nature portfolio

Peer Review File

Alveolar epithelial cells mitigate neutrophilic inflammation in lung injury through regulating mitochondrial fatty acid oxidation

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors report on the role of mitonchondrial long chain fatty acid oxidation in lung epithelial cells and in particular the role of Cpt1 (fatty acid transporter into mitochondria) in the development of (acute) lung inflammation experimentally induced by LPS and fibrosis, experimentally induced by bleomycin. A combination of in vitro studies on cell lines, ex vivo on isolated epithelial cells, and of cell-specific inactivation of mtLFCA-related genes is used. Impact of epithelial cell metabolism on inflammatory immune cells, in particular neutrophils is mechanistically investigated.

Major comments
1. This study provides a wealth of new data and is overall very well performed. Results are technically sound.
2. This manuscript is very long it would benefit from general streamlining, reogranising and proofreading by a native English speaker. This would highlight the originality and "key" message of the manuscript while facilitating the reading
3. Metabolomic analysis are limited. It likely that metabolomic changes induced in the pathological/experimental conditions are not limited to the phospholipid studied. For instance but not limited to amino acid and glycolysis/TCA associated compounds are likely to be altered and should be measured at lest in key experiments
4. How did the authors chose the dose of palmitate used in vitro. Did the authors investigate the metabolic status of the animals/patients? Indeed, One would expect mtLCFAO to also depend on (fatty acids) substrate availability. Hence, while not absolutely required, it would be informative to induce experimental pathology in vivo in a context of high and low fatty acid condition by comparing the outcome in animals fed a normal or a high fat diet.

Minor comments.
1. Fig S9 S14 J. Lung histology seem of poor quality even in the control groups. Alveolae appear heavily damaged. This happens for instance when lungs samples are poorly fixed or when they were inflated with high pressure (eg upon forced ventilation). Use of properly processed tissue would be beneficial. If the authors believe this is due to their experimental model, processing of an unmanipulated lung sample would clearly demonstrate that alveolae rupture result from experimental treatment.
2. Use of LPS intratracheal injection is foremost a model of local endotoxemia rather than acute respiratory syndrome

Reviewer #2 (Remarks to the Author):

The study aims to evaluate the functional and metabolic significance of mitochondrial long-chain fatty acid oxidation (FAO) in alveolar epithelial cells in acute lung injury (ALI). The work has been performed using samples from human subjects with acute respiratory distress syndrome (ARDS), murine ALI models (type 2 alveolar epithelial (AT2) cells from control mice with intratracheal instillation of LPS or bleomycin), tamoxifen-inducible AT2 cell-specific deletion of Cpt1a mice and the AT2 cell line MLE12. The authors demonstrate decreased Cpt1a protein levels in alveolar epithelium in human lungs with ARDS. Mechanistically, they showed that loss of Cpt1a-mediated FAO in murine AT2 cells induces different metabolic and inflammatory adaptations. They conclude that Cpt1a downregulation in alveolar epithelium can impair FAO and mitigate the alveolar inflammation in ALI. They claim that their findings highlight AT2 cell-specific FAO as a potential target to halt persistent alveolar inflammation in ARDS. Overall, this is an interesting comprehensive study. However, there are several problems with the experimental design that seriously affect the interpretation of the results and limit the described conclusions.

Comments
1. Other studies have also demonstrated the role of impaired FAO in alveolar epithelial cells in ALI (ref. 25 by Cui et al. Impairment of fatty acid oxidation in alveolar epithelial cells mediates acute lung injury. Am J Respir Cell Mol Biol, 2019). Thus, the authors should further explain the specific novel contribution of their work.

2. I would be interesting to explain the rationale behind the use of the Cpt1a isoform in their experimental models (tissue-specific expression of Cpt1a in alveolar epithelial cells). In addition, the authors perform experiments to evaluate the subcellular localization of Cpt1a in MLE12 cells and discern whether it is localized in the ER or in the mitochondria. Cpt1c is localized in the endoplasmic reticulum, but Cpt1a and Cpt1b have been widely proved to be localized in the mitochondria. Thus, these experiments are redundant and could be moved to the Supplemental figures.

3. The authors claim that the results from the extracellular flux analyses have been normalized using nucleic acid contents. However, OCR graphs are only represented as pmol/min.

4. The whole manuscript is based on the relevance of FAO and Cpt1a in different experimental models. The authors have evaluated FAO and Cpt1a by measuring extracellular flux analyses and Cpt1a protein and mRNA levels. Both extracellular flux analyses and mRNA or protein levels are indirect methods to evaluate Cpt1 activity. Importantly, to demonstrate palmitate-specific changes in FAO measured by extracellular flux analyses the experiments should be performed by injecting in one port a Cpt1a inhibitor such as the synthetic irreversible inhibitor etomoxir. In addition, in their Cpt1a-depleted models, reminiscent protein levels could still lead to Cpt1 activity. Overall, it would be necessary to directly measure Cpt1 enzymatic activity in all the experimental models. Cpt1a is tightly regulated by its physiological inhibitor, malonyl-CoA. Malonyl-CoA is a key metabolite mainly derived from glucose metabolism but also involved in lipogenesis. Thus, it is highly relevant to know whether Cpt1 enzymatic activity changes under the different nutritional status and cell models used in the study.

5. Changes in mitochondrial activity is frequently linked to the production of reactive oxygen species (ROS) and oxidative damage. Thus, it would be interesting to measure ROS levels in the different experimental models.

6. Lungs are composed by alveolar epithelial cells but also by other cell types such as immune cells. How could the authors discern between the specific contribution of Cpt1a and FAO in alveolar cells vs. the infiltrated immune cells in their results?

7. Is Cpt1a deletion leading to lipid accumulation in the cells of study (AT2, MLE12)? It would be interesting to measure TG content in these models.

8. Paradoxically, the authors show that both FAO and glycolysis are downregulated in their experimental models of Cpt1a depletion (Fig. 2n). They mention that exogenous pyruvate is required to support the in vitro proliferation of cells with electron transport chain defects. Thus, where are the metabolic substrates leading to pyruvate import coming from? i.e. which metabolites upstream or downstream pyruvate are the ones responsible of ATP production for cell survival? E.g.: Krebs cycle anaplerotic substrates (such as the glutamate that the authors add in the extracellular flux analyses medium)? Aminoacid degradation into lactate and then conversion into pyruvate? Pentose phosphate pathway? TG degradation into glycerol? This should be clarified.

Reviewer #3 (Remarks to the Author):

In this paper, Chung et al used AT2 specific Cpt1a mutant mice, patient sample and cell culture to

analyze the relationship between AT2 Cpt1a dependent mitochondrial LC fatty acid oxydation and ALI phenotype. They offer information regarding the change of energy production in Cpt1a-deleted AT2 cells, i.e., from mtLCFAO to the compensated pyruvate mediated one. Further data showed the loss of CPT1a in AT2 cells and changes in phosphor lipids in BAL, and decreases of alveolar inflammation in ALI. However, there is a lack of mechanistic insights regarding the connection among mitochondria fatty acid oxidation, phosphor lipid synthesis and inflammatory responses in AT2 cells. It is not clear whether downregulation of alveolar epithelial mtLCFAO really relates to an altered immune response in ARDS lungs. The manuscript is well written with a lot of data, but the data are largely descriptive.
There are some other issues:
Acadl: the abbreviation was not explained.
Fig1a and Figs1a: they showed correlation of Cpt1a expression and ALI, however they did not include an AT2 marker, therefore it is not known whether the changes in Cpt1a occur in AT2s, especially CD45+ cells express significantly higher levels of Cpt1a.
Sup Fig2: the mRNA level of Cpt1a was decreased after LPS but the protein level was un-changed, how to explain this?
The explanation of Fig 1d, 1f and FigS2f, 3a are unclear and confusing.
Fig1, FigS2.S3, did not explain why the results of bleomycin are different from LPS.
They did not examine the distribution of Cpt1a isoforms in primary AT2s, unclear whether different isoforms will interfere with each other.
It is unclear whether the change of mtLCFAO mediated cellular metabolism in AT2 Cpt1a mice are connected to the change of phosphor lipids in BAL.
They did not observe a change in phosphor lipid in AT2, but it was changed in BAL, unclear if the change in BAL is indirectly caused by macrophage etc.
Fig5A: the figure is unclear, need to show separated channel for AT2 and CD45.
Fig6f,g, there are some differences in lipid components between control and mutant, but the results were not explained.
Fig6m, o, the effect of PG(32:0)_16:0 was not explained.
Fig7: it is not convincing by simply comparing Cpt1a and Acadl1 phenotype to claim that "impairing mtLCFAO in AT2 cells leads to decreased alveolar inflammation in ALI."

Reviewer #4 (Remarks to the Author):

In this manuscript, the authors propose to evaluate the role of mitochondrial long-chain fatty acid oxidation (mtLCFAO) in alveolar epithelial cells, particularly in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Their intent is to add significant insights into the immuno-metabolic mechanisms in respiratory biology. The experimental design appears robust, utilizing both human lung samples and murine models. Techniques like immunohistochemistry, extracellular flux analysis, and transcriptomic profiling are appropriately used.

However, I found several key details on the statistical methods used for data analysis lacking. The results showing the impact of mtLCFAO alterations in type 2 alveolar epithelial (AT2) cells on immune responses in the lungs are compelling. They are well-supported by extensive data and are clearly presented through figures and tables. However, my key concerns are related to lack of significant phenotypic changes in the lungs as a result of deletion of this pathway in the Cpt1aiDAT2 mice. There was no significant change in the long-term fibrosis or acute cellular infiltrates in the lungs in response to the different injuries. Additionally, there was no data that revealed any functional alteration in the injured lungs in these knockout mice. These findings may suggest that this pathway may not be the dominant driver of the lung injury and the injury response. There was no change in the cellular influx as well.

The discussion contextualizes the findings within the broader field, highlighting the potential of mtLCFAO as a therapeutic target in ARDS. However, I did not find compelling evidence that targeting

this pathway would result in a clinical benefit to patients with ARDS.

While the manuscript is well written, there were several typos and grammatical errors. I also found several experiments redundant and the text a bit too verbose, often discussing findings that did not add significant scientific rigor.

A few additional concerns:

Reliability of AT2 cells from injured mice: Given that the AT2 cells are a heterogeneous group it was unclear whether the changes observed in the cell isolated were a result of direct injury response or whether it was a selection bias related to the cells isolated.

While they observe lower levels of certain cytokines, neutrophils numbers are unchanged in the Cpt1aiDAT2 mice. These cytokines are known to be produced by several different types of myeloid and non-myeloid cells.

There is lack of specificity of the responses in the Cpt1a deficient mice. They show a broad decrease in the cytokines but I am not sure if there is sufficient data to establish causality with this pathway. These mice might just be inherently predisposed to a lower cytokine response. The concerns are further highlighted in the fact that there was no demonstratable long-term phenotypic or functional alteration in these mice.

Point-by-point Responses to the Reviewers of Manuscript NCOMMS-23-46729

We appreciate the constructive comments from the four reviewers. Based on the suggestions from the reviewers, we have performed additional experiments and analyses, resulting in 35 new figure panels and 2 new tables, and provide further in-depth discussion. To facilitate readers' comprehension, we have reorganized the manuscript, and also removed data from redundant experiments. We believe that the revisions (denoted by red font in the manuscript) have significantly improved the manuscript. A point-by-point response to each of the comments is enclosed below:

Reviewer #1 (Remarks to the Author):

The authors report on the role of mitonchondrial long chain fatty acid oxidation in lung epithelial cells and in particular the role of CPT1 (fatty acid transporter into mitochondria) in the development of (acute) lung inflammation experimentally induced by LPS and fibrosis, experimentally induced by bleomycin. A combination of *in vitro* studies on cell lines, *ex vivo* on isolated epithelial cells, and of cell-specific inactivation of mtLCFAO-related genes is used. Impact of epithelial cell metabolism on inflammatory immune cells, in particular neutrophils is mechanistically investigated.

We thank Reviewer #1 for the valuable comments. Our detailed point-by-point responses are as follows:

Major comments:

1. This study provides a wealth of new data and is overall very well performed. Results are technically sound.

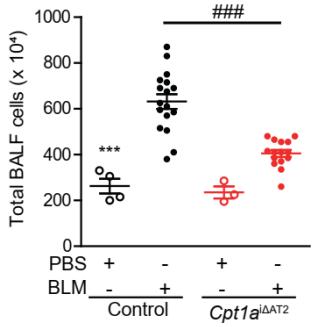
Response 1: We appreciate the comments from the reviewer.

2. This manuscript is very long it would benefit from general streamlining, reogranising and proofreading by a native English speaker. This would highlight the originality and "key" message of the manuscript while facilitating the reading.

