Secreted Extracellular Cyclophilin A is a Novel Mediator of Ventilator Induced Lung Injury

Marissa W. Koh[1], Rhianna F. Baldi[1], Sanooj Soni[1], Rhodri Handslip[1], Ying Ying Tan[1], Kieran P. O'Dea[1], Miroslav Malesevic[2], Daniel F. McAuley[3], Cecilia M. O'Kane[3], Brijesh V Patel[1], Masao Takata[1], Michael R. Wilson[1].

[1] Division of Anaesthetics, Pain Medicine & Intensive Care, Imperial College London, London, UK

[2] Institute of Biochemistry and Biotechnology, Martin-Luther-University Halle-Wittenberg, Halle, Germany

[3] Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, UK

Corresponding author:

Dr Michael R. Wilson, PhD

Division of Anaesthetics, Pain Medicine and Intensive Care, Department of Surgery & Cancer, Imperial College London, Chelsea & Westminster Hospital, Fulham Road, London, UK.

michael.wilson@imperial.ac.uk

tel: (+)44 (0)203 315 8292

Conception and design of the work; MWK, MT, MRW: acquisition, analysis and interpretation of data; MWK, RFB, SS, RH, YYT, KPO, MM, DFM, CMO, BVP, MRW: Manuscript preparation & editing; MWK, MM, MT, MRW: Final manuscript review; all authors.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Support: The study was supported by a grant from the British Journal of Anaesthesia/Royal College of Anaesthetists. MWK is supported by the Agency for Science, Technology and Research (A*STAR) NSS (MBBS-PhD) scholarship (Singapore). SS was recipient of a clinical research training fellowship from the Medical Research Council/British Journal of Anaesthesia. RFB is funded by a studentship from the Biotechnology and Biological Sciences Research Council.

Running Head: Cyclophilin A mediates ventilator induced lung injury

Descriptor: 4.8 Mechanical Ventilation: Physiology & Pathophysiology

Word count: 3490

At a Glance Summary

Scientific Knowledge on the Subject

Cyclophilin A (CypA) is a ubiquitously expressed cytosolic protein most known for its role in intracellular protein folding. The gene responsible for CypA expression has very recently been implicated in susceptibility to COVID-19 infection. A secreted form, extracellular CypA (eCypA) has been identified as having involvement in diseases such as rheumatoid arthritis and coronary artery disease, but little is known regarding any role within the lungs.

What This Study Adds to the Field

We have identified a novel role for secreted eCypA in the pathogenesis of ventilator-induced lung injury (VILI). eCypA is secreted from epithelial cells during VILI in mice and promotes alveolar macrophage activation, such that in vivo inhibition attenuates development of injury. Furthermore eCypA levels are increased in bronchoalveolar

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

lavage fluid of patients with acute respiratory distress syndrome, indicating eCypA as a potential target within critically ill ventilated patients.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

ABSTRACT

Rationale: Mechanical ventilation is a mainstay of intensive care but contributes to the mortality of patients through ventilator induced lung injury. Extracellular Cyclophilin A is an emerging inflammatory mediator and metalloproteinase inducer, and the gene responsible for its expression has recently been linked to COVID-19 infection.

Objectives: Here we explore the involvement of extracellular Cyclophilin A in the pathophysiology of ventilator-induced lung injury.

Methods: Mice were ventilated with low or high tidal volume for up to 3 hours, with or without blockade of extracellular Cyclophilin A signalling, and lung injury and inflammation were evaluated. Human primary alveolar epithelial cells were exposed to in vitro stretch to explore the cellular source of extracellular Cyclophilin A, and Cyclophilin A levels were measured in bronchoalveolar lavage fluid from acute respiratory distress syndrome patients, to evaluate clinical relevance.

Measurements and Main Results: High tidal volume ventilation in mice provoked a rapid increase in soluble Cyclophilin A levels in the alveolar space, but not plasma. In vivo ventilation and in vitro stretch experiments indicated alveolar epithelium as the likely major source. In vivo blockade of extracellular Cyclophilin A signalling substantially attenuated physiological dysfunction, macrophage activation and matrix metalloproteinases. Finally, we found that patients with acute respiratory distress syndrome showed markedly elevated levels of extracellular Cyclophilin A within bronchoalveolar lavage.

Conclusions: Cyclophilin A is upregulated within the lungs of injuriously ventilated mice (and critically ill patients), where it plays a significant role in lung injury.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Extracellular Cyclophilin A represents an exciting novel target for pharmacological intervention.

Abstract word count: 250 words

Key words: mechanical ventilation, acute respiratory distress syndrome, matrix metalloproteinase, cyclosporin, animal model

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

BACKGROUND

Acute respiratory distress syndrome (ARDS) is a frequently fatal condition occurring as a result of a variety of insults including viral and bacterial infection, gastric aspiration, severe trauma and a host of others. No specific therapy for ARDS has been identified, with treatment consisting primarily of supportive care, of which mechanical ventilation is a mainstay. However, mechanical ventilation can itself cause additional morbidity and mortality through the process of ventilator-induced lung injury (VILI). Indeed, the importance of VILI is highlighted by the fact that the majority of guideline recommendations (1) for treatment of ARDS patients, i.e. low tidal volume/pressure ventilation, prone positioning and neuromuscular blockade, primarily limit VILI.

As part of an approach to uncover possible novel targets for intervention, we recently (2) carried out studies into the so-called 'obesity paradox', whereby overweight and obese ventilated patients seemingly suffer lower mortality than normal weight individuals (3, 4). We found that mice made obese through a high fat diet were protected from the development of VILI, which was associated with blunted upregulation of matrix metalloproteinase (MMP) activation within the lungs (2) and decreased expression of the MMP-inducing receptor CD147 (EMMPRIN). While CD147 has previously been implicated in the pathophysiology of VILI (5) its potential as a therapeutic target has been limited by lack of knowledge regarding the mechanism by which it becomes activated to induce downstream events.

Cyclophilin A (CypA) is a ubiquitously expressed 18kDa cytosolic protein, encoded by the *PPIA* gene. It is primarily known for its intracellular roles in protein folding and trafficking (6, 7), and in this compartment is a major binding partner for the immunosuppressive drug cyclosporin (8). However, in recent years CypA has been

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

demonstrated to also have a role as a secreted molecule, which induces target cell responses including MMP induction and leukocyte chemotaxis, through interaction with CD147 (9-11). This extracellular (e)CypA has been implicated in inflammatory disease states such as rheumatoid arthritis, coronary artery disease and sepsis (12-14), but knowledge of any involvement within the lungs is limited to occasional studies in preclinical models of asthma and LPS-induced inflammation (15, 16). Given our previous findings we hypothesised that eCypA plays a pathophysiological role in lung injury. Our current findings suggest that eCypA represents a novel mechanism and therapeutic target during the pathogenesis of VILI. Some of the results of these studies have been previously reported in the form of abstracts (17, 18).

METHODS

Detailed methods are provided in an online supplement.

Animal studies

Studies were carried out under the Animals (Scientific Procedures) Act 1986, UK, using male C57BL6 mice aged 7-11 weeks (weight 22-30g). Model details have been published previously (19). Mice were anaesthetised (80mg/kg ketamine:8mg/kg xylazine), cannulated via the carotid artery for blood pressure and blood gas measurements, and ventilated via tracheostomy. After stabilisation, mice were ventilated with low (8ml/kg) or high (35-40ml/kg) tidal volume (V_T) for up to 3 hours. Experiments were stopped at predetermined time points (1, 2, 3 hours), or if animals met a mortality surrogate of blood pressure <50mmHg and/or peak inspiratory pressure (PIP) increase >20%.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

For intervention studies, mice were administered 50µl of vehicle (10%DMSO in saline) or drug (MM-284, 0.67mM) intratracheally, 30 minutes before high V_T ventilation (30-35ml/kg). Animals were randomly allocated to groups and experiments were carried out by an investigator blinded to treatment. MM-284 is a cell-impermeant non-immunosuppressive cyclosporin derivative, which selectively inhibits extracellular CypA signalling activity (20, 21).

Following termination, the lower lobe of the right lung was removed for wet:dry weight determination, while remaining lobes were saline-lavaged. Lungs were processed to produce single cell suspensions for flow cytometry to identify type I alveolar epithelial cells (AEC) and alveolar macrophages (AM), followed by intracellular CypA staining. In MM-284 studies, lavage fluid was analysed for MMP activity by gelatin zymography, and lungs were processed for leukocyte quantification and cell surface ICAM-1 expression by flow cytometry.

In vitro studies

Human primary AECs (CellBiologics) were cultured until 90-100% confluent and plated onto collagen-coated 6 well plates (BioFlex®, Flexcell International). Cells were exposed to 20% cyclic stretch or left static for 6 hours. eCypA secretion was determined by Western blot of supernatants.

To explore eCypA biological activity, human monocyte-derived macrophages from healthy volunteers and primary AEC were exposed to physiologically-relevant concentrations (1µg/ml) of recombinant CypA. Markers of cell activation were assessed by flow cytometry, and secretion of cytokines was measured by ELISA.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Human studies

eCypA levels were determined by ELISA (Abbexa, UK) in randomly selected bronchoalveolar lavage (BAL) samples from ARDS patients within the control arms of the HARP (22) and KARE (23) studies. Ethical approval was given by ORECNI (Office for Research Ethics Committee Northern Ireland). Samples were taken at baseline, and are compared with samples taken from healthy volunteers as part of the HARP study. See online data supplement for clinical details.

Statistics

Animal numbers were based on power calculations of preliminary experiments indicating that N of 5-7 would achieve 80% power (0.05 alpha). No outliers were removed, and no animals died unexpectedly. Data normality was evaluated by Shapiro-Wilk test and QQ plot of residuals. Normally distributed data were analysed by t-test or one-way ANOVA with Sidak's test for single timepoint studies, and 2-way ANOVA for time course studies. Non-normally distributed data were analysed by Mann-Whitney test or Kruskal-Wallis followed by Dunn's test. Associations between variables were tested by Spearman's non-parametric correlation, while survival was evaluated by Log-rank test. $P<0.05$ was considered statistically significant.

RESULTS

VILI induces secretion of eCypA into the lungs

Initial experiments were carried out to determine a) whether eCypA is secreted into the alveolar space of mice during VILI, and b) whether this occurs before or after the onset of physiological injury. The model characteristics are shown in figures E1-E3 of the online data supplement. Of particular note; i) physiological injury including

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

deteriorated respiratory mechanics and arterial oxygenation occurs, but not before 120 minutes of high V_T ventilation (see **Fig E1** in the online data supplement); ii) the model leads to both increased permeability (raised lavage fluid protein levels and translocation of labelled protein markers from plasma) and inflammation (activation of alveolar macrophages, recruitment of neutrophils, and upregulated lavage fluid cytokines – online **Fig E2**); and iii) the physiological injury is driven by alveolar macrophage function (clodronate depletion of macrophages attenuates injury – online **Fig E3**). Thus the model reflects a true 'inflammatory' VILI.

Following initial validation of the high V_T model, eCypA levels were determined in lung lavage fluid and plasma in ventilated animals. Lavage fluid eCypA (**Fig 1A**) was substantially increased at 2 hours (3.9 fold compared to low V_T) and further increased after 3 hours to very high levels (7.7 fold greater). To explore whether eCypA correlated with injury severity, levels in lavage fluid were compared with markers of injury and permeability. CypA levels showed a significant negative correlation (Spearman's r=-0.47) with arterial oxygenation (**Fig 1B**) and a positive correlation (Spearman's r=0.48) with lavage fluid protein (**Fig 1C**). There was no clear correlation with lung wet:dry weight ratio (not shown) but a significant and stronger (r=0.71) positive correlation between lavage levels of eCypA and sRAGE (**Fig 1D**). We also determined levels of eCypA in plasma during VILI, but these were unchanged during the course of injury (**Fig 1E**). Finally, having shown an early upregulation of eCypA in the alveolar space, we determined levels of the primary receptor CD147 on alveolar macrophages (we have already reported increased expression on AEC early during VILI (2)). CD147 levels were significantly upregulated following 1 hour of high V_T ventilation, and remained somewhat elevated at 2 hours (**Fig 1F**).

