HIF1A-dependent induction of alveolar epithelial PFKFB3 dampens acute lung injury

Christine U. Vohwinkel, …, Rubin M. Tudor, Holger K. Eltzschig

*JCI Insight.* 2022. [https://doi.org/10.1172/jci.insight.157855](https://doi.org/10.1172/jci.insight.157855).

Acute lung injury (ALI) is a severe form of lung inflammation causing acute respiratory distress syndrome in patients. ALI pathogenesis is closely linked to uncontrolled alveolar inflammation. We hypothesize that specific enzymes of the glycolytic pathway could function as key regulators of alveolar inflammation. Therefore, we screened isolated alveolar epithelia from mice exposed to ALI induced by injurious ventilation to assess their metabolic responses. These studies pointed us towards a selective role for isoform 3 of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3). Pharmacologic inhibition or genetic deletion of *Pfkfb3* in alveolar epithelia (*Pfkfb3*<sup>loxp/loxp</sup> SPC-ER-Cre<sup>+</sup> mice) was associated with profound increases in ALI during injurious mechanical ventilation or acid installation. Studies in genetic models linked Pfkfb3 expression and function to hypoxia-inducible factor Hif1a. Intra-tracheal pyruvate instillation not only reconstituted *Pfkfb3*<sup>loxp/loxp</sup> or *Hif1a*<sup>loxp/loxp</sup> SPC ER Cre<sup>+</sup> mice, but pyruvate was also effective in ALI treatment of wild-type mice. Finally, proof-of-principle studies in human lung biopsies confirmed increased PFKFB3 staining in injured lungs and co-localized PFKFB3 to alveolar epithelia. These studies reveal a specific role for PFKFB3 in counter-balancing alveolar inflammation and lay the groundwork for novel metabolic therapeutic approaches during ALI.
HIF1A-dependent induction of alveolar epithelial PFKFB3 dampens acute lung injury

Christine U. Vohwinkel¹,², Nana Burns¹,², Ethan Coit¹,², Xiaoyi Yuan³, Eszter K. Vladar⁴, Christina Sul², Eric P. Schmidt⁴, Peter Carmeliet⁵, Kurt Stenmark¹,², Eva S. Nozik¹,², Rubin M. Tuder¹,⁴, and Holger K. Eltzschig³

¹Cardio Vascular Pulmonary Research Laboratories, University of Colorado School of Medicine, Aurora, CO, USA
²Departments of Pediatrics, Division of Pediatric Critical Care, University of Colorado, School of Medicine, Aurora, CO, USA
³Department of Anesthesiology, Critical Care and Pain Medicine, University of Texas Health Science Center Houston, Houston, TX, USA
⁴Program in Translational Lung Research, Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado School of Medicine, Aurora, CO, USA
⁵Laboratory of Angiogenesis and Vascular Metabolism, Department of Oncology and Leuven Cancer Institute (LKI), KU Leuven, VIB Center for Cancer Biology, VIB, Leuven, Belgium, Laboratory of Angiogenesis and Vascular Heterogeneity, Department of Biomedicine, Aarhus University, Aarhus 8000, Denmark and Center for Biotechnology, Khalifa University of Science and Technology, Abu Dhabi, United Arab Emirates

Corresponding Author:
Christine U Vohwinkel, M.D., Ph.D.
Associate Professor
Developmental Lung Biology, Cardio Vascular Pulmonary Research Laboratories
Division of Pediatric Critical Care
Departments of Medicine and Pediatrics
University of Colorado, Anschutz Medical Campus, Aurora, CO, United States;
Phone: (+1) 720-777-1234
Email: Christine.Vohwinkel@cuanschutz.edu

Running title: PFKFB3 attenuates alveolar inflammation
Word Count: Abstract: 186 words; Manuscript: 11996 words
Keywords: Acute respiratory distress syndrome, ARDS, acute lung injury, ALI, alveolar epithelium, hypoxia-inducible factor, HIF, metabolism, glycolysis, PFKFB3, inflammation
Conflict of Interest Statement:
The co-author PC is named as an inventor on patent applications related to results discussed in this manuscript
Abstract:

Acute lung injury (ALI) is a severe form of lung inflammation causing acute respiratory distress syndrome in patients. ALI pathogenesis is closely linked to uncontrolled alveolar inflammation. We hypothesize that specific enzymes of the glycolytic pathway could function as key regulators of alveolar inflammation. Therefore, we screened isolated alveolar epithelia from mice exposed to ALI induced by injurious ventilation to assess their metabolic responses. These studies pointed us towards a selective role for isoform 3 of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3). Pharmacologic inhibition or genetic deletion of Pfkfb3 in alveolar epithelia (Pfkfb3lox/lox SPC-ER-Cre+ mice) was associated with profound increases in ALI during injurious mechanical ventilation or acid installation. Studies in genetic models linked Pfkfb3 expression and function to hypoxia-inducible factor Hif1a. Intra-tracheal pyruvate instillation not only reconstituted Pfkfb3lox/lox or Hif1alox/lox SPC ER Cre+ mice, but pyruvate was also effective in ALI treatment of wild-type mice. Finally, proof-of-principle studies in human lung biopsies confirmed increased PFKFB3 staining in injured lungs and co-localized PFKFB3 to alveolar epithelia. These studies reveal a specific role for PFKFB3 in counter-balancing alveolar inflammation and lay the groundwork for novel metabolic therapeutic approaches during ALI.
Introduction

Acute lung injury (ALI) is a severe form of lung inflammation with its clinical manifestation as acute respiratory distress syndrome (ARDS) in patients (1, 2). ARDS is characterized by an acute onset of hypoxemia in conjunction with pulmonary edema, which is not explained entirely by fluid overload or cardiac disease (3). The ongoing COVID-19 pandemic highlights the impact of ARDS (2, 4). Moreover, studies demonstrated that ARDS survivors have significant long-term sequelae, which leads to increased utilization of healthcare resources (5). Despite an improved understanding of ARDS pathogenesis, current therapies are mainly symptomatic, which accounts for the plateauing of mortality since the mid 1990’s despite considerable advances in the study of ARDS(6, 7). Therefore, targeting novel therapeutic approaches to dampen or resolve (8, 9) alveolar inflammation as treatment approaches for ARDS are currently areas of intense research (10-13).

The alveolar epithelium is composed of type I and type II alveolar epithelial cells (14), although alveolar type I (AT I) cells comprise a higher percentage of the lung surface area, both cell types are found in similar numbers in the lung (15). Alveolar type II (ATII) cells play a critical part in ALI, which has been established in experimental models of lung insults (16). Recent studies have emphasized their critical role in repair processes of the disrupted epithelial surface, which are initiated immediately after epithelial injury (17). The alveolar epithelium has a critical role in coordinating alveolar inflammation responses (10, 11). Alveolar inflammation plays a key role in the pathogenesis of ALI (18). During alveolar inflammation, profound shifts in the supply and demand ratios for metabolites are known to occur (19, 20). Under normal circumstances, energy demands of pulmonary tissue compartments are met predominantly by oxidative phosphorylation... Alveolar epithelium is known for its capacity to divert pyruvate away from oxidative phosphorylation (12, 20, 21). This metabolic switch to high glycolytic rates and glucose
dependency was first described by Otto Warburg and has gained significant attention in the context of cancer metabolism and hypoxia signaling (22). Emerging evidence indicates that the high demand for biosynthetic precursors as well as the increased energy turnover of cells responding to infection or inflammation is in fact also met by a similar metabolic switch, for example in ARDS (21, 23, 24).

Several recent studies show that during inflammatory conditions such as occur during ARDS, alveolar epithelia can stabilize HIFs to optimize their carbohydrate metabolism (12) or to promote their ability to repair the injured alveolus following injury (25).

We addressed highly specific components of the glycolytic pathway could play selective and functionally critical roles during alveolar inflammation. Consistent with our hypothesis, we found that HIF1A drives the induction of the isoform 3 of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) leading to an enhanced capacity for glycolysis as an endogenous protective pathway by which excessive alveolar inflammation is counterbalanced during ARDS.
Results

Alveolar-epithelial levels of glycolytic enzymes and glycolysis intermediates are elevated during ALI.

Previous studies suggested that alveolar epithelia undergo metabolic adaptation during stress conditions (12). Therefore, we performed studies to gain insight into specific checkpoints of alveolar epithelial carbohydrate metabolism during lung injury. Based on a previously described protocol (12, 26), we exposed mice for 4 hours to injurious mechanical ventilation (pressure controlled ventilation, inspiratory pressures of 45 mbar at 100% FiO\textsubscript{2}; PEEP of 3 mbar) to induce ALI. We isolated ATII cells for molecular analysis of metabolic responses (Figure 1A). For controls, we used isolated alveolar epithelia from mice that were mechanically ventilated in a similar setting using pressure-controlled ventilation at 15 mbar. We performed a targeted qPCR screen of carbohydrate metabolism (84 genes encoding key enzymes of carbohydrate metabolism). Genes encoding the glycolytic pathway experienced the highest degree of transcriptional induction of the metabolic pathways (Figure 1B). Measurements of metabolic intermediates using mass-spectrometry confirmed very robust increases of glycolytic intermediates in murine primary alveolar epithelia exposed to ALI (Figure 1C). In addition, we exposed cultured murine primary alveolar epithelial cells to cyclic mechanical stretch – an in vitro model of alveolar injury resembling injurious mechanical ventilation (12). These in vitro studies of alveolar stretch injury confirmed robust increases in glycolytic intermediates, including glucose-6-phosphate, pyruvate, and lactate (Supplementary Figure 1A). Taken together, these findings demonstrate that during ALI, alveolar epithelia increase transcription of
their glycolytic enzymes and experience elevated concentrations of glycolysis intermediates in response to injury or stretch.

