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Simvastatin mitigates ventilator-induced lung injury in mice with acute respiratory distress syndrome via a mechanism partly dependent on neutrophil extracellular traps

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Abstract

Background Mechanical ventilation (MV) is an essential life support for patients with acute respiratory distress syndrome (ARDS). However, mechanical ventilation in patients with ARDS can cause ventilator-induced lung injury (VILI). Simvastatin can alleviate acute lung injury by anti-inflammatory and enhancing endothelial barrier. The present study aimed to evaluate whether simvastatin could attenuate VILI in mice with ARDS.

Methods Mice were randomized into six groups: the sham (S), LPS (L), MV (V), LPS/MV (LV), LPS/MV/simvastatin (MS) and LPS/MV/GSK484 (MG) groups. The mice in the L group received LPS but not ventilation, the mice in the V group received only MV, and the mice in the LV, MS and MG groups received LPS and MV. Additionally, MS group were treated with simvastatin, MG group were treated with GSK484, and the other mice were injected with saline, starting three days prior to mechanical ventilation. The PaO_2/FiO_2 ratio and wet–dry weight ratio were calculated. Histopathological changes were observed, and injury scores were calculated. Inflammatory factor levels in the bronchoalveolar lavage fluid (BALF) were detected. Peptidylarginine deiminase 4 (PAD4), neutrophil elastase (NE) and citrullinated histone 3 (Cit-H3) in the lung tissue were detected, apoptosis were also evaluated.

Results All indices were improved in group S compared with the other groups. The lung injury score and wet–dry weight ratio were lower, the PaO_2/FiO_2 ratio was greater, inflammatory factor levels in the BALF were lower, PAD4, NE, and Cit-H3 expression was lower, and apoptosis was decreased in the MS and MG groups compared with the LV group.

Conclusions Simvastatin attenuated VILI in mice with ARDS, potentially via reductions in neutrophil extracellular traps (NETs) generation and apoptosis.

Keywords Mechanical ventilation, Neutrophil extracellular traps, Simvastatin, Acute respiratory distress syndrome

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Introduction

Acute respiratory distress syndrome (ARDS) is a clinical syndrome characterized by progressive dyspnea and intractable hypoxemia that accounts for more than 10% of hospitalizations in the intensive care unit, but no established therapeutic agents are available [1]. A recent retrospective analysis of approximately 459 ICUs in 50 countries indicated that the severity of ARDS was mild (30.0%), moderate (46.6%), or severe (23.4%) [2].

Mechanical ventilation (MV) is widely used in patients under clinical anesthesia and in intensive care. For patients with lung injury, especially those with ARDS, MV is a necessary support measure for preventing hypoxemia and alveolar collapse and even reducing pulmonary edema, but it is represents an ongoing danger [3]. MV is an external force that can induce or worsen lung damage by interfering with the natural process of breathing. Clinical studies have shown that approximately 24% of patients under MV have excessively dilated alveoli due to mechanical traction, which causes inflammation and induces or exacerbates lung injury; this condition is known as ventilator-induced lung injury (VILI) [4]. Therefore, the effective prevention and treatment of VILI in ARDS patients are critical for clinical practice.

Neutrophil extracellular traps (NETs), which are released by highly activated neutrophils in response to various stimuli, consist of a network containing DNA skeletons and a variety of proteins, such as neutrophil elastase (NE), myeloperoxidase (MPO) and other granule proteins [5]. NETs are widely involved in the development of various diseases, such as thrombotic, autoimmune and infectious diseases [6]. Relevant studies have proven that NETs play a vital role in the development of acute lung injury [7]. Peptidylarginine deiminase 4 (PAD4), which contains five specific calcium binding sites, is an enzyme that is mainly localized in the nucleus of granulocytes and plays a central role in the formation of NETs [8].

Simvastatin, which suppresses hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme reductase, attenuates ARDS by inhibiting the migration of inflammatory cells and the release of inflammatory factors [9]. In recent years, simvastatin has been shown to reduce asthma severity by decreasing NET production through the inhibition of PAD4 [10]. At the same time, simvastatin has also shown anti-apoptotic effects in lung protection and heart protection studies [11, 12].

Given the critical role of NETs in acute lung injury and VILI, and the possible association of simvastatin with lung disease and NETs, we hypothesized that simvastatin could alleviate VILI in a murine ARDS model by inhibiting NET formation, at least in part via PAD4 suppression. Therefore, we chose to compare the selective PAD4 inhibitor GSK484 with simvastatin to prove its relevant effect.

Methods

Study design

Seventy-two C57BL/6 mice (provided by Jinan Pengyue Experimental Animal Breeding Co. LTD, Jinan, China) were randomized into six groups: the sham group (S), lipopolysaccharide (LPS) group (L), MV group (V), LPS/MV group (LV), LPS/MV/simvastatin group (MS) and LPS/MV/GSK484 group (MG) ($n = 12$).

All mice were anesthetized with 2% pentobarbital sodium (30 mg/kg via intraperitoneal injection). Mice in the MG group were intraperitoneally injected with 4 mg/kg GSK484 (Abcam, USA) once per day three days before modeling [13]; mice in the MS group were intraperitoneally injected with 20 mg/kg simvastatin (Sigma, Germany) once per day three days before modeling [9, 14], and mice in the other groups were given the same amount of saline each day. LPS (5 ug) (Sigma, USA) was injected intratracheally into mice in the L, VL, MG and MS groups to establish the ARDS model, and the same amount of saline was injected into mice in the S and V groups. ARDS establishment was considered successful when the PaO_2/FiO_2 ratio was less than 300 [15]. Two hours later, the mice were intubated, and the mice in groups S and L were autonomously ventilated, while the mice in the other groups were mechanically ventilated (ventilator parameters: tidal volume 20 ml/kg, respiratory rate 45 times/min, inspiratory expiratory ratio 1:2, positive end-expiratory pressure 0 mmHg, inhaled oxygen concentration 21%) for a total of 4 h [16]. During the MV procedure, anesthesia was maintained with 2% pentobarbital sodium (30 mg/kg) and 0.6 mg/kg rocuronium per hour.

