The mitochondrial calcium uniporter of pulmonary type 2 cells determines severity of acute lung injury

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Acute Lung Injury (ALI) due to inhaled pathogens causes high mortality. Underlying mechanisms are inadequately understood. Here, by optical imaging of live mouse lungs we show that a key mechanism is the viability of cytosolic Ca\(^{2+}\) buffering by the mitochondrial Ca\(^{2+}\) uniporter (MCU) in the lung's surfactant-secreting, alveolar type 2 cells (AT2). The buffering increased mitochondrial Ca\(^{2+}\) and induced surfactant secretion in wild-type mice, but not in mice with AT2-specific MCU knockout. In the knockout mice, ALI due to intranasal LPS instillation caused severe pulmonary edema and mortality, which were mitigated by surfactant replenishment prior to LPS instillation, indicating surfactant's protective effect against alveolar edema. In wild-type mice, intranasal LPS, or Pseudomonas aeruginosa decreased AT2 MCU. Loss of MCU abrogated buffering. The resulting mortality was reduced by spontaneous recovery of MCU expression, or by MCU replenishment. Enhancement of AT2 mitochondrial buffering, hence endogenous surfactant secretion, through MCU replenishment might be a therapy against ALI.
Studies in mice involving global\(^8\) or tissue-specific MCU deletion\(^9\), or overexpression of dominant-negative MCU\(^8\) reveal a determining role of the MCU in multiple aspects of organ function, including body size determination\(^8\), exercise tolerance\(^11,12\), myocardial infarction\(^9,13\), pulmonary fibrosis\(^11\), insulin secretion\(^13\), fibroblast differentiation\(^14\), and hepatic lipodosis\(^17\). Despite this extensive evidence for MCU involvement in organ function, the reported data rely on prior genetic modifications that do not replicate natural pathogenesis. For ALI, understanding is lacking as to whether loss of the MCU, hence the resulting loss of buffering, exacerbates ALI by inhibiting surfactant secretion.

Here, we address this issue in LPS and bacterial models of lung infection, using an in situ assay of mitochondrial buffering in AT2 of live alveoli by means of real-time confocal microscopy\(^8\). We could thereby, determine the dynamic Ca\(^2+\) responses in the cytosol and mitochondria in relation to the progression of acute immunity. Our findings indicate that by the first post-infection day, there was marked depletion of both message and protein levels of AT2 MCU, together with loss of buffering and surfactant secretion. Unexpectedly, sustained MCU depletion promoted mortality, which could be mitigated by MCU repletion, revealing the critical role of AT2 MCU in immune alveolar injury.

**Results**

**The MCU and alveolar homeostasis**

We induced ALI by exposing mice to intranasally instilled LPS or *Pseudomonas aeruginosa* (PA), as we indicate below. By live confocal microscopy, we assayed mitochondrial function in AT2 to determine the effect of mitochondrial Ca\(^2+\) buffering on surfactant secretion. We identified AT2 as cells that stained for surfactant containing lamellar bodies (LBS) and for surfactant protein B (Supplementary Fig. 1a). By alveolar micropuncture, we loaded the alveolar epithelium with the dyes, rhod2 and fluo4 to quantify mitochondrial (mCa\(^2+\)) and cytosolic (cCa\(^2+\)) calcium, respectively, as we described\(^15,16\). To confirm compartmental distribution of the dyes, we gave alveolar microinfusion of a mild detergent, which released fluo4, but not rhod2 from the epithelium, affirming that fluo4 was localized to the cytosol and rhod2 to the mitochondrial matrix\(^17\) (Supplementary Fig. 1b, c). Alveolar stretch caused by a 15-second lung hyperinflation induced transient cCa\(^2+\) oscillations in AT2, but no increase of mean cCa\(^2+\) (Fig. 1a, b). Concomitantly, both the mean and the oscillation amplitude of mitochondrial Ca\(^2+\) increased (Fig. 1a, b and Supplementary Movie 1), affirming onset of mitochondrial buffering that protected against cCa\(^2+\) increases.