Selective induction of the PFKFB iso-enzyme during ALI exposure.

We examined the relative contribution of the individual control points of the glycolytic pathway. We studied transcriptional changes for individual glycolytic enzymes in ATII cells of mice exposed to ALI. In agreement with our array studies (Figure 1B), we observed that the glycolytic enzymes glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), enolase (ENO), hexokinase 3 (HK3), and lactate dehydrogenase A (LDHA) were robustly induced. We found the most profound increase in transcript levels for phosphofructokinase (PFK) (Figure 1D). PFK catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate and has been shown to function as a key regulator of the glycolytic pathway(27). PFK forms a regulatory subunit with phosphofructokinase-2/fructose-2,6-bisphosphatase (PFKFB) (28). PFKFB is known for its critical role in enhancing glycolytic flux rates by producing fructose 2,6, bisphosphate, an allosteric activator of glycolysis, leading to an increased activity of downstream glycolytic enzymes (29). Interestingly, we found that injurious mechanical ventilation was associated with a highly selective induction of the isoform 3 of PFKFB in ATII cells (Figure 1E). In contrast, expression of other PFKFB isoforms (PFKFB1,2 or 4) transcription was unaltered in ATII cells isolated from mice exposed to ALI induced by injurious mechanical ventilation. In addition to transcriptional induction, PFKFB3 protein level (Figure 1F-H) were increased after ALI induced by mechanical ventilation. Moreover, in vitro studies of cyclic mechanical stretch of murine alveolar epithelia (MLE-12) were associated with increased PFKFB3 activity (Figure 1I). We previously showed that succinate was protective in ALI (20), we examined whether succinate
supplementation could directly trigger PFKFB3 activation. However, we found that supplementation with cell permeable dimethlysuccinate did not affect PFKFB3 activity (supplementary Figure 1B). Expanding our studies to an additional model of lung injury we used acid-induced ALI, which recapitulates ALI secondary to gastric aspiration in human patients. For these studies, we instilled 50µl of 0.125 M hydrochloric acid (HCl) intratracheally into the lungs of anesthetized mice and harvested the lungs after 3 days. Similar to findings in ALI induced by mechanical ventilation, we found selective induction of PFKFB3 transcript levels in ATII cells isolated 3 days after acid induced ALI (Supplementary Figure 1C). Moreover, PFKFB3 protein were elevated three days after acid installation by Western Blot and immuno-histological staining (Supplementary Figure 1D and E). Taken together, these findings demonstrate a selective induction of alveolar epithelial PFKFB3 following cyclic mechanical stretch exposure in vitro, or during murine ALI induced by injurious mechanical ventilation or acid instillation in vivo.

**Pharmacologic inhibition of PFKFB3 is associated with increased alveolar inflammation.**

We investigated whether pharmacologic PFKFB3 inhibition affects lung inflammation and outcomes in ALI models. We treated wildtype mice with the PFKFB3 inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (30). Based on previous studies (30), mice received 70mg/kg intra-peritoneally 24 hours prior to initiation of ALI by injurious mechanical ventilation (Figure 2A). We found that mice treated with 3PO experienced exacerbation of lung injury including increased protein levels in the bronchoalveolar lavage fluid (BALF), elevated mRNA expression of pro-inflammatory cytokines IL-6 and CXCL-1 in their lungs (Figure 2B-D), leading to exacerbated histologic lung injury, and histologic ALI scores (Figure 2 E, F).
Similarly, we observed that mice subjected to acid aspiration induced ALI experienced a more severe form of ALI when pre-treated with 3PO 24 hours prior to induction of ALI as compared to treatment with vehicle control (Supplementary Figure 2 A-F). Taken together, these pharmacologic studies support that PFKFB3 activation protects against with ALI.

**Pfkfb3lox/lox SPC-Cre-ER+ experience more severe ALI during injurious mechanical ventilation or following acid aspiration.**

We next pursued studies using a genetic model that would allow us to determine specifically the functional contribution of ATII cell specific PFKFB3 during ALI. Since germline deletion of PFKFB3 is associated with embryonic lethality (31), we applied a selective deletion of *Pfkfb3* specifically in ATII cells in adult mice. We crossbred previously described *Pfkfb3lox/lox* mice (32) with an ATII specific driver line (SPC-Cre-ER+) (Supplementary Figure 3A) (32). Successful deletion of PFKFB3 protein was confirmed in isolated alveolar epithelia of *Pfkfb3lox/lox SPC-Cre-ER+* mice following tamoxifen treatment when compared to SPC-Cre-ER+) control mice by Western blotting (Supplementary Figure 3B). When housed in a pathogen-free animal facility, *Pfkfb3lox/lox SPC-Cre-ER+* mice were of normal weight (Supplementary Figure 3C), had normal sized litters, and did not display any apparent immunologic deficits or malformations. Analysis of relative expression levels of *Pfkfb* isoforms in isolated ATII cells confirmed the successful deletion of *Pfkfb3*, while other *Pfkfb* isoforms (Pfkfb1, Pfkfb2 or Pfkfb4) were unaltered in their expressional profile (Figure 3A).

We exposed *Pfkfb3lox/lox SPC-Cre-ER+* mice or littermate SPC-Cre-ER+ controls matched in age, sex and weight to ALI induced by injurious mechanical ventilation following tamoxifen-induction of Cre-recombinase. First, the analysis of glycolytic metabolites in isolated
ATII cells revealed no differences between alveolar epithelia isolated from $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice or controls at baseline (Figure 3B-D). However, the increased levels of the glycolysis intermediates glucose-6-phosphate, pyruvate, or lactate levels in response to injury in control mice were completely abolished in alveolar epithelia isolated from $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice (Figure 3B-D). Moreover, we observed that $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice exhibited a far more severe phenotype during ALI as compared to control mice, as evidenced by a greater increase in BALF protein content (Figure 3E), higher elevations of the transcript levels of IL-6 and CXCL-1 in their lung (Figure 3F-G) and higher levels of pro-inflammatory cytokines in the BALF (Figure 3H-K). We did not find an apparent difference in lung inflammation comparing male or female $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice (supplementary Figure 3D, E), it appears that sex as biological variable does not play a major role in this response. Furthermore, the histologic analysis of ALI-associated alveolar injury revealed that $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice experienced a more severe form of histologic ALI and ALI scoring compared to controls (Figure 3 L, M). Of note, in response to ALI induced by mechanical ventilation, $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice frequently showed worse alveolar hemorrhage than the SPC-Cre-ER controls. Finally, $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice when subjected to ALI induced by mechanical ventilation displayed a marked decrease in survival time compared to control SPC-Cre-ER mice (Figure 3N). Together these findings provide the first genetic evidence for a protective role for $Pfkfb3$ expressed in alveolar epithelia during murine models of ARDS.

We exposed $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice or corresponding littermate SPC-Cre-ER+ controls matched by age, sex and weight to acid-induced ALI, following tamoxifen-mediated induction of Cre-recombinase. Similar to the above studies in ALI induced by mechanical ventilation, we found that $Pfkfb3^{loxp/loxp}$ SPC-Cre-ER+ mice showed a far more severe phenotype.
of alveolar inflammation and lung injury as compared to controls. Pfkfb3\textsubscript{loxp/loxp} SPC-Cre-ER+ mice experienced increased albumin leakage into their BALF after 1 or 3 days following intratracheal installation of hydrochloric acid. Similarly, production of pro-inflammatory cytokines transcript levels (Figure 4B-D) was increased in the lungs. Also, pro-inflammatory cytokine release into the BALF (Figure 4D-G) was elevated. Moreover, we observed a more severe degree of alveolar inflammation and lung injury as evidenced on representative histologic slides (Figure 4H) further confirmed by blinded scoring for lung injury severity in Pfkfb3\textsubscript{loxp/loxp} SPC-Cre-ER+ mice (Figure 4I). Additionally, we investigated whether alveolar epithelial deletion of Pfkfb3 affects the recruitment of inflammatory cells to the lung. We found no difference in neutrophil and monocyte/macrophage recruitment to the BALF in both IMV and acid induced lung injury in Pfkfb3\textsubscript{loxp/loxp} SPC-ER-Cre+ mice compared to SPC-ER-Cre+ controls (Supplementary Figure 3F, G).

Taken together, these studies, provide genetic in vivo evidence for a critical role of alveolar-specific Pfkfb3 in dampening alveolar inflammation during ALI induced by detrimental mechanical ventilation or during acid aspiration.

**Identification of alveolar-epithelial Hif1a as an upstream regulator of Pfkfb3-mediated lung protection.**

Previous studies had shown that the Pfkfb3 gene contains a promotorm sequence for HIF1A (33), and established a functional role for HIF1A as a regulator of the glycolytic pathway - including PFKFB3 - under conditions of limited oxygen availability (19, 34). To examine the potential functional role of HIF1A in regulating PFKFB3 during alveolar inflammation, we first performed studies to address alveolar-epithelial HIF1A protein stabilization during ALI. For this
purpose, we isolated alveolar epithelial cells after ALI induced by either mechanical ventilation or acid aspiration and examined them for HIF stabilization. Consistent with previous studies of alveolar epithelia exposed to cyclic mechanical stretch, or whole lungs of mice exposed to ALI (12), we observed robust stabilization of HIF1A protein in the nuclear fraction derived from alveolar epithelial cell lysate in response to ALI (Figure 5A, B). We performed a targeted mRNA screen of carbohydrate metabolism to examine a role of HIF1A in regulating specific glycolytic enzymes in alveolar epithelial cells during ALI. For this purpose, we utilized previously described mice with tamoxifen-inducible deletion of Hif1a (Hif1a<sup>loxp/loxp</sup> Ubc Cre+ mice) (35), since mice with homozygote deletion of Hif1a die during early embryogenesis (36). Ubc Cre+ mice matched in age, sex and weight were used as controls following tamoxifen induced Cre stabilization (37). To address a potential role for alveolar-epithelial Hif1a in regulating PFKFB3 during ALI, we subjected the mice to ALI induced by injurious mechanical ventilation over 4 hours, and subsequently isolated alveolar epithelia. Pathway analysis demonstrated that the majority of differentially regulated genes were regulators of the glycolysis (Figure 5C). Furthermore, a subsequent analysis using real-time PC confirmed that specific glycolytic enzymes identified in wild type mice (Figure 1D) failed to upregulated in response to IMV in the Hif1a<sup>loxp/loxp</sup> Ubc Cre+ mice (Figure 5D).