All mice were killed via an overdose of anesthetics after 4 h of ventilation, and lung tissue and blood were collected.

Alveolar-capillary permeability

We performed arterial blood gas analysis with a Cobasb123 blood gas analyzer (Roche, Switzerland) to calculate the PaO_2/FiO_2 ratio. Arterial blood was collected from the abdominal aorta after four hours of mechanical ventilation and subjected to blood gas analysis. Moreover, we detected the protein concentration and lung tissue wet/dry weight ratio. Part of the right upper lung tissue was collected, weighed and then dried at 60 °C for 48 h. The lung tissue wet/dry weight ratio was calculated. We also tested the protein levels in BALF collected through the left lung using the bicinchoninic acid (BCA) method.

Histopathologic lung injury

The middle lobe of the right lung was fixed in 4% paraformaldehyde for 48 h, routinely embedded in paraffin, sectioned, and stained with hematoxylin–eosin. Then, the lung tissue sections were observed under a light microscope to determine the histopathological changes in the lungs and to evaluate lung injury. The scoring criteria included four observation items and five levels of scoring. The observation items included alveolar fibrin/edema, alveolar hemorrhage, septal thickening, and cellular infiltration. The sections were evaluated by two pathologists who did not participate in this study. The scoring grades were as follows: no change or slight change, 0; mild change (less than 25% involved), 1; moderate change (25–50% involved), 2; severe change (50–75% involved), 3; and very severe change (more than 75% involved), 4. The sum of the scores of the 4 observations was the lung injury score [17].

Analysis of local and systemic inflammation

The supernatant was frozen at −80 °C and centrifuged, after which the concentrations of IL-1β, IL-6 (Wuhan Hua Mei Bioengineering, China), TNF-α, and MPO (Abcam, USA) in the BALF and peripheral blood samples were determined via ELISA. Furthermore, BALF deposits were stained with Giemsa by an independent pathologist to count the neutrophils.

Western blotting

The lower lobe of the right lung was frozen at −80 °C, placed on an ice box and homogenized using protein lysis buffer and a protease inhibitor. Then, the lung tissues were centrifuged at 4 °C and 2,000 rpm for 10 min (centrifugation radius of 8 cm). The supernatant was collected. The protein concentration was tested with the BCA assay. The corresponding volume of the sample was calculated. SDS-PAGE was performed, and the separated proteins were subsequently transferred to a PVDF membrane, which was blocked with skim milk for 90 min. The PVDF membranes were incubated with the following primary antibodies overnight at 4 °C: PAD4 (dilution 1:1,000, Abcam, USA), NE (dilution 1:1,000, Abcam, USA), Cit-H3 (dilution 1:1,000, Abcam, USA), Bcl-2 (dilution 1:500, Beyotime, China), Bax (dilution 1:1,000, Abcam, USA), and the internal reference β-actin (dilution 1:5,000, Immunoway, USA). The membranes were washed with TBST for 10 min, and the process was repeated three times. The membranes were incubated with a goat anti-rabbit (dilution 1:10,000; Immunoway, USA) secondary antibody for 90 min. The membranes were washed three times with TBST for 10 min each and exposed to the visualizer. After exposure, the gray values were determined by ImageJ software, and the ratio of the gray value of the target protein to the gray value of the internal reference protein was used to determine the protein expression level.

Immunohistochemistry

The expression level of PAD4 was detected via peroxidase-labeled streptavidin staining of paraffin sections. The paraffin sections were dewaxed and hydrated, subjected to antigen retrieval with EDTA, and then incubated overnight at 4 °C with a PAD4 primary antibody (1:1,000 dilution; Abcam, USA). The paraffin sections were washed with PBS for 3 min, and this process was repeated three times. Then, the paraffin sections were incubated with goat anti-rabbit IgG polymer at 37 °C for 30 min and washed 3 times with PBS for 3 min. DAB was added to the paraffin sections, which were incubated at room temperature for 8 min. The paraffin sections were subjected to hematoxylin staining, dehydration, and fixation.

Immunofluorescence analysis

The lung tissue sections were dewaxed and hydrated. Citric acid was used for antigen repair. The lung tissue sections were incubated with Cit-H3 primary antibodies (1:1,000 dilution; Abcam, USA) at 4 °C overnight. Then, the slices were washed three times with PBS for 3 min each. Fluorescent Coralite 488-labeled goat anti-rabbit antibodies (Proteintech, China) were added dropwise, the samples were incubated at 37 °C for 30 min, and the slices were washed three times with PBS for 3 min each. DAPI was added to the slices. The level of protein expression was observed under a fluorescence microscope (Nikon, Japan), and the colocalization of proteins was quantified using Image J software.

Apoptosis assay

According to the instruction manual of the TUNEL assay kit (Beyotime, China), the paraffin sections were dewaxed, hydrated, repaired, blocked, stained, restained and sealed. Then, the paraffin sections were placed under a fluorescence microscope for observation, and images were taken to determine the level of apoptosis.

