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USP14 inhibitor IU1 prevents ventilator-induced lung injury in rats

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Abstract

The pathophysiology of ventilator-induced lung injury (VILI) involves multiple mechanisms including inflammation. USP14 removes the ubiquitin chain of I-κB, therefore inducing I-κB degradation and increasing cytokine release. The purpose of this study was to examine the protecting roles and mechanisms of USP14 inhibitor on I-κB expression and lung injury induced by high tidal volume ventilation in normal rat lung. Male Sprague-Dawley rats were divided into follows: Two ventilation modalities were used: rats in Groups LD (low volume + DMSO) and LI (low volume + IU1) received ventilation with a tidal volume of 8 ml/kg, while the rats in Groups HD (high volume + DMSO) and HI (high volume + IU1) were ventilated with a tidal volume of 40 ml/kg. The levels of lung wet-to-dry weight ratio were used as indicators of water metabolism in lung tissue; the detection of inflammatory cytokines in bronchoalveolar lavage (BAL) fluid was used to indicate inflammatory response, while lung injury was assessed by injury score and morphological changes under light microscope. The USP14 and I-κB protein level was measured in lung tissue by Western blot. Our results indicated that administration of IU1 alleviated ventilator-induced lung injury which was accompanied by reduced MPO activity, wet-to-dry weight ratio, lower TNF-α, IL-1β, IL-6 and IL-8 levels and increased I-κB expression in lung tissue. IU1 could significantly alleviate ventilator-induced rat lung injury by attenuate intrapulmonary inflammatory response.

Key words: Ventilator-induced lung injury, IU1, USP14 inhibitor.

Introduction

Mechanical ventilation (MV), a lifesaving intervention for acute respiratory failure patients, is widely used in general anesthesia (1). However, ventilator meanwhile is associated with unremitting high mortality of acute respiratory distress syndrome (ARDS) and remains a serious problem in the treatment of critically ill patients (2). In patients with severe ARDS, tidal volumes and pressures that exceed recommendations of the ARDS network are often applied, thereby contributing to ventilator-induced lung injury (VILI) (3).

Ventilator-induced lung injury is characterized by the release of inflammatory factors, leukocytes infiltration, alveolar edema, cellular necrosis, and tissue disruption. It is now commonly accepted that increased production of inflammatory factors, especially cytokines including tumor necrosis factor (TNF)-α, interleukin IL-1β, IL-6, and IL-8, plays a critical role in initiating or perpetuating lung injury (4, 5).

The ubiquitin-proteasome system is the major pathway of non-lysosomal intracellular protein degradation, playing an important role in a variety of cellular responses including cell division, proliferation, and apoptosis (6, 7). Ubiquitin-specific protease 14 (USP14) is a component of proteasome regulatory subunit 19S that regulates deubiquitinated proteins entering inside the proteasome core 20 S (8). The role of USP14 in protein degradation is still controversial. Several previous studies suggest that USP14 plays an inhibitory role in protein degradation and loss of USP14 might contribute to several neurodegenerative diseases (9, 10). Recent study reveals that USP14 has an opposite function. For example, USP14 could remove the ubiquitin chain of I-κB, to induce I-κB degradation and promote cytokine release in lung epithelial cells (11).

Our study was designed to evaluate the effect of USP14 inhibitor on lung injury induced by high tidal volume ventilation and I-κB expression. The lung injury was assessed by injury score and morphological changes under light microscope. The levels of lung wet-to-dry weight ratio were used as indicators of water metabolism in lung tissue. The detection of inflammatory cytokines in bronchoalveolar lavage (BAL) fluid was used to indicate inflammatory response. The USP14 and I-κB protein level was measured in lung tissue.

Materials and methods

Animals

This protocol was approved by the Animal Care and Use Committee of our Hospital. Male Sprague Dawley rats weighing 300 - 350 g were maintained in a controlled environment at a temperature of 20–25 and 50 ± 5% relative humidity under a 12-h dark/light cycle, and acclimatized for at least l week before use. The rats were then allocated into four groups: rats in Group LD were given intravenously 2 ml solvent DMSO (Sigma, USA) at 60 min before 4-hour ventilation with low tidal volume and a specific USP14 inhibitor IU1 (100μm) (Abcam, UK), which was dissolved in 2 ml DMSO, were given in Group LI. In Groups HD and HI, the same dose of DMSO and IU1(100μm) were administrated in the same manner as previously described before higher tidal volume ventilation, respectively.