We next performed studies in mice with Hif1a deletion specifically in alveolar epithelial cells of the lungs (20). We had previously generated mice with alveolar specific deletion of Hif1a (Hif1a<sup>loxp/loxp</sup> SPC-Cre-ER+) and found that they were more susceptible to ALI induced by injurious ventilation (20). Consistent with those previously published findings, we show here that when we expose Hif1a<sup>loxp/loxp</sup> SPC-Cre-ER+ mice to ALI induced by installation of hydrochloric acid, the mice experienced more severe lung inflammation (Supplementary Figure 4A, B) and
more severe histologic tissue injury and ALI scoring (Supplementary Figure 4C, D). We examined Pfkfb3 transcript levels Hif1a^loxp/loxp SPC-Cre-ER+ mice in response to ALI induced by either mechanical ventilation or acid aspiration. These studies revealed that increases in Pfkfb3 transcript levels with ALI induced by mechanical ventilation or acid installation were completely abolished in alveolar epithelia isolated from Hif1a^loxp/loxp SPC-Cre-ER+ mice compared to controls (Figure 5E, F). Similarly, elevations of the glycolytic intermediates glucose-6-phosphate, pyruvate or lactate were dampened in alveolar epithelia isolated from Hif1a^loxp/loxp SPC-Cre-ER+ exposed to ALI (Figure 5G-I). Taken together, these findings indicate that PFKFB3 is transcriptionally regulated by HIF1A during ALI, and functions to enhance the glycolytic capacity of alveolar epithelia, thereby dampening alveolar inflammation.

**Reconstitution of mice with alveolar epithelial deletion of Pfkfb3 or Hif1a.**

As proof of principle for the assertion that PFKFB3-driven glycolysis in alveolar epithelia plays a functional role in alveolar inflammation during ARDS, we first pursued studies to reconstitute the phenotype we had observed in Pfkfb3^loxp/loxp SPC-Cre-ER+ mice. PFKFB3 is a critical downstream regulator of glycolysis and Pfkfb3^loxp/loxp SPC-Cre-ER+ mice show decreased glycolytic intermediates downstream of PFKFB3 in alveolar epithelial cells (Figure 3C, D). For the purpose of these studies, we used the glycolysis intermediate pyruvate, which we had found to be deficient in Pfkfb3^loxp/loxp SPC-Cre-ER+ mice exposed to ALI induced by mechanical ventilation (Figure 3C). Moreover, previous studies that used systemic treatment approaches with pyruvate in mice to induce torpor, showed that pyruvate treatment was well tolerated by the experimental animals (38). In order to achieve delivery to alveolar epithelia, we chose the intra-tracheal application route. We used a dose of 200mg/kg of bodyweight i.t. (highest possible concentration that could be dissolved in 50µl, which is the maximum i.t. instillation volume that
is well tolerated (39) 15 min prior to the induction of ALI by injurious mechanical ventilation in $Pfkfb3^{lox/lox}$ SPC-Cre-ER+ mice or littermate Cre+ controls matched in age, sex and weight following tamoxifen induction (Figure 6A). Treatment-free control mice received an equal volume of vehicle, that was pH controlled to that of pyruvate. These studies demonstrated that pyruvate normalized the phenotype of $Pfkfb3^{lox/lox}$ SPC-Cre-ER+ mice, with attenuated lung inflammation (IL-6 and CXCL-1, Figure 6B, C), and attenuated histologic tissue injury and ALI scores (Figure 6D, E). Moreover, these studies also demonstrated the therapeutic effects in control animals demonstrating improvements in all the above assessments of ALI, including improved survival time on the ventilator (Figure 6B-F), suggesting that intra-tracheal treatment with pyruvate attenuates alveolar inflammation during ALI.

We also performed reconstitution studies using intra-tracheal pyruvate treatment of $Hif1a^{lox/lox}$ SPC-ER-Cre+ mice. Similar to mice with alveolar-epithelial deletion of $Pfkfb3$, $Hif1a^{lox/lox}$ SPC-ER-Cre+ mice had more severe alveolar inflammation and ALI (Supplementary Figure 4) (20) and failed to elevate pyruvate levels in response to injurious mechanical ventilation (Figure 5I). Similar to the above studies in mice with alveolar deletion of $Pfkfb3$, $Hif1a^{lox/lox}$ SPC-ER-Cre+ mice or SPC-ER-Cre+ controls received 200mg/kg body weight of intra-tracheal pyruvate prior 15 min prior to the onset of ALI induction by injurious mechanical ventilation or vehicle (Figure 6G). These studies revealed a normalization of lung inflammation with decreased transcript levels for IL-6 and CXCL-1 (Figure 6H-I) and concomitantly improved the ALI histologic score (Figure 6J, L). In addition to the reconstitution of $Hif1a^{lox/lox}$ SPC-ER-Cre+ mice, we confirmed the treatment effects in control animals, thereby confirming the therapeutic effects of intra-tracheal pyruvate during alveolar inflammation. Interestingly unlike the $Pfkfb3^{lox/lox}$ SPC-Cre-ER+ mice, the $Hif1a^{lox/lox}$ SPC-
ER-Cre+ did not display increased mortality in response to IMV. Of note, in both $\text{Hi}f\text{Ia}^{\text{loxp/loxp}}$ SPC-ER-Cre+ and $\text{Pfkfb3}^{\text{loxp/loxp}}$ SPC-ER-Cre+ mice inflammatory gene expression remained above the level of pyruvate treated SPC-Cre-ER+ control mice. One potential reason for i.t. pyruvate only partially rescuing cytokine expression in the $\text{Pfkfb3}^{\text{loxp/loxp}}$ and $\text{Hi}f\text{Ia}^{\text{loxp/loxp}}$ SPC-ER-Cre+ mice, could be that the $\text{Pfkfb3}^{\text{loxp/loxp}}$ and $\text{Hi}f\text{Ia}^{\text{loxp/loxp}}$ SPC-ER-Cre+ mice exacerbated lung injury with an increased capillary leak, which leads to rapid diffusion of intratracheally applied pyruvate in the lung interstitium and therefore decreased availability of pyruvate for the alveolar epithelium. This is in line with our finding that whole lung homogenate from $\text{Pfkfb3}^{\text{loxp/loxp}}$ and $\text{Hi}f\text{Ia}^{\text{loxp/loxp}}$ SPC-ER-Cre+ mice had a higher pyruvate content compared to the SPC-ER-Cre+ controls. Conversely, BALF from SPC-ER-Cre+ controls had higher pyruvate levels than $\text{Pfkfb3}^{\text{loxp/loxp}}$ and $\text{Hi}f\text{Ia}^{\text{loxp/loxp}}$ SPC-ER-Cre+ mice (see Supplementary Figure 5).

Taken together, these studies demonstrate that PFKFB3 and HIF1A resemble critical control point for alveolar inflammation during ARDS and indicate the likelihood that the observed phenotypes in mice deficient in alveolar epithelial Pfkfb3 or Hi1a are related to their inability to increase glycolytic responses during ALI.

**Treatment with intra-tracheal pyruvate after the onset of injury promotes attenuates inflammation in ALI.**

After having shown effective reconstitution of mice with alveolar-epithelial $\text{Pfkfb3}$ or $\text{Hi1a}$ deletion with pyruvate treatment, we next extended those studies into a longer lasting ALI model, as the acid induced ALI model enabled us to treat the mice after the onset of ALI. Six hours after acid installation, we treated littermate mice matched in age, sex and sex with intra-tracheal pyruvate (200 mg/kg body weight) or an equal amount of vehicle control (pH controlled
to pyruvate) in controls (Figure 7A). When analyzing the lungs 24 hours after acid aspiration for lung inflammation or ALI histology, we found attenuated transcript levels of IL-6 or CXCL-1 (Figure 7B, C) in conjunction with attenuated histologic lung injury, or a blinded analysis of lung injury scores (Figure 7D, E). Additionally, animals treated with intra-tracheal pyruvate showed improved oxygenation and improved wet/dry ratio (Figure 7F, G). Together, those studies indicate that treatment with intra-tracheal pyruvate after the onset of acid-induced lung injury promotes attenuation of inflammation in ALI.

