Research Article

Innovative *In Vitro* Method to Study Ventilator Induced Lung Injury

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Abstract

Mechanical ventilation is a life-saving therapy for critically ill patients, alleviating the work of breathing and supporting adequate gas exchange. However, mechanical ventilation can cause ventilator induced lung injury (VILI) by baro/volu- and atelectrauma, lead to acute respiratory distress syndrome (ARDS), and substantially increase mortality. There is a need for specific biomarkers and novel research platforms for VILI/ARDS research to study these detrimental disorders and seek ways to avoid or prevent them. Previous *in vitro* studies on bronchial epithelium, cultured in air-liquid interface (ALI) conditions, have generally utilized static or constant pressure. We have developed a Cyclical Pressure ALI Device (CPAD) that enables cyclical stress on ALI cultured human bronchial cells with the aim of mimicking the effects of mechanical ventilation. Using CPAD we were able to analyze differentially expressed VILI/ARDS and innate immunity associated genes along with increased expression of associated proteins. CPAD provides an easy and accessible way to analyze functional and phenotypic changes that occur during VILI and may provide a platform for future drug testing.

1 Introduction

Mechanical ventilation is life-saving for patients suffering from respiratory failure. Unfortunately, mechanical ventilation can be injurious to lung tissues by mechanical stress through baro/volu- and atelectrauma and by oxidative stress through the high fraction of oxygen in inspired air, collectively called ventilator induced lung injury (VILI), which is a well-known risk of mechanical ventilation (Slutsky, 2005).

During normal inspiration, negative pressure is created inside the thorax through contraction of the diaphragm and intercostal muscles and air flows gently in. During mechanical ventilation the opposite happens: Air is pushed into the lungs with positive pressure that can have aberrant effects on the epithelium in the lungs due to mechanical distortion. The alveoli are heterogeneous in a diseased lung, some are open and others collapse at the end of expiration. During inspiration the open ones can be subjected to harm by overdistension termed volu/barotrauma, while the collapsed ones are vulnerable to shear stress through forced re-opening, termed atelectrauma. These injuries, along with oxidative stress, may lead to activation of resident immune cells in the distal lungs to secrete inflammatory mediators resulting in edema and fibrosis, collectively referred to as biotrauma (Beitler et al., 2016).

All mechanically ventilated patients are at risk for VILI, especially when it becomes challenging to ventilate the patient due to pathology in the lungs, as in acute respiratory distress syndrome (ARDS) (Carrasco Loza et al., 2015). VILI can cause, or augment substantially, damage of lung tissue in ARDS while being a necessary part of its treatment. The onset of ARDS is associated with the activation of the resident alveolar macrophages as a response to an insult or injury. The ensuing secretion of inflammatory mediators attracts neutrophils and monocytes from nearby capillaries, which secrete mediators harmful to endothelium and alveolar epithelium, causing increased permeability of the alveolar-capillary membrane, facilitating edema in the interstitium and air spaces. The following collapse of alveoli, increased dead space, worsening gas exchange and reduced lung compliance necessitate mechanical ventilation (Slutsky and Ranieri, 2013). ARDS is a common and lethal or disabling syndrome that 10% of

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all patients admitted to the intensive care unit (ICU) and 23% of all mechanically ventilated patients develop. The mortality of patients with severe ARDS is 46% and patients that survive are at high risk for cognitive decline, depression, post-traumatic stress disorder, and persistent skeletal muscle weakness (Thompson et al., 2017). It is therefore of high importance to explore methods to avoid or hamper the mechanisms of VILI.

It has been suggested that the lack of specific biomarkers for ARDS/VILI is one of the most important tasks facing researchers and clinicians in this field (Villar and Slutsky, 2017). However, studying VILI can be challenging as lung tissue is not readily available from VILI patients and obtaining a patient sample can be risky. As a result, there is still a limited connection between candidate genes and the susceptibility to VILI.

Most research on the pathogenesis of VILI has been performed in animal models (Hegeman et al., 2013). *In vitro* systems exploring VILI also have provided valuable information on the subject. These methods include applying static pressure on air-liquid interphase (ALI) cultured lung cells (Ressler et al., 2000; Shiomi et al., 2011) or cyclical stress on monolayered cells (Karadottir et al., 2015; Yu and Li, 2017; Zhao et al., 2014). There is also great potential in using lung-on-a-chip for VILI research, although this system has yet to see common use (Huh, 2015). Cellular adaptation to static pressure on ALI cultured cells is a concern and using cyclical stress on monolayered cells is limited to using less differentiated/polarized cells and fails to represent the more specialized cells of the distal bronchi. Using the best of these two systems would generate an *in vitro* system capable of cyclically stressing ALI cultured cells. Such a system could be a platform for finding genetic or biochemical biomarkers for ARDS/VILI. It could also be utilized for inducing VILI for the purpose of drug screening for drugs counteracting VILI and would thus have the potential of facilitating drug discovery and research.

For this purpose, we constructed a Cyclical Pressure ALI Device (CPAD), a novel *in vitro* pressure system capable of challenging ALI cultured lung cells with cyclical hyperbaric pressure. The cell lines used in this research are VA10 and BCi-NS1.1 (Halldorsson et al., 2007; Walters et al., 2013). Both cell lines project a basal cell phenotype, are derived from human lungs and have the potential of forming a pseudostratified-like epithelial layer when grown in ALI conditions. They are of bronchial origin but bronchial cells are, along with alveolar cells, subjected to mechanical strain in the distal and terminal bronchi during mechanical ventilation (Beitler et al., 2016). The A549 adenocarcinoma alveolar cell line has been used extensively in lung research, but it does not produce high TEER (i.e., does not produce tight junctions of high integrity) when grown in ALI conditions (Foster et al., 1998), which is an essential part of establishing the CPAD system.

In this study we used ARDS/VILI associated biomarkers to validate our novel pressure system. Additional markers were tested, including the mechanically sensitive CHI3L and innate immunity markers.

In patients, lung protective ventilation with lower tidal volumes (6 ml/kg), higher positive end-expiratory pressure (PEEP) and a peak inspiratory pressure below 30 cm H_2O are used to limit VILI. These settings are generally adjusted according to individual patients based on bodyweight, severity of injury, lung size and other relevant factors. Two peak inspiratory pressure levels were selected for this study, i.e., 22 and 27 cm H_2O, to reflect a possible gradual increase of VILI with increasing mechanical strain. The frequency was set at 0.27 Hz (16 bpm). For our experiments, positive end-expiratory pressure (PEEP) was set as 5 cm H_2O. These pressure values create driving pressures, i.e., the difference between PEEP and the peak pressure, that are above recommended driving pressure, i.e., 14 cm H_2O, which is associated with increased risk of death in ventilated patients (Amato et al., 2015).