Reagents

IU1 were purchased from Abcam (Cambridge, UK).

Myeloperoxidase (MPO) detection kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and 8 were detected using enzyme-linked immunosorbent assay with commercially available kits (Kangcheng Biotechnology, China) according to the manufacturer's instructions. Total protein level in BAL fluid was measured by method of Coomassie Brilliant Blue.

Experimental protocol

All rats were anesthetized with intraperitoneal administration of 80 mg/kg ketamine and 5 mg/kg of diazepam, then a jugular vein and carotid catheter was then placed. Each rat was orally intubated and mechanically ventilated. Two ventilation modalities were used, for 4 h each, the parameters were set as followings: rats in Groups LD and LI received ventilation with a tidal volume of 8 ml/kg, a respiratory rate of 40/min, I/E ratio 1:2, while the rats in Groups HD and HI were ventilated with a tidal volume of 40 ml/kg, a respiratory rate of 40/min, I/E ratio 1:2. Throughout the experiment, muscular relaxation was achieved by interval intravenous administration of 1.5 mg/kg atracurium, and anesthesia was maintained by interval injection of ketamine and diazepam. The carotid catheterwas kept for continuous sampling and blood pressuremonitoring with a PowerLab electrophysiolograph (AD Instruments, Australia). The ventilated mice received hourly 6-12 ml/kg warm Ringer's solution through jugular catheter to keep blood pressure.

Lung edema by wet-to-dry weight ratio

The left lung was excised, washed in PBS, gently dried using filter paper, and weighed. The tissue was then dried at 60°C for 72 h and reweighed. The change in the ratio of wet-to-dry weight was calculated as an indicator of lung edema formation.

Pulmonary myeloperoxidase (MPO) activity

Frozen lung tissue was homogenized in potassium phosphate buffer (20 mmol/L, pH 7.4) and centrifuged for 40 min. The pellet was resuspended in potassium phosphate buffer (50 mmol/L, pH 6.0) containing cetrimonium bromide. The enzyme activity was determined spectrophotometrically using a MPO detection kit purchased from Jiancheng Bioengineering Institute (Nanjing, China). according to the manufacturer's instructions. The absorbance change at 450 nm was monitored for 3 min with a spectrophotometer.

Histopathological examination

A small portion of lung was fixed with 10% neutral buffered formalin, dehydrated sequentially in 50% to 100% alcohol, and xylene solution was used for clearing samples. Then embedded in paraffin wax, and sectioned for routine histology. 4-um-thick sections were stained with H&E and examined by light microscopy.

Cytokines analysis

The right lung was lavaged 3 times using a single 5 ml aliquot of 4 °C fresh saline. 5 ml bronchoalveolar lavage fluid (BALF) was harvested and then centrifuged (1500 rpm, 10 min, 4 °C), and supernatants were frozen immediately on dry ice and stored at −80 °C for further detections. Single-analyte ELISA assays were performed for the measurement of cytokines (IL-1β, IL-6, IL-8, and TNF-a) in homogenized lung tissue, using enzyme-linked immunosorbent assay with kits purchased from Kangcheng Biotechnology according to the manufacturer's instructions.

Western blot

Lungs were lysed in Laemmli buffer (100 mM Tris-HCl at pH 6.8, 200 mM DTT, 4% SDS [w/v], 20% glycerol), denatured for 10 min at 80°C, and resolved on SDS/PAGE gels. After immunoblotting, the membranes were blocked with 5% nonfat milk. The membranes were then incubated with primary antibodies followed by HRP-linked secondary antibodies. The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit according to manufacturer's instructions. Antibodies directed against USP14, I-κB and β-actin were purchased from Santa Cruz Biotechnology (USA).

Quantitative PCR

Total RNA was extracted from rat lung tissues using the TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA). Reverse transcription of 2 μg RNA was carried out according to the instructions of Prime Script™ 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The real-time PCR reaction was conducted in 20 μl (SYBR® Premix Ex TaqTM, TaKaRa Japan). The result was normalized against GAPDH mRNA signal.

Statistical analyses

Statistical calculations were performed with software SPSS 13.0. Continuous, normally distributed data were presented as mean ±SD, one-way analysis of variance followed by Bonferroni method was used to compare differences between groups. A p value < 0.05 was considered to be statistically significant.

