Inhaled TRIM72 Protein Protects Ventilation Injury to the Lung through Injury-guided Cell Repair

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Abstract

Studies showed that TRIM72 is essential for repair of alveolar cell membrane disruptions, and exogenous recombinant human TRIM72 protein (rhT72) demonstrated tissue-mending properties in animal models of tissue injury. Here we examine the mechanisms of rhT72-mediated lung cell protection *in vitro* and test the efficacy of inhaled rhT72 in reducing tissue pathology in a mouse model of ventilator-induced lung injury. *In vitro* lung cell injury was induced by glass beads and stretching. Ventilator-induced lung injury was modeled by injurious ventilation at 30 ml/kg tidal volume. Affinity-purified rhT72 or control proteins were added into culture medium or applied through nebulization. Cellular uptake and *in vivo* distribution of rhT72 were detected by imaging and immunostaining. Exogenous rhT72 maintains membrane integrity of alveolar epithelial cells subjected to glass bead injury in a dose-dependent manner. Inhaled rhT72 decreases the number of fatally injured

alveolar cells, and ameliorates tissue-damaging indicators and cell injury markers after injurious ventilation. Using *in vitro* stretching assays, we reveal that rhT72 improves both cellular resilience to membrane wounding and membrane repair after injury. Image analysis detected rhT72 uptake by rat alveolar epithelial cells, which can be inhibited by a cholesterol-disrupting agent. In addition, inhaled rhT72 distributes to the distal lungs, where it colocalizes with phosphatidylserine detection on nonpermeabilized lung slices to label wounded cells. In conclusion, our study showed that inhaled rhT72 accumulates in injured lungs and protects lung tissue from ventilator injury, the mechanisms of which include improving cell resilience to membrane wounding, localizing to injured membrane, and augmenting membrane repair.

Keywords: ventilator-induced lung injury; acute respiratory distress syndrome; cell injury and repair; tripartite motif-containing protein 72; biomarkers

Lung cells are regularly subjected to mechanical loads or stress, and respond by changing cell volume, length, or shape (i.e., strain) (1). Stress failure occurs when the local strain is large enough to overcome the adhesion forces among the plasma membrane lipids and those between the plasma membrane and the subcortical cytoskeleton (2). In acute respiratory distress syndrome (ARDS), alveolar-resident

cells may be previously subjected to a barrage of physical and biochemical insults, such as trauma, acid reflux, bacterial toxins, and inflammatory cytokines (3). The frequent need for mechanical ventilation among patients with ARDS also causes additional physical stresses to the lung, manifested as ventilator-induced lung injury (VILI) that is commonly referred to as the

“volutrauma” (4, 5). As a result, membrane blebs, plasma membrane wounds, and loss of cell anchorage to the basement membrane occur (3, 4). Subsequent disruption of normal cellular function, cell death, lung edema, and activation of inflammatory mediators drives the lung into an even more distressed state that is commonly referred to as the “biotrauma” and the “atelectrauma” (6–8). In general,

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the cytoskeleton contributes greatly to the elasticity and overall cell mechanics of a single cell (9). Nonetheless, studies have shown that elasticity of the cell–cell junction area is different from, and significantly influences, stiffness of the single cell (10, 11).

Injury to alveolus-resident cells is the hallmark of ARDS and VILI, in which evidence of epithelial and endothelial cell damage has been well documented (12–15). It is known that all wounded cells have the capacity to restore plasma membrane integrity (16, 17) through orchestrated stepwise cellular processes (3, 4, 18), so manifestations of cell injury are potentially reversible and represent a target for therapeutic interventions. Although a number of cell repair–targeted pharmacologic interventions have proven effective in preclinical models (19–22), the focus in patients remains on lung protective mechanical ventilation (23) as a means to reduce the deforming stress the lung cells experience during ventilation. Clinicians describe these stresses as associated with alveolar overdistension and the cyclic opening and collapse of unstable lung units (24). Although the former is a tangential stress presumably born by cell–cell junctions, the latter is interfacial in nature and is associated with the movement and fracture of liquid bridges in small conducting airways and air spaces.

Our goal is to develop a safe and effective lung cell repair therapy that enhances the cell's intrinsic repair mechanisms. In this regard, we found that tripartite motif family protein 72 (TRIM72) is a critical component of the “repair kit” in alveolar epithelial cells (25, 26), similar to its membrane reparative roles in skeletal muscle and the heart (27–29). In uninjured cells, TRIM72 proteins are thought to dimerize via their leucine zipper motif (30) in the reduced intracellular environment and associate with lipid vesicles at the sub-plasma membrane region (27), aided by its higher affinity to the biological membrane component phosphatidylserine (PS) (31). Upon membrane injury, exposure to the oxidized extracellular environment causes rapid oligomerization of TRIM72 through formation of disulfide bonds among the dimers' cysteine residues (30). These protein–vesicle complexes traffic to the plasma membrane wounds, which may use a nonmuscle myosin type IIA–based motor system (32), to form

repair patches for the membrane wounds (27). Indeed, supplementation with exogenous recombinant human TRIM72 protein (rhT72) intravenously, subcutaneously, or intramuscularly has been shown to protect a variety of organs, including skeletal muscle, heart, kidney, lung, skin, and the brain from toxin, enzyme, strenuous exercise, or ischemic reperfusion–induced injuries (33–38). Studies also showed that rhT72 improves muscle pathology in a few animal models of muscular dystrophies (38–40). Nevertheless, the mechanism for exogenous rhT72–mediated membrane repair is thought to be different from that of the endogenous TRIM72, because rhT72 is often supplied in an oxidized extracellular environment.

In this study, we evaluated the therapeutic potential of inhaled rhT72 for the treatment of VILI using *in vitro* and *in vivo* models of lung injury. We found that rhT72 is readily taken up into the cytosol in a cholesterol-dependent manner, increases the cell's resilience to stretch injury of the plasma membrane, and improves membrane wound repair. The cell-protective efficacy of rhT72 was observed in the micromolar range in both rat and primary human alveolar epithelial cells. *In vivo* studies revealed that inhaled rhT72 accumulated in injured lung regions subjected to high tidal volume (V_T) ventilation, and that it effectively reduced the number of cells with defective membrane repair in the injurious ventilation model. Its use was also associated with reduced biomarkers of lung cell and tissue injury. Our results suggest that inhaled rhT72 distributes to injured lung tissues and protects the lung from various ventilator stressors through improvement of cell resilience to membrane wounding and aid to plasma membrane repair.

Methods

Cell Injury Assays

A total of 9×10^4 rat alveolar epithelial (RLE) cells (ATCC) or primary human alveolar epithelial cells (ScienCell) were aliquoted into 96-well plates in suspension, mixed with 20-mg/well glass beads (Sigma), and horizontally rotated at 180 rpm for 10 minutes. Supernatant lactate dehydrogenase (LDH) was measured using

a Clontech kit. RLE cells were also cultured on BioFlex dishes (FlexCell International) and stretched by maximal 22.5% 0.25-Hz gradient biaxial strain for 10 minutes in medium containing 10 kD fluorescein isothiocyanate–labeled dextran (FDX; Invitrogen). 0.5 $\mu\text{g}/\text{ml}$ propidium iodide (PI; Invitrogen) was added after stretching (25, 38, 41).

