nature portfolio

Peer Review File

Immune protection and persistence in *Pseudomonas aeruginosa* infections by pyruvate cross-feeding

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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)
The manuscript by Lampadariou et al. studies how adaptive mutations acquired by the pulmonary pathogen Pseudomonas aeruginosa in the cystic fibrosis (CF) airway contribute to the establishment of disease tolerance, with a particular focus on alterations in the pyruvate dehydrogenase complex (aceEF). By restricting their capacity to catabolize pyruvate, aceEF mutant strains accumulate and secrete this metabolite, which scavenges oxidants, dampens inflammatory signaling, and promotes intracellular survival within macrophages. This pyruvate-mediated reprogramming of the airway milieu not only supports bacterial persistence but also preserves tissue integrity — hallmarks of disease tolerance. Importantly, the authors extend these observations beyond CF, showing that clinical isolates from other infectious contexts harbor analogous inactivating mutations within the pyruvate dehydrogenase pathway.

Overall, the study is timely and conceptually aligned with the emerging framework of disease tolerance in CF, and the experimental design incorporates appropriate in vitro controls. However, my enthusiasm was tempered by the absence of in vivo evidence supporting the central tolerance hypothesis. Given the intrinsic link between tolerance, organ integrity, and sustained host-pathogen coexistence, validation in an in vivo setting is essential. Below, I outline major points that I hope will help strengthen the manuscript mechanistic.

Major Comments
(1) The proposed disease tolerance model should be tested in vivo, ideally in a lung-based setting that permits host-pathogen coexistence for a few days. One would predict that aceEF mutant P. aeruginosa strains persist alongside improved host tissue integrity compared with WT PAO1, most notably via an immunosuppressed environment. Similar outcomes should be demonstrated for clinical isolates harboring pyruvate dehydrogenase complex mutations.
(2) The airway-derived cues responsible for selecting pyruvate dehydrogenase inactivation remain unclear. While the authors propose plausible hypotheses, like oxidative pressure, these are not supported by strong experimental evidence.
(3) Although the authors thoughtfully examine macrophage responses, CF lung disease is fundamentally neutrophil-dominated. The impact of aceEF mutations on neutrophil recruitment, activation, and protease-mediated tissue damage was not assessed, leaving a critical gap in evaluating airway tolerance. These assays can be done in either CFTR-competent or CFTR-mutant hosts. Furthermore, it is still unclear how pyruvate, mechanistically, dampens macrophage inflammation. Is it via TCA cycle/OXPHOS stimulation? Itaconate induction? M2 polarization via aketoglutarate enrichment?
(4) As indicated by the authors, the association between aceEF mutations and alginate overproduction suggests potential engagement of the itaconate-alginate axis. However, no data are presented to support this link. Direct assessment of itaconate/lrg1 induction by aceEF mutants — and its contribution to pyruvate-mediated tolerance — would clarify whether these mechanisms are independent or synergistic. This is a relevant point by the authors, considered worth of discussion.
(5) Loss or remodeling of surface LPS, often through mutations in lptD or downregulation of LPS biosynthetic clusters, represents a major adaptive strategy promoting tolerance in CF P. aeruginosa — most notably promoted by host immunometabolic cues. Despite the frequent use of LPS as a control, these pathways were not examined in aceEF mutants, which weakens the mechanistic conclusions of how tolerance is achieved.
(6) Figure 1A interpretation: If aceF mutant strains secrete pyruvate, one would expect them to exhibit enhanced resistance to H_2O_2 even in the absence of exogenous pyruvate. However, both WT and mutant strains show a comparable ~50% reduction in CFU — despite initial CFU differences — which appears inconsistent with the stated premise.
(7) Although acetate is mentioned as byproduct of the antioxidant mechanism, its contribution to disease tolerance remains clear. By recovering aceF mutant growth, acetate would increase bacterial biomass and thereby enable abundant LPS

availability, triggering inflammation and compromising the tolerant milieu. This mechanism should be better explained.
(8) Although an exciting hypothesis, authors did not provide evidence that pyruvate acts as a universal mechanism of tolerance to infection by different pathogens. There are no functional studies testing immunomodulation with other organisms than P. aeruginosa.

Minor comments.
— Page 11, lines 262-263. “However, when assessing pyruvate secretion by the aceE and aceF strain under the infection conditions, we found that no pyruvate was produced within the short time frame of the experiment”. There is no evidence provided validating this.
— Fig. 4C: pyruvate reassimilation can also be extracellular catabolism to another product. This is not considered as hypothesis.

Reviewer #2

(Remarks to the Author)
In this study, Lampadariou et al. investigate the biological significance of mutations arising in the bacterial genes *aceE* and *aceF*, which encode components of the pyruvate dehydrogenase complex, proposing that these mutations represent host-driven adaptations that promote immunosuppression and bacterial persistence. This is an interesting and potentially impactful concept. The authors show that although these mutations initially reduce bacterial fitness, they ultimately confer a persistence advantage by increasing pyruvate accumulation and secretion, which is proposed to mitigate oxidative stress and suppress host proinflammatory cytokine production. However, several conclusions are overstated or insufficiently supported by the data, and additional clarification and rigor are required. My specific concerns are outlined below.

1. The use of the ALI model of human airway epithelial cells is well appreciated. However, clarification is needed regarding several aspects of the experimental design.
a. What is the rationale for the 6-hour incubation period prior to H_2O_2 treatment? Given the distinct growth kinetics of WT PAO1 compared with the *aceE* and aceF mutants—as noted by the authors themselves—it is likely that PAO1 reaches higher bacterial burdens during this period. This is expected to result in increased planktonic and surface-associated PAO1 compared to the mutants. Were growth curves for PAO1 and the *aceE/aceF* mutants performed under ALI conditions used in these experiments? In fact, Fig. 1A shows higher PAO1 burdens prior to treatment, and the ratio of PAO1 surviving after versus before H_2O_2 treatment appears comparable to that of the aceF mutant, which is unexpected. This observation seems inconsistent with the conclusions drawn from Fig. 1D and requires further explanation.
b. How much pyruvate is secreted by the aceF mutant under ALI conditions? Was this measured directly, and if so, does this explain the need to add 10 mM exogenous pyruvate? If exogenous supplementation is required, this suggests that endogenous pyruvate levels produced by the aceF mutant are insufficient to confer protection against H_2O_2-mediated killing. In this context, the statements in lines 152–153 appear overstated and should be revised.

2. Fig. 2. Did the authors measure pyruvate levels under the conditions of the ALI experiment? Additionally, were acetyl-CoA levels assessed, and if so, were there differences between strains?

3. The manuscript frequently refers to host–pathogen metabolic crosstalk; however, key mechanistic questions remain unaddressed. Have the authors determined whether pyruvate secreted by the aceF mutant is taken up by host cells and metabolized via host pyruvate dehydrogenase (PDH)? This point is particularly important given that in some assays the authors report that “no pyruvate was produced” (lines 262–263). Is there evidence that host PDH activity or expression is altered (increased) in response to infection? Moreover, is the observed immunosuppressive effect mediated by pyruvate itself, or by downstream metabolites such as acetyl-CoA, potentially through epigenetic mechanisms (e.g., acetylation)? Despite repeated references to “metabolic reprogramming,” no direct assessment of host metabolic changes is presented, and the mechanistic basis for selective suppression of certain cytokines remains unclear.

4. To more rigorously demonstrate that pyruvate secreted by the *aceF* mutant drives host immunosuppression, inclusion of a clean deletion mutant and a complemented strain would be important.

5. While the use of human cell–derived ALI infection models is appreciated, it remains unclear how these findings translate to established in vivo mouse models of pneumonia, where recruited immune cells play a critical role.

6. Finally, the authors make an interesting observation regarding the occurrence of ace gene mutations in other bacterial species, suggesting a potentially conserved persistence mechanism. However, this claim is not experimentally tested, as none of these additional species are examined in the presented models.

Reviewer #3

(Remarks to the Author)

Summary: In this in vitro study, the authors investigated how increased pyruvate secretion conferred by mutations in pyruvate dehydrogenase complex genes (PDHc) alter the interactions between respiratory bacterial pathogens (particularly Pseudomonas aeruginosa) and host cells. Using a combination of bacterial and mammalian cell culture methods, the

investigators show that the addition of exogenous pyruvate mitigates the effects of a key reactive oxygen species, hydrogen peroxide, on bacteria (by protecting from killing and growth arrest) and on host cells (by decreasing provoked inflammation). Building on their previous observation that P. aeruginosa strains with engineered mutations in the PDHc genes aceE and aceF produce relatively high extracellular concentrations of pyruvate, the authors found that these strains had altered survival (compared with the parent strain, PAO1) in both a medium that mimics the cystic fibrosis lung environment (SCFM) and in an air-liquid interface (ALI) cell culture model, although these differences were in opposite directions in these two models. The mutants also induced cultured lung epithelial cell cytotoxicity and cytokine production differently than the parent strain. The authors identified and characterized P. aeruginosa clinical isolates with mutations in either aceE or aceF and found ranges of growth rate and pyruvate secretion that were not obviously related to the type or location of the mutations. Finally, the authors found high-impact mutations in pyruvate dehydrogenase genes in archived sequencing data from other common respiratory pathogen isolates (Haemophilus influenzae, Staphylococcus aureus, and Stenotrophomonas maltophilia), many of which appeared to have a clinical origin. The authors conclude that increased pyruvate secretion conferred by mutations in PHDc genes may help bacteria persist in airway infections, where cytotoxicity to the epithelium and inflammation is decreased but the bacterial load is maintained.

Critique: This manuscript describes novel observations regarding the impact of mutations observed in clinical bacterial isolates on persistence and pathogenicity, at least in in vitro models. The text was generally well-written, authors used appropriate methods that they explained well, performed well-designed experiments, and displayed their results in largely easy-to-understand figures. The topic is likely of interest to the readership of this journal. Despite these positives, we feel that the manuscript would greatly benefit from additional clarification or explanation in key portions. We also found some of the conclusions and interpretations of the authors' findings to not be fully supported by the data as presented; for example, we felt that the analysis of genomic data from H. influenzae, S. aureus, and S. maltophilia did not definitively demonstrate functionally-important mutations in the PDHc similar to their observations with P. aeruginosa, with no analysis (either in silico or in vitro) of PDHc function and/or pyruvate secretion to distinguish from silent, natural variation in coding. It was also unclear whether these isolates came from chronically infected or colonized tissues. In other places, we felt that the limitations of models used (such as epithelial cell cultures or immune cell lines) precluded the interpretations presented (such as inferring immune cell recruitment when this was not explicitly tested). We also would welcome clarification of the rationale and interpretation of differences in experimental conditions (such as reagent concentrations) among otherwise similar experiments. These are further discussed below in addition to various minor points. Therefore, we felt that this manuscript would greatly benefit from revisions before it is ready for publication.

Major points
1) Introduction, page 4, lines 93-98: This section includes both observations made in this work (the effects of pyruvate on IL-8 secretion, for example), some from other work (it reacts non-enzymatically with ROS such as H_2O_2, which was not investigated here), and speculation ("…leading to disease tolerance by limiting airway inflammation). There was no investigation of either whole airways, multi-organ tissues, or animals here, which seemed to be required to make such a statement here, even if the cell-based in vitro data support such a model. Similarly, the statement on line 101-102 that "these results demonstrate that persisting bacteria within the host selects for PDHc mutations to influence disease progression…" seemed entirely speculative and beyond what the authors both could and did observe with their experimental design. This seemed to be more of a hypothesis to be tested (and we don't think the authors meant that persistent bacteria select for PDHc mutations, but rather that the host environment selects for these mutations in the bacteria, line 102).

2) Results, page 5, lines 120-122: "This system allows for the investigation of… immune system recruitment during infection." This statement did not seem to be correct. The authors did not study immune cell recruitment, but rather cytokine production without inclusion of any immune cells in their model.