There are to our best knowledge no other mechanical models in use applying cyclical pressure on ALI cultures; therefore the CPAD may provide a novel approach to study VILI.

2 Materials and methods

3D printed material

3D printed transwell holders were printed with polylactic acid (PLA) and fitted with FKM (Fleuroelastomer) Viton 75Sha O-rings. These were sterilized before each run of the CPA by bathing them in 96% ethanol for 1 h and left to dry in a laminar flow hood. PLA was chosen as this is a known biocompatible material for *in vitro* studies (Ramot et al., 2016).

Cell culture

The E6/E7 viral oncogene immortalized human bronchial epithelial cell line VA10 was cultured in Bronchial/Tracheal Epithelial cell growth medium (Cell Applications, 511A-500) supplemented with retinoic acid (Cell Applications, 511-RA) and penicillin-streptomycin (20 U/ml, 20 µg/ml, respectively, Life Technologies, 15140122) at 37°C and 5% CO_2 (Halldorsson et al., 2007). The other cell line used was BCi-NS1.1, a human airway bronchial cell line immortalized via hTERT expression (Walters et al., 2013). Both cell lines have basal like characteristics and are able to differentiate towards polarized epithelium, generating high transepithelial electrical resistance (TEER). Cells were seeded at passage 10-15. For passaging, cells were trypsinized and spun down in PBS with 10% FBS. PBS/FBS was aspirated and the cell pellet resuspended in medium. All cell cultures were screened bi-monthly for *Mycoplasma* contamination (all negative). Oxygen fraction used in the CPAD experiments was 21%, the same as in the atmosphere. Medium was constantly monitored for color changes, indicating pH level changes, with no coloration changes observed.

In order to establish an ALI culture, VA10 or BCi-NS1.1 cells were seeded onto 12 mm diameter transwell inserts (Corning, Costar) pre-coated with collagen IV from human placenta (Sigma, C7521). Approximately 2.5×10^5 cells were seeded onto each transwell insert. After 2 days, medium was replaced with Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (DMEM/F12, Thermo Fisher Scientific) supplemented with penicillin/streptomycin (20 U/ml and 20 µg/ml, respectively) and 10% fetal bovine serum (FBS), (ThermoFisher Scientific). The next day (3 days after seeding), medium was replaced

with DMEMF/F12 with added penicillin/streptomycin and 2% Ultroser G (PALL Corporation). ALI was established (day 0) by aspirating media from the upper chamber. Medium was changed every other day and TEER was measured. All ALI control samples were maintained at normal air pressure.

Reverse transcription – qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. One µg of RNA was reverse-transcribed to cDNA using 1 µl random primers, 1 µl dNTP mix (Life technologies) and up to 14 µl sterile dH_2O. Using a PCR machine (MJ Research, Peltier Thermal Cycler, PTC-225), the sample was heated to 65°C for 5 min, put on ice for 1 min, and 4 µl 5xSSIV buffer was added along with 1 µl 0.1 MDTT and 1 µl superscript IV (SSIV) (ThermoFisher Scientific). Samples were mixed gently and placed in the PCR machine for 10 min at 23°C, 10 min at 50°C and 10 min at 80°C. One µl of cDNA (50 ng/µl), PowerUp SYBR Green Master Mix (Applied Biosystems, A25742) and 0.5 µM primers listed in Table S1[1] were used for qRT-PCR. qRT-PCR was performed using the LG 7500 Real Time PCR System (Applied Biosystems) with the following cycling conditions: (1) holding stage: 95°C for 10 min, followed by 40 cycles of (2) denatured stage: 95°C for 15 s and (3) annealed/extended stage: 60°C for 1 min. The $2(^{-\Delta\Delta CT})$ Livak method was utilized to calculate fold differences over untreated control (Livak and Schmittgen, 2001).

Live cell imaging – proliferation assay

Cells were incubated in the Incucyte (Incucyte ZOOM, Essen BioScience) for live cell imaging and cell proliferation assay.

Transmission electron microscopy

VA10 or BCi-NS1.1 cells were grown as monolayers on coverslips and under ALI conditions, and prepared for electron microscopy. Medium was removed and cells were washed with PBS. Cells were fixed with 2.5% glutaraldehyde (Ted Pella, Inc.) for 20 min. For ALI transwell filters, glutaraldehyde was as added on top of the cells and also to the bottom of the well. Fixative was removed and cells washed in phosphate buffer (0.075 M with 0.15 M sucrose) twice for 2 min. Fixed coverslips/filters were postfixed in 2% osmium tetroxide (J. B. EM Services, Inc.) for 30 min, followed by a phosphate buffer rinse twice for 3 min. Cells were dehydrated in series with ethanol: 25% ethanol for 2 min, 50% ethanol for 2 min and 70% ethanol for 2 min. Alcohol was replaced with 4% uranyl acetate in 70% ethanol (J. B. EM Services, Inc.) for 7 min, followed by 80% ethanol for 2 min, 90% ethanol for 2 min and 96% ethanol for 2 min and again for 5 min and twice for 7 min. For transwell filters, glass coverslips were placed on a plastic petri dish with a drop of resin (Spurr Resin – Ted Pella, Inc.) on top of them. Coverslips were kept in the plastic petri dish. After the last change of ethanol, the filter was removed from the well and placed on the drop of resin on the coverslip. A few drops of resin were poured on top of cells and left for 30 min. For monolayers of cells, ethanol was removed and a drop of resin was put on the coverslip and left for 30 min. A gelatin capsule was filled with resin and placed upside down on top of the coverslip to create a block. Coverslip/filters were then incubated at 70°C overnight. When capsules were separated from coverslips, ultra-thin (100 nm) sections were cut on an Ultramicrotome (Leica EM UC7) and placed on copper grids (Ted Pella, Inc.). Sections on grids were stained for 10 min with lead citrate (3% J. T. Baker Chemical Co.) and imaged using a JEM-1400PLUS PL Transmission Electron Microscope at different magnifications.

