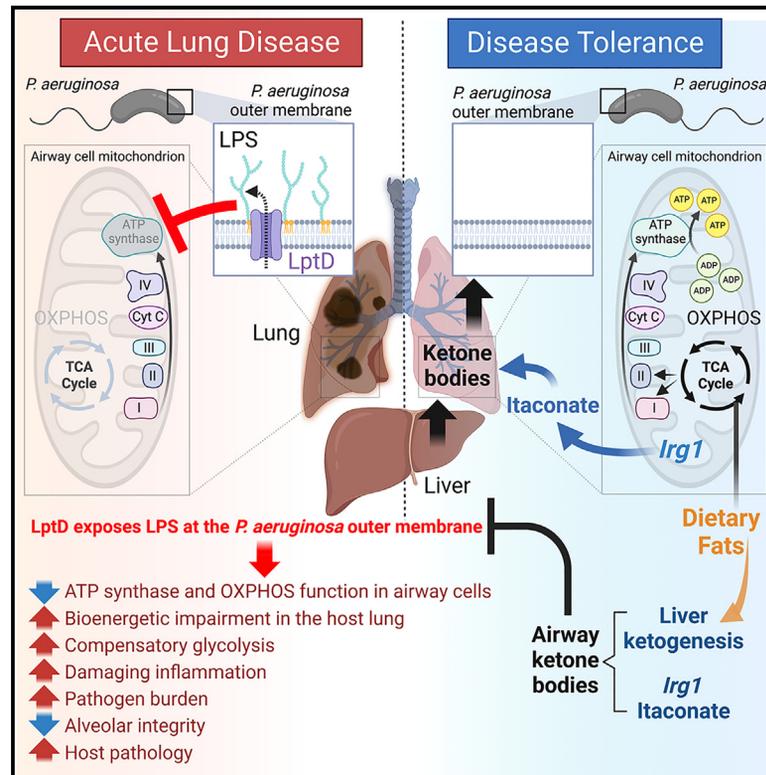


# Cell Metabolism

## Ketogenesis promotes tolerance to *Pseudomonas aeruginosa* pulmonary infection

### Graphical abstract



### Authors

Kira L. Tomlinson, Ying-Tsun Chen, Alex Junker, ..., Robert K. Ernst, Alice Prince, Sebastián A. Riquelme

### Correspondence

sr3302@cumc.columbia.edu

### In brief

Tomlinson et al. report how ketogenesis, a metabolic response to host energy deprivation, promotes disease tolerance during *P. aeruginosa* pneumonia. Ketone bodies select for *P. aeruginosa* strains that are unable to assemble surface LPS, thus preserving the ability of infected airway cells to produce ATP in mitochondria. This favorable microenvironment facilitates host-pathogen coexistence.

### Highlights

- *P. aeruginosa* surface LPS interferes airway cell ATP synthase function
- This bioenergetic impairment triggers detrimental inflammation and alveolar damage
- The host responds to ATP synthase inhibition by upregulating ketogenesis
- Ketone bodies repress *P. aeruginosa* LPS assembly, limiting damaging inflammation



## Article

# Ketogenesis promotes tolerance to *Pseudomonas aeruginosa* pulmonary infection

Kira L. Tomlinson,<sup>1</sup> Ying-Tsun Chen,<sup>1</sup> Alex Junker,<sup>1</sup> AndreaCarola Urso,<sup>1</sup> Tania Wong Fok Lung,<sup>1</sup> Danielle Ahn,<sup>1</sup> Casey E. Hofstaedter,<sup>2</sup> Swikrity U. Baskota,<sup>3</sup> Robert K. Ernst,<sup>2</sup> Alice Prince,<sup>1</sup> and Sebastián A. Riquelme<sup>1,4,\*</sup>

<sup>1</sup>Department of Pediatrics, Columbia University, New York, NY 10032, USA

<sup>2</sup>Department of Microbial Pathogenesis, University of Maryland, Baltimore, MD 21201, USA

<sup>3</sup>Department of Pathology and Cell Biology, Columbia University, New York, NY 10032, USA

<sup>4</sup>Lead contact

\*Correspondence: [sr3302@cumc.columbia.edu](mailto:sr3302@cumc.columbia.edu)

<https://doi.org/10.1016/j.cmet.2023.09.001>

## SUMMARY

*Pseudomonas aeruginosa* is a common cause of pulmonary infection. As a Gram-negative pathogen, it can initiate a brisk and highly destructive inflammatory response; however, most hosts become tolerant to the bacterial burden, developing chronic infection. Using a murine model of pneumonia, we demonstrate that this shift from inflammation to disease tolerance is promoted by ketogenesis. In response to pulmonary infection, ketone bodies are generated in the liver and circulate to the lungs where they impose selection for *P. aeruginosa* strains unable to display surface lipopolysaccharide (LPS). Such keto-adapted LPS strains fail to activate glycolysis and tissue-damaging cytokines and, instead, facilitate mitochondrial catabolism of fats and oxidative phosphorylation (OXPHOS), which maintains airway homeostasis. Within the lung, *P. aeruginosa* exploits the host immunometabolite itaconate to further stimulate ketogenesis. This environment enables host-*P. aeruginosa* coexistence, supporting both pathoadaptive changes in the bacteria and the maintenance of respiratory integrity via OXPHOS.

## INTRODUCTION

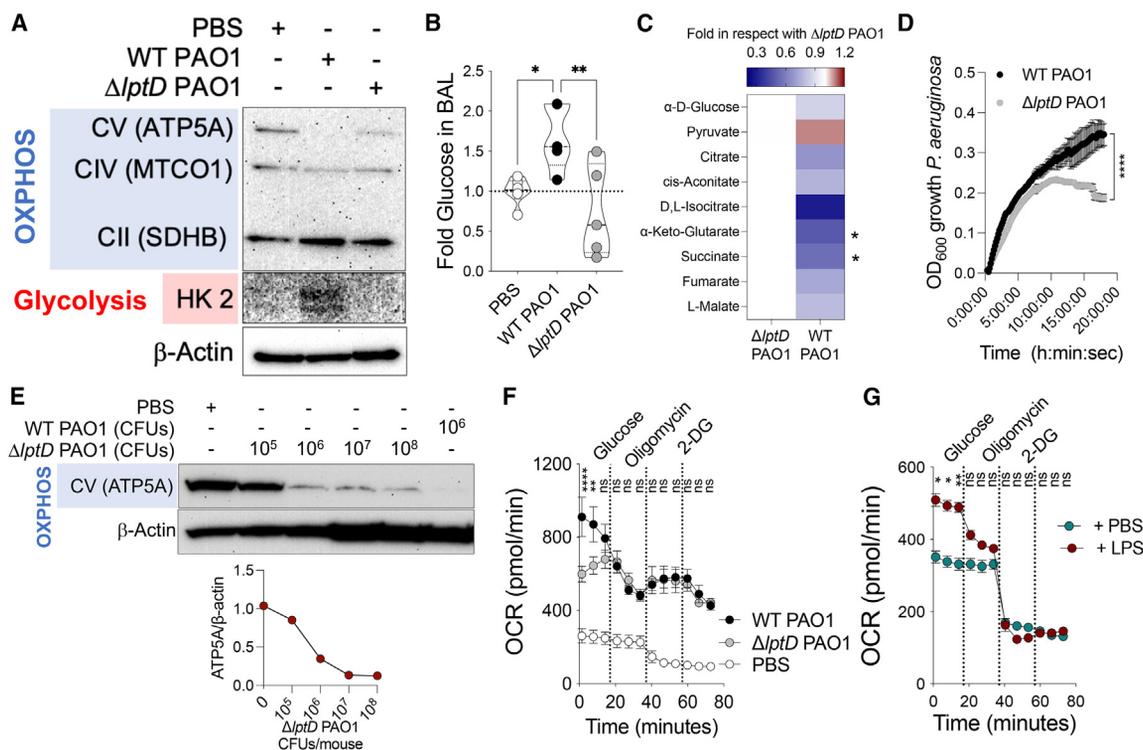
*Pseudomonas aeruginosa* is a feared pulmonary pathogen associated with acute, inflammatory infection resulting in substantial lung destruction caused by its expression of potent toxins and proteases.<sup>1</sup> More commonly, however, it is associated with persistent infection in people with cystic fibrosis (CF),<sup>2,3</sup> bronchiectasis,<sup>4,5</sup> chronic obstructive pulmonary disease<sup>6,7</sup> and primary ciliary dyskinesia,<sup>8,9</sup> subjects without underlying immune dysfunction. These patients develop tolerance to infection, also known as disease tolerance, an evolutionarily conserved defense strategy that controls damage in infected tissues by limiting the inflammatory response against the infectious agent.<sup>10–12</sup> By reducing inflammation, tolerance to infection not only preserves organ function but also enables pathogen coexistence with the host.<sup>10–12</sup> Exactly how the host shifts from an immune response that is highly damaging to one that tolerates bacterial burden in the airway is not well understood.

The physiological processes that normally maintain pulmonary homeostasis are predominantly sustained by oxidative phosphorylation (OXPHOS).<sup>13–15</sup> OXPHOS produces ATP in mitochondria, a process fueled by fats that are normally assimilated from the diet.<sup>16,17</sup> The energy provided by OXPHOS not only supports airway cell viability but also promotes numerous processes essential for normal pulmonary function, such as gas exchange, airway mucus clearance, and surfactant production.<sup>18–22</sup> In response to

an infectious threat, including lipopolysaccharide (LPS) displayed by Gram-negative pathogens such as *P. aeruginosa*, there is a rapid shift from these basal biological activities supported by OXPHOS to glycolysis.<sup>16,17,23–25</sup> This metabolic reprogramming generates inflammatory cytokines to eradicate infection.<sup>23,25</sup> This acute inflammation compromises the integrity of the lung and can be fatal if not regulated.<sup>1,10,11,26,27</sup> However, LPS not only drives local bioenergetic alterations but also systemic host adaptations such as ketogenesis.<sup>28,29</sup> By prompting ketogenesis, hepatic cells redirect fatty acids to produce ketone bodies, which travel to affected organs to stimulate ATP synthesis via OXPHOS.<sup>29,30</sup> By refueling ATP generation, ketone bodies impede tissue necrosis, phagocyte activation, and inflammation.<sup>29</sup> Hosts tolerant of pulmonary infection thus must tightly control the balance between OXPHOS, glycolysis, ketogenesis, and inflammation to safeguard lung integrity.

Bacteria also must adapt to the bioenergetic configuration of the airway. Clinical isolates of *P. aeruginosa* from the lung of tolerant subjects exhibit numerous LPS alterations,<sup>3,31</sup> especially inactivating *lptD* mutations.<sup>32</sup> LptD is a key transporter that enables the display of LPS on the outer membrane of the bacterial surface, and is thus essential for its pro-inflammatory activity.<sup>33,34</sup> Along with changes in cell surface structures, these pathogens readily adapt their own metabolic activity to contribute to the preservation of the local environment.<sup>3,35</sup> In such a favorable milieu, *P. aeruginosa* evolve to persist as discrete communities





**Figure 1. *P. aeruginosa* LPS display inhibits pulmonary ATP synthase**

Mice were treated with PBS, WT PAO1, or  $\Delta lptD$  PAO1.

(A and B) The following was measured: (A) OXPHOS (ATP5A, MTCO1, and SDHB) and glycolysis (HK2) in lung; (B) BAL glucose.

(C) NADH and FADH<sub>2</sub> abundance in WT and  $\Delta lptD$  PAO1 via Biolog plates.

(D) WT and  $\Delta lptD$  PAO1 growth in succinate-rich minimal media.

(E) Lung ATP5A expression in mice exposed to either PBS, WT PAO1, or  $\Delta lptD$  PAO1. ATP5/β-actin ratio is shown.

(F and G) BDMDs oxygen consumption rate (OCR).

Data are shown as average of 2–3 independent experiments ± SEM.

(B)–(C): Student's t test. (D), (F) and (G): two-way ANOVA. \*: p < 0.05; \*\*: p < 0.01; \*\*\*\*: p < 0.0001; ns, non-significant.

See also [Data S1](#).

that engraft within the local microbiome.<sup>2–7,36,37</sup> Adaptive changes observed in *P. aeruginosa* strains from chronically infected patients must synchronize with the bioenergetic setting that conserves pulmonary integrity: OXPHOS.

Here, we establish that tolerance to *P. aeruginosa* lung infection is closely associated with the maintenance of OXPHOS in airway cells. We demonstrate that the infected, tolerant host abrogates glycolysis and the release of airway-damaging cytokines by fueling pulmonary OXPHOS with fat metabolism. Ketogenesis and local immunometabolites in turn drive the selection of *P. aeruginosa* strains with limited display of LPS and enhanced ability to sustain the tolerogenic environment. This milieu not only safeguards respiratory integrity but also promotes pathogen survival, enabling host-*P. aeruginosa* coexistence, which is hallmark of tolerance to infection.

## RESULTS

### *P. aeruginosa* surface LPS drives airway OXPHOS impairment via depletion of ATP synthase

Using a LptD mutant of the laboratory *P. aeruginosa* strain PAO1 unable to display LPS on the surface ( $\Delta lptD$  PAO1),<sup>32,38</sup> we

demonstrated that LPS anchored to the outer membrane induces pulmonary OXPHOS dysfunction. In contrast with mice exposed to  $\Delta lptD$  PAO1 (~10<sup>6</sup> colony-forming units [CFUs]; [Figure S1A](#)), lung protein extracts from animals infected with wild-type (WT) PAO1 exhibited depletion of ATP5A, a key component of ATP synthase (also known as complex V), the OXPHOS machinery that generates ATP<sup>39</sup> ([Figure 1A](#)). We did not observe substantial changes in other mitochondrial complexes involved in OXPHOS, including Sdhb (complex II: succinate dehydrogenase) and Mtco1 (complex IV: cytochrome c oxidase)<sup>40,41</sup> ([Figure 1A](#)). As expected, depletion of ATP synthase in response to *P. aeruginosa* surface LPS correlated with activation of compensatory routes such as glycolysis, illustrated by augmented expression of the pro-glycolytic enzyme hexokinase 2 (HK2)<sup>42</sup> ([Figure 1A](#)) and increased glucose concentration in bronchoalveolar lavage (BAL) fluids ([Figure 1B](#)).

We found that *P. aeruginosa* surface LPS provoked pulmonary depletion of ATP synthase via pathogen burden. Compared with the  $\Delta lptD$  mutant, the WT PAO1 strain more readily consumed the pro-ATP NADH and FADH<sub>2</sub> intermediates that are generated from succinate and its precursor α-ketoglutarate<sup>43</sup> ([Figure 1C](#)). This increased bioenergetic efficiency augmented *P. aeruginosa* biomass generation in succinate ([Figure 1D](#)). To amplify  $\Delta lptD$

PAO1 loads in the host airway, we exposed mice to an increasing inoculum of this strain. At lower doses (e.g.,  $\sim 10^5$  CFUs),  $\Delta lptD$  PAO1 did not provoke substantial depletion of the ATP synthase constituent ATP5A, but at higher inoculum (e.g.,  $> \sim 10^6$  CFUs) it did (Figure 1E).

We confirmed these findings *in vitro*. Bone-marrow-derived macrophages (BMDMs) were exposed to either PBS or a similar inoculum of either WT PAO1 or  $\Delta lptD$  PAO1, and their OXPHOS response was analyzed via Seahorse technology. Compared with BMDMs administered  $\Delta lptD$  PAO1, cells exposed to WT PAO1 had a higher oxygen consumption rate (OCR) (Figure 1F), which is a signature of mitochondrial OXPHOS impairment by LPS stimulation<sup>32,44,45</sup> (Figure 1G). However, when compared with BMDMs treated with PBS, cells exposed to either pathogen elicited significantly higher OCR throughout the course of the assay (Figure 1F), supporting the idea that *P. aeruginosa* LptD causes host OXPHOS interference via both LPS display and bacterial burden.