Response 2: We are grateful for the comments from the reviewer. Under the aids from co-authors Suzanne M Cloonan and Maria Plataki, we largely reorganized the manuscript and incorporated new data from experiments for the revision. We have removed data from redundant experiments (please refer to **response 2.2 to Reviewer #2**). After careful consideration, we also removed data from bleomycin-induced murine lung fibrosis model, which is a translational model frequently applied for exploring mechanisms for human progressive fibrosing lung diseases, such as idiopathic lung fibrosis[1]. Our data from murine ALI models induced through TLR4 or TLR2 signaling have provided evidence supporting the role of AT2 cell-specific CPT1a in regulating the resolution of alveolar inflammation in ALI, and consistently the alveolar inflammation due to bleomycin-induced chemical pneumonitis is

less severe in *Cpt1a*$^{i\Delta AT2}$ mice at 24h after bleomycin instillation (see **Figure included in response 2**, which is Supplementary Fig. 14c in the original manuscript). Removal of the data from murine bleomycin-induced lung fibrosis model thus does not alter the conclusions of our study, but largely improves the data presentation to highlight the key message from our current work. We believe the clarity of the manuscript has been enhanced after the revision.

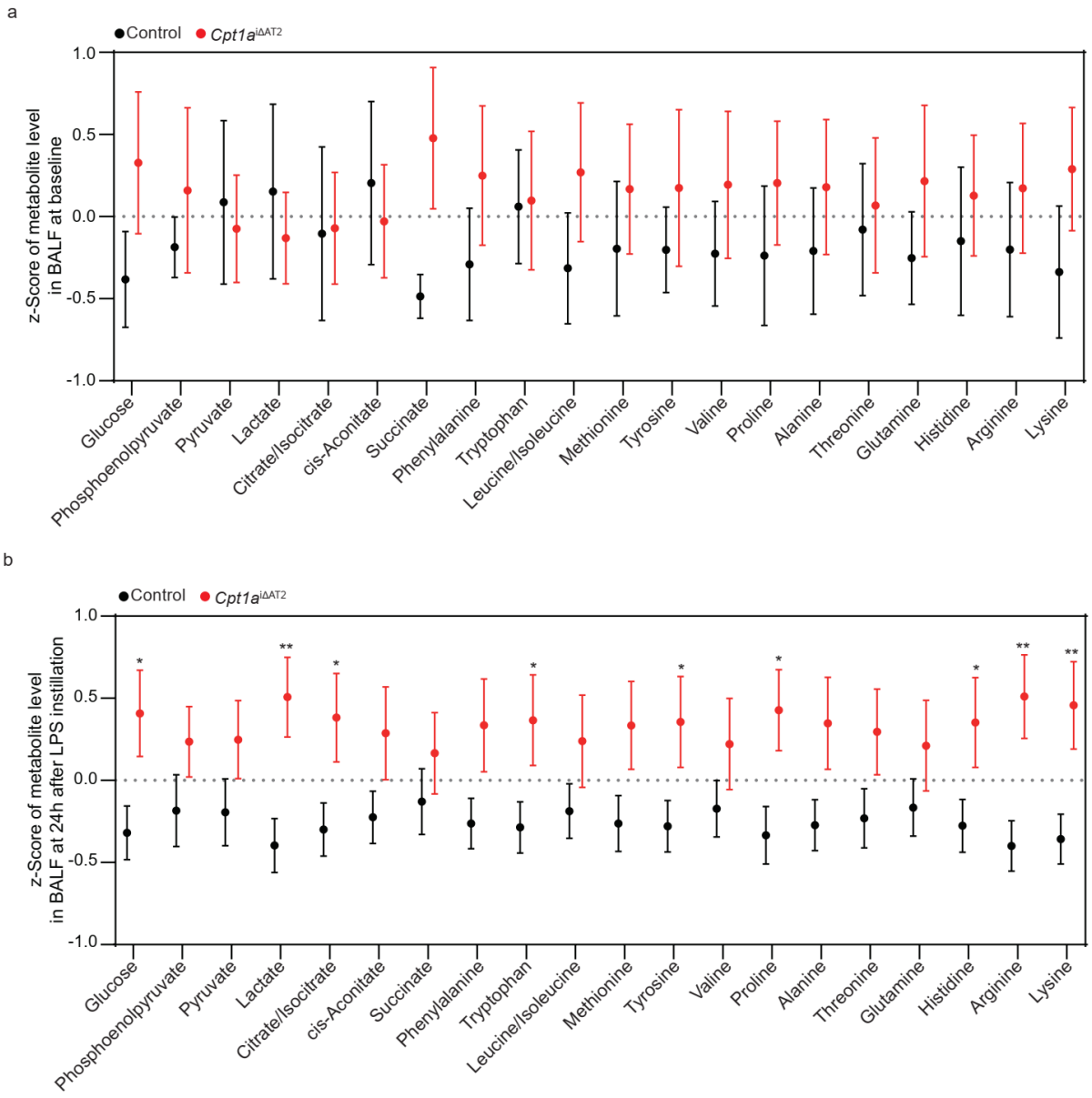
Figure included in response 2 (reviewer #1). BALF cell counts at 24 hours after PBS or bleomycin (BLM) instillation in *Cpt1a*$^{i\Delta AT2}$ and control mice (control mice, n = 4 for PBS, n = 17 for BLM; *Cpt1a*$^{i\Delta AT2}$ mice, n = 3 for PBS, n = 15 for BLM; results of two independent experiments). Data are represented as mean ± SEM (BLM vs. PBS ***p < 0.001; *Cpt1a*$^{i\Delta AT2}$ and control ###p < 0.001, by one-way ANOVA with Bonferroni tests)



3. Metabolomic analysis are limited. It likely that metabolomic changes induced in the pathological/experimental conditions are not limited to the phospholipid studied. For instance but not limited to amino acid and glycolysis/TCA associated compounds are likely to be altered and should be measured at least in key experiments.

Response 3: *Cpt1a* deletion results in metabolic changes in bioenergetics in AT2 cells (**Fig. 2-4**), and, as suggested by the reviewer, the metabolomics in alveoli may thus been altered in *Cpt1a*$^{i\Delta AT2}$ mice at baseline or in ALI model. Through LC-MS/MS platform, we performed metabolomic profiling, specifically focusing on glycolysis- and TCA cycle-related metabolites and amino acids, using BALF samples from *Cpt1a*$^{i\Delta AT2}$ and control mice (**Figure included in response 3**). The alveolar metabolomics at baseline revealed a non-significant trend of increased BALF succinate in *Cpt1a*$^{i\Delta AT2}$ mice. At 24 hours after LPS instillation, the BALF levels of several metabolites and amino acids, in particular lactate, arginine, and lysine, are significantly higher in *Cpt1a*$^{i\Delta AT2}$ mice, when compared to the controls. Based on previous studies, the metabolomic alterations in alveoli in *Cpt1a*$^{i\Delta AT2}$ mice may promote inflammation in alveolar macrophage at the initiation of ALI (**Supplementary Fig. 16**)[2], but form an immuno-modulating milieu to promote resolution of alveolar inflammation[3, 4, 5]. However, the assumption warrants further mechanistic studies to confirm. In addition, the exact mechanism leading to alveolar metabolomic changes in *Cpt1a*$^{i\Delta AT2}$ mice is also unclear. The data are thus premature to be shown. It will be an interesting topic in our future works, and we thank reviewer 1 for the valuable comment.

Figure included in response 3 (reviewer #1). *Cpt1a* deletion in AT2 cells alters alveolar metabolomics in ALI. BALF samples (**a**) at baseline and (**b**) at 24 hours after LPS instillation are collected from *Cpt1a*$^{i\Delta AT2}$ (baseline n = 7; ALI n = 18) and control (baseline n = 6; ALI n = 23) mice for profiling amino acids and metabolites of glycolysis and tricarboxylic acid cycle. The levels of metabolites in BALF samples are standardized, and z-scores are calculated. Data are represented as mean ± SEM (*p < 0.05, **p < 0.01, by unpaired Student's t-tests).

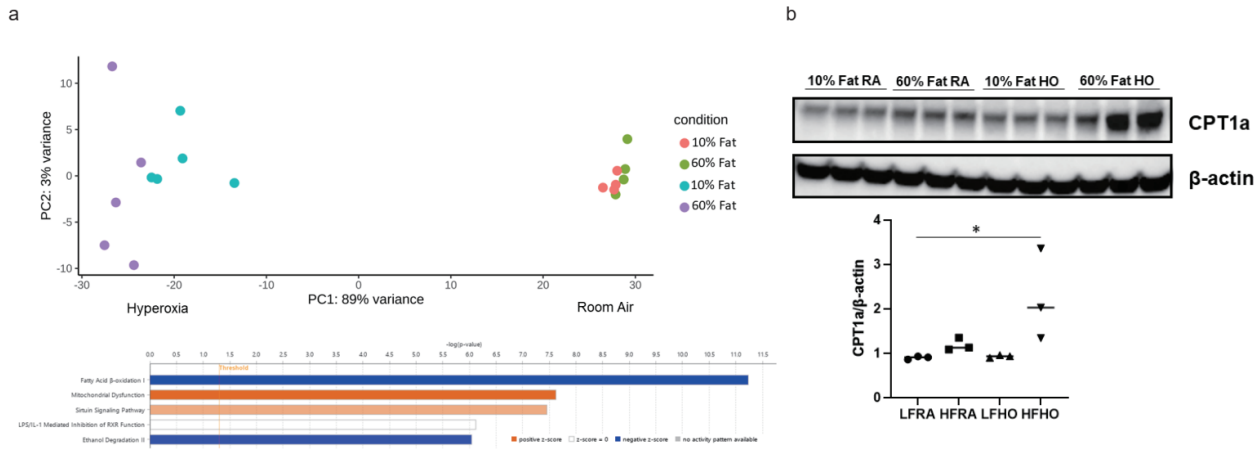


4. How did the authors chose the dose of palmitate used in vitro. Did the authors investigate the metabolic status of the animals/patients? Indeed, one would expect mtLCFAO to also depend on (fatty acids) substrate availability. Hence, while not absolutely required, it would be informative to induce experimental pathology in vivo in a context of high and low fatty acid condition by comparing the outcome in animals fed a normal or a high fat diet.

Response 4: We chose the palmitate dose for extracellular flux analyses based on the protocols reported in several previous studies[6, 7], and the instructions from Agilent Technologies. We did not have data about systemic metabolic status of the mice or the ARDS patients. However, our group has

previously shown that high fat diet is associated with increased free fatty acids in the bronchoalveolar lavage fluid after hyperoxia[8] and that fatty acid synthase is downregulated in the lungs from mice fed with high fat diet. The findings thus indicate that the availability of the substrates, such as fatty acids and lipids, could alter the metabolic activity of the lungs. In our subsequent study, data from RNAseq showed that hyperoxia exposure results in downregulated transcriptomic signature related to mitochondrial fatty acid β-oxidation in AT2 cells from mice fed with high fat diet (see **Figure a included in response 4**) along with more severe mitochondrial bioenergetic failure with high fat diet. Furthermore, immunoblot also revealed that high fat diet increases CPT1a expression in murine AT2 cells after hyperoxia exposure (see **Figure b included in response 4**). This comes as no surprise as prior research has shown that downregulation of mitochondrial fatty acid β-oxidation enzymes downstream to CPT1a increases upstream CPT1a expression in the presence of fatty acids[9]. The findings thus echo the reviewer's comment, suggesting mtLCFAO activity in AT2 cells could be regulated by substrate availability. It remains under investigation how high fat diet together with mtLCFAO activity in AT2 cells affects the phenotypes in murine hyperoxia model and other murine ALI models. We agree with the critical comment from the reviewer, and will clarify more details in our ongoing work.

Figure included in response 4 (reviewer #1). High fat diet feeding and hyperoxia exposure (**a**) downregulate mitochondrial fatty acid β-oxidation in AT2 cells based on RNAseq data, and (**b**) increase CPT1a expression in AT2 cells (*RA* room air, *HO* hyperoxia, *LF* low fat, *HF* high fat; *p < 0.05, by one-way ANOVA with Bonferroni tests)



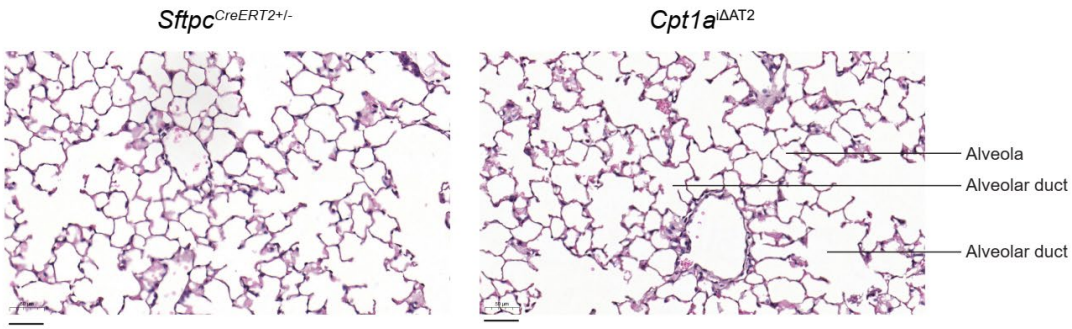
Minor comments.

1. Fig S9 S14 J. Lung histology seem of poor quality even in the control groups. Alveolae appear heavily damaged. This happens for instance when lungs samples are poorly fixed or when they were inflated with high pressure (eg upon forced ventilation). Use of properly processed tissue would be beneficial. If the authors believe this is due to their experimental model, processing of an unmanipulated lung sample would clearly demonstrate that alveolae rupture result from experimental treatment.

