nature portfolio

Peer Review File

Host-Pathogen Pyrimidine Metabolism Drives Cystic Fibrosis Lung Disease

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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)
1. What are the noteworthy results?

The study identifies pyrimidine metabolism as a central pathway in cystic fibrosis (CF) lung disease and links CFTR dysfunction to epithelial cell proliferation and airway remodelling. The work shows that mitochondrial impairment in CF airway epithelial cells induces compensatory activation of DNPPS, which supports proliferation through metabolic rewiring. The findings provide evidence that pharmacological inhibition of the rate-limiting enzyme dihydroorotate dehydrogenase (DHODH) reduces the proliferation of epithelial cells in CF. It reveals that Pseudomonas aeruginosa exhibits convergent metabolic adaptation, activating its own DNPPS machinery to enhance growth and persistence in the CF lung.
Overall, the study links host–pathogen metabolic changes to airway remodelling and bacterial persistence. It highlights pyrimidine signalling as a promising therapeutic target that could complement CFTR modulators and antibiotics, helping preserve lung structure and limit bacterial adaptation.

2. Will the work be of significance to the field and related fields? How does it compare to the established literature?

The work is likely to be significant in advancing the understanding of the complex host-pathogen metabolic relationship in CF disease.
It advances the field by:
• Moving beyond the traditional model of CF lung disease to propose a metabolic driver of pathology.
• Integrating epithelial biology, metabolism, and microbial adaptation into a unified conceptual framework.
• Extending prior literature linking DNPPS to cancer and immune diseases into the context of chronic infection and tissue remodelling.

Compared to established literature:

• Previous studies have shown CFTR-linked mitochondrial dysfunction and altered immune responses. This work builds on that by connecting these defects to pyrimidine metabolism as a functional driver.
• There is growing recognition of pathogen adaptation to host metabolites, but the demonstration of parallel metabolic rewiring in host and pathogen is relatively novel.
The work is both original and innovative, especially in its framing of cystic fibrosis as an immune-metabolic disorder driven by host–pathogen metabolic convergence.

3. Does the work support the conclusions and claims, or is additional evidence needed?

The study uses complementary in vitro, in vivo, metabolic, transcriptomic, and bacterial experiments to show that pyrimidine metabolism consistently drives CF cell growth and pathogen adaptation, strengthening both causality and biological relevance. The work carried out supports the claims and conclusions of the manuscript, it uses multiple experimental approaches to address the research question such as:
• Defining causality in epithelial cells - these experiments demonstrated that changing pyrimidine metabolism directly alters CF epithelial cell proliferation, proving that it actively drives the problem rather than just being linked to it.
• Demonstrating physiological relevance using primary human bronchial epithelial cells - the study shows that CFTR-mutant

cells have increased pyrimidine metabolism and abnormal growth, and that correcting CFTR function or inhibiting DNPPS reverses this.
• Using the publicly available single cell RNA-Seq datasets from human lung tissue with unbiased pathway enrichment analysis to show that genes linked to pyrimidine metabolism are more active in CF airways.
• Demonstrated the metabolic mechanism using bioenergetics and metabolomics to show that CF cells have impaired mitochondria and rely on pyrimidine metabolism.
• In vivo validation using mouse lung infection models with Pseudomonas aeruginosa infection demonstrates that pyrimidine metabolism modulates the airway environment during infection, reducing inflammatory damage and promoting tissue remodelling while also providing conditions that influence bacterial persistence in the CF lung. However, only using P. aeruginosa in this study does not show if metabolic interaction is general across CF pathogens or if it is specific to P. aeruginosa adaptation biology, this could be something to investigate in future work.
• Investigating the role of pyrimidine metabolism in P. aeruginosa showed that knocking out of the key enzyme (pyrD) impairs bacterial growth in the lung, while metabolic tracing demonstrates that this pathway directs nutrients such as glutamine into energy production and biomass expansion.
• The host–pathogen interaction experiments show that changes in host metabolism directly shape the infection environment, creating conditions that both reduce damaging inflammation and promote airway remodelling, while also enabling P. aeruginosa to adapt and persist in the lung.

However, several conclusions would benefit from additional supporting evidence, such as direct in vivo demonstration of airway remodelling, mechanistic evidence for coordinated host–pathogen metabolic interactions, broader testing across CF-relevant pathogens, and stronger clinical validation in patient populations would substantially strengthen the impact and generalisability of the findings.

4. Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

The overall study is well designed and generally robust, with multiple complementary experimental approaches supporting the central conclusions. The data analysis is appropriate and uses standard statistical approaches, but there are some limitations. Many experiments rely on small sample sizes (often n=3), with limited clarity on biological versus technical replicates, which reduces confidence in the robustness of the study. In addition, multiple-testing correction is not clearly described for the high-dimensional datasets such as the metabolomics and transcriptomics, and assumptions of normality are not sufficiently justified.
The following are suggestions to the authors to further improve the manuscript:

Statistics and Methods - statistics section; throughout all figures:
• Clarify biological vs technical replicates (e.g. what does this mean? - “N=3 including all replicates” in Fig. 1) and report exact sample sizes per group.
• Justify small the small number of replicates or increase replication where feasible.
• Avoid assuming normality and justify test selection.

Omics analyses results - Fig. 2, and supplementary Fig. S2; transcriptomics and metabolomics sections:
• Apply and report multiple testing correction (e.g. FDR) for pathway enrichment, scRNA-seq, and metabolomics.
• Update claims such as proportions of pathways altered accordingly (e.g. in lines 206 and 215 ~ is used when showing % of networks, this is ambiguous).

Transcriptomics methods - scRNA-seq analysis and results Fig. 2, supplementary Fig.S2):
• Expand description of batch correction, DEG thresholds, and covariate handling.
• Improve reproducibility by including full analysis parameters and outputs.

In vivo interpretation - results: Fig. 3 and the related text):
• Clarify the apparent inconsistency between reduced inflammation and reduced bacterial burden.
• Temper conclusions or provide additional explanation of infection dynamics.

5. Is the methodology sound? Does the work meet the expected standards in your field?

The study uses a well-integrated, multi-system approach, including:
• Primary human airway epithelial cells
• Established CF cell lines
• Murine infection models
• Metabolomics, transcriptomics, and stable isotope tracing

The methodology used in this study collectively supports a mechanistic framework and aligns well with field standards. The methodology is appropriate, is well executed and meets the expected technical standards in the field. However, to further improve this publication, the authors should:
• Increase biological replication, particularly for the in vitro and omics experiments, to improve reliability of the findings, or acknowledge the current limitations of replicates used.
• Strengthen human data by expanding the cohort or clearly acknowledging the limitations as it currently is.
• Improve the reporting in the transcriptomics, include details of batch correction and analysis thresholds.
• Clarify limitations of the murine model, as it represents acute infection and may not fully represent chronic CF disease.

6. Is there enough detail provided in the methods for the work to be reproduced?

The methods section provides a generally solid level of detail, and most experimental approaches could be reproduced at a broad level. Key techniques such as cell culture conditions, metabolomics workflows, Seahorse assays, and murine infection models are described with sufficient baseline information.

However, some important details are missing or are not described in enough detail, which may limit full reproducibility:

• Statistical analysis: lacks detail on how assumptions were tested such as the normality, and how data were handled across replicates
• Transcriptomics analysis: Insufficient detail on batch correction, filtering criteria, and thresholds used for differential expression.
• Replication clarity: there is limited information on how replicates were defined and combined, it would be useful to clearly define the technical and biological replicates in the manuscript.
• It is not always clear how the data were processed and analysed, especially for transcriptomics and metabolomics, please provide more information on the filtering criteria, thresholds, and normalisation steps used.
• Metabolomics and pathway enrichment: there is limited description of data processing, normalisation, and statistical correction methods. Without these details, another researcher could broadly repeat the experiments but may not be able to reproduce the exact results or analyses.

Overall, this is a strong and impactful study linking host–pathogen metabolic changes to airway remodelling and bacterial persistence in cystic fibrosis. I recommend the manuscript for publication following minor revisions as outlined in my review.

Reviewer #2

(Remarks to the Author)
This is an interesting and well-written manuscript by Lohia et al. that identifies a shared mechanism involving de novo pyrimidine synthesis (DNPPS) in CFTR-mutant airway cells and P. aeruginosa, which drives CF lung remodeling and bacterial persistence. In CF cells, mitochondrial bioenergetic impairment induces upregulation of dihydroorotate dehydrogenase, promoting proliferation and a cytokine-tolerant state that aids P. aeruginosa adaptation via itaconate-mediated mucA inactivation and alginate production. CF-adapted mucoid bacteria (mucA22) further upregulate their DNPPS (pyrD), increasing biomass through glutamine-to-pyrimidine flux. This host-pathogen relationship sustains airway remodeling. CFTR modulators and dihydroorotate dehydrogenase inhibitors, such as leflunomide, can reverse the host phenotype, suggesting potential therapeutic value.

The strengths of the study seem to include: 1) showing that leflunomide, already in use for arthritis, can reduce the abnormal cell growth seen in CF airways, the study points toward repurposing existing medicines rather than developing entirely new ones, which could accelerate translational applicability; 2) giving CF patients glutamine, curiously, worsened their lung infections, but no existing reasoning why. Authors provide a reasonable explanation, which could be an important contribution to patient safety and future design for clinical trials; 3) lastly, authors provide reasoning of why CF lung disease is, in principle, driven by metabolic dysfunction shared between the host and bacteria, which could shift how researchers and clinicians think about the disease more broadly. But provide some sense of how a shared pyrimidine biosynthetic pathway can be targeted to benefit treatment. Thereby, stressing some of the translational/clinical impact this might have to pwCF.