Confocal imaging

Immunofluorescence was captured and visualized using an Olympus FV1200 confocal microscope (Olympus, Tokyo, Japan). Cells were fixed in formalin for 20 min before staining. Antibodies used were host Ac-tubulin (Abcam) and EGFR (Abcam). Alexa Fluor phalloidin (ThermoFisher Scientific) was used to stain for filamentous actin. Counterstaining was done using DAPI (Sigma-Aldrich).

Immunoblotting

Cells were washed with PBS and lysed in RIPA lysis buffer supplemented with Halt Protease and Phosphatase Inhibitor Cocktails (ThermoFisher Scientific) on ice for 30 min. 15-20 µg of total protein was separated using NuPAGE 4-12% Bis-Tris gradient gel (Life Technologies, NP0323) and NuPAGE NuPAGE MES SDS Running Buffer (Life Technologies, NP0002), and the running conditions were 120 V and 275 mA. The proteins were transferred on to a PVDF membrane (0.2 µm pores) using XCell II™ Blot Module (Invirogen, EI9051) and the membrane was blocked with 10% skimmed milk or 5% BSA (Sigma) in TBS-T buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 1 h at RT. Then, the membrane was incubated with primary antibodies: mouse anti-chitinase-3-like protein 1 (YKL-40) (Millipore, MABC196); goat anti-lipocalin 2/NGAL antibody (R&D Systems, AF1757); rabbit anti-surfactant protein B (Abcam, ab40876); rabbit anti-GAPDH (Cell Signaling Technology, 2118) and rabbit anti-β-tubulin (Cell Signaling Technology, 2128) overnight at 4°C using a dilution recommended in the manufacturer's protocol. After washing with TBS-T buffer the membrane was incubated with horse radish peroxidase (HRP) conjugated secondary antibodies (Sigma, A5420 and A0545; Millipore, AP181P) diluted 1:10,000 in 5% skimmed milk or 5% BSA in TBS-T for at least 1.5 h at RT. Immunoblots were developed using Pierce ECL Plus Western blotting substrate (Thermo Scientific, #34095) or Western blotting Luminol reagent (Santa Cruz, sc-2048) and ImageQuant LAS 4000 system (GE Healthcare).

ELISA

Cell culture conditioned medium was analyzed by sandwich enzyme-linked immunosorbent assays (ELISAs) utilizing a human beta defensin-1 (hBD-1), interleukin 8 (*IL-8*), and *TNFα* assay kit according to the manufacturer's instructions (Peprotech, UK).

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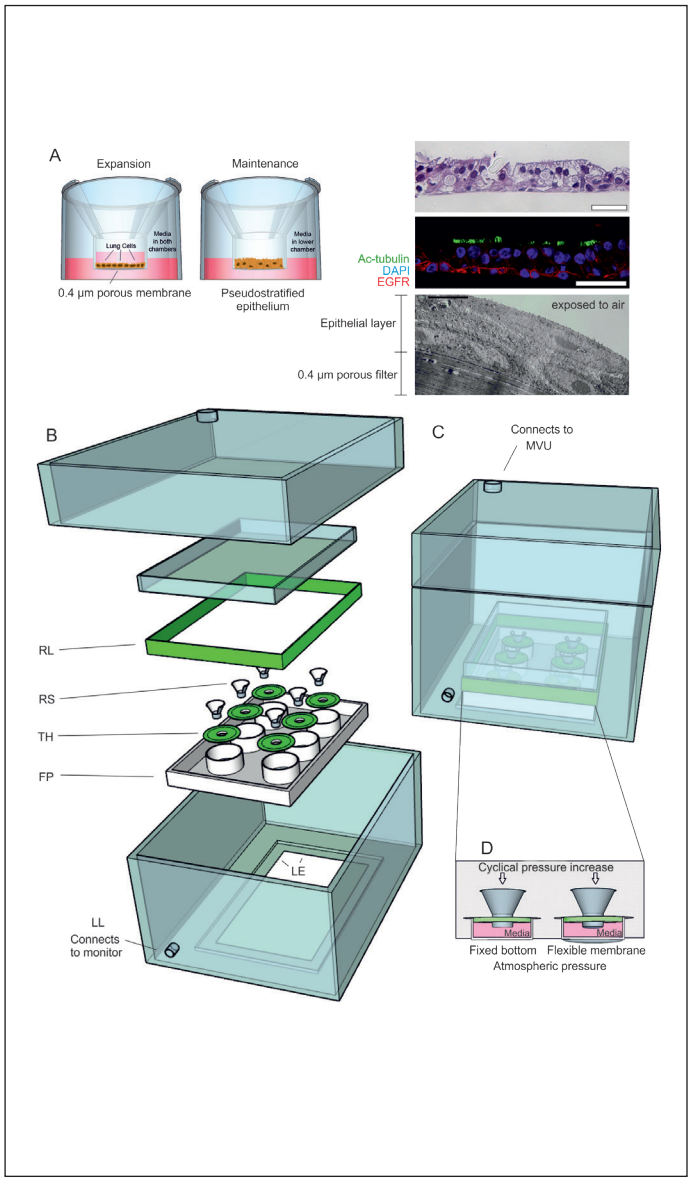

Fig. 1: Construction of ventilator-attached cyclical pressure ALI device (CPAD)
A, Bronchial epithelial cell lines are seeded onto transwell filters. For 2 days they are expanded with medium in the top and bottom chambers. Then the medium is removed from the top chamber and cells are subjected to ALI culture conditions for 21 days with medium being changed every other day. From top to bottom, hematoxylin and eosin staining, scale bar is 50 μm, confocal (Ac-Tubulin and EGFR, counterstained with DAPI), scale bar is 50 μm and electron microscope imaging of cross sectional 21-day ALI cultures (VA10) show the pseudostratified cell layer on top of the 0.4 μm porous filter. Electron microscope image scale bar is 5.0 μm. B, CPAD consists of a lid, which connects to a mechanical ventilation unit (MVU). The bottom part of the pressure chamber has a luer lock outlet (LL), which connects to a monitor where pressure increases can be monitored as well as cycles/sec. The bottom part of the CPAD has an opening with a lowered edge (LE), where a cell culture plate snugly fits. The plate used is a Flexcell plate (FP) with 6 wells and a flexible bottom in each well. The transwell holder is a specifically 3D printed unit that fits inside the well of the Flexcell plate. Transwell filters with differentiated lung cells are moved, under sterile conditions, into a 3D printed transwell holder (TH). The well is filled with appropriate medium. The transwell holder with inserted transwell filter is placed in a well on the Flexcell plates. As transwell filters have raised shoulders (RS), the lid of the Flexcell plate needs to be raised (RL). This is done with a 3D printed scaffold, which is inserted between plate and lid. C, The assembled CPAD is placed into an incubator and connected to a mechanical ventilator unit (MVU) and monitor (LL). D, Flexcell plates have flexible bottoms, therefore the increase in pressure inside the CPAD results in displacement of transwell filters towards the media, which in turn results in displacement of the medium and a slight displacement of the flexible membrane underneath the Flexcell plate towards atmospheric pressure (right). If the cells were placed in plates with a fixed bottom, the cells would be exposed to hydrostatic pressure change and due to the incompressibility of the cells and the medium, less physical deformation would take place (left).