**PFKFB3 is elevated in alveolar epithelial cells during human ARDS.**

We performed proof-of-principle studies in lung biopsies to address the expression and localization of PFKFB3 during human ARDS. For this purpose, we applied immunohistochemistry staining of human lung biopsy samples that showed diffuse alveolar damage, which represents the histologic manifestation of ARDS There is a scarcity of lung biopsy specimens from patients with ARDS, which is a major limitation for translational research in acute lung injury. Patients with ARDS rarely undergo lung biopsies and if a biopsy is obtained, this is frequently due to specific clinical considerations (see Supplementary Figure 5). The patient characteristics of lung biopsy samples analyzed have been described previously (40) and are summarized in Supplementary Figure 5. In control samples (lung biopsy samples obtained from lungs that were evaluated for suitability for transplant), expression of PFKFB3 was only minimal (Figure 8). However, in biopsy samples from patients with diffuse alveolar damage, PFKFB3 expression was significantly increased (Figure 8A, B). Moreover, co-staining staining with alveolar type II marker HT2-280 and PFKFB3 revealed that PFKFB3 co-localizes in the ATII cells (Figure 8C, Supplementary Figure 6B). In summary, these findings indicate the likelihood that human PFKFB3 expression is elevated during ARDS and is located in ATII cells.
Discussion

We examined the functional role of alveolar epithelial PFKFB3 and its transcriptional regulation by HIF1A during alveolar inflammation in experimental models of ARDS. Previous studies had suggested that alveolar epithelia, notably ATII cells, increase their ability for utilizing carbohydrate glycolysis during injurious conditions (12). To gain additional insight into the role of alveolar-epithelial carbohydrate metabolism during ALI, we initially conducted an unbiased screen in murine ATII cells isolated after exposure to ALI. This screen pointed us towards the isoform 3 of PFKFB, which is one of the 4 tissue-specific iso-enzymes. Subsequent pharmacologic and genetic in vivo studies demonstrated a protective role of alveolar-epithelial PFKFB3 during ALI induced by injurious mechanical ventilation or acid aspiration. For example, mice with alveolar-epithelial Pfkfb3 deletion (Pfkfb3loxp/loxp SPC-Cre-ER+ mice) failed to elevate their glycolytic response and concomitantly experienced exacerbated lung injury during murine models of ARDS. Studies to address the upstream mechanism of PFKFB3 regulation showed stabilization of alveolar HIF1a protein levels during ALI leading to elevated PFKFB3 levels. Moreover, reconstitution experiments with intra-tracheal pyruvate given prior to the onset of lung injury partially rescued the phenotype of Pfkfb3loxp/loxp SPC-Cre-ER+ or Hif1aloxp/loxp SPC-Cre-ER+ mice and attenuates inflammation in acid-induced ALI when given 6 hours after the initiation of ALI. Finally, proof-of-principle studies in lung biopsies of patients with histologic diagnosis of ARDS (diffuse alveolar injury) demonstrated elevated PFKFB3 levels in their lungs, while co-staining with the alveolar epithelial markers localized PFKFB3 specifically to alveolar epithelia. In summary, these studies indicate a protective role of ATII
specific PFKFB3 by promoting glycolytic responses during ALI and lay the groundwork for ARDS treatment approaches using glycolytic intermediates such as pyruvate.

In mammals, four PFKFB genes (PFKFB1, PFKFB2, PFKFB3 and PFKFB4) have been described, which code for the respective iso-enzymes. These isoforms share a highly conserved catalytic core domain (85%) but differ greatly in their kinetic properties and responses to regulatory signals (41). PFKFB1 was initially identified in rat liver and muscle tissue, whereas PFKFB2 isoforms are predominantly expressed in the myocardium (42), while PFKFB4 is predominantly expressed in the testis (43). The PFKFB3 isoform is ubiquitously expressed and has been implicated in glucose metabolism during neoplastic disease (44) but also plays a critical role in endothelial cells coordinating angiogenic sprouting during physiologic states or malignancy(29). More recently, it has been appreciated that the initially described tissue-specificity for PFKFB iso-enzymes is not entirely exclusive and more than one isoform can be present in one tissue (45). The current findings suggest a novel role for alveolar ATII cell PFKFB3 in attenuating alveolar inflammation during ALI. Interestingly, studies on the role of endothelial-expressed Pfkfb3 suggest that inducible deletion of Pfkfb3 is protective during pulmonary hypertension (46) or during LPS induced ALI (47). This underscores the cell type and injury specific specific role of Pfkfb3.

Previous studies have implicated other enzymatic control points for alveolar-epithelial carbohydrate metabolism in the regulation of alveolar inflammation during ALI. For example, a study on the role of alveolar carbohydrate metabolism during ALI suggested that the functional activity of succinate dehydrogenase SDH is inhibited in responses to cyclic mechanical stretch, such as occurs during injurious mechanical ventilation (12) and implicated mitogen-activated protein kinase (MAPK) activation (48) or increased levels of the competitive SDH inhibitor
itaconate (49) in the mechanism of SDH inhibition during ALI (20). SDH has been described to have four distinct subunits, SDHA-SDHD, with SDHA carrying the enzymatic activity that catalyzes the conversion of succinate to fumarate (50). Subsequent studies of ALI in mice with inducible alveolar epithelial Sdha deletion (Sdha\textsuperscript{loxp/loxp} SPC-Cre-ER+ mice) revealed reduced lung inflammation, improved alveolar barrier function, and attenuated histologic injury, in conjunction with elevated succinate levels (20). Succinate has previously been shown to function as inhibitor of the prolyl hydroxylases (PHDs) (51), and can thereby function to promote the stabilization of HIF during normoxic conditions. In fact, the protective effects of SDHA inhibition or deletion during ALI, and the concomitant elevations of pulmonary succinate levels have been linked to succinate-mediated HIF1A stabilization (20). To expand on our previous studies and to further investigate the relationship between succinate, we tested whether succinate was able to directly trigger PFKFB3 activity. However, as we found that supplementation with cell permeable succinate did not affect PFKFB3 activity directly, we postulate that succinate stabilizes HIF1A which then, in turn, induces PFKFB3 transcription. Taken together with the current studies it is conceivable that SDHA inhibition and enhanced PFKFB3 function in coordination to provide lung protection during ALI, with SDHA inhibition and elevation of succinate causing PHD inhibition and HIF stabilization. Increased levels of HIF1A can function to transcriptionally induce PFKFB3, and thereby enhance alveolar-epithelial glycolysis resulting in attenuated alveolar inflammation.

Several previous studies are in line with the current findings for a protective role of the transcription factor HIF in dampening mucosal inflammation during ARDS. Furthermore, mice exposed to lower oxygen concentration had increased HIF1A stabilization and subsequently improved outcomes during poly-microbial sepsis (52). The protective role of HIF1A has since
been confirmed in several other models ARDS, including a highly clinically relevant model of viral induced ALI(12, 25, 53). Of note, clinical trials to investigate the role of HIF activators in COVID-19 patients for prevention or treatment of ARDS are currently under way (ClinicalTrials.gov Identifier: NCT04478071). As shown here, HIF can function to optimize alveolar-epithelial glycolysis, although other studies are implicating HIF in the regulation of purinergic signaling events (13, 54) or in the induction of microRNAs that can potentially modulate the expression of alveolar epithelial microRNAs (55) that dampen pro-inflammatory signaling pathways in the alveolar epithelium HIF1A has been shown to regulate the transcription of PFKFB3(45). Based on our observation in animals with genetic and pharmacologic inhibition of PFKFB3, we show that PFKFB3 inhibition is sufficient to exacerbate ALI independently from HIF1A. Taken together, these findings indicate a protective role of HIF signaling during alveolar inflammation and indicate that different molecular mechanisms could contribute to this phenomenon.

In the current studies, we utilized intra-tracheal delivery of pyruvate for reconstitution of a normalized phenotype in Pfkfb3lop/lop SPC-ER-Cre+ or Hif1alop/lop SPC-ER-Cre+ mice and found therapeutic effects on the resolution of ALI in wild-type mice. It is important to keep in mind that the functional outcomes of metabolic reconstitution may likely have cell-specific differences. For example, metabolic intermediates such as succinate have been previously shown to enhance a pro-inflammatory phenotype in cells of the innate immune system (56), which needs to be considered when utilizing metabolites therapeutically. However, the dual role of glycolytic intermediates that quench alveolar epithelial inflammation while simultaneously enhancing myeloid inflammatory response could likely be beneficial for ARDS treatment. For example, reducing alveolar inflammation can function to dampen pulmonary edema and
improving alveolar-capillary function, while at the same time allowing myeloid cells to perform at a high-level during pathogen elimination (55). Pyruvate is metabolized to acetyl-coenzyme A (acetyl-CoA), the entry point into the Krebs cycle by pyruvate dehydrogenase. Pyruvate and its metabolites have been implicated in a hyperoxia induced model of acute lung injury where a differential function between adult and neonatal mice has been reported (57). Furthermore, anti-inflammatory potential with tissue protective effects of its derivate ethylpyruvate has been demonstrated in several in vivo models of acute inflammation (58). Taken together, these finding suggest a potential therapeutic role for metabolic intermediates such as pyruvate as modulators of inflammation in ARDS, furthering our understanding of lung inflammation and innate protective counter-regulatory mechanisms.

Taken together, the present studies identify a selective role for alveolar epithelial PFKFB3 in promoting alveolar integrity through enhancing their capacity for glycolytic carbohydrate metabolism during ARDS. Our findings highlight a unique dependence of alveolar epithelia to respond to stress conditions such as occur during ARDS with elevating their capacity for glycolytic carbohydrate metabolism. Since alveolar epithelial cells represent the first line of pulmonary defense during ARDS, their vulnerability and the extent of alveolar inflammation link them directly to ARDS outcomes (10, 59). As such, the current studies highlight that therapeutic strategies (e.g. through HIF activators or via metabolic reconstitution) should be further explored therapeutically since enhancement of alveolar epithelial glycolysis could represent a new powerful strategy to attenuate lung inflammation during ARDS.
Methods:

Materials: Unless otherwise noted, chemicals were obtained from Sigma (St. Louis/ MO, USA). 3PO was obtained from Callbiochem (San Diego/ CA, USA). Fructose 2,6 bisphosphate was obtained from (Best of Chemicals (BOC) Sciences, New York/NY, USA).

Human lung samples

De-identified lung samples with diffuse alveolar damage (that is the pathologic diagnosis of ARDS) were obtained from the Department of Pathology archives of the University of Colorado. Non-injured controls (donor lungs that were rejected for transplantation) were obtained through the Pulmonary Hypertension Breakthrough Initiative. The patient characteristics of the ALI group has been published previously (40).