Weaknesses are more about the limits of authors' data interpretation. This reader believes the absence of in vivo data on CFTR mutants and the small human cohort are the two issues that weaken the study overall. Nonetheless, with a larger human dataset, in vivo evidence of histological remodeling, and a clearer distinction from the senior author's prior work, the study could be strengthened. There appears to be some slight overlap from prior work, and it is not clear to the reader how much of an advance this study carries from previous studies by the same group.

The broader overall weaknesses include 1) the limited number of only 5 healthy controls and 9 CF patients for sputum metabolomics. However, it is understandable to collect such a large cohort, but these should be noted as specific limitations of the study for readers. 2) Authors use an in vivo model with WT C57BL/6J mice infected with P. aeruginosa and not CFTR-mutant animals. This means the host-pathogen interaction is studied in a non-CF host, which significantly weakens claims about CF-specific disease. Some explanation should be noted; 3) Several important conclusions rest on limited independent experiments n=3 or n=2 for barrier function studies. This reader would've expected greater biological replicate numbers for the authors' primary claims, particularly for in vitro work where variability between patient-derived primary cells can be high.

Minor issues:
Figure 1, panel B demonstrates a western blot with no quantitative value. Is there some statistical value in this? It isn't clear.
Panel H: error bars appear very small for some metabolites, raising some questions about whether individual data points are shown, these should be overlaid as dots.
Figure 2, panel J shows the combined "MitoTempo + leflunomide" condition as absent for WT/WT CFTR cells in the cell number bar graph, which appears incomplete. This might weaken the claim that the effect is "conserved" as stated.
Figure 3, panel B IL-6 demonstrated no significant difference between vehicle and leflunomide groups, yet this is not discussed in the text. This inconsistency with the in vitro IL-6 result (Panel A) should be discussed or acknowledged. In

panel G the authors show that monocyte subpopulations are gated using Ly6C, but the gating strategy is described in the methods and makes challenging to make of their strategy. A supplemental gating figure showing sequential gates would certainly help strengthen assurance of these populations. Just a suggestion.
Figure 5, panel G there are several ND (non-detected) data points that appear for WT PAO1 conditions, but it is not stated whether ND = 0 or below detection threshold, and whether these were included or excluded from statistical analysis. This is slightly confusing to the reader what statistical values are used.

Reviewer #3

(Remarks to the Author)
The manuscript demonstrates the cystic fibrosis pathogen Pseudomonas aeruginosa uses de novo pyrimidine biosynthesis and in particular dihydroorotate dehydrogenase to increase its cellular biomass. This allows P. aeruginosa to create its own niche environment within the lungs of cystic fibrosis patients. The methodology used in the study confirms that de novo pyrimidine biosynthesis is essential for P. aeruginosa survival while the salvage of uridine does not appear to promote bacterial growth. It has been shown by Kim et al. (1991) FEMS Microbiol Lett 77, 175-179 that P. aeruginosa catabolism of uracil and thymine produces ammonia and citric acid cycle intermediates. Beyond de novo pyrimidine biosynthesis, was there any indication that P. aeruginosa cells use pyrimidine base catabolism to sustain bacterial cell biomass? The conclusions presented appear to be validated by the data with the significance of the findings being highly relevant to the treatment of cystic fibrosis patients. The analytical analysis of the data was confirmed by statistical analysis. The results were clearly presented in the manuscript. The references need to be revised since the journal style is not consistent. The style in references 2, 9, 10, 22, 23, 45, 47, 59, 66, 76 and 83 differ for the style used for the other references.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Authors carefully responded to and addressed the criticisms adequately. While there are still some limitations of the study, it does move the CF/CFTR field in a positive way for a better understanding of the influence of pyrimidine metabolism under diseased conditions.

POINT-BY-POINT

Reviewer #1 (Remarks to the Author):

1. What are the noteworthy results?

The study identifies pyrimidine metabolism as a central pathway in cystic fibrosis (CF) lung disease and links CFTR dysfunction to epithelial cell proliferation and airway remodelling. The work shows that mitochondrial impairment in CF airway epithelial cells induces compensatory activation of DNPPS, which supports proliferation through metabolic rewiring. The findings provide evidence that pharmacological inhibition of the rate-limiting enzyme dihydroorotate dehydrogenase (DHODH) reduces the proliferation of epithelial cells in CF. It reveals that Pseudomonas aeruginosa *exhibits convergent metabolic adaptation, activating its own DNPPS machinery to enhance growth and persistence in the CF lung.*

Overall, the study links host–pathogen metabolic changes to airway remodelling and bacterial persistence. It highlights pyrimidine signalling as a promising therapeutic target that could complement CFTR modulators and antibiotics, helping preserve lung structure and limit bacterial adaptation.