3) Results, Figures 1-3 (H_2O_2 and pyruvate concentrations): How did the authors choose the concentrations of H_2O_2 and pyruvate used in Figure 1A, and why do these differ in Figure 1B and 1C? Perhaps reference Figure 1E to rationalize this. Do these differences have any implications for the findings in these different experiments? And while the amount of H_2O_2 added in Figure 1D was in the methods, it would be helpful to similarly add it to this figure legend as it was elsewhere. Similarly, in Figure 2D, what is the final concentration of pyruvate ("10 µL of 10 mM pyruvate" was added, but we don't know the final concentration, or how this would compare with the concentrations used in Figure 1)? And how was the 5 mM concentration in Figure 3C chosen?

4) Results, page 12, Figure 3: In Figure 3A, why do the authors think survival was increased at t0 in the aceF mutant (is there sufficient killing by H_2O_2 or other intracellular conditions expected by then?). Could it be more accurate to label the y-axis as CFU instead of survival? In Figure 3C, why does there appear to be an elevation in IL-8 induced by pyruvate even in the absence of LPS?

5) Results, page 15, Figure 4D: As above, we found it difficult to understand the relationship between the coding variations and clinical relevance of the isolates analyzed when compared with the data on P. aeruginosa (Figures 4A-C). Specifically, how do we know whether the coding variations shown affect pyruvate dehydrogenase activity, or result in changes in pyruvate secretion? And how do we know whether "COPD patient" or "otitis media" or "bloodstream infection" reflects a chronic infection (or, for that matter, how representative is "water" or "horse" of chronic infection isolates), as the authors hypothesize elsewhere that inactivating mutations in the PDHc results from adaptive change over time in P. aeruginosa? In general, we did not feel that this analysis or these data supported the authors' model or conclusions that "… modifications in pyruvate metabolism constitute a recurrent feature of pathogen adaptation during infection." (lines 410-411). To bolster the data the authors present in Figure 4D, we would like to see additional characterization of the isolates (e.g. pyruvate secretion, impact on growth rate, cytotoxicity, pro-inflammatory activity) or some other analysis that suggests an impairment

in PDHc activity which would support the model. Finally, we would like further confirmation that each of the isolates was isolated specifically from a chronic infection or chronically infected tissue and a more detailed explanation of how the clinical nature of the isolate was determined.

Minor points
1) In several places, we felt it would be best to moderate or edit the language of certain results and conclusions. Suggestions are underlined.
a. Line 30: "… with potentially analogous mutations also identified in…"
b. Lines 101-103: see major point 1, the chronic infection environment may select for PDHc mutations.
c. Lines 219: "These results suggest that mutations in the aceE and aceF genes may contribute to persistent infections and establish a potential link between pyruvate metabolism, virulence regulation and modulation of the immune system recruitment in P. aeruginosa infections."
d. Line 330: "additional functionally relevant…" This seemed to imply that the authors knew the coding variations shown were functionally relevant, which they did not show.
e. Line 364: "…it neutralizes ROS through…" The authors did not directly demonstrate the scavenging behavior of pyruvate, although this has been demonstrated previously.
f. Lines 365-367: "It reduces the host immune response by limiting IL-8 production and, therefore, recruitment of immune cells". As above, the authors did not explicitly test recruitment of immune cells, they only investigated specific cell types (even an ALI culture is reductionist compared with an intact animal or even organ or tissue). It seemed more accurate to state what the authors truly observed (and tested) and then hypothesize what would happen in an infected person.
g. Lines 385: "…secreted pyruvate not only neutralizes ROS, but also reduces IL-8 secretion…"
h. Lines 408-411: This sentence does not seem to be accurate given that the authors did not provide evidence that the coding differences in these other bacteria are functionally important. The authors should either remove this sentence or modify their language to be more accurate.

2) A number of typos appear throughout the manuscript.
a. Line 33: "which helps the establishment a more tolerogenic infection microenvironment…"
b. Line 82: "involved in pathogens protection from H2O2…"
c. Lines 95-96: "suppressing immune system cells recruitment. Third, it limits macrophages activation…"
d. Line 143: "mutants showed higher survival than PAO1, respectively…" What does respectively refer to?
e. Line 153: "…infection environments and in laboratory condition."
f. Pluralizing immune cells and cytokines inappropriately. This occurs throughout the manuscript including lines 176, 242, 286, 288, and some Y-axis labels in Figure 3.
g. Using hours time point (line 185) or hours infection (lines 208 and 209).
h. Lines 246-247: "…primary monocytes-derived macrophages…"
i. Line 309: "…all clinical strains secreted high amount of pyruvate…"
j. Line 337: "Type of non-synonymous mutations…"
k. Line 412: "…indicating that similar effect could…"
l. Lines 418-419: "… metabolites with immunomodulatory effect."

3) The manuscript mentions several comparisons without specifying what exactly is being compared:
a. Abstract, page 2, lines 32-33: "Elevated extracellular pyruvate…" Compared to what?
b. Introduction, page 4, line 92: "high secretion" Compared with what?
c. Introduction, page 4, line 98: "…more tolerogenic infection microenvironment" Compared to what? What is tolerating what? What does this mean?
d. Results, page 8, line 200: the aceF mutant strain triggered significantly lower IL-8 secretion compared to what?

4) Introduction, page 3, lines 51-53: "Strikingly, the precise and direct connections between…" We found this sentence difficult to understand.

5) Introduction, page 3, line 55: "several" is not necessary here.

6) Introduction, page 4, line 79: Here, the authors could include that the non-enzymatic reaction between pyruvate and H2O2 forms acetate.

7) Introduction, page 4, lines 86-87: The authors have previously defined disease tolerance in the introduction (lines 62-64), so it is not necessary here.

8) Consider adding new figure panels and/or modifying existing panels.

a. A graphical representation of the mutations in the aceE and aceF genes for the engineered PAO1 mutants. This could be a stand-alone panel or could be included in Figure 4A with the P. aeruginosa clinical isolates.
b. A graphical representation of the ALI cell culture method with labels (apical side/compartment, etc.) and a brief description in the figure caption of where planktonic, attached, and basolateral bacteria were collected from the system. This would be especially helpful for readers that are not familiar with ALI models.

9) Results, page 5, lines 113-117: Can the authors provide more information about the reduced growth rate and pyruvate secretion of aceF and aceE that has been previously reported? Which studies have reported this, is it only the authors' previous study (citation 24)? Which media were used to grow the strains, only SCFM, or were there others? Do the authors

think the growth medium could substantially impact growth rate and/or pyruvate secretion?

10) Results, page 5, lines 122-24: It would be helpful to specify that both the infection and the addition of H2O2 were on the apical side. Currently, only the location of the H2O2 addition is mentioned.

11) Results, page 5, line 138: Is the growth rate mentioned here the maximum growth rate detected in exponential phase? Is it based on the slope of the growth curve? It may be helpful for the authors to briefly describe how these values were determined (using the QurvE software) in the methods.

12) Results, page 6, lines 140-141: "…while pyruvate restored both parameters…" The addition of pyruvate did not completely restore lag time of aceF (Figure S1B). Here were the authors referring to all strains or just PAO1?

13) Lines 142-144 (Figure 1D): There are no statistics comparing PAO1 and aceE in Figure 1D, is that because the comparison is non-significant? Thus, "… aceE and aceF mutants showed higher survival than PAO1…" should be altered accordingly. PAO1 supplemented with aceF supernatant shows comparable protection (line 144) to what (aceE or aceF, or both even though the level of protection is different between aceE and aceF)?

14) Results, page 6, lines 152-153: It is not clear what the authors mean by "…pyruvate protects… both in in vivo-like infection environments and in laboratory conditions." What specifically is pyruvate protecting against H2O2?

15) Results, page 6, lines 142-144: The authors may want to add a description of the concentration of H2O2 used and for how long the strains were treated either here and/or in the Figure 1D caption.

16) Results, page 6, lines 145-146: The authors could specify here that pyruvate secretion was determined by quantifying the pyruvate levels in the supernatants of each culture at the relevant time points.

17) Results, page 6, line 148: The authors may want to include a short explanation of how acetate can complement PDHc mutations here.

18) Figure S1C: The authors should specify the concentration of acetate that was used in the figure caption.

19) Results, page 6, line 150: What do the authors mean by "A similar effect was seen in LB medium…"? Is it that there is also a faster growth rate in LB compared to SCFM2, like how there is a faster growth rate in SCFM supplemented with acetate compared to normal SCFM? Are the authors concluding that the increased growth rate is specific to the acetate present in LB, even when the nutrient profiles of SCFM and LB differ greatly?

20) Results, page 7, Figure 1B: Figures 1A, C, D, and E describe the effects of pyruvate on P. aeruginosa growth and survival in an ALI model, while 1B presents cytokine production data, apparently in macrophages, rather than the ALI model (mentioned in lines 131-136, but not included in figure caption). Therefore, 1B and its data seemed to be more appropriate for Figure 3, which describes the macrophage model.

21) Results, page 8, line 183: The authors cite Figure S1B to show that aceF has a reduced growth rate relative to PAO1, shouldn't this be Figure S1A instead?

22) Results, page 8, Figure 2A and line 194: The authors may want to explain the TEER fold change in the text in a way that makes it easier for readers to interpret the results (e.g. a fold change below 1 indicates that the barrier integrity has worsened). Also, the authors say "TEER…revealed a lower capacity of the aceF mutant…" but the word "capacity" seemed out of place. The authors identified less damage by aceF but did not seem to explore the full capacity of any of the strains to inflict damage.

23) Results, page 9, line 204: "…such an invasive behavior…" Was this observation from Figure 2B of bacteria in the basolateral compartment necessarily due to active invasion, rather than passive diffusion due to disruption of cell layer integrity as shown in Figure 2A?

24) Results, page 9, lines 208-214, Figure 2E: We are not sure what conclusions the authors had drawn from the colonization patterns and staining intensities in this figure.

25) Results, page 11, line 266: "…pyruvate addition suppressed secretion of both cytokines…" Suppressed does not seem like the right word to use here, especially when the reader is given no information for how the "connecting letters" relate to statistical significance in Figure 3C.

26) Results, page 12, Figure 3: In Figures 3B and 3D, we wondered why is there was no indication that the "mock" measurements were significantly different from the others; as shown, they certainly seemed to be different.

27) Results, page 12, Figure 3A: The authors may want to add a simple explanation of how the survival percentages were calculated that are shown at t4. Also, the authors describe t0 in the figure caption of Figure 3A, but not t4. We think it would be beneficial to describe the infection, gentamycin treatment, and incubation timing for t4 as well to help the reader appropriately interpret the results.

28) Results, page 12, Figure 3B: The finding that no pyruvate was produced, or was detected, during the timeframe of the experiment (Figures 3A and 3B) seemed worth further discussion. How do you explain this finding and why do you think you still saw a difference in intracellular survival in Figure 3A? Given the findings in Figure 3D, why include Figure 3B here?

29) Results, page 12, Figure 3C: Instead of asterisks or p-values, this sub-figure had letters to indicate significance that were not defined anyplace we could find. Please either describe this or replace the letters with the same markers as the authors used in other figures.

30) Results, page 12, Figure 3D: The readers are not given any context to interpret the magnitude of differences observed in IL-8, MCP-1, or TNF-alpha in Figure 3(D); are these biologically relevant changes? Would they be expected to change, for example, neutrophil recruitment or activity? Can the authors provide a reference or some other way to interpret these findings?

31) Describing mean fluorescence intensity (MFI): The authors describe MFI in the caption of Figure 3 (lines 291-291) but not when MFI is first presented in Figure 1B. Unless Figure 1B gets moved, MFI should be described in the caption of Figure 1B instead, or in addition to, in the caption of Figure 3.

32) Results, page 13, line 303: The authors mention that the P. aeruginosa isolates "…represent diverse clone types…" Can the authors confirm that "DK" represents a clone type and comment on why there are two "DK12" isolates shown in Figures 4A-C? Or is this not the case because the strains are "…colored by their clone type."? Numerous strains appear to share colors even though they have different DK numbers.