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Epithelial cells are the likely source of eCypA during VILI

Given the clear correlation of eCypA with sRAGE (a known marker of epithelial insult), plus previous findings that AECs are rapidly activated with lung stretch (24-26), we proposed that epithelial cells are the most likely source of secreted eCypA during VILI. To explore this, we firstly carried out VILI experiments in vivo and determined the expression of intracellular CypA by flow cytometry. As shown, $CD45^{-}/CD31^{-}/EPCAM^{-}/T1\alpha^{+}$ type 1 AEC (27) showed a significant decrease in intracellular CypA (**Fig 2A**) whereas $CD45^{+}/CD11c^{+}/F4\text{-}80^{+}/CD11b^{-}$ alveolar macrophages did not (**Fig 2B**), supporting AEC as a likely source of eCypA. To confirm this, human AEC were exposed to in vitro stretch. Levels of IL8 in cell supernatants (**Fig 2C**) were increased indicating activation of the cells by stretch. Levels of secreted eCypA were evaluated by western blot and were also significantly increased (**Fig 2D**). Protein concentration in cell supernatant (579 ± 39 vs $591\pm24\mu g/ml$) and the number of cells recovered from the culture plates after stretching ($1.33\pm0.2x10^{6}$ vs $1.26\pm0.3x10^{6}$) were the same between groups, showing equal loading and experimental conditions. Finally, to determine whether release of eCypA was likely to be a specific secretion process or passive release due to cell damage, cell viability was evaluated by staining of cells for 7-AAD (**Fig 2E**) and determining levels of supernatant LDH (**Fig 2F**). Again these were no different between conditions, indicating that release of eCypA from epithelial cells during in vitro stretch was not due to loss of cell integrity.

eCypA has inflammatory biological activity on macrophages in vitro

Next, to evaluate whether extracellular CypA has relevant inflammatory activity, human monocyte-derived macrophages (HMDM) and primary AEC were stimulated in vitro with recombinant CypA. 6 hours of stimulation induced significant increases in

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

HMDM expression of ICAM-1 (**Fig 3A**) and CD86 **(Fig 3B**). Expression of MHC-II (**Fig 3C**) and CD206 (**Fig 3D**) showed a tendency to increase but not significantly. Stimulation of HMDM with CypA also induced a substantial secretion of IL6 (**Fig 3E**), with no evidence that CypA stimulation led to altered HMDM cell viability (**Fig 3F**). In contrast, CypA stimulation had no effect on primary AEC in culture, in terms of either ICAM-1 expression (**Fig 4A**) or secretion of IL8 or IL6 (**Fig 4B, Fig 4C**). To note, these primary AEC did respond to stimulation with TNF (dotted lines). Similarly, A549 epithelial cells showed no ICAM-1 response to recombinant CypA in vitro (online **Fig E4**). Overall therefore these data strongly suggest that alveolar macrophages would be the primary target of eCypA within the lungs.

eCypA blockade prevents VILI in vivo

Having shown that VILI leads to early upregulation of eCypA levels in the alveolar space, and that eCypA has biological activity on macrophages in vitro, we sought to determine the in vivo biological importance. To do this we instilled mice intratracheally with MM-284, a cell-impermeant, non-immunosuppressive cyclosporin A derivative drug which inhibits signalling activity of extracellular CypA specifically (20, 21). 30 minutes later, animals were ventilated with high V_T for up to 180 minutes or until the mortality surrogate was reached. Importantly, after setting high V_T, both initial PIP (vehicle: 40.2±0.6 cmH_2O vs. MM-284: 40.4±0.3 cmH_2O; N=7-8) and V_T (vehicle: 32.0±0.9 ml/kg vs. MM-284: 31.8±1.7 ml/kg; N=7-8) were identical between groups, indicating that delivered fluids had been equally dispersed and that the MM-284 had no early impact on 'resting' mechanics.

As expected, in vehicle treated groups, high V_T led to a small initial decrease in PIP (**Fig 5A**) followed by a dramatic increase, such that not all animals survived the full 3

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

hour protocol. In contrast, mice treated with MM-284 showed no increase in PIP across the 3 hour experiment (**Fig 5A, Fig 5B**), and all animals survived (**Fig 5C**). Consistent with these observations, alterations in respiratory system elastance (**Fig 5D**) and resistance (**Fig 5E**) were attenuated by MM-284. In addition, MM-284 attenuated the decline of arterial oxygenation (**Fig 5F**). Finally, lung wet:dry weight ratio **(Fig 5G**), lavage fluid total protein (**Fig 5H**) and lavage fluid albumin (**Fig 5I**) were all substantially decreased following inhibition of eCypA signalling.

CypA has previously been described to play roles in leukocyte chemotaxis, cytokine secretion and MMP secretion and activation. We therefore determined whether such processes were inhibited by MM-284 in vivo. Lavage fluid levels of CXCL1 (**Fig 6A**) were significantly decreased following MM-284 treatment, while IL6 (**Fig 6B**) showed a tendency to decrease and IL1β levels (**Fig 6C**) were unaffected. Based on our in vitro data we expected CypA to play a role in alveolar macrophage activation, and indeed ICAM-1 expression on AM was significantly decreased following eCypA inhibition (**Fig 6D**). Interestingly, ICAM-1 expression was also decreased on type 1 AEC (**Fig 6D**) and sRAGE levels were substantially attenuated (**Fig 6E**), suggesting that epithelial cell activation/stress was also affected by MM-284 treatment. Somewhat surprisingly inhibition of eCypA appeared to have no effect on the recruitment of leukocytes into the alveolar space (**Fig 6F**) or lung tissue (**Fig 6G**). Additionally, we determined the levels of active MMPs within the alveolar space by gelatin zymography. While there was very little active MMP9 in lavage fluid with either condition (not shown), levels of active MMP2 were significantly decreased following MM-284 (**Fig 6H**).

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

eCypA is upregulated in ARDS patients

Finally, to confirm the potential relevance of our preclinical findings, baseline (48-72 hours after admission) BAL samples from ARDS patients enrolled onto 2 trials were retrospectively evaluated for levels of eCypA. Compared to healthy volunteers, levels were substantially (~5-6 fold) increased in random samples from both trials (**Fig 7**).

DISCUSSION

Mechanical ventilation, while a life-saving intervention, is also a significant contributor to morbidity and mortality of ARDS through the development of VILI. Due to its widespread use among severely ill patients and iatrogenic nature, pharmacological targeting of VILI represents a highly attractive therapeutic option. In the current study we identify extracellular cyclophilin A (eCypA) as a novel pathogenic mediator in VILI and potential therapeutic target.

Initial experiments were carried out to determine the secretion and kinetics of eCypA during in vivo high V_T ventilation. Levels were found to increase substantially at an early point of VILI before substantial physiological injury was apparent, consistent with a possible role as a mediator of injury. In complete contrast, plasma concentrations were unchanged. Moreover, once the sample dilution introduced by the process of lung lavage is considered, the level of eCypA in the alveolar space is clearly substantially higher than that in plasma. These findings strongly suggest that eCypA was released from cells facing the alveolar space, rather than passively leaking in from the circulation as a result of enhanced permeability (28). We also found in our animal model that eCypA levels correlated with a number of markers of edema and injury. Interestingly, the strongest correlation was with sRAGE levels. sRAGE has been

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

identified as a marker of type I AEC insult (29), implicating epithelial cells as a source for eCypA within the lungs.

To explore the source of eCypA further, lung samples from in vivo ventilated mice were stained for intracellular CypA and analyzed by flow cytometry, which identified type 1 AEC as 'losing' CypA whereas alveolar macrophages did not. The potential for AEC to release CypA in response to stretch was confirmed using human AEC stretched in vitro. Previous studies have demonstrated cellular release of CypA in response to stimuli including LPS (30), thrombin (31) and angiotensin (32), but this is the first time that cell stretch has been identified as an inducer of CypA secretion. The mechanism of CypA release from activated cells is unclear, and potentially depends on cell type and stimulus. CypA is not synthesised with signal peptides typically required for classical protein secretion (9), and its release has been proposed to follow a vesicular pathway (33). We have previously found that mediators packaged and released within microvesicles into the alveolar space can induce responses in target cells and contribute to the pathophysiology of acute lung injury (34, 35), although we did not evaluate here whether eCypA was released in this form. It has also been shown in vascular smooth muscle cells that post-translational modification, in the form of acetylation, is required for CypA secretion, and interestingly leads to enhanced biological activity (32). Alternatively, it has been suggested that CypA is released passively from dead or dying cells as a result of membrane permeabilization (36), although our in vitro data of 7-AAD staining and LDH release suggest that this was not the case in response to cell stretch. Thus we believe that CypA release from alveolar epithelial cells in response to stretch is a specific, regulated process. Studies exploring vascular smooth muscle cells have identified reactive oxygen species, Rho signalling and actin remodelling as playing roles in CypA secretion (33). Given the known

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

involvement of these pathways in VILI (37) we would postulate their involvement in stretch-mediated CypA release, although this requires further exploration. Perhaps surprisingly, little is known regarding the potential influence of steroids on secretion of CypA (or indeed on CD147 expression or CypA:CD147 interaction). Given the increasingly widespread use of steroids in ARDS patients this would be an important area for future consideration.

While eCypA has been found to play a role in various inflammatory conditions, information regarding any role within lung pathologies has been very limited. Mouse models of ovalbumin-induced allergic asthma (16, 38) and LPS-induced pulmonary inflammation (15) have been reported to lead to an upregulation of eCypA levels within the airways. Systemic inhibition of eCypA/CD147 interactions in these models were shown to lead to attenuated leukocyte recruitment, and a direct chemotactic effect of CypA on cells has been widely reported (39). Intriguingly, within our study such a chemotactic effect seems to play a limited role during VILI, as inhibition of eCypA signalling with MM-284 did not influence neutrophil or monocyte recruitment (although we cannot rule out effects on activation status of these cells). In contrast, we found reductions of adhesion molecule expression on lung-resident cells, cytokine production and activity of MMPs, all of which have been previously reported as biological consequences of eCypA in other tissues/conditions (30, 40). Similarly, using human monocyte-derived macrophages in vitro we found that activation markers (including the adhesion molecule ICAM-1) and cytokine (IL6) secretion were upregulated by exposure to CypA. Importantly, the concentration of recombinant (human) CypA we used for these experiments (1μg/ml) was ~10-20 fold higher than the levels we determined in patient BAL samples (50-100ng/ml). However, BAL sampling procedures have been reported to dilute epithelial lining fluid by up to 130

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

fold (41), thus the concentration used for experiments was highly likely to be within the physiologically relevant range.

While the precise position of eCypA within the pathophysiology of VILI remains somewhat unclear, our in vitro and in vivo data would lead us to propose the following paradigm. During ventilation, lung stretch promotes the early secretion of eCypA from alveolar epithelial cells. We propose that eCypA:CD147 interaction on alveolar macrophages then promotes AM activation, inducing secretion of cytokines and active MMP release, leading ultimately to epithelial barrier permeability. Although in vivo MM-284 treatment attenuated AEC activation (in terms of ICAM-1 expression), it is likely that this reflects a secondary consequence of reduced macrophage activation (which plays a crucial role driving physiological dysfunction within VILI – see supplemental Fig E3) rather than a direct effect, as recombinant CypA did not activate AEC in vitro. We did not observe movement of eCypA into plasma, and thus we would postulate that the initial early role of eCypA during VILI is primarily limited to the alveolar space. It should be noted however that previous studies have demonstrated systemic inhibition of CD147 as effective to attenuate pulmonary leukocyte recruitment (15, 16). This could indicate that eCypA may upregulate within the circulation at later time points than studied currently, where it could have direct chemotactic effects on leukocytes. Although we did not confirm that eCypA was definitively signalling through CD147 in the pathophysiology of VILI, we believe it is highly likely. MM-284 specifically inhibits the peptidyl-prolyl *cis-trans* isomerase activity of eCypA (20) which is known to be crucial for signalling through CD147 (42).

Finally, we demonstrated the potential clinical relevance of our preclinical findings by showing that BAL eCypA levels were substantially increased in two populations of

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

ARDS patients compared to healthy controls. eCypA levels have been reported to be increased in patients with sepsis (13) and COPD (43), although these observations were made in plasma samples, and it was not explored whether eCypA levels were a mediator or marker of injury in these studies. Interestingly, while plasma eCypA levels were found to correlate with inflammatory markers in the COPD study, concentrations ranged from just 3-5ng/ml. These levels are much lower than we found within ARDS BAL samples (50-100ng/ml), again highlighting the potential importance of eCypA within the lungs. Intriguingly, a recent meta-analysis of host organism genes implicated in betacoronavirus infection (the family responsible for COVID-19), which identified over 5000 associated genes (44), reported *PPIA*, the gene responsible for CypA expression, as the top ranked gene. This strongly implicates CypA in coronavirus infection-related ARDS, although this was discussed only in terms of the intracellular role of CypA in protein folding, rather than the extracellular signaling role of eCypA demonstrated presently. Thus we believe that the current observations are the first time that eCypA has been clearly identified as a pathological player within the alveolar space, and potential target for therapeutic intervention in critically ill, ventilated patients.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

References

1. Griffiths MJD, McAuley DF, Perkins GD, Barrett N, Blackwood B, Boyle A, Chee N, Connolly B, Dark P, Finney S, Salam A, Silversides J, Tarmey N, Wise MP, Baudouin SV. Guidelines on the management of acute respiratory distress syndrome. *BMJ Open Respir Res* 2019; 6: e000420.

2. Wilson MR, Petrie JE, Shaw MW, Hu C, Oakley CM, Woods SJ, Patel BV, O'Dea KP, Takata M. High-Fat Feeding Protects Mice From Ventilator-Induced Lung Injury, Via Neutrophil-Independent Mechanisms. *Crit Care Med* 2017; 45: e831-e839.

3. Sakr Y, Alhussami I, Nanchal R, Wunderink RG, Pellis T, Wittebole X, Martin-Loeches I, Francois B, Leone M, Vincent JL, Intensive Care Over Nations I. Being Overweight Is Associated With Greater Survival in ICU Patients: Results From the Intensive Care Over Nations Audit. *Crit Care Med* 2015; 43: 2623-2632.

4. Sasabuchi Y, Yasunaga H, Matsui H, Lefor AT, Horiguchi H, Fushimi K, Sanui M. The Dose-Response Relationship Between Body Mass Index and Mortality in Subjects Admitted to the ICU With and Without Mechanical Ventilation. *Respir Care* 2015; 60: 983-991.