We tested these responses in *MCU*\(^−/−\)*-SPC-rtTa-tetO-Cre* mice (rtTa\(^−/−\)*) in which we induced pre-partum Cre recombination in the alveolar epithelium (AE)\(^18,19\) by doxycycline treatment. rtTa\(^−/−\)* mice lacked MCU in AT2 (Supplementary Fig. 1d–f). For control, we withheld doxycycline in rtTa-tetO-Cre mice expressing mice. We imaged alveoli of equal diameters in control and rtTa\(^−/−\)* mice. The hyperinflation-induced mitochondrial responses were inhibited pharmacologically by alveolar treatment with the MCU blocker, ruthenium red and they were absent in the rtTa\(^−/−\)* mice (Supplementary Fig. 1g and Fig. 1b), confirming the MCU role in the buffering. Further, loss of buffering caused the expected increase of mean cCa\(^2+\) (Fig. 1b). The inhibitor of store Ca\(^2+\) release, xestospongin C blocked all Ca\(^2+\) increases (Supplementary Fig. 1g), consistent with the notion that MCU-dependent mCa\(^2+\) increases were due to mitochondrial entry of store-released Ca\(^2+\). Surfactant secretion in intact alveoli is assured by labeling LBS with the fluorescent dye, Lysotracker Red (LTR), then detecting the time-dependent loss of the fluorescence to a secretion stimulus such as hyperinflation\(^18,19\) (Supplementary Fig. 1h–j and Fig. 1c). However, this response was absent in the rtTa\(^−/−\)* mice (Fig. 1c), as also in mice treated with ruthenium red, indicating that blocking mitochondrial Ca\(^2+\) entry blocked surfactant secretion (Supplementary Fig. 1i, j).

These findings indicated that the AT2 MCU is a major determinant of surfactant secretion.

To rule out possible toxicity of the rtTa-tetO system\(^20\), we bred *MCU*\(^−/−\)*-SPC-Cre-ERT2 mice (ERT2\(^−/−\)) as an alternative system for activation of Cre recombinase in AT2. We affirmed that the hyperinflation-induced AT2 responses, namely mitochondrial calcium buffering and surfactant secretion, were absent (Fig. 1d, e). Thus, our key findings could be replicated by two different strategies of Cre recombinase activation, ruling out non-specific effects of Cre recombination as a complicating factor.

**ALL causes MCU loss**

We determined time dependent responses of AT2 mitochondria in the LPS-induced mouse model of ALI\(^14,21\). Since mouse strains differ in susceptibility to LPS\(^22\), we strain adjusted the LPS dose as we indicate (Supplementary Table 3). A nonlethal LPS dose (1 mg/kg) in the *Swiss Webster* strain markedly increased leukocyte counts in the bronchoalveolar lavage (BAL) within Day 1 (Supplementary Fig. 2a). The counts recovered to baseline by Day 5 (Supplementary Fig. 2a). On Day 1, surfactant secretion was repressed (Fig. 2a), and extravascular lung water, a measure of pulmonary edema, increased (Supplementary Fig. 2b).

In lungs derived from these LPS-treated mice, the AT2 physiologic responses, namely mCa\(^2+\) increase and surfactant secretion, were present at 4 h post-LPS, but repressed at 24 h (Fig. 2a, b). Immunoblots of AT2 mitochondria indicated that MCU expression progressively decreased and was significantly lower than baseline by 16 h (Fig. 2c). MCU RNA decreased to about 50% of baseline levels (Fig. 2d). These findings indicated that LPS decreased MCU and mitochondrial buffering by Day 1.

We applied an optogenetic approach as an alternative approach for increasing cCa\(^2+\)\(^−/−\). We expressed the light sensitive, cation channel, channelrhodopsin-2 (ChR2) in the alveolar epithelium (Supplementary Fig. 2c). Excitation of ChR2 by blue light increased AT2 mitochondrial Ca\(^2+\) markedly less in LPS- than PBS-treated lungs (Supplementary Fig. 2d, e and Fig. 2e). Hence, the hyperinflation and optogenetics approaches together denoted loss of MCU-induced buffering in AT2 following LPS treatment.

At Day 1 post-LPS, the mitochondrial outer membrane proteins, TOM-20 and VDAC, and the matrix protein HSP60 were well expressed (Supplementary Fig. 2f, g). The proteins of the mitochondrial electron transport chain (ETC) were also well expressed and their activities were not diminished (except for a small decrease in Complex II activity) (Supplementary Fig. 2h, i). Therefore, the MCU loss was not due to a non-specific loss of mitochondrial proteins.

The mitochondrial membrane potential informs mitochondrial fitness. Mitochondrial depolarization stabilizes PINK1 on the outer membrane. PINK1 recruits the E3-ubiquitin ligase, parkin, which initiates mitophagy\(^21\). To determine whether the MCU impacted mitochondrial potential, we loaded the alveolar epithelium with the potentiometric dye, TMRE, fluorescence of which decreases with mitochondrial depolarization\(^22,23\). Our findings indicate that despite the loss of the MCU, TMRE fluorescence was unchanged (Supplementary Fig. 2j, k), indicating that the AT2 mitochondria were not depolarized, hence they were unlikely to be mitophagy targets of the PINK1-parkin mechanism. To test this hypothesis, we carried out immunoblots on mitochondria derived from AT2 24 h after LPS treatment. These studies failed to reveal association of parkin (Supplementary Fig. 2i), suggesting that the MCU loss did not induce a mitophagy signal.