Answer: We appreciate the reviewer for their positive evaluation of our work. Below, we have addressed all comments and suggestions made, which has substantially improved the quality of our work.

2. Will the work be of significance to the field and related fields? How does it compare to the established literature?

The work is likely to be significant in advancing the understanding of the complex host-pathogen metabolic relationship in CF disease.
It advances the field by:
• Moving beyond the traditional model of CF lung disease to propose a metabolic driver of pathology.
• Integrating epithelial biology, metabolism, and microbial adaptation into a unified conceptual framework.
• Extending prior literature linking DNPPS to cancer and immune diseases into the context of chronic infection and tissue remodelling.
Compared to established literature:
• Previous studies have shown CFTR-linked mitochondrial dysfunction and altered immune responses. This work builds on that by connecting these defects to pyrimidine metabolism as a functional driver.
• There is growing recognition of pathogen adaptation to host metabolites, but the demonstration of parallel metabolic rewiring in host and pathogen is relatively novel.
The work is both original and innovative, especially in its framing of cystic fibrosis as an immune-metabolic disorder driven by host–pathogen metabolic convergence.

Answer: We appreciate the reviewer for their positive comments on our work.

3. Does the work support the conclusions and claims, or is additional evidence needed?

The study uses complementary in vitro, in vivo, metabolic, transcriptomic, and bacterial experiments to show that pyrimidine metabolism consistently drives CF cell growth and pathogen adaptation, strengthening both causality and biological relevance. The work carried out supports the claims and conclusions of the manuscript, it uses multiple experimental approaches to address the research question such as:
• Defining causality in epithelial cells - these experiments demonstrated that changing pyrimidine metabolism directly alters CF epithelial cell proliferation, proving that it actively drives the problem rather than just being linked to it.
• Demonstrating physiological relevance using primary human bronchial epithelial cells - the study shows that CFTR-mutant cells have increased pyrimidine metabolism and abnormal growth, and that correcting CFTR function or inhibiting DNPPS reverses this.
• Using the publicly available single cell RNA-Seq datasets from human lung tissue with unbiased pathway enrichment analysis to show that genes linked to pyrimidine metabolism are more active in CF airways.
• Demonstrated the metabolic mechanism using bioenergetics and metabolomics to show that CF cells have impaired mitochondria and rely on pyrimidine metabolism.
• In vivo validation using mouse lung infection models with Pseudomonas aeruginosa *infection demonstrates*

that pyrimidine metabolism modulates the airway environment during infection, reducing inflammatory damage and promoting tissue remodelling while also providing conditions that influence bacterial persistence in the CF lung. However, only using P. aeruginosa *in this study does not show if metabolic interaction is general across CF pathogens or if it is specific to* P. aeruginosa *adaptation biology, this could be something to investigate in future work.*

Response: We agree with the Reviewer that future work should address how pyrimidine metabolism could influence lung disease by other CF pathogens. We have considered this point in the new section of our updated manuscript entitled "Limitations of the Study", which can be found on Pages 9-10, line 478-493.

• *Investigating the role of pyrimidine metabolism in* P. aeruginosa *showed that knocking out of the key enzyme (pyrD) impairs bacterial growth in the lung, while metabolic tracing demonstrates that this pathway directs nutrients such as glutamine into energy production and biomass expansion.*
• *The host–pathogen interaction experiments show that changes in host metabolism directly shape the infection environment, creating conditions that both reduce damaging inflammation and promote airway remodelling, while also enabling P. aeruginosa to adapt and persist in the lung. However, several conclusions would benefit from additional supporting evidence, such as direct in vivo demonstration of airway remodelling, mechanistic evidence for coordinated host–pathogen metabolic interactions, broader testing across CF-relevant pathogens, and stronger clinical validation in patient populations would substantially strengthen the impact and generalisability of the findings.*

Response: We thank the Reviewer for this thoughtful comment and agree that additional studies examining airway remodeling, broader pathogen representation, and expanded clinical validation would further strengthen the generalizability of our findings. However, we believe the current study already provides substantial mechanistic and translational evidence supporting our central conclusions through the integration of human samples, primary airway epithelial cells, bacterial genetics, metabolomics, and *in vivo* infection models. While additional pathogens and validation approaches would broaden the scope of the work, they would primarily extend rather than alter the principal biological concepts established here. To ensure full transparency, we have incorporated the Reviewer considerations into a new "Limitations of the Study" section (Pages 9-10, lines 478–493).

4. Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
• *The overall study is well designed and generally robust, with multiple complementary experimental approaches supporting the central conclusions. The data analysis is appropriate and uses standard statistical approaches, but there are some limitations. Many experiments rely on small sample sizes (often n=3), with limited clarity on biological versus technical replicates, which reduces confidence in the robustness of the study. In addition, multiple-testing correction is not clearly described for the high-dimensional datasets such as the metabolomics and transcriptomics, and assumptions of normality are not sufficiently justified.*

The following are suggestions to the authors to further improve the manuscript:
Statistics and Methods - statistics section; throughout all figures:
• *Clarify biological vs technical replicates (e.g. what does this mean? - "N=3 including all replicates" in Fig. 1) and report exact sample sizes per group.*
• *Justify small the small number of replicates or increase replication where feasible.*
• *Avoid assuming normality and justify test selection.*

Response: We thank the reviewer for these important comments and suggestions. In response, we have revised the Methods section and figure legends to provide all additional details regarding biological replicates, statistical analyses, normality and test selection, multiple-testing correction, and assessment of data distribution where appropriate. These changes can be found in our revised manuscript on Page 11 lines 559-592; Page 16, lines 805-812; Page 25, lines 210-211, Page 26, lines 234- 235; Page 27, lines 251-252; Page 28, lines 270-273; Page 29, lines 284-285 of the revised Main Manuscript and Page 2, line 65; Page 3, lines 87-88; Page 4, lines 100-101; Page 5, lines 120-122; Page 6, lines 141-142; and Page 7, line 173-174 of the Supplementary

Information. Furthermore, we have indicated all limitations mentioned in our new section "Limitations of the Study", Pages 9-10, lines 478–493 of the revised Main Manuscript.

Omics analyses results - Fig. 2, and supplementary Fig. S2; transcriptomics and metabolomics sections:
• *Apply and report multiple testing correction (e.g. FDR) for pathway enrichment, scRNA-seq, and metabolomics.*

Response: We used multiple testing correction in our initial pathway enrichment analyses and OMICs approaches. As suggested by the Reviewer, we now include a detailed explanation of these analyses in the Methods section. These changes can be found on Page 11, lines 563-592 and Page 16, lines 805-812.

• Update claims such as proportions of pathways altered accordingly (e.g. in lines 206 and 215 ~ is used when showing % of networks, this is ambiguous).

Response: These were typos and we apologies for this. We have removed the symbol "~" in both lines. This change can now be found on Page 4, lines 209 and 218.

Change in the main material and method
Transcriptomics methods - scRNA-seq analysis and results Fig. 2, supplementary Fig.S2):
• *Expand description of batch correction, DEG thresholds, and covariate handling.*
• *Improve reproducibility by including full analysis parameters and outputs.*

Response: We have addressed these comments by including more detailed descriptions of batch correction, DEG thresholds, and covariate handling. These changes can now be found on Page 11, lines 563-574 and Page 16, lines 805-812 of the revised main manuscript.

In vivo interpretation - results: Fig. 3 and the related text):
• *Clarify the apparent inconsistency between reduced inflammation and reduced bacterial burden.*
• *Temper conclusions or provide additional explanation of infection dynamics.*

Response: We and others have previously reported that *P. aeruginosa* exploits IL-1β signaling to worsen airway damage and increase bacterial burden (PMID: 40253414; PMID: 37793346; PMID: 26984603; PMID: 11792633; PMID: 23478406). Our current results in Fig. 3 are consistent with this dynamic, revealing now a crucial role for DHODH limiting *P. aeruginosa* infection and respiratory destruction by restricting IL-1β production. We have clarified this concept in Page 6, line 303-304 and Page 6, lines 310-312.

5. Is the methodology sound? Does the work meet the expected standards in your field?
The study uses a well-integrated, multi-system approach, including:
• *Primary human airway epithelial cells*
• *Established CF cell lines*
• *Murine infection models*
• *Metabolomics, transcriptomics, and stable isotope tracing*

The methodology used in this study collectively supports a mechanistic framework and aligns well with field standards. The methodology is appropriate, is well executed and meets the expected technical standards in the field. However, to further improve this publication, the authors should:
• *Increase biological replication, particularly for the in vitro and omics experiments, to improve reliability of the findings, or acknowledge the current limitations of replicates used.*
• *Strengthen human data by expanding the cohort or clearly acknowledging the limitations as it currently is.*

Response: We thank the Reviewer for this suggestion. With the exception of a small number of complementary assays presented in the Supplemental Figures, all experiments in this study were performed using at least three independent biological experiments. To improve clarity, we have explicitly stated the number of independent experimental and technical replicates per assay in the Materials and Methods section and figure legends (Page 11 lines 559-592; Page 16, lines 805-812; Page 25, lines 210-211, Page 26, lines 234- 235; Page 27, lines 251-252; Page 28, lines 270-273; Page 29, lines 284-285 of the revised Main Manuscript and Page 2, line 65; Page

3, lines 87-88; Page 4, lines 100-101; Page 5, lines 120-122; Page 6, lines 141-142; and Page 7, lines 173-174 of the Supplementary Information). In addition, as suggested, we have acknowledged the limitations associated with certain experimental and patient sample sizes in the newly added "Limitations of the Study" section (Pages 9-10, lines 478–493), where we discuss both assay constraints and the challenges associated with obtaining suitable clinical specimens.