Statistical analysis

Statistical analyses were performed, and graphs were generated using GraphPad Prism 7.0 software (GraphPad, La Jolla, CA). All normally distributed data are presented as the means ± standard deviation (SD). Analysis of variance with the Tukey honestly significant difference test for post hoc analyses was used to compare data between more than 2 groups. Comparisons between groups were made using one-way ANOVA. All statistical

analyses were per formed using SPSS 26.0 for Windows (SPSS, Inc., USA). $P < 0.05$ was considered to indicate statistical significance.

Results

Effects of simvastatin on alveolocapillary permeability

The PaO_2/FiO_2 ratio, total protein concentration in the BALF and lung tissue wet/dry weight ratio were calculated to evaluate the effect of simvastatin on alveolocapillary permeability. As expected, compared with those in group S, the wet/dry weight ratio and protein concentration were greater, and the PaO_2/FiO_2 ratio was lower in group L, group V, group LV, group MS and group MG ($P < 0.05$). Compared with those in group L and group V, the wet/dry weight ratio and protein concentration were greater, and the PaO_2/FiO_2 ratio was lower in group LV ($P < 0.05$). As expected, compared with those in the LV group, the wet/dry weight ratio and protein concentration in the MS and MG groups were lower, and the PaO_2/FiO_2 ratio was greater ($P < 0.05$) (Fig. 1).

Effects of simvastatin on lung histopathology

Hematoxylin–eosin staining of the lung tissue sections was examined under a light microscope. There was no significant damage to the lung tissue in group S, and the thickness of the alveolar wall was evenly distributed, with few inflammatory secretions. In group L, group V and group LV, severe inflammatory reactions were observed, with obvious thickening of the bronchial and alveolar walls, the rupture of part of the alveolar wall, and the exudation of erythrocytes and inflammatory secretions in the alveolar lumen. However, compared with that in group L, group V and group LV, the damage in group MS and group MG was alleviated, and there was less alveolar destruction and less erythrocyte and inflammatory secretion exudate in the alveolar lumen (Fig. 2).

Effects of simvastatin on local and systemic inflammation

First, we detected the inflammatory cell counts and cytokine concentrations in the BALF to estimate the effect of simvastatin on local inflammation.

Compared with those in group S, the concentrations of IL-1β, IL-6 and TNF-α and neutrophil counts were greater in group L, group V, group LV, group MS and group MG ($P < 0.05$); compared with those in group L and group V, the concentrations of IL-1β, IL-6 and TNF-α and neutrophil counts were greater in group LV ($P < 0.05$). As expected, the concentrations of IL-1β, IL-6 and TNF-α and the neutrophil count were lower in the MS and MG groups than in the LV group ($P < 0.05$) (Fig. 3).

Second, we detected serum cytokine levels to observe the effect of simvastatin on systemic inflammation. Similar to the results obtained by analysis of the BALF, the serum IL-1β, IL-6 and TNF-α levels were significantly increased after ventilation and LPS injection, and this increase was decreased by simvastatin.

The production of NETs was reduced by simvastatin

The purpose of our study was to examine the effects of simvastatin on the production of NETs. Therefore, the concentration of PAD4 was determined in lung tissue sections by immunohistochemistry (Fig. 4a). In addition, the levels of Cit-H3 were measured by immunofluorescence analysis to determine the effect of PAD4 on histone citrullination (Fig. 4b).

For further verification, the expression of PAD4, NE and Cit-H3 in lung tissue was detected by Western

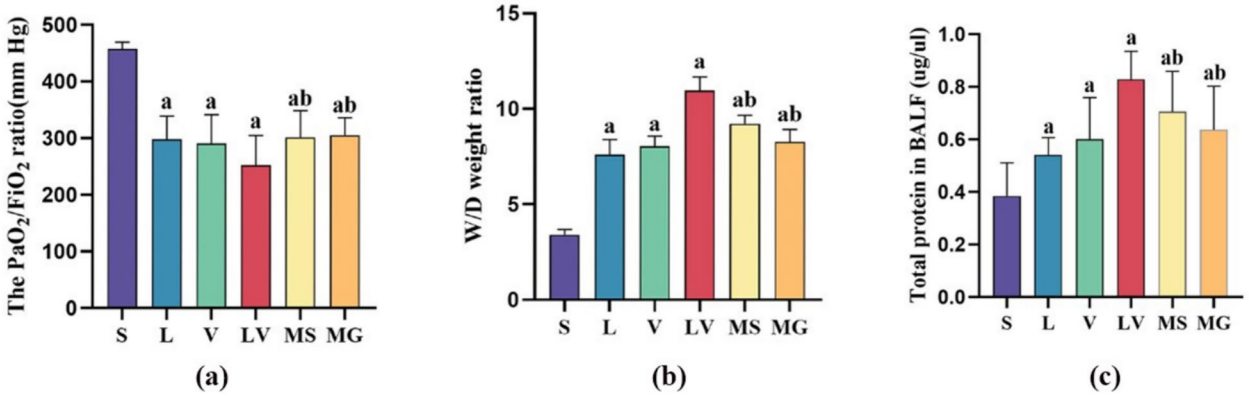


Fig. 1 Effects of simvastatin on alveolocapillary permeability. **a** The PaO_2/FiO_2 ratio were assessed at the end of 4 h ventilation. **b** The wet/dry weight ratio were assessed. **c** The total protein concentration in the BALF were assessed. The data are presented as the means ± SD. **a** $P < 0.05$ vs. the S group; **b** $P < 0.05$ vs. the LV group. $N = 12$ per group