Mice

Male and female mice were used for the studies and were matched for weight, age and sex. All animals were housed under a 12-hour light/12-hour dark cycle and experiments were conducted (age- and weight-matched) between 10 and 16 weeks of age. Wild type mice (C57BL/6), Hif1a^loxp/loxp (B6.129-Hif1a^tm3Rsjo/J, JAX stock #007561) and Ubc-Cre (UBC-cre/ERT2, JAX Stock #007001) mice were purchased from Jackson laboratories. Mice with Cre exclusively expressed in alveolar type 2 cells SPC-ER-Cre (Sftpc^tm1(cre/ERT2)Blh, JAX stock #028054) were generously provided by from Bridget Hogan (Duke University, Durham NC, USA) (32). Pfkfb3^loxp/loxp mice (Pfkfb3^tm1Pec) were provided by Peter Carmeliet (Katholieke Universiteit Leuven, Belgium) (29). Whole body Hif1a^loxp/loxp mice were produced by crossing Hif1a^loxp/loxp mice with the Ubc-Cre+ (35). For tissue specific knockout in the ATII cells Hif1a^loxp/loxp respectively Pfkfb3^loxp/loxp were
crossed with SPC-ER-Cre+ animals. Hif1a\textsuperscript{lox/lox} SPC-ER-Cre+ and Hif1a\textsuperscript{lox/lox} SPC-ER-Cre+ mice were described previously\citep{20, 35}. Conditional knock-out was induced by 75mg/kg/d tamoxifen over 5 days intraperitoneally (ip) as described before \citep{60}. Genotyping PCR from tails was used to confirm specific deletion of the floxed area of Pfkfb3 and Hif1a, as well as Cre and flox expression (GeneTyper, New York, NY). As controls for the Hif1a\textsuperscript{lox/lox} Ubc-Cre+ served Ubc-Cre+ animals and SPC-ER-Cre+ animals for the tissue specific knockout animals (of note one SPC-ER-Cre control group for injurious mechanical respectively acid aspiration was utilized for Figure 3-7). Control animals also received i.p tamoxifen. All genetically modified mice strains had litter sizes and frequencies comparable to wild-type mice. We did not observe an increased frequency of congenital malformation in the genetically modified mice (C57BL/6 background) compared C57BL/6 wild type mice.

**Murine Models of acute lung injury**

Murine models for acid aspiration and injurious mechanical ventilation (IMV) were performed as described previously \citep{61}. Briefly, IMV was induced with pressure-controlled ventilation using high inspiratory pressure of 45 mbar for the experimental group and 15 mbar for the control group. FiO2 (100%), rate (80/min) and positive end expiratory pressure-PEEP (3 mbar) were kept the same in IMV and control group. Prior to connecting to the ventilator, animals were anesthetized with pentobarbital (70 mg/kg ip for induction and 20 mg/kg per hour for maintenance). After tracheotomy, a tracheal tube was connected to a mechanical ventilator (Siemens Servo 900C and Draeger Evita 2 Dura, with pediatric tubing). Mice were ventilated for 4 hours or until a cardiac standstill was observed. For acid aspiration, animals were intubated with a 22 G catheter via guide wire using a small animal laryngoscope (Penncentury,
Wyndmoor/PA, USA) and 50µl of 0.125 M hydrochloric acid (HCl) was instilled. Control animals received 50µl of 0.9 M NaCl (pH controlled to HCl).

**Isolation of alveolar epithelial cells**

AT II cells were isolated (62). Briefly, mice were deeply anesthetized with 70 mg/kg ip pentobarbital. Mice were exsanguinated by and lungs were then lavaged with sterile PBS via the right ventricle. Corning dispase (Fisher Scientific, Waltham/MA,USA) was then instilled intratracheally followed by a low-melting point agarose plug. En-bloc removed and incubated for 45 min at room temperature. Tissue was teased apart and passed through a 70 micron strainer (BD Bioscience, San Jose/CA, USA). Cell mixture was then labelled with mixture of CD16/32 (cat.#553143), TER119 (cat. #553672), CD 45 (cat. 3553078) and CD90 (cat. #554896) (all from BD Bioscience) and subsequently incubated with streptavidin labeled with magnetic beads to negative select for AT II cells. As a final step, fibroblasts were removed by adherence to a petri dish for 2 hours. To control the purity of the isolated cells, cells were stained with EpCAM expression with immunofluorescence microscopy.

**Metabolite Analysis of alveolar epithelial cells**

Metabolites from frozen cell pellets were extracted using ice-cold methanol/acetonitrile/water (5:3:2) at a ratio of 2⁶ cells per mL by vortexing 30 min at 4 °C. Samples were clarified through centrifugation (10 min at 10,000 rpm, 4 °C) and 10 µl of supernatant was analyzed using a 5 min C18 gradient on a Thermo Vanquish UHPLC coupled online to a Thermo Q Exactive mass spectrometer operating in positive and negative ion modes (separate runs) as previously described in detail (63). Samples were normalized to cell count.
**Cell Culture and Treatment**

MLE-12 cells (obtained from ATCC, CRL-2110) and primary alveolar epithelial type 2 cells were cultured as described previously (64, 65) in DMEM with 4.5 g/L glucose and stable L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mix (all from Corning Cellgro, Manassas/VA, USA). Cells were incubated in a humidified atmosphere of 5% CO2/95% air at 37 °C.

**In vitro stretch model**

To recapitulate cyclic mechanical stretch, we utilized a previously described in vitro model (12) resembling mechanical ventilation. Briefly, cells were plated on BioFlex cultures plates (Flexcell, International, Burlington, NC, USA) that were coated with collagen type1 (MLE-12 cells) or fibronectin (primary isolated AT II cells), and cells were allowed to attach. In case of the MLE-12 cells were grown to 80% confluence, AT II cells were seeded with a density of 3 x 10^6/well (6 well plate). All cells were cultured in 4ml medium (DMEM, 4.5 g/L glucose, 10% FBS, 0.02% L-Glutamine). Plates were then placed on a FlexCell FX_4000T tension Plus System. Cells were stretched at 30% and sine wave 5s on, 5s off. Cells were then collected at specified time point and processed for further analysis. For controls cells were cultured under identical conditions without application of cyclic mechanical stretch.

**PFKFB3 activity**

PFKFB3 activity was assessed by measuring intracellular fructose 2,6 bisphosphate levels as previously described (28, 66). In brief, cell pellets were obtained by centrifugation at 2000 rpm
for 10 min at 4°C. The pellets were resuspended in 50 mM Tris acetate (pH 8.0) and 100 mM NaOH, incubated at 80°C for 5 minutes, and placed on ice. Cell lysates were neutralized to pH 7.2 with 1 M acetic acid and 1 M HEPES and then incubated at 25°C for 2 minutes in 50 mM Tris, 2 mM Mg2+, 1 mM F6P, 0.15 mM NAD, 10 U/L PPI-dependent PFK-1, 0.45 kU/L aldolase, 5 kU/L triosephosphate isomerase, and 1.7 kU/L glycerol-3-phosphate dehydrogenase. Pyrophosphate (0.5 mM) was added and the rate of change in absorbance (OD = 339 nm) per minute over 5 minutes was determined with a Thermo Scientific GENESIS 40-Vis spectrophotometer (Thermo Fisher Scientific, Waltham/MA, USA). A calibration curve using 0.1 to 1 pmol of F2,6BP was used to calculate F2,6BP, which was then normalized to total protein.

Sample Collection

Lungs were lavaged 3 times (each time 1 ml of PBS) to obtain bronchoalveolar lavage fluid. Retrieved lavage fluid was the at 300g for 5 min at 4°C and resulting cell-free BAL was immediately snap-frozen for subsequent measurement of protein and ELISA studies. For pulmonary tissues, the lungs were flushed with 10 ml saline via the right ventricle, and either snap-frozen in liquid nitrogen and stored at −80°C or conserved in formalin for histologic analysis.

Measurement of BALF protein content and cytokine concentrations

Protein content of BALF was measured via Bradford Assay as described previously (67). For measurements of cytokines via ELISA assay BAL fluid samples were thawed to 4°C, and specific cytokine concentrations (IL-1β, IL-6, KC/GRO (which is the CXCL-1 equivalent), and
TNF-α) in BAL fluid were determined using a V-Plex Pro-inflammatory multiplex ELISA panel (Mesoscale, Rockville MD). Cytokine concentration were normalized to BALF protein content.

**Wet/dry ratio and atrial oxygen partial pressure (paO₂) measurement**

Animals were euthanized and 100 µl blood was taken from the aorta and arterial blood gas was measured with an i-STAT1 blood gas analyzer (Abbott Laboratories, Chicago, IL, USA). Wet/dry ratio of lungs was obtained as previously described (68).

**Cell Differential in BALF**

Cell differential was performed by transferring undiluted BALF onto slides (Cytospin, ThermoFisher Scientific). Slides were then stained with Hema 3 Stat Pack (ThermoFisher Scientific). Ten random images were obtained at 20x magnification. Neutrophils and monocytes/macrophages cells were counted manually, with the experimenter blinded.

**Measurement of pyruvate in BALF and whole lung tissue**

Pyruvate was measured in whole lung tissue and BALF with a commercially available colorimetric assay (Sigma Aldric, St.Louis, MO, USA) according to the manufacture’s instructions. Briefly, enzyme mix, and dye reagent were added to BALF or homogenized whole lung tissue and absorbance was measured at 579nm.