### ***P. aeruginosa* inhibition of host ATP synthase triggers damaging inflammation**

We confirmed the damaging consequences of *P. aeruginosa* interference with host ATP synthase in a model of acute pneumonia. We treated animals with a sublethal dose of oligomycin ( $\sim 0.2$  mg/kg), which is a specific inhibitor of ATP synthase,<sup>46</sup> prior to infection with  $\sim 10^5$  CFUs of WT PAO1. Oligomycin amplified the accumulation of IL-1 $\beta$  and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (Figure 2A), cytokines associated with tissue damage, as well as increased WT PAO1 burden (Figure 2B). To further demonstrate that blockade of host mitochondrial bioenergetics exacerbates lung inflammatory stress during infection, we blocked fatty acid  $\beta$ -oxidation (FAO), a pathway upstream of OXPHOS that fuels host ATP synthase activity.<sup>47</sup> FAO metabolizes fatty acids that are trafficked into mitochondria by the transporter *Cpt1a*.<sup>47</sup> These imported metabolites are then  $\beta$ -oxidized, generating intermediates that enter the tricarboxylic acid (TCA) cycle to fuel OXPHOS and ATP synthase function. To interfere with host FAO, we treated mice with a specific *Cpt1a* blocker, etomoxir.<sup>48</sup> In contrast with controls, mice treated with etomoxir and infected with WT PAO1 had significantly higher levels of IL-6 and TNF $\alpha$  (Figure 2C) and increased bacterial burden (Figure 2D). Similar findings were observed in animals exposed to  $\Delta lptD$  PAO1 (Figures 2E and 2F), confirming that impairment of pathways that fuel host ATP synthase intensifies airway disease.

We observed that cytokines produced during host ATP synthase interference triggered an inflammatory feedback loop that worsened pulmonary integrity. Compared to *Il1r*<sup>-/-</sup> mice, which are unable to signal through IL-1 $\beta$ , *Il1r*<sup>+/+</sup> animals exposed to WT PAO1 had significantly increased airway levels of IL-6 ( $\sim 2.7$ -fold) and TNF $\alpha$  ( $\sim 1.8$ -fold) (Figure S1B), augmented bacterial burden in BAL ( $\sim 27$ -fold) (Figure S1C), and greater albumin leakage into the airway fluid (Figure S1D), which is an indicator of vascular barrier damage.<sup>49,50</sup> These findings were further supported using WT and  $\Delta lptD$  PAO1 strains (Figures S1E–S1G).

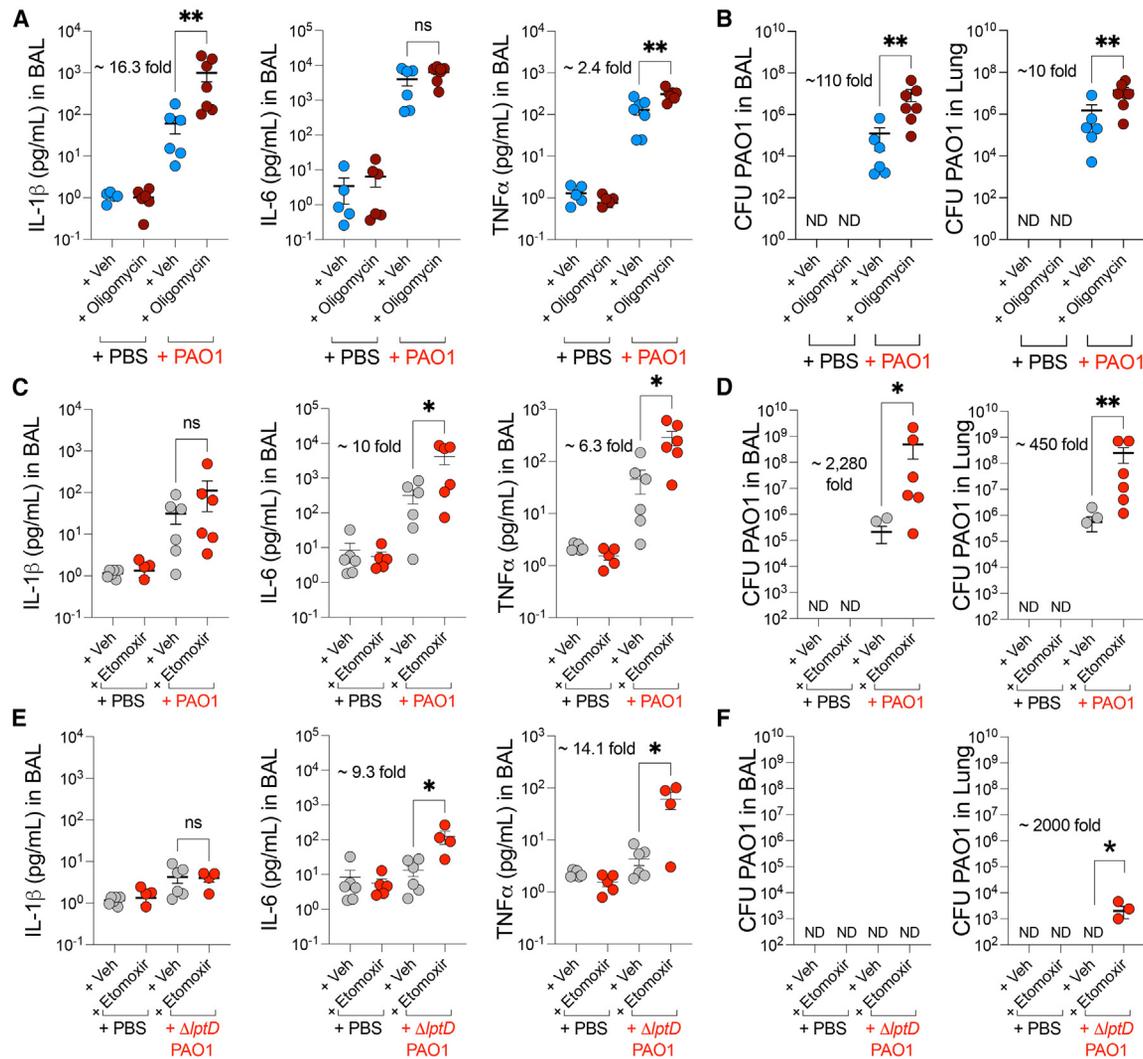
### ***P. aeruginosa* strains from tolerant hosts preserve lung OXPHOS during pneumonia**

We next addressed whether clinical isolates of *P. aeruginosa* from tolerant subjects, in fact, maintain host OXPHOS and if

this is linked to the repression of LptD. We found that  $\sim 85\%$  of *P. aeruginosa* strains from the airways of 13 subjects in the intensive care unit (ICU) exhibited substantially lower mRNA expression of *lptD*, in contrast with WT PAO1 (Figure 3A). Similar findings were observed with longitudinal collections of *P. aeruginosa* isolates from the lungs of two chronically infected individuals with CF (CF#1 and CF#2). Analysis of 14 *P. aeruginosa* strains from CF#1 showed that, at advanced stages of infection, 100% of these pathogens exhibited low *lptD* expression (Figure 3B, left). We validated these observations by examining 17 *P. aeruginosa* strains from CF#2, which showed null *lptD* expression (Figure 3B, right). These data confirmed the association between tolerance to *P. aeruginosa* infection and LptD inhibition.

We next analyzed how *P. aeruginosa* isolates from the lung of tolerant subjects affected pulmonary ATP synthase. Compared with animals exposed to WT PAO1, mice infected with a mixture of the 17 isolates from CF#2 (hereafter referred to as “*P. aeruginosa* isolates”) (Figure 3B, right; Figure S1H) preserved ATP5A expression in the lung tissue (Figure 3C). They did not trigger any changes in mitochondrial complexes II and IV, nor did they increase pro-inflammatory glycolytic metabolism via HK2 (Figure 3C).

To better appreciate how the stress of bacterial infection impacts mitochondrial bioenergetics at the cellular level, we performed single-cell RNA-sequencing (scRNA-seq) of whole lung tissue from animals exposed to either PBS, WT PAO1, or the *P. aeruginosa* isolates. We were more specifically interested in delineating changes in bioenergetics in immune versus stromal cell populations. Initial clustering of our integrated scRNA-seq data identified 23 populations with distinctive transcriptional signatures, including cells in direct contact with the airway lumen, such as alveolar macrophages, type 1 and 2 pneumocytes, ciliated cells, fibroblasts, and vascular and capillary endothelial cells, as well as cells that are not directly exposed to the airway, such as mesothelial cells and smooth muscle cells (Figures 3D, S1I, and S1J; Table S1). We computed OXPHOS scores for every cell using 171 genes associated with the pathway, including ATP synthase and complexes I–IV in the electron transport chain (ETC) (Table S2).<sup>51</sup> We then calculated a  $\Delta$ OXPHOS score compared with uninfected, PBS-treated mice and created volcano plots to visualize how the different *P. aeruginosa* strains affected this bioenergetic pathway in each cell cluster. As anticipated, WT PAO1, but not the *P. aeruginosa* isolates, significantly compromised OXPHOS in key respiratory cells. Although WT PAO1 suppressed OXPHOS in 13 out of the 23 populations identified ( $\sim 57\%$ ) (Figure 3E), the *P. aeruginosa* isolates only caused OXPHOS downregulation in 5 ( $\sim 22\%$ ) (Figure 3F). One of the most highly impacted populations in WT PAO1-infected mice were alveolar macrophages, followed by cell populations required to maintain pulmonary gas exchange and surfactant production, including type 2 pneumocytes, vascular, and capillary endothelial cells<sup>13</sup> (Figure 3E). Conversely, the *P. aeruginosa* isolates did not compromise OXPHOS in these cell subsets (Figure 3F). Instead, the *P. aeruginosa* isolates impaired OXPHOS in recruited phagocytes ( $\sim 60\%$  of cells affected), including peripheral blood (PB) neutrophils, granulocytes, and regulatory myeloid cells, indicating that the



**Figure 2. Host OXPHOS impairment worsens lung inflammation and *P. aeruginosa* burden**

(A and B) Mice were administered either vehicle or oligomycin (ATP synthase inhibitor) and exposed to PBS or WT PAO1. The following were analyzed: (A) BAL cytokines and (B) bacterial burden in BAL and lung. Animals were treated with either vehicle or etomoxir (FAO blocker) and exposed to PBS, WT PAO1, or  $\Delta ptD$  PAO1.

(C–F) The following were examined: (C) BAL cytokines for WT PAO1; (D) bacterial burden in BAL and lung for WT PAO1; (E) BAL cytokines for  $\Delta ptD$  PAO1; and (F) bacterial burden in BAL and lung for  $\Delta ptD$  PAO1.

Data are shown as average of 2–3 independent experiments  $\pm$  SEM, 4–7 mice in total.

(A)–(F): one-way ANOVA. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; ns, non-significant.

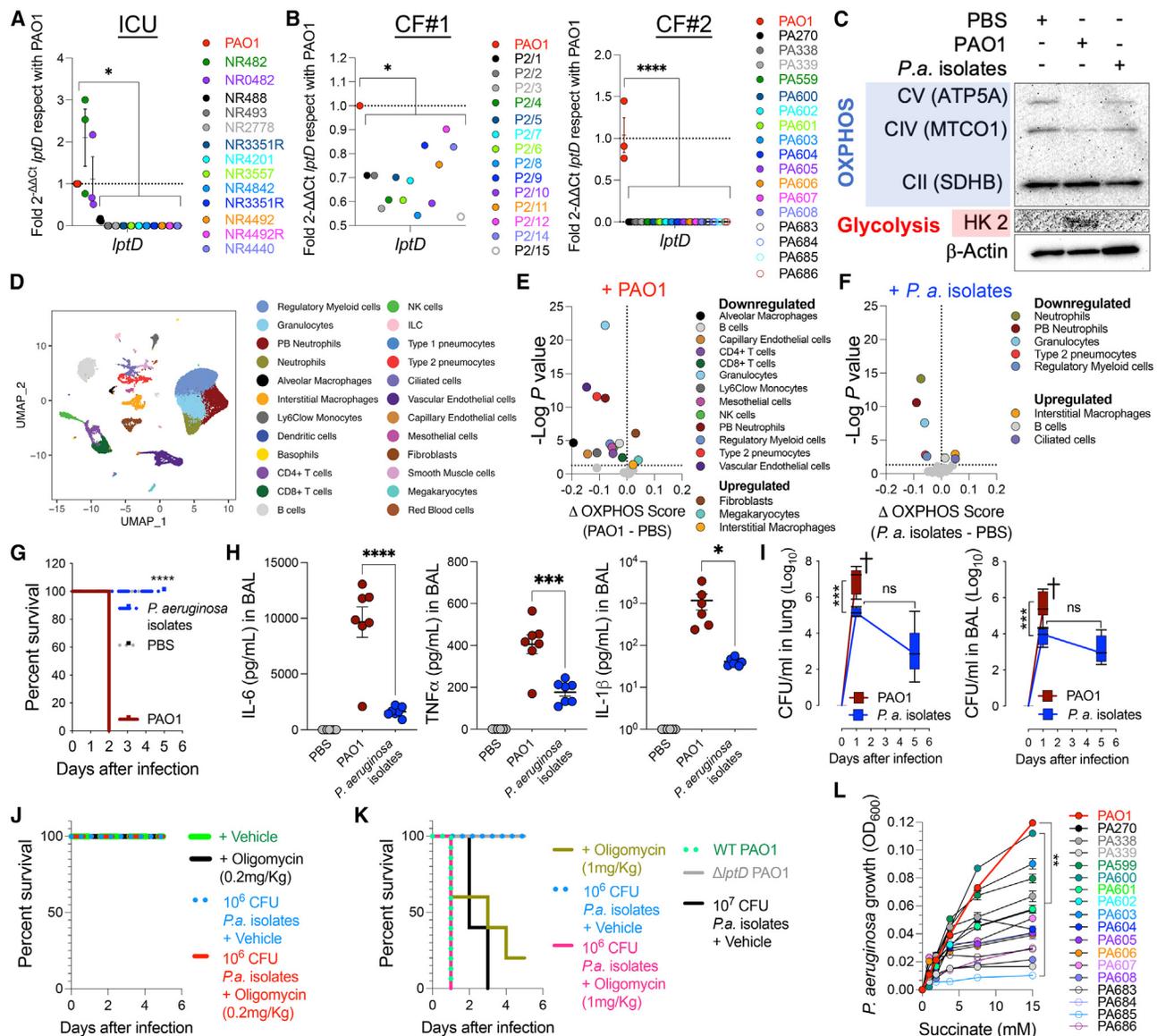
See also [Figure S1](#) and [Data S1](#).

*P. aeruginosa* isolates preserve the bioenergetic homeostasis of resident respiratory cells.

### Preservation of host ATP synthase function enables disease tolerance

Our findings thus far indicated that the host responded to infection by the *P. aeruginosa* isolates with disease tolerance<sup>10–12,52</sup>; in contrast with WT PAO1, the clinical strains were not lethal (Figure 3G), provoked reduced inflammation (Figure 3H), restricted hypothermia (Figure S2A), and limited vascular dysfunction (Figure S2B). The isolates exhibited ~10-fold lower pathogen burden than WT PAO1 after 24 h, which correlated with prolonged host-pathogen coexistence (Figures 3I and 3G).