33) Results, page 13, line 304: In what way are the 12 isolates representative of the 89 isolates in the collection? Do the 12 feature all the mutations represented in the 89 isolates?

34) Results, page 13, line 308: The authors present the range of growth rates, which should probably have the units (hr-1), of the 12 clinical isolates but do not include the growth rates of the engineered mutants to compare to. This comparison would be helpful for the reader.

35) Results, page 13, lines 310-311: The authors say that pyruvate secretion levels were "…comparable to those of the recombinant strains…" but do not provide any values to compare. Again, providing a range of the pyruvate secretion of the clinical isolates and comparing it to the secretion of the recombinant strains would be helpful for the reader.

36) Results, page 13, lines 313-314: "…characterized by continuous secretion." This observation seemed to involve only 24 hours of measurement, and given that many of these isolates had relatively slow growth rates in 4B, it seems possible that longer incubation times could show these isolates to have similar patterns (with "re-assimilation") to the faster-growing isolates. Also, how do the authors know the patterns of increases followed by decreases reflect re-assimilation, rather than enzymatic degradation?

37) Results, page 13, lines 317-318: It is not clear what conclusion the authors are making here.

38) Results, page 14, lines 322: The authors mention PDHc subunits 1 and 2 here but don't explicitly link the subunits with the genes aceE and aceF. It would be helpful to explicitly state this to help the reader interpret the data in Figure 4C and Figure S2.

39) Results, page 15, Figure 4D: This figure was difficult to interpret. What do dark purple, light purple, and white shading mean? Are all of the isolates/strains in Figure 4D from unique people or sources or infections, or might some of the isolates have come from the same source (such as the two cheek skin S. aureus sequences)?

40) Discussion, page 17, lines 406-407: "…immune modulation is a strategy for…" seems like deterministic language. Do bacteria have strategies? It would be more accurate for the authors to describe what the effects of pyruvate are, rather than implying bacteria use it purposely.

41) Discussion, page 18, line 421: The authors mention that "counteracting" the effects of pyruvate, among other things, may offer a novel strategy for managing chronic infections. Wouldn't counteracting its effects increase inflammation? How would this be advantageous?

42) Discussion, page 18, Figure 5: They authors may want to add "model" or "diagram" to the title of this figure. The authors do not fully address their model in the discussion; thus, it was surprising to see that the model started with the T3SS that was only briefly mentioned in lines 389-392 of the discussion. The authors may want to more thoroughly address their complete model in the discussion, so the readers are not surprised when they get to the model figure.

Reviewer #4

(Remarks to the Author)
Chronic infections in CF patients are characterised by persistent infection and oxidative stress, but the mechanisms of persistence of bacteria are not fully understood. The authors identified a novel persistence mechanism that is mediated by secretion of pyruvate leading to a more tolerogenic environment and promoting bacterial persistence. By framing their work

in the context of disease tolerance of CF and bacteria-host crosstalk, the authors make a good argument for why this question is important for current research. The manuscript is of high quality, conceptually strong and well written.

The authors show that secreted pyruvate scavenges reactive oxygen species, suppresses epithelial and macrophage immune activation, and enhances bacterial survival, thereby promoting long-term persistence and a more tolerogenic infection microenvironment. They therefore used a broad spectrum of appropriate experiments and technologies. By doing so, they integrated the results of mutations with ability to induce inflammation, using ALI cultures and macrophages for further deeper characterization. The methods are state-of-the-art. It is logical and well executed.
The results were analysed and interpreted correctly. The evidence supports the authors conclusions.
It is still an open research quesiton.
This study represents a substantial advance in our understanding of chronic bacterial infections. It identifies pyruvate as a previously underappreciated bacterial immunometabolite and provides a clear mechanistic link between metabolic adaptation, immune modulation, and persistence.
The importance of the advance is well aligned with the standards of high-quality journals in infection biology, host–pathogen interactions, and immunometabolism. The work has both mechanistic depth and translational relevance. It will be of interest to researchers in the field of CF, scientists working on the pathogen-immune-host-crosstalk
The manuscript fit together very well. It clearly describe what was done, why it was done and what the results mean. The manuscirpt is written well and I enjoyed reading it.
It still has some mistakes. The readability of the manuscript would benefit from reducing the use of non-standard or less commonly used abbreviations (for example, “pwCF”), especially for readers outside the immediate cystic fibrosis research community.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed several of my initial comments; however, key conceptual and mechanistic concerns remain unresolved. Most notably, the absence of in vivo validation of the proposed mechanism significantly limits the interpretation of the findings, particularly with respect to disease tolerance. Without this level of analysis, it is not possible to determine whether the immunosuppressive effects attributed to pyruvate during P. aeruginosa infection reflect a bona fide tolerance mechanism, or simply a consequence of impaired infection resistance.

This concern is supported by the authors’ own data (Figure 3), where exogenous pyruvate — as well as infection with pyruvate-secreting P. aeruginosa strains — results in increased bacterial burden. Such outcomes are more consistent with reduced antimicrobial efficacy via resistance evasion than with a coordinated tolerance program. Notably, this issue was raised by multiple reviewers — where all requested in vivo validation.

The authors’ response further underscores this limitation. They cite recent work by Zhou et al. (2025, Cell Reports), which demonstrates that pyruvate secretion driven by gavR mutations enhances local bacterial persistence via evasion of infection resistance. However, rather than strengthening the present study, this comparison diminishes its novelty, and more worringly, point to inadequate data interpretation. Indeed, Zhou et al. already provide in vivo evidence that perturbations in central metabolism (e.g., aceEF inactivation) compromise infection resistance, thereby challenging the interpretation of pyruvate as a driver of tolerance in the current manuscript. In this context, additional in vivo experimentation would not be merely confirmatory — it would be essential to discriminate between resistance failure and tolerance induction.

Although the authors state that they have tempered their interpretation of disease tolerance, this is not consistently reflected in the revised manuscript. The abstract, in particular, continues to advance a strong tolerance-centric narrative, for example:

1) These mutations lead to elevated extracellular pyruvate, which helps establish a more tolerogenic infection microenvironment and favors bacterial persistence.
2) This metabolic crosstalk promotes bacterial persistence while limiting host tissue damage.
3) Our findings reveal pyruvate as a bacterial immunometabolite that mimics host antioxidant defenses, reshaping the infection niche to favor long-term colonization.

These statements extend beyond the evidence presented and risk conflating persistence with tolerance without sufficient mechanistic support.

Overall, while the updated study addresses an interesting aspect of host-pathogen metabolic crosstalk, substantial revision

is still required to clarify its mechanistic claims and to establish genuine conceptual novelty. At present, the work appears to largely reinforce previously published findings rather than provide a definitive advance. It is unclear if such a study can be considered for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
We appreciate the authors' careful attention to detail in revising their manuscript. In general, we felt the authors were very responsive to the critiques from all reviewers; there remain only a few minor issues that we thought would be worth addressing.
1) We were confused why pyruvate levels were sometimes presented in OD and in absolute concentration in others. We were confused why, in the revision (lines 131-134 clean copy/151-154 edited copy) the new text states that aceF made no detectable pyruvate after 6 hours, while Figure S1A has a missing 6hr data point for aceE and not aceF. Later, the authors also say that no pyruvate could be quantified but then reference Figure S1H, where it looks like there is pyruvate that can be quantified although it is displayed as an OD measurement instead of an absolute quantification. Can the authors clarify this?
2) In line 272 clean/326 edited, we suggest the authors might want to soften "confirming the role of PDHc…" to "suggesting a role of PDHc…". Similarly, "confirm" may be too strong a word for line 364 clean/437 edited.
3) In line 342 clean/409 edited, the authors reference both Figure 4C and D, but it appears as though only Figure 4C should be referenced.
4) We found figure 4D difficult to interpret in its current form.
a. Can the authors use the same phrasing from the Figure 3C figure legend to explain the interpretation of the statistical results using letters? We found the explanation for 3C to be clearer than the explanation for 4D.
b. Can the authors add a key within the graph that specifies which strains have PDHc mutations and which do not? The information is in the figure caption, but including it in the graph itself would increase readability.
5) We are not sure what the authors meant in lines 362-363 clean/435-436 edited by newly-added phrase "the historical contingency of the individual evolutionary trajectories of the strains."
6) We recommend that the authors provide a citation in lines 428-429 clean/513-514 edited to support their statement regarding the role of the T3SS in infection and immune recruitment.
7) We didn't understand what the authors meant in line 433 clean/518 edited by "altering such infection pattern".
8) Line 513 edited: "During infections…, bacteria use the T3SS to damage the host epithelium…" This new phrase seems to imply intent on the part of the bacteria- that they "use" a system for an intended purpose, which we didn't think the authors meant to imply. Similarly, lines 107-108 edited (…"PDHc mutations are selected for…to influence disease progression within the host") seems to imply that bacterial mutations are selected for their influence on disease severity, which also seemed unlikely.
9) Lines 97-98 edited: Suggest the authors specify the PDHc mutations are in P aeruginosa, not in the host or other bacteria (apologies for missing this in the first review).

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are grateful for the time and effort dedicated by the reviewers in critically evaluating our work. Their comprehensive feedback has been very useful for shaping the revised version of this manuscript. Our responses to all points raised by the reviewers are detailed below in blue.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):
The manuscript by Lampadariou et al. studies how adaptive mutations acquired by the pulmonary pathogen Pseudomonas aeruginosa in the cystic fibrosis (CF) airway contribute to the establishment of disease tolerance, with a particular focus on alterations in the pyruvate dehydrogenase complex (aceEF). By restricting their capacity to catabolize pyruvate, aceEF mutant strains accumulate and secrete this metabolite, which scavenges oxidants, dampens inflammatory signaling, and promotes intracellular survival within macrophages. This pyruvate-mediated reprogramming of the airway milieu not only supports bacterial persistence but also preserves tissue integrity — hallmarks of disease tolerance. Importantly, the authors extend these observations beyond CF, showing that clinical isolates from other infectious contexts harbor analogous inactivating mutations within the pyruvate dehydrogenase pathway.

Overall, the study is timely and conceptually aligned with the emerging framework of disease tolerance in CF, and the experimental design incorporates appropriate in vitro controls. However, my enthusiasm was tempered by the absence of in vivo evidence supporting the central tolerance hypothesis. Given the intrinsic link between tolerance, organ integrity, and sustained host-pathogen coexistence, validation in an in vivo setting is essential. Below, I outline major points that I hope will help strengthen the manuscript mechanistic.

We thank the reviewer for the positive comments on the manuscript and for the critique on the in vivo validation which we find relevant and that we have addressed below.

Major Comments
(1) The proposed disease tolerance model should be tested in vivo, ideally in a lung-based setting that permits host-pathogen coexistence for a few days. One would predict that aceEF mutant P. aeruginosa strains persist alongside improved host tissue integrity compared with WT PAO1, most notably via an immunosuppressed environment. Similar outcomes should be demonstrated for clinical isolates harboring pyruvate dehydrogenase complex mutations.

We agree with the reviewer that in vivo validation of the disease tolerance concept would provide additional relevance to our study. However, as noted by Reviewer #3, our discussion of disease tolerance, which we agree with the reviewer requires a whole organism to be fully demonstrated, was more speculative than our data and experimental approach could provide. We acknowledge our oversight in not making this clearer in the manuscript and in not providing a clearer perspective on the persistence phenotype that guided our experimental approach. Recently, Zhou et al. (2025, Cell Reports) reported that pyruvate secretion caused by *gavR* mutations enhances local persistence without systemic spread in mice. We believe that adding a mouse experiment in our work would likely be confirmatory rather than changing the mechanistic interpretation derived from

our human-based models. For these reasons, and in agreement with the editor, we have substantially revised the manuscript to refocus on bacterial persistence mechanisms, and now we present disease tolerance strictly as an untested hypothesis rather than a conclusion. Importantly, focusing on bacterial persistence has strengthened the manuscript narrative and its conclusions.