VILI

Wild-type mice were ventilated at 30 ml/kg V_T , 3 cm H_2O positive end-expiratory pressure, with room air for 2 hours. Controls received 6 ml/kg V_T ventilation. A total of 200 μl of 1.03 μM rhT72 (or BSA) was nebulized at 6 ml/kg V_T for 5 minutes every 30 minutes through an aeroneb-pro nebulizer (SCIREQ). Elastance was recorded using FlexiWare (SCIREQ). After over-ventilation (OV), PI was injected into the pulmonary circulation, and lungs were occluded at 30 ml/kg with room air. Confocal images at 405 and 561 nm were taken on a Zeiss LSM 810 confocal microscope in a blinded fashion. BAL fluid (BALF) and lung tissues were collected in separate experiments for detection of BALF protein, LDH, biomarkers, histology, and wet/dry ratio (26).

Cellular Uptake of rhT72

RLE cells were incubated with 6.25 nmol red fluorescent protein (RFP)-TRIM72 (or RFP) plus 5 μM FM1-43 (Invitrogen). Fluorescent signals at 562 and 488 nm were monitored for 50 minutes, and the percentage of cells internalizing RFP-TRIM72 was quantified. To examine mechanisms of uptake, RLE cells were pretreated with 5 mM methyl- β -cyclodextrin (M β CD; Sigma) at 37°C for 30 minutes before treated with RFP-TRIM72.

In Vivo Distribution of rhT72

RFP-TRIM72 or maltose binding protein (MBP)-TRIM72 was administered to mouse lungs via nebulizer under normal ventilation (NV) for 5 minutes after 30 minutes of OV. RFP-TRIM72-treated lungs were submerged in optimal cutting temperature compound (OCT) and imaged at 563 nm on an Olympus IX73 fluorescent microscope. Fluorescent intensity was quantified using ImageJ (NIH). MBP-TRIM72-treated lungs were fixed in 4% paraformaldehyde and processed for immunostaining with anti-MBP (Thermo Fisher) and anti-PS (Millipore) antibodies,

followed by secondary antibodies and DAPI counterstaining. Lung slices were either permeabilized with Triton X-100 or not. Colocalization of fluorescence images was quantified using CellSens software.

Statistical Analysis

Data were analyzed using Origin 6.0 (OriginLab) and SPSS Statistics v25 (IBM), and are presented as mean $\pm$ SEM. Significance was assumed at P less than 0.05. See the data supplement for details.

Results

Recombinant TRIM72 Protein Ameliorates Alveolar Epithelial Cell Injury

As alveolar epithelial cells are a cell type that is susceptible to VILI (3), we first tested the cell-protective efficacy of rhT72 on these cells *in vitro*. MBP-tagged rhT72 was used, because it showed good efficacy in reducing

skeletal muscle injuries in dystrophic mouse models (38). The molecular size of MBP-rhT72 is 97.4 kD, with a calculated isoelectric point of 5.4. Colloidal blue staining showed greater than 95% purity of affinity-purified rhT72, and approximate yield of 32.03 μ M in the first 1-ml elution (Figure 1A). Glass bead compression and shear stress induced the release of intracellular enzyme LDH into the cell culture medium (Figure 1), which was used for quantitative evaluation of cell wounding. As shown in Figure 1B and 1C, rhT72 reduced LDH release in both RLE and primary human alveolar epithelial cells in a dose-dependent manner, whereas such an effect was not seen in control cells treated with equal molar concentration of BSA. The calculated dose for half-maximal response is 0.626 μ M (59.45 μ g/ml) for RLE cells and 1.17 μ M (64.49 μ g/ml) for primary human alveolar epithelial cells (Figures 1B and 1C).

Recombinant TRIM72 Protein Reduces Fatal Alveolar Cell Wounding after Injurious Ventilation

To test the protective effects of rhT72 *in vivo*, we administrated rhT72 via a ventilator-driven intermittent mesh nebulizer (Figure 1D) and applied *ex vivo* quantification of fatally injured alveolar cells (Figures 1E and 1F) (16). Our results showed that lungs received BSA inhalation and subjected to injurious ventilation at 30 ml/kg V_T for 2 hours had PI⁺ cells lining the alveolar wall (BSA-OV), while intermittent inhalation of 1.03 μ M rhT72 through the OV process significantly reduced the proportion of PI⁺ unrepaired alveolar resident cells (41, 42) in the subpleural lung regions as compared with BSA-treated controls. Although the *in vitro* and *in vivo* data in Figure 1 cannot distinguish between the cells' resistance to biophysical injury and enhanced repair of injured cells, it would seem that the latter is

Figure 1. Recombinant tripartite motif family protein (TRIM) 72 protein ameliorates alveolar epithelial cell injury due to mechanical stresses. (A) Affinity purification of recombinant human TRIM72 protein (rhT72). E1–E6 = elution fractions; FT = flow through; M = protein size marker. Dose–response curve of rhT72 reduction in lactate dehydrogenase (LDH) leak into the cell culture medium in glass bead-injured (B) rat alveolar epithelial (RLE) cells and (C) primary human alveolar epithelial cells. EC50 = dose for half-maximal response; solid line = BSA controls; dashed line = rhT72-treated cells; $n = 6$ for BSA, $n = 6–14$ for rhT72; $*P < 0.05$ compared with BSA controls. (D) Protocol for the 2-hour injurious ventilator-induced lung injury (VILI) model and intermittent nebulization delivery of rhT72, normal ventilation (NV) at 6 ml/kg tidal volume (V_T), over-ventilation (OV) at 30 ml/kg tidal volume. (E) Representative images ($\times 400$ magnification) of fatally wounded cells (propidium iodide [PI]⁺) at subpleural lung regions in the BSA control group (BSA-OV) and rhT72-treated group (rhT72-OV). (F) Quantification of PI⁺ cells per alveolus in the subpleural lung regions after OV. Open bar = BSA controls; solid bar = rhT72-treated lungs; $n = 5$; $*P < 0.05$ compared with BSA controls. Data are presented as mean $\pm$ SEM.