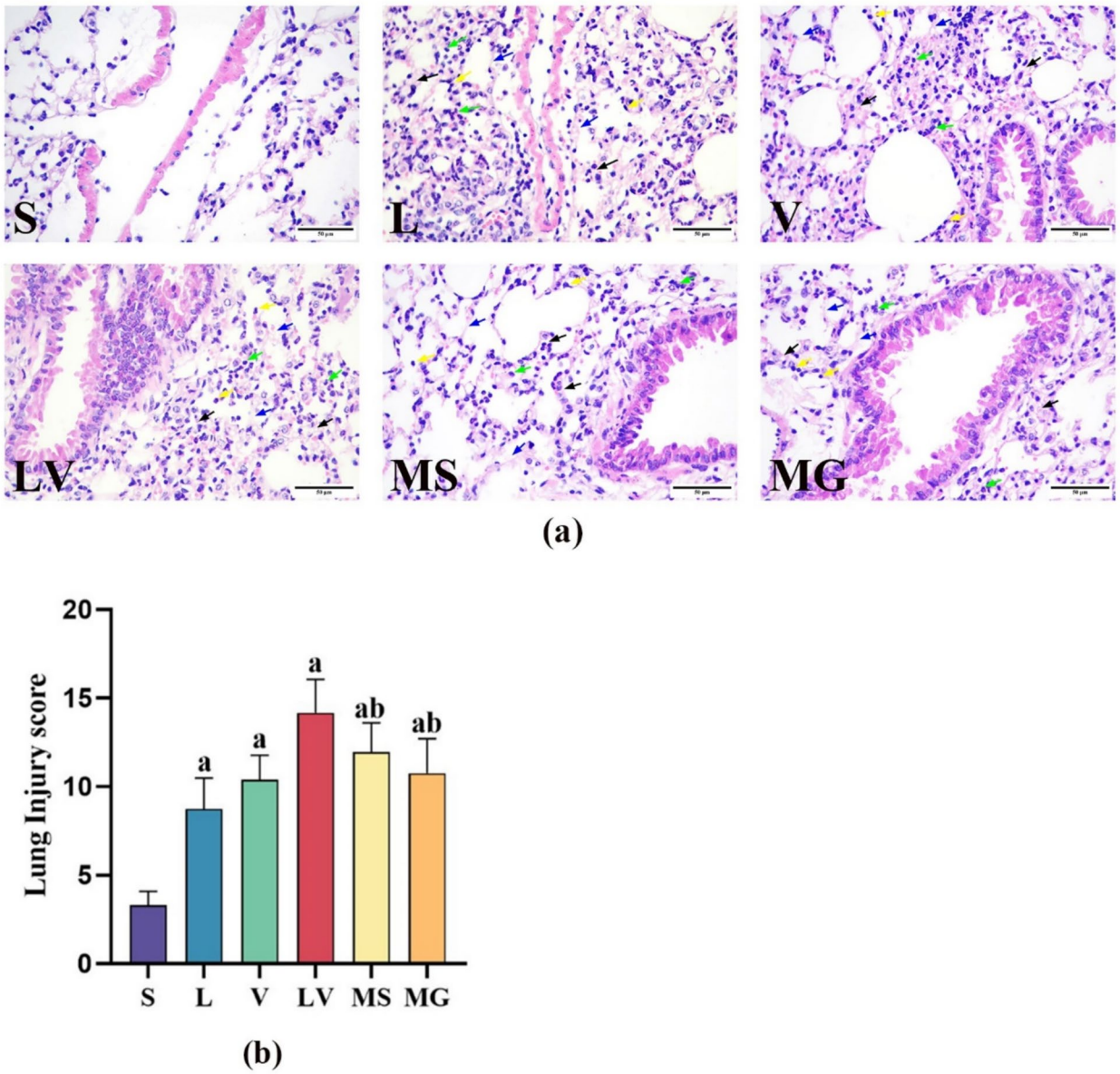


Fig. 2 Effects of simvastatin on lung histopathology. **a** Lung tissue injury was evaluated by HE staining (scale bar: 50 μm). **b** Lung injury scores were assessed. The data are presented as the means ± SD. **a** $P < 0.05$ vs. the S group; **b** $P < 0.05$ vs. the LV group. $N = 12$ per group

blot analysis. The expression of MPO in the BALF was also determined. Compared with those in group S, the expression levels of PAD4, NE, Cit-H3 and MPO were increased in group L, group V, group LV, group MS and group MG ($P < 0.05$). Compared with those in group L and group V, the expression levels of PAD4, NE, Cit-H3 and MPO were increased in group LV ($P < 0.05$). As expected, the expression of PAD4, NE, Cit-H3 and MPO was lower in the MS and MG groups than in the LV group ($P < 0.05$) (Fig. 4c).

Effects of simvastatin on apoptosis

To clarify the effects of simvastatin on apoptosis, lung tissue sections were analyzed by TUNEL staining. The results indicated that cell death was severe in the lung tissue of the LV group. The severity of apoptosis was significantly reduced in the MS and MG groups (Fig. 5a).

