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ORIGINAL ARTICLE

Dihydromyricetin Alleviates Sepsis-Induced Acute Lung Injury through Inhibiting NLRP3 Inflammasome-Dependent Pyroptosis in Mice Model

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Abstract— Increasing evidence demonstrates that pyroptosis, pro-inflammatory programmed cell death, is linked to acute lung injury (ALI). Dihydromyricetin (DHM) has been reported to exert anti-inflammatory effects by inhibiting NLRP3 inflammasome activation in vascular endothelial cells. However, the effects of DHM on NLRP3 inflammasome-induced pyroptosis in ALI remain elusive. In the present study, male BALB/c mice were subjected to cecal ligation and puncture (CLP), and DHM (50, 100, 150 mg/kg) was orally administered (once per day, for 3 days) 2 h after CLP. After 72 h, lung histopathology was examined, and the wet/dry (W/D) ratio, inflammatory infiltration, total protein concentration, total cell, and neutrophil counts were detected. Myeloperoxidase (MPO), interleukin (IL)-6, TNF-α, IL-1β, and IL-18 levels in bronchoalveolar lavage fluid (BALF) were measured by ELISA. Additionally, the expression of NLRP3 signaling pathway proteins were detected by Western blotting. The results revealed that in BALF, DHM (150 mg/kg) treatment significantly reduced the CLP-induced lung histopathological injury, inflammatory cell infiltration, total cell and neutrophil number, and total protein and albumin concentration. DHM treatment significantly inhibited the CLP-induced NLRP3 inflammasome pathway (NLRP3, ASC, caspase-1, gasdermin D (Gsdmd), IL-1β, and IL-18). In conclusion, these results demonstrate that DHM protects against CLP-induced ALI by inhibiting NLRP3 inflammasome activation and subsequent pyroptosis.

KEY WORDS: dihydromyricetin; NLRP3 inflammasome; pyroptosis; acute lung injury; sepsis.

INTRODUCTION

Sepsis is a complex systemic inflammatory response syndrome (SIRS) triggered in patients with infections and is associated with multiple organ dysfunction syndrome (MODS) and mortality in critically ill patients [1]. Acute lung injury (ALI), a common complication of sepsis, has been reported to have a high morbidity and mortality rate in critically ill patients [2]. ALI is characterized by excessive, uncontrolled activation of the lung inflammatory response, which includes many types of inflammatory cells [3, 4]. Despite considerable effort and progress in several ALI fields, no effective drugs or therapies exist for curing ALI/acute respiratory distress syndrome (ARDS) [5].

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Abbreviations: *DHM*, Dihydromyricetin; *NLRP3*, NLR pyrin domain containing 3; *ASC*, Apoptosis-associated speck-like protein containing CARD; *ALI*, Acute lung injury; *CLP*, Cecal ligation and puncture; *W/D*, Wet/dry; *MPO*, Myeloperoxidase; *IL*, Interleukin; *BALF*, Bronchoalveolar lavage fluid; *Gsdmd*, Gasdermin D; *SIRS*, Systemic inflammatory response syndrome; *MODS*, Multiple organ dysfunction syndrome; *ARDS*, Acute respiratory distress syndrome; *CMC-Na*, Carboxymethylcellulose; *H&E*, Hematoxylin and eosin; *DAPI*, *4′*, 6-Diamidino-2-phenylindole; *PVDF*, Polyvinylidene fluoride; *SEM*, Standard error of the mean; *ANOVA*, Analysis of variance

Canonical pyroptosis is caspase-1-dependent programmed cell death, which features rapid plasma membrane rupture and pro-inflammatory cytokine production [6]. Recent evidence has indicated that pyroptosis in lung vascular endothelial cells and alveolar macrophages plays a critical role in ALI development [7–9]. In addition, the specific caspase-1 inhibitor Ac-YVAD-CMK attenuates LPS-induced lung injury in mice by inhibiting caspase-1 activation and pyroptotic cell death [10]. Therefore, inhibiting pyroptosis may be a future approach to the clinical prevention or treatment of ALI/ARDS in patients.