Statistical analysis
As pressure values (22 and 27 cm H_2O) were compared to the same control, Student's t-test was used for statistical analysis. GraphPad Prism 7.04 was used to calculate significance. Values are given as mean ±SD.

3 Results

3.1 Construction of Cyclical Pressure ALI Device (CPAD)

To simulate cyclical stress seen *in vivo* during mechanical ventilation and to address the lack of a cyclical stress inducing system of ALI cultures, we designed an airtight chamber for culture plates that connects to a mechanical ventilator. This enables positive pressure cyclical stress of ALI cultured cells.

A two-piece chamber was designed. The bottom part has an opening at its base and an insulated groove to fit a Flexcell culture plate. The top part consists of a lid with a connector to a mechanical ventilator. Locking mechanisms on the sides of the chamber and insulation between the bottom part of the chamber and the lid ensures air-tight conditions in the chamber once the cell culture plate is inserted (Fig. 1A,B). BioFlex 6-well culture plates with flexible, silicone membranes in the bottom (Dunn Labortechnik GmbH) were used. Transwell insert holders were 3D printed to fit into the wells of the BioFlex plates. They were lined with O-rings on the outside to tightly fit into the wells and on the inside to ensure full sealing of inserts (Fig. 1B).

Cells were cultured on transwell inserts (Corning, Costar) in ALI culture conditions for 21 days to allow differentiation of the airway epithelium (Fig. 1A). Transwell ALI culture inserts were

subsequently placed in the 3D printed transwell holders and inserted into BioFlex plates containing medium. A printed 3D scaffold to raise the BioFlex plate lid was placed on the plate, which was necessary because of the height of the transwell inserts in the holders. The BioFlex plate was then placed into the groove in the base of the CPAD (Fig. 1B). The CPAD was sealed off, connected to a mechanical ventilator (Elisée™ 150, ResMed) and placed in a standard cell incubator (Fig. 1C). Internal chamber pressure was monitored with a separate sensor and monitor.

The cells were mechanically influenced by the pressure change resulting from the pressure difference between the inside of the chamber and atmospheric pressure. The flexible membranes under the wells extend downwards toward atmospheric pressure once pressure is increased inside the chamber during inspiration. The medium on top of the flexible membrane is pushed towards atmospheric pressure and so is the transwell filter (Fig. 1D, right panel). This creates increased stress on the epithelium, whereas if there was no flexible membrane (Fig. 1D, left panel), the cells would mostly experience a change in hydrostatic pressure due to the incompressibility of the cells and the medium; we hypothesized that in that case less physical deformation would take place. In our model the cells were therefore simultaneously subjected to apical pressure from above and lateral stretch on the semipermeable transwell filter.

The transwell holders were 3D printed using polylactic acid (PLA) and fitted with FKM viton O-rings in order to seal better when the transwell holder, with the fitted transwell filter, is inserted into the Flexcell plate well. Even though PLA is the most commonly used biodegradable polymer in clinical use (Da Silva et al., 2018), we performed proliferation studies to test the biocompatibility of the 3D printed components. Cells were incubated in the presence of sterilized PLA and sterilized FKM Viton O-ring, separately and combined (Fig. S1A[1]). No difference in proliferation was observed and all samples reached full confluency (Fig. S1B[1]).

3.2 Phenotypic alterations of airway epithelium *in vitro* subsequent to cyclical pressure

The airway epithelial cells were differentiated in ALI culture for 21 days and subsequently challenged with cyclical hyperbaric stress. Figure 2A shows a representative graph from 4 different ALI cultures with VA10 cell lines; these values are comparable to published TEER values for differentiated VA10 cell line at day 21 (Halldorsson et al., 2007). When TEER values were measured before and after cyclical hyperbaric stress, no consistent trend was observed, as TEER would increase or decrease after stressing the cells.

We observed phenotypic alterations as a result of cyclical strain as shown by confocal and electron microscope (EM) imaging (Fig. 2B,C). Actin stress fiber expression was increased and aggregates were observed indicating increased tensional stress when cultures were subjected to 27 cm H_2O cyclical pressure for 24 h (Fig. 2B). Cross sectional EM images show the difference between cultures subjected to 27 cm H_2O pressure for 24 h and controls, where stressed cell layers show a disrupted phenotype when compared to the more uniform control cell lay-

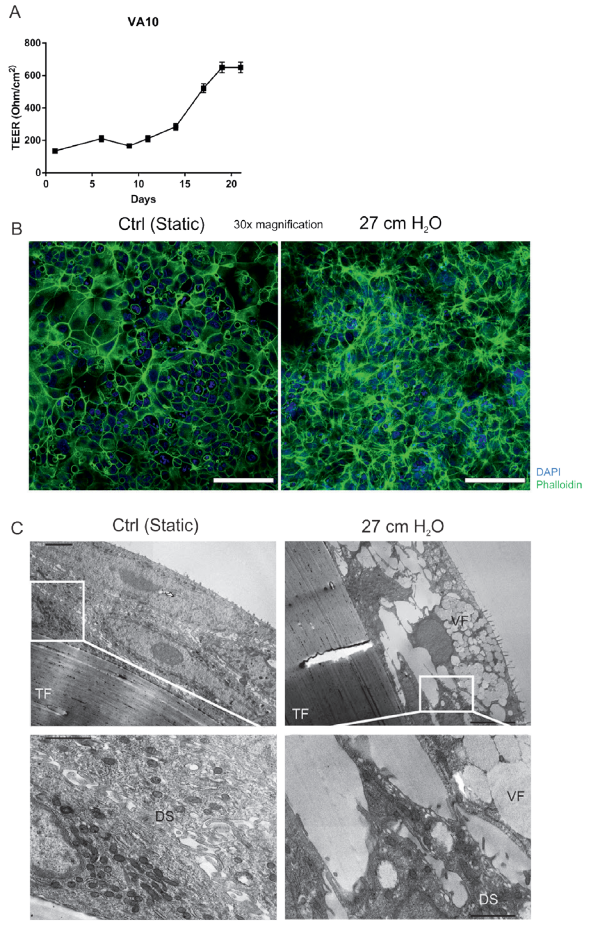

Fig. 2: Phenotypic alterations of *in vitro* airway epithelium subsequent to cyclical pressure