We have presented this limitation in the discussion of the manuscript (lines 496-502).

To further strengthen the clinical relevance of our work, we have performed macrophage infection assays with two matched pairs of clinical isolates with and without pyruvate dehydrogenase (PDH) mutations, confirming increased intracellular survival of the mutant strains (lines 351-365).

(2) The airway-derived cues responsible for selecting pyruvate dehydrogenase inactivation remain unclear. While the authors propose plausible hypotheses, like oxidative pressure, these are not supported by strong experimental evidence.

We agree with the reviewer that the precise selective pressure driving PDHc inactivation remains unknown. However, our longitudinal analysis of clinical isolates shows that *aceEF* mutations arise repeatedly and independently during chronic airway infections, strongly supporting their adaptive value for persistence. While oxidative stress may be one possible explanation, previous work has shown that overexpression of catalases is one of the main adaptive responses in in vitro settings (da Cruz Nizer et al., 2021 Pathogens). This suggests that PDHc mutations may reflect a broader adaptation to the host environment rather than a direct response to oxidative stress alone. We have clarified this point in the discussion and highlighted it as an important direction for future investigation (lines 452-460 and 468-475).

(3) Although the authors thoughtfully examine macrophage responses, CF lung disease is fundamentally neutrophil-dominated. The impact of aceEF mutations on neutrophil recruitment, activation, and protease-mediated tissue damage was not assessed, leaving a critical gap in evaluating airway tolerance. These assays can be done in either CFTR-competent or CFTR-mutant hosts. Furthermore, it is still unclear how pyruvate, mechanistically, dampens macrophage inflammation. Is it via TCA cycle/OXPHOS stimulation? Itaconate induction? M2 polarization via aketoglutarate enrichment?

We agree with the reviewer that CF lungs are dominated by neutrophils. However, macrophages are an important component of long-term airway immunity, and, therefore, better represent the persisting immune pressure in CF airway. Additionally, Zhou et al. (2025, Cell Reports) reported improved survival in neutrophils of adapted *P. aeruginosa* strains secreting pyruvate. For these reasons, we chose macrophages in our study. We have explained this rationale in the results section (lines 137-140).

We also agree with the reviewer that the molecular aspects underlying the remodeling of both ALI cultures and macrophages remain unclear. Addressing these mechanisms in detail would require a comprehensive multi-omics approach beyond the scope of the current study. Nonetheless, to partially address this point, we quantified extracellular pyruvate dynamics in both ALI cultures and macrophages and observed a decrease in extracellular pyruvate over time compared to cell-free controls, indicating active cellular uptake. While this does not resolve the intracellular fate of

pyruvate, it provides direct evidence that extracellular pyruvate is metabolically accessible to host cells, supporting its role as an immunometabolite.

We have included both the limitations of our study and these additional results in the results and discussion sections (lines 222-226, 282-285, and 502-506).

(4) As indicated by the authors, the association between aceEF mutations and alginate overproduction suggests potential engagement of the itaconate-alginate axis. However, no data are presented to support this link. Direct assessment of itaconate/Irg1 induction by aceEF mutants — and its contribution to pyruvate-mediated tolerance — would clarify whether these mechanisms are independent or synergistic. This is a relevant point by the authors, considered worth of discussion.

We acknowledge that there may be a link between PDHc mutations, alginate production and the itaconate axis. However, in our previous proteomic analysis carried out in synthetic cystic fibrosis medium (SCFM2) (Pedersen et al., 2024, PLOS Biology), only a few proteins were significantly up-regulated (AlgA, AlgF, and MucP) in the PAO1 derived recombinant *aceE* and *aceF* strains. In addition, phenotypic characterization did not reveal the mucoid phenotype characteristic of alginate overproduction, suggesting that PDHc mutations alone are insufficient to drive this axis. While the adapted clinical strains previously analyzed show increased expression of alginate-related pathways (Pedersen et al., 2024, PLOS Biology), their complex mutational backgrounds prevent direct attribution of this phenotype to PDHc mutations alone.

We have presented the limitation of our work including the molecular aspects of the pyruvate/alginate/itaconate axis in the discussion section of the manuscript (lines 481-484 and 502-506).

(5) Loss or remodeling of surface LPS, often through mutations in lptD or downregulation of LPS biosynthetic clusters, represents a major adaptive strategy promoting tolerance in CF P. aeruginosa — most notably promoted by host immunometabolic cues. Despite the frequent use of LPS as a control, these pathways were not examined in aceEF mutants, which weakens the mechanistic conclusions of how tolerance is achieved.

We agree with the reviewer that loss or remodeling of LPS is a well-described adaptive mechanism in clinical isolates during chronic CF infection. However, our previous proteomic analysis of PAO1 derived recombinant *aceE* and *aceF* strains in SCFM2 medium (Pedersen et al., 2024, PLOS Biology) does not indicate clear downregulation or remodeling of the LPS, O-antigen, or Lpt transport system caused by these mutations. The only changes observed were increases in Wzt, ArnB, and AlgA and a decrease in LpxO1 expression, which do not constitute either an enriched pathway or a canonical LPS remodeling pattern. These data suggest that LPS remodeling is unlikely to be the primary mechanism underlying the phenotypes associated with PDHc mutations in our system, even though changes in proteome composition and LPS remodeling may occur during infection, driven by immunometabolic cues.

We have clarified this point in the revised manuscript.

(6) Figure 1A interpretation: If aceF mutant strains secrete pyruvate, one would expect them to

exhibit enhanced resistance to H2O2 even in the absence of exogenous pyruvate. However, both WT and mutant strains show a comparable ~50% reduction in CFU — despite initial CFU differences — which appears inconsistent with the stated premise.

We agree with the reviewer that even in the *aceF* strain one would expect increased resistance to H_2O_2 without the addition of exogenous pyruvate. However, at 6 hours post-infection (when the H_2O_2 killing assay in ALI infections was carried out), no pyruvate was detected on the apical side of the ALI cultures, explaining the absence of increased resistance in *aceF* mutants. Due to the fast dynamics of metabolic processes, this may be due to limited pyruvate secretion within this timeframe at a level within the detection limit, or a rapid assimilation by epithelial cells. Nevertheless, we have quantified pyruvate disappearance from ALI models when spiked on the apical side, supporting that pyruvate, if produced by *aceEF* mutants, can be readily uptaken.

We have specifically addressed this point, indicating these possibilities in the results section (lines 130-136 and 222-226). Additionally, we have included the pyruvate quantification data in Supplementary Figure 1A and 1F and in the source data file.

(7) Although acetate is mentioned as byproduct of the antioxidant mechanism, its contribution to disease tolerance remains clear. By recovering aceF mutant growth, acetate would increase bacterial biomass and thereby enable abundant LPS availability, triggering inflammation and compromising the tolerant milieu. This mechanism should be better explained.

We agree with the reviewer and have included this point in the discussion and clarified its potential contribution to bacterial metabolism and host response (lines 446-451).

(8) Although an exciting hypothesis, authors did not provide evidence that pyruvate acts as a universal mechanism of tolerance to infection by different pathogens. There are no functional studies testing immunomodulation with other organisms than P. aeruginosa.

We agree with the reviewer that our comparative analysis of WGS data is limited to the presence of high-impact mutations in the PDHc in other bacterial species than *P. aeruginosa*. However, it provides preliminary evidence of convergent evolution which requires functional validation. We have expanded our bioinformatic analysis comparing the functional domains in *P. aeruginosa* with those in other species, which showed that mutations localize in the same functional domain regardless of the species suggesting similar metabolic outcomes. Additionally, we have requested clinical isolates of *Haemophilus influenzae* harboring PDHc mutations identified in our analysis from other international research groups, however, the strains could not be shared.

We have specifically indicated the limitation of our analysis in the results and discussion (lines 366-391 and 506-515).

Minor comments.
— Page 11, lines 262-263. "However, when assessing pyruvate secretion by the aceE and aceF strain under the infection conditions, we found that no pyruvate was produced within the short time frame of the experiment". There is no evidence provided validating this.

We have included the pyruvate quantification data in Supplementary Figure 1 and in the source data file to support this statement. We also discuss two possible explanations of why we could not quantify pyruvate under the used experimental conditions. Additionally, we quantified pyruvate assimilation by LPS-stimulated macrophages in the presence of pyruvate, confirming that pyruvate disappears from the medium, indicating cellular uptake (lines 277-288).

— Fig. 4C: pyruvate reassimilation can also be extracellular catabolism to another product. This is not considered as hypothesis.

We agree with the reviewer, and we have indicated this possibility in the results section. Now we refer to the observed pattern as "transient secretion" to better reflect these dynamics (lines 344-347).

Reviewer #2 (Remarks to the Author):
In this study, Lampadariou et al. investigate the biological significance of mutations arising in the bacterial genes *aceE* and *aceF*, which encode components of the pyruvate dehydrogenase complex, proposing that these mutations represent host-driven adaptations that promote immunosuppression and bacterial persistence. This is an interesting and potentially impactful concept. The authors show that although these mutations initially reduce bacterial fitness, they ultimately confer a persistence advantage by increasing pyruvate accumulation and secretion, which is proposed to mitigate oxidative stress and suppress host proinflammatory cytokine production. However, several conclusions are overstated or insufficiently supported by the data, and additional clarification and rigor are required. My specific concerns are outlined below.

We thank the reviewer for their interest in our manuscript and for the critiques, which we appreciate and address below.

1. The use of the ALI model of human airway epithelial cells is well appreciated. However, clarification is needed regarding several aspects of the experimental design.
a. What is the rationale for the 6-hour incubation period prior to H_2O_2 treatment? Given the distinct growth kinetics of WT PAO1 compared with the *aceE* and aceF mutants—as noted by the authors themselves—it is likely that PAO1 reaches higher bacterial burdens during this period. This is expected to result in increased planktonic and surface-associated PAO1 compared to the mutants. Were growth curves for PAO1 and the *aceE/aceF* mutants performed under ALI conditions used in these experiments? In fact, Fig. 1A shows higher PAO1 burdens prior to treatment, and the ratio of PAO1 surviving after versus before H_2O_2 treatment appears comparable to that of the aceF mutant, which is unexpected. This observation seems inconsistent with the conclusions drawn from Fig. 1D and requires further explanation.

As indicated in the text, we use a 6-hour incubation period prior to the addition of H_2O_2 because a small bacterial population is required to evaluate the neutralizing potential of pyruvate. This time point has also been used in previous publications (Laborda et al., 2024, Nat Commun; Goltermann et al., 2024, Nat Commun) as the pre-treatment time point before antibiotic addition. Therefore, we applied the same approach and protocol for the H_2O_2 assay. As pointed out by the reviewer, there is a difference in growth kinetics between PAO1 and the *aceE* and *aceF* strains in ALI models, and indeed lower bacterial counts were observed for the *aceF* strain. Nevertheless, since pyruvate reacts non-enzymatically with H_2O_2, the protective effect occurs independently of the strain employed and bacterial count. Thus, rather than comparing PAO1 with the *aceF* strains, the conditions with and without pyruvate within each strain should be compared. We did not perform detailed infection timelines (growth curves) in ALI models, as the aim of the assay was to evaluate the chemical neutralization of H_2O_2 by pyruvate rather than bacterial growth dynamics. The reason why the survival rates appear comparable between the two strains is that, because pyruvate was not detected on the apical side of the ALI models in the *aceF* strain (see explanation below), we added exogenous pyruvate in both assays. Therefore, the assays shown in Fig. 1A and Fig. 1D are not directly comparable.