5. Foda HD, Rollo EE, Drews M, Conner C, Appelt K, Shalinsky DR, Zucker S. Ventilator-induced lung injury upregulates and activates gelatinases and EMMPRIN: attenuation by the synthetic matrix metalloproteinase inhibitor, Prinomastat (AG3340). *Am J Respir Cell Mol Biol* 2001; 25: 717-724.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

6. Gothel SF, Marahiel MA. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell Mol Life Sci* 1999; 55: 423-436.

7. Wang P, Heitman J. The cyclophilins. *Genome Biol* 2005; 6: 226.

8. Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* 1984; 226: 544-547.

9. Bukrinsky MI. Cyclophilins: unexpected messengers in intercellular communications. *Trends Immunol* 2002; 23: 323-325.

10. Xu Q, Leiva MC, Fischkoff SA, Handschumacher RE, Lyttle CR. Leukocyte chemotactic activity of cyclophilin. *J Biol Chem* 1992; 267: 11968-11971.

11. Yang Y, Lu N, Zhou J, Chen ZN, Zhu P. Cyclophilin A up-regulates MMP-9 expression and adhesion of monocytes/macrophages via CD147 signalling pathway in rheumatoid arthritis. *Rheumatology (Oxford)* 2008; 47: 1299-1310.

12. Wang L, Wang CH, Jia JF, Ma XK, Li Y, Zhu HB, Tang H, Chen ZN, Zhu P. Contribution of cyclophilin A to the regulation of inflammatory processes in rheumatoid arthritis. *J Clin Immunol* 2010; 30: 24-33.

13. Tegeder I, Schumacher A, John S, Geiger H, Geisslinger G, Bang H, Brune K. Elevated serum cyclophilin levels in patients with severe sepsis. *J Clin Immunol* 1997; 17: 380-386.

14. Nigro P, Satoh K, O'Dell MR, Soe NN, Cui Z, Mohan A, Abe J, Alexis JD, Sparks JD, Berk BC. Cyclophilin A is an inflammatory mediator that promotes

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med* 2011; 208: 53-66.

15. Arora K, Gwinn WM, Bower MA, Watson A, Okwumabua I, MacDonald HR, Bukrinsky MI, Constant SL. Extracellular cyclophilins contribute to the regulation of inflammatory responses. *J Immunol* 2005; 175: 517-522.

16. Gwinn WM, Damsker JM, Falahati R, Okwumabua I, Kelly-Welch A, Keegan AD, Vanpouille C, Lee JJ, Dent LA, Leitenberg D, Bukrinsky MI, Constant SL. Novel approach to inhibit asthma-mediated lung inflammation using anti-CD147 intervention. *J Immunol* 2006; 177: 4870-4879.

17. Koh MW, Takata M, Wilson MR. Cyclophilin A as a Novel Mediator in Acute Lung Injury. *Am J Respir Crit Care Med* 2019; 199: A7318.

18. Koh MW, Takata M, Wilson MR. Cyclophilin A as a Novel Mediator in Ventilator-Induced Lung Injury. *FASEB J* 2020; 34.

19. Wilson MR, Patel BV, Takata M. Ventilation with "clinically relevant" high tidal volumes does not promote stretch-induced injury in the lungs of healthy mice. *Crit Care Med* 2012; 40: 2850-2857.

20. Malesevic M, Gutknecht D, Prell E, Klein C, Schumann M, Nowak RA, Simon JC, Schiene-Fischer C, Saalbach A. Anti-inflammatory effects of extracellular cyclosporins are exclusively mediated by CD147. *J Med Chem* 2013; 56: 7302-7311.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

21. Malesevic M, Kuhling J, Erdmann F, Balsley MA, Bukrinsky MI, Constant SL, Fischer G. A cyclosporin derivative discriminates between extracellular and intracellular cyclophilins. *Angew Chem Int Ed Engl* 2010; 49: 213-215.

22. Craig TR, Duffy MJ, Shyamsundar M, McDowell C, O'Kane CM, Elborn JS, McAuley DF. A randomized clinical trial of hydroxymethylglutaryl- coenzyme a reductase inhibition for acute lung injury (The HARP Study). *Am J Respir Crit Care Med* 2011; 183: 620-626.

23. McAuley DF, Cross LM, Hamid U, Gardner E, Elborn JS, Cullen KM, Dushianthan A, Grocott MP, Matthay MA, O'Kane CM. Keratinocyte growth factor for the treatment of the acute respiratory distress syndrome (KARE): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Respir Med* 2017; 5: 484-491.

24. Copland IB, Reynaud D, Pace-Asciak C, Post M. Mechanotransduction of stretch-induced prostanoid release by fetal lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L487-495.

25. Oudin S, Pugin J. Role of MAP kinase activation in interleukin-8 production by human BEAS-2B bronchial epithelial cells submitted to cyclic stretch. *Am J Respir Cell Mol Biol* 2002; 27: 107-114.

26. Woods SJ, Waite AA, O'Dea KP, Halford P, Takata M, Wilson MR. Kinetic profiling of in vivo lung cellular inflammatory responses to mechanical ventilation. *Am J Physiol Lung Cell Mol Physiol* 2015; 308: L912-921.

27. Wang Y, Tang Z, Huang H, Li J, Wang Z, Yu Y, Zhang C, Li J, Dai H, Wang F, Cai T, Tang N. Pulmonary alveolar type I cell population consists of two

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

distinct subtypes that differ in cell fate. *Proc Natl Acad Sci U S A* 2018; 115: 2407-2412.

28. Dorr AD, Wilson MR, Wakabayashi K, Waite AC, Patel BV, van Rooijen N, O'Dea KP, Takata M. Sources of alveolar soluble TNF receptors during acute lung injury of different etiologies. *J Appl Physiol (1985)* 2011; 111: 177-184.

29. Uchida T, Shirasawa M, Ware LB, Kojima K, Hata Y, Makita K, Mednick G, Matthay ZA, Matthay MA. Receptor for advanced glycation end-products is a marker of type I cell injury in acute lung injury. *Am J Respir Crit Care Med* 2006; 173: 1008-1015.

30. Kim SH, Lessner SM, Sakurai Y, Galis ZS. Cyclophilin A as a novel biphasic mediator of endothelial activation and dysfunction. *Am J Pathol* 2004; 164: 1567-1574.

31. Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, Cahill DJ, Emili A, Fitzgerald DJ, Maguire PB. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* 2004; 103: 2096-2104.

32. Soe NN, Sowden M, Baskaran P, Kim Y, Nigro P, Smolock EM, Berk BC. Acetylation of cyclophilin A is required for its secretion and vascular cell activation. *Cardiovasc Res* 2014; 101: 444-453.

33. Suzuki J, Jin ZG, Meoli DF, Matoba T, Berk BC. Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ Res* 2006; 98: 811-817.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

34. Soni S, O'Dea KP, Tan YY, Cho K, Abe E, Romano R, Cui J, Ma D, Sarathchandra P, Wilson MR, Takata M. ATP redirects cytokine trafficking and promotes novel membrane TNF signaling via microvesicles. *FASEB J* 2019; 33: 6442-6455.

35. Soni S, Wilson MR, O'Dea KP, Yoshida M, Katbeh U, Woods SJ, Takata M. Alveolar macrophage-derived microvesicles mediate acute lung injury. *Thorax* 2016; 71: 1020-1029.

36. Christofferson DE, Yuan J. Cyclophilin A release as a biomarker of necrotic cell death. *Cell Death Differ* 2010; 17: 1942-1943.

37. Gawlak G, Tian Y, O'Donnell JJ, 3rd, Tian X, Birukova AA, Birukov KG. Paxillin mediates stretch-induced Rho signaling and endothelial permeability via assembly of paxillin-p42/44MAPK-GEF-H1 complex. *FASEB J* 2014; 28: 3249-3260.

38. Balsley MA, Malesevic M, Stemmy EJ, Gigley J, Jurjus RA, Herzog D, Bukrinsky MI, Fischer G, Constant SL. A cell-impermeable cyclosporine A derivative reduces pathology in a mouse model of allergic lung inflammation. *J Immunol* 2010; 185: 7663-7670.

39. Dawar FU, Wu J, Zhao L, Khattak MN, Mei J, Lin L. Updates in understanding the role of cyclophilin A in leukocyte chemotaxis. *J Leukoc Biol* 2017; 101: 823-826.

40. Kim H, Kim WJ, Jeon ST, Koh EM, Cha HS, Ahn KS, Lee WH. Cyclophilin A may contribute to the inflammatory processes in rheumatoid arthritis through

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

induction of matrix degrading enzymes and inflammatory cytokines from macrophages. *Clin Immunol* 2005; 116: 217-224.

41. Zedtwitz-Liebenstein K, Schenk P, Apfalter P, Fuhrmann V, Stoiser B, Graninger W, Schuster E, Frass M, Burgmann H. Ventilator-associated pneumonia: Increased bacterial counts in bronchoalveolar lavage by using urea as an endogenous marker of dilution. *Crit Care Med* 2005; 33: 756-759.

42. Yurchenko V, Zybarth G, O'Connor M, Dai WW, Franchin G, Hao T, Guo H, Hung HC, Toole B, Gallay P, Sherry B, Bukrinsky M. Active site residues of cyclophilin A are crucial for its signaling activity via CD147. *J Biol Chem* 2002; 277: 22959-22965.

43. Zhang M, Tang J, Yin J, Wang X, Feng X, Yang X, Shan H, Zhang Q, Zhang J, Li Y. The clinical implication of serum cyclophilin A in patients with chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* 2018; 13: 357-363.

44. Parkinson N, Rodgers N, Head Fourman M, Wang B, Zechner M, Swets MC, Millar JE, Law A, Russell CD, Baillie JK, Clohisey S. Dynamic data-driven meta-analysis for prioritisation of host genes implicated in COVID-19. *Sci Rep* 2020; 10: 22303.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure Legends

Figure 1. eCypA levels and correlations. eCypA levels (A) were determined in lung lavage fluid at the end of experiments lasting 1, 2 or 3 hours (or when mortality surrogates were reached if earlier than 3 hours). Levels were shown to be correlated with markers of injury, i.e. PaO_2 (B), lung lavage total protein (C) and soluble RAGE (D). eCypA levels in plasma were also determined at the end of experiments lasting 1, 2 or (up to) 3 hours (E). Alveolar macrophage (AM) cell surface expression of the principal eCypA receptor CD147 was determined by flow cytometry of lung cell suspensions at either 1 or 2 hours of ventilation (F). All data were determined to be non-normally distributed and are displayed as individual data points, with solid line indicating median value. Data in panels A, E and F were evaluated by Kruskal-Wallis followed by Dunn's multiple comparisons test. N=5-6 for panels A & E, and 4-5 for panel F. * $p<0.05$, ** $p<0.01$. In panels B, C & D associations were evaluated with non-parametric Spearman's correlation (r and p-values given on panels).

Figure 2. Source of eCypA. Mice were ventilated with high tidal volume (V_T) for 3 hours (or until mortality surrogates were met) and intracellular levels of CypA were determined by flow cytometry on type I alveolar epithelial cells (A) and alveolar macrophages (B), and compared to levels in non-ventilated (control) mice. Data are expressed as mean fluorescence intensity (MFI). Human AEC in culture were exposed to 6 hours of 20% stretch or held static, and levels of secreted IL8 (C) were determined by ELISA. Levels of secreted eCypA were determined in cell supernatants by Western blotting (D). Upper portion of panel D shows a representative blot with lanes from left to right showing: 1 – 20kDa molecular weight marker, 2 – stretch, 3 static, 4 – stretch, 5 - static, 6 – stretch. Lower portion of panel D shows relative band

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

intensity normalised to a 'standard' sample loaded onto every gel. Positive cell staining for 7-AAD (E) and supernatant levels of lactate dehydrogenase (LDH – panel F) were determined to evaluate cell viability. Data in all panels except E were determined to be normally distributed and evaluated by Student's t-test. Data in panel E were evaluated by Mann-Whitney test. Data are displayed as individual points, with solid line indicating either mean or median value for normal or non-normal data respectively. N= 6 for each dataset. * $p<0.05$, ** $p<0.01$.

Figure 3. Biological effect of CypA on macrophages in vitro. Human monocyte-derived macrophages were stimulated with 1μg/ml recombinant human CypA for 6 hours in vitro. Cell surface expression of ICAM-1 (A), CD86 (B), MHC-II (C) and CD206 (D) were determined by flow cytometry. Levels of secreted IL6 within culture supernatants were determined by ELISA (E). Cell viability was evaluated via 7-AAD staining by flow cytometry (F). Data in panels A, D, E & F were determined to be normally distributed and were evaluated by Student's t-test, while data in panels B & C were non-normal and evaluated by Mann-Whitney test. Data are displayed as individual data points with solid line indicating mean or median value for normal or non-normal data respectively. N=6-7 for each dataset. * $p<0.05$, *** $p<0.001$, ****$p<0.0001$.

Figure 4. Biological effect of CypA on alveolar epithelial cells in vitro. Human primary AEC were stimulated with 1μg/ml recombinant human CypA for 6 hours in vitro. Cell surface expression of ICAM-1 (A) was determined by flow cytometry. Levels of secreted IL8 (B) and IL6 (C) within culture supernatants were determined by ELISA. Cell viability was evaluated via 7-AAD staining by flow cytometry (D). Data in panels A & D were determined to be normally distributed and evaluated by Student's t-test,

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

while data in panels B & C were non-normal and evaluated by Mann-Whitney test. Data are displayed as individual points, with solid line indicating either mean or median value for normal or non-normal data respectively. Dotted line represents positive control data from cells stimulated with 1ng/ml TNF to demonstrate that cells were functionally responsive. N= 6 for each dataset.