We hypothesized that the tolerogenic response to infection by the *P. aeruginosa* isolates was enabled by preservation of ATP synthase function. Compared with mice that were infected with the clinical strains and treated with a sublethal dose of the ATP synthase inhibitor oligomycin (~0.2 mg/kg) (Figure 3J), hosts infected with the same inoculum but treated with a higher dose of oligomycin (~1.0 mg/kg) rapidly developed disease, including hypothermia (Figure S2C) and death (Figure 3K), indicating that it is critical for the host to conserve functional OXPHOS during infection with these clinical pathogens. The indolent infection produced by the *P. aeruginosa* isolates more closely resembled the disease caused by  $\Delta ptD$  PAO1 (Figure 3K). Indeed, and as observed



**Figure 3. *P. aeruginosa* isolates preserve airway OXPHOS and enable host-pathogen coexistence**

(A and B) *LptD* mRNA levels in *P. aeruginosa* strains from: (A) 13 patients in ICU and (B) tolerant people with CF. CF#1: 14 strains; CF#2: 17 strains. Animals were exposed to PBS, WT PAO1, or a mixture of the 17 *P. aeruginosa* isolates from CF#2.

(C–I) The following were analyzed: (C) OXPHOS (ATP5A, MTCO1, SDHB) and glycolysis (HK2) in lung; (D) pulmonary cell subsets identified by scRNA-seq; (E) and (F)  $\Delta$ OXPHOS transcriptomic score per lung population in response to each pathogen; (G) mouse survival; (H) BAL cytokines; and (I) bacterial burden in lung and BAL (black cross indicates all animals are dead).

(J and K) Survival of animals exposed to either  $\sim 10^6$  or  $\sim 10^7$  CFUs of the *P. aeruginosa* isolates and then treated with either a sublethal (J) or lethal dose (K) of oligomycin. WT and  $\Delta$ *lptD* PAO1 were also studied.

(L) Bacterial endpoint growth in increasing succinate.

Data are shown as average  $\pm$  SEM.

(C)–(I): 2–3 independent experiments, with 3–7 animals in total.

(J) and (K): 2 independent experiments, with 5 mice in total. (I) and (L): two-way ANOVA; (G), (J), and (K): Kaplan-Meier; (H): one-way ANOVA. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; \*\*\*\*:  $p < 0.0001$ ; ns, non-significant.

See also [Figures S1 and S2](#), [Tables S1 and S2](#), and [Data S1](#).

with this mutant (Figure 1D), the clinical strains were not as proficient at growth as WT PAO1 (Figure 3L), suggesting that tolerance to these organisms might be linked to reduced pathogen burden. When we increased the pathogen biomass in our *in vivo* model by administering a 10-fold higher

inoculum of the *P. aeruginosa* isolates ( $\sim 10^7$  CFUs), we observed more disease, illustrated by hypothermia and death (Figures S2D and 3K). Thus, the tolerogenic response to the *P. aeruginosa* isolates is supported by maintenance of host ATP synthase.



### ***P. aeruginosa* isolates facilitate generation of energy in airway cells via catabolism of dietary fats**

We postulated that to stimulate pulmonary ATP synthase and thus mitigate airway inflammation, the *P. aeruginosa* isolates would enable host catabolism of fats. Fats are typically assimilated from the diet and are used to fuel OXPHOS.<sup>16,17</sup> During infection by the clinical pathogens, host catabolism of dietary fats would prevent the mobilization of glucose into the lung, restricting the synthesis of cytokines that rely on glycolysis. The opposite would occur during WT PAO1 infection, which depletes pulmonary ATP synthase; dietary fats would be unable to fuel energy production in pulmonary mitochondria, prompting compensatory glycolysis and cytokine release.

To stimulate OXPHOS via diet, we fed mice *ad libitum* for 1 week with a fatty acid-rich, carbohydrate-low diet (fatty acid: ~89%; carbohydrates: ~0.1%; protein: ~10%). As control, we used a fatty acid-low, carbohydrate-rich intake (fatty acid: ~10%; carbohydrates: ~80%; protein: ~10%), which induced similar animal weight as the fatty acid-rich diet (Figure S3A). We then exposed these mice to either WT PAO1 or the *P. aeruginosa* isolates. As predicted, the levels of airway glucose in fatty acid-fed hosts varied with the ability of the pathogen to preserve host ATP synthase: during WT PAO1 infection, BAL carbohydrate levels were significantly increased, whereas the opposite was observed during infection with the *P. aeruginosa* isolates (Figure 4A). The differences in airway glucose abundance were not due to variations in pathogen burden (Figures 4B and 4C) or glycemia (Figure 4D), confirming that airway glucose concentrations depend on the ability of *P. aeruginosa* to preserve host OXPHOS.

Increased airway glucose levels in mice fed with fats and exposed to WT PAO1 correlated with the accumulation of numerous pro-inflammatory cytokines fueled by glycolysis,<sup>17,23,25,53,54</sup> including IL-17, MCP-1, MIP-2, IL-1 $\beta$ , IL-1 $\alpha$ , TNF $\alpha$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , lipopolysaccharide-induced CXC chemokine (LIX), regulated on activation, normal T cell expressed and secreted (RANTES), and monokine induced gamma interferon (MIG) (Figure 4E). Conversely, during infection with the *P. aeruginosa* isolates, the reduced airway glucose levels during host fat intake limited the production of key cytokines such as TNF $\alpha$ , MIP-1 $\beta$ , IL-6, IL-12p70, and G-CSF (Figure 4F).

Using the  $\Delta$ *lptD* PAO1 strain, we confirmed that glucose abundance and cytokine release in the airway of fat-fed mice were modulated by the maintenance of pulmonary ATP synthase. Compared with animals fed with the control diet, animals on the fatty acid-rich intake and exposed to  $\Delta$ *lptD* PAO1 exhibited reduced airway glucose (Figures 4A and 4D) and cytokines, including IL-17, MCP-1, MIP-2, IL-6, and keratinocyte chemoattractant (KC)<sup>17,23,25,53,54</sup> (Figure 4G). This nutritional

approach demonstrates that conservation of host ATP synthase during *P. aeruginosa* infection is central not only to airway energy generation via catabolism of fats but also to limit inflammatory glycolysis.

### **Ketogenesis induced by dietary fats limits inflammatory disease during *P. aeruginosa* pneumonia**

The fatty acid-rich, carbohydrate-low diet that we used is a “ketogenic diet” that drives the generation of ketone bodies, such as  $\beta$ -hydroxybutyrate (BHB).<sup>29,55</sup> In addition to stimulating OXPHOS in peripheral tissues, ketones also suppress pathways linked to inflammatory disease, such as myeloid cell recruitment<sup>56,57</sup> and hypothermia.<sup>28</sup> We observed that the ketogenic diet significantly increased systemic BHB levels during infection with both the *P. aeruginosa* isolates and WT PAO1 (Figure 4H). During infection with the *P. aeruginosa* isolates, this diet reduced the levels of monocytes in the BAL and neutrophils in the lungs (Figures 4I and S3B). During WT PAO1 infection, there was a significant reduction in BAL monocytes (Figures 4J and S3C). To confirm this decrease in immune cell recruitment, we analyzed hematoxylin-eosin (H&E)-stained lung sections. The ketogenic diet mitigated myeloid cell accumulation in the perivascular zone (Figure 4K, black arrows) and in the perialveolar area (Figure 4K, blue arrows) during WT PAO1 infection. This reduced phagocytic burden correlated with decreased lung edema (Figure 4K, blue arrows). Similar findings were observed during infection with the *P. aeruginosa* isolates (Figure 4K, black/blue arrows).

In addition to suppressing phagocytic influx into the lung, we found that the ketogenic diet preserved host body temperature during pneumonia by both pathogens at early time points (Figures 4L and 4M). However, the benefits of body temperature regulation were dependent on the degree of *P. aeruginosa* adaptation to the human lung, as animals infected with WT PAO1 still exhibited high mortality (Figures 4N and 4O).

### **Ketone bodies are mobilized to the airway in response to the inhibition of host ATP synthase**

Given the importance of ketones in fueling OXPHOS and suppressing inflammation in the lung, we assessed how the host produced these metabolites in response to *P. aeruginosa* pneumonia. Animals fed a regular chow responded to *P. aeruginosa* airway infection by significantly increasing BHB release into the blood, which is a proxy for hepatic ketogenesis,<sup>29</sup> even more so when the pathogen expressed LptD (Figure 5A). Hepatic BHB synthesis is typically triggered by anorexia, hypoglycemia, or liver infection.<sup>51</sup> However, animals infected with either WT or  $\Delta$ *lptD* PAO1 exhibited similar food consumption (Figure S4A), blood glucose levels (Figure S4B), weight loss (Figure S4C) and pathogen burden in the liver (Figure S4D), indicating that

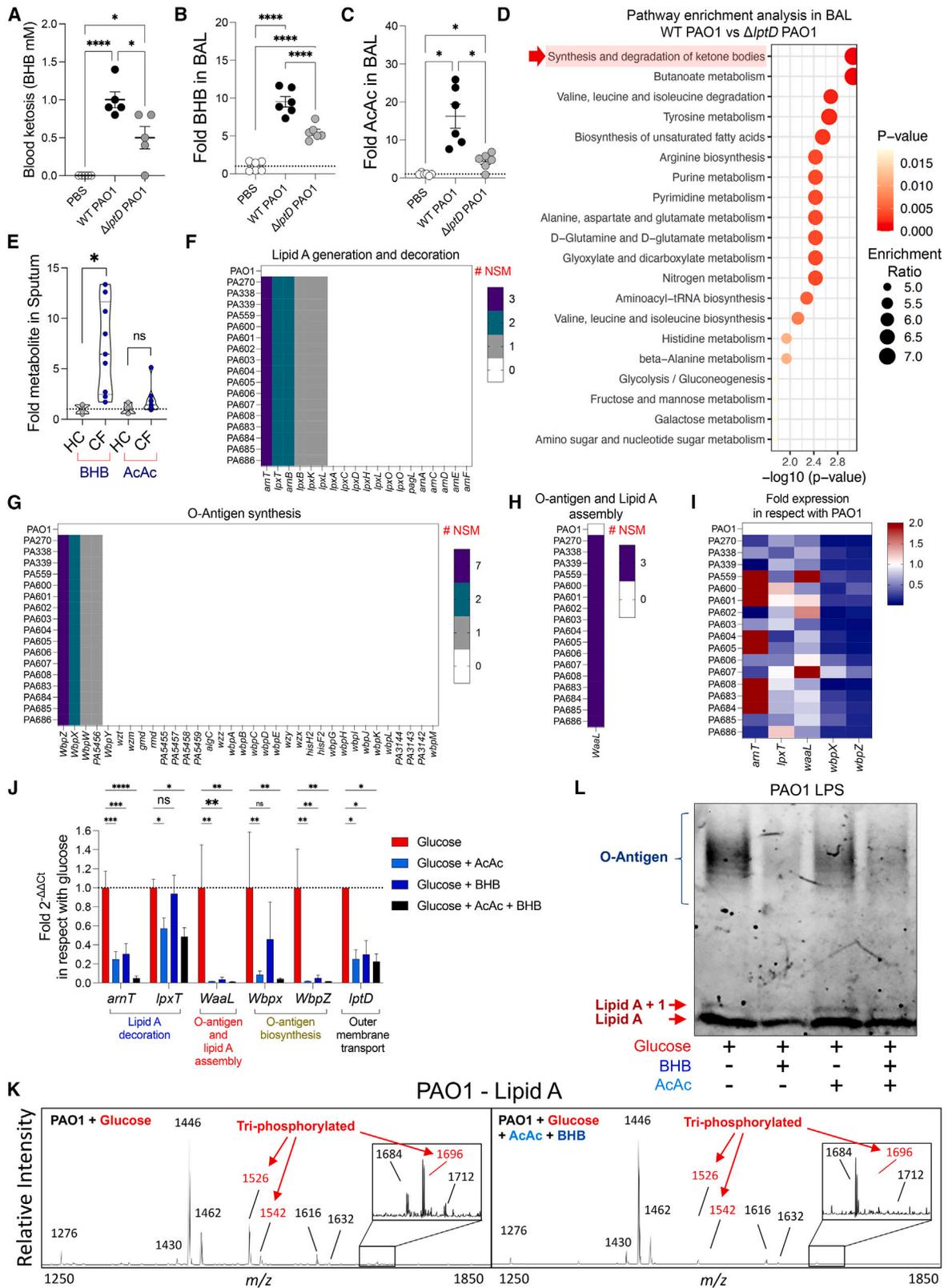
#### **Figure 4. Dietary fats modulate host tolerance to *P. aeruginosa* lung infection**

Mice fed with either a fatty acid-rich (F. Acids) or a carbohydrate-rich (Carbs) diet were exposed to PBS, WT PAO1,  $\Delta$ *lptD* PAO1, or the *P. aeruginosa* isolates. The following were examined: (A) BAL glucose; (B) and (C) bacterial burden; (D) glycemia; (E)–(G) BAL cytokines enrichment; (H) blood BHB (beginning of the light cycle); (I) and (J) phagocyte recruitment in BAL and lung; (K) lung H&E; (L) and (M) body temperature; and (N) and (O) animal survival.

Data are from 2 to 3 independent assays, 4–8 mice in total, and shown as average  $\pm$  SEM.

(A)–(D), (H)–(J): one-way ANOVA. (L) and (M): two-way ANOVA. (N) and (O): Kaplan-Maier. (E)–(G): Student’s t test. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; ns, non-significant.

See also Figure S3 and Data S1.



(legend on next page)

LptD stimulated ketone release via alternative routes. Another mechanism by which LptD might induce hepatic BHB production is by enabling LPS to enter circulation and systemically impact ATP synthase activity.<sup>58</sup> After being trafficked to the bacterial surface by LptD, LPS is shed as either free molecules or as part of microvesicles,<sup>59–61</sup> accumulating in the airway of infected subjects.<sup>62,63</sup> This soluble LPS translocates into the bloodstream,<sup>62,63</sup> where it can impact many tissues, including hepatocytes.<sup>28,58</sup> To determine if the *P. aeruginosa* LptD-LPS axis triggered hepatic ketogenesis via blockade of host ATP synthase, we treated mice with a sublethal dose of oligomycin. As anticipated, animals exposed to the ATP synthase inhibitor increased blood levels of BHB (Figure S4E), confirming that the increase in hepatic ketogenesis during WT PAO1 infection is in response to the bioenergetic impairment imposed by LPS.

We found that hepatic ketones accumulate in the infected airway. Unbiased pathway enrichment analysis of BAL metabolites demonstrated that ketones, including BHB and acetoacetate (AcAc), were the most significantly increased metabolites in the airway of WT PAO1-infected mice compared with  $\Delta$ *lptD* PAO1-exposed animals (Figures 5B–5D). This ketone accumulation was facilitated by blockade of host ATP synthase activity, as animals treated with oligomycin also exhibited greater levels of ketones in their BAL fluid (Figure S4F).

To determine if ketones accumulate in the lung of tolerant hosts, we quantified ketone bodies in airway fluids of people with CF.<sup>2,3</sup> These individuals exhibited long-term colonization by different pathogens, including *P. aeruginosa* (Figure S4G). Compared with healthy controls (HCs), sputum from patients with CF displayed significantly higher levels of BHB (Figure 5E), confirming that ketone bodies accumulate in the airway of tolerant individuals.