We have provided an extended explanation and clarification in the results section and added an additional supplementary figure (Figure S1A and S1F) with the pyruvate quantification (lines 130-136 and 222-226).

b. How much pyruvate is secreted by the aceF mutant under ALI conditions? Was this measured directly, and if so, does this explain the need to add 10 mM exogenous pyruvate? If exogenous supplementation is required, this suggests that endogenous pyruvate levels produced by the aceF mutant are insufficient to confer protection against H_2O_2-mediated killing. In this context, the statements in lines 152–153 appear overstated and should be revised.

We measured pyruvate secretion during PAO1, *aceE*, and *aceF* infections in ALI at both 6 and 16 hours. However, the concentrations were below the detection limit of the assay used (Pyruvate Assay Kit, Sigma-Aldrich). Due to the fast dynamics of metabolic processes, this likely reflects secreted concentrations below the detection threshold of the kit or rapid uptake by epithelial cells. Nevertheless, the reduced IL-8 secretion observed during *aceF* infections, the reduced IL-8 secretion in the presence of LPS plus pyruvate, and the assimilation of pyruvate by epithelial cells (see fig S1F) is consistent with pyruvate being secreted and assimilated by epithelial cells. Importantly, in macrophage assays both *aceE* and *aceF* strains show higher survival than PAO1, indicating efficient neutralization of H_2O_2 within the phagolysosome by pyruvate.

We have incorporated these possible interpretations into the results section and have toned down the original statement accordingly (lines 130-136 and 160-161). Additionally, we have added four additional supplementary figures (Supplementary Figure 1 A, F, H, I) with the pyruvate quantification from both ALI and macrophage infections.

2. Fig. 2. Did the authors measure pyruvate levels under the conditions of the ALI experiment? Additionally, were acetyl-CoA levels assessed, and if so, were there differences between strains?

As indicated above, we did not manage to reliably quantify pyruvate in ALI infection models. This could indicate that pyruvate is secreted by the *aceE*, and *aceF* mutants at a concentration below the detection threshold of the kit, or that it is rapidly uptaken by epithelial cells or macrophages. We did not quantify acetyl-CoA levels either in the ALI infection models nor the bacterial intracellular one.

We have indicated these possibilities in the result section (lines 130-136).

3. The manuscript frequently refers to host–pathogen metabolic crosstalk; however, key mechanistic questions remain unaddressed. Have the authors determined whether pyruvate secreted by the aceF mutant is taken up by host cells and metabolized via host pyruvate dehydrogenase (PDH)? This point is particularly important given that in some assays the authors report that "no pyruvate was produced" (lines 262–263). Is there evidence that host PDH activity or expression is altered (increased) in response to infection? Moreover, is the observed immunosuppressive effect mediated by pyruvate itself, or by downstream metabolites such as acetyl-CoA, potentially through epigenetic mechanisms (e.g., acetylation)? Despite repeated references to "metabolic reprogramming," no direct assessment of host metabolic changes is presented, and the mechanistic basis for selective suppression of certain cytokines remains unclear.

We agree with the reviewer that the molecular mechanisms underlying the metabolic crosstalk and reprogramming in both the ALI and macrophage models remain incompletely addressed. As also

noted by reviewer 1, several mechanisms could be involved in the modulation of epithelial cells and macrophages, including epigenetics and acetylation. A detailed investigation of these mechanisms is beyond the scope of the present work and would require an in-depth multi-omics approach to elucidate the specific role of pyruvate. However, to support the existence of metabolic crosstalk, we quantified pyruvate assimilation in both the ALI and macrophage models. In both systems, exogenous pyruvate was assimilated by the cells, confirming that this metabolite is readily internalized when available. Regarding why we were unable to quantify pyruvate secretion by the *aceE* and *aceF* mutant strains, several explanations are possible: pyruvate concentrations may have been below the detection limit, or pyruvate may have been rapidly assimilated by the host cells as it was produced. Nevertheless, altogether our results support a central role for pyruvate during infection.

We have incorporated this information into the results section (lines 222-226, 282-288).

4. To more rigorously demonstrate that pyruvate secreted by the *aceF* mutant drives host immunosuppression, inclusion of a clean deletion mutant and a complemented strain would be important.

We acknowledge that a clean deletion mutant would provide additional confirmation of the PDHc-associated phenotypes. However, beyond its metabolic role, the PDHc also has other functions that would be lost in a full deletion mutant (Pfuger-Grau et al., 2011 BBA; Hallstrom et al., 2015, PLOS One; Glasser et al., 2017 JBC). For this reason, we introduced clinically relevant point mutations in the PAO1 background instead. In our previously published study (Pedersen et al., 2024, PLOS Biology), we complemented the *aceE* strain by inserting a single chromosomal copy under its native promoter at the Tn7 site, thereby avoiding changes in gene dosage or overexpression artefacts. This strategy could not be applied to the *aceF* mutant, as *aceE* and *aceF* form an operon. Nevertheless, we used the complemented *aceE(rev)* strain in macrophage infection assays, which showed a 20% decrease in intracellular survival compared to the *aceE* mutant strain. Considering that the complemented strain carries both a functional and a non-functional PDHc, this reduction is biologically and statistically significant. These data have now been added to the results section (lines 269-273) and to supplementary figure 1F. Additionally, we quantified the intracellular survival capacity of two independent pairs of clinical isolates from the same lineage, collected within months of each other and differing by the presence or absence of PDHc mutations, which confirmed the increased survival of the mutant clinical strains compared with the isolates carrying wild type *aceEF* genes (lines 351-365; Fig. 4D).

5. While the use of human cell–derived ALI infection models is appreciated, it remains unclear how these findings translate to established in vivo mouse models of pneumonia, where recruited immune cells play a critical role.

We agree with the reviewer that in vivo validation using mouse models of pneumonia would provide additional relevance to our study. However, as noted by Reviewer #3, our discussion of disease tolerance, which we agree with the reviewer requires a whole organism to be fully demonstrated, was more speculative than our data and experimental approach could provide. We acknowledge our oversight in not making this clearer in the manuscript and in not providing a clearer perspective on the persistence phenotype that guided our experimental approach. Recently, Zhou et al. (2025, Cell Reports) reported that pyruvate secretion caused by *gavR* mutations enhances local

persistence without systemic spread in mice. We believe that adding a mouse experiment in our work would likely be confirmatory rather than change our in vitro interpretation. For these reasons, and in agreement with the editor, we have reframed the manuscript around the persistence mechanism, leaving disease tolerance as an untested hypothesis. Importantly, focusing on bacterial persistence has strengthened the manuscript narrative and its conclusions.

We have presented this limitation in the discussion of the manuscript (lines 496-502).

6. Finally, the authors make an interesting observation regarding the occurrence of ace gene mutations in other bacterial species, suggesting a potentially conserved persistence mechanism. However, this claim is not experimentally tested, as none of these additional species are examined in the presented models.

We agree with the reviewer that our comparative analysis of WGS data is limited to the presence of high-impact mutations in the PDHc in other bacterial species than *P. aeruginosa*. However, it provides preliminary evidence of convergent evolution which requires functional validation. We have expanded our bioinformatic analysis comparing the functional domains in *P. aeruginosa* with those in other species, which showed that mutations localize in the same functional domain regardless of the species, suggesting similar metabolic outcomes. Additionally, we have requested clinical isolates of *Haemophilus influenzae* harboring PDHc mutations identified in our analysis from other international research groups, however, the strains could not be shared.

We have specifically indicated the limitation of our analysis in the results and discussion (lines 366-391 and 506-515).

Reviewer #3 (Remarks to the Author):
Summary: In this in vitro study, the authors investigated how increased pyruvate secretion conferred by mutations in pyruvate dehydrogenase complex genes (PDHc) alter the interactions between respiratory bacterial pathogens (particularly Pseudomonas aeruginosa) and host cells. Using a combination of bacterial and mammalian cell culture methods, the investigators show that the addition of exogenous pyruvate mitigates the effects of a key reactive oxygen species, hydrogen peroxide, on bacteria (by protecting from killing and growth arrest) and on host cells (by decreasing provoked inflammation). Building on their previous observation that P. aeruginosa strains with engineered mutations in the PDHc genes aceE and aceF produce relatively high extracellular concentrations of pyruvate, the authors found that these strains had altered survival (compared with the parent strain, PAO1) in both a medium that mimics the cystic fibrosis lung environment (SCFM) and in an air-liquid interface (ALI) cell culture model, although these differences were in opposite directions in these two models. The mutants also induced cultured lung epithelial cell cytotoxicity and cytokine production differently than the parent strain. The authors identified and characterized P. aeruginosa clinical isolates with mutations in either aceE or aceF and found ranges of growth rate and pyruvate secretion that were not obviously related to the type or location of the mutations. Finally, the authors found high-impact mutations in pyruvate dehydrogenase genes in archived sequencing data from other common respiratory pathogen isolates (Haemophilus influenzae, Staphylococcus aureus, and Stenotrophomonas maltophilia), many of which appeared to have a clinical origin. The authors conclude that increased pyruvate secretion conferred by mutations in PHDc genes may help bacteria persist in airway infections, where cytotoxicity to the epithelium and inflammation is decreased but the bacterial load is maintained.

Critique: This manuscript describes novel observations regarding the impact of mutations observed in clinical bacterial isolates on persistence and pathogenicity, at least in in vitro models. The text was generally well-written, authors used appropriate methods that they explained well, performed well-designed experiments, and displayed their results in largely easy-to-understand figures. The topic is likely of interest to the readership of this journal. Despite these positives, we feel that the manuscript would greatly benefit from additional clarification or explanation in key portions. We also found some of the conclusions and interpretations of the authors' findings to not be fully supported by the data as presented; for example, we felt that the analysis of genomic data from H. influenzae, S. aureus, and S. maltophilia did not definitively demonstrate functionally-important mutations in the PDHc similar to their observations with P. aeruginosa, with no analysis (either in silico or in vitro) of PDHc function and/or pyruvate secretion to distinguish from silent, natural variation in coding. It was also unclear whether these isolates came from chronically infected or colonized tissues. In other places, we felt that the limitations of models used (such as epithelial cell cultures or immune cell lines) precluded the interpretations presented (such as inferring immune cell recruitment when this was not explicitly tested). We also would welcome clarification of the rationale and interpretation of differences in experimental conditions (such as reagent concentrations) among otherwise similar experiments. These are further discussed below in addition to various minor points. Therefore, we felt that this manuscript would greatly benefit from revisions before it is ready for publication.

We thank the reviewer for the positive and thorough evaluation of our manuscript and for the critiques raised.

Major points

1) Introduction, page 4, lines 93-98: This section includes both observations made in this work (the effects of pyruvate on IL-8 secretion, for example), some from other work (it reacts non-enzymatically with ROS such as H2O2, which was not investigated here), and speculation ("...leading to disease tolerance by limiting airway inflammation). There was no investigation of either whole airways, multi-organ tissues, or animals here, which seemed to be required to make such a statement here, even if the cell-based in vitro data support such a model. Similarly, the statement on line 101-102 that "these results demonstrate that persisting bacteria within the host selects for PDHc mutations to influence disease progression..." seemed entirely speculative and beyond what the authors both could and did observe with their experimental design. This seemed to be more of a hypothesis to be tested (and we don't think the authors meant that persistent bacteria select for PDHc mutations, but rather that the host environment selects for these mutations in the bacteria, line 102).

We agree with the reviewer that whole airways, multi-organ tissues, or animal experiments would provide additional validation of the disease tolerance concept. Indeed, as correctly noted by the reviewer, our discussion of disease tolerance, which we agree requires a whole organism to be fully demonstrated, was more speculative than our data and experimental approach could provide. We acknowledge our oversight in not making this clearer in the manuscript and in not providing a clearer perspective on the persistence phenotype that guided our experimental approach. Recently, Zhou et al. (2025, Cell Reports) reported that pyruvate secretion caused by *gavR* mutations enhances local persistence without systemic spread in mice. We believe that adding a mouse experiment in our work would likely be confirmatory rather than change our in vitro interpretation. For these reasons, and in agreement with the editor, we have reframed the manuscript around the persistence mechanism, leaving disease tolerance as an untested hypothesis. Importantly, focusing on bacterial persistence has strengthened the manuscript narrative and its conclusions.