**Targeted carbohydrate metabolism mRNA screen**

mouse RT2 Profiler™ PCR Array Glucose Metabolism (PAMM-006Z, Qiagen, Germantown/PA, USA) was used to screen a panel of 84 genes involved in glucose metabolism
in ATI cells. Total RNA was isolated and quantified by a Nanodrop 2000 (Gene Company Limited, Hong Kong, China) and c-DNA was generated using RT2 first strand kit (Qiagen). cDNA was mixed with 2 × RT2 SYBR Green qPCR Master Mix and ddH2O. The qPCR was performed on a Biorad iCycler according to the RT2 Profiler PCR Array instructions under the PCR running parameters: 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 1 min. Each array contained five separate housekeeping genes (Actb, B2m, Gapdh, Gusb and Hsp90ab1) – we utilized Actb for normalization of the sample data. Microarray data was normalized against the housekeeping genes by calculating the ΔCt for each gene of interest in the plate. Fold changes of gene expression were analyzed and generated by using RT2 PCR array data analysis web portal version 3.5(http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Genes that had fold changes of more than two in expression against control group were considered significant. The candidate genes were validated by individual qPCR. Supplementary Table 1 contains lists of genes and their respective p-values.

**Transcriptional analysis (qPCR)**

Total RNA was isolated from primary alveolar epithelial cells, MLE-12 cells or murine lung tissue using Qiagen RNeasy Mini Kit by following manufacturer’s protocol, and c-DNA was generated using iScript cDNA Synthesis Kit (Bio-Rad, Hercules/CA, USA). RNA transcript levels were determined by real-time RT-PCR (iCycler, Bio-Rad Laboratories Inc.). Primers were obtained from Quantitect (Qiagen). Mouse: Actinb (Qt01136772), IL-6 (QT00098875), CXCL-1(QT00115647), PFKFB1 (QT00285096), PFKFB2 (QT 00154000), PFKFB3 (QT 00109284),
PKF (QT00136738), GAPDS (QT0097146), PFK (QT00155911), ENO1 (QT00260442), LDHA (QT02325414), HK3 (QT01561252)

**Immunoblotting**

Western-Immunoblotting was used to measure HIF1A or PFKFB3 protein content. For Western-Immunoblotting cells were washed twice with ice-cold PBS and lysed with 1x cell lysis buffer (Cell Signaling) including protease inhibitor PMSF (effective concentration 100µg/ml). For immunoblotting of HIF1A, nuclear protein fraction was extracted from alveolar epithelial cells using a NE-PER Nuclear and Cytoplasmic Extraction Reagents Complete Protease Inhibitor (Thermo Fisher, Waltham/MA, USA). Protein concentrations were determined using Quickstart Bradford dye reagent (Biorad, Hercules/CA, USA) and equal protein amounts were denatured in sample buffer, separated by SDS-electrophoresis, transferred to nitrocellulose membrane, blocked in 5 % skim-milk [w/v] in TBS incl. 1 % Tween-20 [v/v], washed 3 times with TBST and then and probed with the respective primary antibodies at 4°C overnight. After incubation with primary antibodies membranes were washed with TBST 3 10min and then incubated secondary HRP anti-rabbit (Cell Signaling cat. #7074) or HRP anti-mouse (Cell Signaling cat. #7076) for 1 hour. Proteins were detected by ECL (Thermo Fisher Scientific). Nuclear loading control was Lamin A/C and cytoplasmatic loading control was b-Actin. Densitometry was performed with Image J (NIH). Primary Antibodies were obtained from: primary PFKFB3 (cat. # 181861, Abcam, Cambridge, UK), HIF-1a (cat. # NB100-105 clone: H1alpha67, Novus Biologicals, Centennial /CO, USA), b-Actin (cat. # A5441, Sigma Aldrich), Lamin A/C (cat. # sc-4777, Santa Cruz)
**Histopathological evaluation of acute lung injury**

Lungs were explanted and prepared for paraffin embedding as described (61). 5 μm sections were stained with H&E. Assessment of histological lung injury was performed by grading as follows (61): infiltration or aggregation of inflammatory cells in air space or vessel wall: 1, only wall; 2, few cells (one to five cells) in air space; 3, intermediate; 4, severe (air space congested); interstitial congestion and hyaline membrane formation: 1, normal lung; 2, moderate (<25% of lung section); 3, intermediate (25–50% of lung section); 4, severe (>50% of lung section); hemorrhage: 0, absent; 1, present. Six representative images were obtained by from each animal and were analyzed by 2 investigators blinded to group assignments.

**Immunohistochemistry Staining**

Detection of PFKFB3 by immunohistochemistry in human and mouse lungs was performed after deparaffinization and antigen retrieval with citrate solution (pH 6.0; 30 min) as described previously (69). Primary antibody against PFKFB3 (cat.# 181861; 1:300; 1h, Abcam), followed by treatment with secondary Dako EnVision rabbit system K 4010 (Agilent DAKO, Santa Clara/CA, USA). Sections were then counterstained with Light green (StatLab Medical Products, McKinney/TX, USA)). Slides were then coded, and images of the lung parenchyma were captured in an unbiased fashion, using the 40x objective. The intensity of expression was then quantified on coded images, using a macro developed for Metamorph software (Molecular Devices, San Jose/CA, USA), which integrates intensity (in pixels) and area (in pixels) of the positive immunohistochemistry staining, generating arbitrary units. Following decoding, data were analyzed by comparison among experimental groups.
**Immunofluorescence Staining**

Sections of lung from patients with ARDS and control lungs were stained with both the anti-PFKFB3 antibody (cat. # 181861, Abcam) and an anti-HT2-280 antibody (cat. # TB-27AHT2-280, Terrace Biotech, San Francisco/CA, USA) to identify alveolar type II cells (39). The sections were treated with Antigen Unmasking Solution, Citrate-Based H-3300-250 (Vector Laboratories, Burlingame/CA, USA) and then blocked with a mixture of 10% donkey serum, 10% goat serum, reconstituted in antibody diluent for 1h at room temperature. The slides were incubated with a combination of anti-PFKFB3 (1:100) and anti-HT2-280 antibody (1:200) or rat IgG (negative control) at a dilution of 1:200 applied for 1 h at room temperature. A combination of secondary antibodies of Alexa fluor 488 donkey anti mouse (cat. # A-21202, Invitrogen, ThermoFisher Scientific) and Alexa fluor 555 goat anti-rabbit (cat. # A-21428, Invitrogen, ThermoFisher Scientific - each diluted 1:200 applied for 1 h and DAPI. Sections then treated with Vector® TrueVIEW® Autofluorescence Quenching Kit (cat. #SP-8400-15, Vector Laboratories). Images from the double stained slides were examined under a Zeiss LSM900 confocal microscope. Images were obtained utilizing ZEN 3.1 (Zeiss, White Plains/NY, USA) software.

**Data Analysis**

We assessed the severity of ALI by determining mRNA expression levels of pro-inflammatory cytokines, pulmonary barrier function, protein content in the bronchoalveolar lavage fluid (BALF) and histology. For analysis of alveolar epithelial cells each data point was isolated from a different animal. For cell line experiments each experiment was carried out independently. For analysis of histology two investigators, who were blinded to genotype and treatment, performed scoring independently. All statistical analyses were carried out using GraphPad Prism 8.1.2
We conducted Grubbs’ tests on all eligible data to examine outliers. No outliers were identified. All data approximately followed normal distributions and were summarized as means ± SD. For comparison between two groups, student’s t-test was used. In cases of comparison between more than two groups, ANOVA with Tukey’s correction for multiple comparison adjustments was applied. The Mantel-Cox test was used for analysis of survival curves. p values less than 0.05 were considered statistically significant. The authors had full access to the data and have read and agree to the manuscript as written.

**Study Approval**

*Mice*

The local institutional animal care and use committee (University of Colorado, Anschutz Medical Campus) approved all animal experiments (protocol numbers B104914(06)1D B104917(04)E and 00128). Animal experiments were carried out in accordance with the US Law on the Protection of Animals and the National Institutes of Health guidelines for use of live animals.

*Humans*

The lung samples with the diffuse alveolar damage diagnosis were obtained through the pathology archives of the University of Colorado. The de-identified human lungs control samples were obtained through the Pulmonary Hypertension Breakthrough Initiative Research Network (Philadelphia/PA, USA). The Colorado Multiple Institutions Review Board approved all human protocols and waived the requirement for informed consent for the use of archived, de-identified paraffin-embedded lung samples.
Author Contributions:

CUV, RMT and HKE designed the research studies, CUV, EC, CS, EKV and NB conducted the experiments, CUV, NB, XY analyzed the data. PC provided knock out animals. RMT and EPS access to patient samples. All original research data were reviewed by CUV, EC, NB, XY, RMT and HKE. CUV wrote the manuscript and provided all figures. RMT, ESN, XY, KS and HKE revised the manuscript.

Acknowledgements:

We would like to thank Aneta Gandjeva (CVP Laboratories) for technical assistance with the histology staining, Dr. Ryan Fredericks and Dr. Angelo D’Alessandro for their generous support in performing the mass spectroscopy experiments and Dr. Yanyu Wang for reviewing all preliminary data prior to manuscript submission.

Funding Statement:

This work was supported by National Institute of Health Grant K12HD068372, K08HL130586 and Parker B. Francis Fellowship (CUV). National Institute of Health Grants R01HL154720, R01DK122796, R01DK109574, R01HL133900 and Department of Defense Grant W81XWH2110032 (HKE). P01HL152961 to (KS and RMT)
References


67. Maeda M, Ozaki T, Yasuoka S, and Ogura T. [Role of alveolar macrophages and neutrophils in the defense system against infection of Pseudomonas aeruginosa in the...