Figure 2. Recombinant TRIM72 improves lung injury indicators and reduces selective cell injury markers in VILI. (A) Representative hematoxylin and eosin histology images ($\times 200$ magnification) of NV BSA-treated control lungs, BSA-treated OV lungs, and rhT72-treated OV lungs. Scale bars: 100 μm . (B) BAL fluid (BALF) LDH levels. (C) Total BALF protein. (D) Lung wet-to-dry ratio (wet/dry). (E) ELISA quantification of BALF levels of receptor for advanced glycation end-products (RAGE), (F) surfactant protein-D (SP-D), (G) angiopoietin 2 (ANGPT2), (H) CXC chemokine ligand (CXCL) 1/keratinocyte chemoattractant (KC), (I) CXCL2/macrophage inflammatory protein (MIP)-2, (J) von Willebrand factor A2 domain (vWFA2), (K) IL-6, (L) IL-1 β , and (M) TNF- α . Gray bar = NV controls treated with BSA; white bar = OV treated with BSA; black bar = OV treated with rhT72; $n = 5$; $^*P < 0.05$ compared with OV BSA controls, $^{\#}P > 0.05$ compared with NV BSA controls. Data are presented as mean $\pm$ SEM.

more likely based on our previous studies (25, 26).

Recombinant TRIM72 Improves Lung Injury Indicators in VILI

To examine if the cytoprotective effect of rhT72 corresponds to reduced tissue injury indicators in VILI, we characterized tissue histology, lung mechanic parameters, and biochemical indicators (41) (Figures 2A–2D). As seen in Figure 2A, mild histopathological changes were seen in BSA-treated lungs after 2 hours of OV, including areas of atelectasis and thickened alveolar septa. In contrast, rhT72-treated lungs had fewer of these histopathological changes and were similar to BSA-treated control lungs subjected to low V_T ventilation at 6 ml/kg (Figure 2A). Injurious ventilation was associated with a significant increase in elastance, which was mitigated by rhT72 inhalation (Table 1). Furthermore, rhT72 inhalation reduced the levels of BALF total protein, LDH, and wet-to-dry ratio, which were elevated after 2 hours of injurious ventilation (Figure 2B–2D).

Recombinant TRIM72 Reduces Selective Cell Injury Markers in VILI

ARDS and VILI biomarkers have been shown to aid in the diagnosis of lung injury, predict outcome, and reveal the type of injured cells (43–46). We measured the levels of several of these biomarkers in BALF of the VILI mice, including: receptor for advanced glycation end-products (RAGE); surfactant protein-D (SP-D); angiopoietin 2 (ANGPT2); von Willebrand factor A2 domain; CXC chemokine ligand (CXCL) 1 (or keratinocyte chemoattractant); CXCL2 (or macrophage inflammatory protein 2); IL-1; and TNF- α . These markers indicate the presence of major pathogenic mechanisms for VILI, such as type I alveolar epithelial cell injury, type II alveolar epithelial cell injury, endothelial cell injury, coagulation pathway activation, neutrophil recruitment, and the development of biotrauma (45). Consistent with previous biomarker studies (43–46), all of these markers were significantly elevated after injurious ventilation (Figures 2E–2M), although the increases in proinflammatory cytokines IL-6 and IL-1 β were approximately 10–1,000 times less than we previously reported in a bacterial pneumonia model (47). Interestingly, rhT72 significantly reduced the BALF levels

Table 1. Elastance of BSA- and Recombinant Human Tripartite Motif Family Protein 72–treated Lungs during Over-Ventilation (cm H₂O/ml)

Ventilation duration	0 min	60 min	120 min
BSA-OV	21.847 $\pm$ 0.794	44.484 $\pm$ 1.557	62.813 $\pm$ 2.282
rhT72-OV	21.464 $\pm$ 0.885	36.960 $\pm$ 0.904*	52.983 $\pm$ 1.262*

Definition of abbreviations: OV = over-ventilation; rhT72 = recombinant human tripartite motif family protein 72.

* $P < 0.05$ compared to BSA-OV at the same time point, $n = 5$ for each group.

of RAGE, SP-D, ANGPT2, and CXCL1 as compared with the BSA controls (Figure 2F–2I). This suggests that inhaled rhT72 ameliorates VILI by repairing injuries to multiple types of lung cells. It was not surprising to also observe reduced CXCL1 in rhT72-treated mice, as it is well known that damage-associated molecular patterns released from damaged cells can activate the innate immune system (6). Therefore, although no substantial inflammatory cell recruitment was observed

in our one-hit sterile 2-hour ventilation model, we expect that inflammatory cell infiltration to the lung, and thus biotrauma, would be subsequently ameliorated by rhT72 inhalation.

Recombinant TRIM72 Protein Increases Cell Resilience to Stretch Injury and Promotes Repair

To determine whether rhT72 protects cells from membrane wounding or simply improves membrane repair afterward, we

Figure 3. rhT72 increases cell resilience to stretch injury and promotes repair. (A) Representative images of fluorescein isothiocyanate-labeled dextran (FDX)⁺ (green) and PI⁺ (red) RLE cells treated with BSA (stretch + BSA) or rhT72 (stretch + rhT72) after 22.5% stretching. Scale bars: 100 μ m. (B) Representative images of nonstretched control RLE cells incubated with equal amount of FDX⁺ (green) and PI⁺ (red). Scale bar: 50 μ m. (C) Quantification of total wounded cells (FDX⁺ + PI⁺/total cells) and percentage of nonrepaired cells (PI⁺/total wounded cells) in controls (–) and rhT72-treated groups (+); $n = 8$; * $P < 0.05$ compared with control groups. Data are presented as mean $\pm$ SEM.

Figure 4. rhT72 is taken up by alveolar epithelial cells. Fluorescent images of red fluorescent protein (RFP; red) and FM14-3-labeled plasma membrane (green) at 5, 15, 20, and 25 minutes after RFP or RFP-TRIM72 proteins were applied to RLE cells. (A) RFP controls, red = RFP, overlay = RFP + FM14-3. (B) RFP-tagged TRIM72 protein (RFP-T72), $\times 1,000$ magnification. Red = RFP-T72; overlay = RFP-T72 + FM14-3; arrows = RFP-TRIM72 uptake. (C) Enlarged red and overlay images of an RLE cell taking up RFP-T72 at 15 and 25 minutes. Experiments were repeated three times each.

used a previously established assay to differentiate these two possibilities (48). RLE cells were stretched to up to 22.5% at 0.25 Hz and 75% duty cycle in the presence of FDX dye to label wounded and repaired cells. PI was added after stretching to label cells that were wounded, but unrepaired (41). As shown in Figure 3, in the presence of exogenous rhT72, there were significantly fewer wounded cells (FDX⁺ plus PI⁺/total cells), as well as a lower percentage of nonrepaired cells (PI⁺/total wounded cells) than the BSA controls. This suggests that pretreatment of cells with rhT72 protein not only improves membrane repair, but also increases the cells' resistance to membrane wounding. This unexpected finding suggests the intriguing possibility that rhT72 pretreatment increases deformation-induced lipid trafficking (49, 50) at the cell membrane to counter for the plasma membrane lytic tension, as discussed subsequently.