### Ketone bodies abrogate LPS assembly and transportation to the bacterial surface via LptD

The accumulation of ketones in the airway presents a metabolic pressure for the bacteria. We postulated that the adaptive changes in the LPS machinery found in *P. aeruginosa* isolates from tolerant subjects were in response to the ketones enriched in the respiratory tract. LPS is formed by two covalently bound subunits: lipid A and O-antigen.<sup>64</sup> While O-antigen serves as a polysaccharide shield that interacts with the most immediate environment of the pathogen, lipid A attaches the toxin to the bacterial surface by embedding it in the outer membrane.<sup>64</sup> To efficiently anchor LPS to the outer membrane, lipid A requires “decorations” at positions 1' and 4', including

arabinose molecules via *arnT* or phosphate groups via the kinase *lpxT*.<sup>64,65</sup> We used genome sequencing analysis of the *P. aeruginosa* isolates to examine how adaptation to the ketone-rich lung affected clusters involved in lipid A generation and decoration. Identification of non-synonymous mutations (NSMs) indicated that both *arnT* and *lpxT* were the most affected loci, harboring 3 and 2 NSMs, respectively (Figure 5F). These NSMs correlated with decreased *arnT* and *lpxT* mRNA expression in the *P. aeruginosa* isolates (Figure 5I). To synthesize O-antigen and covalently attach it to lipid A, *P. aeruginosa* employs many glycosyltransferases and ligases.<sup>64</sup> In the *P. aeruginosa* isolates, we observed that the most mutated genes in these routes were *WbpZ* and *WbpX*, which polymerize O-antigen, as well as *WaaL*, which binds it to lipid A, with 7, 2, and 3 NSMs, respectively (Figures 5G and 5H). Most of these genetically inactivated clusters were also transcriptionally suppressed in the *P. aeruginosa* isolates (Figure 5I).

We established that exposure of WT PAO1 to ketone bodies induces alterations in the LPS assembly pathway that are consistent with the adaptive changes found in *P. aeruginosa* isolates from tolerant subjects. Addition of AcAc, BHB, or both to glucose-rich minimal media, which facilitates LPS production,<sup>64</sup> significantly restricted the ability of WT PAO1 to express the *arnT* locus involved in lipid A decoration (Figure 5J). These ketones also suppressed translation of the lipid A kinase *lpxT* (Figure 5J). This impaired *lpxT* function was corroborated by MALDI-TOF, which demonstrated smaller mass-to-charge ratio peaks at  $m/z = 1526$ ,  $m/z = 1542$ , and  $m/z = 1696$  (Figure 5K, red arrows), a hallmark of reduced *lpxT* activity.<sup>65</sup> Similarly, WT PAO1 exposure to ketones restricted O-antigen synthesis (Figure 5L) and prevented its attachment to lipid A. *WaaL*, the ligase that covalently binds these LPS components,<sup>64</sup> was significantly depleted in the presence of AcAc, BHB, or both (Figure 5J). Ketogenic pressure also suppressed *WpbX* and *WbpZ*, the glycosyltransferases involved in O-antigen polymerization (Figure 5J). This impaired LPS assembly corresponded with decreased expression of *lptD* (Figure 5J).

### *P. aeruginosa* isolates from tolerant subjects exploit host immunometabolites to enrich ketone bodies in the lung

Many metabolites that could impose selective pressure on *P. aeruginosa* accumulate in the airway along the course of infection. Itaconate, produced by immunoresponsive gene 1 (*Irg1*), is a major host immunoregulatory carboxylate that drives FAO<sup>66</sup> and tolerance to LPS-driven inflammation.<sup>67–70</sup> During infection

#### Figure 5. Airway ketones drive *P. aeruginosa* LPS pathoadaptation

(A–D) Mice were exposed to PBS, WT PAO1, or  $\Delta$ *lptD* PAO1. The following were measured at the beginning of the light cycle: (A) BHB in blood; (B)–(D) BHB and AcAc abundance in BAL. In (D), pathway enrichment analysis was performed via Metaboloanalyst 5.0 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) platform as database.

(E) Ketones in sputum of HCs and individuals with CF.

(F–H) Number of non-synonymous mutations (NSMs) found in each *P. aeruginosa* isolate in routes linked to LPS biogenesis.

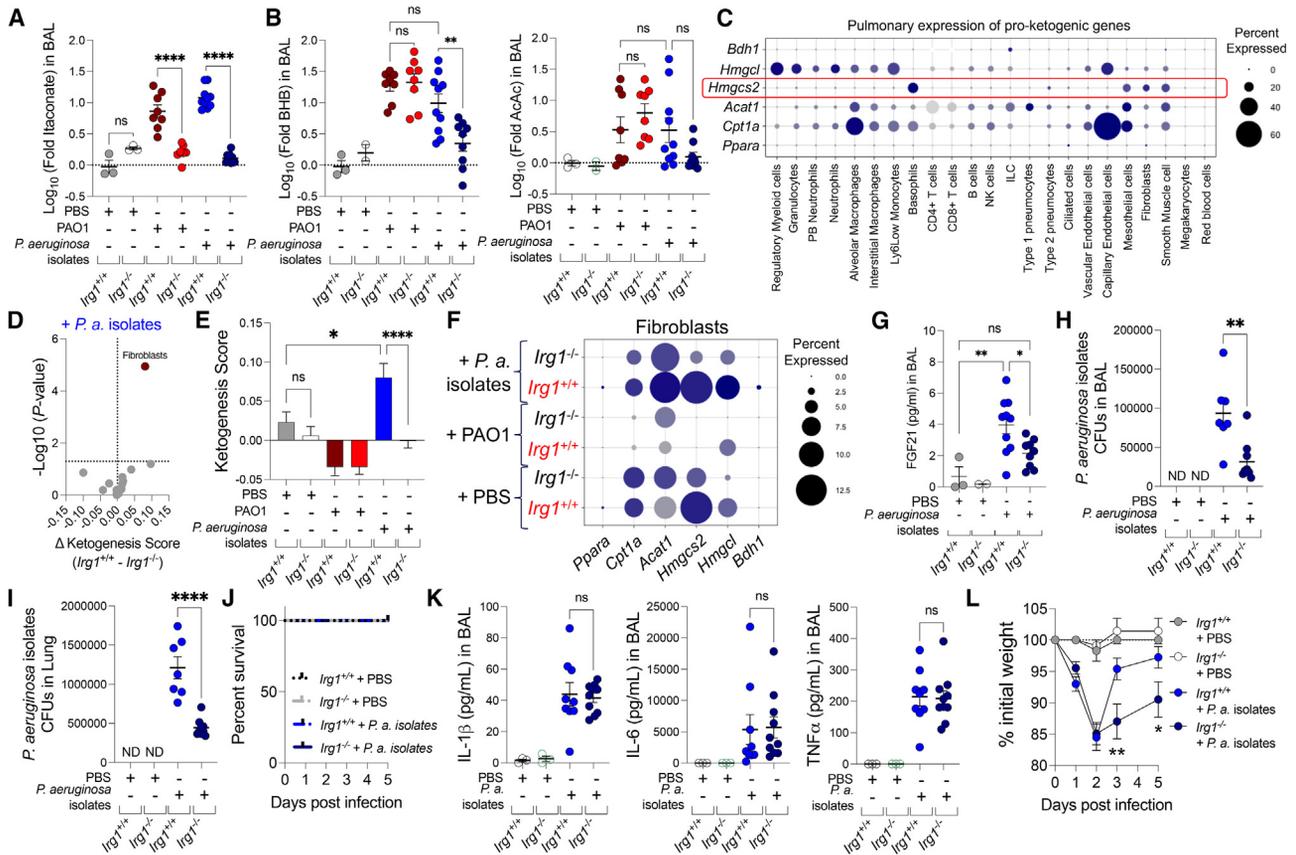
(I) mRNA levels of LPS assembly genes. WT PAO1 was grown in glucose-rich minimal media and complemented with AcAc, BHB, or both ketones.

(J–L) The following were measured: (J) mRNA levels of gene clusters involved in LPS assembly; (K) lipid A phosphorylation via MALDI-TOF; and (L) O-antigen abundance.

Data are shown as average  $\pm$  SEM from 2 to 3 independent assays.

(A)–(D): 5–6 mice in total per group. (A)–(C) and (J): one-way ANOVA. (D) and (E): Student's t test: \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ; \*\*\*\*:  $p < 0.0001$ ; ns, non-significant.

See also Figure S4 and Data S1.



**Figure 6. Itaconate promotes ketone enrichment in the lung during *P. aeruginosa* infection**

(A–L) *Irg1*<sup>+/+</sup> and *Irg1*<sup>-/-</sup> mice were exposed to PBS, WT PAO1, or the *P. aeruginosa* isolates. The following were measured: (A) and (B) BAL metabolites; (C) mRNA expression of ketogenic clusters in each lung cell subset identified by scRNA-seq; (D) volcano plot of  $\Delta$ ketogenesis scores (“*Irg1*<sup>+/+</sup>”–“*Irg1*<sup>-/-</sup>”) for each lung cell subset during infection with the *P. aeruginosa* isolates; (E) ketogenesis transcriptomic score in fibroblasts; (F) expression of ketogenic clusters in fibroblasts; (G) BAL FGF21; (H–I) respiratory pathogen burden during infection with the *P. aeruginosa* isolates; (J) animal survival; (K) BAL cytokines; and (L) weight change.

Data are shown as average  $\pm$  SEM from 2 to 3 independent experiments, with 3–10 animals in total.

(A), (B), (E), and (G)–(I): one-way ANOVA; (L): two-way ANOVA; (D): Student’s t test. (J): Kaplan-Maier. \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*\*:  $p < 0.0001$ ; ns, non-significant.

See also Figures S5 and S6, Table S2, and Data S1.

with the *P. aeruginosa* isolates, but not WT PAO1, ketone bodies clustered with itaconate as the most upregulated metabolites in the BAL fluid (Figures S5A and S5B), suggesting that these isolates might exploit this immunometabolite to promote ketone concentration and pathoadaptation.

Using animals that are unable to produce itaconate (*Irg1*<sup>-/-</sup>) and controls (*Irg1*<sup>+/+</sup>) (Figure 6A), we found that accumulation of BHB in the airway is *Irg1*-dependent during infection with the *P. aeruginosa* isolates, but not WT PAO1 (Figure 6B). This *Irg1*-associated BHB accumulation was not driven by increased systemic ketones, as both *Irg1*<sup>+/+</sup> and *Irg1*<sup>-/-</sup> mice showed comparable BHB blood levels (Figure S5C). Indeed, in contrast to hosts exposed to WT PAO1, infection with the *P. aeruginosa* isolates was associated with less BHB in circulation (Figure S5D), which was not related to differences in food consumption, hypoglycemia, weight loss, and liver infection (Figures S5E–S5H). These findings confirmed that strains from the lungs of tolerant subjects respond to *Irg1* by concentrating ketones in the respiratory tract.

### Itaconate promotes altered expression of ketogenic genes in lung fibroblasts

To identify local adjustments consistent with ketone metabolism during infection with the *P. aeruginosa* isolates, we analyzed ketogenic gene expression in each lung cell population. Ketogenesis is regulated by *Hmgcs2*, which is the rate-limiting step of this pathway and is used to identify ketogenic cells.<sup>29,71</sup> Working in concert with other ketogenic genes such as *Cpt1a*, *Ppara*, *Acat1*, *Hmgcl*, and *Bdh1*, *Hmgcs2* drives the synthesis of AcAc and BHB.<sup>29</sup> In our scRNA-seq analysis (Figure S6A), we noted that fibroblasts, basophils, type 2 pneumocytes, mesothelial cells, and smooth muscle cells expressed *Hmgcs2* (Figure 6C), suggesting ketogenic metabolism in these subsets. We analyzed scRNA-seq data from *Irg1*<sup>+/+</sup> or *Irg1*<sup>-/-</sup> mice exposed to either PBS or the *P. aeruginosa* isolates, calculating a ketogenesis score for each cell subset (Table S2). We then computed a  $\Delta$ ketogenesis score (“*Irg1*<sup>+/+</sup>”–“*Irg1*<sup>-/-</sup>”). Volcano plots of these scores showed that *Irg1* signaling significantly enriched expression of ketogenic genes in fibroblasts during infection with the

*P. aeruginosa* isolates (Figures 6D and 6E). This increased ketogenic score was driven by augmented expression of *Acat1*, *Hmgcs2*, and *Hmgcl*, which generate AcAc, as well as *Bdh1*, which converts AcAc into BHB (Figure 6F).<sup>29</sup> During WT PAO1 infection, however, fibroblasts exhibited lower expression of many of the ketogenic genes, including *Hmgcs2*, *Cpt1a*, and *Bdh1*, independently of *Irg1* (Figures 6E and 6F). Thus, only *P. aeruginosa* isolates that evolved in the lung of tolerant subjects co-opted itaconate to drive expression of ketogenic genes in fibroblasts.

Ketogenesis is promoted by fibroblast growth factor 21 (FGF21).<sup>72,73</sup> *Irg1* enabled airway FGF21 accumulation during infection with the *P. aeruginosa* isolates (Figure 6G), but not with WT PAO1 (Figure S6B), corroborating the link between itaconate and fibroblast pathways associated with ketogenesis in a tolerogenic environment.

### **Irg1 enables disease tolerance during *P. aeruginosa* lung infection**

Given its role in promoting *P. aeruginosa* adaptation to the airway through ketogenesis, we postulated that itaconate facilitates host tolerance to *P. aeruginosa* disease. Compared with *Irg1*<sup>-/-</sup> mice, *Irg1*<sup>+/+</sup> animals had an increased pathogen burden during infection with the *P. aeruginosa* isolates (Figures 6H and 6I). Despite this increased bacterial load, *Irg1*<sup>+/+</sup> mice did not exhibit worse inflammatory pathology, evidenced by identical animal survival (Figure 6J), similar IL-1 $\beta$ , IL-6, and TNF $\alpha$  levels (Figure 6K), comparable albumin infiltration into the airway (Figure S6C) and equivalent body temperature regulation (Figure S6D). *Irg1* signaling improved host weight recovery over the course of infection with the *P. aeruginosa* isolates (Figure 6L). We studied the impact of *Irg1* on WT PAO1 infection. Although itaconate did not affect pathogen burden, IL-6, TNF $\alpha$ , or albumin infiltration (Figures S6E–S6H), it suppressed IL-1 $\beta$  release (Figure S6I). However, this attenuated inflammatory signaling was not sufficient to prevent immunopathology, as both *Irg1*<sup>+/+</sup> and *Irg1*<sup>-/-</sup> mice rapidly succumbed to WT PAO1 infection (Figure S6J).

### **DISCUSSION**

Our findings strongly suggest that disease tolerance in the human lung is a bilateral process based in host-pathogen metabolic remodeling. During infection with environmental *P. aeruginosa* strains that display LPS on their surface, airway cells fail to produce ATP via OXPHOS, triggering severe inflammation and alveolar destruction. This detrimental environment aggravates pathogen burden, worsening the bioenergetic interference and accelerating host demise. In response to the interruption of OXPHOS provoked by *P. aeruginosa* LPS, hepatic cells release ketone bodies into circulation, which enter the highly permeable airway compartment. These ketones restrict *P. aeruginosa* LPS assembly, selecting for less metabolically active strains compatible with airway cell OXPHOS function. Such keto-adapted *P. aeruginosa* strains limit the production of inflammatory mediators, safeguarding the alveolar tone and extending host survival. In this favorable milieu, *P. aeruginosa* exploits local immunometabolites to maintain ketone abun-

dance, reinforcing bacterial adaptation, host-pathogen coexistence, and disease tolerance.

Maintenance of OXPHOS is an essential component of pulmonary integrity. In healthy hosts, this platform is fueled by FAO, which we show is fundamental during *P. aeruginosa* pneumonia to restrict both glycolysis and damaging inflammation.<sup>11,12,52</sup> In tolerant subjects, this bioenergetic circuit is preserved by ketogenesis, which typically functions to rescue the ATP homeostasis of peripheral organs. Ketogenesis selects for *P. aeruginosa* strains incapable of assembling the toxins that impair OXPHOS, preventing the metabolo-inflammatory pathways that compromise the infected tissue: i.e., glycolysis. Ketogenesis is thus central in the conservation of the lung bioenergetic configuration that permits tolerance to *P. aeruginosa* infection.<sup>10–12</sup>

Our findings indicate not only that the host actively responds to the pulmonary bioenergetic stress initiated by acute infection, but also the pathogen. In response to the airway ketogenic reconfiguration provoked during pneumonia, *P. aeruginosa* alters its own surface architecture, generating slow-growing strains that preserve mitochondrial bioenergetics. Other phenotypic adaptations sustain chronic *P. aeruginosa* persistence, including pro-biofilm polysaccharides, quorum-sensing regulators, and drug efflux pumps.<sup>2,3,31</sup> These changes may reflect exposure to ketones and other tolerogenic metabolites in the airway. As we show, the diet also impacts the composition of the airway metabolome, indicating that nutrition might regulate the evolution and persistence of diverse pathogens in the human lung.