A, TEER is measured regularly during 3 weeks of differentiation. The graph represents results from four different 3-week ALI cultures (N = 4). B, Confocal microscope analyses using phalloidin staining of actin, counterstained with DAPI. Control cell layer (left, no pressure) and cells after 24 h cyclical stress with 27 cm H_2O pressure (right). Images shown are representative of 3 different experiments (N = 3). Scale bar is 100 μm. C, Electron microscope analyses. The control cell layer presents differentiated lung epithelial cells with visible desmosomes and tight junctions (left). Image on right shows cell layer subsequent to 24 h cyclical stress with 27 cm H_2O pressure. Images shown are representative of 3 different experiments (N = 3). Shown are desmosomes (DS), vesicle formations (VF) and transwell filter membrane (TF). The top left image scale bar is 2 μm, top right scale bar is 5 μm and lower image scale bars are 1 μm.

er (Fig. 2C). These phenotypic changes were also observed in BCi-NS1.1 derived airway epithelium (Fig. S2[1]).

In summary, mechanical stress generated by our pressure system affects cell morphology.

3.3 Cyclical pressure applied to airway epithelium *in vitro* induces differential mRNA expression of VILI/ARDS and innate immunity associated markers

Although ARDS or VILI do not have a definite clinically suitable biomarker that predicts their onset and clinical outcome, there are a number of biomarkers that are associated with ARDS/VILI (Blondonnet et al., 2016; Meyer and Christie, 2013). Table S2[1] summarizes biomarkers selected for this research. Using the CPAD to cyclically increase and decrease pressure over ALI cultured lung cells (VA10) for 24 hours using our aforementioned pressure values of 22 cm H_2O and 27 cm H_2O, respectively, showed that expression of ARDS associated biomarkers was affected. Vascular endothelial growth factor A (*VEGFA*) and hypoxia-inducible factor 1-alpha (*HIF1A*) were increased in a pressure-dependent manner (Fig. 3A), However, only *VEGFA* showed a significant increase. Receptor for advanced glycation endproducts (*RAGE*) was significantly suppressed and surfactant protein B (*SFPB*) showed a significant upregulation at the 22 cm H_2O pressure range and lower upregulation trend at 27 cm H_2O (Fig. 3A).

Because the innate immunity components are closely associated with ARDS/VILI, we selected markers of innate immunity that might have implications in ARDS/VILI, i.e., β-defensin 2 (*HBD2*), cathelicidin antimicrobial peptide LL-37 (*CAMP*), interleukin 10 (*IL-10*), and tumor necrosis factor alpha (*TNFα*) (Fig. 3B). With the exception of *IL-10*, an induced expression was apparent for all of these markers upon cyclical stress of ALI cultured cells (Fig. 3B). Similar results were observed when using the BCi-NS1.1 cell line (Fig. S3[1]).

Our findings provide evidence that mechanical stress generated by our pressure system affects the gene expression profile of ARDS/VILI associated biomarkers and innate immunity genes.

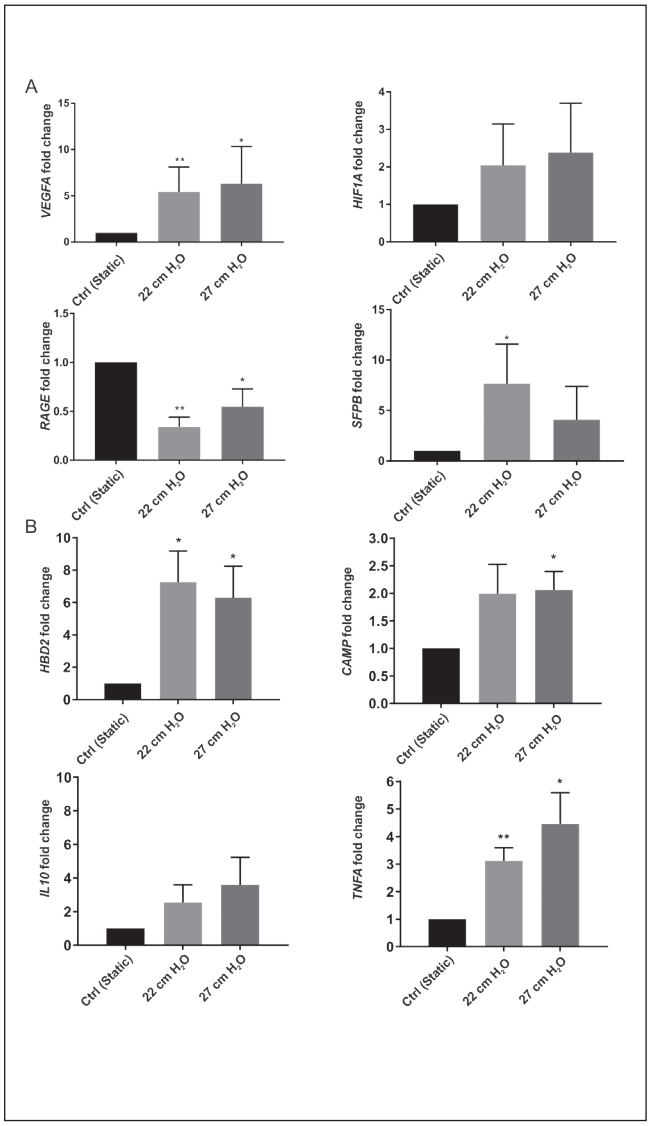

Fig. 3: Cyclical pressure applied to *in vitro* airway epithelium shows differential expression of VILI/ARDS and innate immunity associated markers

A, Expression of ARDS/VILI associated biomarkers *VEGFA*, *HIF1A*, *RAGE*, and *SFPB* by qRT-PCR, N = 5, P ≤ 0.05 = * and P ≤ 0.01 = **. B, Expression of innate immunity genes *HBD2*, *CAMP*, *IL10*, and *TNFα* analyzed in the novel pressure system. N = 5, P ≤ 0.05 = * and P ≤ 0.01 = **.