Recombinant TRIM72 Protein Is Taken Up by Alveolar Epithelial Cells

A previous study showed that endogenous TRIM72 binds to intracellular vesicles, which then migrates to areas requiring membrane repair (27). In the present study, we examine whether exogenous rhT72 can enter the cytosol and associate

with intracellular vesicles. The plasma membrane of the RLE cells was labeled with lipophilic Styryl dye FM14-3, which emits fluorescence once, inserting into the outer leaflet of the plasma membrane and is taken up into cytosol via endocytosis (51). As shown in Figure 4 and movies in the data supplement, FM14-3 signal is visible after 5 minutes of contact with the cell membrane, and dye internalization was seen at approximately 15–25 minutes. Figures 4B and 4C show that extracellular RFP-TRIM72 comigrates with these lipid vesicles and gets internalized, whereas a minimal amount of RFP control protein internalization was observed (Figure 4A), suggesting that the majority of the RFP-TRIM72 uptake was not due to nonspecific endocytosis. To test the specific mechanism for rhT72 uptake, we incubated RLE cells with M β CD to deplete membrane cholesterol (52) before incubation with rhT72. As shown in Figure 5, M β CD significantly reduced the percentage of RLE cells taking up recombinant proteins. This could be due to the possibility that inhibition of the cholesterol-rich caveolae disrupts the previously reported TRIM72 and caveolae interaction (25, 26). However, we could not exclude the broader impact of cholesterol depletion on other types of endocytosis (53, 54).

Inhaled Recombinant TRIM72 Distributes to the Distal Lung Regions in OV

To examine tissue distribution of inhaled rhT72, we used RFP-TRIM72 for imaging. RFP-TRIM72 or RFP only was administered through nebulization for 5 minutes after 30 minutes of injurious ventilation or normal V_T ventilation (Figure 6A). To preserve the fluorescence of exogenous RFP-TRIM72 after nebulization, the lungs were inflated with air (30 ml/kg volume), the airways occluded, and the lungs submerged into OCT *en bloc*. RFP-TRIM72 distribution on OCT lung slices was then observed under a fluorescence microscope (Figures 6B–6D). Our results showed that, when the lungs were ventilated with low V_Ts, RFP-TRIM72 was mainly distributed to large airways and, occasionally, small airways (Figure 6B). In contrast, significant amounts of RFP-TRIM72 were seen throughout the lungs receiving OV, including distal alveolar regions (Figure 6B–C, Table 2), whereas we did not observe any fluorescence in RFP protein-inhaled lungs even in over-ventilated lungs (Figure 6D, Table 2). This suggests that rhT72 has a higher affinity to lung tissues wounded by OV, consistent with a previous study showing accumulation of rhT72 at wounded skeletal muscle cells (38).

Figure 5. Cholesterol depletion inhibits rhT72 uptake by alveolar epithelial cells. (A) Fluorescent images of RFP (RFP-TRIM72 or RFP control, red) and overlay of RFP and FM14-3-labeled plasma membrane (green) in the presence or absence of methyl-β-cyclodextrin (M β CD). Images were taken at 25 minutes after incubation of RLE cells with RFP or RFP-TRIM72 proteins at $\times 1,000$ magnification. Arrows = RLE cells taking up RFP or RFP-TRIM72. (B) Quantification of the percentage of RLE cells taking up RFP-tagged protein at 0, 5, 15, and 25 minutes after adding RFP, RFP-T72, or RFP-T72 with M β CD to the cultured cells; $n = 3$; * $P < 0.05$ compared with RFP control, # $P < 0.05$ compared with RFP-T72 groups. Data are presented as mean $\pm$ SEM.

Figure 6. Inhaled rhT72 distributes to the distal lung regions in OV. (A) Protocol for postinjury (or control) intermittent nebulization delivery of rhT72 in NV and OV. (B) Representative images ($\times 200$ magnification) of transmission, RFP, and RFP + transmission of optimal cutting temperature compound-embedded wild-type lungs receiving RFP-TRIM72 nebulization and ventilation (NV or OV). Insets = enlarged area; lower row (C) = enlarged images of enclosed area to show distribution of RFP-TRIM72 to distal lung areas in the OV groups. (D) RFP nebulization and OV. Experiments were repeated three times. Scale bars: 100 μ m.

We further examined the affinity of rhT72 to wounded lung tissues by performing coimmunostaining of inhaled rhT72 with cell injury markers. In this regard, we used an anti-PS antibody to stain nonpermeabilized lung slices. This should label both apoptotic cells where the plasma membrane inner leaflet PS is flipped outside, and all wounded cells bearing plasma membrane defects where plasma membrane PS can then become accessible through the membrane wounds. As shown in Figure 7A, OV induced broadly distributed cell wounding (PS^+ cells) in distal alveolar

spaces in both post-injury MBP-TRIM72 and MBP control protein-treated lungs. Immunostaining detection of inhaled MBP-TRIM72 revealed broad distribution of MBP-TRIM72 in the injured lungs, and a significant level of colocalization of MBP-TRIM72 with PS^+ cells. In contrast, MBP control protein-treated lungs showed diffuse staining without preference for PS^+ cells (Table 3). These data suggest that inhaled rhT72, in contrast with the control protein, is able to accumulate to wounded lung regions. Validation of the PS immunostaining on nonpermeabilized lung

slices as a nonspecific marker for cell wounding is shown in Figure 7B, as PS^+ cells only account for a percentage of DAPI $^+$ cells on the nonpermeabilized lung slices, but a near-complete overlapping was observed on the permeabilized lung slices.

Discussion

In this study, we tested the therapeutic efficacies of rhT72 on ameliorating stress-induced lung cell injury *in vitro* and *in vivo*, and characterized the inhalation route of rhT72 delivery to the lung tissue. Our results revealed significant efficacy of rhT72 in the repair of injured alveolar epithelial cells, enhanced distribution of inhaled rhT72 to lung tissues wounded following high V_T ventilation, and a correlation between its cytoprotective efficacy and reduction in lung pathology and biomarkers of lung injury.

Injury to alveolus-resident cells is an important pathophysiological mechanism of ARDS and VILI (6, 55). Although the causes for cellular injury are diverse and complex in ARDS, *in vitro* and *in vivo* studies demonstrated that several stages of lung cell membrane repair can be modulated by various agents. Notably, hypertonic saline can stimulate caveolar endocytosis (20), stiffen the alveolar epithelial cells, and promote tethering of the subcortical cytoskeleton to the plasma membrane (56), and thereby increase cell resilience to injurious interfacial stresses (20, 21). Indeed, nebulized hypertonic saline was proven to be cytoprotective in rodent models of trauma and hemorrhagic shock-induced acute lung injury (22). Another membrane repair agent that had been examined in the context of lung injury is poloxamer 188 (P188), a biocompatible, nonionic, amphiphilic copolymer. P188 inserts into the lipid bilayer (19), and thus may aid lipid trafficking-mediated plasma membrane repair (49, 50). Studies showed that P188 was effective in improving a variety of cell injuries and tissue pathologies, such as muscular dystrophy, heart failure, neurodegenerative disorders, and electroporation damage (19). In the lung, studies reported that P188 can not only protect alveolar-resident cells from ventilation injury, but also improve conventional lung injury indicators, such as lung mechanics or vascular leakage in

Table 2. Quantification of *In Vivo* Red Fluorescent Protein Fluorescence Distribution in the Lung

Groups	RFP-TRIM72 NV	RFP-TRIM72 OV	RFP OV
Average mean intensity	2.016 ± 0.259	15.406 ± 1.373*, #	0.599 ± 0.040

Definition of abbreviations: NV = normal ventilation; OV = over-ventilation; RFP = red fluorescent protein; TRIM72 = tripartite motif family protein 72.