To evaluate the effect of inflammation severity, we exposed the *Swiss Webster* mice to intranasal LPS at nonlethal (1 mg/kg) or lethal (10 mg/kg) doses (Supplementary Table 3). For the nonlethal dose, the MCU loss on Day1 was followed by recovery of MCU expression (Fig. 2f). By contrast, for the lethal dose the expression did not recover (Fig. 2f). Similarly, although both doses caused loss of body weight by...
Day 1, the weight recovered for the nonlethal, but not the lethal dose (Fig. 2g). These findings suggested that adequacy of AT2 MCU expression was a determinant of survival.

Since the effects of purified LPS might not be entirely representative of those induced by Gram-negative bacteria, we infected mice lacking the MCU in the alveolar epithelium. Bars: mean ± SEM. n = 4 mice per group. Groups were compared using one-way ANOVA with Bonferroni correction.

Rescue strategies against LPS-induced mortality

In ERT2MCU−/− mice MCU was specifically deleted in AT2 through post-partum tamoxifen activation of the Cre recombinase (Fig. 3a). To determine the effects of MCU addback, we transduced MCU in the knockout mice by intranasal plasmid delivery in ERT2MCU−/− mice. Thus, MCU expression, which was absent in AT2 of the knockout mice, was reinstated by the transduction (Fig. 3a). An LPS dose that caused moderate mortality in Cre-inactivated, littermate controls, induced severe mortality in ERT2MCU−/− mice (Fig. 3b). Similarly, the high mortality following LPS was also evident in rtTaMCU−/− mice (Supplementary Fig. 3a). Thus, loss of MCU in AT2 mitochondria enhanced mortality in a strain-
and Cre-independent manner. Further, MCU transduction markedly abrogated the mortality (Fig. 3b). Taken together, we interpret that loss of MCU in AT2 was of systemic homeostatic significance.

In our previous studies we noted that wild-type mice of the Swiss Webster strain18 are more susceptible to LPS-induced mortality as compared with other strains21. To determine whether the susceptibility is reversed by MCU enrichment, we transfected MCU in Swiss Webster mice to increase AT2 MCU expression (Supplementary Fig. 3b, c). A subsequent intranasal instillation of LPS decreased MCU expression in 24 h (Fig. 3c), as before. However, despite this decrease, the expression remained sufficiently high (60% of baseline) that buffering was retained (Fig. 3c, d). Although LPS caused the expected decrease of

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4
ATP in controls, and the expected high mortality, MCU-transfection protected ATP production (Fig. 3e), surfactant secretion (Fig. 3f), and survival (Fig. 3g). Together, these studies indicated that MCU overexpression ensured adequate protection of AT2 buffering and surfactant secretion, thereby protecting survival.

By contrast, post-LPS MCU transfection, which we initiated 12 h after LPS instillation, did not increase MCU expression in AT2 mitochondria; although the expression was evident in the AT2 cytosol (Fig. 3h). Since the increased cytosolic expression attests to success of transfection, we are uncertain as to why the mitochondrial expression failed. Failure of mitochondrial import of the MCU remains a possibility. Together, these studies indicated that the transfection approach for enriching AT2 MCU was likely to succeed as a protective (pre-ALI), but not a therapeutic (post-ALI) strategy for ALI.

Clements proposed that deficiency of secreted surfactant increases the alveolar air-liquid surface tension, causing as a result, decrease of the interstitial pressure, hence increase of microvascular filtration and edema. We tested the Clements hypothesis in ERT2MCU−/− mice that failed to secrete surfactant in response to hyperinflation. In these knockout mice, LPS caused greater increases of lung water (Fig. 3i), and severe mortality than controls (Fig. 3b), although the increases in BAL protein levels were similar (Fig. 3i). The LPS-induced lung injury was markedly abrogated by a single intranasal dose of purified surfactant (Curosurf) given 1 h prior to LPS instillation (Fig. 3b, i). Thus consistent with Clements’ proposal, LPS-induced mortality in ERT2MCU−/− mice resulted from severe edema and respiratory failure due to absent surfactant secretion.

As a possible therapeutic approach for enriching AT2 MCU in ALI, we considered a mitochondrial transfer strategy. Mitochondrial transfer from bone marrow-derived mesenchymal stromal cells (BMSCs) to the alveolar epithelium protects against ALI. However, the protective mechanisms are not understood. To determine the role of the AT2 MCU in this protection, we transfected BMSCs with plasmids encoding GFP-tagged, wild-type MCU (pMUCwt) (Supplementary Fig. 4a), or a mutant MCU (pMUCmt) that inhibits mitochondrial Ca2+ entry. We intranasally instilled BMSCs expressing the MCU plasmids 4 h after instilling LPS. In lungs obtained from these mice 24 h after LPS, we confirmed that BMSC mitochondria were transferred to AT2 (Supplementary Fig. 4b, c), and that the transfer increased MCU expression in AT2 (Fig. 4a). Overexpression of MUCunt protected MCU function and abrogated inflammation, increasing survival (Fig. 4b–e).