3.4 Cyclical pressure applied to ALI cultures affects expression of mechanically sensitive biomarker YKL-40 (*CHI3L*) and innate immunity markers IL-8 (*IL8*) and NGAL (*LCN2*)

Chitinase-like protein YKL-40, encoded by the *CHI3L* gene, has been shown to be upregulated in ALI cultured bronchial epithelial cells when they are mechanically stressed by 30 cm H_2O static compressive stress (Park et al., 2010). Therefore, we analyzed the effect on *CHI3L* gene expression and the YKL-40 protein in our system. Cyclically stressing the cells (VA10) for 24 h at 22 and 27 cm H_2O, respectively, resulted in over 2-fold upregulation of *CHI3L* expression (Fig. 4A). YKL-40 expression was significantly increased (Fig. 4B; Fig. S4[1]).

Interleukin 8 (*IL-8*) is an innate immunity associated biomarker and when we tested the gene expression of *IL-8*, we saw a significant upregulation at both pressure values (Fig. 4C). However, changes at the protein level of IL-8 measured by ELISA were not significant (Fig. 4D). We also analyzed expression level of neutrophil gelatinase-associated lipocalin (*NGAL*) as it is a suggested biomarker for VILI (Xiao and Chen, 2017). We did not detect a significant alteration of NGAL (*LCN2*) expression in our model for VA10 and BCi-NS1.1 cell lines (Fig. 4E; Fig. S3[1]) although there was a tendency towards increased protein expression for VA10 cells (Fig. 4F; Fig. S5A[1]), but not for BCi-NS1.1 (Fig. S5B[1]).

In summary, using the novel pressure system on bronchial epithelial cell layers affects expression of candidate biomarkers.

4 Discussion

ALI culture is an *in vitro* approach used by many researchers as a way to induce and capture *in vivo*-like differentiation of airway epithelial cells towards a pseudostratified/polarized epithelial layer. In order to address responses to cyclical pressure in mature bronchial epithelium, a novel pressure chamber (CPAD) was designed. We have previously utilized the Flexcell 5000 tension system (Karadottir et al., 2015) to study cyclic stretch on monolayer (undifferentiated) cells. However, it was not possible to apply that system to study cyclical hyperbaric stress on ALI cultured cells. Ressler and coworkers (2000) used static stress to address tension caused by pressure in ALI cultured cells. The CPAD was developed to combine the two approaches. While using established components like transwell inserts and Flexcell 5000 tension system culture plates, we also designed and 3D printed components as complements (Fig. 1). Transwell filter membranes were observed to displace downward towards the media in a pressure-dependent manner. Transwell membranes were frequently seen to detach from transwell inserts when using pressure values over 30 cm H_2O.

Through a series of experiments we validated the CPAD as a novel platform for VILI research. VA10 and BCi-NS1.1 cell lines used in this research form polarized, differentiated epithelial layers in ALI culture (Halldorsson et al., 2007, 2010; Karadottir et al., 2015; Walters et al., 2013). We showed that the unaffected airway epithelium presented organized actin structures, (Fig. 2B, left panel), but when challenged by cyclical stress, the actin fibers showed a marked increase, disassociation and bundling, without apparent loss of cells as seen when the cell layer was counterstained with DAPI (Fig. 2B, right panel). VILI is characterized by epithelial barrier disruption (Carrasco Loza et al., 2015). By electron microscopy we confirmed that the *in vitro* airway epithelium exposed to 27 cm H_2O cyclical stress shows a disrupted phenotype as compared to the static control (Fig. 2C). The affected cell layer shows a stressed phenotype with a disrupted barrier. Cells appear to separate without completely disassociating the epithelial layer. Along with this disruption, vesicle formation was observed in the stressed cell layers. This is an apparent stress response which could be linked to selective differentiation of the basal cells in the cell layer. Park and colleagues (2012) recently reported that compressive stress of bronchial cells induces tissue factor-bearing exosomes, which could be a possible explanation for the vesicle formation in our pressure system.

As a proof of principle, we wanted to test if known ARDS/VILI associated biomarkers (Blondonnet et al., 2016; Meyer and Christie, 2013) are modulated in the CPAD. As a reference gene, we used GAPDH, which is considered a relatively unaffected gene in cyclical mechanical stress (Pinhu et al., 2008). We compared B2M, UBC, and GAPDH as control genes and found GAPDH to be the most stable. Although there were consistently slightly higher CT values (difference of approx. 1.5) of GAPDH in the pressurized samples, this could not explain the differences observed in our qPCRs. This was confirmed in the YKL-40 Western blots, where β-tubulin was used as a reference protein and there was a clear increase in protein expression which was

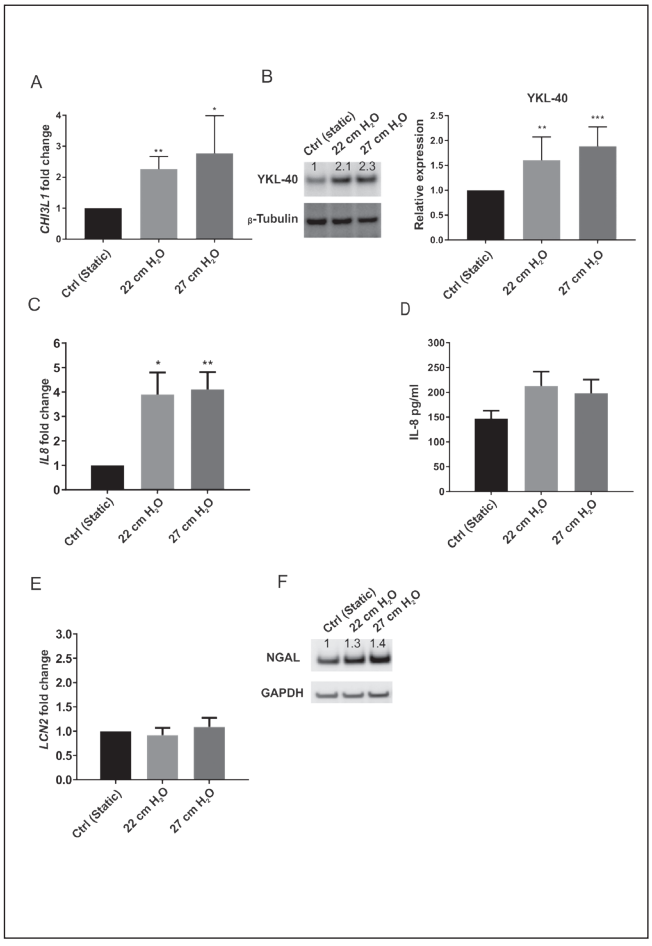

Fig. 4: Cyclical pressure applied to ALI cultures affects expression of mechanically sensitive biomarker YKL-40 (*CHI3L*) and innate immunity markers IL-8 (*IL8*) and NGAL (*LCN2*)