* $P < 0.05$ compared to RFP OV, $n = 3$ for each group.

$P < 0.05$ compared to RFP-TRIM72 NV, $n = 3$ for each group.

isolated ventilated rat lungs (41). However, such protective effects were not seen in the whole-rat injurious ventilation model (41), indicating bioaccessibility issues of P188 into the lung. Thus, the search for effective lung repair therapies continues.

In the context of VILI, the most relevant cell injury mechanisms are biophysical stresses, such as over-distention and interfacial stress. It is worth noting that complete separation of over-distention and interfacial stresses is impossible, due to the dual effect of lung water (edematous fluid), which both increases interfacial stress, and by increasing stiffness in some lung zones, causes over-distention in other areas. The *in vivo* injurious ventilation model used in this study is relevant for both types of biophysical stresses, as lung edema does develop as a result of OV. Previous studies on the mechanisms of endogenous TRIM72-mediated membrane repair have suggested that the association of TRIM72 with intracellular vesicles and the mobilization of the TRIM72-vesicle complex are important steps in membrane repair (27). Studies from our group also examined the mechanisms of endogenous TRIM72-mediated repair of alveolar epithelial cells after injury (25, 47). A major contribution of the current work is to establish that aerosolized delivery of rhT72 can provide benefits similar to that previously shown for TRIM72 overexpression (25). Using a variety of experimental models, we had demonstrated that rhT72 protects lung cell injury caused by glass bead compression and shear stress, stretching, as well as combined *in vivo* volutrauma, atelectrauma, and biotrauma. As previous studies suggest that the cytoprotective effect of certain repair compounds may depend on the nature of the injurious stresses (56, 57), it is significant that our data showed that rhT72 effectively reduces fatally injured cells in multiple cell injury models, demonstrating

a broad target of its cytoprotective effect for different mechanisms of cellular injury. Notably, we showed that *in vivo* nebulization delivery of rhT72 after the start of tissue injury still provides significant clinical benefits in decreasing histopathological markers of lung injury, improving lung mechanics, and decreasing VILI-associated biomarkers.

Although aerosol delivery was thought to be problematic in ventilated patients (58, 59), we showed that nebulized rhT72 can be successfully delivered to the lung. In our study, increased distribution of rhT72 via postinjury delivery was visualized in the distal lung regions of the over-ventilated lungs, but mostly in the proximal airways of the NV lungs. In contrast, RFP controls did not demonstrate significant deposition in the lung, even under OV. Coimmunostaining results showed that rhT72 highly localized to regions positive for a broad cell injury marker (e.g., PS), suggesting that rhT72 is attracted to wounded lung regions (38). Nevertheless, our current inhalation protocol cannot ensure protein delivery to all wounded lung areas, which may explain why only partial tissue protection was observed in our *in vivo* model of lung injury, suggesting that optimal inhalation strategy is key to further improve the therapeutic efficacy of rhT72 therapy for VILI. However, it is rational to reason that, in more severely wounded lungs, or lungs subjected to regional over-distension, localization of rhT72 to areas of greater injury may be further increased so potential side effects could be lowered. We didn't see significant amounts of rhT72 cellular uptake in NV lungs treated with RFP-TRIM72, possibly due to the short time of protein exposure (a total of 15 min). As *in vivo* cytosolic uptake of rhT72 and possible effect of cyclic stretching on the ratio of uptake are of interest for optimal rhT72 inhalation delivery, we will further investigate in future studies.

In addition, although rhT72 has been shown to have similar repair efficacy as endogenous TRIM72 *in vitro* and *in vivo* (28, 29, 38, 60), before our study, it was not clear whether rhT72 worked via these same mechanisms. In this study, we further elucidated the membrane repair mechanisms of exogenous rhT72 from two aspects. First, we demonstrated that, when incubated with alveolar epithelial cells, rhT72 is taken up into the cytosol where it associates with intracellular lipid vesicles in approximately 20 minutes. FM14-3 is taken up by endocytic pathways (51). Given the overlap of the FM14-3 and the rhT72 signals during this process, it is likely that rhT72 is also taken up via endocytosis. Indeed, cholesterol depletion by M β CD significantly reduced rhT72 cellular uptake, suggesting a significant role of clathrin-independent endocytosis in this process. Considering our previous reports identifying a physical interaction between TRIM72 and caveolin 1, and the intimate interplay between TRIM72 and caveolar endocytosis (25, 26), we speculate that rhT72 uptake is through the caveolar endocytic pathway. However, more specific pathway inhibitors are required to further confirm the specific pathway for rhT72 uptake into the cells (53, 54).

Furthermore, we examined whether exogenous rhT72 protects lung tissue from injury by increasing cell resilience to membrane wounding or by improving cellular repair (41, 42). Although effects of rhT72 in over-ventilated murine lungs on markers of cell injury and inflammation cannot be attributed to cell repair to the exclusion of cytoprotection, this distinction is possible in the *in vitro* cell stretch system. In that system, the majority of injured cells typically repair in less than 90 seconds, and can be identified by their cytoplasmic retention of large fluorescent molecules, such as Dextran. Cells that fail to repair within 2 minutes can be identified *post hoc* by PI-positive staining. Accordingly, an intervention that promotes repair of injured cells would be expected to increase the fraction of Dextran-retaining cells. Although rhT72 reduced the fraction of permanently damaged PI-positive cells as expected, it also reduced the fraction of cells with retained FDX. This observation can only mean that rhT72 exposure had protected alveolar epithelial cells from stretch-related wounding. The protective effect of rhT72 on the probability of

Figure 7. Inhaled rhT72 colocalizes with wounded lung cells. (A) Representative immunostaining images ($\times 400$ magnification) of anti-maltose binding protein (MBP; green), anti-phosphatidylserine (PS; red), DAPI (blue), and overlay on wild-type lung slices after 30 minutes of OV and postinjury nebulization of MBP-TRIM72 (MBP-T72) or MBP control protein. Experiments were repeated in five mice for MBP-T72 group and in three mice for MBP control group. (B) Overlay images of anti-PS immunostaining (red) with DAPI on nonpermealized lung slices and permeabilized lung slices. Scale bars: 50 μm .

stretch-induced plasma membrane wounding (Figure 3) was unexpected. After all, the known effects of TRIM72 on membrane traffic (27, 61) and as chaperone in caveolar endocytosis (26) suggested that its principle mode of action is to facilitate plasma membrane repair. This unexpected finding warrants a broader consideration of enhanced membrane traffic as a defense mechanism against stretch-related plasma membrane stress failure. Because the plasma membrane is virtually inelastic, membrane tension can only be maintained

at sublytic levels through a combination of membrane unfolding, enhanced lipid trafficking, and fusion of intracellular membrane-derived lipids to the cell surface (4, 49, 50, 56, 62). Such a mechanism had been proposed as an explanation of the cytoprotective effects of secretagogues, such as ATP and β agonists (63), and could apply to rhT72 as well. Because rhT72 can be taken up into the cytosol where it associates with intracellular vesicles, the amount of rhT72-vesicle complex to traffic to the plasma membrane

during deformation should increase correspondingly, which may explain the improvement in both the cell's resilience to membrane wounding and ability of membrane repair when exogenous rhT72 was applied.