Our work provides another layer of complexity to our understanding of how ketone bodies safeguard mucosal OXPHOS during infectious diseases. This tolerogenic mechanism likely works in concert with previously established roles for ketogenesis, including inhibition of the inflammasome during intoxication with soluble LPS<sup>58,74</sup> and stimulation of anti-oxidant receptors such as *Hcar2* (*Gpr109a*).<sup>75</sup> Importantly, the impact of ketones on immunopathology varies by pathogen. In COVID-19, for example, ketone bodies support the generation of competent lymphocytes that contain the infection and promote mucosal repair.<sup>76</sup> Additional studies are needed to better understand how ketones influence progression of different inflammatory disease states.

By switching from systemic ketogenesis to concentration of ketones in the airway, the host response to the *P. aeruginosa* isolates limited the detrimental consequences of prolonged ketonemia.<sup>77–79</sup> The local augmentation of ketones promoted by the *P. aeruginosa* isolates correlated with expression of ketogenic genes in lung fibroblasts. These findings are consistent with the ketogenic role of these cells in other long-term inflammatory settings, such as cancer, where tumor-associated fibroblasts release ketone bodies as consequence of catabolic metabolism.<sup>80,81</sup> In the airway, fibroblasts modulate tissue repair and fibrosis, processes regulated by itaconate.<sup>82</sup> These findings suggest that lung fibroblasts, along with itaconate, could be targeted for the development of novel therapies to mitigate pulmonary injury during infectious diseases.

We established a link between ketogenesis, pulmonary OXPHOS maintenance, and tolerance to *P. aeruginosa* lung infection. Our findings suggest that novel therapeutic strategies for inflammatory airway diseases should consider how

respiratory cells produce ATP during infection, and how this bioenergetic configuration could be targeted by either the diet or other approaches that influence the balance between OXPHOS and glycolysis.

### Limitations of study

We appreciate that the animal models of pneumonia used in this work might not necessarily reflect the complex immunometabolic matrix of the human lung. The concentration of many metabolites found in murine fluids, such as in blood and airway, might not necessarily represent the abundance of these determinants in human tissues. Similarly, the enrichment of ketones in sputum of either healthy individuals or people with CF might be also result of different degrees of mucus accumulation, myeloid cell infiltration, dehydration, and pathogen burden. The LPS adaptation induced by ketones on *P. aeruginosa* isolates is indicative of many collaborative processes occurring in the human respiratory tract, suggesting that future studies should examine these parallel routes for a better comprehension of disease tolerance. Likewise, our assays are also limited by examining *in vitro*, but not *in vivo*, how ketones impact *P. aeruginosa* LPS assembly and transport to the surface. Although our work considered the interaction between ketogenesis and other metabolic networks, like itaconate, fatty acid oxidation, and nutrition, we did not study how these pathways associated with additional immunoregulatory platforms involved in disease tolerance. The identification of alterations in ketogenic gene expression in pulmonary fibroblasts of mice exposed to tolerogenic *P. aeruginosa* strains does not discard that many other respiratory subsets might also display similar changes in the human lung. A more comprehensive study evaluating the crosstalk between tolerance, ketogenesis, and *P. aeruginosa* infection would support the conclusions of this manuscript.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
  - Mice experiments
  - Human samples
  - *P. aeruginosa* strains
- **METHOD DETAILS**
  - Oxygen consumption rate measurements in macrophages
  - *P. aeruginosa* consumption of NADH and FADH2
  - Infection of mice with *P. aeruginosa* strains
  - Food consumption during infection of mice with *P. aeruginosa* strains
  - Airway metabolomics
  - Lung tissue histopathology studies
  - Diets for *in vivo* assays

- Mouse lung processing for single-cell RNA-sequencing analysis
- Single cell RNA-sequencing analysis
- qRT-PCR for genes in *P. aeruginosa* exposed to ketones
- LPS assembly studies in WT PAO1
- Pathway analysis of airway fluids
- *P. aeruginosa* lipid A isolation and analysis
- Lung protein extraction and western blots
- Whole-genome sequencing and single-nucleotide polymorphism (SNP) calling in *P. aeruginosa* isolates

### ● QUANTIFICATION AND STATISTICAL ANALYSIS

### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cmet.2023.09.001>.

### ACKNOWLEDGMENTS

This study was funded by grants R35GM146776 (NIH), RIQUEL2110 (CFF), and GT007268 (Innovation Nucleation Fund) awarded to S.A.R. K.L.T. was supported by NIH grant 1F30AI161801, T.W.F.L. was supported by NIH grant K99HL157550, and A.P. was supported by NIH grant 1R35HL135800 and CFF grant 003028G221. We thank the Single Cell Core at the Columbia University Genome Center and the Molecular Pathology Core at Columbia University for their technical help with single-cell RNA-sequencing and histology studies. We thank Dr. Ian Lewis and Marija Drikk at University of Calgary, Alberta, Canada, for their help with metabolomic studies. We thank Dr. Paul Planet and Dr. Ahmed Moustafa at Children's Hospital of Philadelphia, USA, for their help on bacterial genome sequencing analyses. We thank Dr. Clemente Britto-Leon at Yale University, USA, for providing sputum samples. This publication is also supported by the CCTI Flow Cytometry Core at CUIMC (NIH S10RR027050).

### AUTHOR CONTRIBUTIONS

Conceptualization, K.L.T. and S.A.R.; methodology, K.L.T. and S.A.R.; software, K.L.T. and S.A.R.; validation, K.L.T. and S.A.R.; formal analysis, K.L.T., Y.-T.C., S.U.B., C.E.H., and S.A.R.; investigation, K.L.T., Y.-T.C., A.J., A.U., D.A., T.W.F.L., S.U.B., C.E.H., R.K.E., and S.A.R.; resources, S.A.R., R.K.E., and A.P.; data curation, K.L.T. and S.A.R.; writing – original draft, K.L.T. and S.A.R.; writing – review & editing, K.L.T., A.P., and S.A.R.; visualization, K.L.T. and S.A.R.; supervision, S.A.R. and A.P.; project administration, K.L.T. and S.A.R.; funding acquisition, S.A.R. and A.P.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: May 26, 2022

Revised: June 14, 2023

Accepted: September 5, 2023

Published: October 3, 2023

### REFERENCES

1. Lin, C.K., and Kazmierczak, B.I. (2017). Inflammation: A Double-Edged Sword in the Response to *Pseudomonas aeruginosa* Infection. *J. Innate Immun.* 9, 250–261. <https://doi.org/10.1159/000455857>.
2. Faure, E., Kwong, K., and Nguyen, D. (2018). *Pseudomonas aeruginosa* in Chronic Lung Infections: How to Adapt Within the Host? *Front. Immunol.* 9, 2416. <https://doi.org/10.3389/fimmu.2018.02416>.

3. Winstanley, C., O'Brien, S., and Brockhurst, M.A. (2016). *Pseudomonas aeruginosa* Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. *Trends Microbiol.* *24*, 327–337. <https://doi.org/10.1016/j.tim.2016.01.008>.
4. Hilliam, Y., Moore, M.P., Lamont, I.L., Bilton, D., Haworth, C.S., Foweraker, J., Walshaw, M.J., Williams, D., Fothergill, J.L., De Souza, A., and Winstanley, C. (2017). *Pseudomonas aeruginosa* adaptation and diversification in the non-cystic fibrosis bronchiectasis lung. *Eur. Respir. J.* *49*, 1602108. <https://doi.org/10.1183/13993003.02108-2016>.
5. Chalmers, J.D., and Hill, A.T. (2013). Mechanisms of immune dysfunction and bacterial persistence in non-cystic fibrosis bronchiectasis. *Mol. Immunol.* *55*, 27–34. <https://doi.org/10.1016/j.molimm.2012.09.011>.
6. Martínez-Solano, L., Macia, M.D., Fajardo, A., Oliver, A., and Martínez, J.L. (2008). Chronic *Pseudomonas aeruginosa* infection in chronic obstructive pulmonary disease. *Clin. Infect. Dis.* *47*, 1526–1533. <https://doi.org/10.1086/593186>.
7. Rodrigo-Troyano, A., Melo, V., Marcos, P.J., Laserna, E., Peiro, M., Suarez-Cuartin, G., Perea, L., Feliu, A., Plaza, V., Faverio, P., et al. (2018). *Pseudomonas aeruginosa* in Chronic Obstructive Pulmonary Disease Patients with Frequent Hospitalized Exacerbations: A Prospective Multicentre Study. *Respiration* *96*, 417–424. <https://doi.org/10.1159/000490190>.
8. Wijers, C.D., Chmiel, J.F., and Gaston, B.M. (2017). Bacterial infections in patients with primary ciliary dyskinesia: Comparison with cystic fibrosis. *Chron. Respir. Dis.* *14*, 392–406. <https://doi.org/10.1177/1479972317694621>.
9. Cohen-Cymbberknoh, M., Weigert, N., Gileles-Hillel, A., Breuer, O., Simanovsky, N., Boon, M., De Boeck, K., Barbato, A., Snijders, D., Collura, M., et al. (2017). Clinical impact of *Pseudomonas aeruginosa* colonization in patients with Primary Ciliary Dyskinesia. *Respir. Med.* *131*, 241–246. <https://doi.org/10.1016/j.rmed.2017.08.028>.
10. Martins, R., Carlos, A.R., Braza, F., Thompson, J.A., Bastos-Amador, P., Ramos, S., and Soares, M.P. (2019). Disease Tolerance as an Inherent Component of Immunity. *Annu. Rev. Immunol.* *37*, 405–437. <https://doi.org/10.1146/annurev-immunol-042718-041739>.
11. Schneider, D.S., and Ayres, J.S. (2008). Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nat. Rev. Immunol.* *8*, 889–895. <https://doi.org/10.1038/nri2432>.
12. Soares, M.P., Teixeira, L., and Moita, L.F. (2017). Disease tolerance and immunity in host protection against infection. *Nat. Rev. Immunol.* *17*, 83–96. <https://doi.org/10.1038/nri.2016.136>.
13. Liu, G., and Summer, R. (2019). Cellular Metabolism in Lung Health and Disease. *Annu. Rev. Physiol.* *81*, 403–428. <https://doi.org/10.1146/annurev-physiol-020518-114640>.
14. Tsitoura, E., Vasarmidi, E., Bibaki, E., Trachalaki, A., Koutoulaki, C., Papastratigakis, G., Papadogiorgaki, S., Chalepakis, G., Tzanakis, N., and Antoniou, K.M. (2019). Accumulation of damaged mitochondria in alveolar macrophages with reduced OXPHOS related gene expression in IPF. *Respir. Res.* *20*, 264. <https://doi.org/10.1186/s12931-019-1196-6>.
15. Cloonan, S.M., Kim, K., Esteves, P., Trian, T., and Barnes, P.J. (2020). Mitochondrial dysfunction in lung ageing and disease. *Eur. Respir. Rev.* *29*, 200165. <https://doi.org/10.1183/16000617.0165-2020>.
16. Mills, E.L., Kelly, B., Logan, A., Costa, A.S.H., Varma, M., Bryant, C.E., Tourlomis, P., Däbritz, J.H.M., Gottlieb, E., Latorre, I., et al. (2016). Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell* *167*, 457–470.e13. <https://doi.org/10.1016/j.cell.2016.08.064>.
17. Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGettrick, A.F., Goel, G., Frezza, C., Bernard, N.J., Kelly, B., Foley, N.H., et al. (2013). Succinate is an inflammatory signal that induces IL-1 $\beta$  through HIF-1 $\alpha$ . *Nature* *496*, 238–242. <https://doi.org/10.1038/nature11986>.
18. Cloonan, S.M., and Choi, A.M.K. (2016). Mitochondria in lung disease. *J. Clin. Invest.* *126*, 809–820. <https://doi.org/10.1172/JCI81113>.
19. Nam, H.S., Izumchenko, E., Dasgupta, S., and Hoque, M.O. (2017). Mitochondria in chronic obstructive pulmonary disease and lung cancer: where are we now? *Biomark. Med.* *11*, 475–489. <https://doi.org/10.2217/bmm-2016-0373>.
20. Kandasamy, J., Rezonzew, G., Jilling, T., Ballinger, S., and Ambalavanan, N. (2019). Mitochondrial DNA variation modulates alveolar development in newborn mice exposed to hyperoxia. *Am. J. Physiol. Lung Cell Mol. Physiol.* *317*, L740–L747. <https://doi.org/10.1152/ajplung.00220.2019>.
21. Aghapour, M., Remels, A.H.V., Pouwels, S.D., Bruder, D., Hiemstra, P.S., Cloonan, S.M., and Heijink, I.H. (2020). Mitochondria: at the crossroads of regulating lung epithelial cell function in chronic obstructive pulmonary disease. *Am. J. Physiol. Lung Cell Mol. Physiol.* *318*, L149–L164. <https://doi.org/10.1152/ajplung.00329.2019>.
22. Mason, R.J. (2006). Biology of alveolar type II cells. *Respirology* *11* (Suppl.), S12–S15. <https://doi.org/10.1111/j.1440-1843.2006.00800.x>.
23. Everts, B., Amiel, E., Huang, S.C.C., Smith, A.M., Chang, C.H., Lam, W.Y., Redmann, V., Freitas, T.C., Blagih, J., van der Windt, G.J.W., et al. (2014). TLR-driven early glycolytic reprogramming via the kinases TBK1-IKK $\nu$  supports the anabolic demands of dendritic cell activation. *Nat. Immunol.* *15*, 323–332. <https://doi.org/10.1038/ni.2833>.
24. Everts, B., Amiel, E., van der Windt, G.J.W., Freitas, T.C., Chott, R., Yarasheski, K.E., Pearce, E.L., and Pearce, E.J. (2012). Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. *Blood* *120*, 1422–1431. <https://doi.org/10.1182/blood-2012-03-419747>.
25. Ryan, D.G., and O'Neill, L.A.J. (2020). Krebs Cycle Reborn in Macrophage Immunometabolism. *Annu. Rev. Immunol.* *38*, 289–313. <https://doi.org/10.1146/annurev-immunol-081619-104850>.
26. Cohen, T.S., and Prince, A.S. (2013). Activation of inflammasome signaling mediates pathology of acute *P. aeruginosa* pneumonia. *J. Clin. Invest.* *123*, 1630–1637. <https://doi.org/10.1172/JCI66142>.
27. Riquelme, S.A., Hopkins, B.D., Wolfe, A.L., DiMango, E., Kitur, K., Parsons, R., and Prince, A. (2017). Cystic Fibrosis Transmembrane Conductance Regulator Attaches Tumor Suppressor PTEN to the Membrane and Promotes Anti *Pseudomonas aeruginosa* Immunity. *Immunity* *47*, 1169–1181.e7. <https://doi.org/10.1016/j.immuni.2017.11.010>.
28. Ganeshan, K., Nikkanen, J., Man, K., Leong, Y.A., Sogawa, Y., Maschek, J.A., Van Ry, T., Chagwedera, D.N., Cox, J.E., and Chawla, A. (2019). Energetic Trade-Offs and Hypometabolic States Promote Disease Tolerance. *Cell* *177*, 399–413.e12. <https://doi.org/10.1016/j.cell.2019.01.050>.
29. Puchalska, P., and Crawford, P.A. (2017). Multi-dimensional Roles of Ketone Bodies in Fuel Metabolism, Signaling, and Therapeutics. *Cell Metab.* *25*, 262–284. <https://doi.org/10.1016/j.cmet.2016.12.022>.
30. Cahill, G.F., Jr. (2006). Fuel metabolism in starvation. *Annu. Rev. Nutr.* *26*, 1–22. <https://doi.org/10.1146/annurev.nutr.26.061505.111258>.
31. Planet, P.J. (2022). Adaptation and Evolution of Pathogens in the Cystic Fibrosis Lung. *J. Pediatric Infect. Dis. Soc.* *11*, S23–S31. <https://doi.org/10.1093/jpids/piac073>.
32. Riquelme, S.A., Liimatta, K., Wong Fok Lung, T., Fields, B., Ahn, D., Chen, D., Lozano, C., Sáenz, Y., Uhlemann, A.C., Kahl, B.C., et al. (2020). *Pseudomonas aeruginosa* Utilizes Host-Derived Itaconate to Redirect Its Metabolism to Promote Biofilm Formation. *Cell Metab.* *31*, 1091–1106.e6. <https://doi.org/10.1016/j.cmet.2020.04.017>.
33. Lo Sciuto, A., Martorana, A.M., Fernández-Piñar, R., Mancone, C., Polissi, A., and Imperi, F. (2018). *Pseudomonas aeruginosa* LptE is crucial for LptD assembly, cell envelope integrity, antibiotic resistance and virulence. *Virulence* *9*, 1718–1733. <https://doi.org/10.1080/21505594.2018.1537730>.
34. Konovalova, A., Kahne, D.E., and Silhavy, T.J. (2017). Outer Membrane Biogenesis. *Annu. Rev. Microbiol.* *71*, 539–556. <https://doi.org/10.1146/annurev-micro-090816-093754>.
35. Huus, K.E., Joseph, J., Zhang, L., Wong, A., Aaron, S.D., Mah, T.F., and Sad, S. (2016). Clinical Isolates of *Pseudomonas aeruginosa* from