Figure 5. Physiological effects of eCypA inhibition during VILI in vivo. Mice were ventilated for up to 3 hours with high tidal volume (V_T) following intratracheal delivery of either eCypA inhibitor MM-284 or vehicle (10% DMSO in saline). Changes in peak inspiratory pressure (PIP) were determined over time (A) and at end-point (B). Note, 'end' reflects the final measurement taken regardless of the length of ventilation as some animals in the vehicle group met the mortality surrogate before 3 hours. Survival (C) was evaluated over time of ventilation. Changes in respiratory system elastance (Ers; D) and resistance (Rrs; E) were calculated as percentage increase at the end versus start of ventilation. Arterial oxygenation (PaO_2; F) was evaluated over time, while lung wet:dry weight ratio (G), lung lavage protein (H) and lavage fluid albumin (I) were measured as end-point markers of oedema and permeability. Data in panels A, B, D, F, G & H were determined to be normally distributed while data in panels E and I were non-normally distributed. Panels A & F showing time courses are displayed as mean±SD and were evaluated by 2-way ANOVA, with significant differences shown as time x treatment group interaction. Survival in panel C was evaluated by Log-rank test. Data in other panels are displayed as individual points with solid lines indicating mean or median value for normal or non-normal data respectively. Data in panels B, D, G & H were evaluated by Student's t-test, while data in panels E and I were evaluated by Mann-Whitney test. N= 7-8 for each dataset. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

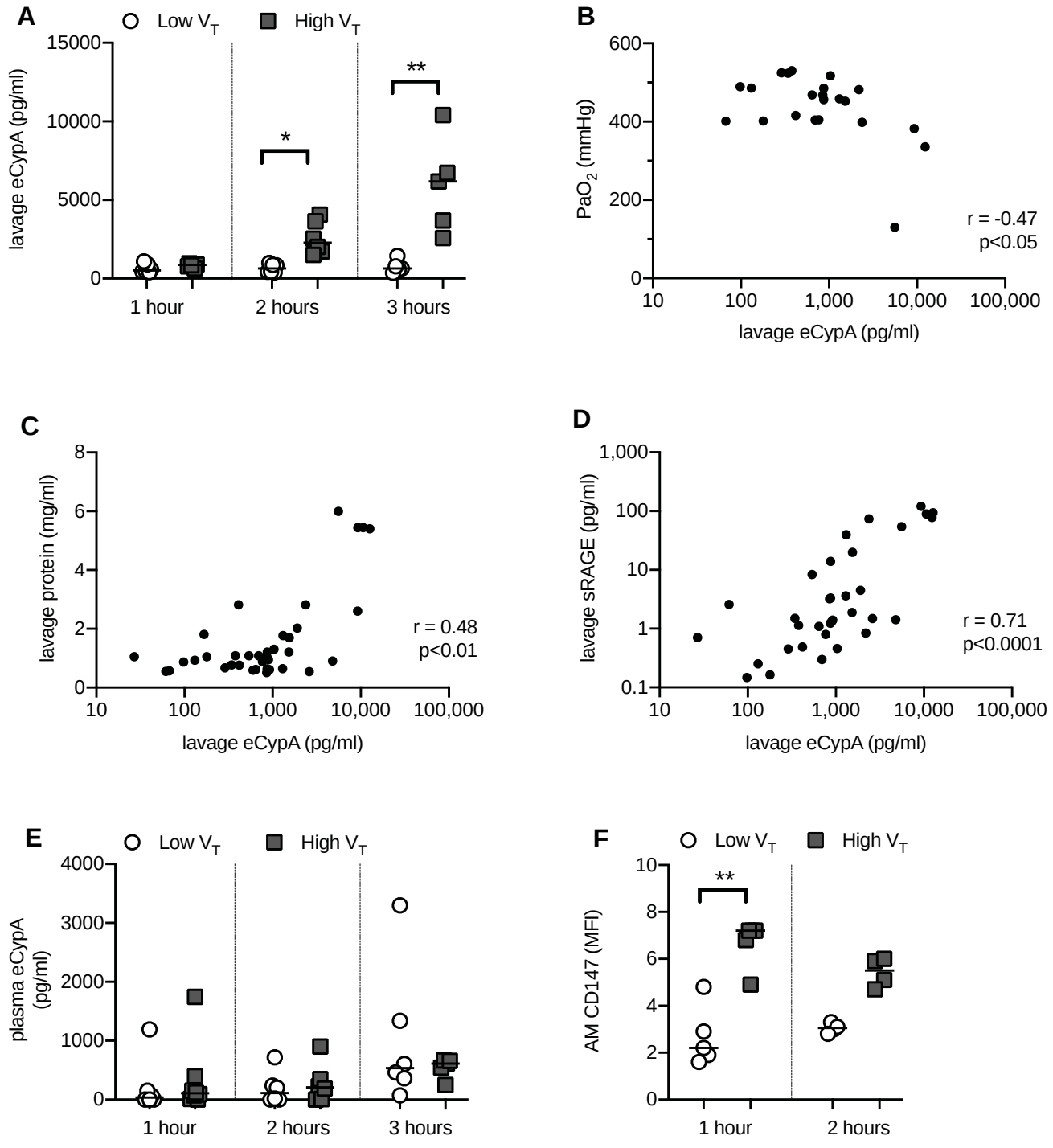
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 6. Anti-inflammatory effects of eCypA inhibition during VILI in vivo. Mice were ventilated for up to 3 hours with high tidal volume (V_T) following intratracheal delivery of either eCypA inhibitor MM-284 or vehicle (10% DMSO in saline). Lavage fluid levels of CXCL1 (A), IL6 (B), IL1β (C) and soluble RAGE (E) were determined by ELISA. Cell surface expression of ICAM-1 (D) was determined on alveolar macrophages (AM) and type 1 alveolar epithelial cells (AEC) by flow cytometry of lung cell suspensions. Numbers of neutrophils and inflammatory $Ly6C^+$ monocytes were quantified in lavage fluid (F) and lung tissue (G) by flow cytometry. Amount of active MMP2 in lavage fluid was determined by gelatin zymography (H). Upper portion of panel H shows representative gel with lanes from left to right showing: Lane 1 – 57kDa molecular weight marker, lanes 2-5 high V_T + vehicle, lanes 6-8 high V_T + MM-284, lane 9 – MMP2 standard. Lower portion of panel H shows relative band intensity normalised to a 'standard' sample loaded onto every gel. Data in all panels except H were determined to be non-normally distributed and were evaluated by Mann-Whitney test or Kruskal-Wallis followed by Dunn's multiple comparisons test (D, F & G). Data in panel H were evaluated by Student's t-test. Data are displayed as individual points with solid lines indicating mean or median value for normal or non-normal data respectively. N= 6-8 for each dataset. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 7. Human data. eCypA levels were determined by ELISA in randomly selected baseline BAL samples from ARDS patients within the control arms of the previously published HARP and KARE studies, and compared to samples from healthy volunteers. Data were assumed to be non-normally distributed and displayed as individual data points with line indicating median value. Data were evaluated by Kruskal-Wallis followed by Dunn's multiple comparisons test. ** $p<0.01$.

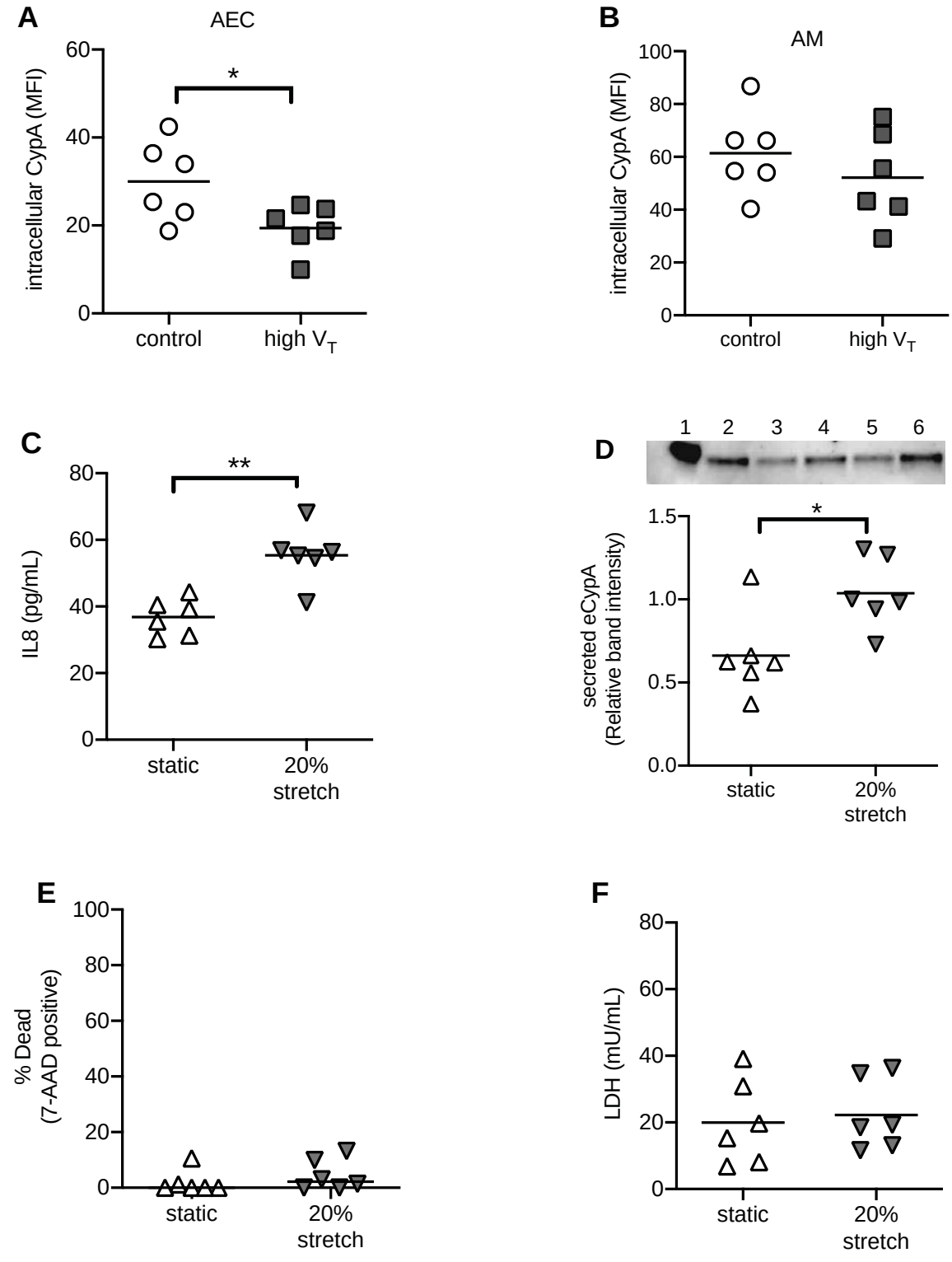
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 1



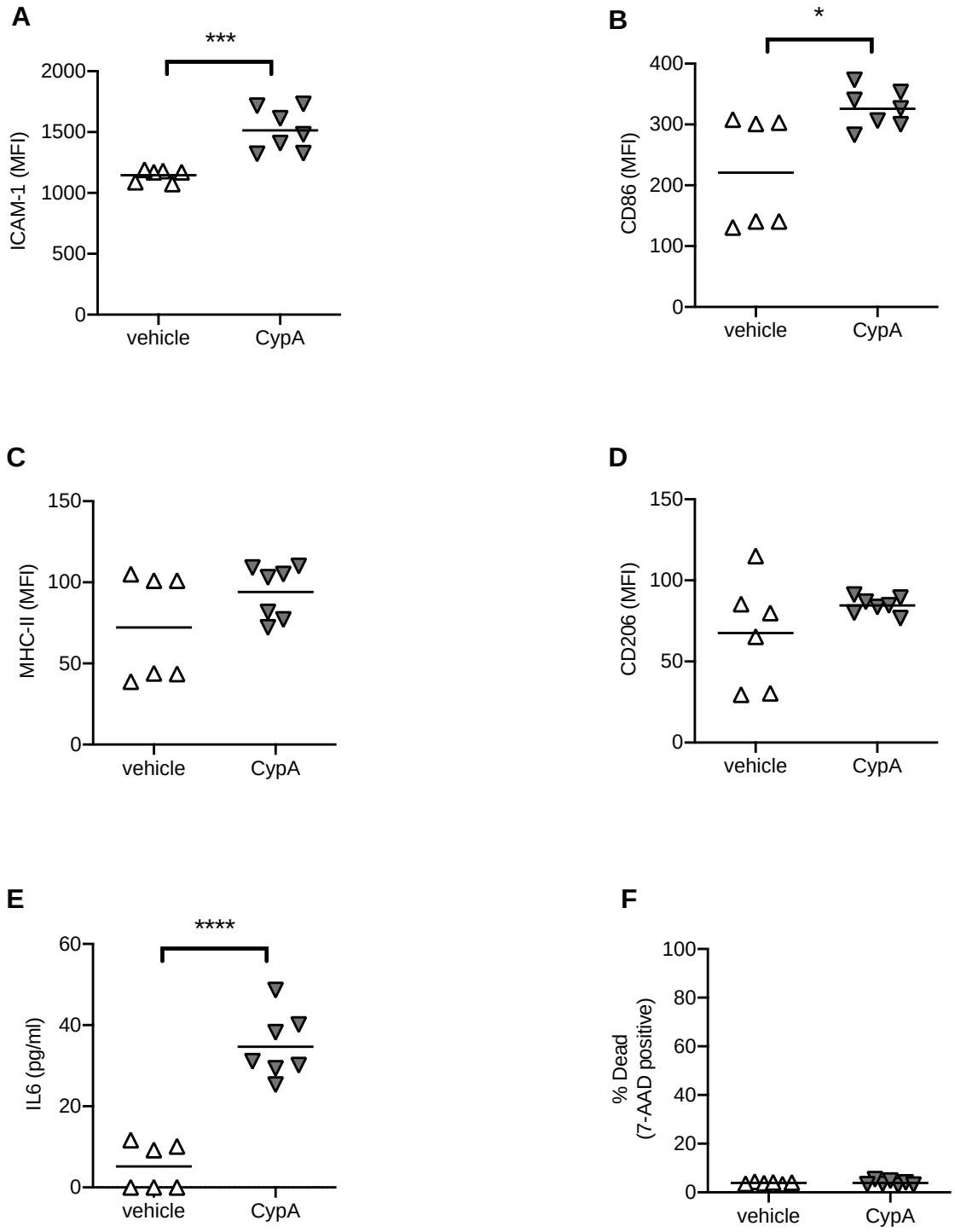
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 2