A, Expression of CHI3L analyzed by qRT-PCR at 22 cm and 27 cm H_2O pressure values. N = 5, P ≤ 0.05 = * and P ≤ 0.01 = **. B, Western blot of YKL-40 at 22 cm and 27 cm H_2O pressure values and quantification. N = 5, P ≤ 0.05 = *, P ≤ 0.01 = ** P ≤ 0.001 = ***. C, Expression of IL-8 at 22 cm and 27 cm H_2O pressure values. N = 5, P ≤ 0.05 = * and P ≤ 0.01 = **. D, IL-8 ELISA at 22 cm and 27 cm H_2O pressure values. E, Expression of LCN2 at 22 cm and 27 cm H_2O pressure values. F, Western blot of NGAL at 22 cm and 27 cm H_2O pressure values.

observed to be approximately the same as the gene level increase determined where GAPDH was used.

We selected *VEGFA*, *HIF1A*, *RAGE*, and *SFPB*. *VEGFA* and *HIF1A* responded in a stepwise pressure-dependent manner; the *SFPB* response was most prominent at the 22 cm H_2O pressure value. This suggests that a careful selection of the pressure value is of importance and might indicate different lung responses to an individual degree of pressure insult. Furthermore, *RAGE* was significantly downregulated. *RAGE* is an ARDS associated biomarker (Jabaudon et al., 2018) and downregulation of

RAGE has been associated with pulmonary fibrosis (Queisser et al., 2008).

To see if selected innate immunity associated genes are affected by the cyclical stress of the CPAD, we selected initially *HBD2* and *CAMP*, both established innate immunity markers (Cederlund et al., 2011). A clear upregulation was seen in *HBD2* (Fig. 3B), an NF-κB regulated antimicrobial peptide. The cathelicidin (*CAMP*) response was not as pronounced but still significant when 27 cm H_2O was applied, while the same trend was observed for the 22 cm H_2O pressure value (Fig. 3B). *IL-10* and *TNFα* were also analyzed but only *TNFα* showed a significant response in a pressure-dependent manner (Fig. 3B).

Next we evaluated if YKL-40 (*CHI3L1*), a known mechanical stress biomarker (Park et al., 2010), is affected by the CPA unit. We observed a significant response (Fig. 4A), which was confirmed on protein level where cyclical stress was shown to significantly increase expression of YKL-40 (Fig. 4B). Innate immunity marker *IL-8* showed a clear upregulation in stressed cell layers on the gene expression level, however on the protein level, there was no significant difference (Fig. 4C, D). NGAL (*LCN2*) expression was not significantly altered, possibly due to pressure value selection (Fig. 4 E, F). Western blot analysis with SFPB showed no clear protein expression trend (Fig. S6[1]).

Pro-inflammatory chemokines/cytokines IL-8, TNFα and also human β-defensin 2 antimicrobial peptide are components regulated by the NF-κB pathway (Schutte and McCray, 2002; Liu et al., 2017). NF-κB induction by pressure has been shown (Lemarie et al., 2003). Interestingly, the observed expression increases in *IL-8*, *HBD2*, and *TNFα* in a pressure-dependent manner could indicate activation of the NF-κB pathway in our model.

An interesting next step for this system would be the co-culturing of macrophages and/or fibroblasts and bronchial/alveolar cell lines before introducing them to the CPAD. Kletting et al. (2018) showed that co-culturing macrophages and a newly developed alveolar type I cell line is a viable option. Another interesting venue would be to mimic normal breathing in the control cells, that is to introduce low cyclical pressure increases on the control cells.

We have constructed a pressure chamber that can cyclically stress ALI cultured cells and induce a VILI-like phenotype while expression of ARDS/VILI and other innate immunity and mechanically sensitive genes and proteins are significantly affected. This novel *in vitro* system can be a powerful tool in ARDS/VILI research and as a potential drug discovery platform. The fact that *in vitro* lung models are gaining popularity among researchers that are increasingly avoiding animal testing, and the difficulty in gaining human samples for lung research make this pressure system a very relevant tool for use in lung and drug testing research.

References

Amato, M. B., Meade, M. O., Slutsky, A. S. et al. (2015). Driving pressure and survival in the acute respiratory distress syndrome. *N Engl J Med 372*, 747-755. doi:10.1056/NEJMsa1410639

Beitler, J. R., Malhotra, A. and Thompson, B. T. (2016). Ventilator-induced lung injury. *Clin Chest Med 37*, 633-646. doi:10.1016/j.ccm.2016.07.004

Blondonnet, R., Constantin, J. M., Sapin, V. et al. (2016). A pathophysiologic approach to biomarkers in acute respiratory distress syndrome. *Dis Markers 2016*, 3501373. doi:10.1155/2016/3501373

Carrasco Loza, R., Villamizar Rodriguez, G. and Medel Fernandez, N. (2015). Ventilator-induced lung injury (VILI) in acute respiratory distress syndrome (ARDS): Volutrauma and molecular effects. *Open Respir Med J 9*, 112-119. doi:10.2174/1874306401509010112

Cederlund, A., Gudmundsson, G. H. and Agerberth, B. (2011). Antimicrobial peptides important in innate immunity. *FEBS J 278*, 3942-3951. doi:10.1111/j.1742-4658.2011.08302.x

Da Silva, D., Kaduri, M., Poley, M. et al. (2018). Biocompatibility, biodegradation and excretion of polylactic acid (PLA) in medical implants and theranostic systems. *Chem Eng J 340*, 9-14. doi:10.1016/j.cej.2018.01.010

Foster, K. A., Oster, C. G., Mayer, M. M. et al. (1998). Charactes rization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism. *Exp Cell Res 243*, 359-366. doi:10.1006/excr.1998.4172

Halldorsson, S., Asgrimsson, V., Axelsson, I. et al. (2007). Differentiation potential of a basal epithelial cell line established from human bronchial explant. *In Vitro Cell Dev Biol Anim 43*, 283-289. doi:10.1007/s11626-007-9050-4