• *Improve the reporting in the transcriptomics, include details of batch correction and analysis thresholds.*

Response: As suggested, we have now included these additional details in the revised main manuscript. These can be found on Page 11 lines 568-574 and Page 16, lines 805-812.

• *Clarify limitations of the murine model, as it represents acute infection and may not fully represent chronic CF disease.*

Response: In addition to clarify the use of this model in the Results section in Page 6, lines 299-301, we have addressed any limitation with a detailed discussion in the new section "Limitations of the Study" (Pages 9-10, lines 478-493 of the revised main manuscript).

6. Is there enough detail provided in the methods for the work to be reproduced?
The methods section provides a generally solid level of detail, and most experimental approaches could be reproduced at a broad level. Key techniques such as cell culture conditions, metabolomics workflows, Seahorse assays, and murine infection models are described with sufficient baseline information. However, some important details are missing or are not described in enough detail, which may limit full reproducibility:
• Statistical analysis: lacks detail on how assumptions were tested such as the normality, and how data were handled across replicates

Response: We now provide with more detailed explanation on assumptions on normality as well as how data were studied across replicates (Page 11, lines 559-592 of the revised main manuscript).

• *Transcriptomics analysis: Insufficient detail on batch correction, filtering criteria, and thresholds used for differential expression.*

Response: We have added this information, which can be found in Page 11, lines 562-574 and Page 16, lines 805-812 of the revised main manuscript.

• *Replication clarity: there is limited information on how replicates were defined and combined, it would be useful to clearly define the technical and biological replicates in the manuscript.*

Response: We regret for the lack of clarity. We have now provided this information in both the Methods section of the revised main manuscript (Page 11, lines 559-567; Page 16, lines 805-812) and figure legends (Page 25, lines 210-211, Page 26, lines 234- 235; Page 27, lines 251-252; Page 28, lines 270-273; Page 29, lines 284-285 of the revised Main Manuscript and Page 2, line 65; Page 3, lines 87-88; Page 4, lines 100-101; Page 5, lines 120-122; Page 6, lines 141-142; and Page 7, lines 173-174 of the Supplementary Information).

• *It is not always clear how the data were processed and analysed, especially for transcriptomics and metabolomics, please provide more information on the filtering criteria, thresholds, and normalisation steps used.*
• *Metabolomics and pathway enrichment: there is limited description of data processing, normalisation, and statistical correction methods. Without these details, another researcher could broadly repeat the experiments but may not be able to reproduce the exact results or analyses.*

Response: For transcriptomics, we have complemented the Methods section with more information regarding filtering criteria, thresholds, and statistical analysis (Page 11, lines 568-574 of revised main manuscript). Furthermore, for metabolomics studies we have also completed the Methods section explaining filtering criteria, thresholds, and normalization steps (Page 11, lines 575-592 of revised main manuscript).

Overall, this is a strong and impactful study linking host–pathogen metabolic changes to airway remodelling and bacterial persistence in cystic fibrosis. I recommend the manuscript for publication following minor revisions as outlined in my review.

We sincerely thank the reviewer for the time and effort dedicated to handling our manuscript. We believe that these comments and suggestions have substantially strengthened the quality, rigor, and clarity of our work.

Reviewer #2 (Remarks to the Author):

This is an interesting and well-written manuscript by Lohia et al. that identifies a shared mechanism involving de novo pyrimidine synthesis (DNPPS) in CFTR-mutant airway cells and P. aeruginosa, which drives CF lung remodeling and bacterial persistence. In CF cells, mitochondrial bioenergetic impairment induces upregulation of dihydroorotate dehydrogenase, promoting proliferation and a cytokine-tolerant state that aids P. aeruginosa adaptation via itaconate-mediated mucA inactivation and alginate production. CF-adapted mucoid bacteria (mucA22) further upregulate their DNPPS (pyrD), increasing biomass through glutamine-to-pyrimidine flux. This host-pathogen relationship sustains airway remodeling. CFTR modulators and dihydroorotate dehydrogenase inhibitors, such as leflunomide, can reverse the host phenotype, suggesting potential therapeutic value.

Response: We thank the reviewer for their positive assessment of our work. We have addressed all concerns raised and incorporated the corresponding changes into the revised manuscript. We believe that these revisions have substantially improved the quality and rigor of our study.