In addition, the expression of Bcl-2 and Bax in the lung tissue was detected by Western blot analysis. Compared with that in group S, the expression of Bax was increased in group L, group V, group LV, group MS and group MG ($P < 0.05$). Compared with that in group L and group V, the expression of Bax was increased in group LV ($P <$

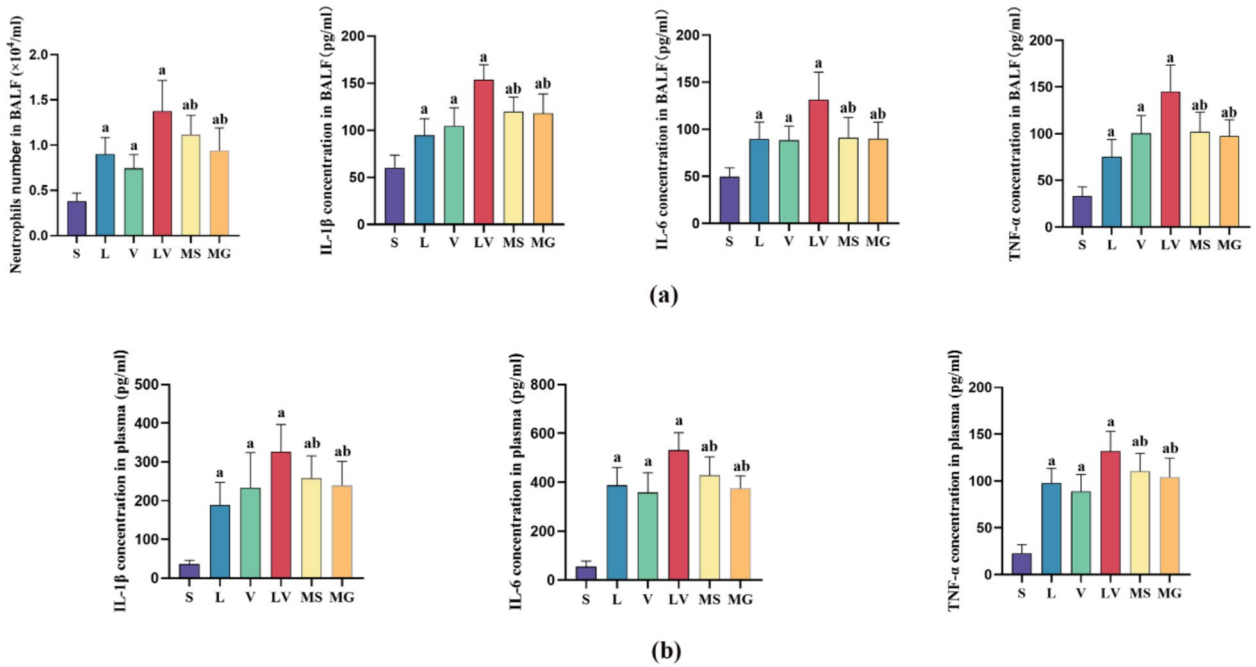


Fig. 3 Effects of simvastatin on inflammation. **a** The levels of TNF-α, IL-1β, and IL-6 in BALF were determined via ELISA. The number of neutrophils in the BALF was also detected. **b** The levels of TNF-α, IL-1β, and IL-6 in serum were determined via ELISA. The data are presented as the means ± SDs. **a** $P < 0.05$ vs. the S group; **b** $P < 0.05$ vs. the LV group. $N = 12$ per group

0.05). As expected, the expression of Bax was lower in the MS and MG groups than in the LV group ($P < 0.05$). Moreover, the change in the expression of Bcl-2 was the opposite of that of Bax (Fig. 5b).

Discussion

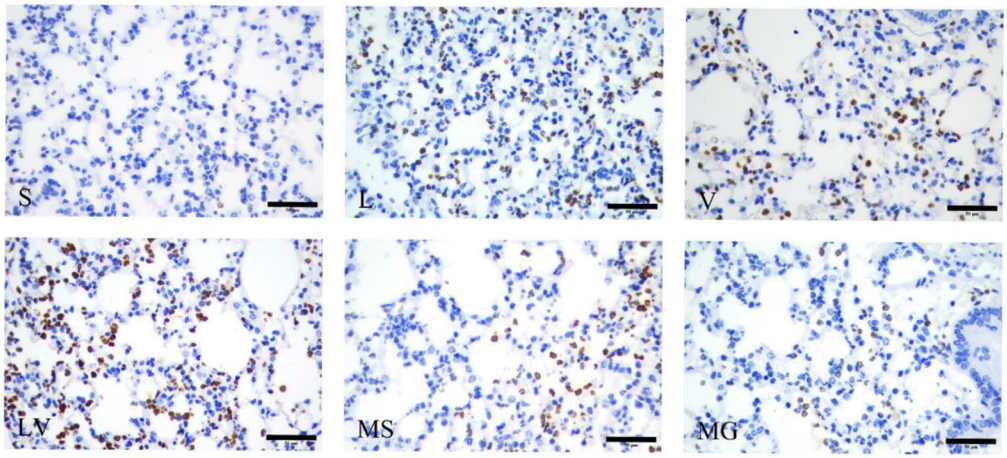
The results of this study suggest that pretreatment with simvastatin can alleviate VILI in mice with ARDS and that this effect may be achieved via a reduction in NETs. Simvastatin significantly improved alveolar capillary permeability and endothelial function in mice, alleviated inflammation and inhibited VILI-induced apoptosis; furthermore, simvastatin significantly reduced NET levels by inhibiting PAD4 expression.