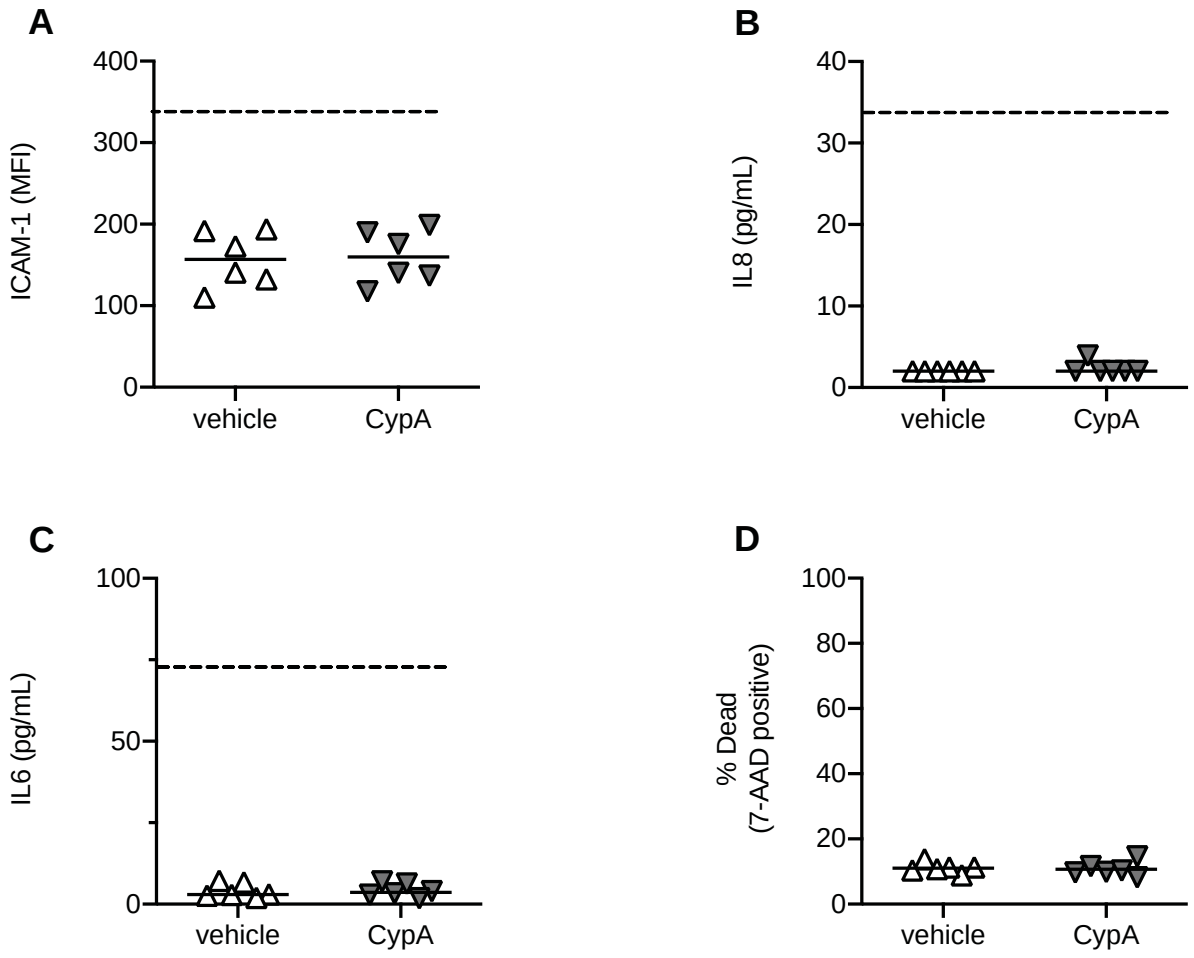
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 3



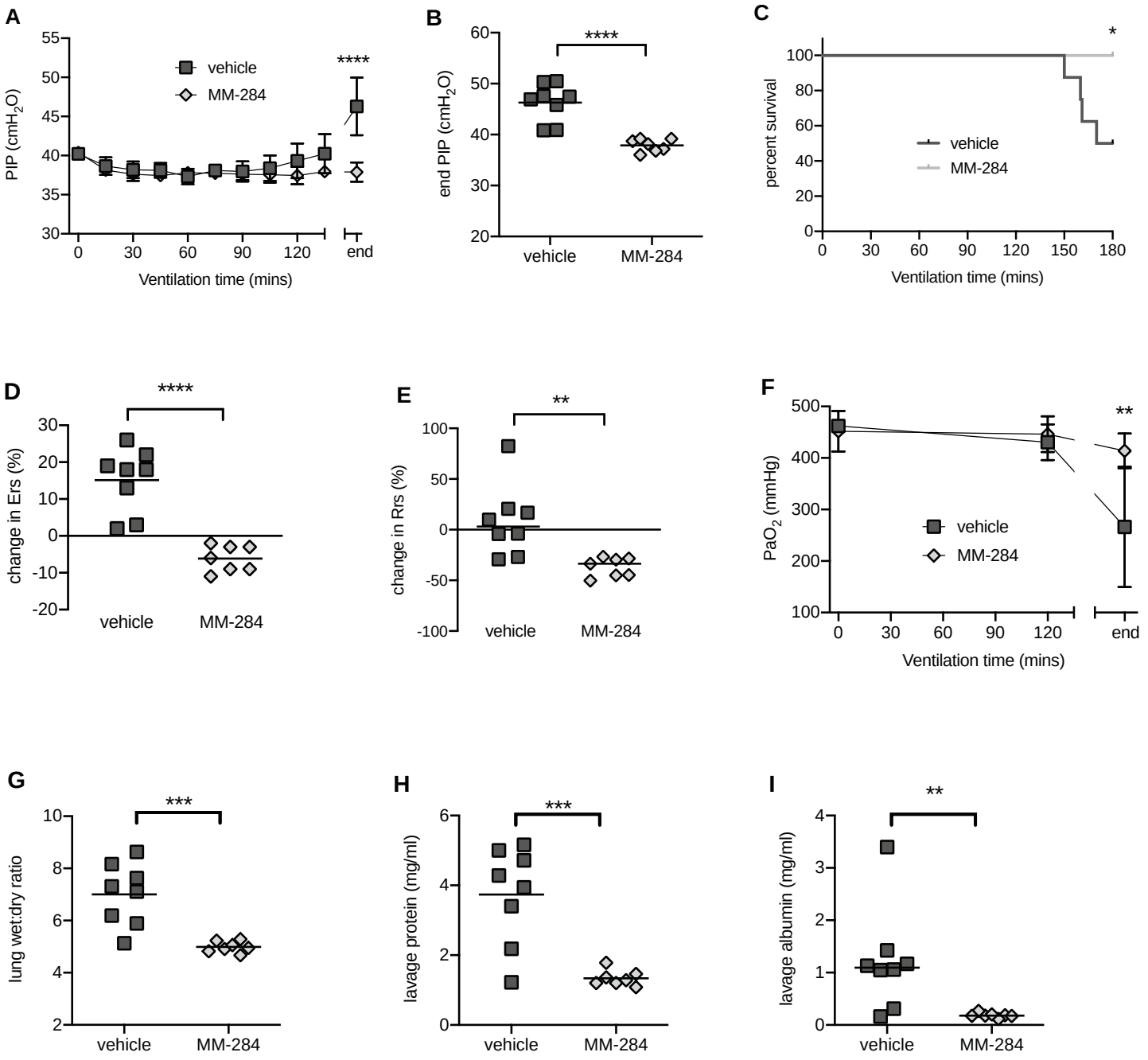
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 4



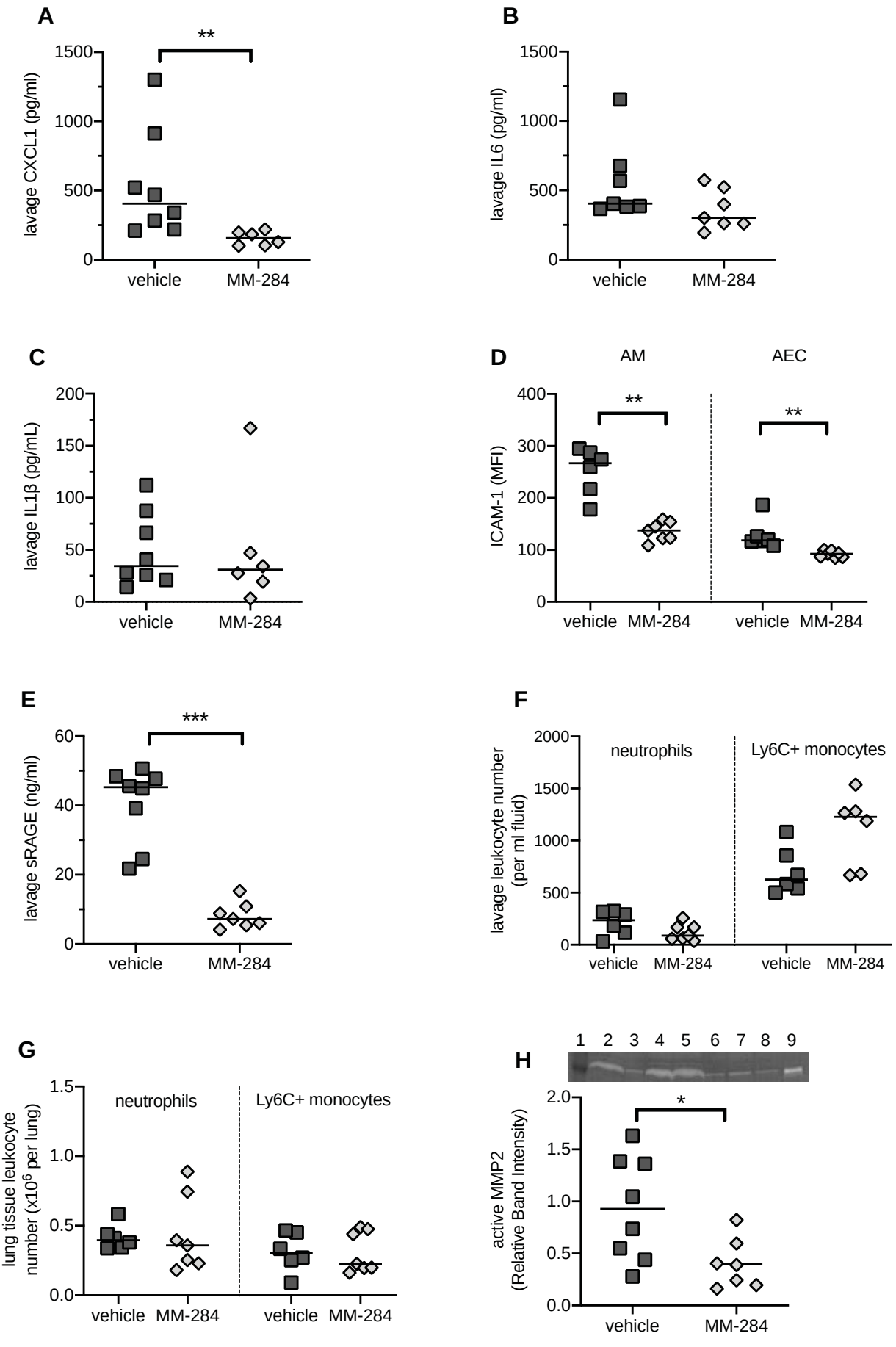
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 5