Overexpression of MUCunt failed to achieve the protections (Fig. 4b–e). Taking our findings together, we conclude that primary lack of the AT2 MCU was sufficient to tilt the inflammatory outcome towards ALI, and that rescue of MCU function by mitochondrial transfer protected inflammation resolution and survival.

LPS-induced mitochondrial H2O2 degrades MCU of AT2 mitochondria

To determine the role of mitochondrial H2O2 production in these responses, we expressed the matrix-targeted, H2O2 sensor roGFP. Transfection of roGFP by intranasal delivery of liposomal plasmid avoids potential difficulties due to mitochondrial uptake of diffusible H2O2 sensing dyes. Intranasal LPS instillation progressively increased H2O2 in AT2 mitochondria (Supplementary Fig. 5a and 5a). To block this response, we expressed catalase in AT2 mitochondria by crossing inducible SPC-rTa-Cre mice with mice expressing mitochondria-targeted human catalase downstream of a floxed STOP codon. Post-partum induction of SPC-Cre caused catalase expression in AT2 mitochondria of these mice (AT2CAT+/+), as confirmed by alveolar immunofluorescence and immunoblot (Supplementary Fig. 5b, c). LPS treatment of AT2CAT+/+ mice failed to increase mitochondrial H2O2, or induce loss of AT2 MCU (Fig. 5b, c). Accordingly, MCU function was retained (Fig. 5d, e), alveolar inflammation was mitigated (Fig. 5j), and mortality was reduced (Fig. 5g). Mice pre-treated with the mitochondria-specific anti-oxidant, MitoQ were also protected from the MCU loss (Supplementary Fig. 5d), indicating efficacy of pharmacologic intervention. Together, these findings indicated that LPS-induced increase in AT2 mitochondrial H2O2 was a critical mediator of the MCU loss.

We considered the possibility that mitochondrial H2O2 might induce mitochondrial fragmentation, and thereby impair mitochondrial Ca2+ uptake. Mitochondrial H2O2 can activate mitochondrial fragmentation by inducing dynamin-related protein-1 (Drp1). Since mitochondrial distribution has not been determined in intact alveolar epithelium, we imaged AT2 in alveoli of PhAMfloxed:E2a-Cre mice that globally express the mitochondria-targeted fluorescent protein, Dendra-2 (Supplementary Fig. 6a, and Fig. 6a). AT2 mitochondria aggregated at peri-nuclear poles, co-existing with surfactant-
containing lamellar bodies. This polarized aggregation was lost by Day 1 after LPS, as fragmented mitochondria distributed throughout the cytosol (Fig. 6a, b). However, following intranasal delivery of the Drp1 inhibitor, Mdivi-138, LPS failed to disrupt the polarized mitochondrial distribution (Fig. 6a, b). Thus, Drp1 inhibition blocked LPS-induced mitochondrial disaggregation.

We confirmed that Drp1 was activated, as revealed by Drp1 phosphorylation at Ser-616 through immunoblots of AT2 mitochondria with a specific antibody (Fig. 6c). Immunoblots also revealed Drp1 localization to mitochondria in fixed littermate controls, but not in AT2CAT+/+ mice (Fig. 6d). Thus Drp1 activation was H2O2 dependent.

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Fig. 3 | Effects of wild-type MCU overexpression in alveolar epithelium.

A Immunoblot and densitometric analysis of MCU expression in AT2 mitochondrial fractions derived from indicated strains. The Western blot of MCU (0.85 kDa, middle) with mouse IgG (1 mg/kg) mAb shows increased expression of MCU in the AT2Cx43−/− mice as compared to control mice (AT2Drp1−/−). The Western blot of MCU (0.85 kDa, middle) with mouse IgG (1 mg/kg) mAb shows increased expression of MCU in the AT2Cx43−/− mice as compared to control mice (AT2Drp1−/−).

B Western blot and densitometric analysis of the mitochondrial protein content of lungs from indicated strains. Results were expressed as the percentage of wild-type expression (LPS = 100%). Western blot of mitochondrial fractions from control mice (AT2Cx43−/−, WT) and AT2Drp1−/− mice (MCUadd, red) revealed an increase in MCU expression in AT2Cx43−/− mice. Western blot and densitometric analysis of the mitochondrial protein content of lungs from indicated strains. Results were expressed as the percentage of wild-type expression (LPS = 100%). Western blot of mitochondrial fractions from control mice (AT2Cx43−/−, WT) and AT2Drp1−/− mice (MCUadd, red) revealed an increase in MCU expression in AT2Cx43−/− mice.

