimed to determine whether Dpp3 deficiency intrinsically alters immune programming prior to pathogen exposure. We have re-organized the Results section to explicitly clarify this rationale and to better distinguish baseline priming from infection-induced responses. **Revisions made:** Result section
4. **Fig. 4:** Panels j and k were mislabeled in the original submission. The figure has been reorganized for clarity, all panels are now correctly labeled, and the corresponding legend has been updated.
5. **Fig. 5i:** Statistical analysis is now included, with the appropriate test (Mann–Whitney) indicated in the figure legend. We believe these revisions improve the clarity, accuracy, and presentation of the manuscript.

Point-by-point response to Reviewer #4

Reviewer: *Dpp3 has been a suggested marker for patients with sepsis and thus could play a role in immune regulation during infection. In the manuscript “Dipeptidyl peptidase 3 sets the threshold for immune activation and survival during experimental bacterial infection” by Facchetti et al., the authors investigated the role of Dpp3 as a regulator of the immune response during pulmonary infection using a K. pneumoniae infection model. They use Dpp3 -/- mice and found that it increased bacterial clearance and reduced tissue damage during infection. They also leverage a P. aeruginosa infection model, and show that Dpp3 -/- mice have lower pulmonary bacterial burden as compared to WT mice, suggesting that this phenotype is conserved during different pulmonary bacterial infections. Using publicly available scRNA-seq sets they demonstrate that Dpp3 is upregulated in immune cells during K. pneumoniae infection. RNA-seq of CD3+ T cells, CD19+ B cells, and CD11b + cells were used to investigate the altered immune response in Dpp3 -/- mice. The authors found enrichment in immune related pathways, specifically cytokine mediated signaling, leukocyte activation, and antigen presentation. Pathways that were “down” were immune tolerance and regulatory signaling, suggesting an overall more pro-inflammatory state. The authors go on to further investigate the how the loss of Dpp3 contributes to bacterial clearance and tolerance. Again using the Dpp3 KO, the authors found that upon LPS stimulation of splenocytes Dpp3 KO cells failed to induce Nrf2 compared to WT suggesting that Dpp3 acts upstream of Nrf2 signaling. They found that this was associated with altered intracellular redox and metabolism as well as cytokines that are associated with NF-KB activation. Understanding how Dpp3 function in the immune response to pulmonary infection is important topic in the field. Overall, the authors did an extremely good job detailing how Dpp3 deficiency impacts the susceptibility to infection and the immune response during pulmonary infection. However, the experiments implicating modulation of Nrf2 are generally correlative and do not support the authors claims. Additionally, the molecular mechanism by which Dpp3 suppresses the inflammatory/antimicrobial response is largely missing. Does Dpp3 modulate Nrf2? How does it do this? Is low Nrf2 actually responsible for improved infection outcomes in the Dpp3 KO? How does it mechanistically impact these responses that are observed in the Dpp3 -/- mice?*

We thank the reviewer for the careful and comprehensive evaluation of our work and for the positive assessment of the overall experimental framework. We appreciate the recognition that our study provides a detailed characterization of how Dpp3 deficiency impacts host susceptibility, bacterial clearance, and immune activation during pulmonary infection.

We also acknowledge the reviewer’s concerns regarding the mechanistic depth of the Nrf2-related findings. In the original version of the manuscript, our intention was to highlight Nrf2 dysregulation as one mechanistic layer contributing to the phenotype observed in Dpp3-deficient mice, rather than to propose a linear or exclusive Dpp3-Nrf2 axis. We agree that this distinction was not sufficiently emphasized.

To address these concerns, we have strengthened the mechanistic component of the manuscript by incorporating additional biochemical, functional, and network-level analyses. Taken together, the revised data support a model in which Dpp3 functions as a systems-level modulator of immune activation thresholds, integrating redox

regulation, proteostasis, and inflammatory signaling, rather than acting solely through Nrf2 suppression. We have revised the manuscript accordingly to clarify this conceptual framework and to avoid overinterpretation of Nrf2 as the sole mediator of the phenotype. We respond in detail to the specific mechanistic concerns below.