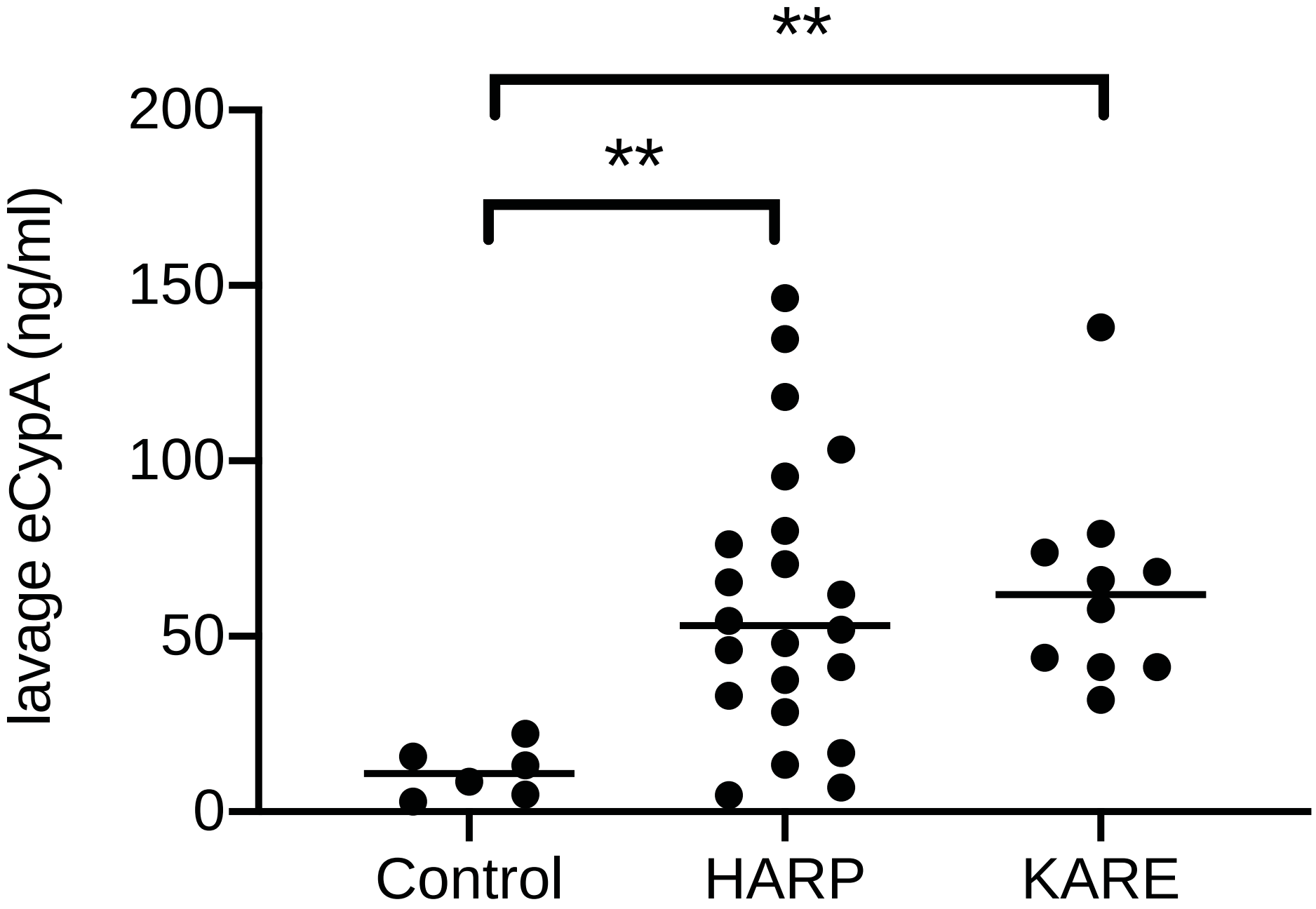
AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 6



AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure 7



AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Secreted Extracellular Cyclophilin A is a Novel Mediator of Ventilator Induced Lung Injury

Marissa W. Koh, Rhianna F. Baldi, Sanooj Soni, Rhodri Handslip, Ying Ying Tan, Kieran P. O'Dea, Miroslav Malesevic, Daniel F. McAuley, Cecilia M. O'Kane, Brijesh V Patel, Masao Takata, Michael R. Wilson

Online supplement – extended methods

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Animal studies

In vivo ventilation model

All animal studies were approved by the Imperial College London ethical review board, and were carried out under the Animals (Scientific Procedures) Act 1986, UK. Male C57BL6 mice aged 7-11 weeks (weight 22-30g) were anaesthetised by intraperitoneal injection of 80mg/kg ketamine and 8mg/kg xylazine. Once surgical anaesthesia was achieved, mice were tracheostomised and connected to a custom-made mouse ventilator/pulmonary function testing system (E1). The left carotid artery was cannulated for fluid infusion (10U/ml heparin in saline, 0.3ml/hour), arterial blood pressure monitoring and withdrawal of samples for blood gas analysis. An intraperitoneal line was placed to enable administrations of maintenance anaesthesia (bolus injections of 40mg/kg ketamine and 4mg/kg xylazine every 20-25 minutes throughout the protocol). Temperature was maintained at 36.5–37.5ºC by external heat sources, monitored by a rectal temperature probe.

During surgery, mice were ventilated with non-injurious, low tidal volume (V_T) settings, i.e. 8ml/kg V_T, 3cmH_2O positive end-expiratory pressure (PEEP) and 120 breaths per minute respiratory rate (RR), using 100%O_2. Once surgery was complete, a recruitment manoeuvre was performed (sustained inflation at 35cm H_2O for 5 seconds) to recruit any atelectatic areas and standardise the volume history of the lungs. Mice were then randomised to receive either low (same settings) or high V_T ventilation (35-40ml/kg V_T, 3cmH_2O PEEP and 80 breaths per minute RR, using 96%O_2:4%CO_2 to avoid hypocapnia). Low V_T mice received a recruitment manoeuvre every 30 minutes throughout the protocol to avoid the development of ongoing derecruitment (E1).

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Mice were then ventilated for predetermined times of 1, 2, or up to 3 hours. Some high V_T animals met a series of mortality surrogates (i.e. blood pressure <50mmHg and/or increase in peak inspiratory pressure >20%) from 150 minutes onwards. Data from these animals is still included and referred to either as 'end' (for time course studies) or '3 hours' (for end point analyses).

Peak inspiratory pressure and mean arterial blood pressure were monitored continuously throughout experiments. Respiratory mechanics (elastance, resistance) were determined by end-inflation occlusion at the start of ventilation protocols and every 30 minutes thereafter. Samples for blood gas analysis were taken at predetermined time points, depending on the intended length of ventilation protocol: for 1 hour studies samples were taken after 15 minutes and at the end (1 hour); for 2 hour studies samples were taken after 15 minutes and at the end (2 hours); for 3 hour studies samples were taken after 15 minutes, 2 hours, and at the end (3 hours or when mortality surrogates were reached if earlier). Sample volumes removed were 60-70µl each time, with fluid volume replaced with ~150µl saline (containing 10U/ml heparin).

In a separate set of '3 hour' ventilation experiments, mice were injected intravenously with a 50µl bolus of fluorescent-conjugated albumin (Alexa-Fluor 594-BSA, Invitrogen) after 1 hour of high or low V_T. At the end of experiments, blood and lavage fluid samples were collected, and the ratio of lavage:plasma fluorescence determined as reported previously (E2).

Alveolar macrophage depletion

In studies designed to validate the inflammatory nature of the model, clodronate-loaded liposomes were used to deplete alveolar macrophages. Mice were briefly anaesthetised and dosed intratracheally with 50µl of saline (vehicle) or clodronate-

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

loaded liposomes 48 hours prior to high tidal volume mechanical ventilation, a time period demonstrated to induce substantial alveolar macrophage depletion (E2, E3). We and others have previously demonstrated that alveolar macrophage depletion using clodronate-loaded liposomes leads to attenuated inflammation and injury, within models of VILI and other acute aetiologies of lung injury (E2, E4, E5). Of note, we utilised saline alone as a control, rather than saline-loaded liposomes, which have been postulated as an alternative or additional control for such studies (E6). However, the aforementioned papers utilised empty or saline-loaded liposomes as controls, demonstrating that it is depletion of alveolar macrophages specifically that influences progression of injury in such scenarios. Moreover, when directly compared, it has been shown that animals dosed intratracheally with either saline alone or saline-loaded liposomes show no differences in alveolar cytokines, alveolar protein levels, pressure volume curves, macrophage counts, or surfactant phospholipid levels or composition (E7). Thus we believe it is very unlikely that the differences ultimately observed (see online data supplement Fig E3) were influenced by the presence of liposomes per se, but rather were due to the depletion of macrophages.

MM-284 inhibition studies

For in vivo studies exploring the impact of inhibiting extracellular CypA signaling, mice were anaesthetised and instrumented as described above. After completing surgery, animals were placed on non-injurious low V_T settings for 5 minutes. They were then disconnected from the ventilator and 50 µL of either vehicle (10% DMSO; Sigma-Aldrich) or MM-284 (0.67 mM) was instilled into the lungs through the endotracheal tube. After re-attachment to the ventilator, 3 recruitment manoeuvres were performed to aid in the distribution of fluid within the airspaces. Animals were then ventilated with

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

these same low V_T settings for a further 30 minutes to ensure fluid distribution and stability of mechanics.

After this 30 minute stabilisation period, respiratory mechanics were determined and mice were switched to high V_T settings, which were the same as described above with the exception that V_T was slightly lower (30-35 cmH_2O). Animals were then ventilated for 3 hours (or were terminated earlier if mortality surrogates were reached) and monitored/sampled as described above.

Sample harvest and processing

At the end of ventilation experiments mice were sacrificed and samples were collected. Blood samples were collected via exsanguination and centrifuged at *1500g* for 5 minutes at 4ºC. The right lower lobe was tied off and removed for wet:dry weight determination, and the remaining lobes were lavaged by flushing and gently aspirating 650 µL of saline in and out of the lungs via the endotracheal tube three times. The recovered lavage fluid was centrifuged at *300g* for 5 minutes at 4ºC and the supernatant was collected. In some experiments, the remaining lobes were then processed for flow cytometry.

ELISA

Mouse specific ELISA kits were used to determine levels of CypA in lavage fluid and plasma (mouse cyclophilin A ELISA; Elabscience, China), and levels of CXCL1, IL-6, MCP-1 (all R&D systems) and albumin (abcam) in lavage fluid. The assays were conducted as per manufacturer's instructions and plates were read at 450 nm in an absorbance microplate reader (Biotex ELx800).

Intracellular staining for CypA

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

A flow cytometry based staining protocol was used to measure intracellular CypA, adapted from our previous studies (E2). Lungs were harvested and briefly rinsed with saline and blotted dry before mechanical homogenisation for 1 minute using a GentleMACS tissue dissociator (Miltenyi Biotec Ltd.) with 2 mL of cold FACS wash buffer (FWB) containing phosphate buffered saline (PBS), 2% fetal calf serum (FCS), 0.1% sodium azide, and 5mM EDTA (Invitrogen). The lung homogenate was then sieved through a 40μm nylon strainer (BD Falcon) to produce a single-cell suspension which was then centrifuged at 2,000 rpm for 5 minutes at 4°C. Supernatants were discarded and cell pellets were washed by resuspension in FWB and centrifugation, following which the final cell pellets were incubated with 250 μL of BD Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4ºC. Cells were then washed twice by re-suspension in 4 mL of permeabilisation wash buffer (PWB) containing PBS with 0.2% saponin, 0.5% bovine serum albumin (BSA), 0.1% sodium azide and centrifugation. The final cell pellets were re-suspended in 50 μL of PWB.

A three-step staining procedure was performed, with samples washed twice with PWB between each step: (1) Permeabilised single cell suspension was incubated for one hour at 4ºC with primary anti-cyclophilin A antibody (10 μg/mL; Abcam); (2) 30 minute incubation at 4ºC with rabbit IgG APC-conjugated antibody (80 ng/mL; R&D); (3) 30 minute incubation at 4ºC with fluorochrome-conjugated antibodies against surface antigen markers. The following panel of antibodies for lung parenchymal cells was used: EpCAM (G8.8; BioLegend); CD31 (Mec13.3; BD Bioscience); MHC-II (M5/114.15.2; BioLegend), T1α (8.1.1; BioLegend); CD45 (30-F11; BioLegend). The following panel of antibodies for leukocyte markers was used: F4/80 (BM8; BioLegend); CD11b (M1/70; BioLegend); CD45 (30-F11; BioLegend); Ly6C (HK1.4; BioLegend); Ly6G (1A8; BioLegend); CD11c (BU15; eBioscience). The final cell

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

pellets were resuspended in PWB for analysis on a 7-channel CyAn ADP flow cytometer (Beckman Coulter). All flow cytometric data were analysed with FlowJo™ software (Becton, Dickinson and Company).

Type I alveolar epithelial cells (AEC) were defined as CD45-/CD31-/ EpCAM-/ T1α+/ MHC-II- cells. Alveolar macrophages (AM) were defined as CD45+/CD11c+/CD11b-/F4-80+ cells.

Leukocyte quantification and ICAM-1 expression

In MM-284 studies, lungs were processed for flow cytometry to evaluate cell surface ICAM-1 expression on AEC and AM. Lungs were homogenised as above to yield single cell suspensions. Cells were incubated in the leukocyte and parenchymal antibody panels as described above with the additional of surface activation marker ICAM-1 (YN1/1.7.4; BioLegend).

In addition, recruited leukocytes were quantified in lung tissue and lavage samples. Lung single cell suspensions were processed and stained as described above, while lavage fluid samples were directly stained with the aforementioned antibody cocktail. Neutrophils were identified as CD45+/CD11b+/CD11c-/Ly6G+ events, while inflammatory monocytes were identified as CD45+/CD11b+/CD11c-/Ly6G-/Ly6C+ events. Immediately prior to analysis on the flow cytometer, AccuCheck counting beads (Invitrogen) were added. For lavage samples, leukocyte quantification is calculated as leukocytes per ml lavage fluid, while lung sample leukocytes are expressed as cells per lung.