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content. As we show here and have reported, LPS increases alveolar epithelial cCa²⁺ that could activate Ca²⁺-induced barrier-weakening mechanisms, such as calpain activation, or actin depolymerization. Oxidant release by recruited leukocytes may damage the air-blood barrier causing hyperpermeability. Oxidant release by recruited leukocytes may damage the air-blood barrier causing hyperpermeability. Here, we propose that enhancement of endogenous surfactant secretion mechanisms, and hyperpermeability resulted from the combined effects of LPS-induced barrier-weakening mechanisms, such as calpain activation, or actin depolymerization. We conclude, mitochondrial H₂O₂ as a determinant of MCU expression. We further showed that AE-derived H₂O₂ mediates paracrine proinflammatory effects on the adjoining endothelial. Here, the time course of MCU expression was unchanged from baseline for about 8 h post-LPS. Subsequently the expression progressively decreased by Day 1. In parallel, MCU function was intact early but not late after LPS exposure, while mitochondrial H₂O₂ progressively increased. AT2-specific catalase expression blocked the MCU loss, implicating mitochondrial H₂O₂ as a determinant of MCU expression. We conclude, increased production of mitochondrial H₂O₂ destabilized mitochondrial buffering, contributing to ALI lethality. Increase of mitochondrial H₂O₂ in AT2 can occur from Complex 1 catalysis blocked the MCU loss, implicating mitochondrial H₂O₂ as a determinant of MCU expression. We conclude, increased production of mitochondrial H₂O₂ destabilized mitochondrial buffering, contributing to ALI lethality. Increase of mitochondrial H₂O₂ in AT2 can occur from Complex 1 catalysis, or by Ca²⁺ communication across Cx43-containing GJs in the AE. We confirmed these mechanisms, in that heterozygous ECSIT knockout, or AT2-specific
Cx43 knockout each markedly abrogated the LPS-induced MCU loss. These mechanisms may have sustained H2O2 increase. H2O2 production failed, the ECSIT-induced effect may have dominated. Together, these mitochondrial buffering was present. In the later phase, as buffering effect operating in the early phase of the immune response when small amount of mitochondrial H2O2 abrogates AT2 MCU depletion. a, b Group data are for baseline AT2 mitochondrial H2O2 production following non-lethal LPS (1 mg/kg) instillations. mCAT+/+, mice fixed for mitochondrial catalase (mCAT): AT2mCAT+/+, mice expressing mCAT in AT2. In b, determinations were made 24 h after indicated instillations. LPS was instilled at a nonlethal dose (1 mg/kg). Bars: mean ± SEM. n = 4 lungs for each group. Groups were compared using one-way ANOVA with Bonferroni correction. c MCU immunoblots and densitometry of AT2 mitochondria from lungs given intranasal PBS or nonlethal LPS (1 mg/kg). Lungs were excised and AT2 isolated 24 h after instillations. Bars: mean ± SEM. n = 3 lungs for each group. Groups were compared using one-way ANOVA with Bonferroni correction. d-f Group data show in situ determinations of AT2 cytosolic (cCa2+) and mitochondrial (mCa2+) calcium (d), surfactant secretion (e) and lung inflammation (f). All determinations were made 24 h after intranasal instillations. LPS was instilled at a nonlethal dose (1 mg/kg). Bars: mean ± SEM. n = 4 lungs each bar.

A limitation of our study is that the mechanisms underlying the ALI-induced lethal LPS (50 mg/kg). n = 10 mice in each group. *p = 0.027 versus mCAT+/+, p value is calculated by Log-Rank test.
several proteins of the mitochondrial outer and inner (IMM) membranes, the matrix, and the electron transport chain were not lost. Notably, in the post-LPS period, MCU mRNA was about 50% of control levels, indicating that MCU transcription was substantially active. This finding was further affirmed in that MCU transfection in the post-LPS period increased cytosolic MCU, but not mitochondrial MCU. One possibility is that LPS impaired mitochondrial import mechanisms. Since LPS caused mitochondrial redistribution, we speculate that mitochondrial import may have been impaired by loss of ER-mitochondrial tethering due to mitochondrial fragmentation or Drp1 activation. These proposed mechanisms need to be further understood in the ALI context.

Mitochondrial proteases known as ATPases associated with diverse cellular activities (AAA) may also have contributed to the LPS-induced MCU loss. In this regard, attention has focused on the AAA proteases, AFG3L2 and SPG7. These proteases curate MCU function by degrading unassembled subunits of the MCU gatekeeper, EMRE. Interestingly, knockouts of these proteases do not modify MCU expression, diminishing their possible role in the present MCU loss. Identification of new MCU-specific proteases might clarify the issue.

In conclusion, an important understanding that emerges relates to the role of MCU-dependent surfactant secretion in the pathophysiology of ALI. It is widely held that inflammatory cells, such as activated neutrophils that are rapidly recruited by the pathogen-challenged lung, cause barrier hyperpermeability and ALI. However, lung infection in leukopenic patients can also cause ALI. Hence, non-neutrophil mechanisms of ALI must also be considered, such as the MCU-surfactant mechanism we propose here. Further studies are required to better understand the role of the MCU mechanism in ALI under multiple predisposing conditions, including viral infections and malignant leukopenia.