This unexpected finding also prompts a discussion about the relevance of monolayer cell stretch as a model of OV-associated lung cell injury. The shape change associated with cell stretch is associated with an increase in cell surface area, and, as such, would increase plasma membrane tension to lytic levels unless this change is counteracted. Changes in cytoskeletal mechanics have also been linked to the risk of deformation injury (50, 57). It stands to reason that an increase in cell/cytoskeletal stiffness must increase the stress that a given stretch transmits to cell-cell junctions. Unless cytoskeletal stiffening is associated with a compensatory reinforcement in cell-cell adhesive interactions and tight junctions, an agent that increases cytoskeletal stiffness would be expected to disrupt the epithelial barrier (64, 65), whereas an agent that softens the cell would be barrier protective. To our knowledge, the effects of rhT72 on cell elastic moduli and epithelial barrier properties have not been measured to date. Although cell stiffening may increase the risk of epithelial barrier damage in over-distended alveolar spaces, there is compelling evidence that it will protect cells from damage by interfacial stress (50, 57). This mechanism has been detailed in the context of lung protection afforded by hypertonic saline inhalation (20, 56). The complex interplay between cell mechanical properties and the diverse and topographically heterogeneous stresses in the injured, mechanically ventilated lung pose a challenge when trying to attribute effect on an intervention to a single mechanism.

In addition, given the diversity and distinct functions of different lung cell types, revealing the cell types that were injured during ARDS and VILI will provide insights into the pathogenic mechanisms of the disease. Early studies demonstrate the presence of epithelial and endothelial cell membrane injury on transmission electron microscopy (14, 15). Recent biomarker association studies for ARDS also revealed the clinical relevance of specific cell injury markers. For example, in a retrospective case-control study of 100 patients, Ware and colleagues (43) found that plasma

Table 3. Quantification of Maltose Binding Protein–tagged Recombinant Human Tripartite Motif Family Protein 72 Distribution to Wounded Cells

Groups	MBP-T72	MBP
Pearson's colocalization efficiency (r)	0.540 ± 0.040	0.094 ± 0.013*

Definition of abbreviations: MBP = maltose binding protein; T72 = tripartite motif family protein 72.

* $P < 0.05$ compared to MBP control, $n = 5$ mice for MBP-T72 and $n = 3$ mice for MBP group. Average value from four to five images was used for each lung.

levels of SP-D, RAGE, IL-8, CC-16, and IL-6 could help to differentiate ARDS from sepsis, among which three are lung epithelium-specific markers. Another study by Ware and colleagues (46) analyzed plasma biomarkers of 549 patients in the ARDSNet trial who received low or high positive end-expiratory pressure ventilation. They found that neutrophil chemotactic factor, IL-8, and SP-D are the best-performing biomarkers to predict patient mortality when combined with clinical predictors. Calfee and colleagues (44) used a new risk classification method and found that a three-biomarker set, which includes IL-8, soluble TNF receptor-1, and SP-D can independently predict patient mortality when combined with clinical indicators. Collectively, these studies highlight the significance of lung epithelial cell injury in the pathogenesis of ARDS and the correlation between serum markers and mortality. In this study, we showed that injurious ventilation significantly elevated levels of SP-D, RAGE, ANGPT2, and CXCL1 in BALF. These findings confirm that VILI is associated with injury to alveolar epithelial and endothelial cells, as

well as activation of the innate immune system. Importantly, rhT72 inhalation significantly reduced these biomarkers, suggesting that the *in vivo* lung protection efficacy of rhT72 is through its cytoprotective effects.

It is worth noting here that we used a one-hit sterile injurious ventilation model to interrogate the protective effect of rhT72 on *de novo* volutrauma/atelectrauma and subsequent biotrauma, whereas a percentage of clinical ARDS is caused by bacterial pneumonia (66). In that context, our recent study (47) showed that TRIM72 in alveolar macrophage maintains the quiescence of complement receptor of the Ig family, and thus overexpression of TRIM72 led to reduction in complement-mediated phagocytosis and enhanced NF- κ B activation in the presence of bacteria, whereas TRIM72 knockout improves mortality due to bacterial pneumonia. Thus, we do not recommend the use of rhT72 in ARDS associated with active bacterial pneumonia. Whether TRIM72 has a proinflammatory effect in the absence of bacterial infection has not been tested yet. Nevertheless, even if TRIM72 itself has a

direct proinflammatory role, it is likely that rhT72 needs to be taken up into the cell for such an effect. Our results indicate that there is rhT72 uptake into epithelial cells, but the amount does not seem high. Indeed, when BALF levels of IL-6, IL-1, and TNF- α were tested, we found that rhT72-treated lungs had similar cytokine levels to those in BSA-treated lungs, which argues against that direct application of rhT72 has a significant proinflammatory effect. Nevertheless, in our current study, rhT72 improved lung injury without inhibiting IL-6, IL-1 β , TNF- α , and CXCL2 elevation in the VILI model, suggesting that moderate increases in these proinflammatory mediators are not substantial drivers for tissue injury in VILI. In fact, this reconciles with an interesting concept that some inflammatory cytokines, such as IL-6, are beneficial for the repair of acute lung injury through stimulating reparative cell proliferation (67, 68).