Bradford assay

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

The protein concentrations of lavage fluid samples and lung homogenate supernatants were measured via the Bradford assay (Bio-Rad Laboratories), with absorbances measured at 595 nm.

Gelatin zymography

Equal volumes of BAL fluid (12.5 µL) were mixed 1:1 with 1X Novex™ Tris-Glycine SDS Sample Buffer (Invitrogen). (Equal volume loading is a commonly used technique to standardise cell-free samples for zymography or Western blotting (E8, E9)). Samples were loaded into Novex™ 10% zymogram (gelatin) protein gels (Invitrogen) for electrophoresis separation under non-reducing conditions. After, gels were washed with renaturing buffer and then incubated overnight in developing buffer to induce digestion of gelatin by any MMP-2 and MMP-9 present in the samples. Gels were then stained with colloidal blue to allow visualisation of denatured areas. To allow comparison between gels, an internal standard – a sample known to contain high levels of MMPs – was loaded on to every gel. The density of this sample was given an arbitrary unit of 1 and the density of all bands were expressed as a value relative to it. Gels were scanned using a flat-bed scanner and analyses of gels were performed using ImageJ software (National Institutes of Health, USA).

In vitro studies

Cell stretch studies

For stretch experiments, human primary alveolar epithelial cells (AEC; CellBiologics) were cultured until 90-100% confluent and plated onto collagen-coated 6 well plates (BioFlex®, Flexcell International) in complete human epithelial cell medium supplemented with 0.5 mL Insulin-Transferrin-Selenium, 0.5 mL EGF, 0.5 mL

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

hydrocortisone, 5 mL L-Glutamine, 5 mL antibiotic-antimycotic solution, and 25 mL FCS (CellBiologics). Immediately before stretch experiments were commenced, media was replaced with fresh low-serum (5% FCS) media. Cells were exposed to cyclic mechanical stretch at 0.25 Hz and duty cycle of 33.3% using a computer-controlled vacuum strain apparatus (Flexcell Tension System) with a vacuum pressure sufficient to generate a maximum of 20% elongation. For the duration (6 hours) of the experiment, cells were placed in an incubator maintained at 37ºC in a humidified 5% CO2 atmosphere. Static control cells were also seeded and cultured on the same BioFlex® culture plates and placed into the same incubator for the same stretch exposure time.

At the end of stretch experiments, conditioned culture media were collected and centrifuged at *300g* for 5 minutes at 4ºC to yield cell-free supernatants. Supernatants were analysed for LDH levels using a lactate dehydrogenase assay kit (Abcam), IL8 levels using ELISA (R&D), and eCypA secretion by western blotting. Cells were recovered from plates using Cell Dissociation Buffer (ThermoFisher Scientific) and underwent flow cytometric analysis to determine cell numbers and evaluate cell viability using 7-amino-actinomycin D (7-AAD; eBioscience) staining. Human primary alveolar epithelial cells were identified based on their expression of EpCAM.

Western blotting

Equal volumes of cell-free supernatants were loaded on NuPAGE 12% Bis-Tris gels (Invitrogen), separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (ThermoFisher Scientific). Membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 hour at room temperature with

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

gentle agitation. Membranes were washed three times with TBS-T and incubated with anti-cyclophilin A antibody (1:500; Abcam) in TBS-T overnight at 4ºC.

The following day, membranes were rinsed in TBS-T three times before incubation with anti-rabbit HRP-conjugated secondary antibody (1:1000; Abcam) for 1 hour. Membranes were washed three times for 5 minutes each and blots were visualised with Luminol reagent (Santa Cruz Biotechnology). Blots were captured with GeneSnap (Syngene) and analysed with ImageJ (National Institutes of Health).

Human monocyte derived macrophage (HMDM) isolation and culture

50 mL Leucosep tubes containing 15 mL Histopaque-1077 (Sigma-Aldrich) were centrifuged at 28,000 rpm for 1 minute. Blood was collected from healthy volunteers in EDTA containing BD Vacutainer tubes. To each Leucosep tube, 20 mL of blood was added and centrifuged at 28,000 rpm for 1 minute. Following separation, the peripheral blood mononuclear cell (PBMC) layer was gently aspirated and washed twice in sterile PBS. The final cell pellet was resuspended in 1 mL of cell sorting buffer (PBS, 1% FCS, 2 mM EDTA, 1% penicillin-streptomycin). Cell counts were determined using flow cytometry. Monocytes were then isolated from PBMCs using a pan-monocyte isolation kit (Miltenyi Biotec). The protocol was performed as per manufacturer's instructions. Monocytes were then seeded into 55 cm^2 dishes and cultured for five days in incubators maintained at 37ºC in a humidified 5% CO_2 atmosphere. Media was first replaced on day 3, and then every other day subsequently. Cells were ready for use from day 7 onwards.

Stimulation of HMDM and AEC with recombinant CypA

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

HMDM were seeded in 48-well plates at a density of 9 x 10^4 cells/well in RPMI media supplemented with 10% FCS and 1% penicillin-streptomycin-glutamine (PSG) and allowed to adhere overnight. The following day, media was replaced with serum-free RPMI media for stimulation experiments. In separate experiments, human primary AEC (CellBiologics) were seeded in 48-well plates at a density of 7 x 10^4 cells/well in complete human epithelial cell medium supplemented with 0.5 mL Insulin-Transferrin-Selenium, 0.5 mL EGF, 0.5 mL hydrocortisone, 5 mL L-Glutamine, 5 mL antibiotic-antimycotic solution, and 25 mL FCS (CellBiologics). Upon confluence, media was replaced with fresh serum-free media for stimulation experiments. Both cell types were exposed to either media alone (vehicle) or were stimulated with 1µg/ml recombinant human CypA (R&D Systems) for 6 hours.

Following stimulation, media was collected and centrifuged at *300g* for 5 minutes at 4ºC to yield cell-free supernatant. Supernatants were analysed using ELISA for IL8 or IL6 levels (R&D Systems). Cells were harvested, processed, and stained for analysis with flow cytometry. Noise and cell debris were excluded based on auto-fluorescence and size. HMDM were identified as CD45+/CD16+ cells, while AEC were identified based on their expression of EpCAM. Cell surface expression of ICAM-1, CD86, MHC-II, and CD206 were evaluated as appropriate. Cell viability was also assessed by flow cytometric analysis of 7-AAD staining.

In a small set of experiments, recombinant CypA stimulation was also tested in A549 epithelial cells. These were seeded in 48-well plates at 1x10^5 cells/well in DMEM supplemented with 10% FCS and 1% PSG. As above, media was replaced with fresh serum-free media for stimulation experiments involving exposure to either media alone

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

(vehicle) or 1μg/ml recombinant human CypA for 6 hours. Cell surface expression of ICAM-1 was determined and cell viability was assessed via 7-AAD staining.

Human studies

Human ARDS samples were kindly provided by Professor Danny McAuley. All samples were collected and stored in accordance with the protocol and ethics of the 'Keratinocyte growth factor for the treatment of the acute respiratory distress syndrome' (KARE) and 'A randomised clinical trial of hydroxymethylglutaryl-coenzyme A reductase inhibition for acute lung injury' (HARP) studies. These studies and their protocols have been published (E10, E11). All samples were derived from patients that were randomised to the control (placebo) group. Control samples were from healthy volunteers enrolled in the HARP study (trial no: ISRCTN70127774) who underwent BAL as controls. BAL fluid levels of CypA were evaluated using a human cyclophilin A ELISA kit (Abbexa, UK). The assay was conducted as per manufacturer's instructions.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

References

E1. Wilson MR, Patel BV, Takata M. Ventilation with "clinically relevant" high tidal volumes does not promote stretch-induced injury in the lungs of healthy mice. *Crit Care Med* 2012; 40: 2850-2857.

E2. Patel BV, Wilson MR, O'Dea KP, Takata M. TNF-induced death signaling triggers alveolar epithelial dysfunction in acute lung injury. *J Immunol* 2013; 190: 4274-4282.

E3. Dorr AD, Wilson MR, Wakabayashi K, Waite AC, Patel BV, van Rooijen N, O'Dea KP, Takata M. Sources of alveolar soluble TNF receptors during acute lung injury of different etiologies. *J Appl Physiol (1985)* 2011; 111: 177-184.

E4. Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA. Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury. A*m J Physiol Lung Cell Mol Physiol* 2006; 291 :L1191-8.

E5. Eyal FG, Hamm CR, Parker JC. Reduction in alveolar macrophages attenuates acute ventilator induced lung injury in rats. *Intensive Care Med* 2007; 33 :1212-1218.

E6. Weisser SB, van Rooijen N, Sly LM. Depletion and reconstitution of macrophages in mice. *J Vis Exp* 2012; 66: 4105.

E7. Forbes A, Pickell M, Foroughian M, Yao LJ, Lewis J, Veldhuizen R. Alveolar macrophage depletion is associated with increased surfactant pool sizes in adult rats. J Appl Physiol (1985) 2007; 103: 637-45.

E8. Albaiceta GM, Gutierrez-Fernandez A, Garcia-Prieto E, Puente XS, Parra D, Astudillo A, Campestre C, Cabrera S, Gonzalez-Lopez A, Fueyo A, Taboada F, Lopez-

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Otin C. Absence or inhibition of matrix metalloproteinase-8 decreases ventilator-induced lung injury. *Am J Respir Cell Mol Biol* 2010; 43: 555-563.

E9. Blazquez-Prieto J, Lopez-Alonso I, Amado-Rodriguez L, Huidobro C, Gonzalez-Lopez A, Kuebler WM, Albaiceta GM. Impaired lung repair during neutropenia can be reverted by matrix metalloproteinase-9. *Thorax* 2018; 73: 321-330.

E10. Craig TR, Duffy MJ, Shyamsundar M, McDowell C, O'Kane CM, Elborn JS, McAuley DF. A randomized clinical trial of hydroxymethylglutaryl- coenzyme a reductase inhibition for acute lung injury (The HARP Study). *Am J Respir Crit Care Med* 2011; 183: 620-626.

E11. McAuley DF, Cross LM, Hamid U, Gardner E, Elborn JS, Cullen KM, Dushianthan A, Grocott MP, Matthay MA, O'Kane CM. Keratinocyte growth factor for the treatment of the acute respiratory distress syndrome (KARE): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Respir Med* 2017; 5: 484-491.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Supplementary Table E1. Summary clinical data of human lavage sample patients.

	HARP study	KARE study	volunteers
Sex (% male)	78	40	83
Age (yrs)	54.8±20.2	59.5±19.3	27.0±4.1
P/F ratio (mmHg)	169±59	189±72	-
Cause (% direct)	52	80	-
Sepsis, number (%)	-	1 (10)	-
Trauma, number (%)	-	1 (10)	-
Pneumonia, number (%)	-	4 (40)	-
Aspiration pneumonia, number (%)	-	3 (30)	-
Smoke inhalation, number (%)	-	1 (10)	-

Samples were randomly chosen from patients allocated to the vehicle arms of the relevant study, at baseline. Note that in the 'HARP' study, aetiology was only recorded as direct or indirect.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Supplementary Figure E1. Time course of injury in VILI model. Peak inspiratory pressure (A), mean arterial blood pressure (B) and arterial PaO_2 (C) were measured across the course of experiments lasting either 3 hours or until surrogate mortality markers were met if that occurred sooner (high V_T animals started to meet mortality surrogate from 150 minutes onwards). Note 'end' reflects the final measurement taken regardless of the length of ventilation. As 'end-point' markers of injury lavage fluid protein (D), lung wet:dry weight ratio (E) and lavage fluid soluble RAGE levels (F) were measured following termination of experiments at 1, 2, and 3 hours (or when mortality surrogates were reached if earlier than 3 hours). Data in panels A, B, C and E were normally distributed while data in panels D & F were not. Data in panels A-C are displayed as mean±SD, while other data are displayed as individual points with solid line indicating either mean or median value for normal or non-normal data respectively. Data in Panel C were evaluated by 2-way ANOVA, with significant differences shown as time x treatment group interaction. Data in panel E were evaluated by one-way ANOVA followed by Sidak's multiple comparisons test, while data in D & F were evaluated by Kruskal-Wallis followed by Dunn's multiple comparisons test. N=5-7 for each dataset (at each time point for time course studies). * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

Supplementary Figure E2. Permeability and inflammation following 3 hours high or low V_T ventilation (or when mortality surrogates were reached if earlier than 3 hours). (A) Ratio of AlexaFluor 594-BSA fluorescence in lavage fluid:plasma as a marker of permeability. Quantification of (B) alveolar macrophages, (C) Ly6C high monocytes, and (D) neutrophils within lung tissue was carried out by flow cytometry. ICAM-1 expression on alveolar macrophages (E) and epithelial cells (F) was evaluated by flow cytometry as a marker of cell activation. Lavage fluid levels of CXCL1 (G), IL6 (H) and

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

MCP-1 (I) were determined by ELISA. Data in panels B-H were normally distributed and analysed by student's t-test, while non-normally distributed data in panels A & I were analysed by Mann-Whitney test. Data are displayed as individual points, with solid line indicating either mean or median value for normal or non-normal data respectively. N=6 for each dataset. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.