Methods
Reagents, plasmids, primers, and antibodies
A list is provided in Supplementary Tables 1 and 2.

Animals
Animal procedures were approved by the Institutional Animal Care and Use Committee of Vagelos College of Physicians and Surgeons at Columbia University. All animals were cared for according to the NIH guidelines for the care and use of laboratory animals. Mice were housed under a 12 h light/dark cycle with ad libitum access to water and food. All experimental animals were 5–10 weeks old and age- and sex-matched. Mice used are listed in Supplementary Table 1. Briefly, the mice used were: Wild type: C57BL/6J, FVB/NJ (The Jackson Laboratories); Swiss Webster (Taconic). Cre recombinase expressing mice: SPC-
rtTa-tetO-Cre, SPC-ERT2-Cre. Mice with floxed genes: MCU\(^{f/f}\); mCAT\(^{f/f}\); Cx43\(^{f/f}\); Drp1\(^{f/f}\). Other: Dendra-2 expressing mice. To delete MCU in the alveolar epithelium (rtTa\(^{+/-}\)), we crossed MCU\(^{f/f}\) mice\(^{c}\) with SPC-rtTa-Cre mice\(^{d}\). The deletion was induced pre-partum through SPC-Cre induction by doxycycline treatment\(^{e}\). To delete MCU in AT2 cells (ERT2\(^{+/-}\)), we crossed MCU\(^{f/f}\) mice with the SPC-ERT2-Cre mice, then induced Cre by post-partum tamoxifen treatment (75 mg/kg body weight, i.p. for 5 d\(^{e}\)). Controls (ERT2\(^{+/-}\)) were mice given corn oil. To overexpress mitochondrial catalase (mCAT) in AT2 (AT2\(^{f/f}\)), we crossed SPC-rtTa-Cre mice with mCAT\(^{f/f}\) mice\(^{e}\), then activated the Cre post-partum by doxycycline treatment. To delete Cx43 (AT2\(^{+/-}\)) and Drp1 (AT2\(^{+/-}\)) in AT2, we crossed, respectively, Cx43\(^{−/−}\) and Drp1\(^{−/−}\) mice with SPC-rtTa-Cre. To achieve AT2-specific deletion, we induced the SPC-Cre post-partum\(^{e}\).

**Acute Lung Injury (ALI)**

ALI was induced in anesthetized (ketamine-100 mg/kg and xylazine 5 mg/kg, i.p.) animals by airway instillation of LPS (E.coli 0111:B4) in sterile PBS. Control animals were instilled an equal volume of sterile PBS. Since multiple mouse strains were used, and since the lung injury-causing LPS dose varies between mouse strains, we gave different LPS doses to simulate sublethal, moderate and lethal ALI-inducing that caused mortality of 0, 30 and 80%, respectively, in control groups (Supplementary Table 3).

**Isolated, blood-perfused lungs**

Using our reported methods\(^{9,20,21}\), lungs were excised from anesthetized mice, then perfused with autologous blood through cannulas in the pulmonary artery and left atrium. The blood was diluted in 4% dextran (70 kDa), 1% fetal bovine serum and buffer (150 mmol/l Na\(^+\), 5 mmol/l K\(^+\), 1.0 mmol/l Ca\(^{2+}\), 1 mmol/l Mg\(^{2+}\), and 20 mmol/l HEPES at pH 7.4). The perfusion flow rate was 0.5 ml/min at 37 °C at osmolarity of 300 mosM (Fiske Micro-Osmometer, Fiske\(^{e}\) Associates, Norwood, MA), and hematocrit 10%. The lung was inflated through a tracheal cannula with a gas mixture (30% O\(_2\), 6% CO\(_2\), balance N\(_2\)). Vascular (artery and vein at 10 and 3 cmH\(_2\)O) and airway (5 cmH\(_2\)O) pressures were held constant during microscopy.

**Alveolar microinfusion and imaging**

To load the alveolar epithelium with fluorescent dyes or antibodies, we micropunctured single alveoli with glass micropipettes (tip diameter 3–5 μm) and microinfused -10 neighboring alveoli\(^{19,21}\). After the microinfusions, the free liquid in the alveolar lumen drained in seconds re-establishing air-filled alveoli\(^{19}\). This rapid clearance indicates that the micropuncture does not rupture the alveolar wall, and that the micropunctured membrane rapidly reseals as reported for other cells\(^{22}\). We selected non-micropunctured alveoli for imaging. To confirm that the fluorescence was intracellular, we microinfused alveoli for 10 min with trypan blue (0.01% w/v), which eliminates extracellular fluorescence\(^{21}\). In all experiments in which we infused multiple dyes, we confirmed absence of bleed-through between fluorescence emission channels. We imaged intact alveoli of live lungs with laser scanning microscopy (LSM 510 META, Zeiss and TCS SP8, Leica).