- Chronically Infected Cystic Fibrosis Patients Fail To Activate the Inflammasome during Both Stable Infection and Pulmonary Exacerbation. *J. Immunol.* 196, 3097–3108. <https://doi.org/10.4049/jimmunol.1501642>.
36. Bhagirath, A.Y., Li, Y., Somayajula, D., Dadashi, M., Badr, S., and Duan, K. (2016). Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. *BMC Pulm. Med.* 16, 174. <https://doi.org/10.1186/s12890-016-0339-5>.
  37. Muhlebach, M.S., Zorn, B.T., Esther, C.R., Hatch, J.E., Murray, C.P., Turkovic, L., Ranganathan, S.C., Boucher, R.C., Stick, S.M., and Wolfgang, M.C. (2018). Initial acquisition and succession of the cystic fibrosis lung microbiome is associated with disease progression in infants and preschool children. *PLoS Pathog.* 14, e1006798. <https://doi.org/10.1371/journal.ppat.1006798>.
  38. Balibar, C.J., and Grabowicz, M. (2016). Mutant Alleles of IptD Increase the Permeability of *Pseudomonas aeruginosa* and Define Determinants of Intrinsic Resistance to Antibiotics. *Antimicrob. Agents Chemother.* 60, 845–854. <https://doi.org/10.1128/AAC.01747-15>.
  39. Song, J., Steidle, L., Steymans, I., Singh, J., Sanner, A., Böttinger, L., Winter, D., and Becker, T. (2023). The mitochondrial Hsp70 controls the assembly of the F(1)F(O)-ATP synthase. *Nat. Commun.* 14, 39. <https://doi.org/10.1038/s41467-022-35720-5>.
  40. Smeitink, J., van den Heuvel, L., and DiMauro, S. (2001). The genetics and pathology of oxidative phosphorylation. *Nat. Rev. Genet.* 2, 342–352. <https://doi.org/10.1038/35072063>.
  41. Wilson, D.F. (2017). Oxidative phosphorylation: regulation and role in cellular and tissue metabolism. *J. Physiol.* 595, 7023–7038. <https://doi.org/10.1113/JP273839>.
  42. Wolf, A.J., Reyes, C.N., Liang, W., Becker, C., Shimada, K., Wheeler, M.L., Cho, H.C., Popescu, N.I., Coggeshall, K.M., Arditi, M., and Underhill, D.M. (2016). Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan. *Cell* 166, 624–636. <https://doi.org/10.1016/j.cell.2016.05.076>.
  43. Meylan, S., Porter, C.B.M., Yang, J.H., Belenky, P., Gutierrez, A., Lobritz, M.A., Park, J., Kim, S.H., Moskowitz, S.M., and Collins, J.J. (2017). Carbon Sources Tune Antibiotic Susceptibility in *Pseudomonas aeruginosa* via Tricarboxylic Acid Cycle Control. *Cell Chem. Biol.* 24, 195–206. <https://doi.org/10.1016/j.chembiol.2016.12.015>.
  44. Jones, N., Blagih, J., Zani, F., Rees, A., Hill, D.G., Jenkins, B.J., Bull, C.J., Moreira, D., Bantan, A.I.M., Cronin, J.G., et al. (2021). Fructose reprograms glutamine-dependent oxidative metabolism to support LPS-induced inflammation. *Nat. Commun.* 12, 1209. <https://doi.org/10.1038/s41467-021-21461-4>.
  45. Vijayan, V., Pradhan, P., Braud, L., Fuchs, H.R., Gueler, F., Motterlini, R., Foresti, R., and Immenschuh, S. (2019). Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - A divergent role for glycolysis. *Redox Biol.* 22, 101147. <https://doi.org/10.1016/j.redox.2019.101147>.
  46. Symersky, J., Osowski, D., Walters, D.E., and Mueller, D.M. (2012). Oligomycin frames a common drug-binding site in the ATP synthase. *Proc. Natl. Acad. Sci. USA* 109, 13961–13965. <https://doi.org/10.1073/pnas.1207912109>.
  47. Schlaepfer, I.R., and Joshi, M. (2020). CPT1A-mediated Fat Oxidation, Mechanisms, and Therapeutic Potential. *Endocrinology* 161, bqz046. <https://doi.org/10.1210/endo/bqz046>.
  48. Divakaruni, A.S., Hsieh, W.Y., Minarrieta, L., Duong, T.N., Kim, K.K.O., Desousa, B.R., Andreyev, A.Y., Bowman, C.E., Caradonna, K., Dranka, B.P., et al. (2018). Etomoxir Inhibits Macrophage Polarization by Disrupting CoA Homeostasis. *Cell Metab.* 28, 490–503.e7. <https://doi.org/10.1016/j.cmet.2018.06.001>.
  49. Kowalski, M.L., Didier, A., and Kaliner, M.A. (1989). Neurogenic inflammation in the airways. I. Neurogenic stimulation induces plasma protein extravasation into the rat airway lumen. *Am. Rev. Respir. Dis.* 140, 101–109. <https://doi.org/10.1164/ajrccm/140.1.101>.
  50. Minakata, Y., Nakanishi, M., Hirano, T., Matsunaga, K., Yamagata, T., and Ichinose, M. (2005). Microvascular hyperpermeability in COPD airways. *Thorax* 60, 882. <https://doi.org/10.1136/thx.2005.045765>.
  51. Tirosh, I., Izar, B., Prakadan, S.M., Wadsworth, M.H., 2nd, Treacy, D., Trombetta, J.J., Rotem, A., Rodman, C., Lian, C., Murphy, G., et al. (2016). Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* 352, 189–196. <https://doi.org/10.1126/science.aad0501>.
  52. McCarville, J.L., and Ayres, J.S. (2018). Disease tolerance: concept and mechanisms. *Curr. Opin. Immunol.* 50, 88–93. <https://doi.org/10.1016/j.coi.2017.12.003>.
  53. Bechara, R., McGeachy, M.J., and Gaffen, S.L. (2021). The metabolism-modulating activity of IL-17 signaling in health and disease. *J. Exp. Med.* 218, e20202191. <https://doi.org/10.1084/jem.20202191>.
  54. Shanmugam, N., Reddy, M.A., Guha, M., and Natarajan, R. (2003). High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. *Diabetes* 52, 1256–1264. <https://doi.org/10.2337/diabetes.52.5.1256>.
  55. Crosby, L., Davis, B., Joshi, S., Jardine, M., Paul, J., Neola, M., and Barnard, N.D. (2021). Ketogenic Diets and Chronic Disease: Weighing the Benefits Against the Risks. *Front. Nutr.* 8, 702802. <https://doi.org/10.3389/fnut.2021.702802>.
  56. Song, Y., Yang, Y., Zeng, W., Loo, J.J., Jiang, Q., Peng, Z., Li, Y., Jiang, S., Feng, X., Du, X., et al. (2022). beta-Hydroxybutyrate impairs neutrophil migration distance through activation of a protein kinase C and myosin light chain 2 signaling pathway in ketotic cows. *J. Dairy Sci.* 105, 761–771. <https://doi.org/10.3168/jds.2021-20875>.
  57. Dong, Z., Sun, X., Tang, Y., Luo, S., Jia, H., Xu, Q., Jiang, Q., Loo, J.J., Xu, W., and Xu, C. (2022). beta-hydroxybutyrate impairs monocyte function via the ROS-NLR family pyrin domain-containing three inflammasome (NLRP3) pathway in ketotic cows. *Front. Vet. Sci.* 9, 925900. <https://doi.org/10.3389/fvets.2022.925900>.
  58. Wang, A., Huen, S.C., Luan, H.H., Yu, S., Zhang, C., Gallezot, J.D., Booth, C.J., and Medzhitov, R. (2016). Opposing Effects of Fasting Metabolism on Tissue Tolerance in Bacterial and Viral Inflammation. *Cell* 166, 1512–1525.e12. <https://doi.org/10.1016/j.cell.2016.07.026>.
  59. Kadurugamuwa, J.L., and Beveridge, T.J. (1995). Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J. Bacteriol.* 177, 3998–4008. <https://doi.org/10.1128/jb.177.14.3998-4008.1995>.
  60. Ellis, T.N., Leiman, S.A., and Kuehn, M.J. (2010). Naturally produced outer membrane vesicles from *Pseudomonas aeruginosa* elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. *Infect. Immun.* 78, 3822–3831. <https://doi.org/10.1128/IAI.00433-10>.
  61. Vanaja, S.K., Russo, A.J., Behl, B., Banerjee, I., Yankova, M., Deshmukh, S.D., and Rathinam, V.A.K. (2016). Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. *Cell* 165, 1106–1119. <https://doi.org/10.1016/j.cell.2016.04.015>.
  62. McElvaney, O.J., Zaslona, Z., Becker-Flegler, K., Palsson-McDermott, E.M., Boland, F., Gunaratnam, C., Gulbins, E., O'Neill, L.A., Reeves, E.P., and McElvaney, N.G. (2019). Specific Inhibition of the NLRP3 Inflammasome as an Antiinflammatory Strategy in Cystic Fibrosis. *Am. J. Respir. Crit. Care Med.* 200, 1381–1391. <https://doi.org/10.1164/rccm.201905-1013OC>.
  63. del Campo, R., Martínez, E., del Fresno, C., Alenda, R., Gómez-Piña, V., Fernández-Ruiz, I., Siliceo, M., Jurado, T., Toledano, V., Arnalich, F., et al. (2011). Translocated LPS might cause endotoxin tolerance in circulating monocytes of cystic fibrosis patients. *PLoS One* 6, e29577. <https://doi.org/10.1371/journal.pone.0029577>.
  64. King, J.D., Kocincová, D., Westman, E.L., and Lam, J.S. (2009). Review: Lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *Innate Immun.* 15, 261–312. <https://doi.org/10.1177/1753425909106436>.