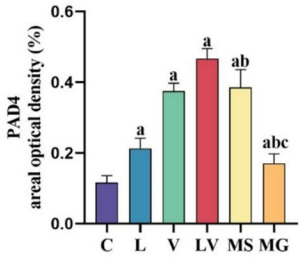
In clinical practice, patients with lung injury often require MV support. Approximately 75% of ARDS patients need MV, but MV can also aggravate lung damage in patients, leading to VILI [2]. Despite lung protection strategies, the mortality rate of ARDS patients is still as high as 45% [18]. In this study, we used three trauma groups: group L (LPS only), group V (MV only) and group LV (LPS+ ventilation). The results showed that the L and V groups did not exhibit injury as severe as that in the LV group, which could verify how LPS and high tidal volume mechanical ventilation synergistically aggravate lung injury and is more clinically relevant.

Statins exert anti-inflammatory and immunomodulatory effects on several diseases [19], and simvastatin has been shown to be beneficial in the treatment of both severe asthma and sepsis. Therefore, we studied the effect of simvastatin on VILI and the possible underlying mechanism by administering large tidal volume ventilation to mice with ARDS.

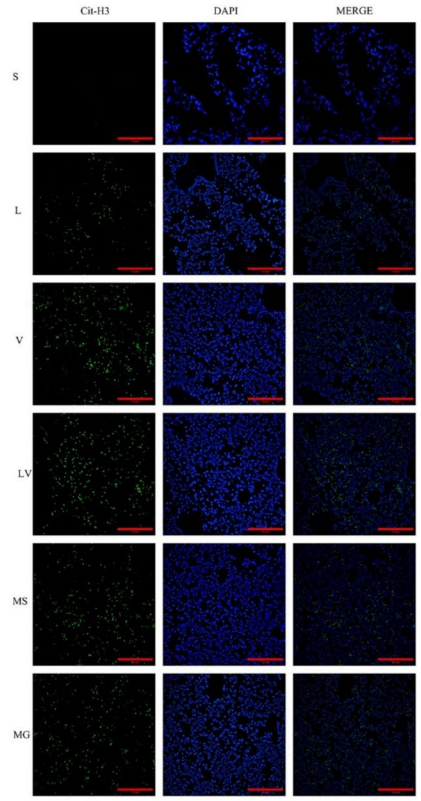
In the present study, after early administration of simvastatin, the PaO_2/FiO_2 ratio significantly increased, while the lung wet–dry weight ratio decreased. Furthermore, we found that simvastatin significantly reduced lung tissue damage. These results indicated that simvastatin significantly improved alveolar capillary permeability, alleviated pulmonary edema and improved alveolar structural damage in ARDS mice with VILI.

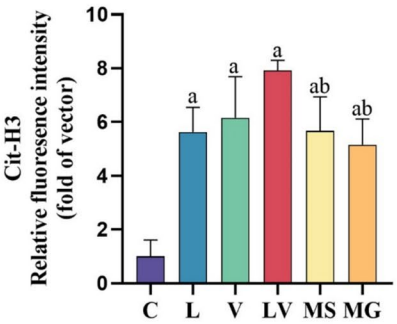
During inflammation associated with VILI and ARDS, neutrophils are recruited to the lung tissue, where they release large amounts of NETs, damaging lung endothelial and epithelial cells and promoting the secretion of inflammatory factors such as TNF-α, IL-1β and IL-6 [20]. Moreover, these inflammatory factors further recruit inflammatory cells from the peripheral blood to injured lung tissue. These cells infiltrate the injured lung tissue, release more cytokines into the lung tissue or peripheral blood, and cause systemic inflammatory response syndrome [10]. The results showed that simvastatin significantly reduced the





(a)





(b)

Fig. 4 The production of NETs was reduced by simvastatin. **a** The expression of PAD4 in lung tissue sections was detected by immunohistochemistry (scale bar: 50 μm). **b** Representative immunofluorescence images of CitH3 and DAPI staining (scale bar: 50 μm). **c** The NET-associated proteins PAD4, NE, Cit-H3 and MPO were determined. The data are presented as the means ± SD. **a** $P < 0.05$ vs. the S group; **b** $P < 0.05$ vs. the LV group; **c** $P < 0.05$ vs. the MS group. $N = 12$ per group