Response 5: The protocol for lung inflation and fixation in this study has been established in our lab.

We have thoroughly checked the original slides and confirmed that alveolar break does not appear. For the histology image in **Fig. S9**, the lungs were inflated with 1.2 ml 4% paraformaldehyde, and were fixed in 4% paraformaldehyde for 24 hours. The image under high power field was shown (see **Figure included in response 5**, which is Fig. S9 in original manuscript), and alveolar ducts may thus resemble alveoli with disrupted septa. To avoid misunderstanding, we showed image under low power field in the revised manuscript (**Supplementary Fig. 9b** in revised manuscript). As for the immunofluorescent staining image in **Fig. S14J**, the lungs were inflated with 1.5ml 1:2 OCT:1xPBS solution, and were then fixed in 4% paraformaldehyde. This inflation method will compress the alveolar septa (interstinum) and is preferred for observing alveoli lined by differentiated tdTomato-epxressing AT2 cells, as shown in previous studies[10, 11]. The image was acquired at small confocal plane (~425μm x 425μm) under high power field using a confocal microscope. Similarly, image resembling alveolar rupture is thus generated due to partial volume effect and the structure of alveolar ducts. Data from murine bleomycin-induced lung fibrosis model are removed in the revised manuscript (please refer to **response 2**), and the confocal microscopic image with improved quality was thus not shown. We thank the reviewer for the comment, which improved our presentation of murine lung histology data (*please see Supplementary Fig. 9b and the related figure legends*).

Figure included in response 5 (reviewer #1). Histology of murine lungs at 8 months after tamoxifen injection under high power field (scale bar = 50 μm).



2. Use of LPS intratracheal injection is foremost a model of local endotoxemia rather than acute respiratory syndrome

Response 6: Several murine models of ALI, including LPS-induced ALI, have been proposed based on official American Thoracic Society workshop reports[12, 13]. However, we totally agree with reviewer as for the translational gap from murine ALI models to human ARDS. Indeed, there are no perfect murine models of ALI recapitulating all the pathophysiological features and the course of human ARDS. Therefore, the results from murine models should be very carefully interpreted in terms of clinical implications, and we address this limitation in the discussion in the revised manuscript (*please see page 24 of the revised manuscript*).

Reviewer #2 (Remarks to the Author):

The study aims to evaluate the functional and metabolic significance of mitochondrial long-chain fatty acid oxidation (FAO) in alveolar epithelial cells in acute lung injury (ALI). The work has been performed using samples from human subjects with acute respiratory distress syndrome (ARDS), murine ALI models (type 2 alveolar epithelial (AT2) cells from control mice with intratracheal instillation of LPS or bleomycin), tamoxifen-inducible AT2 cell-specific deletion of *Cpt1a* mice and the AT2 cell line MLE12. The authors demonstrate decreased Cpt1a protein levels in alveolar epithelium in human lungs with ARDS. Mechanistically, they showed that loss of *Cpt1a*-mediated FAO in murine AT2 cells induces different metabolic and inflammatory adaptations. They conclude that *Cpt1a* downregulation in alveolar epithelium can impair FAO and mitigate the alveolar inflammation in ALI. They claim that their findings highlight AT2 cell-specific FAO as a potential target to halt persistent alveolar inflammation in ARDS. Overall, this is an interesting comprehensive study. However, there are several problems with the experimental design that seriously affect the interpretation of the results and limit the described conclusions.

We thank Reviewer #2 for the insightful comments. We have performed additional experiments to address the concerns of Reviewer #2. Below are detailed point-by-point responses:

Comments

1. Other studies have also demonstrated the role of impaired FAO in alveolar epithelial cells in ALI (ref. 25 by Cui et al. Impairment of fatty acid oxidation in alveolar epithelial cells mediates acute lung injury. Am J Respir Cell Mol Biol, 2019). Thus, the authors should further explain the specific novel contribution of their work.

Response 1: We have cited this reference in the introduction in our manuscript (**reference 23**)[14]. In that paper, they found that deleting *Ppargc1a* in AT2 cells exacerbates the severity LPS-induced ALI. Consistent with findings in a previous study[15], the study by Huachun Cui et al. revealed that *Cpt1a* expression is decreased in *Ppargc1a*-deficient AT2 cells, and the *in vitro* experiments showed that etomoxir (100μM) enhances LPS-induced death of MLE12 cell line. Based on the *in vitro* data, the authors conclude that impaired mitochondrial fatty acid oxidation in *Ppargc1a* $^{-/-}$ AT2 cells accounts for the phenotypic deterioration in LPS-induced ALI. Overall, *the assumption is based on indirect evidence generated from in vitro experiments and is not examined in in vivo system.* In addition, the dosage of etomoxir in the study by Huachun Cui et al. will result in inhibition of mitochondrial respiratory complex I[6], leading to increased cell death in *in vitro* experiments. In our study, we provide direct evidence and the mechanisms for the role of mtLCFAO in regulating the immunomodulating function of AT2 cells, and also demonstrate that deleting *Cpt1a* would not decrease the viability of AT2 cells in LPS-induced ALI (**Supplementary Fig. 18**). PGC1α, encoded by *Ppargc1a*, is a mass

transcription coregulator crucial for mitochondrial biogenesis, mitochondrial dynamics, and cellular metabolism[16], and our study suggests that deleting *Ppargc1a* in AT2 cells may aggravate LPS-induced ALI through mechanisms other than perturbation of mtLCFAO (*please see page 4 of the revised manuscript*).

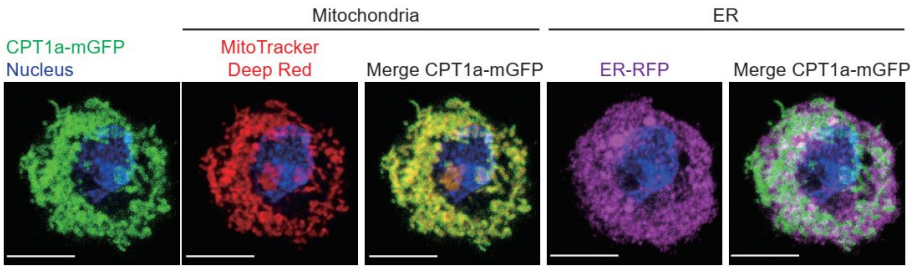
2.1 It would be interesting to explain the rationale behind the use of the *Cpt1a* isoform in their experimental models (tissue-specific expression of *Cpt1a* in alveolar epithelial cells).

Response 2.1: According to the literature[17], *Cpt1b* is mainly expressed in myocardium and skeletal muscle, and *Cpt1c* is expressed in neurons and gonads. In addition, data from The Human Protein Atlas[18] show that CPT1a is the main CPT1 isoform expressed in human lungs, while CPT1b and CPT1c are not expressed. Our RNAseq data further confirm that *Cpt1a* is the principal isoform expressed in murine AT2 cells, and, as the findings in T lymphocytes[6], no remarkable upregulation of *Cpt1b* or *Cpt1c* expression in AT2 cells occurred after *Cpt1a* deletion (**Supplementary Fig. 4**). We add the above information in the revised manuscript as the rationale supporting the investigations of *Cpt1a*, but not *Cpt1b* or *Cpt1c*, in alveolar epithelium, AT2 cells and the experimental models (*please see page 5 and 7 of the revised manuscript, and also Supplementary Fig. 4 and the related figure legends*).

2.2 In addition, the authors perform experiments to evaluate the subcellular localization of *Cpt1a* in MLE12 cells and discern whether it is localized in the ER or in the mitochondria. *Cpt1c* is localized in the endoplasmic reticulum, but *Cpt1a* and *Cpt1b* have been widely proved to be localized in the mitochondria. Thus, these experiments are redundant and could be moved to the Supplemental figures.

Response 2.2: In the process of our investigations, we examined the subcellular localization of CPT1a in MLE12 cells, after we found that CPT1a regulates the phospholipid synthesis activity in MLE12 cell line (**Fig. 4a-e**). Since phospholipid synthesis mainly occurs in the endoplasmic reticulum and mitochondria[19], we thus examined the subcellular localization of CPT1a in the MLE12 cell line, and consistently found that CPT1a is localized in mitochondria (see **Figure included in Response 2.2**, which are Supplementary Fig. 4a, b in the original manuscript). We agree with the reviewer that CPT1a has been widely shown to be localized in mitochondria[17], and have therefore removed the redundant data in the revised manuscript.

Figure included in response 2.2 (reviewer #2). Representative live cell images (n = 5 cells) through confocal microscopydemonstrate the sub-cellular localization of CPT1a-mGFP fusion protein in MLE12 cells (ER, endoplasmic reticulum; scale bar = 10 μm).



3. The authors claim that the results from the extracellular flux analyses have been normalized using nucleic acid contents. However, OCR graphs are only represented as pmol/min.

Response 3: Extracellular flux analyses were performed in this study using either MLE12 cell line or AT2 cells. For AT2 cells, extracellular flux analyses were performed immediately after isolation of the cells, and 2 x 10^5 cells were added to each well in a Seahorse 24-well microplate. Therefore, the results were not normalized using nucleic acid contents, since cell numbers were the same in all the wells. However, for MLE12 cells, 4 x 10^4 cells were plated per well in a Seahorse 24-well microplate, and the analyses were performed after culture overnight. Therefore, the cell numbers may not be the same in all the wells, and nucleic acid contents were used for data normalization per plate. Normalized OCR and ECAR per plate were calculated using the equation below:

$$Normalized\ OCR\ (or\ normalized\ ECAR)$$

$$= \frac{OCR\ (or\ ECAR) \times \sum_{i=1}^{n} NA_i}{n \times NA} = \frac{OCR\ (or\ ECAR) \times \overline{NA}}{NA}$$

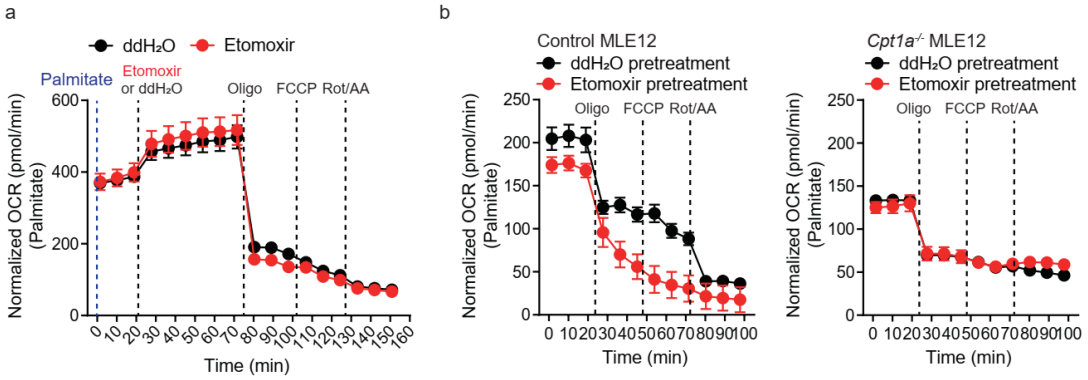
, where NA is the nucleic acid content in each well, n is the total well number in the plate, and $\overline{NA}$ is the average nucleic acid content of all the wells in the plate. We revised the axis lables for figures about extracellular flux analyses in MLE12 cells and addressed the details about normalization in the revised manuscript (*please see page 37 of the revised manuscript, and also Supplementary Fig. 6b-i and the related figure legends*).

4.1 The whole manuscript is based on the relevance of FAO and CPT1a in different experimental models. The authors have evaluated FAO and CPT1a by measuring extracellular flux analyses and CPT1a protein and mRNA levels. Both extracellular flux analyses and mRNA or protein levels are indirect methods to evaluate CPT1 activity. Importantly, to demonstrate palmitate-specific changes in FAO measured by extracellular flux analyses the experiments should be performed by injecting in one port a CPT1a inhibitor such as the synthetic irreversible inhibitor etomoxir.

Response 4.1: When we developed the protocols for evaluating mtLCFAO activity through

extracellular flux analyses, the methods in various papers[6, 7] and the instructions by Agilent Technologies were reviewed in detail. First, in the assay buffer in our study, glucose is not added, and palmitate is the only substrate used for extracellular flux analyses. As shown in our study, metabolic changes in glycolysis and ATP production occur in *Cpt1a*$^{-/-}$ MLE12 and AT2 cells. We found that glucose addition, even in very low concentrations, in the assay medium would cause interference to the results. Therefore, the OCRs measured in our assay of mtLCFAO activity are directly related to utilization of exogenous palmitate and endogenous substrates. Second, we performed extracellular flux analyses in MLE12 cells using palmitate and added 40μM etomoxir during the analyses. The data again showed that palmitate-relate OCRs cannot be suppressed by 40μM etomoxir in MLE12 cells (see **Figure a included in response 4.1**). According to a *Nat Immunol* paper by Mitsunori Nomura et al.[7], etomoxir pretreatment (40μM) before palmitate addition successfully suppresses CPT1a activity during the analyses. We then tried the protocol in the *Nat Immunol* paper, and found it works well to suppress CPT1a-mediated OCRs utilizing palmitate in MLE12 cells (see **Figure b included in response 4.1**). We think the palmitate binding sites of CPT1a may be saturated with palmitate if palmitate is added first, and CPT1a can only be effectively inhibited when the cells are pretreated with etomoxir. Collectively, our data above thus indicate that etomoxir pretreatment for mtLCFAO extracellular flux analyses with palmitate can be applied to measure palmitate-dependent OCRs mediated by CPT1a activity (*please see page 36-37 of the revised manuscript*).