We have presented this limitation in the discussion of the manuscript (lines 496-502). Additionally, we have toned down the sentence in line 101-102 (lines 100-102) to account for the reviewer's comment, and to present it as a possible hypothesis for the selection of PDHc mutations.

2) Results, page 5, lines 120-122: "This system allows for the investigation of... immune system recruitment during infection." This statement did not seem to be correct. The authors did not study immune cell recruitment, but rather cytokine production without inclusion of any immune cells in their model.

We agree with the reviewer's comment and have modified the text (lines 119-121).

3) Results, Figures 1-3 (H2O2 and pyruvate concentrations): How did the authors choose the concentrations of H2O2 and pyruvate used in Figure 1A, and why do these differ in Figure 1B and 1C? Perhaps reference Figure 1E to rationalize this. Do these differences have any implications for the findings in these different experiments? And while the amount of H2O2 added in Figure 1D was in the methods, it would be helpful to similarly add it to this figure legend as it was elsewhere. Similarly, in Figure 2D, what is the final concentration of pyruvate ("10 µL of 10 mM pyruvate" was added, but we don't know the final concentration, or how this would compare with the concentrations used in Figure 1)? And how was the 5 mM concentration in Figure 3C chosen?

We have now revised the figure legends to explicitly state the concentrations of both H_2O_2 and pyruvate in the relevant panels (lines 239-241).
The differences in pyruvate concentrations across experiments reflect the distinct experimental configurations used in this study. In the macrophage assays, cells were cultured in submerged conditions and pyruvate was therefore added directly to the culture medium at a defined final concentration (5 mM), a concentration commonly used in cellular metabolism studies and within the range reported in the literature for evaluating metabolic effects of extracellular pyruvate. By contrast, in the airway epithelial ALI model, pyruvate was applied apically as a small droplet (10 µL of a 10 mM solution), consistent with the physiological configuration of this system. For clarity, we now report the total amount of pyruvate applied in this condition (100 nmol) in the figure legend.

Importantly, these methodological differences do not affect the interpretation of the results, as each experiment was designed to address a specific aspect of pyruvate's activity (ROS scavenging or host cell modulation) within the constraints of the corresponding experimental system.

4) Results, page 12, Figure 3: In Figure 3A, why do the authors think survival was increased at t0 in the aceF mutant (is there sufficient killing by H2O2 or other intracellular conditions expected by then?). Could it be more accurate to label the y-axis as CFU instead of survival? In Figure 3C, why does there appear to be an elevation in IL-8 induced by pyruvate even in the absence of LPS?

Intramacrophage killing begins within 15–30 minutes after bacterial engulfment, as the newly formed phagosome rapidly undergoes maturation, acidification, and initiation of the oxidative burst. Although we define t0 as the reference time point for the assay, the protocol includes a 1 hour macrophage–bacteria incubation followed by a 1 hour gentamicin treatment to eliminate all extracellular bacteria. The observation that the *aceF* strain shows increased CFUs relative to PAO1 at t0 likely reflects early differences in intracellular survival during the infection steps prior to this time point. We have underlined this in the result section (lines 260-266).

We prefer to retain "survival" (renamed "Intracellular survival") on the y-axis, as this highlights the primary outcome of the assay.

The increased level of IL-8 observed in Fig. 3C, even in the absence of LPS, may be due to the mild acidity of pyruvate, which can acidify the medium and alter the pH, or due to an increase in osmolarity. Macrophages are highly sensitive to such environmental shifts, which could contribute to a baseline level of activation. Importantly, this effect remains clearly distinct from the response induced by LPS stimulation.

5) Results, page 15, Figure 4D: As above, we found it difficult to understand the relationship between the coding variations and clinical relevance of the isolates analyzed when compared with the data on P. aeruginosa (Figures 4A-C). Specifically, how do we know whether the coding variations shown affect pyruvate dehydrogenase activity, or result in changes in pyruvate secretion? And how do we know whether "COPD patient" or "otitis media" or "bloodstream infection" reflects a chronic infection (or, for that matter, how representative is "water" or "horse" of chronic infection isolates), as the authors hypothesize elsewhere that inactivating mutations in the PDHc results from adaptive change over time in P. aeruginosa? In general, we did not feel that this analysis or these data supported the authors' model or conclusions that "… modifications in pyruvate metabolism constitute a recurrent feature of pathogen adaptation during infection." (lines

410-411). To bolster the data the authors, present in Figure 4D, we would like to see additional characterization of the isolates (e.g. pyruvate secretion, impact on growth rate, cytotoxicity, pro-inflammatory activity) or some other analysis that suggests an impairment in PDHc activity which would support the model. Finally, we would like further confirmation that each of the isolates was isolated specifically from a chronic infection or chronically infected tissue and a more detailed explanation of how the clinical nature of the isolate was determined.

We agree with the reviewer that our analysis does not provide experimental validation of the identified mutations. In this study, we reported only high-impact variants, including nonsynonymous mutations, insertions, and deletions, all of which fall within the same functional domains identified in *P. aeruginosa*. This suggests they may have comparable functional consequences, although we cannot confirm any effect on PDHc activity. Because all protein sequences were retrieved from the NCBI database, only limited clinical metadata were available. Although some isolates are indicated as originating from acute infections (bloodstream infection, otitis media), their infection chronicity and evolutionary history cannot be determined from these annotations. We also attempted to obtain clinical isolates of *Haemophilus influenzae* harboring PDHc mutations identified in our analysis from other international research groups, however, the strains could not be shared.

We have now clarified these limitations in the results and discussion sections and have tempered our conclusions, emphasizing that our findings highlight the occurrence of PDHc mutations across pathogens, rather than providing a direct clinical effect (lines 366-391 and 506-515).

Minor points
1) In several places, we felt it would be best to moderate or edit the language of certain results and conclusions. Suggestions are underlined.
a. Line 30: "… with potentially analogous mutations also identified in…"

Changed

b. Lines 101-103: see major point 1, the chronic infection environment may select for PDHc mutations.

Changed

c. Lines 219: "These results suggest that mutations in the aceE and aceF genes may contribute to persistent infections and establish a potential link between pyruvate metabolism, virulence regulation and modulation of the immune system recruitment in P. aeruginosa infections."

Changed

d. Line 330: "additional functionally relevant…" This seemed to imply that the authors knew the coding variations shown were functionally relevant, which they did not show.

We have modified the result section to account for the reviewer's comment and included a more in-depth analysis of the potential effect of the identified mutations.

e. Line 364: "…it neutralizes ROS through…" The authors did not directly demonstrate the scavenging behavior of pyruvate, although this has been demonstrated previously.

We have included the original reference in which it was first demonstrated that pyruvate reacts with H_2O_2.

f. Lines 365-367: "It reduces the host immune response by limiting IL-8 production and, therefore, recruitment of immune cells". As above, the authors did not explicitly test recruitment of immune cells, they only investigated specific cell types (even an ALI culture is reductionist compared with an intact animal or even organ or tissue). It seemed more accurate to state what the authors truly observed (and tested) and then hypothesize what would happen in an infected person.

We agree with the reviewer and have modified the sentence accordingly to be more precise about the tested results and the hypotheses.

g. Lines 385: "…secreted pyruvate not only neutralizes ROS, but also reduces IL-8 secretion…"

Changed

h. Lines 408-411: This sentence does not seem to be accurate given that the authors did not provide evidence that the coding differences in these other bacteria are functionally important. The authors should either remove this sentence or modify their language to be more accurate.

We have removed the sentence and modified the result section to account for the limitations of our study.

2) A number of typos appear throughout the manuscript.
a. Line 33: "which helps the establishment a more tolerogenic infection microenvironment…"

Corrected

b. Line 82: "involved in pathogens protection from H2O2…"

Corrected

c. Lines 95-96: "suppressing immune system cells recruitment. Third, it limits macrophages activation…"

Corrected

d. Line 143: "mutants showed higher survival than PAO1, respectively…" What does respectively refer to?

Corrected

e. Line 153: "…infection environments and in laboratory condition."

Corrected

f. Pluralizing immune cells and cytokines inappropriately. This occurs throughout the manuscript including lines 176, 242, 286, 288, and some Y-axis labels in Figure 3.

Corrected

g. Using hours time point (line 185) or hours infection (lines 208 and 209).

Corrected

h. Lines 246-247: "…primary monocytes-derived macrophages…"

Corrected

i. Line 309: "…all clinical strains secreted high amount of pyruvate…"

Corrected

j. Line 337: "Type of non-synonymous mutations…"

Corrected

k. Line 412: "…indicating that similar effect could…"

Corrected

l. Lines 418-419: "… metabolites with immunomodulatory effect."

Corrected

3) The manuscript mentions several comparisons without specifying what exactly is being compared:
a. Abstract, page 2, lines 32-33: "Elevated extracellular pyruvate…" Compared to what?

We used elevated implying that it is compared to wild type.

b. Introduction, page 4, line 92: "high secretion" Compared with what?

Relative to the absence of mutations.

c. Introduction, page 4, line 98: "…more tolerogenic infection microenvironment" Compared to what? What is tolerating what? What does this mean?

The sentence explains the role of pyruvate relative to its absence.

d. Results, page 8, line 200: the aceF mutant strain triggered significantly lower IL-8 secretion compared to what?

Modified

4) Introduction, page 3, lines 51-53: "Strikingly, the precise and direct connections between…" We found this sentence difficult to understand.

In the sentence, we want to underline that the relationship between specific metabolic pathways, bacterial pathogenicity and the host response is still unclear. Modified

5) Introduction, page 3, line 55: "several" is not necessary here.

Removed

6) Introduction, page 4, line 79: Here, the authors could include that the non-enzymatic reaction between pyruvate and H2O2 forms acetate.

Added

7) Introduction, page 4, lines 86-87: The authors have previously defined disease tolerance in the introduction (lines 62-64), so it is not necessary here.

Removed

8) Consider adding new figure panels and/or modifying existing panels.

a. A graphical representation of the mutations in the aceE and aceF genes for the engineered PAO1 mutants. This could be a stand-alone panel or could be included in Figure 4A with the P. aeruginosa clinical isolates.

We have included in figure 4A the specific representation of the mutations in the recombinant strains.

b. A graphical representation of the ALI cell culture method with labels (apical side/compartment, etc.) and a brief description in the figure caption of where planktonic, attached, and basolateral bacteria were collected from the system. This would be especially helpful for readers that are not familiar with ALI models.

Thank you for the suggestion. We recently published a paper (Colque et al., 2026 *Cell Reports*) comparing clinical isolates and airway cell types, where the ALI system is clearly explained. We have added a reference to this paper in the text to help readers unfamiliar with ALI models.

9) Results, page 5, lines 113-117: Can the authors provide more information about the reduced growth rate and pyruvate secretion of aceF and aceE that has been previously reported? Which studies have reported this, is it only the authors' previous study (citation 24)? Which media were

used to grow the strains, only SCFM, or were there others? Do the authors think the growth medium could substantially impact growth rate and/or pyruvate secretion?

Since we previously performed a full phenotypic characterization of the *aceE* and *aceF* strains under laboratory conditions (Pedersen et al., 2024, PLOS Biology), we prefer not to repeat this information here to avoid redundancy. For the reviewer's reference, in our prior study we used only SCFM2, as it closely mimics the nutritional composition of CF sputum. Importantly, pyruvate secretion is strongly influenced by nutrient composition, since the presence of acetate re-establishes TCA cycle activity and thereby increases growth rate.

10) Results, page 5, lines 122-24: It would be helpful to specify that both the infection and the addition of H2O2 were on the apical side. Currently, only the location of the H2O2 addition is mentioned.