**Alveolar immunofluorescence**

We used our reported methods to detect intracellular immunofluorescence in live alveoli\(^{19,21}\). Briefly, we gave alveoli successive 20-minute microinfusions of 4% paraformaldehyde and 0.1% triton X-100. Then, we microinfused fluorescence-conjugated antibodies (40 ng/ml) for 10 min, followed by microinfusion of fluorescence-conjugated...
Fig. 8 | Mechanisms of mitochondrial H$_2$O$_2$ generation. a Immunoblots in AT2 mitochondria (left) and densitometric quantification of immunoblots (right) show MCU expression 24 h after indicated treatments. LPS was instilled at a nonlethal dose (1 mg/kg). Cx43, connexin 43; Cx43$^{−/−}$, mice floxed for Cx43; AT2$^{Cx43−/−}$, mice lacking Cx43 in AT2. Bars: mean ± SEM. $n$ = 3 lungs each bar. Groups were compared using one-way ANOVA with Bonferroni correction. b Determinations of mitochondrial Ca$^{2+}$ responses to hyperinflation 24 h after indicated intranasal instillations. LPS was instilled at a nonlethal dose. Bars: mean ± SEM. $n$ = 4 lungs each bar. Groups were compared using one-way ANOVA with Bonferroni correction. c Immunoblot (left), densitometric determinations (right) from AT2 mitochondria. AT2 were isolated 24 h after instillations of either PBS or nonlethal LPS. ECSIT, evolutionarily conserved signaling intermediate in Toll pathway; ECSIT$^{−/−}$, heterozygous knockout mice for ECSIT. Bars: mean ± SEM. $n$ = 3 lungs each bar. Groups were compared using one-way ANOVA with Bonferroni correction.

Bacterial culture
We cultured single colonies of *Pseudomonas aeruginosa* (strain K, provided by Dr. A. Prince, Columbia University, New York, NY, USA), overnight in 4 ml of Luria–Bertani (LB) medium at 37 °C and 250 rpm (Innova42, New Brunswick Scientific). *P. aeruginosa* were selected with Carbenicillin (300 μg/ml). On the day of the experiment, overnight cultures were diluted 1:100 in fresh LB and grown in a shaking incubator to optical density (OD) 0.5 at 600 nm (SPECTRAmax Plus, Molecular Devices).

In vivo bacterial instillation
For intranasal instillations in mice, 1 ml bacterial culture was centrifuged and resuspended in 10 ml sterile PBS. In anesthetized animals, we airway instilled 50 μl suspension to deliver “high” inoculum (1 × 10$^6$ CFU) per mouse. For “low” inoculum instillation, we diluted (x10) the inoculum suspension, then airway instilled 50 μl suspension to deliver 1 × 10$^5$ CFU per mouse.

Bone marrow-derived stromal cell (BMSC) isolation, purification and culture
We isolated mouse BMSCs according to our reported methods.$^{18}$ Briefly, in anesthetized animals, bone lumens of excised femurs and tibia were infused with MSC growth medium to recover marrow. Three days after marrow plating on tissue culture flasks, the supernatant was rejected and adherent BMSCs were incubated (37 °C) under subconfluent conditions to prevent cell differentiation. BMSCs from passages 5 to 12 were used in this study. In accordance with established criteria,$^{73}$ BMSCs were characterized as we reported.$^{18}$ BMSCs were cultured in 5% CO$_2$ at 37 °C in DMEM containing 10% FBS and 1% of an antibiotic mixture.

AT2 isolation by flow cytometry
We isolated AT2 by our reported methods.$^{18}$ Briefly, isolated lungs were buffer perfused through vascular cannulas to clear blood, then we exposed the lungs to intratracheal dispase (0.2 U/ml, 2 ml, 45 min) at room temperature. The tissue was suspended in PBS and sieved and the sieved sample was then centrifuged (300 g x 5 min). The pellet was resuspended and incubated together with the AT2 localizing dye, lysotracker red (LTR)$^{42}$, the nuclear dye, Hoechst 33342, and fluorescence Allophycocyanin (APC)-conjugated Ab against the leukocyte antigen, CD45. The suspension was then subjected to cell sorting (Influx cell sorter) to recover AT2.

Mitochondria isolation
We used a commercially available isolation kit (Thermo Scientific)$^{133}$. Briefly, we homogenized lungs and AT2 (Tissue Tearor; Biospec Products, Bartlesville, OK), on ice with buffers provided in the kit. The buffers were supplemented with protease and phosphatase inhibitors. We centrifuged the homogenate (800 g x 10 min) and collected the supernatants, which were further centrifuged at 17,000 g for 15 min at 4 °C. The pellets and the supernatants contained respectively, the mitochondrial and the cytosolic fractions. Purity of cell fractionation was determined by secondary antibodies (40 ng/ml). To washout unbound fluorescence, we microin fused buffer for 10 min and commenced imaging after a further 10 min.
immunoblotting for voltage-dependent anion channel (VDAC) in mitochondrial and cytosolic fractions.