Figure included in response 4.1 (reviewer #2). Extracellular flux analyses in MLE12 cells using palmitate with (**a**) etomoxir added after palmitate or (**b**) etomoxir added as pretreatment. Data are represented as mean ± SEM.

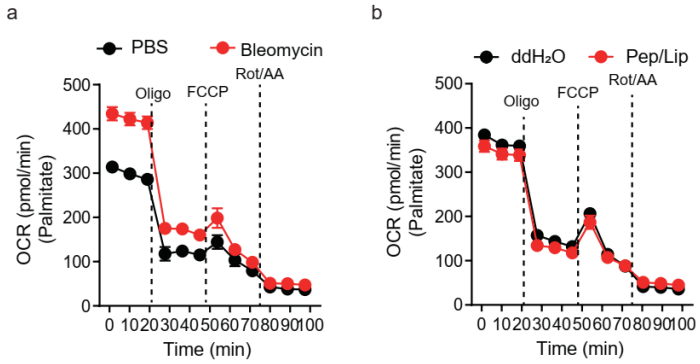


4.2 In addition, in their CPT1a-depleted models, reminiscent protein levels could still lead to CPT1 activity. Overall, it would be necessary to directly measure CPT1 enzymatic activity in all the experimental models.

Response 4.2: We found that etomoxir pretreatment eliminates the differences in palmitate-dependent OCRs in *Cpt1a*$^{-/-}$ and control AT2 cells (**Figure 2g, h**). Based on **response 4.1**, the data confirm that downregulated palmitate-dependent OCRs in *Cpt1a*$^{-/-}$ AT2 cells is due to decreased CPT1a activity. To investigate whether CPT1a activity is downregulated in AT2 cells in LPS-induced ALI model, our results demonstrated that palmitate-dependent OCRs for ATP production in AT2 cells

were not significantly different at baseline or in LPS-induced ALI after etomoxir pretreatment (**right panels in Figure 1e, f**). While cell energy production with glucose, pyruvate, and glutamine was decreased in AT2 cells in LPS-induced ALI (**Supplementary Fig. 3a, b**), our new data indicate that downregulated palmitate-dependent OCRs for ATP production in AT2 cells in LPS-induced ALI (**left panels in Figure 1e, f**) are related to decreased CPT1a activity in AT2 cells. To avoid out of focus data presentation (please refer to **response 2 to Reviewer #1** and **response 5 to Reviewer #3**), we removed data of extracellular flux analyses using palmitate in AT2 cells after instillation of bleomycin or peptidoglycan/lipoteichoic acid (see **Figure a, b included in response 4.2**, which are respectively Fig. 1f, g and Supplementary Fig. 13c, d in the original manuscript), since it is not our focus to examine the changes of CPT1a and mtLCFAO activity after instillation of different stimulants (*please see page 6 and 7 of the revised manuscript, and also Fig. 1e, 1f, 2g, 2h and the related figure legends*).

Figure included in response 4.2 (reviewer #2). Extracellular flux analyses in primary AT2 cells at 24 hours after intra-tracheal instillation of (**a** bleomycin or (**b**) peptidoglycan/lipoteichoic acid (Pep/Lip). Data are represented as mean ± SEM.



4.3 CPT1a is tightly regulated by its physiological inhibitor, malonyl-CoA. Malonyl-CoA is a key metabolite mainly derived from glucose metabolism but also involved in lipogenesis. Thus, it is highly relevant to know whether CPT1 enzymatic activity changes under the different nutritional status and cell models used in the study.

Response 4.3: Our data confirm that mtLCFAO in AT2 cells is downregulated in LPS-induced ALI, due to decreased CPT1a activity in AT2 cells (**Fig. 1e, f**). While the study by Huachun Cui et al.[14] demonstrated CPT1a protein expression is decreased in AT2 cells in LPS-induced ALI, we fail to reproduce the data and cannot show robust decrease of CPT1a protein expression in AT2 cells in LPS-induced ALI (**Supplementary Fig. 3e-g**). As mentioned by the reviewer, CPT1a activity has been shown to be regulated by malonyl-CoA. Furthermore, a recent study by Fengqi Hao et al.[20] revealed that butyryl-CoA can upregulate CPT1a activity. These findings thus indicate that CPT1a activity may be differently affected by metabolic intermediates of lipogenesis, and metabolites regulating CPT1a activity may not fully explored. Therefore, as mentioned by the reviewer, the CPT1a protein level may not be equivalent to CPT1a activity, and our data from extracellular flux analyses and immunoblots (**Fig. 1e, f** and **Supplementary Fig. 3e-g**) thus suggest that downregulated mtLCFAO in AT2 cells in

LPS-induced ALI may be related to altered regulation of CPT1a activity. Furthermore, our recent data from immunoblots revealed that high fat diet increases CPT1a expression in murine AT2 cells after hyperoxia exposure (please see **response 4 to Reviewer #1**). The mechanisms as for how nutritional status alters CPT1a activity in AT2 cells are not yet completely explored, and our ongoing work will clarify more details (*please see page 6 of the revised manuscript*).

5. Changes in mitochondrial activity is frequently linked to the production of reactive oxygen species (ROS) and oxidative damage. Thus, it would be interesting to measure ROS levels in the different experimental models.

Response 5: Through mitoSOX™ (Thermo Fisher Scientific) staining and flow cytometric analyses, we evaluated whether deleting *Cpt1a* in AT2 cells alters mitochondrial ROS production. Our data showed that loss of CPT1a does not alter mitochondrial ROS production in AT2 cells (**Supplementary Fig. 5**) (*please see page 8 and 44 of the revised manuscript, and also Supplementary Fig. 5 and the related figure legends*).

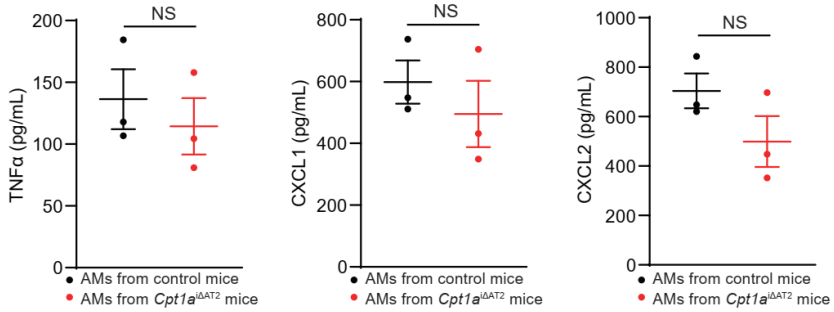
6. Lungs are composed by alveolar epithelial cells but also by other cell types such as immune cells. How could the authors discern between the specific contribution of *Cpt1a* and FAO in alveolar cells vs. the infiltrated immune cells in their results?

Response 6: In this study, the expression Cre^{ERt2} recombinase is under the control of *Sftpc* promoter, and *Sftpc* is exclusively expressed in AT2 cells, but not in immune cells[11]. Therefore, the phenotypes in murine ALI models are directly linked to CPT1a loss in AT2 cells, since *Cpt1a* is deleted only in AT2 cells after tamoxifen injection, but not in other immune cells. Our flow cytometric analyses of BALF cells showed that alveolar macrophages are the majority at baseline, and neutrophils are the main immune cells in ALI models (**Fig. 5b** and **Supplementary Fig. 10b, 11b, and 13b**). Mechanistic exploration suggests that deleting *Cpt1a* in AT2 cells may affect the inflammatory responses in alveolar macrophages and neutrophils in the alveoli in ALI. The RNAseq data in alveolar macrophages from $Cpt1a^{i\Delta AT2}$ and control mice revealed increased inflammatory responses in alveolar macrophages after *Cpt1a* deletion in AT2 cells (**Supplementary Fig. 16c-h**). *In vitro* experiments demonstrated that LPS-induced cytokine production is not different between alveolar macrophages from $Cpt1a^{i\Delta AT2}$ mice and those from the control (see **Figure included in response 6**), and the results suggest extrinsic factors in alveoli cause increased inflammatory responses in alveolar macrophages in $Cpt1a^{i\Delta AT2}$ mice (please refer to **response 3 to Reviewer #1**). While detailed mechanisms require further investigations, the findings may explain why $Cpt1a^{i\Delta AT2}$ mice do not show less alveolar inflammation at 24h after LPS instillation. In addition, our qPCR analyses revealed less *Cxcl1* and *Cxcl2* expression in alveolar neutrophils in LPS-induced ALI (**Fig. 7j, k**), and the findings may be due to downregulated CXCL2 production in $Cpt1a^{-/-}$ AT2 cells (**Fig. 7h, i**)[21]. Therefore, while *Cpt1a* deletion only occurs in AT2 cells in our transgenic mouse model, it also results in transcellular regulation of inflammatory

responses in other alveolar immune cells.

Notably, functional enrichment analysis of RNAseq data in alveolar macrophages revealed annotation of "lipid localization" (GO: 0010876) (**Supplementary Fig. 19b**). However, whether CPT1a and mtLCFAO are crucial to the function of alveolar macrophages or other immune cells requires further investigations through cell-specific murine models (*please see page 13-15 and 19 of the revised manuscript, and also Fig. 7h-k, Supplementary Fig. 16 and the related figure legends*).

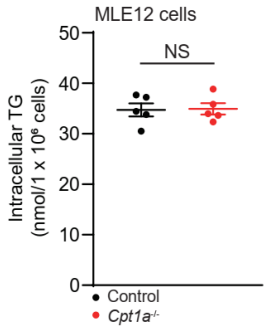
Figure a included in response 6 (reviewer #2). Alveolar macrophages (AM) are isolated from *Cpt1a*$^{i\Delta AT2}$ (n = 5) and control (n = 5) mice, and are added to a 96-well culture plate (2 x 10^5 cells/well, 3 wells each group). After incubation for 24 hours, LPS (2 ng/mL) was used to stimulate the cells, and the medium was obtained at four hours for cytokine measurements by ELISA. Data are represented as mean ± SEM (NS non-significant, by unpaired Student's t-tests).



7. Is *Cpt1a* deletion leading to lipid accumulation in the cells of study (AT2, MLE12)? It would be interesting to measure TG content in these models.

Response 7: Although intracellular TG contents are not increased in *Cpt1a*-deficient MLE12 cell line (see **Figure included in response 7**), our results revealed that deleting *Cpt1a* significantly increases intracellular TG contents in AT2 cells (**Supplementary Fig. 8**). In contrast, although mtLCFAO is impaired in *Acadl*$^{-/-}$ AT2 cells (**Fig. 8f, g**), intracellular TG contents and phospholipid synthesis are not increased in *Acadl*$^{-/-}$ AT2 cells (**Supplementary Fig. 22a-c**). The data together highlight the critical role of CPT1a in regulating lipid metabolism in AT2 cells (*please see page 10, 19-20 of the revised manuscript, and also Supplementary Fig. 8, 22 and the related figure legends*).

Figure included in response 7 (reviewer #2). Intracellular TG levels in CPT1a-deficient and control MLE12 cells. Data are represented as mean ± SEM (NS non-significant, by a unpaired Student's t-test).



8.1 Paradoxically, the authors show that both FAO and glycolysis are downregulated in their

experimental models of *Cpt1a* depletion (Fig. 2n).

Response 8.1: We thank for the critical perspectives from the reviewer. In both *Cpt1a*$^{-/-}$ MLE12 and *Cpt1a*$^{-/-}$ AT2 cells, the downregulated glucose-related ECAR is associated with increased OCRs using glucose, pyruvate and glutamine (**Fig. 3b-e**, and **Supplementary Fig. 6d-g**). Stable isotope resolved metabolomics with ^{13}C uniformly labeled glucose ([U-^{13}C] glucose) is indicated to clarify the glycolysis activity in AT2 and MLE12 cells with deficiency of CPT1a. Assuming similar metabolic changes between *Cpt1a*$^{-/-}$ AT2 cells and *Cpt1a*$^{-/-}$ MLE12 cells, we performed SIRM experiments using *Cpt1a*$^{-/-}$ and control MLE12 cells (**Supplementary Figure 7**). The results showed that ^{13}C labeled metabolites of glycolysis and TCA cycle are significantly decreased in *Cpt1a*$^{-/-}$ MLE12 cells, and confirm that glycolysis is downregulated in *Cpt1a*$^{-/-}$ MLE12 cells (*please see page 9, 49-51 of the revised manuscript, and also Supplementary Fig. 7 and the related figure legends*).