1) The strengths of the study seem to include: 1) showing that leflunomide, already in use for arthritis, can reduce the abnormal cell growth seen in CF airways, the study points toward repurposing existing medicines rather than developing entirely new ones, which could accelerate translational applicability; 2) giving CF patients glutamine, curiously, worsened their lung infections, but no existing reasoning why. Authors provide a reasonable explanation, which could be an important contribution to patient safety and future design for clinical trials; 3) lastly, authors provide reasoning of why CF lung disease is, in principle, driven by metabolic dysfunction shared between the host and bacteria, which could shift how researchers and clinicians think about the disease more broadly. But provide some sense of how a shared pyrimidine biosynthetic pathway can be targeted to benefit treatment. Thereby, stressing some of the translational/clinical impact this might have to pwCF.

Response: We appreciate the positive evaluation of our work to the field.

1) Weaknesses are more about the limits of authors' data interpretation. This reader believes the absence of in vivo data on CFTR mutants and the small human cohort are the two issues that weaken the study overall. Nonetheless, with a larger human dataset, in vivo evidence of histological remodeling, and a clearer distinction from the senior author's prior work, the study could be strengthened. There appears to be some slight overlap from prior work, and it is not clear to the reader how much of an advance this study carries from previous studies by the same group. The broader overall weaknesses include 1) the limited number of only 5 healthy controls and 9 CF patients for sputum metabolomics. However, it is understandable to collect such a large cohort, but these should be noted as specific limitations of the study for readers.

Response: We thank the Reviewer for these thoughtful comments and agree that larger patient cohorts and additional *in vivo* studies would further strengthen the work. As suggested, we have incorporated these considerations into a new "Limitations of the Study" section (Pages 9-10, lines 478-493 of the revised main manuscript), where we discuss both the limited availability of clinical samples and the constraints of the *in vivo* models used.

Regarding overlap with our previous work, we respectfully note that the central hypothesis examined here — the role of pyrimidine metabolism in shaping host–pathogen interactions in the cystic fibrosis lung — has not been addressed in our prior studies. While certain conceptual themes are shared, reflecting a broader effort to understand how immunometabolic pathways influence pulmonary infection, we view this as a strength of a coherent and progressive research program. Importantly, the current study extends these previous observations by establishing a novel framework linking host and bacterial pyrimidine metabolism to airway remodeling, inflammatory regulation, and pathogen adaptation in cystic fibrosis.

2) Authors use an in vivo model with WT C57BL/6J mice infected with P. aeruginosa *and not CFTR-mutant animals. This means the host-pathogen interaction is studied in a non-CF host, which significantly weakens claims about CF-specific disease. Some explanation should be noted;*

Response: We thank the Reviewer for raising this important point. As described in the manuscript, WT C57BL/6J mice were used as a proof-of-principle model to establish the role of pyrimidine metabolism in regulating infection burden, inflammatory signaling, and airway remodeling *in vivo* (Page 6, lines 299-301). To specifically address the relevance of these findings to cystic fibrosis, we complemented these studies with CFTR-mutant airway epithelial models, which allowed us to investigate host-pathogen interactions within a disease-relevant context. We believe that the integration of these complementary experimental systems strengthens the overall conclusions of the study. To provide additional clarity, we have now discussed the scope and limitations of these models in the new section "Limitations of the Study", which can be found on Pages 9-10, lines 478-493 of the revised main manuscript.

3) Several important conclusions rest on limited independent experiments n=3 or n=2 for barrier function studies. This reader would've expected greater biological replicate numbers for the authors' primary claims, particularly for in vitro work where variability between patient-derived primary cells can be high.

Response: We agree that biological replication is an important consideration, particularly when working with primary human cells. In the case of the barrier function assays, we used N=2 independent experiments because our mechanistic studies did not identify epithelial barrier dysfunction as a major pathway linking pyrimidine signaling to airway remodeling or host-pathogen interactions. Accordingly, these experiments were included as supportive data and are presented in Supplementary Figure 3. Importantly, the studies were independently repeated twice, with each data point representing the average of a total of 24 technical replicates, yielding highly consistent results across experiments.

To facilitate better interpretation between independent experiments and their corresponding technical replicates, we have added to the Methods section and Figure legends this detailed information. This can be found in Page 11 lines 559-592; Page 16, lines 805-812; Page 25, lines 210-211, Page 26, lines 234- 235; Page 27, lines 251-252; Page 28, lines 270-273; Page 29, lines 284-285 of the revised Main Manuscript and Page 2, line 65; Page 3, lines 87-88; Page 4, lines 100-101; Page 5, lines 120-122; Page 6, lines 141-142; and Page 7, lines 173-174 of the Supplementary Information.

Minor issues:

1) Figure 1, panel B demonstrates a western blot with no quantitative value. Is there some statistical value in this? It isn't clear.

Response: We now provide with densitometry analysis of the western blot provided in Figure 1B. This can now be found in the Figure 1B itself (fold DHODH/actin) and described on Page 25, line 206-207 of the revised main manuscript.