Reviewer: *The authors point to Nrf2 failing to be expressed in Dpp3^{-/-} splenocytes by IF. If Nrf2 is indeed reduced in Dpp3^{-/-} splenocytes compared to WT then one would expect that Nrf2 targets to be also reduced in the KO so this is something to measure at the transcriptional level (beyond just Nrf2 in nuclei). Beyond this, the results in Figure 6 are largely correlative and the authors cannot conclude (based on the results presented) that an increase in NF-κB genes in the Dpp3 KO are indeed due failure to increase Nrf2 expression. Experiments comparing WT, Dpp3^{-/-} and Nrf2^{-/-} splenocytes /mice are needed to fully implicate Nrf2 as the culprit. [The expectation is that would Nrf2^{-/-} would phenocopy the Dpp3^{-/-} mice (and a Dpp3^{-/-}Nrf2^{-/-} mouse would also have the same phenotype as Dpp3^{-/-} mice)*

Response: We thank the Reviewer for this thoughtful and important comment. We fully agree that distinguishing correlative from causal relationships within the Dpp3–Nrf2 axis is essential.

In response to the suggestion that Nrf2 target genes should be evaluated at the transcriptional level, we have now quantified canonical Nrf2 target genes (e.g., Nqo1, Hmox1, Gclc) in WT and *Dpp3*^{-/-} splenocytes under basal and LPS-stimulated conditions. Consistent with the Nrf2 DNA-binding ELISA assay, inducible expression of a subset of Nrf2 target genes is reduced in *Dpp3*^{-/-} cells following LPS stimulation, whereas basal expression is not uniformly suppressed. These data indicate that Dpp3 deficiency does not result in global loss of Nrf2 function, but rather impairs stimulus-dependent activation dynamics.

We also agree that increased NF-κB-associated gene expression in *Dpp3*^{-/-} cells cannot be attributed solely to reduced Nrf2 activity. Our quantitative proteomic and network analyses (**new Supplementary Figs. 17–19**) demonstrate coordinated remodeling not only of the Keap1–Nrf2 regulatory axis, but also of IKK–NF-κB signaling and proteostasis-related pathways. Together, these findings support a systems-level model in which Dpp3 modulates inflammatory and redox signaling thresholds rather than acting exclusively upstream of Nrf2.

Importantly, we do not expect *Nrf2*^{-/-} mice to phenocopy *Dpp3*^{-/-} animals. Complete Nrf2 deficiency is known to impair host defense and exacerbate oxidative stress, whereas *Dpp3*^{-/-} mice exhibit enhanced bacterial clearance with reduced tissue injury. This divergence indicates that Dpp3 deficiency does not mimic global Nrf2 ablation, but instead alters the kinetics and balance of inducible redox responses. Consistent with this interpretation, we observe detectable basal nuclear Nrf2 in *Dpp3*^{-/-} cells but impaired inducible stabilization following stimulation.

We agree that epistasis experiments using *Nrf2*^{-/-} or compound *Dpp3*^{-/-} *Nrf2*^{-/-} models would provide additional mechanistic resolution. However, within the scope of the present study, the combination of biochemical interaction data (Dpp3–Keap1), functional assays (Nrf2 transcriptional activity), transcriptional target analysis, and systems-level proteomic remodeling provides substantial mechanistic support while appropriately avoiding overstatement of causality. The revised manuscript now explicitly frames Nrf2 as one component of a broader Dpp3-regulated immunometabolic network.

Revisions made: Results, page 14, lines 421-435; Discussion, page 15, lines 457-466.

Reviewer: “Because Dpp3 is a metalloprotease and has many targets/substrates this increase in inflammatory response could be Nrf2 independent and it’s possible that the responses being observed are due to another mechanism. Some additional experiments getting at molecular mechanism to explain the *in vivo* phenotypes would help bolster the manuscript. How does Dpp3 function mechanistically to limit the inflammatory/antimicrobial responses? The manuscript presents data that is largely phenomenological—i.e. what happens in the absence of Dpp3.”

Response: We thank the reviewer for this important and insightful comment. We agree that Dpp3, as a metalloprotease with multiple potential substrates, may influence inflammatory responses through mechanisms that extend beyond Nrf2 regulation. Our intention was not to propose a single linear pathway, but rather to identify Dpp3 as a modulator of immune activation thresholds.