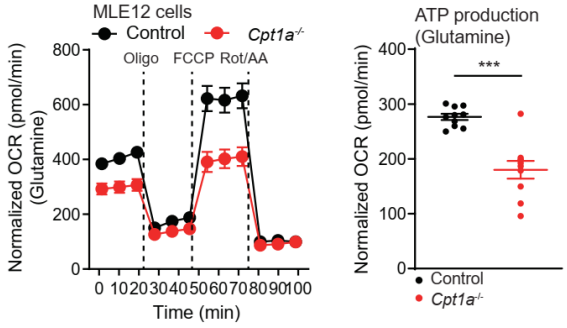
8.2 They mention that exogenous pyruvate is required to support the *in vitro* proliferation of cells with electron transport chain defects. Thus, where are the metabolic substrates leading to pyruvate import coming from? i.e. which metabolites upstream or downstream pyruvate are the ones responsible of ATP production for cell survival? e.g.: Krebs cycle anaplerotic substrates (such as the glutamate that the authors add in the extracellular flux analyses medium)? Amino acid degradation into lactate and then conversion into pyruvate? Pentose phosphate pathway? TG degradation into glycerol? This should be clarified.

Response 8.2: OCRs for ATP production using glucose, pyruvate, and glutamine are significantly increased in *Cpt1a*$^{-/-}$ MLE12 and *Cpt1a*$^{-/-}$ AT2 cells. Our data above indicate that glucose is not the key substrate for maintaining cellular energy production in MLE12 and AT2 cells after *Cpt1a* deletion (**response 8.1**), and glutamine anaplerosis or exogenous pyruvate supplement has been shown to support energy production under glucose deprivation[22, 23]. We thus investigated whether either glutamine or pyruvate alone is enough to fully support cellular energy production in *Cpt1a*-deficient MLE12 and AT2 cells. However, we found that both *Cpt1a*$^{-/-}$ MLE12 cells (see **Figure included in response 8.2**) and *Cpt1a*$^{-/-}$ AT2 cells (**Fig. 3f, g**) showed significantly less OCRs for ATP production utilizing glutamine alone. The results confirm that cellular energy production in *Cpt1a*-deficient MLE12 and AT2 cells is not maintained by glutamine anaplerosis. Our data further demonstrate that pyruvate-dependent OCRs for ATP production are not significantly different between *Cpt1a*$^{-/-}$ and control AT2 cells (**Fig. 3h, i**). Therefore, pyruvate and glutamine are both required to support ATP production in *Cpt1a*-deficient MLE12 and AT2 cells (**Fig. 3j, k and Supplementary Fig. 6h, i**). Collectively, our results indicate that adaptative metabolism is activated to maintain energy production in *Cpt1a*$^{-/-}$ AT2 cells (**Fig. 3l**).

Our data do not preclude substrates other than pyruvate/glutamine can be used by *Cpt1a*$^{-/-}$ AT2 cells to maintain energy production. We apologize for wrong interpretation of the metabolic responses as “pyruvate-dependent” in *Cpt1a*-deficient AT2 cells, and thus did not explore whether deleting *Cpt1a*

results in upregulation of *de novo* pyruvate synthesis through metabolites other than glucose, since primary AT2 cells can acquire exogenous pyruvate from the circulation. However, the exact mechanisms underlying increased pyruvate/glutamine-dependent OCRs for ATP production in *Cpt1a*-deficient MLE12 and AT2 cells warrant further explorations in our future work. We thank Reviewer #2 again for the important comment, and included the new data and the related discussion in the revised manuscript (*please see page 9-10 and 21-22 of the revised manuscript, and also Fig. 3 and the related figure legends*).

Figure included in response 8.2 (reviewer #2). Etracellular flux analyses with glutamine alone in MLE12 cells. Data are presented as mean ± SEM. (***p < 0.001, by a unpaired Student's t-test).



Reviewer #3 (Remarks to the Author):

In this paper, Chung et al used AT2 specific Cpt1a mutant mice, patient sample and cell culture to analyze the relationship between AT2 Cpt1a dependent mitochondrial LC fatty acid oxydation and ALI phenotype. They offer information regarding the change of energy production in *Cpt1a*-deleted AT2 cells, i.e., from mtLCFAO to the compensated pyruvate mediated one. Further data showed the loss of CPT1a in AT2 cells and changes in phosphor lipids in BAL, and decreases of alveolar inflammation in ALI. However, there is a lack of mechanistic insights regarding the connection among mitochondria fatty acid oxidation, phosphor lipid synthesis and inflammatory responses in AT2 cells. It is not clear whether downregulation of alveolar epithelial mtLCFAO really relates to an altered immune response in ARDS lungs. The manuscript is well written with a lot of data, but the data are largely descriptive.

We thank Reviewer #3 for the comments. We have reorganized the manuscript and performed additional experiments to address the reviewer's concerns. Below are detailed point-by-point responses:

There are some other issues:

1. *Acadl*: the abbreviation was not explained.

Response 1: *Acadl* is the abbreviation of "acyl-coenzyme A dehydrogenase long chain" gene, and

has bee explained in the abstract and the introduction in the revised manuscript (*please see page 2 and 3 of the revised manuscript*).

2. Fig1a and Figs1a: they showed correlation of CPT1a expression and ALI, however they did not include an AT2 marker, therefore it is not known whether the changes in CPT1a occur in AT2s, especially CD45+ cells express significantly higher levels of CPT1a.

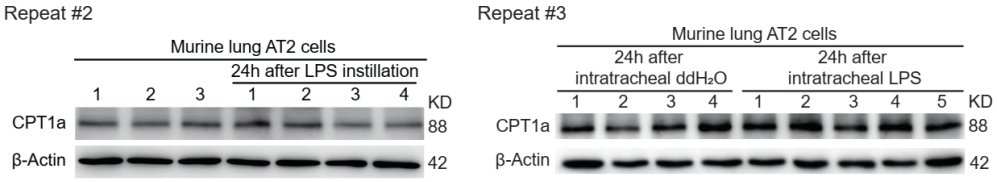
Response 2: In our IHC staining study using human surgical samples, we had included an epithelial cell marker CK for identifying the alveolar epithelium (**Fig. 1a**). During the process of revision, pathologists (co-authors Y.L.H. and M.S.H.) helped to review the original slides again. The CPT1a staining involved both type 1 and type 2 alveolar epithelial cells, and we could not find good antibodies for immunofluorescent co-staining for both CPT1a and SFTPC using formalin-fixed paraffin-embedded samples. To obtain further insights regarding the alterations of mitochondrial fatty acid β-oxidation in AT2 cells in human ARDS lungs, we performed additional analyses using online available single cell RNA sequencing (scRNAseq) datasets generated from individuals with coronavirus disease 2019 (COVID-19)-associated ARDS and control subjects (**Supplementary Fig. 2a**)[24, 25]. AT2 cell clusters were identified through the positive expression of *SFTPA1*, *SFTPB*, and *SFTPC*, and negative expression of *AGER* and *PDPN* (**Supplementary Fig. 2b, c**). Gene set enrichment analyses revealed downregulated expression scores for the gene ontology pathway (GO) fatty acid β-oxidation (GO:0006635) in AT2 cells from individuals with COVID-19-associated ARDS (**Fig. 1d**). The results are thus consistent with our findings and suggest that mtLCFAO is downregulated in the alveolar epithelium, in particular AT2 cells, in ARDS (*please see page 5-6 of the revised manuscript, and also Fig. 1d, Supplementary Fig. 2 and the related figure legends*).

3. Sup Fig2: the mRNA level of *Cpt1a* was decreased after LPS but the protein level was unchanged, how to explain this?

Response 3: Similar to the study by Huachun Cui et al.[14], our data showed downregulated *Cpt1a* mRNA expression in AT2 cells in LPS-induced ALI. However, while the study by Huachun Cui et al. demonstrated CPT1a protein expression is decreased in AT2 cells in LPS-induced ALI, we fail to reproduce the data (**Supplementary Fig. 3e-g**). We have tried the immunoblots using samples from several independent experiments, including another experiment during the revision process (see **Figure included in response 3**), but cannot show robust decrease of CPT1a protein expression in AT2 cells in LPS-induced ALI. The exact reason for data discrepancy between studies is unclear. However, both mRNA translation and protein turnover rate affects the protein expression amounts[26]. A recent study also demonstrated that the stability of CPT1a could be regulated by itaconate[27]. The findings indicate that the turnover rate of CPT1a may be altered under different biological states and suggest that CPT1a protein levels in AT2 cells in LPS-induced ALI may not be determined solely by mRNA translation. Meanwhile, our data confirm that mtLCFAO in AT2 cells is downregulated in LPS-

induced ALI, due to decreased CPT1a activity in AT2 cells (**Fig. 1e, f**) (please refer to **response 4.3 to Reviewer #2**). CPT1a activity has been shown to be regulated by malonyl-CoA and butyryl-CoA[20, 28]. Therefore, the CPT1a protein level may not be equivalent to CPT1a activity, and our data from extracellular flux analyses and immunoblots (**Fig. 1e, f** and **Supplementary Fig. 3e-g**) thus suggest that downregulated mtLCFAO in AT2 cells in LPS-induced ALI may be related to altered regulation of CPT1a activity (*please see page 6 of the revised manuscript*).

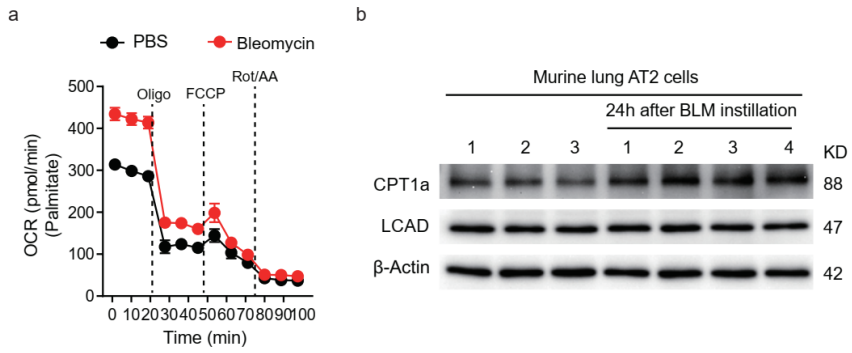
Figure included in response 2 (reviewer #3). Immunoblots for CPT1a protein expression in AT2 cells at baseline and in LPS-induced ALI. The data are from two independent experiments



4. The explanation of Fig 1d, 1f and FigS2f, 3a are unclear and confusing.

Response 4: In LPS-induced ALI, our data showed palmitate-dependent OCRs are significantly decreased in AT2 cells at 24h after LPS instillation (left panels in **Fig. 1e, f in the revised manuscript**, which are in Fig. 1d, e in the original manuscript). We performed additional experiments and pretreated AT2 cells with etomoxir, which is an irreversible inhibitor of CPT1a, 2h before extracellular flux analyses using palmitate (please refer to **response 4.1 and 4.2 to Reviewer #2** for details). The results showed that etomoxir pretreatment eliminates the differences in palmitate-dependent OCRs in AT2 cells from ALI model and the control (right panels in **Fig. 1e, f**). Therefore, while OCRs with glucose, pyruvate, and glutamine are decreased in AT2 cells in LPS-induced ALI (**Supplementary Fig. 3a, b**), our new data (right panels in **Fig. 1e, f**) indicate that downregulated CPT1a activity accounts for decreased palmitate-dependent OCRs in AT2 cells after LPS instillation. Meanwhile, our data showed upregulated palmitate-dependent OCRs and increased CPT1a protein expression in AT2 cells isolated at 24h after bleomycin instillation (see **Figure included in response 4**, which is Fig. 1f-g and Supplementary Fig. 3c in the original manuscript). As we mentioned in **response 3**, The interpretation of the data needs further extracellular flux analyses with etomoxir pretreatment to confirm upregulation of CPT1a activity. However, to improve data presentation and to highlight key message in our current work, we removed data from murine bleomycin-induced lung fibrosis model in the revised manuscript (please refer to **response 2 to Reviewer #1** and **response 4.2 to Reviewer #2**), and thus did not perform additional experiments to evaluate CPT1a activity in AT2 cells isolated after bleomycin instillation. We have modified the related contents in the revised manuscript (*please see page 6 of the revised manuscript*).

Figure included in response 4 (reviewer #3). (**a**) Extracellular flux analyses with palmitate and (b) immunblots for CPT1a and LCAD in AT2 cells at 24 hours after instillation of PBS or bleomycin



5. Fig1, FigS2.S3, did not explain why the results of bleomycin are different from LPS.

Response 5: While both LPS and bleomycin activate inflammatory responses in AT2 cells through TLR4 signaling[29], bleomycin also induces DNA damage in nuclear and mitochondrial genomes[30]. Although the different biologic effects caused by LPS and bleomycin may account for different results in palmitate-dependent OCRs in AT2 cells after exposure (Fig. 1e, f and also **Figure included in response 4**), the assumption requires further investigations to confirm. However, our aim in **Fig. 1** is to illustrate CPT1a expression and mtLCFAO activity in alveolar epithelium in human ARDS and in AT2 cells from murine models of ALI, and it is not our focus to examine the changes of CPT1a and mtLCFAO activity in AT2 cells after instillation of different stimulants to murine lungs. As mentioned in **response 4**, to avoid distraction, we have removed data from murine model of bleomycin-induced lung fibrosis in the revised manuscript (please refer to **response 2 to Reviewer #1** and **response 4.2 to Reviewer #2**).