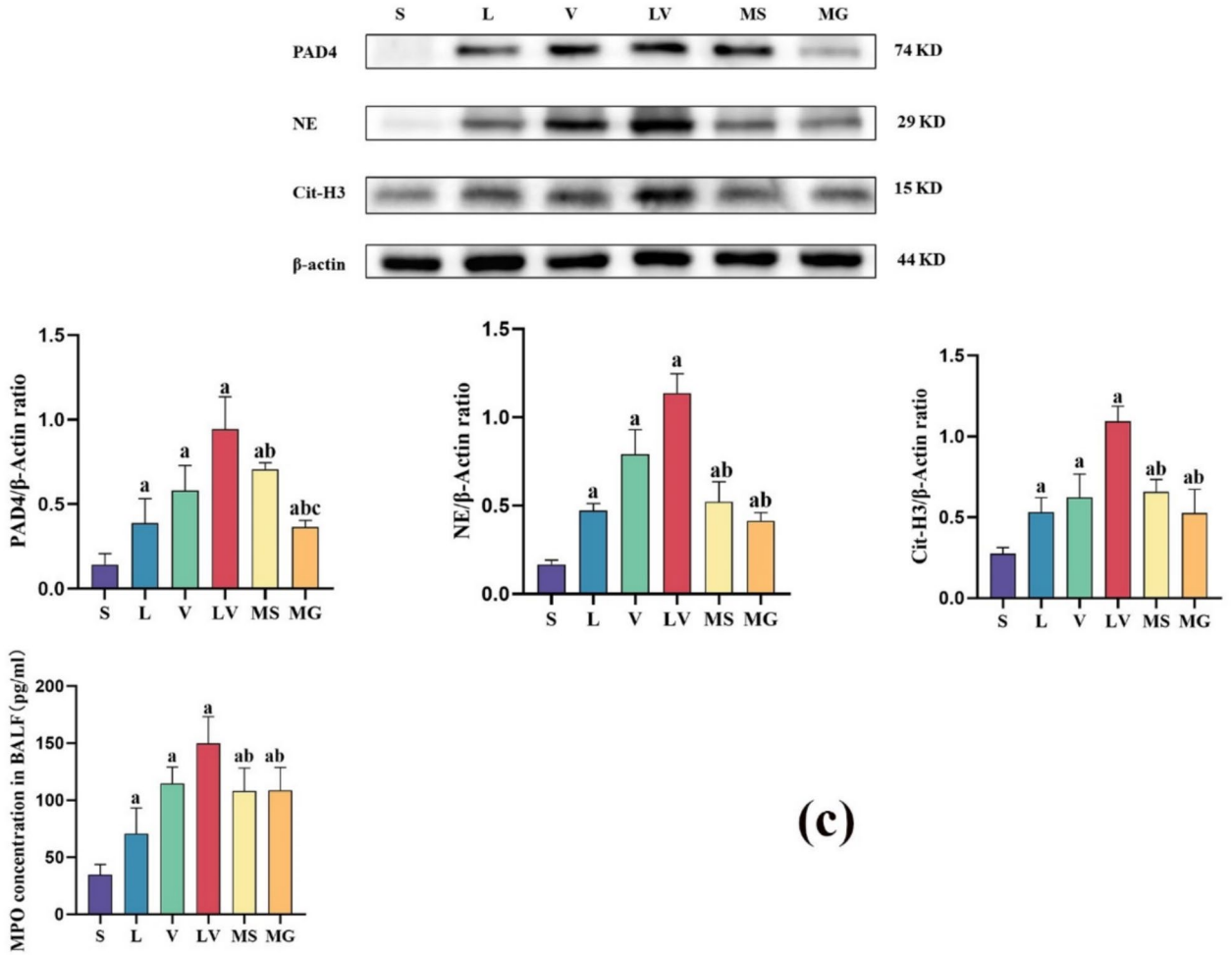


Fig. 4 continued

expression of the proinflammatory factors TNF-α, IL-1β and IL-6. The critical role of NETs in the inflammatory response to lung injury has been demonstrated by several studies [21, 22]. In our study, compared with those in the LV group, the expression levels of PAD4, Cit-H3 and NE, which are hallmark products of NET formation, in the MS group were significantly lower, and the MPO concentration in the BALF was significantly lower. Therefore, we concluded that simvastatin reduced VILI in mice with ARDS by reducing neutrophil infiltration and NET production.

Endothelial and epithelial cell apoptosis play important roles in VILI and ARDS. During VILI and ARDS, the proinflammatory factors TNF-α and ROS, which are released from neutrophils, and proinflammatory cytokines lead to apoptosis by activating endogenous and exogenous apoptotic pathways [23, 24]. As the most important apoptosis regulatory protein, Bax plays a crucial role in apoptosis, and Bcl-2 is an important anti-apoptotic protein that can inhibit apoptosis mediated by Bax [25]. The results showed that simvastatin significantly decreased the expression of the proapoptotic protein Bax and increased the expression of the anti-apoptotic protein Bcl-2. Therefore, the inhibitory effect of simvastatin on apoptosis may partly depend on the regulation of apoptosis-related proteins. Moreover, TUNEL results showed that the number of apoptotic cells in MG and MS groups was significantly lower than that in LV group, while the degree of apoptosis in MG and MS groups was similar. This suggests that simvastatin may alleviate apoptosis in VILI in ARDS mice by reducing NET production.

In this study, the expression of PAD4, Cit-H3 and NE, which are hallmark products of NET formation, was upregulated in group L, group V and group LV, and the MPO concentration in the BALF was increased, indicating that the mass production of NETs was related to the exacerbation of the inflammatory response, which was consistent with the results of previous studies [10].