To address the concern that our data were primarily phenomenological, we have now strengthened the mechanistic component of the manuscript in several ways:

1. **Biochemical evidence:** We demonstrate a physical interaction between Dpp3 and Keap1 by co-immunoprecipitation, detectable under basal conditions and enhanced upon LPS stimulation. This provides direct evidence linking Dpp3 to the Keap1–Nrf2 regulatory axis in primary immune cells.
2. **Functional evidence:** Nrf2 transcriptional activity assays reveal that inducible Nrf2 activation is significantly impaired in Dpp3^{-/-} cells following LPS stimulation, despite comparable basal activity. This supports a role for Dpp3 in fine-tuning stimulus-dependent redox responses.
3. **Proteomic network analysis:** Quantitative proteomics (Figs. S13–S15) reveal coordinated remodeling of the Keap1–Cul3–proteasome complex, the IKK–NF-κB signaling module, and ribosomal/metabolic networks in Dpp3-deficient cells. These data indicate that Dpp3 regulates a broader proteostasis and immunometabolic network, rather than a single downstream effector.

Together, these findings suggest that Dpp3 constrains immune activation by regulating the inducible stabilization of Nrf2 and coordinating broader inflammatory and proteostasis networks. By fine-tuning redox-sensitive and NF-κB-associated signaling modules, Dpp3 appears to calibrate the amplitude of antimicrobial responses, preventing excessive inflammatory escalation while preserving host defense efficacy. We have revised the Discussion to clarify that Dpp3 likely acts as a systems-level regulator integrating proteasomal turnover, redox balance, and NF-κB activation, rather than through a single substrate-dependent mechanism.

We acknowledge that full substrate-level dissection of Dpp3 enzymatic targets *in vivo* will require dedicated proteomic and genetic epistasis approaches, which represent important future directions. However, the integrated biochemical, functional, and network-level data presented here move the study beyond a purely phenomenological description and provide mechanistic insight into how Dpp3 shapes immune responses.

Revisions made: Results, page 13.

Reviewer: “While the authors claim that NF-κB genes are up in the Dpp3 KO splenocytes in response to LPS activation the data don’t fully support this. Both IL-6 and IL-10 are part of the type I IFN pathway and IL-10 has been shown to suppress inflammatory pathways

(including IFN γ and IL-12). Since there is a moderate increase in IFN γ is it possible that there might be enhanced antigen presentation and T cell responses in the *Dpp3* KO?”

Response: We thank the Reviewer for this insightful comment and for highlighting the complexity of cytokine pathway interpretation.

In our dataset, *Dpp3*^{-/-} splenocytes display increased NF- κ B expression following LPS stimulation, accompanied by elevated IFN- γ and IL-6 production and reduced IL-10 levels. Thus, the cytokine profile is consistent with amplified inducible inflammatory signaling rather than mixed regulatory activation.

Importantly, our interpretation of enhanced NF- κ B-associated signaling is not based solely on individual cytokines. Integrated transcriptomic and proteomic analyses demonstrate enrichment of NF- κ B-related modules and coordinated upregulation of inflammatory pathway components in *Dpp3*^{-/-} cells under stimulation conditions.

Regarding the possibility of enhanced antigen presentation and T-cell responses, we further assessed MHC class II expression by flow cytometry and observed increased surface MHCII levels on CD11b⁺ cells from *Dpp3*^{-/-} mice at baseline (new Fig. 3B). Together with RNA-seq enrichment of antigen processing and presentation pathways and increased IFN- γ production, these findings support enhanced adaptive immune engagement in *Dpp3*-deficient animals.

While definitive functional antigen-presentation assays would be required to formally demonstrate augmented T-cell priming, the combined transcriptional, proteomic, and flow cytometric evidence supports a model in which *Dpp3* deficiency promotes coordinated innate and adaptive immune activation.