6. They did not examine the distribution of Cpt1a isoforms in primary AT2s, unclear whether different isoforms will interfere with each other.

Response 6: Previous studies have widely proved that CPT1a is located in mitochondria[17], and we have removed the redundant data in MLE12 cells from the revised manuscript (please refer to **response 2.2 to Reviewer #2**). In addition, data from The Human Protein Atlas[18] show that CPT1a is the main CPT1 isoform expressed in human lungs, and our RNAseq data further confirm that *Cpt1a* is the principal isoform expressed in murine AT2 cells, and deleting *Cpt1a* does not result in increased *Cpt1b* or *Cpt1c* expression in AT2 cells (**Supplementary Fig. 4a-d**) (please refer to **response 2.1 to Reviewer #2**). We add the above information in the revised manuscript (*please see page 5 and 7 of the revised manuscript, and also Supplementary Fig. 4 and the related figure legends*).

7. It is unclear whether the change of mtLCFAO mediated cellular metabolism in AT2 *Cpt1a* mice are connected to the change of phosphor lipids in BAL. They did not observe a change in phosphor lipid in AT2, but it was changed in BAL, unclear if the change in BAL is indirectly

caused by macrophage etc.

Response 7: In this study, through CLICK labeling of newly synthesized choline-containing phospholipids[31], we provided data to show increased phospholipid synthesis activity in AT2 cells (**Fig. 4f, g**). Meanwhile, the expression Cre^{ERt2} recombinase is under the control of *Sftpc* promoter[11], and *Cpt1a* is only deleted in AT2 cells after tamoxifen injection, but not in other immune cells. However, we agree with the reviewer that alveolar macrophages are critical in consuming and recycling surfactant phospholipids[32], and defects in GM-CSF signaling in alveolar macrophages can lead to pulmonary alveolar proteinosis with surfactant phospholipid accumulation in alveoli. Therefore, through flow cytometric sorting of BALF cells (**Supplementary Fig. 14**), we isolated alveolar macrophages from *Cpt1a*$^{i\Delta AT2}$ and control mice for RNAseq. The transcriptomic profiles in alveolar macrophages from *Cpt1a*$^{i\Delta AT2}$ and control mice were not remarkably different (**Supplementary Fig. 15a**) with gene set enrichment analyses demonstrating that a loss of *Cpt1a* in AT2 cells did not alter GM-CSF signaling (GO: 0097012), fatty acid oxidation (GO: 0019395) and lipid biosynthetic process (GO: 0008610) in alveolar macrophages (**Supplementary Fig. 15b-e**). Our new data thus exclude the possibility that the observed alterations in alveolar phospholipids are attributed to alterations in alveolar macrophages after *Cpt1a* deletion in AT2 cells, and support that *Cpt1a* deletion in AT2 cells results in altered phospholipid contents in the alveoli (*please see page 13-14 of the revised manuscript, and also Supplementary Fig. 15 and the related figure legends*).

8. Fig5A: the figure is unclear, need to show separated channel for AT2 and CD45.

Response 8: We provide enlarged image of **Fig. 7a in the revised manuscript** (**Fig. 5a in the original manuscript**) in **Supplementary Fig. 19**, with the channel for tdTomato (AT2 cells) and that for CD45 (immune cells) separated. In addition, the size of **Supplementary Fig. 20a** (Supplementary 16a in the original manuscript) was also enlarged with tdTomato and CD45 channels separated (*please see page Supplementary Fig. 19, 20a and the related figure legends*).

9. Fig6f,g, there are some differences in lipid components between control and mutant, but the results were not explained.

Response 9: To assess if increased abundance of PG species in the alveoli, as observed in the *Cpt1a*$^{i\Delta AT2}$ mice at baseline (**Fig. 4k**), may be a mechanism by which these mice have lower inflammation under ALI, we carried out lipidomic analyses using BALF isolated from *Cpt1a*$^{i\Delta AT2}$ and control mice at 24 hours after LPS instillation. Total BALF levels of measured PG species in ALI were similarly decreased in controls and *Cpt1a*$^{i\Delta AT2}$ mice (**Fig. 6b**) with the PG species PG(34:2)_18:2 and PG(36:4)_20:4 significantly lower in the *Cpt1a*$^{i\Delta AT2}$ mice when compared to controls (**Fig. 6c**, which is Fig. 6f in the original manuscript). Loss of PG species abundance may result from increased phospholipase activity leading to increased levels of lysophospholipids[33, 34], and our data showed BALF LPG(16:0) levels were significantly increased in *Cpt1a*$^{i\Delta AT2}$ mice when compared to controls

upon ALI (**Fig. 6f**, which is Fig. 6g in the original manuscript). These findings, which show a significant loss of PG abundance in the *Cpt1a*$^{i\Delta AT2}$ mice in ALI, may be related to enhanced PG breakdown in *Cpt1a*$^{i\Delta AT2}$ mice in LPS-induced ALI. We address the data about differences in BALF PG and LPG compositions in ALI between *Cpt1a*$^{i\Delta AT2}$ and control mice in the revised manuscript (*please see page 15-16 of the revised manuscript*).

10. Fig6m, o, the effect of PG(32:0)_16:0 was not explained.

Response 10: To confirm that inflammatory resolution in *Cpt1a*$^{i\Delta AT2}$ mice is not be related to increased alveolar PG species at baseline (**Fig. 6a**), LPS was instilled into *Cpt1a*$^{i\Delta AT2}$ and control mice together with high-dose PG(32:0)_16:0 or PG(34:1)_18:1 to evaluate if high-dose PG supplement at baseline results in similar alveolar inflammation between *Cpt1a*$^{i\Delta AT2}$ and control mice (**Fig. 6g**). Although instillation of PG(32:0)_16:0 obliterated the differences in alveolar CXCL1 and TNFα levels (**Fig. 6j, l**, which are respectively Fig. 6m, o in the original manuscript), alveolar cell numbers and CXCL2 levels remained significantly higher in control than those in *Cpt1a*$^{i\Delta AT2}$ mice (**Fig. 6h, k**). The data indicate that high-dose PG instillation may affect the mechanisms regulating alveolar cytokine levels in LPS-induced ALI, but does not reduce alveolar cellular infiltrates in controls. Furthermore, our data in *Acadl*$^{i\Delta AT2}$ mice (please refer to **response 11**) also support that decreased alveolar inflammation in *Cpt1a*$^{i\Delta AT2}$ mice is not related to altered alveolar PG contents at baseline. We modified the presentation of the data in the revised manuscript (*please see page 16-17 of the revised manuscript*).

11. Fig7: it is not convincing by simply comparing *Cpt1a* and *Acadl* phenotype to claim that "impairing mtLCFAO in AT2 cells leads to decreased alveolar inflammation in ALI."

Response 11: Impairing mtLCFAO in AT2 cells through deleting *Cpt1a* or *Acadl* restricts alveolar inflammation in ALI at 72 hours after LPS stimulation by hindering the production of the neutrophilic chemokine CXCL2 (**Fig. 5f** and **8l**). However, deleting *Cpt1a* results in metabolic changes, including decreased glycolysis (**Fig. 3d, e**), and upregulated phospholipid synthesis in AT2 cells (**Fig. 4f, g**). It is unknown whether downregulated CXCL2 production in *Cpt1a*-deficient AT2 cells in ALI is related to metabolic responses after *Cpt1a* deletion or is related to impaired mtLCFAO. For investigation, we performed additional experiments to characterize the metabolism in *Acadl*$^{-/-}$ AT2 cells. In contrast to metabolic changes in *Cpt1a*$^{-/-}$ AT2 cells, loss of LCAD does not upregulate OCRs with glucose, pyruvate and glutamine, or alter glycolysis activity in AT2 cells (**Supplementary Fig. 21**). In addition, *Acadl* deletion neither alters intracellular TG contents or phospholipid synthesis in AT2 cells, nor leads to remarkable changes in alveolar PC and PG compositions (**Supplementary Fig. 22**; **Supplementary Table 7, 8**). The findings indicate that the metabolic changes in *Cpt1a*$^{-/-}$ AT2 cells are specific to CPT1a loss, and further strengthen the role of mtLCFAO activity in tuning CXCL2 production in AT2 cells. We included the data above in the revised manuscript. In addition, since the metabolic changes are not completely the same between *Acadl*-deficient AT2 cells and MLE12 cell

line, we removed data from *Acadl*-deleted MLE12 cells in the original manuscript (*please see page 19-20 of the revised manuscript, and also Supplementary Fig. 21, 22 and the related figure legends*).

Reviewer #4 (Remarks to the Author):

In this manuscript, the authors propose to evaluate the role of mitochondrial long-chain fatty acid oxidation (mtLCFAO) in alveolar epithelial cells, particularly in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). Their intent is to add significant insights into the immuno-metabolic mechanisms in respiratory biology. The experimental design appears robust, utilizing both human lung samples and murine models. Techniques like immunohistochemistry, extracellular flux analysis, and transcriptomic profiling are appropriately used.

We thank Reviewer #4 for the thoughtful comments. We have performed additional experiments and revised the manuscript to improve the delivery of the key message in this study. Below are detailed point-by-point responses:

1.1 However, I found several key details on the statistical methods used for data analysis lacking.

Response 1.1: The statistical analyses for calculating each *p* value are all addressed in details in Figure Legends. The detailed methods for analyzing RNAseq data are described in detail in ONLINE METHODS. Due to the word count limit for the main test, the information above is not addressed in the main text in the result section (*please see page 46-47 and 58 of the revised manuscript, and the related figure legends*).

1.2 The results showing the impact of mtLCFAO alterations in type 2 alveolar epithelial (AT2) cells on immune responses in the lungs are compelling. They are well-supported by extensive data and are clearly presented through figures and tables. However, my key concerns are related to lack of significant phenotypic changes in the lungs as a result of deletion of this pathway in the *Cpt1a*$^{i\Delta AT2}$ mice. There was no significant change in the long-term fibrosis or acute cellular infiltrates in the lungs in response to the different injuries.

Response 1.2: Loss of CPT1a in AT2 cells is not associated with histopathological changes in murine lungs at 8 months (~37 weeks old) after tamoxifen injection (**Supplementary Fig. 9b**). In our previous work, we demonstrated no histopathological changes in lungs of *Mfn1*$^{i\Delta AT2}$ (*Mfn1*$^{loxP/loxP}$*Sftpc*$^{CreERt2+/-}$) and *Mfn2*$^{i\Delta AT2}$ (*Mfn2*$^{loxP/loxP}$*Sftpc*$^{CreERt2+/-}$) mice at 8 months after tamoxifen injection, but deleting either *Mfn1* or *Mfn2* in AT2 cells aggravate lung fibrosis development after bleomycin instillation[35]. The findings indicate that genetic defects in AT2 cells may lead to altered phenotypes to lung injury without altering lung histology at baseline.

Cpt1a$^{i\Delta AT2}$ mice do not have less alveolar cellular infiltrates at 24 hours after TLR2 or TLR4 stimulation (**Fig. 5c** and **Supplementary 13c**). Mechanistic exploration suggests that deleting *Cpt1a* in AT2 cells may affect the inflammatory responses in alveolar macrophages (AMs), and the RNAseq data revealed increased inflammatory responses in alveolar macrophages in LPS-induced ALI after *Cpt1a* deletion in AT2 cells (**Supplementary Fig. 16c-h**). The finding may account for a lack of anti-inflammatory phenotypes in *Cpt1a*$^{i\Delta AT2}$ mice at 24 hours after LPS instillation (please see **response 6 to Reviewer #2**). Furthermore, AM pool is depleted at 24 hours in LPS-induced ALI, and is subsequently replenished and reorganized through AM proliferation and monocyte recruitment after 72 hours[36]. The transcellular regulation from *Cpt1a*$^{-/-}$ AT2 cells to AMs may affect the reorganization of AM pool in in *Cpt1a*$^{i\Delta AT2}$ mice, as suggested by our new data (see **response 1.3**). A recent study showed that the reorganization of AM population after ALI can be long-lasting[37], and it requires further studies to clarify whether *Cpt1a* deletion in AT2 cells alters the restoration of AM pool after ALI. Meanwhile, the severity of bleomycin-induced lung fibrosis is not aggravated in young *Cpt1a*$^{i\Delta AT2}$ mice in our study (Supplementary Fig. 14 in the original manuscript). Young mice are more resistant to bleomycin-induced lung fibrosis than aged mice, and it remains unknown whether deleting *Cpt1a* in AT2 cells will affect bleomycin-induced lung fibrosis in aged mice[38].

Of note, we provide extensive data and direct evidence to support the findings that deleting *Cpt1a* in AT2 cells results in improved resolution of alveolar cellular infiltrates (**Fig. 5f** and **Supplementary Fig. 13h**), and the results challenge previous assumptions about potential detrimental effects of mtLCFAO impairment in AT2 cells in ALI[14, 39]. Although this study has advanced our understandings about the effects of *Cpt1a* deletion to the metabolism and function of AT2 cells, the phenotypic impacts after *Cpt1a* deletion in AT2 cells are not fully explored in this study and deserve further explorations. We thank Reviewer #4 for the comment, and address details in the discussion of the revised manuscript. Since the data about bleomycin-induced murine fibrosis model are removed from revised manuscript (please see **response 2 to Reviewer #1**), the effects of *Cpt1a* deletion in AT2 cells to bleomycin-induced lung fibrosis are not discussed (*please see page 14-15 and 22-23 of the revised manuscript, and also Supplementary Fig. 15, 16 and the related figure legends*).