Specified

11) Results, page 5, line 138: Is the growth rate mentioned here the maximum growth rate detected in exponential phase? Is it based on the slope of the growth curve? It may be helpful for the authors to briefly describe how these values were determined (using the QurvE software) in the methods.

The growth rates were calculated during the exponential phase based on the slope of the curve. We have included this information in the Material and Methods section and added the reference for the software used.

12) Results, page 6, lines 140-141: "…while pyruvate restored both parameters…" The addition of pyruvate did not completely restore lag time of aceF (Figure S1B). Here were the authors referring to all strains or just PAO1?

Here, we refer to all strains, as the lag phase was restored in each of them, although not completely in the *aceF* strain.

13) Lines 142-144 (Figure 1D): There are no statistics comparing PAO1 and aceE in Figure 1D, is that because the comparison is non-significant? Thus, "… aceE and aceF mutants showed higher survival than PAO1…" should be altered accordingly. PAO1 supplemented with aceF supernatant shows comparable protection (line 144) to what (aceE or aceF, or both even though the level of protection is different between aceE and aceF)?

We used a one-way ANOVA to compare the different strains. Since the difference between PAO1 and *aceE* is smaller (approximately 2-fold) than the difference observed for *aceF* or PAO1 supplemented with *aceF* supernatant (approximately 3-fold), this comparison is not statistically significant. However, the trend remains biologically relevant. There is no significant difference between PAO1 (supplemented) and either *aceE* or *aceF*, and indeed the p values were > 0.05. We had by mistake indicated the statistical comparison incorrectly, and this has now been corrected.

14) Results, page 6, lines 152-153: It is not clear what the authors mean by "…pyruvate protects… both in in vivo-like infection environments and in laboratory conditions." What specifically is pyruvate protecting against H2O2?

As indicated in the full sentence, "pyruvate protects against H_2O_2 oxidative stress…"

15) Results, page 6, lines 142-144: The authors may want to add a description of the concentration of H2O2 used and for how long the strains were treated either here and/or in the Figure 1D caption.

We have now indicated it in the figure legend.

16) Results, page 6, lines 145-146: The authors could specify here that pyruvate secretion was determined by quantifying the pyruvate levels in the supernatants of each culture at the relevant time points.

We prefer to omit this information from the main results section, since it is already provided in the figure legend.

17) Results, page 6, line 148: The authors may want to include a short explanation of how acetate can complement PDHc mutations here.

Since we have already provided two references for acetate complementation (Pedersen et al., 2024 PLOS Biology and Jeyaseelan et al., 1980 J Gen Microbiol), we prefer not to repeat the same concepts in the main results section.

18) Figure S1C: The authors should specify the concentration of acetate that was used in the figure caption.

Added

19) Results, page 6, line 150: What do the authors mean by "A similar effect was seen in LB medium…"? Is it that there is also a faster growth rate in LB compared to SCFM2, like how there is a faster growth rate in SCFM supplemented with acetate compared to normal SCFM? Are the authors concluding that the increased growth rate is specific to the acetate present in LB, even when the nutrient profiles of SCFM and LB differ greatly?

Yes, the similar effect refers to the increase in growth rate described in the previous sentence. The increased growth rate depends on the presence of acetate, which is converted to acetyl-CoA and re-establishes a functional TCA cycle.

20) Results, page 7, Figure 1B: Figures 1A, C, D, and E describe the effects of pyruvate on P. aeruginosa growth and survival in an ALI model, while 1B presents cytokine production data, apparently in macrophages, rather than the ALI model (mentioned in lines 131-136, but not included in figure caption). Therefore, 1B and its data seemed to be more appropriate for Figure 3, which describes the macrophage model.

Since this section of the results focuses on the neutralizing effect of pyruvate against H_2O_2, we prefer to keep Figure 1B and its data in this section. We have included in the figure legend that the cytokine measurements were obtained from macrophages.

21) Results, page 8, line 183: The authors cite Figure S1B to show that aceF has a reduced growth rate relative to PAO1, shouldn't this be Figure S1A instead?

Corrected

22) Results, page 8, Figure 2A and line 194: The authors may want to explain the TEER fold change in the text in a way that makes it easier for readers to interpret the results (e.g. a fold change below 1 indicates that the barrier integrity has worsened). Also, the authors say "TEER…revealed a lower capacity of the aceF mutant…" but the word "capacity" seemed out of place. The authors identified less damage by aceF but did not seem to explore the full capacity of any of the strains to inflict damage.

We have provided an explanation for the TEER fold-change in the Materials and Methods section. The extent of epithelial damage depends on the virulence of the strain. The *aceF* strain shows a limited damaging capacity (Fold change TEER ~0.9) compared with PAO1 (Fold change TEER ~0.3), which reflects a substantially higher damaging capacity.

23) Results, page 9, line 204: "…such an invasive behavior…" Was this observation from Figure 2B of bacteria in the basolateral compartment necessarily due to active invasion, rather than passive diffusion due to disruption of cell layer integrity as shown in Figure 2A?

We agree with the reviewer that the presence of bacteria on the basolateral side could result from either active invasion or passive diffusion. However, given that the WT PAO1 strain is highly virulent (see Colque et al., 2026, Cell Reports), we believe that in this case the most likely explanation is active invasion.

24) Results, page 9, lines 208-214, Figure 2E: We are not sure what conclusions the authors had drawn from the colonization patterns and staining intensities in this figure.

The conclusions related to the indicated paragraph are drawn from the quantitative data in Fig. 2A–C. Figure 2E qualitatively illustrates the virulence and growth capabilities of the different strains.

25) Results, page 11, line 266: "…pyruvate addition suppressed secretion of both cytokines…" Suppressed does not seem like the right word to use here, especially when the reader is given no information for how the "connecting letters" relate to statistical significance in Figure 3C.

We use the term "suppress" as a synonym for "repress". We have explained the connecting letters statistical significance in the figure legend.

26) Results, page 12, Figure 3: In Figures 3B and 3D, we wondered why is there was no indication that the "mock" measurements were significantly different from the others; as shown, they certainly seemed to be different.

The data presented in Figures 3B and 3D were generated in two independent experiments, and MFI values are therefore shown only for within-experiment comparisons. Because fluorescence

measurements obtained by flow cytometry can vary between experiments, absolute MFI values should not be directly compared across experiments.

27) Results, page 12, Figure 3A: The authors may want to add a simple explanation of how the survival percentages were calculated that are shown at t4. Also, the authors describe t0 in the figure caption of Figure 3A, but not t4. We think it would be beneficial to describe the infection, gentamycin treatment, and incubation timing for t4 as well to help the reader appropriately interpret the results.

We have expanded the explanation provided in the figure legend.

28) Results, page 12, Figure 3B: The finding that no pyruvate was produced, or was detected, during the timeframe of the experiment (Figures 3A and 3B) seemed worth further discussion. How do you explain this finding and why do you think you still saw a difference in intracellular survival in Figure 3A? Given the findings in Figure 3D, why include Figure 3B here?

We have now provided in the text two possible explanations for why we could not detect pyruvate. While low concentrations of pyruvate may be ineffective in the extracellular milieu, even small amounts within the phagolysosome could readily neutralize the H_2O_2 produced. We have included this explanation in the results section. We believe that Figures 3B and 3D illustrate different aspects of pyruvate activity, and therefore we prefer to retain both.

29) Results, page 12, Figure 3C: Instead of asterisks or p-values, this sub-figure had letters to indicate significance that were not defined anyplace we could find. Please either describe this or replace the letters with the same markers as the authors used in other figures.

We have now included an explanation of the meaning of the connecting letters in the figure legend.

30) Results, page 12, Figure 3D: The readers are not given any context to interpret the magnitude of differences observed in IL-8, MCP-1, or TNF-alpha in Figure 3(D); are these biologically relevant changes? Would they be expected to change, for example, neutrophil recruitment or activity? Can the authors provide a reference or some other way to interpret these findings?

Even if modest in magnitude, reductions in cytokine release can still have biologically meaningful consequences in vivo. Our in vitro model does not allow assessment of downstream effects such as neutrophil recruitment or activity, so these results should be interpreted as direct comparisons between the normal inflammatory profile elicited by a virulent PAO1 strain and the dampened response observed when pyruvate is present.

31) Describing mean fluorescence intensity (MFI): The authors describe MFI in the caption of Figure 3 (lines 291-291) but not when MFI is first presented in Figure 1B. Unless Figure 1B gets moved, MFI should be described in the caption of Figure 1B instead, or in addition to, in the caption of Figure 3.

We have now described MFI in Figure 1.

32) Results, page 13, line 303: The authors mention that the P. aeruginosa isolates "…represent diverse clone types…" Can the authors confirm that "DK" represents a clone type and comment on

why there are two "DK12" isolates shown in Figures 4A-C? Or is this not the case because the strains are "…colored by their clone type."? Numerous strains appear to share colors even though they have different DK numbers.

During the revision of the paper, we realized that we had mistakenly color coded some of the isolates incorrectly. The colors and symbols are now clearly indicated. It is correct that "DK" refers to the clone type designation. Two independent mutations were identified in distinct isolates belonging to the DK12 clone type, and these are now clearly distinguished. We have added this information to the figure legend.

33) Results, page 13, line 304: In what way are the 12 isolates representative of the 89 isolates in the collection? Do the 12 feature all the mutations represented in the 89 isolates?

The 12 clinical isolates harbor all 12 distinct and independent high impact mutations identified in the 89 mentioned isolates.

34) Results, page 13, line 308: The authors present the range of growth rates, which should probably have the units (hr-1), of the 12 clinical isolates but do not include the growth rates of the engineered mutants to compare to. This comparison would be helpful for the reader.

As pointed out by the reviewer, the growth rate is indeed indicated as hr^{-1}. The growth rates of the recombinant strains are presented in Figure S1B, so we prefer to present the clinical isolates separately from the recombinant ones.

35) Results, page 13, lines 310-311: The authors say that pyruvate secretion levels were "…comparable to those of the recombinant strains…" but do not provide any values to compare. Again, providing a range of the pyruvate secretion of the clinical isolates and comparing it to the secretion of the recombinant strains would be helpful for the reader.

We have now provided in the text the amount of pyruvate secreted by the recombinant strains, as well as the corresponding figure reference.

36) Results, page 13, lines 313-314: "…characterized by continuous secretion." This observation seemed to involve only 24 hours of measurement, and given that many of these isolates had relatively slow growth rates in 4B, it seems possible that longer incubation times could show these isolates to have similar patterns (with "re-assimilation") to the faster-growing isolates. Also, how do the authors know the patterns of increases followed by decreases reflect re-assimilation, rather than enzymatic degradation?

We agree with the reviewer that, at longer time points, the secretion pattern could become similar across all strains. However, we do not observe a direct correlation between growth rate and pyruvate secretion pattern. We have now included the possibility that the apparent re-assimilation may instead reflect extracellular catabolism into another metabolite, and we have renamed this group as "transient secretion".

37) Results, page 13, lines 317-318: It is not clear what conclusion the authors are making here.

Since the clinical strains were isolated from different patients with distinct immune system activity, we believe that pyruvate secretion may represent a key metabolic crosstalk mechanism across diverse infection scenarios and lineages.

38) Results, page 14, lines 322: The authors mention PDHc subunits 1 and 2 here but don't explicitly link the subunits with the genes aceE and aceF. It would be helpful to explicitly state this to help the reader interpret the data in Figure 4C and Figure S2.

We have provided a more detailed analysis of the PDHc genes *aceE* and *aceF*, including their subunit composition and function.

39) Results, page 15, Figure 4D: This figure was difficult to interpret. What do dark purple, light purple, and white shading mean? Are all of the isolates/strains in Figure 4D from unique people or sources or infections, or might some of the isolates have come from the same source (such as the two cheek skin S. aureus sequences)?

The color code of the alignment is based on the BLOSUM62 matrix, and this information is now indicated in the figure legend. We are unable to verify whether the isolates originate from the same patients. The sequences were retrieved from the NCBI public database, including the associated metadata. In some cases, no peer-reviewed publication was available, so we cannot verify the origin of the samples in all instances.