Results

Treatment with IU1 alleviated high volume ventilator-induced morphologic lesions in lung tissue

The lung specimens from groups receiving lower tidal volume showed no obvious morphological changes (Groups LD and LI) (Fig. 1A,B), while lung specimens from higher tidal volume treated with DMSO alone (Groups HD) animals displayed significant histological abnormalities, including infiltration of leukocytes into the interstitial spaces, hemorrhage, and marked swelling of the alveolar walls (Fig. 1C). These histological alterations were attenuated in IU1 treated mice (Groups HI), where less neutrophil infiltration and alveolar wall deformation were found, and interstitial edema was improved (Fig. 1D). Taken together, these data suggested that USP14 inhibitor IU1 could alleviate ventilator-induced morphologic lesions in lung tissue.

Treatment with IU1 reduced lung edema

One of the typical features of ventilator-induced lung injury is edema (4). In the present study, we found that higher tidal volume caused a marked increase in lung water content, showed that wet-to-dry weight ratio in Group HD was larger than Groups LD and LI. Howe-

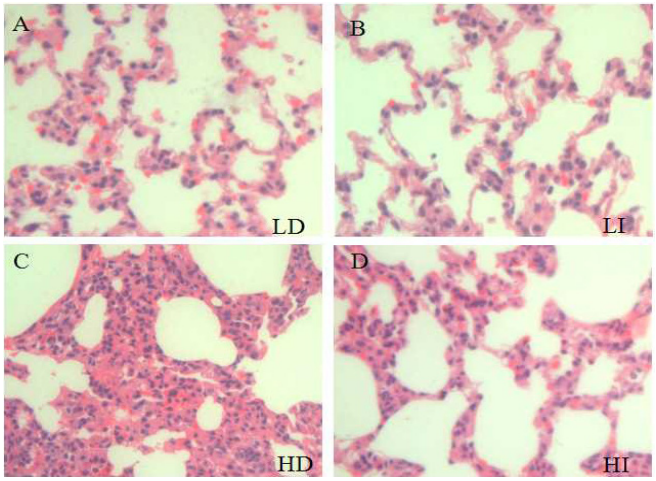

Figure 1. Treatment with USP14 inhibitor IU1 alleviated ventilator-induced morphologic lesions in rat lung tissue

Histopathological changes in the lung. Lung samples harvested after mechanical ventilation were processed and stained with hematoxylin and eosin (HE) for morphological evaluation. (A) LD group. (B) LI group. (C) HD group. (D) HI group. Representative lung sections of each group are shown.

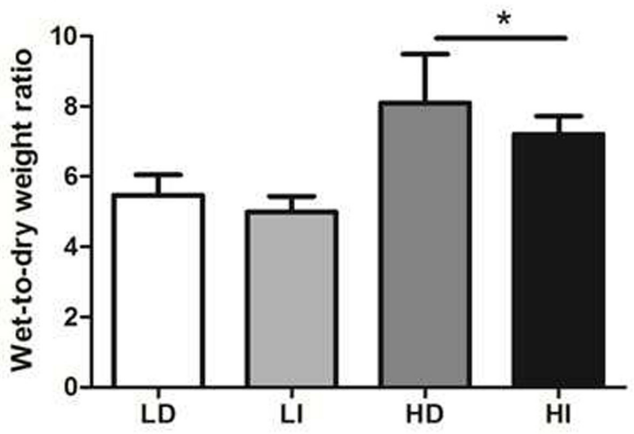

Figure 2. Treatment with IU1 reduced lung edema

The Wet/dry weight ration of lung tissues from rats with indicated treatment was measured. Data are expressed as mean ± SEM of the values of 5 rat of each group. * p<0.05 compared with the HD group.

ver, IU1 treatment (Group HI) significantly decreased the wet-to-dry weight ratio (HI vs HD, p <0.05) (Fig. 2). These data suggested that IU1 could reduce lung edema caused by ventilator-induced lung injury.

Treatment with IU1 reduced leukocyte infiltration

Next, the infiltration of neutrophils into the lung tissue was determined by measuring the activities of MPO, a reliable marker of neutrophil infiltration. In Group HD, the activity of MPO was obviously high than Groups LD and LI (Fig. 3). However, IU1 treatment (Group HI) significantly reduced the MPO activities in lung compared with Group HD (HI vs HD, p <0.05) (Fig. 3). These data suggested that IU1 could reduce leukocyte infiltration in lung caused by ventilator-induced lung injury.