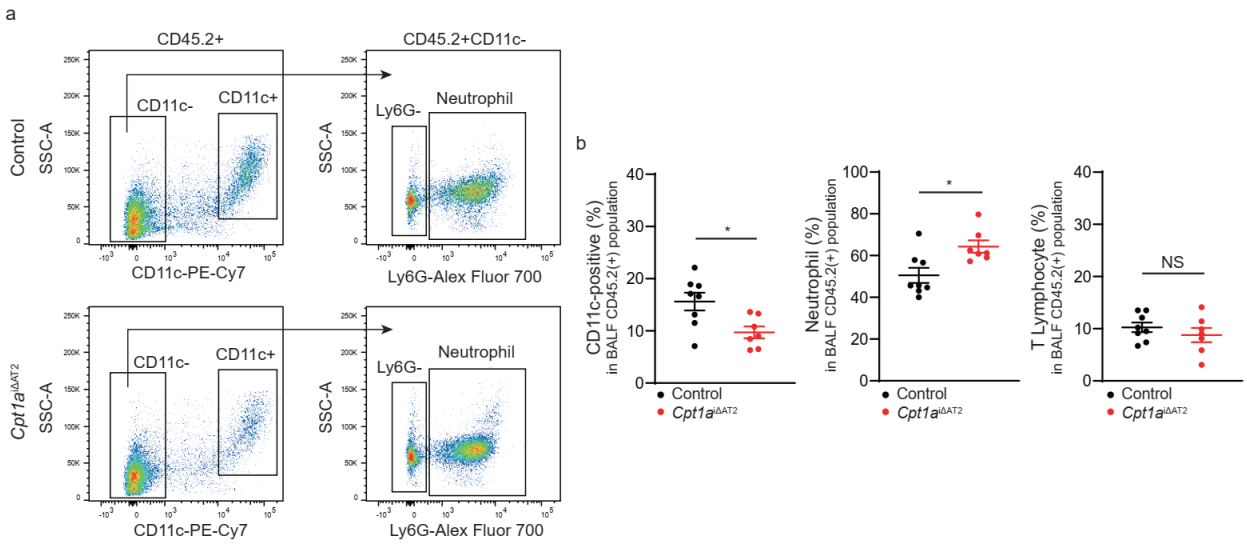
1.3 Additionally, there was no data that revealed any functional alteration in the injured lungs in these knockout mice. These findings may suggest that this pathway may not be the dominant driver of the lung injury and the injury response. There was no change in the cellular influx as well.

Response 1.3: We did not measure lung function in ALI. However, the protein content in BALF is a surrogate to evaluate the barrier function of alveolar-capillary membrane[13], and our data show that *Cpt1a*$^{i\Delta AT2}$ mice do not have altered BALF protein levels in ALI. While AT2 cells are responsible for alveolar fluid clearance[40], the alveolar permeability changes in murine ALI models are also regulated by type 1 alveolar epithelial cells and endothelial cells[41, 42], and are not solely determined by alveolar

neutrophilic influx. Various cell populations in lungs are involved in lung injury[42]. In this study, *Cpt1a* is solely deleted in AT2 cells in murine lungs. Our aim is to explore the role of mtLCFAO in regulating the function of AT2 cells at baseline and in ALI, but is not to prove that mtLCFAO in AT2 cells is the driver of complex pathophysiologic process in ARDS (please see **response 2** for clinical implications).

As for the alveolar influx of immune cells in LPS-induced ALI, we find that the principal immune cells in the BALF at baseline are alveolar macrophages, whereas, at 24 hours, there is a significant dominance of neutrophils which begin to diminish or resolve 72 hours post LPS instillation (**Supplementary Fig. 10**). Although *Cpt1a*$^{i\Delta AT2}$ mice and the control both have similar neutrophil percentages in BALF immune cells in ALI at 24 hours after LPS instillation (**Fig. 5b)**, our new data indicate the percentages of neutrophils and CD11c-positive innate immune cells are different between the control and *Cpt1a*$^{i\Delta AT2}$ mice at 72 hours (please see **Figure included in response 1.3**; SiglecF is not stained due to shortage of antibody for the experiment). As we mentioned in **response 1.2**, AM restoration by AM proliferation and monocyte recruitment begins at 72 hours after LPS instillation[36]. In addition, neutrophilic inflammation has been shown critical to monocytic emigration to the inflammatory sites[43]. The resolution of alveolar cellular infiltrates in *Cpt1a*$^{i\Delta AT2}$ mice may not be solely due to decreased alveolar influx of neutrophils, as suggested by our data, but may be also related to perturbed AM pool restoration after ALI. Although this study reveals the transcellular regulation between *Cpt1a*$^{-/-}$ AT2 cells and neutrophils (**Fig. 7h-k**), the transcellular regulation from *Cpt1a*$^{-/-}$ AT2 cells to AMs in ALI warrants further exploration. We thank Reviewer #4 again for the insightful comment, and will address more details in our future work (*please see page 22-23 of the revised manuscript, and also Supplementary Fig. 10, 15, 16 and the related figure legends*).

Figure included in response 1.3 (reviewer #4). (**a**) Flow cytometric ananlyses of BALF immune cells in *Cpt1a*$^{i\Delta AT2}$ and control mice at 72 hours after LPS instillation (*Cpt1a*$^{i\Delta AT2}$ mice n = 7, control mice n = 8), and (**b**) the percentages of CD11c(+) immune cells, neutrophils, and T lymphocytes in BALF CD45.2-positive population were compared between groups. Data are represented as mean ± SEM (*p < 0.05, NS non-significant, by unpaired Student's t-tests)



2. The discussion contextualizes the findings within the broader field, highlighting the

potential of mtLCFAO as a therapeutic target in ARDS. However, I did not find compelling evidence that targeting this pathway would result in a clinical benefit to patients with ARDS.

Response 2: We apologize for overstating mtLCFAO as a potential beneficial therapeutic target in ARDS. A recent study by Ly Pham et al. showed that intraperitoneal administration of etomoxir, an irreversible inhibitor of CPT1a, reduced neutrophilic influx to murine lungs after bacterial inoculation[44]. However, the restricted neutrophilic inflammation results in poor clearance of bacteria and increased mortality of the murine host. Therefore, while inhibiting mtLCFAO in AT2 cells may decrease alveolar neutrophilic inflammation in ARDS, this may also suppress the alveolar immunity against invading pathogen and potentially may lead to worse clinical outcomes. Furthermore, none of the murine ALI models can similar all the pathophysiological aspects and clinical courses of human ARDS. Considering the translation gaps from murine ALI modes to human ARDS, the interpretation about clinical implications of this study should be cautious. We thus modified the contents in the discussion (*please see page 23-24 of the revised manuscript*).

3. While the manuscript is well written, there were several typos and grammatical errors. I also found several experiments redundant and the text a bit too verbose, often discussing findings that did not add significant scientific rigor.

Response 3: We thank reviewer #4 for this critical comment, which was also raised by Reviewer #1 and Reviewer #2 (Please refer to **response 2 to Reviewer #1** and **response 2.2 to Reviewer #2**). We have thoroughly reorganized the data presentation and removed redundant data in the revised manuscript. Murine model of bleomycin-induced lung fibrosis is a key model frequently applied in the field for investigating the mechanisms leading to lung fibrosis development[1]. To highlight the key message about mtLCFAO in AT2 cells and alveolar inflammation in ALI, we removed data from murine model of bleomycin-induced lung fibrosis in the revised manuscript. We believe the coherence and logical flow have been improved in the revised manuscript.

A few additional concerns:

4. Reliability of AT2 cells from injured mice: Given that the AT2 cells are a heterogeneous group it was unclear whether the changes observed in the cell isolated were a result of direct injury response or whether it was a selection bias related to the cells isolated.

Response 4: We isolate AT2 cells from whole murine lungs, and the method for AT2 cell isolation is based on a paper by Elise M Messier, et al.[45]. The purity of the AT2 cells based on our previous study is about 94% by flow cytometric analysis[35], and the finding is similar to that in the paper by Elise M Messier, et al.[45]. While the population of AT2 cells is heterogenous, we isolated whole lung AT2 cells from all transgenic mice using the same method, and theoretically all subgroups of AT2 cells will be isolated. Therefore, the results should be due to direct injury to AT2 cells in the model. However, in a *Science* paper by Ahmad Nabhan et al.[46], while only *Axin2*-positive AT2 cells can be progenitor cells

of the alveoli, both *Axin2*-positive and *Axin2*-negative AT2 cell subpopulations can produce alveolar surfactant. It is unknown whether the role of mtLCFAO in regulating CXCL2 production exists in a specific subpopulation of AT2 cells or in the whole AT2 cell population. The question is beyond the scope of this study but is an interesting field for future research.

5. While they observe lower levels of certain cytokines, neutrophils numbers are unchanged in the *Cpt1a*$^{i\Delta AT2}$ mice. These cytokines are known to be produced by several different types of myeloid and non-myeloid cells.

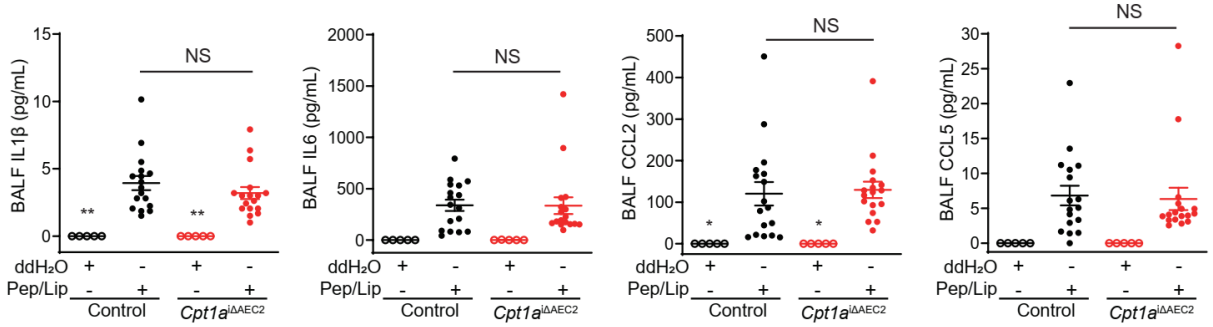
Response 5: In this study, neutrophil *percentages* in BALF immune cells are unchanged in *Cpt1a*$^{i\Delta AT2}$ mice at 24 hours in ALI (please refer to the **2nd paragraph in response 1.3**). Our results showed that *Cpt1a* deletion in AT2 cells alters early immune responses in alveolar macrophages (4 hours after LPS instillation), leading to upregulation of several proinflammatory cytokines (**Supplementary 16c-h**) (please refer to **response 6 to Reviewer #2**). The findings indicate that alveolar macrophages are not the key cells leading to less alveolar cytokine levels in ALI in *Cpt1a*$^{i\Delta AT2}$ mice. On the other hand, neutrophil self-recruitment by CXCL2 has been shown critical in maintaining neutrophilic inflammation[47]. Our results revealed that deleting *Cpt1a* in AT2 cells results in *decreased CXCL1 and CXCL2 production from neutrophils* (**Fig. 7j, k**), which may be related to decreased CXCL2 production from *Cpt1a*$^{-/-}$ AT2 cells (**Fig. 7h**). Since alveolar macrophages and neutrophils are respectively the main immune cells at baseline and in murine models of ALI (**Supplementary Fig. 10**), we did not explore cytokine production from the minority population of alveolar immune cells in this study (*please see page 14-15, 18-19, and 20 of the revised manuscript, and also Fig. 7j, 7k, 8i-8k, Supplementary Fig. 15, 16 and the related figure legends*).

6. There is lack of specificity of the responses in the *Cpt1a* deficient mice. They show a broad decrease in the cytokines but I am not sure if there is sufficient data to establish causality with this pathway. These mice might just be inherently predisposed to a lower cytokine response. The concerns are further highlighted in the fact that there was no demonstratable long-term phenotypic or functional alteration in these mice.