Figure 1

A. Ventilator blood gas analysis IMV 4hours

AT II isolation

Targeted mRNA screen
Carbohydrate metabolism Metabolomics

B. Alveolar epithelial carbohydrate metabolism pathways affected by IMV

- **Glycolysis**
- **TCA cycle**
- **Pentosephosphate Pathway**
- **Glycogen Metabolism**
- **Regulatory Genes**
- **Gluconeogenesis**

C. Alveolar Epithelial Metabolites

D. mRNAs fold Δ alveolar epithelial cells

E. mRNAs fold Δ alveolar epithelial cells

F. Pfkfb3 expression

G. PFKFB3 activity alveolar epithelial cells [pmol/l]

H. Isotype Control Ctrl IMV

I. Isotype Control Ctrl IMV
Figure 1: Glycolysis is upregulated in ATII cells in response to acute lung injury

(A) Schematic overview of experiment: mice (matched for age, sex and weight) were ventilated with the following parameters: peak inspiratory pressure (PIP) 45 mbar, respiratory rate (RR) 80, positive end-expiratory pressure (PEEP) 3 mbar and FiO\textsubscript{2} 100% in injurious ventilation (IMV), control group (Ctrl) were ventilated with the same parameters but with a PIP of 15 mbar. After ventilation for 4 hours alveolar epithelial cells were isolated for RNA or metabolomics analysis. (B) mRNA isolated from alveolar epithelial cells after IMV (n=4) and Ctrl (n=3) was analyzed using a targeted carbohydrate metabolism screen (80 genes). 17 genes were found to be differentially regulated in the IMV group and different pathways of carbohydrate metabolism were analyzed. Proportions of the pie diagram represent (number of genes / out of total significant genes. (C) alveolar epithelial cells were isolated after animals were ventilated (n=4/group) and glycolytic intermediates were determined by mass spectrometry. (D) Validation of the most differentially regulated genes in alveolar epithelial cells by qPCR (n=4/group). (E) mRNA expression of PFKFB subunits was determined by qPCR (n=4/group). (F, G) PFKFB3 protein levels in alveolar epithelial cells by Western blot (n=3/group). (H) Immunohistochemical expression of PFKFB3 in mouse lungs after IMV and control ventilation. Rabbit IgG was used as a negative isotype control. (i) Measurement of PFKFB3 activity in alveolar epithelial MLE-12 cell line in response to cyclic stretch (n=4/group). Panel A, D and E: 6 male and 5 female. Panel C: 4 male and 4 female. Panel F, G: 2 male and 4 female, Panel H: 2 male and 2 female. GAPDH-glycerolaldehyde-3- dehydrogenase, ENO1-enolase1, HK3=hexokinase 3, LDHA- lactate dehydrogenase A, PFK- phosphofructokinase. Data are represented as mean ± SD, ns-not significant, * p, 0.05, ** p, 0.01, ***p<0.001, ****p<0.0001. Data were analyzed with two-tailed, unpaired, student’s t-test.
Figure 2

A) Timeline of the experiment:
- 70mg/kg 3 PO i.p.
- Start IMV
- Whole lung BAL, histology
- 24 h timeline

B) BAL protein levels:
- Ctrl vs IMV
- Δ[relative units]
- **

C) Whole lung IL6 mRNA fold:
- Ctrl vs IMV
- Δ[relative units]
- ***

D) Whole lung CXCL-1 mRNA fold:
- Ctrl vs IMV
- Δ[relative units]
- **

E) Ventilator 3PO PFKFB3
- Ctrl vs IMV
- ALI score
- Relative values
- 82μm
- **

F) Whole lung mRNA BAL, histology
- Ctrl vs IMV
- ALI score
- Relative values
- 82μm
- **
- n.s

Legend:
- ● vehicle
- ○ 3PO
Figure 2: Pharmacological inhibition of PFKFB3 exacerbates lung injury

(A) Schematic of the experiment: C57BL/6 mice (matched for age, sex and weight) received 70mg/kg of PFKFB3 inhibitor 3PO i.p 24 hours prior to ventilation: Ctrl group being ventilated with a PIP of 15 mbar and IMV group with PIP of 45 mbar. Animals were matched for age, sex and weight. The groups that were not treated with 3PO received vehicle (DMSO). After 4 hours of ventilation the lungs were removed. (B) BALF protein concentration was measured with Bradford-Assay qPCR (n=4/group). (C, D) IL-6 and CXCL-1 mRNA expression in whole lung tissue was determined by qPCR (n=4/group). (E,F) Representative images of H&E stained lungs; cumulative lung injury score which is a combined score of cellular infiltrates, interstitial congestion and hyaline membrane formation and hemorrhage (n=4/IMV, IMV+3PO, Ctrl group, n=3 Ctrl+3PO group). Panel B: 8 male and 8 female. Panel C-F: 7 male and 8 female. Data are represented as mean ± SD, n.s= not significant, ** p< 0.01, ****p<0.0001. Data were analyzed with 1-way ANOVA with Tukey’s correction for multiple comparisons.
Figure 3: Functional Consequences of AT II specific deletion of \textit{Pfkfb3} in lung injury induced by injurious ventilation

\textit{Pfkfb3}^{loxp/loxp} Surfactant Cre+ (\textit{Pfkfb3}^{loxp/loxp} \textit{SPC-ER-Cre+}) mice and controls (\textit{SPC-ER-Cre+}) were exposed to injurious (IMV- PIP 45 mbar) or control ventilation (Ctrl-PIP 15 mbar) (age, sex and weight matched). (A) Alveolar epithelial cells were isolated from \textit{Pfkfb3}^{loxp/loxp} \textit{SPC-ER-Cre+} mice and PFKFB isoform mRNA expression was determined via qPCR (n=4/group). (B-D) Glycolytic intermediates from alveolar epithelial cells isolated from \textit{Pfkfb3}^{loxp/loxp} \textit{SPC-ER-Cre+} and \textit{SPC-ER-Cre+} after IMV and control ventilation were determined with mass spectrometry (n=4/group). (E, F) IL-6 and CXCL-1 mRNA expression in whole lung tissue was determined by qPCR (n=4/group). (G) Protein concentration was measured in BALF with Bradford-Assay (n=4/ \textit{SPC-ER-Cre+}, n=5 \textit{Pfkfb3}^{loxp/loxp} \textit{SPC-ER-Cre+} group). (H-K) Concentration of cytokines in BALF was measured by ELISA (n=4/group). (L,M) Representative images of H&E stained lungs; cumulative lung injury score which is a combined score of cellular infiltrates, interstitial congestion and hyaline membrane formation and hemorrhage (n=3/ \textit{SPC-ER-Cre+}, n=4 \textit{Pfkfb3}^{loxp/loxp} \textit{SPC-ER-Cre+} group). (N) Survival curve in response to IMV for \textit{SPC-ER-Cre+}\textit{Pfkfb3}^{loxp/loxp} and \textit{SPC-ER-Cre+}. Panel A: 4 male and 4 female. Panel B-D: 8 male and 8 female. Panel F-G: 8 male and 8 female. Panel E and H-K: 8 male and 8 female. Panel L, M: 8 male and 6 female. Panel N: 31 male and 31 female. Survival curve was analyzed with Log-Rank (Mantel-Cox) test. Other data are represented as mean ± SD and were analyzed with 1-way ANOVA with Tukey’s correction for multiple comparisons. n.e= not expressed, n.s= not significant, * p< 0.05, ** p<0.01, ***p<0.001, ****p<0.0001.
Figure 4

A-C

Whole lung IL6 mRNA fold [relative units]

Δ [relative units]

n.s

* **

Ctrl Acid 1d Acid 3d

Whole lung CXCL-1 mRNA fold [relative units]

Δ [relative units]

n.s

* **

Ctrl Acid 1d Acid 3d

C

BALF protein [µg/ml]

n.s

* **

Ctrl Acid 1d Acid 3d

D-G

BAL IL1β [pg/ml]

n.s

* 

Ctrl Acid 1d Acid 3d

BAL IL-6 [pg/ml]

n.s

* 

Ctrl Acid 1d Acid 3d

BAL CXCL-1 [pg/ml]

* **

Ctrl Acid 1d Acid 3d

ALI score [relative values]

ns

* **

Ctrl Acid 1d Acid 3d

H-I

SPC-Cre-ER+ Pfkfb3^{loxp/loxp} SPC-Cre-ER+
Figure 4: Functional consequences of ATII specific deletion of Pfkfb3 in acid induced ALI.

Lung injury was induced in Pfkfb3^loxp/loxp Surfactant Cre+ (Pfkfb3^loxp/loxp SPC-ER-Cre+) mice and age, sex and weight matched controls (SPC-ER-Cre+) by acid instillation with i.t. HCl. The control groups received pH controlled NaCl. After 1 and 3 days, the lungs were removed. (A, B) IL-6 and CXCL-1 mRNA expression in whole lung tissue was determined by qPCR (n=4/group). (C) Protein concentration measured in BALF with Bradford-Assay (n=4/group). (D-G) Concentration of cytokines-α in BALF was determined by ELISA (n=4/group). (H, I) Representative images of H&E stained lungs; cumulative lung injury score which is a combined score of cellular infiltrates, interstitial congestion and hyaline membrane formation and hemorrhage (n=3/group). Panel A-G: 11 male and 13 female. Panel H, I: 6 male and 6 female. Data are represented as mean ± SD, n.s= not significant,  * p< 0.05, ** p< 0.01, *** p< 0.001, ****p<0.0001. Data were analyzed with 1-way ANOVA with Tukey’s correction for multiple comparisons.
Figure 5

**A**

ALVEOLAR EPITHELIAL CELLS

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>IMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamin A/C</td>
<td>120 kDa</td>
<td>74/63 kDa</td>
</tr>
</tbody>
</table>

**B**

ALVEOLAR EPITHELIAL CELLS

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>1d</th>
<th>3d</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamin A/C</td>
<td>120 kDa</td>
<td>74/63 kDa</td>
<td></td>
</tr>
</tbody>
</table>