Treatment with IU1 prevented high volume ventilator-induced inflammatory cytokines release in BALF

Cytokines, including TNF-α, IL-1β, IL-6, and IL-8 play critical roles in the pathogenesis of VILI (12, 13). We could obvious observed increased TNF-α, IL-1β, IL-6, and IL-8 release in Groups HD when compared with Groups LD and LI. However, IU1 treatment (Group HI) significantly prevented high volume ventilator-induced those inflammatory cytokines release in BALF (HI vs HD, p <0.05) (Figure. 4). These data indicated that IU1 could alleviate high volume ventilator-induced inflammatory response in lung tissue.

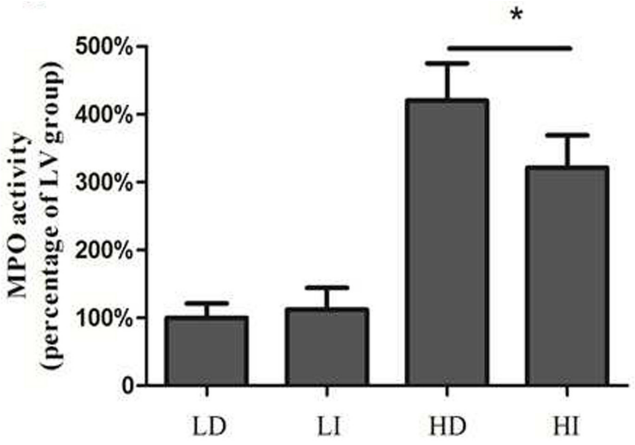

Figure 3. Treatment with IU1 reduced leukocyte infiltration

The myeloperoxidase (MPO) activity in lung tissues from rats with indicated treatment was measured. * p<0.05 compared with the HD group.

Treatment with IU1 blocked high volume ventilator-induced I-κB protein accumulation

To investigate the underline mechanism of the protective role of IU1 in high volume ventilator-induced inflammatory cytokines release, we determine the expression of I-κB protein in lung tissues. As expected, I-κB protein expression was obviously decreased in Group HD when compared with Groups LD and LI (p <0.05). However, IU1 treatment significantly blocked high volume ventilator-induced I-κB protein decrease (HI vs HD, p <0.05) (Figure.5A, 5B). However, the mRNA levels of I-κB were almost unchanged in all the groups (Figure.5C), suggesting that a post-translational regulation happened in Groups HD and HI. Interestingly, the mRNA levels of USP14 were also almost unchanged in all the groups (Figure.5D), suggesting that groups treated with high tidal volume, the activity of USP14, but not the protein level of USP14, may be increased that caused the decrease of IκB protein and increase of cytokine release, while IU1 treatment could reverse these events. Taken together, our data suggested that IU1-induced I-κB protein degradation might contribute to its protective role in high volume ventilator-induced inflammatory cytokines release.

Discussion

Neutrophil rolling along the endothelium may initiate a cascade of cellular interactions, resulting in endothelial damage and subsequent development of lung damage. In consistent with previous reports, our data showed that a marker for neutrophil influx, MPO activity, was greatly enhanced in ventilator-induced lung injury rats. Moreover, we could also observer neutrophil infiltration in histological sections of ventilator-induced lung injury rats. USP14 inhibitor IU1 not only decreased the activity of MPO but also alleviated ventilator-induced morphologic lesions in lung tissue of ventilator-induced lung injury rats. These results showed an

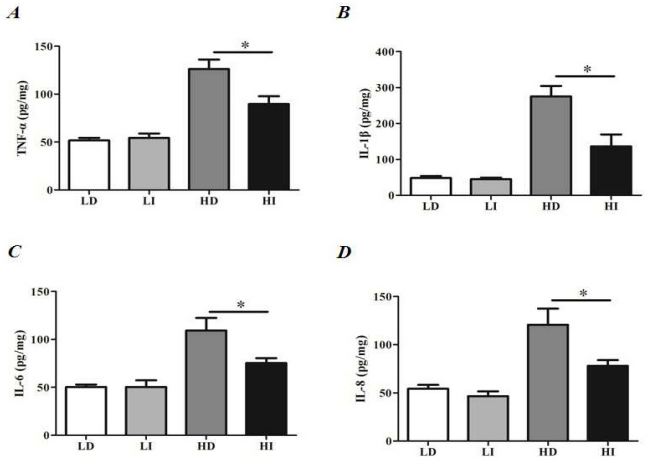

Figure 4. Treatment with IU1 prevented high volume ventilator-induced inflammatory cytokines release in BALF