**Immunoblotting/Immunoprecipitation**

For immunoprecipitation and immunoblotting, mitochondrial fractions were lysed in 2% SDS. For immunoprecipitation, lysates containing 2 mg total protein were precleared with appropriate control IgG for 30 min at 4 °C with 20 μl of prewashed protein A/G. Equal amounts (100 μg) of protein from lysates were separated by SDS-PAGE, electro-transferred onto nitrocellulose membrane overnight at 4 °C and blocked in Starting Block Blocking Buffer (Pierce) for 1 h then subjected to immunoblotting. Densitometry was performed using ImageJ software.

**RNA determination**

RNA was extracted from isolated AT2 using the RNeasy Micro Kit. The purity of the RNA was assessed by absorbance at 260 and 280 nm using a Thermo Scientific NanoDrop spectrophotometer. Using RNA with a 260/280 ratio of >1.8, cDNA was synthesized using oligo (dt) and Superscript II. Quantitative RT-PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems) and SYBR Green Master Mix.

**Lung transfection and knockdown**

A list of plasmids and oligonucleotides used are listed in Supplementary Table I. We purchased siRNA against MCU and RISP. For knockdown experiments, we intranasally instilled mice with 50 ng siRNA complexed with freshly extruded liposomes. Stock solutions of plasmids (2.5 μg/μl) were similarly complexed with freshly extruded unilamellar liposomes (20 μg/μl, 100 nm pore size) in sterile PBS to a final concentration of 1 μg oligonucleotide/μl. Mice were intranasally instilled (50-75 μl) the nucleic acid-liposome mixture. Lungs from transected animals were excised 48 h later. Protein expression or knockdown was confirmed by immunoblotting.

**Channelrhodopsin activation**

To activate ChR2 in the alveolar epithelium, imaged alveolar fields were excited by mercury lamp illumination directed through an FITC filter. The duration of illumination was 10 seconds.

**Mitochondrial complex activity assay**

To determine complex I-V activity, purified mitochondria were resuspended in complexes I-V activity assay buffers. Activity of complexes I and II were measured as the rate of decrease in absorbance at 600 nm. Whereas, activity of complexes III and IV were measured respectively, as the rate of increase and decrease in absorbance at 550 nm. For each assay, absorbance was measured at the given wavelength every 10 s for 30 min at 25 °C using a SpectraMax microplate spectrophotometer. Enzymatic activities were normalized to protein concentrations. Complex I activity was calculated as the difference between activities measured when samples were incubated with 2 μM Rotenone or ethanol.

**Extravascular lung water**

We determined blood-free lung water content by our reported methods. Briefly, we determined the wet and dry weights of excised lungs and quantified hemoglobin concentrations in lung homogenates to correct for blood water content in the wet/dry ratio.

**Lung ATP determination**

We used our reported methods. Briefly, excised lungs were immediately immersed in liquid N2. Lung samples (~10 mg) were subsequently subjected to colorimetric assays for determinations of ATP and protein content.

**Bronchoalveolar lavage (BAL) neutrophil count**

BAL fluid was obtained following intratracheal instillations of 1 ml ice-cold sterile PBS. The BAL was centrifuged (400 g x 5 min at 4 °C), then the pellet resuspended in PBS supplemented with 1% BSA. To count neutrophils in the resuspended sample, we removed RBCs by exposing the sample to BCA lysis buffer. Then, we added fluorescent Ly-6G antibody to the sample, which we viewed by fluorescence microscopy in a Neubauer chamber (Olympus AX70).

**Quantification and statistical analysis**

All major groups comprised a minimum of 3 mice each. Age and sex-matched groups were randomly assigned. All data analysis was carried out by blinded protocol. Mean±SE was calculated on a per-lung basis for each group. Differences between groups were analyzed by the Student’s t-test for two groups, and ANOVA with the Bonferroni correction for multiple groups. Survival comparisons were analyzed by the Logrank test. Significance was accepted at P < 0.05.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Source Data for all figures are provided. All data supporting the findings described in this manuscript are available in the article and in the Supplementary Information and from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions
M.N.I. planned, carried out and analyzed all experiments. G.A.G. contributed to alveolar type 2 cell isolation and imaging experiments. L. L., E.M. and L.M. contributed to alveolar hyperinflation experiments. S.Q. contributed to experiments with Pseudomonas aeruginosa. M.A. and E.O-A. performed mitochondrial complex activity determinations. S.D. carried out immunoblotting. S.B. contributed to the plan. J.B. designed the overall project. All authors contributed to the writing.

Competing interests
The authors declare no competing interests.

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