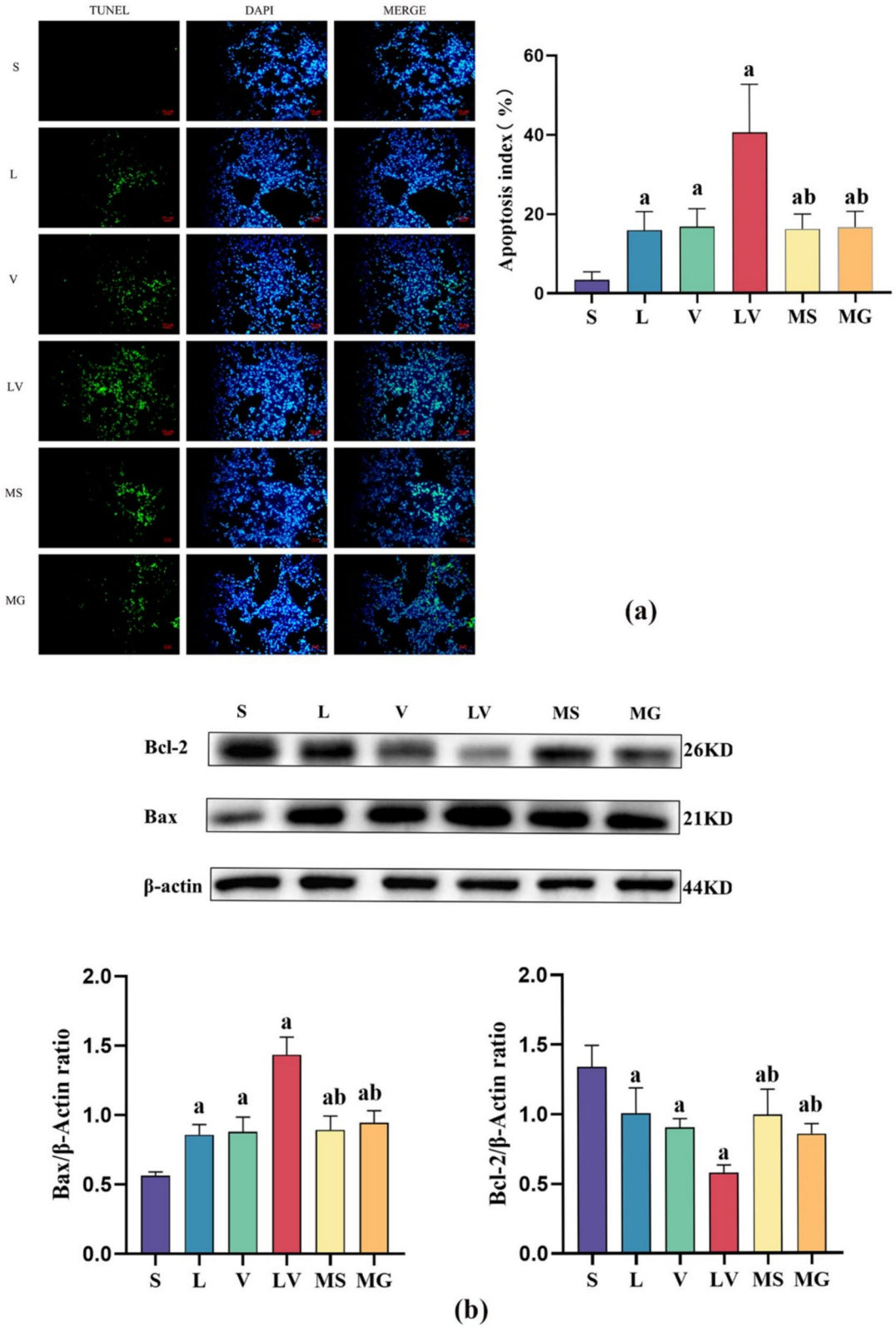


Fig. 5 Effects of simvastatin on apoptotic cell death. **a** TUNEL staining was performed on lung tissues from different treatment groups (scale bar: 10 μm). Quantification of TUNEL staining was also calculated. **b** The apoptotic proteins Bax and Bcl-2 measured by western blotting. The data are presented as the means ± SD. **a** $P < 0.05$ vs. the S group; **b** $P < 0.05$ vs. the LV group. $N = 12$ per group

Furthermore, PAD4 has been shown to promote the formation of NETs and aggravate damage to the alveolar epithelium and endothelial cells both in vivo and in vitro [26]. Therefore, we used GSK484, a selective inhibitor of PAD4, to compare its effect with that of simvastatin. To explore whether simvastatin might reduced VILI in mice with ARDS by reducing NET production. The results showed that simvastatin and GSK484 induced similar decreases in NET production and had the same overall effects on VILI in mice with ARDS, suggesting that simvastatin alleviated VILI in mice with ARDS by decreasing NET production. Meanwhile, GSK484 was superior to simvastatin in some indicators, which may be because GSK484 is a specific inhibitor of PAD4, which has been shown to play a key role in NETs [27, 28]. Simvastatin has a more extensive effect and a more complex mechanism of action, so it is not as good as GSK484 in NETs-related indicators. However, simvastatin has been used in cardiac therapy in clinical practice, so it may show better therapeutic effects on multi-system diseases, which is also the direction of our future research. Moreover, the specific inhibition of PAD4 by GSK484 is also one of our research directions for the treatment of NETs-involved diseases.

Compared with other treatments for lung injury, simvastatin is still in the stage of basic research, and because of its basic properties of cholesterol-lowering and heart-related diseases, it may take a long time for simvastatin to be used for the treatment of other systemic diseases. At the same time, simvastatin is a mature clinical drug, and it will be more convenient for clinical use after perfect clinical research. We believe that simvastatin has a good research prospect.

Limitations

To explore the mechanism by which simvastatin affected VILI in mice with ARDS, we administered the PAD4 inhibitor GSK484. However, no direct data related to the mechanistic relationship between simvastatin and NETs are available. We will continue to explore the specific underlying mechanism that are involved.

Conclusions

The results of this study suggest that pretreatment with simvastatin could attenuate VILI in mice with ARDS and that the protective effect of simvastatin may be associated with its anti-inflammatory and anti-apoptotic effects. This anti-inflammatory response and anti-apoptotic response may occur through a reduction in NETs. Considering the clinical application of simvastatin for lung disease [29, 30], we hypothesize that simvastatin may be an additional new preventive treatment for patients with ARDS who require MV support.

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Author contributions
CM was involved in study design, data collection, interpretation and writing of manuscript. XW reviewed data interpretation and writing of manuscript. YD as independent statistician was responsible for data management, analysis and interpretation. WQ contributed to design, data interpretation and writing of manuscript. WH and LS reviewed the manuscript. All authors read and approved the fnal manuscript.

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Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Laboratory Animal Committee of Qingdao Municipal Hospital (2021-118).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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