Halldorsson, S., Gudjonsson, T., Gottfredsson, M. et al. (2010). Azithromycin maintains airway epithelial integrity during Pseudomonas aeruginosa infection. *Am J Respir Cell Mol Biol 42*, 62-68. doi:10.1165/rcmb.2008-0357OC

Hegeman, M. A., Hemmes, S. N., Kuipers, M. T. et al. (2013). The extent of ventilator-induced lung injury in mice partly depends on duration of mechanical ventilation. *Crit Care Res Pract 2013*, 435236. doi:10.1155/2013/435236

Huh, D. D. (2015). A human breathing lung-on-a-chip. *Ann Am Thorac Soc 12, Suppl 1*, S42-44. doi:10.1513/AnnalsATS.201410-442MG

Jabaudon, M., Berthelin, P., Pranal, T. et al. (2018). Receptor for advanced glycation end-products and ARDS prediction: A multicentre observational study. *Sci Rep 8*, 2603. doi:10.1038/s41598-018-20994-x

Karadottir, H., Kulkarni, N. N., Gudjonsson, T. et al. (2015). Cyclic mechanical stretch down-regulates cathelicidin antimicrobial peptide expression and activates a pro-inflammatory response in human bronchial epithelial cells. *PeerJ 3*, e1483. doi:10.7717/peerj.1483

Kletting, S., Barthold, S., Repnik, U. et al. (2018). Co-culture of human alveolar epithelial (hAELVI) and macrophage (THP-1) cell lines. *ALTEX 35*, 211-222. doi:10.14573/altex.1607191

Lemarie, C. A., Esposito, B., Tedgui, A. and Lehoux, S. (2003). Pressure-induced vascular activation of nuclear factor-κB: Role in cell survival. *Circ Res 93*, 207-212. doi:10.1161/01.RES.0000086942.13523.88

Liu, T., Zhang, L., Joo, D. and Sun, S. C. (2017). NF-κB sig-

naling in inflammation. *Signal Transduct Target Ther 2*, doi:10.1038/sigtrans.2017.23

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods 25*, 402-408. doi:10.1006/meth.2001.1262

Meyer, N. J. and Christie, J. D. (2013). Genetic heterogeneity and risk of acute respiratory distress syndrome. *Semin Respir Crit Care Med 34*, 459-474. doi:10.1055/s-0033-1351121

Park, J. A., Drazen, J. M. and Tschumperlin, D. J. (2010). The chitinase-like protein YKL-40 is secreted by airway epithelial cells at base line and in response to compressive mechanical stress. *J Biol Chem 285*, 29817-29825. doi:10.1074/jbc.M110.103416

Park, J. A., Sharif, A. S., Tschumperlin, D. J. et al. (2012). Tissue factor-bearing exosome secretion from human mechanically stimulated bronchial epithelial cells in vitro and in vivo. *J Allergy Clin Immunol 130*, 1375-1383. doi:10.1016/j.jaci.2012.05.031

Pinhu, L., Park, J. E., Yao, W. and Griffiths, M. J. (2008). Reference gene selection for real-time polymerase chain reaction in human lung cells subjected to cyclic mechanical strain. *Respirology 13*, 990-999. doi:10.1111/j.1440-1843.2008.01396.x

Queisser, M. A., Kouri, F. M., Konigshoff, M. et al. (2008). Loss of RAGE in pulmonary fibrosis: Molecular relations to functional changes in pulmonary cell types. *Am J Respir Cell Mol Biol 39*, 337-345. doi:10.1165/rcmb.2007-0244OC

Ramot, Y., Haim-Zada, M., Domb, A. J. and Nyska, A. (2016). Biocompatibility and safety of PLA and its copolymers. *Adv Drug Deliv Rev 107*, 153-162. doi:10.1016/j.addr.2016.03.012

Ressler, B., Lee, R. T., Randell, S. H. et al. (2000). Molecular responses of rat tracheal epithelial cells to transmembrane pressure. *Am J Physiol Lung Cell Mol Physiol 278*, L1264-1272. doi:10.1152/ajplung.2000.278.6.L1264

Schutte, B. C. and McCray, P. B., Jr. (2002). β-defensins in lung host defense. *Annu Rev Physiol 64*, 709-748. doi:10.1146/annurev.physiol.64.081501.134340

Shiomi, T., Tschumperlin, D. J., Park, J. A. et al. (2011). TNF-α-converting enzyme/a disintegrin and metalloprotease-17 mediates mechanotransduction in murine tracheal epithelial cells. *Am J Respir Cell Mol Biol 45*, 376-385. doi:10.1165/rcmb.2010-0234OC

Slutsky, A. S. (2005). Ventilator-induced lung injury: From barotrauma to biotrauma. *Respir Care 50*, 646-659.

Slutsky, A. S. and Ranieri, V. M. (2013). Ventilator-induced lung injury. *N Engl J Med 369*, 2126-2136. doi:10.1056/NEJMra1208707

Thompson, B. T., Chambers, R. C. and Liu, K. D. (2017). Acute respiratory distress syndrome. *N Engl J Med 377*, 562-572. doi:10.1056/NEJMra1608077

Villar, J. and Slutsky, A. S. (2017). GOLDEN anniversary of the acute respiratory distress syndrome: Still much work to do! *Curr Opin Crit Care 23*, 4-9. doi:10.1097/MCC.0000000000000378

Walters, M. S., Gomi, K., Ashbridge, B. et al. (2013). Generation of a human airway epithelium derived basal cell line with multipotent differentiation capacity. *Respir Res 14*, 135. doi:10.1186/1465-9921-14-135

Xiao, R. and Chen, R. (2017). Neutrophil gelatinaseassociated lipocalin as a potential novel biomarker for ventilatorassociated lung injury. *Mol Med Rep 15*, 3535-3540. doi:10.3892/mmr.2017.6442

Yu, Q. and Li, M. (2017). Effects of transient receptor potential canonical 1 (TRPC1) on the mechanical stretch-induced expression of airway remodeling-associated factors in human bronchial epithelioid cells. *J Biomech 51*, 89-96. doi:10.1016/j.jbiomech.2016.12.002

Zhao, T., Liu, M., Gu, C. et al. (2014). Activation of c-Src tyrosine kinase mediated the degradation of occludin in ventilator-induced lung injury. *Respir Res 15*, 158. doi:10.1186/s12931-014-0158-2

Conflict of interest

The authors declare no conflict of interest regarding the publication of this article.

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