Supplementary Figure E3. Impact of alveolar macrophage depletion by clodronate-liposomes in VILI model. Peak inspiratory pressure (A) and arterial PaO_2 (B) were measured across the course of experiments lasting either 3 hours or until surrogate mortality markers were met if that occurred sooner. Note 'end' reflects the final measurement taken regardless of the length of ventilation. Changes in respiratory system elastance (Ers; C) and resistance (Rrs; D) were calculated as percentage increase at the end versus start of ventilation. Lung wet:dry weight ratio (E) and lavage fluid protein levels (F) were measured following termination of experiments. Data in panels A, B, C, D & F were normally distributed while data in panel E were not. Data in panels A & B are displayed as mean±SD, while other data are displayed as individual points with solid line indicating either mean or median value for normal or non-normal data respectively. Data in panels A & B were evaluated by 2-way ANOVA, with significant differences shown as time x treatment group interaction. Data in panels C, D & F were evaluated by students t-test, while data in E were evaluated by Mann-Whitney test. N=6 for each dataset (at each time point for time course studies). ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

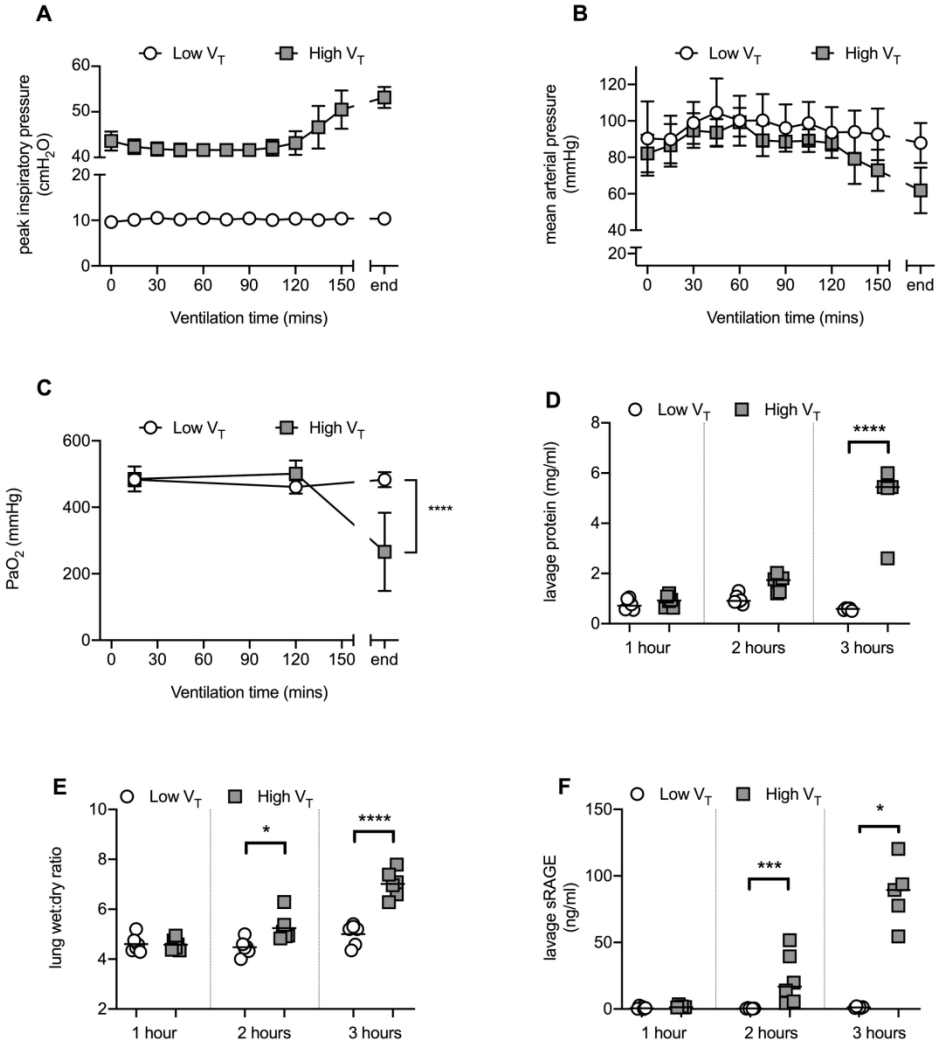
Supplementary Figure E4. A549 AEC were stimulated with 1μg/ml recombinant human CypA for 6 hours in vitro. Cell surface expression of ICAM-1 (A) was determined by flow cytometry as a marker of activation, while cell viability was

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

evaluated via 7-AAD staining by flow cytometry (B). Data were treated as non-normally distributed and were evaluated by Mann-Whitney test. Data are displayed as individual points, with solid line indicating median value. N= 5 for each dataset.

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

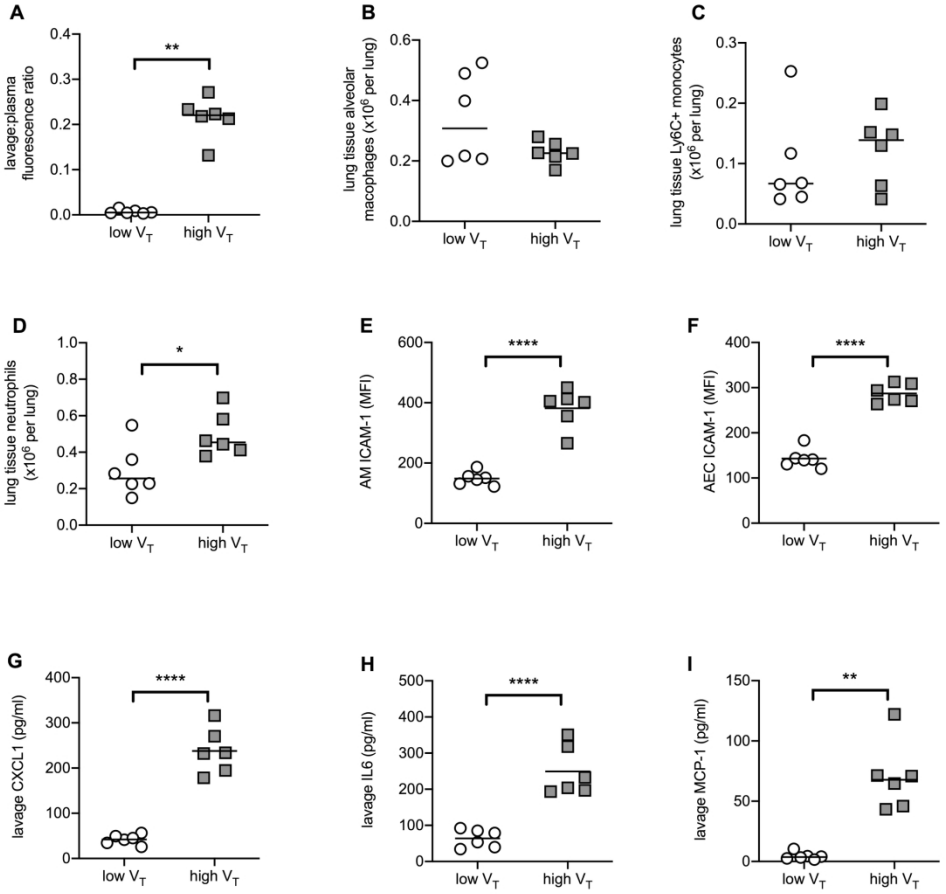
Figure E1



177x202mm (300 x 300 DPI)

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

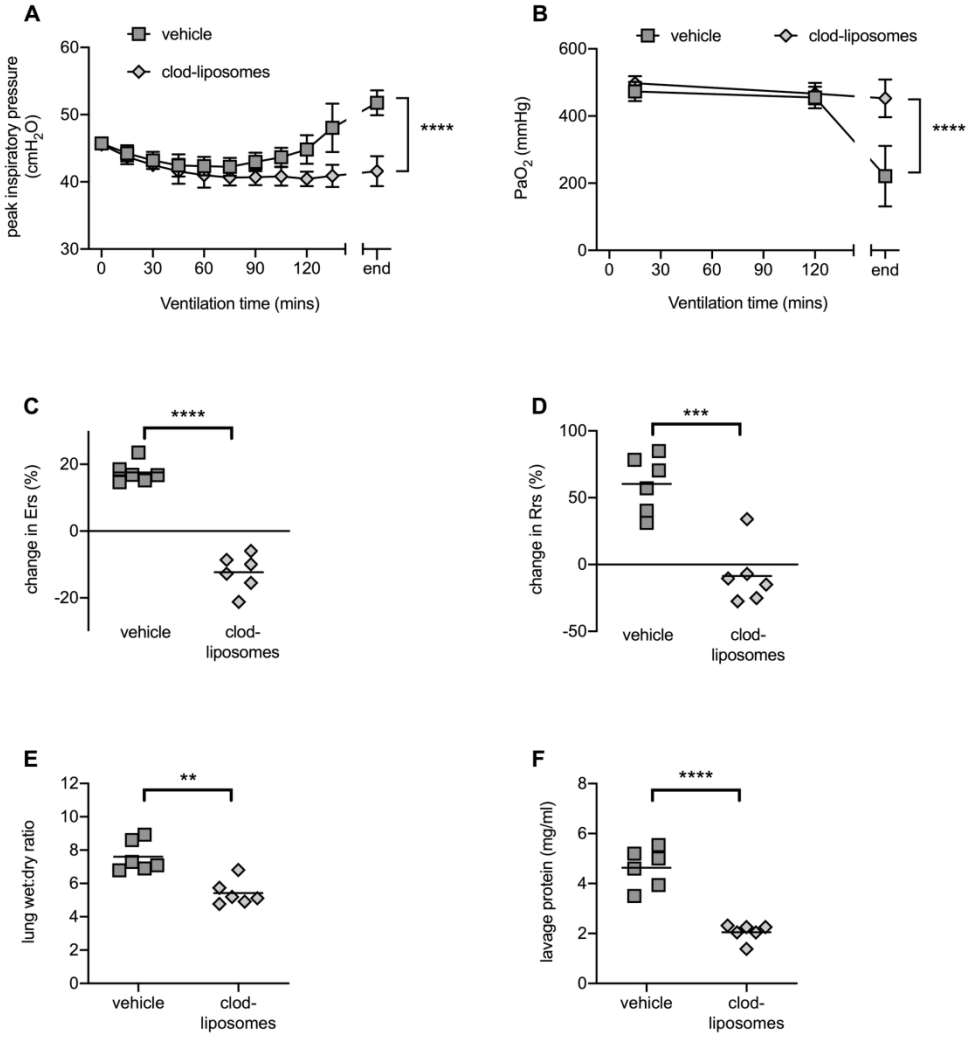
Figure E2



198x190mm (300 x 300 DPI)

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

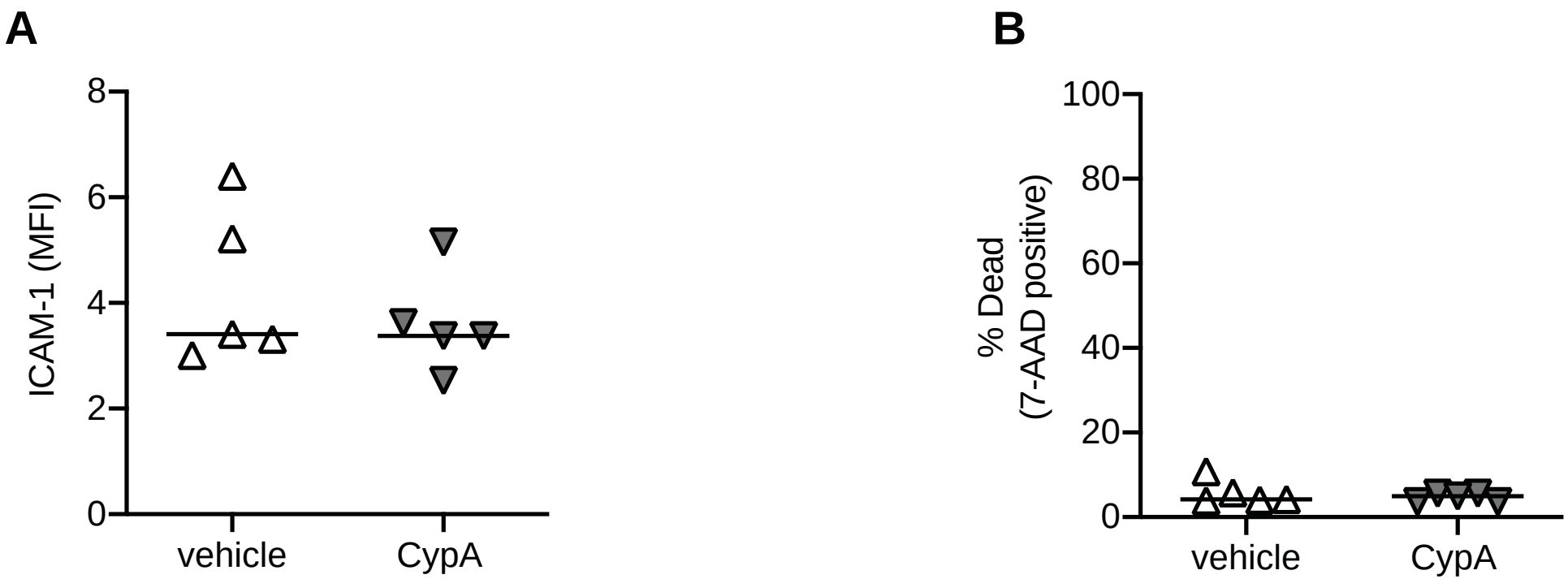
Figure E3



183x204mm (300 x 300 DPI)

AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC

Figure E4



AJRCCM Articles in Press. Published April 13, 2021 as 10.1164/rccm.202009-3545OC