2) Panel H: error bars appear very small for some metabolites, raising some questions about whether individual data points are shown, these should be overlaid as dots.

Response: Given their spatial proximity, datapoints are overlaid. To avoid this visual limitation, we have increased datapoints size and represented every one of them as empty circles. This can be found in the new panel 1H (Page 25 of revised main manuscript).

3) Figure 2, panel J shows the combined "MitoTempo + leflunomide" condition as absent for WT/WT CFTR cells in the cell number bar graph, which appears incomplete. This might weaken the claim that the effect is "conserved" as stated.

Response: We apologize for any confusion. The combined "MitoTempo + leflunomide" treatment is included in Figure 2J and corresponds to the fourth bar from left to right in the WT/WT CFTR group.

4) Figure 3, panel B IL-6 demonstrated no significant difference between vehicle and leflunomide groups, yet this is not discussed in the text. This inconsistency with the in vitro IL-6 result (Panel A) should be discussed or acknowledged.

Response: We thank the reviewer for this comment. We have better explained these differences in Page 6, line 307 of the revised main manuscript. We note that, whereas the *in vitro* experiments in Panel A assess cytokine production exclusively by epithelial cells, IL-6 levels measured *in vivo* in Panel B reflect the combined contribution of multiple inflammatory cell populations. As a result, the epithelial-specific effects of pyrimidine signaling on IL-6 production may be masked within the more complex inflammatory environment of the lung, including many other IL-6 producing myeloid cells that replenish the airway.

5) In panel G the authors show that monocyte subpopulations are gated using Ly6C, but the gating strategy is described in the methods and makes challenging to make of their strategy. A supplemental gating figure showing sequential gates would certainly help strengthen assurance of these populations. Just a suggestion.

Response: To facilitate experiment reproducibility, we have improved our gating strategy explanation in the methods section. These changes can be found on Page 12, Lines 631–636 of the revised main manuscript. Given that this manuscript already contains a substantial amount of data and Figures, we refrained of adding more illustrations.

6) Figure 5, panel G there are several ND (non-detected) data points that appear for WT PAO1 conditions, but it is not stated whether ND = 0 or below detection threshold, and whether these were included or excluded from statistical analysis. This is slightly confusing to the reader what statistical values are used.

Response: We believe the reviewer is referring to Figure 5H rather than Figure 5G. In this panel, "ND" denotes "Not Detected" and corresponds to uninfected control samples, where bacterial burden is expected to be zero. To avoid confusions, we have removed "ND" in the revised figure.

We sincerely thank the reviewer for their careful assessment of our work and for the valuable feedback provided throughout the review process. We believe that addressing these comments has improved the clarity, rigor, and overall quality of the manuscript.

Reviewer #3 (Remarks to the Author):

The manuscript demonstrates the cystic fibrosis pathogen Pseudomonas aeruginosa *uses de novo pyrimidine biosynthesis and in particular dihydroorotate dehydrogenase to increase its cellular biomass. This allows* P. aeruginosa *to create its own niche environment within the lungs of cystic fibrosis patients. The methodology used in the study confirms that de novo pyrimidine biosynthesis is essential for* P. aeruginosa *survival while the salvage of uridine does not appear to promote bacterial growth. It has been shown by Kim et al. (1991) FEMS Microbiol Lett 77, 175-179 that* P. aeruginosa *catabolism of uracil and thymine produces ammonia and citric acid cycle intermediates.*

1)Beyond de novo pyrimidine biosynthesis, was there any indication that P. aeruginosa *cells use pyrimidine base catabolism to sustain bacterial cell biomass?*

Response: We acknowledge the relevance of this point. However, in this study we focused on *de novo* pyrimidine generation, and not pyrimidine catabolism for bioenergetics and production of nitrogenated byproducts for growth. Given the previous work cited, we anticipate that *P. aeruginosa* could exploit their own generated pyrimidines to increase biomass, particularly during periods of nutritional constraints and starvation.

2) The conclusions presented appear to be validated by the data with the significance of the findings being highly relevant to the treatment of cystic fibrosis patients. The analytical analysis of the data was confirmed by statistical analysis. The results were clearly presented in the manuscript.

Response: We thank the reviewer for their enthusiasm and interest in our work.

3) The references need to be revised since the journal style is not consistent. The style in references 2, 9, 10, 22, 23, 45, 47, 59, 66, 76 and 83 differ for the style used for the other references.

Response: We have now updated the reference section as per the journal style. These changes can now be found in the revised manuscript in the References section between Pages 20 and 23.

We thank the reviewer for their thoughtful evaluation of our manuscript and for their constructive suggestions. We believe that the revisions made in response to these comments have substantially strengthened the quality, clarity, and rigor of our study.