In summary, our results demonstrate that rhT72 effectively protects wounded lung regions and had less accumulation in healthy lung regions. This is ideal for the treatment of VILI and ARDS, due to the heterogeneous nature of tissue injury in these syndromes. In addition, as lung cell wounding is widespread in VILI and ARDS, and is often an upstream event for biotrauma, the use of rhT72 to target cell wounding should have broader efficacy than therapies that only target a single or limited signaling pathways. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Moeendarbary E, Harris AR. Cell mechanics: principles, practices, and prospects. *Wiley Interdiscip Rev Syst Biol Med* 2014;6:371–388.
- Vlahakis NE, Hubmayr RD. Invited review: plasma membrane stress failure in alveolar epithelial cells. *J Appl Physiol* (1985) 2000;89:2490–2496. [Discussion, p. 2497.]
- Cong X, Hubmayr RD, Li C, Zhao X. Plasma membrane wounding and repair in pulmonary diseases. *Am J Physiol Lung Cell Mol Physiol* 2017;312:L371–L391.
- Vlahakis NE, Hubmayr RD. Cellular stress failure in ventilator-injured lungs. *Am J Respir Crit Care Med* 2005;171:1328–1342.
- Slutsky AS, Ranieri VM. Ventilator-induced lung injury. *N Engl J Med* 2013;369:2126–2136.
- Andrews NW. Membrane repair and immunological danger. *EMBO Rep* 2005;6:826–830.
- Slutsky AS. Ventilator-induced lung injury: from barotrauma to biotrauma. *Respir Care* 2005;50:646–659.
- Curley GF, Laffey JG, Zhang H, Slutsky AS. Biotrauma and ventilator-induced lung injury: clinical implications. *Chest* 2016;150:1109–1117.
- Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. *Nature* 2010;463:485–492.
- Stroka KM, Aranda-Espinoza H. Effects of morphology vs. cell–cell interactions on endothelial cell stiffness. *Cell Mol Bioeng* 2011;4:9–27.
- Harris AR, Peter L, Bellis J, Baum B, Kabla AJ, Charras GT. Characterizing the mechanics of cultured cell monolayers. *Proc Natl Acad Sci USA* 2012;109:16449–16454.
- Dreyfuss D, Basset G, Soler P, Saumon G. Intermittent positive-pressure hyperventilation with high inflation pressures produces pulmonary microvascular injury in rats. *Am Rev Respir Dis* 1985;132:880–884.
- Dreyfuss D, Saumon G, Hubmayr R. Ventilator-induced lung injury. New York: Taylor & Francis; 2006.
- Costello ML, Mathieu-Costello O, West JB. Stress failure of alveolar epithelial cells studied by scanning electron microscopy. *Am Rev Respir Dis* 1992;145:1446–1455.
- Fu Z, Costello ML, Tsukimoto K, Prediletto R, Elliott AR, Mathieu-Costello O, et al. High lung volume increases stress failure in pulmonary capillaries. *J Appl Physiol* (1985) 1992;73:123–133.