Response 6: In LPS-induced ALI, BALF levels of several cytokines/chemokines related to innate immune responses, such as IL1β, CCL2, CCL5 and IL6, are not different between *Cpt1a*$^{i\Delta AT2}$ and control mice. In addition, in ALI induced by instillation of peptidoglycan and lipoteichoic acid, only BALF levels of CXCL2, but not TNFα, CXCL1, IL1β, CCL2, CCL5 and IL6, are decreased in *Cpt1a*$^{i\Delta AT2}$ mice (**Supplementary Fig. 13e-g**, and **Figure included in response 6**). The above results thus suggest that *Cpt1a*$^{i\Delta AT2}$ mice are not inherently predisposed to a lower cytokine response. However, it is unknown whether the metabolic changes in *Cpt1a*$^{-/-}$ AT2 cells are CPT1a-specific or related to mtLCFAO impairment. Moreover, it is also unclear whether downregulated CXCL2 production in *Cpt1a*-deficient AT2 cells in ALI is CPT1a-dependent, transgenic mouse strain dependent, or can be

suppressed by impairing mtLCFAO (**Fig. 8a**). For investigation, we generated *Acadl*$^{loxP/loxP}$*Sftpc*$^{CreERt2+/-}$ mice (referred to as *Acadl*$^{i\Delta AT2}$ mice) (**Fig. 8b-d**). Our data confirm that impairing mtLCFAO by deleting *Acadl* in AT2 cells also suppresses CXCL2 production from AT2 cells in LPS-induced ALI (**Fig. 8j**). The data from *Acadl*$^{i\Delta AT2}$ mice indicate that the findings about improved resolution of alveolar cellular infiltrates in *Cpt1a*$^{i\Delta AT2}$ mice are not transgenic mouse strain dependent, and support the regulation between mtLCFAO and CXCL2 production in AT2 cells. Lastly, as we mentioned in **response 1.2**, the phenotypic impacts after *Cpt1a* deletion in AT2 cells are not fully explored in this study, and deserve further explorations (*please see page 19-20 the revised manuscript, and also Fig. 8, Supplementary Fig. 21-22 and the related figure legends*).

Figure included in response 6 (reviewer #4). BALF cytokine levels at 24 hours after intra-tracheal instillation of peptidoglycan and lipoteichoic acid (Pep/Lip). Data are represented as mean ± SEM (Pep/Lip vs. ddH₂O **p < 0.01, *p < 0.05; *Cpt1a*$^{i\Delta AT2}$ vs. control NS non-significant; by one-way ANOVA with Bonferroni tests).



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors did perform additionnal measurements to substantiate their findings, removed data from one experimental model and streamlined their manuscript.

Yet, in many of their replies to this reviewer (as well as to some others) they chose to keep a significant number of results for reviewer purpose only or they mention that fully answering to the reviewers comment requires further investigations.

Thus, it would be useful to include more data from the point by point reply in the manuscript, at least as supplementary data.

It is also important to include a formal paragraph related to the limitation of the study at the end of the discussion

Reviewer #2 (Remarks to the Author):

I thank the authors for their time preparing the rebuttal letter and related experiments. My questions have been partially answered.

Reviewer #3 (Remarks to the Author):

The revised version is significantly improved and the authors have addressed many of my concerns. However, there are still some points need minor revisions:
1) They used AT2 specific promoter Sftpc to knock-out Cpt1a, but in "Figure a included in response 6 (reviewer #2)", the values of alveolar macrophages from AT2-Cpt1a KO mice showed decrease in all three panels, even though the difference is N.S., which could be due to small sample sizes. In Figure S15, they showed that transcriptomics in alveolar macrophages is not significantly altered by deleting Cpt1a in AT2 cells, they also need to show levels of Cpt1a in macrophage and confirm that their knock-out is specific to AT2s.
2) For Rev#3: they mentioned "we could not find good antibodies for immunofluorescent co-staining for both CPT1a and SFTPC using formalin-fixed paraffin-embedded samples." There is a good pro-SFTPC antibody in Millipore, and many other AT2 markers can be used such as HTII280, ABCA3 etc. They should do co-staining of CPT1 and AT2 markers. The scRNA-seq data they mentioned is indirect evidence.
3) Fig7a: the color of AT2-tomato and CD45 are similar and difficult to distinguish between each other.

Reviewer #4 (Remarks to the Author):

Thank you for being responsive for the critiques.

Point-by-point Responses to the Reviewers of Manuscript NCOMMS-23-46729B

We appreciate the comments from the reviewers. We have performed additional experiments and analyses, resulting in 8 new figure panels, 1 new table, and 3 revised figure panels, and provide further discussion to address the study limitation. We believe that the revisions (denoted by red font in the manuscript) have significantly improved the manuscript. A point-by-point response to each of the comments is enclosed below:

Reviewer #1 (Remarks to the Author):

The authors did perform additional measurements to substantiate their findings, removed data from one experimental model and streamlined their manuscript. Yet, in many of their replies to this reviewer (as well as to some others) they chose to keep a significant number of results for reviewer purpose only or they mention that fully answering to the reviewers comment requires further investigations. Thus, it would be useful to include more data from the point by point reply in the manuscript, at least as supplementary data.

Response: We appreciate the comments from the reviewers. After cautious consideration, we decide to include the data about alveolar metabolomic profiling at baseline and in ALI in the revised manuscript (**Supplementary Fig. 14; Supplementary Table 6**). In addition, we also include the complete BALF cytokine profiling data at 24 hours after instillation TLR2 agonists (**Supplementary Fig. 15h-k**). The results about high diet feeding and mitochondrial fatty acid β-oxidation in AT2 cells were included in a manuscript by our co-author Maria Plataki, and are not included in the revised manuscript. Meanwhile, data from MLE12 cell line are not included, since we have shown results in primary AT2 cells in the manuscript. Lastly, we believe thorough investigations are required to conclude details regarding the regulation of alveolar macrophages in *Cpt1a*$^{i\Delta AT2}$ mice, and the results about alveolar macrophages in the revised manuscript are thus not included in the revised manuscript (please see **response 1.1 to reviewer #3**) (*please see page 13-14, 54-55 in the revised manuscript, Supplementary Fig. 14, 15h-k and related figure legends, and Supplementary Table 6*).

2. It is also important to include a formal paragraph related to the limitation of the study at the end of the discussion.

Response 2: This study reveals that *Cpt1a* deletion in AT2 cells results in remarkable changes of alveolar metabolome in LPS-induced ALI murine model (**Supplementary Fig. 14; Supplementary Table 6**), yet the link between alveolar metabolome and altered metabolism in *Cpt1a*$^{-/-}$ AT2 cells in ALI remains to be determined. In addition, the role of alveolar metabolome in *Cpt1a*$^{i\Delta AT2}$ mice in regulating inflammatory responses in ALI is not fully explored in this study, and warrants further mechanistic clarification. We thank the reviewer for the suggestion, and address the limitations of this study in a paragraph in discussion (*please see page 26-27 in the revised manuscript*).

Reviewer #2 (Remarks to the Author):

I thank the authors for their time preparing the rebuttal letter and related experiments. My questions have been partially answered.

We thank Reviewer #2 for the comments.

Reviewer #3 (Remarks to the Author):

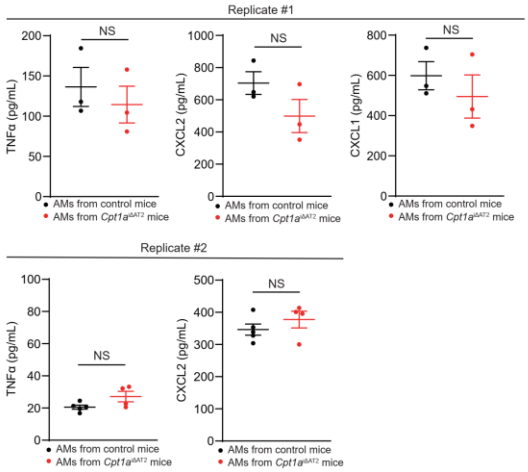
The revised version is significantly improved and the authors have addressed many of my concerns. However, there are still some points need minor revisions.

We appreciate Reviewer #3 for the comments. We performed additional experiments and analyses for the revision. Our detailed point-by-point responses are as the below:

1.1 They used AT2 specific promoter *Sftpc* to knock-out *Cpt1a*, but in "Figure a included in response 6 (reviewer #2)", the values of alveolar macrophages from AT2-Cpt1a KO mice showed decrease in all three panels, even though the difference is N.S., which could be due to small sample sizes.

Response 1.1: The results in "Figure a included in response 6 to reviewer #2" are representative data of two independent experiments, and here we show the data from another replication (please see **Figure included in response 1.1**; replicate #1 results are from "Figure a included in response 6 to Reviewer #2", and replicate #2 results are from replicative experiment). As mentioned by Reviewer #3, we also had the same assumptions that LPS-induced cytokine/chemokine production may be less in alveolar macrophages (AMs) from *Cpt1a*$^{i\Delta AT2}$ mice, when we saw replicate #1 results. However, data from replicative experiment did not suggest that *Cpt1a* deletion in AT2 cells alters LPS-induced inflammatory responses in AMs. Because we cannot obtain the same CXCL1 ELISA kit used in replicate #1 experiment (R&D Systems, MKC00B), CXCL1 levels were not measured in replicate #2 experiment. However, as pointed out by the reviewer, small sample size in our experiments is a critical issue. In our experiments, AMs were isolated, cultured overnight, and were then stimulated by LPS. Through this method, only small numbers of AMs can be obtained for experiments, unless several mice are sacrificed for AM isolation. A recent study by Anna-Dorothea Gorki et al. showed a better protocol for AM maintenance and culture[1], which will be applied in our ongoing work exploring the regulation of AMs in *Cpt1a*$^{i\Delta AT2}$ mice.

Figure included in response 1.1 (reviewer #3). Alveolar macrophages (AM) are isolated from *Cpt1a*$^{i\Delta AT2}$ (n = 5 for replicate #1; n = 7 for replicate #2) and control (n = 5 for replicate #1; n = 10 for replicate #2) mice, and are added to a 96-well culture plate (2 x 10^5 cells/well, 3 wells each group for replicate #1; control 5 wells, *Cpt1a*$^{i\Delta AT2}$ 4 wells for replicate #2). After incubation for 24 hours, LPS (2 ng/mL) was used to stimulate the cells, and the medium was obtained at four hours for cytokine measurements by ELISA. Data are represented as mean ± SEM (NS non-significant, by unpaired Student's t-tests).



1.2 In Figure S15, they showed that transcriptomics in alveolar macrophages is not significantly altered by deleting *Cpt1a* in AT2 cells, they also need to show levels of Cpt1a in macrophage and confirm that their knock-out is specific to AT2s.

Response 1.2: Both *Sftpc*- and *Lyz2*-driven Cre recombinase expression can be applied to delete target genes in AT2 cells[2, 3]. However, *Lyz2*-driven Cre recombinase expression may also lead to gene deletion in myeloid lineage. In this study, tamoxifen-inducible *Cpt1a* deletion in AT2 cells is induced through *Sftpc*-driven CreERt2 expression. Theoretically, Cpt1a in myeloid lineage, such as AMs, will not be affected. To address the concern of the reviewer, we showed *Cpt1a* mRNA expression in AMs from *Cpt1a*$^{i\Delta AT2}$ mice and those from the control mice in the revised manuscript (*please see page 15 and Supplementary Fig. 17a*).

2. For Rev#3: they mentioned "we could not find good antibodies for immunofluorescent co-staining for both CPT1a and SFTPC using formalin-fixed paraffin-embedded samples." There is a good pro-SFTPC antibody in Millipore, and many other AT2 markers can be used such as HTII280, ABCA3 etc. They should do co-staining of CPT1 and AT2 markers. The scRNA-seq data they mentioned is indirect evidence.

Response 2: In this study, we used the pro-SFTPC antibody from Millipore (catalog ABC99) for immunoblots. However, both CPT1a and pro-SFTPC antibodies are generated from the rabbit, and so we cannot use pro-SFTPC for immunofluorescent staining. However, we appreciate the critical suggestion from the reviewer, and find HTII-280 antibody from Terrace Biotech (catalog TB-27AHT2-280), generated from mice, is suitable for the experiments. Therefore, immunofluorescent staining of CPT1a and HTII-28027 was performed using diffuse alveolar damage (DAD) lung samples with low IHC CPT1a H scores and control lung samples. The results confirmed that CPT1a expression was remarkably decreased in AT2 cells in DAD (**Supplementary Fig. 2**). We showed the data and

the detailed methods for the staining in the revised manuscript (*please see page 5, 34-36, Supplementary Fig. 2 and the related figure legend*).

3. Fig7a: the color of AT2-tomato and CD45 are similar and difficult to distinguish between each other.

Response 3: We changed the pseudo-color (from orange to yellow) to label CD45-postive cells in **Fig. 7a** and **Supplementary Fig. 21** and **22a**. Furthermore, we have deposited all the microscopic images in the manuscript, including **Fig. 7a**, and **Supplementary Fig. 21** and **22a**, in Mendeley online data repository (doi: 10.17632/343cym3z8p.1), and so that full size images with high resolution can be obtained by the readers for reference. We thank the reviewer for the comment (*please see page 63, Fig. 7a, and Supplementary Fig. 21 and 22a*).

Reviewer #4 (Remarks to the Author):

Thank you for being responsive for the critiques.

We thank Reviewer #4 for the comments.

References

1. Gorki AD, *et al.* Murine Ex Vivo Cultured Alveolar Macrophages Provide a Novel Tool to Study Tissue-Resident Macrophage Behavior and Function. *Am J Respir Cell Mol Biol* **66**, 64-75 (2022).

2. Desai TJ, Brownfield DG, Krasnow MA. Alveolar progenitor and stem cells in lung development, renewal and cancer. *Nature* **507**, 190-194 (2014).

3. Rock JR, *et al.* Multiple stromal populations contribute to pulmonary fibrosis without evidence for epithelial to mesenchymal transition. *Proc Natl Acad Sci U S A* **108**, E1475-1483 (2011).

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

The authors have addressed my concerns.