(A) The concentration of TNF-α in BALF of rats with indicated treatment was monitored by ELISA. (B) The concentration of IL-1β in BALF of rats with indicated treatment was monitored by ELISA. (C) The concentration of IL-6 in BALF of rats with indicated treatment was monitored by ELISA. (D) The concentration of IL-8 in BALF of rats with indicated treatment was monitored by ELISA.

* $p<0.05$ compared with the HD group

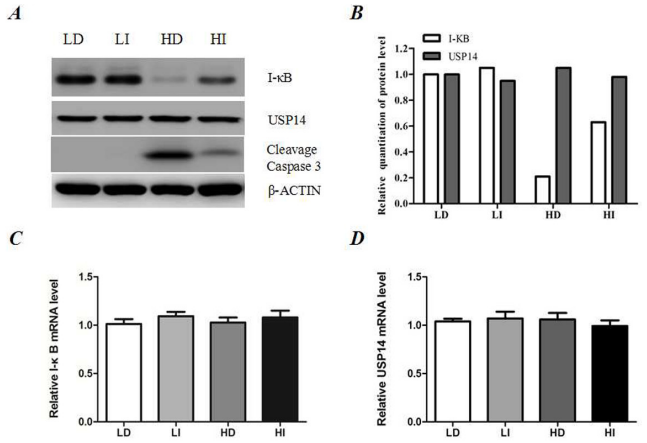

Figure 5. Treatment with IU1 blocked high volume ventilator-induced I-κB protein accumulation

(A) The proteins of rat lung tissues with indicated treatment were measured by Western blot with indicated antibodies. β-actin was used as loading control. (B) The protein levels of IκB and USP14 in (A) were quantified by the Quantity one software. (C) The mRNA levels of I-κB in rat lung tissues with indicated treatment were measured by Quantitative PCR. (D) The mRNA levels of USP14 in rat lung tissues with indicated treatment were measured by Quantitative PCR.

activated inflammatory response during injurious ventilation and IU1 could alleviate inflammatory and largely prevent ventilator-induced lung injury.

It's well accepted that activation of inflammatory mediators, including TNF-α, IL-1β, IL-6, and IL-8, play critical roles in the pathogenesis of VILI (13). Several studies suggest that TNF-α and IL-1β shared some effects, including neutrophil recruitment, stimulation of chemokine release, and upregulation of adhesion molecules (14). IL-6 plays a role in the development of distal organ failure in ventilator-induced lung injury (15). IL-8 has also been proposed to play an important role in the progression of VILI by activating neutrophils (16).

The nuclear factor NF-κB pathway has long been considered a proinflammatory signaling pathway that regulates the expression of proinflammatory genes including cytokines and chemokines. In an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE) to activate downstream genes transcription and cytokines secretion (17). Our data showed that high volume ventilator-induced lung injury resulted in decreased expression of I-κB and enhanced inflammatory cytokines expression. It has been reported that USP14 regulates cytokine release via modulating I-κB stability (11). Indeed, our data agreed with previous study that IU1 could induce I-κB protein degradation. The administration of USP14 inhibitor IU1 resulted in decreased activation of NF-κB signaling pathway that may contribute to its anti-VILI roles.

In conclusion, our study described the anti-inflammatory effect of USP14 specific-inhibitor IU1 in high volume ventilator-induced lung injury. IU1 treatment could significantly alleviate high volume ventilator-induced lung injury. Our results suggest that the administration of IU1 seems a promising anti-inflammatory strategy to regulate the release of inflammatory factors and control ventilator-induced lung injury.