16. Gajic O, Lee J, Doerr CH, Berrios JC, Myers JL, Hubmayr RD. Ventilator-induced cell wounding and repair in the intact lung. *Am J Respir Crit Care Med* 2003;167:1057–1063.
17. Hussein O, Walters B, Stroetz R, Valencia P, McCall D, Hubmayr RD. Biophysical determinants of alveolar epithelial plasma membrane wounding associated with mechanical ventilation. *Am J Physiol Lung Cell Mol Physiol* 2013;305:L478–L484.
18. McNeil PL, Kirchhausen T. An emergency response team for membrane repair. *Nat Rev Mol Cell Biol* 2005;6:499–505.
19. Moloughney JG, Weisleder N. Poloxamer 188 (P188) as a membrane resealing reagent in biomedical applications. *Recent Pat Biotechnol* 2012;6:200–211.
20. Wang S, Singh RD, Godin L, Pagano RE, Hubmayr RD. Endocytic response of type I alveolar epithelial cells to hypertonic stress. *Am J Physiol Lung Cell Mol Physiol* 2011;300:L560–L568.
21. Godin LM, Vergen J, Prakash YS, Pagano RE, Hubmayr RD. Spatiotemporal dynamics of actin remodeling and endomembrane trafficking in alveolar epithelial type I cell wound healing. *Am J Physiol Lung Cell Mol Physiol* 2011;300:L615–L623.
22. Wohlaue M, Moore EE, Silliman CC, Fragoso M, Gamboni F, Harr J, et al. Nebulized hypertonic saline attenuates acute lung injury following trauma and hemorrhagic shock via inhibition of matrix metalloproteinase-13. *Crit Care Med* 2012;40:2647–2653.
23. Laffey JG, Kavanagh BP. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury. *N Engl J Med* 2000;343:812; author reply 813–814.
24. Platakis M, Hubmayr RD. The physical basis of ventilator-induced lung injury. *Expert Rev Respir Med* 2010;4:373–385.
25. Kim SC, Kellett T, Wang S, Nishi M, Nagre N, Zhou, et al. TRIM72 is required for effective repair of alveolar epithelial cell wounding. *Am J Physiol Lung Cell Mol Physiol* 2014;307:L449–L459.
26. Nagre N, Wang S, Kellett T, Kanagasabai R, Deng J, Nishi M, et al. TRIM72 modulates caveolar endocytosis in repair of lung cells. *Am J Physiol Lung Cell Mol Physiol* 2016;310:L452–L464.
27. Cai C, Masumiya H, Weisleder N, Matsuda N, Nishi M, Hwang M, et al. MG53 nucleates assembly of cell membrane repair machinery. *Nat Cell Biol* 2009;11:56–64.
28. Cao CM, Zhang Y, Weisleder N, Ferrante C, Wang X, Lv F, et al. MG53 constitutes a primary determinant of cardiac ischemic preconditioning. *Circulation* 2010;121:2565–2574.
29. Wang X, Xie W, Zhang Y, Lin P, Han L, Han P, et al. Cardioprotection of ischemia/reperfusion injury by cholesterol-dependent MG53-mediated membrane repair. *Circ Res* 2010;107:76–83.
30. Hwang M, Ko JK, Weisleder N, Takeshima H, Ma J. Redox-dependent oligomerization through a leucine zipper motif is essential for MG53-mediated cell membrane repair. *Am J Physiol Cell Physiol* 2011;301:C106–C114.
31. Kim S, Seo J, Ko YG, Huh YD, Park H. Lipid-binding properties of TRIM72. *BMB Rep* 2012;45:26–31.
32. Lin P, Zhu H, Cai C, Wang X, Cao C, Xiao R, et al. Nonmuscle myosin IIA facilitates vesicle trafficking for MG53-mediated cell membrane repair. *FASEB J* 2012;26:1875–1883.
33. Li H, Duann P, Lin PH, Zhao L, Fan Z, Tan T, et al. Modulation of wound healing and scar formation by MG53 protein-mediated cell membrane repair. *J Biol Chem* 2015;290:24592–24603.
34. Liu J, Zhu H, Zheng Y, Xu Z, Li L, Tan T, et al. Cardioprotection of recombinant human MG53 protein in a porcine model of ischemia and reperfusion injury. *J Mol Cell Cardiol* 2015;80:10–19.
35. Jia YL, Chen K, Lin PH, Lieber G, Nishi M, Yan R, et al. Treatment of acute lung injury by targeting MG53-mediated cell membrane repair. *Nat Commun* 2014;5:4387.
36. Duann P, Li HC, Lin PH, Tan T, Wang Z, Chen K, et al. MG53-mediated cell membrane repair protects against acute kidney injury. *Sci Transl Med* 2015;7:279ra36.
37. Yao Y, Zhang B, Zhu H, Li H, Han Y, Chen K, et al. MG53 permeates through blood–brain barrier to protect ischemic brain injury. *Oncotarget* 2016;7:22474–22485.
38. Weisleder N, Takizawa N, Lin P, Wang X, Cao C, Zhang Y, et al. Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. *Sci Transl Med* 2012;4:139ra85.
39. He B, Tang RH, Weisleder N, Xiao B, Yuan Z, Cai C, et al. Enhancing muscle membrane repair by gene delivery of MG53 ameliorates muscular dystrophy and heart failure in δ -sarcoglycan-deficient hamsters. *Mol Ther* 2012;20:727–735.
40. Gushchina LV, Bhattacharya S, McElhanon KE, Choi JH, Manning H, Beck EX, et al. Treatment with recombinant human MG53 protein increases membrane integrity in a mouse model of limb girdle muscular dystrophy 2B. *Mol Ther* 2017;25:2360–2371.
41. Platakis M, Lee YD, Rasmussen DL, Hubmayr RD. Poloxamer 188 facilitates the repair of alveolus resident cells in ventilator-injured lungs. *Am J Respir Crit Care Med* 2011;184:939–947.
42. Unal Cevik I, Dalkara T. Intravenously administered propidium iodide labels necrotic cells in the intact mouse brain after injury. *Cell Death Differ* 2003;10:928–929.
43. Ware LB, Koyama T, Zhao Z, Janz DR, Wickersham N, Bernard GR, et al. Biomarkers of lung epithelial injury and inflammation distinguish severe sepsis patients with acute respiratory distress syndrome. *Crit Care* 2013;17:R253.
44. Caffee CS, Ware LB, Glidden DV, Eisner MD, Parsons PE, Thompson BT, et al.; National Heart, Blood, and Lung Institute Acute Respiratory Distress Syndrome Network. Use of risk reclassification with multiple biomarkers improves mortality prediction in acute lung injury. *Crit Care Med* 2011;39:711–717.
45. Barnett N, Ware LB. Biomarkers in acute lung injury—marking forward progress. *Crit Care Clin* 2011;27:661–683.
46. Ware LB, Koyama T, Billheimer DD, Wu W, Bernard GR, Thompson BT, et al.; NHLBI ARDS Clinical Trials Network. Prognostic and pathogenetic value of combining clinical and biochemical indices in patients with acute lung injury. *Chest* 2010;137:288–296.
47. Nagre N, Cong X, Terrazas C, Pepper I, Schreiber JM, Fu H, et al. Inhibition of macrophage complement receptor CR1g by TRIM72 polarizes innate immunity of the lung. *Am J Respir Cell Mol Biol* 2018;58:756–766.
48. Belete HA, Godin LM, Stroetz RW, Hubmayr RD. Experimental models to study cell wounding and repair. *Cell Physiol Biochem* 2010;25:71–80.
49. Vlahakis NE, Schroeder MA, Pagano RE, Hubmayr RD. Deformation-induced lipid trafficking in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L938–L946.
50. Vlahakis NE, Schroeder MA, Pagano RE, Hubmayr RD. Role of deformation-induced lipid trafficking in the prevention of plasma membrane stress failure. *Am J Respir Crit Care Med* 2002;166:1282–1289.
51. Amaral E, Guatimosim S, Guatimosim C. Using the fluorescent styryl dye FM1-43 to visualize synaptic vesicles exocytosis and endocytosis in motor nerve terminals. *Methods Mol Biol* 2011;689:137–148.
52. Mahammad S, Parmryd I. Cholesterol depletion using methyl- β -cyclodextrin. *Methods Mol Biol* 2015;1232:91–102.
53. Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci USA* 1999;96:6775–6780.
54. Rodal SK, Skretting G, Garred O, Vilhardt F, van Deurs B, Sandvig K. Extraction of cholesterol with methyl- β -cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell* 1999;10:961–974.
55. Manicone AM. Role of the pulmonary epithelium and inflammatory signals in acute lung injury. *Expert Rev Clin Immunol* 2009;5:63–75.
56. Oeckler RA, Lee WY, Park MG, Kofler O, Rasmussen DL, Lee HB, et al. Determinants of plasma membrane wounding by deforming stress. *Am J Physiol Lung Cell Mol Physiol* 2010;299:L826–L833.
57. Yalcin HC, Hallow KM, Wang J, Wei MT, Ou-Yang HD, Ghadiali SN. Influence of cytoskeletal structure and mechanics on epithelial cell injury during cyclic airway reopening. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L881–L891.
58. Ari A, Fink JB, Dhand R. Inhalation therapy in patients receiving mechanical ventilation: an update. *J Aerosol Med Pulm Drug Deliv* 2012;25:319–332.
59. MacIntyre NR, Silver RM, Miller CW, Schuler F, Coleman RE. Aerosol delivery in intubated, mechanically ventilated patients. *Crit Care Med* 1985;13:81–84.

60. Zhang Y, Lv F, Jin L, Peng W, Song R, Ma J, *et al.* MG53 participates in ischaemic postconditioning through the RISK signalling pathway. *Cardiovasc Res* 2011;91:108–115.
61. Weisleder N, Takeshima H, Ma J. Mitsugumin 53 (MG53) facilitates vesicle trafficking in striated muscle to contribute to cell membrane repair. *Commun Integr Biol* 2009;2:225–226.
62. Vlahakis NE, Hubmayr RD. Response of alveolar cells to mechanical stress. *Curr Opin Crit Care* 2003;9:2–8.
63. Belete HA, Hubmayr RD, Wang S, Singh RD. The role of purinergic signaling on deformation induced injury and repair responses of alveolar epithelial cells. *PLoS One* 2011;6:e27469.
64. Krishnan R, Klumpers DD, Park CY, Rajendran K, Treppe X, van Bezu J, *et al.* Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces. *Am J Physiol Cell Physiol* 2011;300:C146–C154.
65. Roan E, Wilhelm K, Bada A, Makena PS, Gorantla VK, Sinclair SE, *et al.* Hyperoxia alters the mechanical properties of alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L1235–L1241.
66. Bauer TT, Ewig S, Rodloff AC, Müller EE. Acute respiratory distress syndrome and pneumonia: a comprehensive review of clinical data. *Clin Infect Dis* 2006;43:748–756.
67. Yang ML, Wang CT, Yang SJ, Leu CH, Chen SH, Wu CL, *et al.* IL-6 ameliorates acute lung injury in influenza virus infection. *Sci Rep* 2017;7:43829.
68. Liang J, Zhang Y, Xie T, Liu N, Chen H, Geng Y, *et al.* Hyaluronan and TLR4 promote surfactant-protein-C-positive alveolar progenitor cell renewal and prevent severe pulmonary fibrosis in mice. *Nat Med* 2016;22:1285–1293.