65. Nowicki, E.M., O'Brien, J.P., Brodbelt, J.S., and Trent, M.S. (2014). Characterization of *Pseudomonas aeruginosa* LpxT reveals dual positional lipid A kinase activity and co-ordinated control of outer membrane modification. *Mol. Microbiol.* *94*, 728–741. <https://doi.org/10.1111/mmi.12796>.
66. Weiss, J.M., Davies, L.C., Karwan, M., Ileva, L., Ozaki, M.K., Cheng, R.Y., Ridnour, L.A., Annunziata, C.M., Wink, D.A., and McVicar, D.W. (2018). Itaconic acid mediates crosstalk between macrophage metabolism and peritoneal tumors. *J. Clin. Invest.* *128*, 3794–3805. <https://doi.org/10.1172/jci99169>.
67. Bambouskova, M., Potuckova, L., Paulenda, T., Kerndl, M., Mogilenko, D.A., Lizotte, K., Swain, A., Hayes, S., Sheldon, R.D., Kim, H., et al. (2021). Itaconate confers tolerance to late NLRP3 inflammasome activation. *Cell Rep.* *34*, 108756. <https://doi.org/10.1016/j.celrep.2021.108756>.
68. Lampropoulou, V., Sergushichev, A., Bambouskova, M., Nair, S., Vincent, E.E., Loginicheva, E., Cervantes-Barragan, L., Ma, X., Huang, S.C.C., Griss, T., et al. (2016). Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. *Cell Metab.* *24*, 158–166. <https://doi.org/10.1016/j.cmet.2016.06.004>.
69. O'Neill, L.A.J., and Artyomov, M.N. (2019). Itaconate: the poster child of metabolic reprogramming in macrophage function. *Nat. Rev. Immunol.* *19*, 273–281. <https://doi.org/10.1038/s41577-019-0128-5>.
70. Peace, C.G., and O'Neill, L.A. (2022). The role of itaconate in host defense and inflammation. *J. Clin. Invest.* *132*, e148548. <https://doi.org/10.1172/JCI148548>.
71. Asif, S., Kim, R.Y., Fatica, T., Sim, J., Zhao, X., Oh, Y., Denoncourt, A., Cheung, A.C., Downey, M., Mulvihill, E.E., and Kim, K.H. (2022). Hmgcs2-mediated ketogenesis modulates high-fat diet-induced hepatosteatosis. *Mol. Metab.* *67*, 101494. <https://doi.org/10.1016/j.molmet.2022.101494>.
72. Badman, M.K., Pissios, P., Kennedy, A.R., Koukos, G., Flier, J.S., and Maratos-Flier, E. (2007). Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab.* *5*, 426–437. <https://doi.org/10.1016/j.cmet.2007.05.002>.
73. Badman, M.K., Koester, A., Flier, J.S., Kharitonov, A., and Maratos-Flier, E. (2009). Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. *Endocrinology* *150*, 4931–4940. <https://doi.org/10.1210/en.2009-0532>.
74. Youm, Y.H., Nguyen, K.Y., Grant, R.W., Goldberg, E.L., Bodogai, M., Kim, D., D'Agostino, D., Planavsky, N., Lupfer, C., Kanneganti, T.D., et al. (2015). The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat. Med.* *21*, 263–269. <https://doi.org/10.1038/nm.3804>.
75. Graff, E.C., Fang, H., Wanders, D., and Judd, R.L. (2016). Anti-inflammatory effects of the hydroxycarboxylic acid receptor 2. *Metabolism* *65*, 102–113. <https://doi.org/10.1016/j.metabol.2015.10.001>.
76. Karagiannis, F., Peukert, K., Surace, L., Michla, M., Nikolka, F., Fox, M., Weiss, P., Feuerborn, C., Maier, P., Schulz, S., et al. (2022). Impaired ketogenesis ties metabolism to T cell dysfunction in COVID-19. *Nature* *609*, 801–807. <https://doi.org/10.1038/s41586-022-05128-8>.
77. Armeni, E., Aziz, U., Qamar, S., Nasir, S., Nethaji, C., Negus, R., Murch, N., Beynon, H.C., Bouloux, P., Rosenthal, M., et al. (2020). Protracted ketonaemia in hyperglycaemic emergencies in COVID-19: a retrospective case series. *Lancet Diabetes Endocrinol.* *8*, 660–663. [https://doi.org/10.1016/S2213-8587\(20\)30221-7](https://doi.org/10.1016/S2213-8587(20)30221-7).
78. Kanikarla-Marie, P., and Jain, S.K. (2016). Hyperketonemia and ketosis increase the risk of complications in type 1 diabetes. *Free Radic. Biol. Med.* *95*, 268–277. <https://doi.org/10.1016/j.freeradbiomed.2016.03.020>.
79. Elsheikh, M., and Conway, G.S. (1998). The impact of obesity on cardiovascular risk factors in Turner's syndrome. *Clin. Endocrinol.* *49*, 447–450. <https://doi.org/10.1046/j.1365-2265.1998.00552.x>.
80. Martinez-Outschoorn, U.E., Peiris-Pagés, M., Pestell, R.G., Sotgia, F., and Lisanti, M.P. (2017). Cancer metabolism: a therapeutic perspective. *Nat. Rev. Clin. Oncol.* *14*, 113. <https://doi.org/10.1038/nrclinonc.2017.1>.
81. Martinez-Outschoorn, U.E., Lin, Z., Whitaker-Menezes, D., Howell, A., Sotgia, F., and Lisanti, M.P. (2012). Ketone body utilization drives tumor growth and metastasis. *Cell Cycle* *11*, 3964–3971. <https://doi.org/10.4161/cc.22137>.
82. Ogger, P.P., Albers, G.J., Hewitt, R.J., O'Sullivan, B.J., Powell, J.E., Calamita, E., Ghai, P., Walker, S.A., McErlan, P., Saunders, P., et al. (2020). Itaconate controls the severity of pulmonary fibrosis. *Sci. Immunol.* *5*, eabc1884. <https://doi.org/10.1126/sciimmunol.abc1884>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-mouse CD45 AF700	Biologend	Cat 103128; RRID:AB_493715
Anti-mouse CD11c Bv605	Biologend	Cat 117333; RRID:AB_11204262
Anti-mouse SiglecF	Biologend	Cat 562680; RRID:AB_2687570
Anti-mouse CD11b AF594	Biologend	Cat 101254; RRID:AB_2563231
Anti-mouse MHC II APC/Cy7	Biologend	Cat 107628; RRID:AB_2069377
Anti-mouse Ly6C Bv421	Biologend	Cat 128032; RRID:AB_2562178
Anti-mouse Ly6G PerCP/Cy5.5	Biologend	Cat 127616; RRID:AB_1877271
anti- $\beta$ -actin	Sigma	Cat A5441; RRID:AB_476744
OXPHOS rodent WB antibody cocktail	Thermo Fisher Scientific	Cat 45-8099; RRID:AB_2533835
hexokinase 2 polyclonal antibody	Thermo Fisher Scientific	Cat 22029-1-AP; RRID:AB_11182717
Goat anti-Ms IgG HRP	Santa Cruz	Cat sc-2005; RRID:AB_631736
Goat anti-Rb IgG HRP	Santa Cruz	Cat sc-2004; RRID:AB_631746
<b>Biological samples</b>		
CF sputum samples	This study	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
Oligomycin	Selleckchem	S1478
Etomoxir	Sigma Aldrich	E1905
Succinic acid	Sigma Aldrich	14160
D-Glucose	Sigma Aldrich	G8270
Trizol™ Reagent	Thermo Fisher Scientific	15596026
Novex™ 10–20% Tricine Protein Gels	Thermo Fisher Scientific	EC6625BOX
Pro-Q™ Emerald 300 Lipopolysaccharide Gel Stain Kit	Thermo Fisher Scientific	P20495
Live/Dead DAPI dye	Thermo Fisher Scientific	Cat L34962
Formalin-free tissue fixative	Sigma Aldrich	Cat A5472
Paraformaldehyde 32%	Electron Microscopy Sciences	Cat 15714-S
Methanol	Thermo Fisher Scientific	Cat A456-1
Methanol	Alpha Aesar	Cat 22909
Trypan blue stain	Invitrogen	Cat T10282
Glucose	Agilent	Cat 103577-100
collagenase I	Gibco	1100-017
Elastase	Worthington	LS006365
Dispase	Gibco	17105-041
BSA	Thermo Fisher Scientific	BP9705
<b>Critical commercial assays</b>		
Mouse Cytokine Array/Chemokine Array 31-Plex	Evetechnologies	MD31
Seahorse XFe24 FluxPaks	Agilent Technologies	102340-100
RNeasy Mini Kit	Qiagen	Cat 74104
E.Z.N.A.® Total RNA Kit I	Omega Biotek	R6834-01
DNA free removal kit	Thermo Fisher Scientific	AM1906
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	4368813
Power SYBR™ Green PCR Master Mix	Applied Biosystems	4368577
Biolog MitoPlate S-1	Biolog	Cat 14105
Biolog Redox Dye Mix MC	Biolog	Cat 74353

(Continued on next page)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Continued</b>		
<b>Experimental models: Organisms/strains</b>		
Mouse: C57BL/6	Jackson Laboratories	JAX: 000664
Mouse: C57BL/6J/N	Jackson Laboratories	JAX: 005304
Mouse: C57BL/6 <i>Irg1</i> <sup>-/-</sup> ( <i>Acod1</i> <sup>-/-</sup> )	Jackson Laboratories	JAX: 029340
Mouse: B6.129S7-Il1r1tm1Imx/J ( <i>Il1r</i> <sup>-/-</sup> )	Jackson Laboratories	JAX: 003245
<b>Bacterial and virus strains</b>		
<i>P. aeruginosa</i> WT PAO1	Our laboratory	N/A
<i>P. aeruginosa</i> WT PAO1	Provided by Dr. CJ Balibar; Balibar and Grabowicz <sup>38</sup>	N/A
<i>P. aeruginosa</i> $\Delta$ <i>lptD</i> PAO1 (mutant 4213)	Provided by Dr. CJ Balibar; Balibar and Grabowicz <sup>38</sup>	N/A
CF subject 2 <i>P. aeruginosa</i> isolates	Riquelme et al. <sup>32</sup>	N/A
CF subject 1 <i>P. aeruginosa</i> isolates	Provided by Dr. Barbara Kahl	N/A
ICU <i>P. aeruginosa</i> isolates	Provided by Dr. A.C. Uhlemman	N/A
<b>Oligonucleotides</b>		
<i>rpsL</i> -F: CGGCACTGCGTAAGGTATGC	Riquelme et al. <sup>32</sup>	N/A
<i>rpsL</i> -R: CGTACTTCGAACGACCCTGCT	Riquelme et al. <sup>32</sup>	N/A
<i>lptD</i> -F: CCGTCCCTACAACCCAGGTG	Riquelme et al. <sup>32</sup>	N/A
<i>lptD</i> -R: ATGCTGCCGTCGTCATTGAA	Riquelme et al. <sup>32</sup>	N/A
<i>waaL</i> -F: CTACGCCAGATCAGCGAGCA	Riquelme et al. <sup>32</sup>	N/A
<i>waaL</i> -R: CCTCCAGCGAAAAGCACACC	Riquelme et al. <sup>32</sup>	N/A
<i>wbpX</i> -F: GAGACCATCCGCGACGAAGT	Riquelme et al. <sup>32</sup>	N/A
<i>wbpX</i> -R: TCCTCCACCAGGTCCAGCTC	Riquelme et al. <sup>32</sup>	N/A
<i>wbpZ</i> -F: GCTCCGCCAGTACCGAGAAA	Riquelme et al. <sup>32</sup>	N/A
<i>wbpZ</i> -R: ATCACCCCGACGAACAGGAA	Riquelme et al. <sup>32</sup>	N/A
<i>lpxT</i> -F: CTGACCTTCGGCTTCATCGT	Nowicki et al. <sup>65</sup>	N/A
<i>lpxT</i> -R: TGGAGCGGTCCTTGATTTCC	Nowicki et al. <sup>65</sup>	N/A
<i>amT</i> -F: GGCTATGCCAACCTCGACCC	Nowicki et al. <sup>65</sup>	N/A
<i>amT</i> -R: GCGAGGAAGCCCTTGGTCAG	Nowicki et al. <sup>65</sup>	N/A
<b>Software and algorithms</b>		
GraphPad Prism 9	GraphPad Software	<a href="https://www.graphpad.com/">https://www.graphpad.com/</a>
FlowJo X Flow cytometry	FlowJo	<a href="https://www.flowjo.com">https://www.flowjo.com</a>
FIJI	FIJI	<a href="http://imagej.net">http://imagej.net</a>
Seurat v4, Seurat v5	Seurat library	<a href="https://satijalab.org">https://satijalab.org</a>
MetaboloAnalyst 5.0	MetaboloAnalyst	<a href="https://www.metaboloanalyst.ca">https://www.metaboloanalyst.ca</a>
Kyoto Encyclopedia of Genes and Genomes	KEGG	<a href="https://www.genome.jp/kegg/">https://www.genome.jp/kegg/</a>
Adobe Photoshop 24.5.0	Adobe	<a href="https://www.adobe.com/">https://www.adobe.com/</a>
<b>Deposited data</b>		
scRNA-seq lung tissue	GEO	Access number: GSE203352
Raw metabolomics BAL tissue	MetaboloLights	Access numbers: MTBLS4922, MTBLS4923, and MTBLS4924
<i>P. aeruginosa</i> isolates genomes	NIH SRA	Access numbers: PA270 (SRR8775051), PA338 (SRR8775050), PA339 (SRR8775058), PA599 (SRR8775057), PA600 (SRR8775065), PA601 (SRR8775054), PA602 (SRR8775055), PA603 (SRR8775053), PA604 (SRR8775056), PA605 (SRR8775052), PA606 (SRR8775060), PA607 (SRR8775059), PA608 (SRR8775062), PA683 (SRR8775061), PA684 (SRR8775064), PA685 (SRR8775063) and PA686 (SRR8775066).
Raw Data	Data S1	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
RPMI 1640 1x with glutamine	Corning	10-040-CV
Antibiotic Penicillin-Streptomycin	Corning	30-002-CI
Fetal Bovine Serum	Gibco	26140-079
KetoBM Ketone Strips	KBM	ASIN: B087JYC6JD
KetoBM Blood Ketone Meter Kit for Keto Diet Testing	KetoBM	ASIN: B07QWMM4M6
AUVON I-QARE DS-W Draw-in Blood Glucose Test Strips	AUVON	ASIN: B09T6CHZVZ
AUVON DS-W Diabetes Sugar Testing Meter	AUVON	ASIN: B081ZY1LHV
Irradiated Fatty Acid rich diet	Research Diets, Inc	D1007080i
Irradiated Carbohydrate rich diet	Research Diets, Inc	D19082304i
Irradiated Purina regular chow	Purina	5053

**RESOURCE AVAILABILITY**

**Lead contact**

Requests for materials and resources should be directed to and will be fulfilled by the lead contact, Sebastián A. Riquelme ([sr3302@cumc.columbia.edu](mailto:sr3302@cumc.columbia.edu)).

**Materials availability**

Bacterial strains used in this study are available from the lead contact without restriction.

**Data and code availability**

- single-cell RNA-seq data have been deposited at GEO (accession numbers GEO: GSE203352) and are publicly available as of the date of publication. Metabolomics data are available at Metabolights (accession numbers: MTBLS4922, MTBLS4923, and MTBLS4924). Genomes of isolates are available at NIH SRA (access numbers: PA270 (SRR8775051), PA338 (SRR8775050), PA339 (SRR8775058), PA599 (SRR8775057), PA600 (SRR8775065), PA601 (SRR8775054), PA602 (SRR8775055), PA603 (SRR8775053), PA604 (SRR8775056), PA605 (SRR8775052), PA606 (SRR8775060), PA607 (SRR8775059), PA608 (SRR8775062), PA683 (SRR8775061), PA684 (SRR8775064), PA685 (SRR8775063) and PA686 (SRR8775066). The accession numbers are also listed in the [key resources table](#).
- This paper does not report original code.
- [Data S1](#) represents an Excel file containing the values that were used to create all the graphs in the paper, as well as gels in [Figures 1A, 1E, 3C, and 5L](#). Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

**EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**

**Mice experiments**

All animal experiments were performed following institutional guidelines at Columbia University. Mice studies were approved by protocol IACUC AABE8600. 8–10-week-old WT C57bl/6 mice (20–25grs) were obtained from The Jackson Laboratories. *Irg1*<sup>-/-</sup> (*Acod1*<sup>-/-</sup>) and *Ilr1*<sup>-/-</sup> mice were also obtained from The Jackson Laboratories and bred in our facilities at Columbia University Medical Center. WT, *Irg1*<sup>-/-</sup>, and *Ilr1*<sup>-/-</sup> mice are immunocompetent animals, and they received neither medical nor drug treatments prior to infection. Each *in vivo* experiment was performed using 50% female and 50% male animals, and results were not expected to be influenced by sex. Animals were randomly assigned to cages, and maintained under regular rodent light/dark cycles, 18°C–23°C. Unless otherwise indicated, mice were fed with a regular irradiated chow, Purina 5053 (~25% protein, ~62% carbohydrate, ~13% fat).

**Human samples**

Ketones in human samples were measured in sputum from 5 healthy controls (HCs) and 9 subjects with CF, all adults. Male and female samples were collected in an approximate 50%–50% ratio. These samples were kindly provided by Dr. Clemente Britto-Leon from Yale University. An informed consent was signed by all subjects providing samples. These studies were performed under our approved IRB protocol AAAR1395.

### ***P. aeruginosa* strains**

WT PAO1 and  $\Delta$ *lptD* PAO1 were grown in LB overnight and sub-cultured until exponential phase for studies. *P. aeruginosa* strains from CF#1 were from a 10-year chronically infected CF patient. *P. aeruginosa* strains from CF#2 were recovered from a 12-year chronically infected CF individual. All *P. aeruginosa* isolates from CF#1 and CF#2 were part of longitudinal collections. *P. aeruginosa* isolates from 10 ICU patients were obtained at Columbia University Irving Medical Center facilities. These subjects were acutely infected for at least 3–5 days. These studies were performed under our approved IRB protocols AAAR1395 and AAAS6553. All these strains were plated in LB agar and their phenotypes were characterized regarding small colony variants or mucoid morphology. Infection experiments with these clinical isolates were performed from LB subcultures on exponential phase of growth, which derived from overnight grown stocks. Overnight cultures and subcultures were grown at 37°C under shaking.

## **METHOD DETAILS**

### **Oxygen consumption rate measurements in macrophages**

Mouse bone-marrow derived macrophages (BMDMs) were exposed at 37°C for 3h to either PBS, 50ug/ml LPS, WT PAO1, or  $\Delta$ *lptD* PAO1 at multiplicity of infection (MOI) = 10. Then, via Seahorse technology, oxygen consumption rate (OCR) was tracked in these cells using commercially obtained plates as recommended by the manufacturer.

### ***P. aeruginosa* consumption of NADH and FADH2**

Both WT PAO1 and  $\Delta$ *lptD* PAO1 were grown until exponential phase and applied at same concentration to a Biolog 96-well plate array where each well contained a single carbon source (Mitoplate S1). For these assays, we followed the instructions provided by the manufactures. Plates were incubated at 37°C for 48h–72 h and then absorbances were measured at OD<sub>400nm</sub>.