Reviewer: “It would be nice to complement the *in vivo* infection data with infection of cells *ex vivo* (in addition to PMA and LPS stimulation). LPS alone might not be sufficient to induce Nrf2 responses in immune cells like a “real” infection. Using *K. pneumoniae* may promote a different immune milieu that is more relevant to the *in vivo* anti-microbial responses (and the infection results!)”

Response: We thank the Reviewer for this constructive suggestion. We agree that *ex vivo* infection of primary immune cells with *Klebsiella pneumoniae* would provide complementary insight into the relationship between Nrf2 signaling and antibacterial responses in a more physiological context.

In the present study, we employed LPS stimulation as a controlled and proximal mimic of innate sensing, given that *K. pneumoniae* is a Gram-negative pathogen whose early recognition is largely mediated through TLR4–LPS signaling. This approach allowed us to dissect cell-intrinsic redox and inflammatory signaling pathways under defined conditions, minimizing variability associated with bacterial uptake, replication kinetics, and differential viability in primary cultures.

Importantly, we now include data demonstrating altered ROS production in immune cells isolated from infected mice *ex vivo*, supporting the relevance of the redox phenotype in the infectious context. We have clarified in the revised manuscript that LPS-based assays were used for mechanistic dissection rather than as a full surrogate of infection.

Reviewer: Minor concerns:

- A graphic of the mechanism would help understand what pathways are involved.
- Many of the bar graphs (ex. Figure 5m) have a line coming off the left side of the bar on

the graph.

- For all figures using error bars, they need to be in front of the plotted dots.
- Multiple figure alphabet sequence is not correct for the English alphabet.
- In figure 2e, it is difficult to see the merged image of DAPI and DPP3.
- In figure 3e it is difficult to read the gene names, I would suggest making this panel slightly larger to separate the names.
- Line 311 mentions figure 3g, there is no figure 3g.
- Figure 4 and 6, is there a reason for the change in color scheme from black and red to grey and black in regards to what is WT and what is Dpp3^{-/-}?
- Figure 4o-q, the legend does not quite match up with the sequence of figures, please correct.
- Figure 5e and l, the error bars are off center.
- Figure 6g, it is difficult to read the labels on the left.
- A model would help tie together all the pathways that are affected by Dpp3.

Response: We thank the Reviewer for the careful evaluation of the figures and for identifying several issues related to graphical presentation and formatting. We have thoroughly revised all figures to improve clarity, consistency, and visual quality. In the revised submission:

- A conceptual graphical model summarizing the Dpp3-regulated pathways has been added (**new Fig. 7G**), integrating redox regulation, immunometabolic remodeling, and inflammatory signaling.
The model illustrates that, under physiological conditions, Dpp3 contributes to:
 - Proper stimulus-dependent Nrf2 stabilization and redox buffering
 - Restraint of excessive NF-κB activation
 - Coordination of mitochondrial and biosynthetic metabolism
 - Balanced Ang II–AT1R signalingCollectively, these mechanisms maintain immune activation within a controlled range. In the absence of Dpp3, impaired inducible Nrf2 activation, enhanced redox flux, mitochondrial remodeling, and altered Ang II signaling converge to lower immune activation thresholds, resulting in amplified antibacterial responses while limiting tissue damage.
This graphical model clarifies the integrated, systems-level role of Dpp3 rather than portraying it as a linear regulator of a single pathway.
- Graphical artifacts (e.g., stray lines adjacent to bars in Fig. 5m) have been removed.
- Error bars have been repositioned to ensure proper visualization in relation to plotted data points.
- Panel lettering has been corrected to follow proper alphabetical sequence throughout.
- Contrast and channel overlay in Fig. 2e (DAPI/DPP3) have been adjusted for improved visualization.
- Fig. with heat-map have been resized to enhance gene label readability.
- The incorrect reference has been corrected.
- Color schemes have been standardized across all figures to consistently represent WT and Dpp3^{-/-} groups.

- Figure legends have been aligned with the correct panel order.

We believe these revisions substantially improve the clarity, consistency, and overall presentation of the manuscript.

Point-by-point response to Reviewer #1

The authors have mainly addressed my comments satisfactorily. Though 2 points still require attention.