References

1. Durbin, C. J., Blanch, L., Fan, E., and Hess, D. R., Respiratory care year in review 2013: airway management, noninvasive monitoring, and invasive mechanical ventilation. *Respir Care* 2014, **59**: 595-606. doi: 10.4187/respcare.03199

2. Cabrera-Benitez, N. E., Laffey, J. G., Parotto, M., Spieth, P. M., Villar, J., Zhang, H., and Slutsky, A. S., Mechanical Ventilation-associated Lung Fibrosis in Acute Respiratory Distress Syndrome: A Significant Contributor to Poor Outcome. *Anesthesiology* 2014, **1**: 189-198. doi: 10.1097/ALN.0000000000000264

3. Helmi, M., and Gommers, D., Less invasive mechanical ventilation strategies in ARDS: the future? *Acta Med Indones* 2013, **45**: 329-336.

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

5. Marini, J. J., Ventilator-associated problems related to obstructive lung disease. *Respir Care* 2013, **58**: 938-949. doi: 10.4187/respcare.02242

6. Amm, I., Sommer, T., and Wolf, D. H., Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. *Biochim Biophys Acta* 2014, **1843**: 182-196. doi: 10.1016/j.bbamcr.2013.06.031

7. Chitra, S., Nalini, G., and Rajasekhar, G., The ubiquitin proteasome system and efficacy of proteasome inhibitors in diseases. *Int J Rheum Dis* 2012, **15**: 249-260. doi: 10.1111/j.1756-185X.2012.01737.x

8. Borodovsky, A., Kessler, B. M., Casagrande, R., Overkleeft, H. S., Wilkinson, K. D., and Ploegh, H. L., A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. *EMBO J* 2001, **20**: 5187-5196. doi: 10.1093/emboj/20.18.5187

9. Wilson, S. M., Bhattacharyya, B., Rachel, R. A., Coppola, V., Tes-

sarollo, L., Householder, D. B., Fletcher, C. F., Miller, R. J., Copeland, N. G., and Jenkins, N. A., Synaptic defects in ataxia mice result from a mutation in Usp14, encoding a ubiquitin-specific protease. *Nat Genet* 2002, **32**: 420-425. doi: 10.1038/ng1006

10. Lee, B. H., Lee, M. J., Park, S., Oh, D. C., Elsasser, S., Chen, P. C., Gartner, C., Dimova, N., Hanna, J., Gygi, S. P., Wilson, S. M., King, R. W., and Finley, D., Enhancement of proteasome activity by a small-molecule inhibitor of USP14. *Nature* 2010, **467**: 179-184. doi: 10.1038/nature09299

11. Mialki, R. K., Zhao, J., Wei, J., Mallampalli, D. F., and Zhao, Y., Overexpression of USP14 protease reduces I-kappaB protein levels and increases cytokine release in lung epithelial cells. *J Biol Chem* 2013, **288**: 15437-15441.

12. Dechert, R. E., Haas, C. F., and Ostwani, W., Current knowledge of acute lung injury and acute respiratory distress syndrome. *Crit Care Nurs Clin North Am* 2012, **24**: 377-401. doi: 10.1016/j.ccell.2012.06.006

13. van Wessem, K. J., Hennus, M. P., van Wagenberg, L., Koenderman, L., and Leenen, L. P., Mechanical ventilation increases the inflammatory response induced by lung contusion. *J Surg Res* 2013, **183**: 377-384. doi: 10.1016/j.jss.2012.12.042

14. Leal, M. C., Casabona, J. C., Puntel, M., and Pitossi, F. J., Interleukin-1beta and tumor necrosis factor-alpha: reliable targets for protective therapies in Parkinson's Disease? *Front Cell Neurosci* 2013, **7**: 53. doi: 10.3389/fncel.2013.00053

15. Gurkan, O. U., He, C., Zielinski, R., Rabb, H., King, L. S., Dodd-o, J. M., D'Alessio, F. R., Aggarwal, N., Pearse, D., and Becker, P. M., Interleukin-6 mediates pulmonary vascular permeability in a two-hit model of ventilator-associated lung injury. *Exp Lung Res* 2011, **37**: 575-584. doi: 10.3109/01902148.2011.620680

16. Iwaki, M., Ito, S., Morioka, M., Iwata, S., Numaguchi, Y., Ishii, M., Kondo, M., Kume, H., Naruse, K., Sokabe, M., and Hasegawa, Y., Mechanical stretch enhances IL-8 production in pulmonary microvascular endothelial cells. *Biochem Biophys Res Commun* 2009, **389**: 531-536. doi: 10.1016/j.bbrc.2009.09.020

17. Lawrence, T., The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 2009, **1**: a001651.