### **Infection of mice with *P. aeruginosa* strains**

Animals used were WT, *Irg1*<sup>-/-</sup> (*Acod1*<sup>-/-</sup>), or *Il1r*<sup>-/-</sup> C57bl/6 mice. Animals were exposed to either PBS, WT PAO1,  $\Delta$ *lptD* PAO1 or the *P. aeruginosa* isolates. When indicated, the bacterial dose varied accordingly with the experiment performed. We used different inoculum:  $\sim 10^5$ ,  $\sim 10^6$  or  $\sim 10^7$  CFUs or PBS alone (non-infected); in the mix of the *P. aeruginosa* isolates, equal parts of each individual strain were used to form the desired inoculum. When indicated, animals were administered or not during the infection either 0.2 mg/kg or 1.0 mg/kg of oligomycin. Similarly, when indicated, 24h prior and during the moment of the infection, animals were treated or not with 20 mg/kg of etomoxir. Survival of mice was monitored for 5 days. When indicated, animal temperature was tracked with an infrared thermometer that measured surface and internal temperature. To evaluate the host immune response to infection, mice were sacrificed 24h after exposure to the pathogens, and BAL and lungs were collected. CFU amounts were quantified by LB agar plating. CFUs below limit of detection ( $10^2$  CFU per tissue) were represented in graphs as “non-detected” (ND). Immune cells (flow cytometry) and cytokines (ELISA) were quantified in BAL and in lungs. Flow cytometry: alveolar macrophages were CD45<sup>+</sup>CD11b<sup>low/-</sup>SiglecF<sup>high</sup>CD11c<sup>+</sup>CD193<sup>-</sup>Ly6G<sup>-</sup>Ly6C<sup>-</sup>, neutrophils were CD45<sup>+</sup>CD11b<sup>high</sup>SiglecF<sup>low/-</sup>CD11c<sup>-</sup>MHCII<sup>-</sup>CD193<sup>-</sup>Ly6G<sup>+</sup>Ly6C<sup>low/-</sup>, and monocytes were CD45<sup>+</sup>CD11b<sup>high</sup>SiglecF<sup>low/-</sup>CD11c<sup>-</sup>MHCII<sup>-</sup>CD193<sup>-</sup>Ly6G<sup>-</sup>Ly6C<sup>low/high</sup>. Samples were analyzed with FlowJo, vX. Cell viability was determined by using live/dead DAPI staining (Thermo Fisher Scientific, L34962). These animals were controlled for ketosis via quantification of  $\beta$ -hydroxybutyrate (BHB) at the beginning of the light cycle (between 8:00a.m.–9:00a.m.) using KetoBM Ketone Strips and the KetoBM Blood Ketone Meter Kit for Keto Diet Testing. We also measured glycemia by using the AUVON I-QARE DS-W Draw-in Blood Glucose Test Strips system together with the AUVON DS-W Diabetes Sugar Testing Meter. When indicated, BAL were analyzed by Mass-Spec for metabolomics studies.

### **Food consumption during infection of mice with *P. aeruginosa* strains**

To track mouse food consumption and weight, mice were individually placed in separated cages (1 animal/cage). After exposure of these animals to PBS or infected either with WT PAO1,  $\Delta$ *lptD* PAO1, or the *P. aeruginosa* isolates (from CF#2), we monitored food consumption by weighting the amount of food before and after infection, every 24h. We performed an exhaustive examination of each cage to confirm that the food counted as consumed was not found in the cage. Similarly, we tracked daily animal weight using a digital scale.

### **Airway metabolomics**

BAL from either non-infected or 24h-infected mice were collected with 3mL of sterile PBS. Samples were immediately placed on ice. Then, samples were diluted with 100% methanol in a 1:1 proportion, mixed and stored at  $-80^\circ\text{C}$  for future metabolomics analysis. Just prior to mass spectrometry, samples were thawed and dried under a stream of N<sub>2</sub> and were resuspended in HPLC-grade water at a 4:1 dilution (relative to the original BAL volume). High-resolution mass spectrometry data were acquired on a Thermo Fisher Exactive Mass spectrometer in negative mode using 25 min reverse phase gradients and ion-pairing chromatography. Metabolites were identified using the known chromatographic retention times of standards, and metabolite signals were quantified using MAVEN. Metabolite signal intensities were used to quantify difference between treatments in BAL and respect to PBS-treated animals.

### **Lung tissue histopathology studies**

Mice aged 8–10 weeks were intranasally exposed to either PBS, WT PAO1, or the *P. aeruginosa* isolates. Then, 24h later, animals were sacrificed and a canula was inserted into the trachea and tied with suture. The thorax was opened, and the lungs

were fixed by gentle infusion of formalin-free tissue fixative through the canula from a syringe positioned 5cm above the mouse. The whole lungs were excised and placed in formalin-free tissue fixative for 24h before transfer to 70% ethanol. The tissue was paraffinized, and two 5mm sections were taken 25mm apart. The slices were then stained with hematoxylin and eosin and scored by a pathologist, who was blinded to the sample identities. Immune cell infiltration and edema were examined by light microscopy.

### Diets for *in vivo* assays

When indicated, animals were fed a ketogenic diet (D10070801i, Research Diets, Inc) or a carbohydrate-rich control diet (D19082304i, Research diets, Inc) for 1 week (*ad libitum*). Ketogenic diet contained ~10% protein, ~0.1% carbohydrate, ~88.9% fat (%Kcal). Control diet contained ~10% protein, ~80% carbohydrate, ~10% fat (%Kcal). After one week, mice were intranasally infected with ~10<sup>6</sup> CFU of the *P. aeruginosa* isolates, WT PAO1, or  $\Delta$ *lptD* PAO1. 24h later, animals were processed. When indicated, blood was obtained from the tail of these animals and glycemia and ketosis were measured by using draw-in blood test strips for glucose and  $\beta$ -hydroxybutyrate, respectively.

### Mouse lung processing for single-cell RNA-sequencing analysis

WT or *Irg1*<sup>-/-</sup> C57bl/6 mice (8–10 week old) were treated with PBS or infected either with WT PAO1 or the *P. aeruginosa* clinical isolates (~10<sup>6</sup> total CFU per mouse in 50 $\mu$ L). 24h after infection, mice were euthanized, their lungs were harvested, and single cell suspensions of the lungs were prepared as described. Briefly, the lungs were placed in an Eppendorf tube containing an enzymatic digestion solution of collagenase I (2 mg/mL), dispase (20 mg/mL), elastase (1 mg/mL), and DNase (1  $\mu$ L/mL) in PBS. The lungs were minced within the tube and then incubated with shaking at 37°C for 30 min. 4 volumes of PBS supplemented with 10% FBS was added to quench the digestion, and the digestion solution was strained over a 70-micron filter. The cell suspension was spun down at 4°C and 1400 rpm for 7 min. Red blood cell lysis was performed using the Invitrogen RBC lysis buffer. The resulting cell pellet was resuspended in PBS supplemented with 0.04% BSA before being loaded onto the 10X Genomics Chromium Single Cell Controller. Cell viability analysis was performed before loading the samples, and was above 95% per sample. A total of ~8000 cells were analyzed per sample. FASTQ file generation, alignment, filtering, barcode counting, and UMI counting were done using the 10X Cell Ranger software.

### Single cell RNA-sequencing analysis

Matrix data generated by scRNA-seq were analyzed using the Seurat library (<https://satijalab.org>) in R Studio (<https://www.rstudio.com>). Data were cleaned for mitochondrial RNA (less than 5% per sample), and unbiased cell clustering was performed. The Seurat algorithm identified 23 cell clusters with unique transcriptomic signatures. To determine the biological nature of each one of these clusters, we did comparative analyses of the transcriptomic profile of each cluster with available databases using the SingleR algorithm (using ImmGen as the reference dataset). In addition, this cluster characterization was manually cross-checked with cell markers from publicly available datasets using the top 10 markers for each cluster. Scores for ketogenic routes were calculated using the AddModuleScore function in Seurat. Briefly, ketogenic scores included the following genes: *Hmgcs2*, *Bdh1*, *Hmgcl*, *Acat1*, *Ppara* and *Cpt1a*. We calculated ketogenic scores for each either WT or *Irg1*<sup>-/-</sup> pulmonary subset, exposed or not to WT PAO1 or the *P. aeruginosa* isolates. Then, via a MAST statistical test, we determined differences between treatments in the same pulmonary subset, and computed a “-Log(p-value)”. To establish if *Irg1* promoted enrichment of decrease of this ketogenic score in a specific pulmonary subset, we subtracted the ketogenic score obtained in the *Irg1*<sup>-/-</sup> background from WT controls ( $\Delta$ ketogenesis score (“*Irg1*<sup>+/+</sup>”-“*Irg1*<sup>-/-</sup>”). Then, for each pulmonary cell type, we performed volcano plots illustrating in the Y axis the “-Log(p-value)” and in the X coordinate the  $\Delta$ ketogenesis score.

### qRT-PCR for genes in *P. aeruginosa* exposed to ketones

WT PAO1 was grown in LB overnight and then subcultured until exponential phase in M9 supplemented with glucose, glucose + BHB, glucose + AcAc, or glucose + BHB + AcAc (7mM each metabolite). Total RNA was extracted using either the E.Z.N.A. Total RNA Kit1 or RNeasy Mini Kit. The RNA was then processed with DNase. The RNA concentration was measured using NanoDrop One. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and qPCR was performed with a StepOnePlus Real-Time PCR System (Applied Biosystems) using POWER SYBR Green PCR Master Mix (Applied Biosystems). Sequences of the primers used for qRT-PCRs are listed in Key Resource Table. Relative gene expression was calculated by the 2<sup>- $\Delta\Delta$ CT</sup> method. The *rpsL* transcript was used as a reference housekeeping gene, and the WT PAO1 strain grown in glucose was used as a calibrator. When studying clinical isolates, these strains were grown in LB overnight and then subcultured in the same conditions until exponential phase. Then, total RNA extracted and qRT-PCR were performed.

### LPS assembly studies in WT PAO1

To visualize the O-antigen and lipid A in WT PAO1 exposed to ketones, LPS was first isolated using a hot phenol extraction from overnight cultures normalized to OD<sub>600</sub> = 0.5 after 1:10 dilution. Samples were treated with DNase and RNase for 30 min at 37°C and Proteinase K at 59°C overnight. After hot phenol extraction with Trizol, the isolated LPS was run on a Tricine 10%–20% gel. The gel was then stained using Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit and imaged using UV light on a Protein Simple imager.

### Pathway analysis of airway fluids

Curated metabolomics data from airway fluids of both control and bacteria-exposed mice were submitted to MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>). Data were analyzed through Enrichment Analysis, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as reference. To maintain unbiased interpretation of the data, we utilized the graphs provided by the platform.

### *P. aeruginosa* lipid A isolation and analysis

*P. aeruginosa* WT PAO1 was cultured overnight in LB, then washed, and subcultured in M9 media containing either glucose (7mM) alone or glucose (7mM), BHB (7mM), and AcAc (7mM). Bacteria were grown until exponential phase at 37°C with shaking. Lipid A was extracted from cell pellets using an ammonium hydroxide-isobutyric acid-based procedure. Approximately 5 mL of cell culture was pelleted and resuspended in 400  $\mu$ L of 70% isobutyric acid (Sigma, I1754) and 1 M ammonium hydroxide (Sigma, 221228), (5:3 v/v). Samples were incubated for 1 h at 100°C and centrifuged at 8,000  $\times$  g for 5 min. Supernatants were collected, added to endotoxin-free water (1:1 v/v), snap-frozen on dry ice, and lyophilized overnight. The resultant material was washed twice with 1 mL methanol (Thermo Fisher Scientific, A456-1), and lipid A was extracted using 80  $\mu$ L of a mixture of chloroform (Thermo Fisher Scientific, C606SK-4), methanol, and water (3:1:0.25 v/v/v). Once extracted, 1  $\mu$ L of the concentrate was spotted on a steel re-usable MALDI plate (Hudson Surface Technology, PL-PD-000040-P) followed by 1  $\mu$ L of 10 mg/mL norharmane matrix (Sigma, NG252) in chloroform-methanol (2:1 v/v, Sigma) and air-dried. All samples were analyzed on a Bruker Microflex mass spectrometer (Bruker Daltonics) in the negative-ion mode with reflectron mode. An electrospray tuning mix (Agilent, G2421A) was used for mass calibration. Spectral data were analyzed with Bruker Daltonics FlexAnalysis software (v4.30). The resulting spectra were used to estimate the lipid A structures present in each condition based on their predicted structures and molecular weights.

### Lung protein extraction and western blots

Total lung tissue was removed from WT mice exposed for 24h to either PBS, WT PAO1,  $\Delta$ *lptD* PAO1 or the *P. aeruginosa* isolates. Single cell fractions were prepared, washed, and resuspended for 30min in 300 $\mu$ L of RIPA buffer containing protease/phosphatase inhibitors at 4°C. Samples were centrifuged for 25 min at 15,000g and the supernatant was recovered and stored at  $-80^{\circ}$ C. To perform western blots, samples were denatured at 37°C for 20min and ran in 4–12% gels in MES buffer. To stain actin, we used a monoclonal anti- $\beta$ -actin antibody produced in mouse (Sigma, Cat A5441). To mark OXPHOS components, we used an OXPHOS rodent WB antibody cocktail (Thermo Fisher Scientific, 45–8099). To detect hexokinase-2, we used a hexokinase 2 polyclonal antibody (Thermo Fisher Scientific, 22029-1-AP). Membranes were transferred using iblot and stained overnight with primary antibodies. Then, samples were washed and incubated for 1h with the HRP-tagged secondary antibody. Membranes were revealed and analyzed for protein expression.

### Whole-genome sequencing and single-nucleotide polymorphism (SNP) calling in *P. aeruginosa* isolates

*P. aeruginosa* isolates were grown overnight in LB broth at 37°C. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer instructions. Genomic DNA libraries were prepped using the Nextera XT kit and sequenced on a HiSeq 2500 sequencer (Illumina) with 125-bp paired-end reads. Genome assembly was done using the paired-end implementation of ABySS. For single-nucleotide polymorphism (SNP) calling, we compared SNPs between isolates and WT PAO1. This was done by means of short-read alignment to the genome of PAO1 as reference (GenBank: AE004091), using the Burrows–Wheeler alignment (bwa) tool (available at: <http://bio-bwa.sourceforge.net>). SNP calls were made using samtools (available at: <http://samtools.sourceforge.net>). SNPs that were not listed as heterozygous and had a per base Q score  $\geq 20$  were defined as high quality. Samples can be found at NIH SRA with the following codes: PA270 (SRR8775051), PA338 (SRR8775050), PA339 (SRR8775058), PA599 (SRR8775057), PA600 (SRR8775065), PA601 (SRR8775054), PA602 (SRR8775055), PA603 (SRR8775053), PA604 (SRR8775056), PA605 (SRR8775052), PA606 (SRR8775060), PA607 (SRR8775059), PA608 (SRR8775062), PA683 (SRR8775061), PA684 (SRR8775064), PA685 (SRR8775063) and PA686 (SRR8775066).

### QUANTIFICATION AND STATISTICAL ANALYSIS

We modeled the number of independent experiments required to reach significance between groups by using JMP, a computer program dedicated for statistical analysis. We based this simulation on preliminary data, experimental design, and past experience. These analyses were done assuming a 20% standard deviation (SD), and equivalent variances within groups. Significance ( $p < 0.05$ ) with power 0.8 was used. Experiments in this study were not performed in a blinded fashion. All analyses and graphs were performed using the GraphPad Prism 9 software. Data in graphs are shown as average  $\pm$ SEM and data were assumed to fit normal distribution. For comparison between average values for more than 2 groups, we performed One-Way ANOVA with a multiple posteriori comparison. When studying two or more group along time, data were analyzed using two-Way ANOVA with a multiple posteriori comparison. Differences between two groups in samples' average values were analyzed using analysis of variance (parametric) or Student's *t* test for normally distributed data or Mann-Whitney or Kruskal-Wallis test otherwise. Differences were considered significant when *p* value (two-sides) was under 0.05 ( $p < 0.05$ ). Significances *p* values and amounts of independent experiments and replicates are indicated in each Figure Legend.