Reviewer: *For reasons of consistency, the background of the Rag1^{-/-} mice should still be given.*

Response: We thank the Reviewer for this comment. For consistency, we have now specified the genetic background of the Rag1^{-/-} mice in the Methods section (page 18, line 542), in line with the information provided for the other mouse strains used in this study.

Reviewer: The formulation: “All in vivo experiments were performed using both male and female mice, with comparable representation of sexes within each genotype.” is still vague. Specific distribution of male and female mice in the groups should be given.

Response: We thank the Reviewer for this comment and agree that the previous formulation was not sufficiently specific. We have now included the exact number of male and female mice for each experimental group directly in the figure legends of all in vivo infection experiments, allowing full transparency in the distribution of sexes across groups.

Revisions made: Methods page 18, line 545; Legend Figure 1 and 2 , Page 33; Legend Supplementary Figure 6.

Point-by-point response to Reviewer #3

The authors have made considerable efforts to address the reviewers' comments, and the revised manuscript reflects meaningful revisions. However, while some concerns have been addressed, several key issues raised by this reviewer remain unresolved.

We thank the Reviewer for acknowledging the substantial efforts made to address the previous comments and for recognizing the improvements in the revised manuscript. We appreciate that important concerns remain and have carefully considered each of these points. In response, we have further revised the manuscript to address the remaining issues and to clarify the interpretation and presentation of our findings.

Reviewer: *I remain unconvinced that the mechanisms / pathways identified using LPS-stimulated splenic cultures adequately explain resistance mechanisms operating in the lungs. The cellular composition of naïve spleens differs substantially from that of infected lungs, with B cells representing the predominant population in the former setting. In contrast, infected lungs exhibit a markedly distinct immune landscape. To strengthen the physiological relevance of the findings, T and B cell responses should be examined directly in infected lungs or lung-draining lymph nodes rather than in spleens (Fig. 3). Similarly, Figures 6 and 7 are difficult to interpret, as the composition of myeloid populations in naïve spleens and inflamed lungs differs significantly. For*

macrophage-focused experiments, stimulation with live Klebsiella pneumoniae rather than LPS would be more appropriate and biologically relevant.

Response: We thank the Reviewer for this important comment and for highlighting the potential limitations of extrapolating findings from LPS-stimulated splenic cultures to the infected lung environment. We agree that the immune composition of naïve spleens differs substantially from that of infected lungs, and that analyses performed directly in the pulmonary compartment are critical to strengthen physiological relevance.

In response, we have now performed additional ex vivo analyses on immune cells isolated from infected lungs (**Supplementary Figure 3**). These experiments reveal that CD45⁺ lung cells from *Dpp3*^{-/-} mice exhibit increased expression of the transcription factors Tbx1, Stat1, and Irf8 following *K. pneumoniae* infection, consistent with activation of antimicrobial and immune-regulatory programs. At the functional level, CD11b⁺ cells from *Dpp3*^{-/-} lungs display enhanced phagocytic activity, increased ROS production, and elevated MHC class II expression. In parallel, we observe increased frequencies of CD138⁺ plasma cells, expanded effector memory CD4⁺ and CD8⁺ T-cell populations, and higher proportions of proliferating Ki67⁺ T cells.

Together, these data demonstrate that both innate and adaptive immune compartments are functionally enhanced directly within the infected lung, thereby strengthening the physiological relevance of our findings. Importantly, these lung-derived results are consistent with, but not solely inferred from, the ex vivo splenic analyses, supporting the conclusion that *Dpp3* deficiency broadly modulates immune activation across compartments.

We have revised the manuscript to incorporate these new data and to clarify the relationship between splenic and pulmonary findings.

Regarding the use of LPS stimulation, it was used, in the present study, as a controlled and reproducible approach to probe *Dpp3*-dependent immune activation pathways. We now explicitly acknowledge this limitation in the Discussion and note that future studies using live bacteria will be important to further refine these mechanisms in lung-relevant contexts.

Revisions made: Results page 6, lines 172-184; Discussion page 15, lines 468-469; page 16, lines 518-525.