40) Discussion, page 17, lines 406-407: "…immune modulation is a strategy for…" seems like deterministic language. Do bacteria have strategies? It would be more accurate for the authors to describe what the effects of pyruvate are, rather than implying bacteria use it purposely.

We have modified the text accordingly.

41) Discussion, page 18, line 421: The authors mention that "counteracting" the effects of pyruvate, among other things, may offer a novel strategy for managing chronic infections. Wouldn't counteracting its effects increase inflammation? How would this be advantageous?

We agree with the reviewer that counteracting the effect of pyruvate could potentially increase inflammation. However, blocking its activity may also enhance H_2O_2-mediated killing and macrophage function. Therefore, although counterintuitive, the potential benefits may outweigh the drawbacks.

42) Discussion, page 18, Figure 5: They authors may want to add "model" or "diagram" to the title of this figure. The authors do not fully address their model in the discussion; thus, it was surprising to see that the model started with the T3SS that was only briefly mentioned in lines 389-392 of the discussion. The authors may want to more thoroughly address their complete model in the discussion, so the readers are not surprised when they get to the model figure.

We have provided an extended description of the model, including the left panel and the role of the T3SS, in the discussion.

Reviewer #4 (Remarks to the Author):
Chronic infections in CF patients are characterised by persistent infection and oxidative stress, but the mechanisms of persistence of bacteria are not fully understood. The authors identified a novel persistence mechanism that is mediated by secretion of pyruvate leading to a more tolerogenic environment and promoting bacterial persistence. By framing their work in the context of disease tolerance of CF and bacteria-host crosstalk, the authors make a good argument for why this question is important for current research. The manuscript is of high quality, conceptually strong and well written.

The authors show that secreted pyruvate scavenges reactive oxygen species, suppresses epithelial and macrophage immune activation, and enhances bacterial survival, thereby promoting long-term persistence and a more tolerogenic infection microenvironment. They therefore used a broad spectrum of appropriate experiments and technologies. By doing so, they integrated the results of mutations with ability to induce inflammation, using ALI cultures and macrophages for further deeper characterization. The methods are state-of-the-art. It is logical and well executed.
The results were analysed and interpreted correctly. The evidence supports the authors conclusions.
It is still an open research quesiton.
This study represents a substantial advance in our understanding of chronic bacterial infections. It identifies pyruvate as a previously underappreciated bacterial immunometabolite and provides a clear mechanistic link between metabolic adaptation, immune modulation, and persistence.
The importance of the advance is well aligned with the standards of high-quality journals in infection biology, host–pathogen interactions, and immunometabolism. The work has both mechanistic depth and translational relevance. It will be of interest to researchers in the field of CF, scientists working on the pathogen-immune-host-crosstalk
The manuscript fit together very well. It clearly describe what was done, why it was done and what the results mean. The manuscirpt is written well and I enjoyed reading it.
It still has some mistakes. The readability of the manuscript would benefit from reducing the use of non-standard or less commonly used abbreviations (for example, "pwCF"), especially for readers outside the immediate cystic fibrosis research community.

We thank the reviewer for the positive consideration of our work. We have carefully revised the manuscript to correct the identified errors. We understand that the abbreviation may be confusing for the broader research community, and we have therefore reduced the use of less common abbreviations where possible and ensured that all abbreviations, including "pwCF", are clearly defined upon first use. As "pwCF" is widely used within the field, we have retained it for consistency.

Reviewer #5 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for reviewing the manuscript.

We are grateful for the time and effort dedicated by the reviewers in critically evaluating our work. Their comprehensive feedback has been very useful for shaping the revised version of this manuscript. Our responses to all points raised by the reviewers are detailed below in blue.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):
The authors have addressed several of my initial comments; however, key conceptual and mechanistic concerns remain unresolved. Most notably, the absence of in vivo validation of the proposed mechanism significantly limits the interpretation of the findings, particularly with respect to disease tolerance. Without this level of analysis, it is not possible to determine whether the immunosuppressive effects attributed to pyruvate during P. aeruginosa infection reflect a bona fide tolerance mechanism, or simply a consequence of impaired infection resistance.

This concern is supported by the authors' own data (Figure 3), where exogenous pyruvate — as well as infection with pyruvate-secreting P. aeruginosa strains — results in increased bacterial burden. Such outcomes are more consistent with reduced antimicrobial efficacy via resistance evasion than with a coordinated tolerance program. Notably, this issue was raised by multiple reviewers — where all requested in vivo validation.

The authors' response further underscores this limitation. They cite recent work by Zhou et al. (2025, Cell Reports), which demonstrates that pyruvate secretion driven by gavR mutations enhances local bacterial persistence via evasion of infection resistance. However, rather than strengthening the present study, this comparison diminishes its novelty, and more worringly, point to inadequate data interpretation. Indeed, Zhou et al. already provide in vivo evidence that perturbations in central metabolism (e.g., aceEF inactivation) compromise infection resistance, thereby challenging the interpretation of pyruvate as a driver of tolerance in the current manuscript. In this context, additional in vivo experimentation would not be merely confirmatory — it would be essential to discriminate between resistance failure and tolerance induction.

Although the authors state that they have tempered their interpretation of disease tolerance, this is not consistently reflected in the revised manuscript. The abstract, in particular, continues to advance a strong tolerance-centric narrative, for example:

1) These mutations lead to elevated extracellular pyruvate, which helps establish a more tolerogenic infection microenvironment and favors bacterial persistence.
2) This metabolic crosstalk promotes bacterial persistence while limiting host tissue damage.
3) Our findings reveal pyruvate as a bacterial immunometabolite that mimics host

antioxidant defenses, reshaping the infection niche to favor long-term colonization.

These statements extend beyond the evidence presented and risk conflating persistence with tolerance without sufficient mechanistic support.

Overall, while the updated study addresses an interesting aspect of host-pathogen metabolic crosstalk, substantial revision is still required to clarify its mechanistic claims and to establish genuine conceptual novelty. At present, the work appears to largely reinforce previously published findings rather than provide a definitive advance. It is unclear if such a study can be considered for publication in Nature Communications.

We thank the reviewer for the additional comments and for reiterating the important conceptual distinction between disease tolerance and impaired infection resistance. We agree that disease tolerance cannot be demonstrated in the absence of whole-organism experiments and that distinguishing between tolerance and resistance requires in vivo validation. In fact, we accepted this criticism during the previous revision and substantially reframed the manuscript accordingly. Our intention in the revised version is no longer to present disease tolerance as a demonstrated outcome of PDHc mutations, but rather to focus on the experimentally supported observations of bacterial persistence, pyruvate-mediated host modulation, and host–pathogen metabolic crosstalk. Throughout the revision process, we removed disease tolerance as a central interpretation and now present it only as a possible conceptual framework that would require future in vivo investigation. As requested by multiple reviewers, we deliberately restricted our conclusions to phenomena that can be directly supported by the experimental systems employed.

We nevertheless appreciate the reviewer's concern that certain statements in the Abstract may still be interpreted as implying disease tolerance. To avoid any ambiguity, we have further revised the wording throughout the Abstract, Introduction, and Discussion. In particular, we have removed terminology such as "tolerogenic microenvironment" and replaced broader conceptual interpretations with direct descriptions of the observed experimental outcomes.

Regarding the comparison with Zhou et al. (2025), we view this work as complementary rather than contradictory to our study. While Zhou et al. demonstrate that pyruvate secretion associated with gavR mutations promotes bacterial persistence in vivo, our work investigates a distinct adaptive mechanism involving PDHc mutations and provides mechanistic insight into how pyruvate affects epithelial and macrophage responses. Importantly, we cite Zhou et al. not as evidence for disease tolerance, but rather as independent support for the broader concept that altered pyruvate metabolism can contribute to bacterial persistence during infection.

Finally, we would like to emphasize that the principal conclusion of the revised manuscript is that adaptive mutations in the pyruvate dehydrogenase complex promote pyruvate-associated persistence phenotypes and modulate host-pathogen interactions. We fully agree that determining whether these effects ultimately represent disease tolerance,

impaired resistance, or a combination of both will require dedicated in vivo studies, and we now explicitly identify this as an important direction for future work.

Reviewer #3 (Remarks to the Author):
We appreciate the authors' careful attention to detail in revising their manuscript. In general, we felt the authors were very responsive to the critiques from all reviewers; there remain only a few minor issues that we thought would be worth addressing.

We thank the reviewer for acknowledging our efforts to improve the manuscript in response to their comments.

1) We were confused why pyruvate levels were sometimes presented in OD and in absolute concentration in others. We were confused why, in the revision (lines 131-134 clean copy/151-154 edited copy) the new text states that aceF made no detectable pyruvate after 6 hours, while Figure S1A has a missing 6hr data point for aceE and not aceF. Later, the authors also say that no pyruvate could be quantified but then reference Figure S1H, where it looks like there is pyruvate that can be quantified although it is displayed as an OD measurement instead of an absolute quantification. Can the authors clarify this?

We thank the reviewer for this comment and apologize for the confusion.
Pyruvate was measured using a colorimetric assay. When OD values were at or below the detection limit, reliable conversion to absolute concentrations was not possible. In Figure S1A, the OD values at 6 h (≈0.065) are too close to background to allow quantification. In Figure S1H, OD values are within the readable range of the spectrophotometer but below the lowest standard curve point and were not converted to absolute concentrations. Nevertheless, in all cases no statistically significant differences were observed between the blank and the samples.
We have included this explanation in the figure legend for the figure S1.

2) In line 272 clean/326 edited, we suggest the authors might want to soften "confirming the role of PDHc…" to "suggesting a role of PDHc…". Similarly, "confirm" may be too strong a word for line 364 clean/437 edited.

We acknowledge that the term "confirm" might sound too strong, but those sentences summarize several results presented in the text. We therefore prefer to maintain the sentences as they are.

3) In line 342 clean/409 edited, the authors reference both Figure 4C and D, but it appears as though only Figure 4C should be referenced.

Modified.

4) We found figure 4D difficult to interpret in its current form.
a. Can the authors use the same phrasing from the Figure 3C figure legend to explain the interpretation of the statistical results using letters? We found the explanation for 3C to be clearer than the explanation for 4D.

We have modified the figure legend following the reviewer's comment to enhance clarity.

b. Can the authors add a key within the graph that specifies which strains have PDHc mutations and which do not? The information is in the figure caption, but including it in the graph itself would increase readability.

We have included the specific mutation of the strains in the graph.

5) We are not sure what the authors meant in lines 362-363 clean/435-436 edited by newly-added phrase "the historical contingency of the individual evolutionary trajectories of the strains."

Historical contingency refers to the dependence of the evolutionary history of clinical strains on prior evolutionary events.

6) We recommend that the authors provide a citation in lines 428-429 clean/513-514 edited to support their statement regarding the role of the T3SS in infection and immune recruitment.

Added.

7) We didn't understand what the authors meant in line 433 clean/518 edited by "altering such infection pattern".

We refer to the previous sentence where the wt infection pattern is explained.

8) Line 513 edited: "During infections..., bacteria use the T3SS to damage the host epithelium..." This new phrase seems to imply intent on the part of the bacteria- that they "use" a system for an intended purpose, which we didn't think the authors meant to imply. Similarly, lines 107-108 edited (..."PDHc mutations are selected for...to influence disease progression within the host") seems to imply that bacterial mutations are selected for their influence on disease severity, which also seemed unlikely.

Modified.

9) Lines 97-98 edited: Suggest the authors specify the PDHc mutations are in P aeruginosa, not in the host or other bacteria (apologies for missing this in the first review).

We indicate in the sentence that PDHc mutations are present in several clinical isolates of *P. aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Stenotrophomonas maltophilia*. So, we are not sure about what the reviewer refers to.

Reviewer #5 (Remarks to the Author):
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for reviewing the manuscript.