**C**

Hif1a dependent carbohydrate metabolism pathways in alveolar epithelial cells

- Red: Glycolysis
- Green: Glycogen Metabolism
- Purple: Regulatory Genes
- Blue: TCA cycle

**D**

- Black circle: Ubc-Cre+
- Open circle: Hif1a^{lox/lox} Ubc-Cre+

**E**

Whole lung mRNA fold Δ in IMV

<table>
<thead>
<tr>
<th></th>
<th>PFKFB1</th>
<th>PFKFB2</th>
<th>PFKFB3</th>
<th>PFKFB4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td>IMV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**F**

Whole lung mRNA fold Δ in Acid

<table>
<thead>
<tr>
<th></th>
<th>PFKFB1</th>
<th>PFKFB2</th>
<th>PFKFB3</th>
<th>PFKFB4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td>Acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**G**

Alveolar Epithelial Glucose-6-phosphate [relative abundance]

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>IMV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0×10^6</td>
<td>5.0×10^6</td>
</tr>
</tbody>
</table>

**H**

Alveolar Epithelial Pyruvate [relative abundance]

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>IMV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2×10^5</td>
<td>2×10^5</td>
</tr>
</tbody>
</table>

**I**

Alveolar epithelial Lactate [relative abundance]

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>IMV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0×10^5</td>
<td>5.0×10^5</td>
</tr>
</tbody>
</table>
Figure 5: Alveolar epithelial glycolysis induced by ALI is controlled by HIF1A

(A, B) ALI was induced in C57BL/6 mice (matched for age, sex and weight) by IMV (A) or acid instillation (B). Alveolar epithelial cells were isolated and HIF1A protein expression was determined in nuclear fraction via Western Blot (each blot lane is from alveolar epithelial cells of an individual animal). (C) mRNA was isolated from alveolar epithelial cells isolated from Hif1alox/loxp Ubc-Cre+ mice (n=3/group) and Ubc-Cre+ (n= 4/group) control mice after IMV and a targeted carbohydrate metabolism screen (80 gens) was performed. 12 genes were found to be differentially regulated in the Ubc-Cre+ mice and different pathways of carbohydrate metabolism were analyzed. Proportions of the pie diagram represent (number of genes / out of total significant genes. (D) Validation of the most differentially regulated genes in alveolar epithelial cells and LDHA by qPCR (n=3/ Hif1alox/loxp Ubc-Cre+ group and n=4/ Ubc-Cre+ group). (E, F) Pfkfb3 mRNA expression was measured in whole lungs from Hif1alox/loxp SPC-ER-Cre+and SPC-ER-Cre+ mice after IMV and acid instillation (n= 6/group). (G, I) Isolation of alveolar epithelial cells from Hif1alox/loxp SPC-ER-Cre+and SPC-ER-Cre+ after IMV and control ventilation followed by determination of glycolytic intermediates with mass spectrometry (n= 4/group, except Hif1alox/loxp SPC-ER-Cre+ group n=3). Panel A, B: 7 male and 7 female. Panel C, D: 9 male and 11 female. Panel E, F: 17 male and 19 female. Data are represented as mean ± SD.n.s= not significant. * p< 0.05, ** p< 0.01, *** p< 0.001, ****p<0.0001. Data were analyzed with two-tailed, unpaired, student's t-test.
Figure 6

A

<table>
<thead>
<tr>
<th>200mg/kg pyruvate i.t.</th>
<th>Start IMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15 min</td>
<td>0</td>
</tr>
</tbody>
</table>

Whole lung mRNA, histology

B

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole lung IL-6 mRNA fold Δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative values

C

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole lung CXCL-1 mRNA fold Δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative units

D

IMV | IMV+ pyruvate

SPC-Cre-ER+ | Pfkfb3<sup>loxp/loxp</sup>

SPC-Cre-ER+ | Hif1a<sup>loxp/loxp</sup>

92μm | 92μm

E

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALI score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative values

F

<table>
<thead>
<tr>
<th>Ventilator Survival (hours)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IMV+ pyruvate | IMV

G

<table>
<thead>
<tr>
<th>200mg/kg pyruvate i.t.</th>
<th>Start IMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15 min</td>
<td>0</td>
</tr>
</tbody>
</table>

Whole lung mRNA, histology

H

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole lung IL-6 mRNA fold Δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative values

I

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole lung CXCL-1 mRNA fold Δ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative units

J

IMV | IMV+ pyruvate

SPC-Cre-ER+ | Pfkfb3<sup>loxp/loxp</sup>

SPC-Cre-ER+ | Hif1a<sup>loxp/loxp</sup>

92μm | 92μm

K

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALI score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative values

L

<table>
<thead>
<tr>
<th>Ventilator Survival (hours)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent survival</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IMV+ pyruvate | IMV
Figure 6: Locally delivered pyruvate reconstitutes $Hif1a^{loxp/loxp}$ SPC-ER-Cre+ and $Pfkfb3^{loxp/loxp}$ SPC-ER-Cre+ animals

(A, G) Schematic of experiment: C57BL/6 wild-type mice with matched weight and sex were used in all experiments. Alveolar epithelial cell specific conditional knockout mice ($Hif1a^{loxp/loxp}$ SPC-ER-Cre+ and $Pfkfb3^{loxp/loxp}$ SPC-ER-Cre+) or control animals (SPC-ER-Cre+) received 200mg/kg i.t. pyruvate 15 min prior to induction of IMV. After 4 hours lung tissue was harvested for analysis. (B, C and H, I) IL-6 and CXCL-1 mRNA expression was determined in whole lung tissue by qPCR (n=4/group, expect SPC-ER=Cre+ pyruvate n=3/group). (D, E and J, K) Representative images of H&E stained lungs from mice subjected to IMV and controls and cumulative lung injury score which is a combined score of cellular infiltrates, interstitial congestion and hyaline membrane formation and hemorrhage PCR (n=3/group, expect SPC-ER IMV n=4/group). Same SPC-ER-Cre+ control mice were utilized for histologic controls (D, E and I, J). Survival curve in response to IMV with and without i.t. pyruvate treatment (F, K). Panel B, C: 11 male and 15 female. Panel D, E: 13 male and 17 female. Panel G, H: 12 male and 13 female. Data are represented as mean ± SD. **p<0.01, ***p<0.001, ****p<0.0001. Data were analyzed with 1-way ANOVA with Tukey's correction for multiple comparisons.
Figure 7

A timeline

Vehicle
Pyruvate
200mg/kg
i.t.
HCl

Whole lung mRNA histology

0 6h 24h

B Whole lung IL-6 mRNA fold [relative values]

Veh Pyr

C Whole lung CXCL-1 mRNA fold [relative units]

Veh Pyr

D Acid Acid + pyruvate

92μm 92μm

E ALI score [relative values]

Veh Pyr

F PaO₂ [mmHg]

Veh Pyr

G Wet/dry weight [relative values]

Veh Pyr
Figure 7: Locally delivered pyruvate is therapeutic in acid induced lung injury.

(A) Schematic of experiment. C57BL/6 mice were matched for age, weight and sex. ALI was induced by acid instillation. The mice in the treatment group received 200mg/kg i.t. pyruvate 6 hours after induction of lung injury (Pyr). Control animals received pH-controlled PBS (Ctrl). Lung tissue was harvested for analysis 24 hours after induction of ALI. (B, C) IL-6 and CXCL-1 mRNA expression in whole lung tissue was determined with q PCR (n=5 in control group, n=3 in pyruvate group). (D, E) Representative images of H&E stained lungs and cumulative lung injury score were obtained which consists of a combined score of cellular infiltrates, interstitial congestion and hyaline membrane formation and hemorrhage (n=3/group). (F) Partial pressure of arterial oxygen (paO₂) was measured from blood samples obtained from the aorta, n=5/group. (G) Wet/dry ratio was calculated 4 days after instillation. Panel B, C: 8 male and 7 female n=6/5. Panel D, E: 3 male and 3 female. Panel E, F: 5 male and 6 female. Data are represented as mean ± SD. * p<0.05, ** p<0.01, *** p<0.001, ****p<0.0001. Data were analyzed with two-tailed, unpaired, student’s t-test.
Figure 8

A. 

Normal Control Tissue

Diffuse alveolar damage

80 μm 80 μm

45 μm 45 μm

B. 

PFKFB3/reference area

Control tissue Diffuse alveolar damage

C. 

BF merged DAPI HT2-280 PFKFB3

Normal Control Tissue

Diffuse alveolar damage

20 μm 20 μm
Figure 8: PFKFB3 is apparent in patients with diffuse alveolar damage

(A) Representative immunohistochemical staining of lung biopsy tissue specimen from a patient with diffuse alveolar damage (the histologic manifestation of ARDS) and control specimen. (B) Quantification of PFKFB3 expression in lung tissue of patients with diffuse alveolar damage (n=9) compared to control patients (n=6). Control specimens were lung biopsies from lungs rejected for transplants. Box and whisker plots illustrating the significant difference in mean PFKFB3 expression observed between control and patients with diffuse alveolar damage. For each plot, box bounds represent the first quartile (lower bound) and third quartile (upper bound). Lines within the box represent the median. Whiskers represent the difference from the minimum value observed in the data set to the first quartile (lower whisker) and the difference from the third quartile to the maximum value observed (upper whisker). * p< 0.05. Data were analyzed with two-tailed, unpaired, student's t-test. (C) Representative images of lungs from control lungs and patients with diffuse alveolar damage, that were stained with AT II cell marker anti-HT2-280 and anti-Pfkfb3 antibody. The anti-HT2-280 antibody colocalizes with Pfkfb3 within many alveolar epithelial cells (white arrowheads). Images were obtained with 20x objective. All scale bars are 20 μm.