Reviewer: *I also do not agree with the authors' assertion that "adoptive transfer experiments demonstrate that Dpp3-deficient immune cells are sufficient to confer protection in vivo, thereby directly linking splenic phenotypes to pulmonary infection outcomes." Unless it can be demonstrated that T cells from lymph nodes do not transfer protection, the proposed link between splenic phenotypes and pulmonary immunity remains insufficiently supported. It is well established that adoptively transferred immunity in RAG models can be mediated by T lymphocytes independently of their tissue of origin, which weakens the authors' interpretation.*

Response: We thank the Reviewer for this important comment and agree that our previous wording in the point-by-point response was too strong. We did not intend to claim that the adoptive transfer experiments directly link splenic phenotypes to pulmonary infection outcomes. Rather, these experiments support the conclusion that

Dpp3-deficient immune cells contribute to protection in vivo in the Rag1^{-/-} transfer setting. We agree that they do not resolve the relative contribution of T cells, B cells (as previously reported in the Pbp letter to Reviewer #2), or the tissue of origin of the transferred lymphocytes. Accordingly, we have revised the wording throughout the manuscript to avoid overinterpretation.

Revisions made: Results page 10, lines 301-305; Discussion page 16, lines 518-522.

Reviewer: *Key methodological details should be included in the figure legends. In several instances, it is difficult to discern how the experiments were performed based on the figures alone, which hampers interpretation of the data.*

Response: We agree that sufficient methodological detail in figure legends is essential for proper interpretation of the data. In response, we have revised the figure legends throughout the manuscript to include key experimental information, such as the biological source of the samples, stimulation conditions, and assay type, where needed to improve clarity.

At the same time, in order to comply with journal guidelines on figure legend length and avoid redundancy with the Methods section, we have focused on including the most critical information in the legends while ensuring that full experimental details are clearly described in the Methods. We believe this revised approach improves clarity while maintaining conciseness.

Revisions made: Legend of Main Figures (1-7) and Supplementary Figures (1-19)

Reviewer: Related to the point above, it is unclear how the mean fluorescence intensity (MFI) data shown in the revised Figure 4F were quantified. Please clarify whether MFI was determined by flow cytometry or by image-based analysis. If flow cytometry was used, this should be explicitly stated in both the figure legend and the Methods section. If image-based quantification was performed, the authors should comment on the accuracy and limitations of this approach, particularly given that DPP3 signals in many images appear saturated. The same concern applies to the quantification of NRF2 expression. In addition, representative staining for both WT and Dpp3^{-/-} cells should be shown. Inclusion of Dpp3^{-/-} cells as negative controls in immunofluorescence experiments throughout the manuscript would further strengthen the rigor of the study.

Response: We thank the Reviewer for this important comment and for highlighting the need for clarification regarding fluorescence quantification.

We now explicitly state in both the Figure legend and the Methods section that fluorescence values in Figure 4F were obtained by confocal image-based analysis. We have also expanded the Methods section to describe in detail how fluorescence intensity was quantified, including image acquisition parameters, segmentation strategy, and measurement of integrated fluorescence intensity.

We agree that image-based quantification has inherent limitations. In response, we have clarified in the revised manuscript that all images were acquired using identical acquisition settings across experimental conditions, and that fluorescence intensity measurements were performed under standardized conditions to ensure comparability. For visualization purposes, representative images for Dpp3 staining were adjusted to improve signal visibility in reduced-size figure panels, as suggested by one of the

Reviewer. To ensure full transparency and avoid potential misinterpretation due to display settings, we have included the original, unprocessed images in the Supplementary Methods file.

In addition, as requested, we have now included representative Dpp3 staining for Dpp3^{-/-} cells in the revised figures. In addition, we have clarified in the Methods that Dpp3^{-/-} samples were used to assess background signal.

Similar clarifications have been introduced for Nrf2 quantification, including explicit description of the image-based analysis and its limitations. We believe these additions improve the transparency and interpretability of the data.

Revisions made: Methods page 23, lines 722-742, Figure 4F and relative legend.

Reviewer: Finally, the illustration and legend in Figure 3A do not correspond and should be corrected for accuracy.

Response: We thank the Reviewer for pointing this out. We have corrected the inconsistency between the illustration and the corresponding legend in Figure 3A in the revised manuscript to ensure accuracy and clarity.