Dihydromyricetin (DHM) is isolated from the tender stem and leaves of the *Ampelopsis grossedentata* species [11]. Recent studies have suggested that DHM has multiple pharmacological functions, such as anti-inflammatory, antioxidation, and anticancer effects [12, 13]. Xie et al. demonstrated that in mice, DHM alleviates tetrachloride-induced acute liver injury by inhibiting inflammatory reactions [14]. A study by Hu et al. showed that DHM inhibits NLRP3 inflammasome-dependent pyroptosis in vascular endothelial cells [15]. However, whether and how DHM inhibits the activation of NLRP3 inflammasome-dependent pyroptosis in ALI requires further investigation. Therefore, the aim of this study was to investigate the effects of DHM on cecal ligation and puncture (CLP)-induced ALI in mice.

MATERIALS AND METHODS

Ethics Statement

Animals were maintained in accordance with the guidelines of the experimental animal center of Tongji Medical College, and protocols were approved by the medical ethics committee of Tongji Hospital at the Tongji Medical College of Huazhong University of Science and Technology.

Animals

Male Balb/c mice (25–30 g) were obtained from the experimental animal center of Tongji Medical College, China. Before the experiment, mice were housed in a specific pathogen-free (SPF) environment (room temperature 25 °C, 12 h light/dark cycle) for at least a week and received standard chow and water. All experimental procedures were performed in accordance with the Provision and General Recommendation of Chinese Experimental Animal Administration Legislation and were approved by the Ethical Committee on Animal Research at Huazhong University of Science and Technology.

CLP Model of Sepsis

Sepsis-induced ALI models were established by CLP as previously described with minor modifications [12]. Mice were anesthetized using sodium pentobarbital (intraperitoneally, 40 mg/kg). Sham-operated mice underwent the same procedure without CLP. Finally, mice were resuscitated with subcutaneously injected saline (50 mL/kg). The upper portion of the left lung was fixed in 4% paraformaldehyde for histological examination. The lower portion of the left lung was used for the lung wet/dry (W/D) weight ratio assay. The right lung was stored at −80 °C for ELISAs, MPO activity detection, and Western blot analysis.

Experimental Protocols

First, Balb/c mice were randomly divided into five groups: (1) the vehicle-treated sham group, (2) vehicle-treated CLP group, (3) CLP + DHM (50 mg/kg) group, (4) CLP + DHM (100 mg/kg) group, and (5) CLP + DHM (150 mg/kg) group. The DHM dose was selected according to previous research. DHM was purchased from Chengdu MUST Bio-Technology (Sichuan, China). The dose of DHM was selected according to previous research [14]. Lung histological changes were evaluated to find the most effective concentration of DHM for further experiments.

Next, Balb/c mice were randomly divided into three groups: (1) the vehicle-treated sham group, (2) vehicle-treated CLP group, and (3) CLP + DHM (150 mg/kg) group. Inflammatory cell infiltration and inflammatory cytokine and protein expression levels were detected in this step.

DHM was dissolved in 0.5% sodium carboxymethylcellulose (CMC-Na) and diluted in ultrapure water to a final concentration of 37.5 mg/mL. Then, the mice were treated with DHM (once per day, for 3 days) 2 h after the CLP procedure.

Pulmonary Histopathology and ALI Score

The lung tissues (upper portion) were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5-μm-thick slices. Then, hematoxylin and eosin (H&E) staining was performed. Histopathological examinations were carried out using a microscope (RX51, Olympus Optical Co., Ltd., Tokyo, Japan).

A modified VILI histological scoring system was used [24]. Each tissue received the following scores based on the damage level: 1, mild damage; 2, moderate damage; 3, severe damage; 4, maximal damage. The following parameters were assessed: (I) alveolar capillary congestion, (II) hemorrhage, (III) neutrophil infiltration into the airspace or vessel wall and alveolar wall thickness, and (IV) alveolar wall thickness/hyaline membrane formation. A score of 0 represented normal findings, while scores of 1–4 represented mild (< 25%), moderate (25–50%), severe (50–75%), and very severe (> 75%) lung involvement, respectively.

Lung W/D Weight Ratio

The fresh lower portion of left lung was separated and weighed to obtain the wet weight. Subsequently, the lungs were dried in an oven at 80 °C for at least 48 h and then weighed to determine the dry weight of each lung. The lung wet/dry weight (W/D) ratio was calculated to assess lung edema.

Elevation of the Protein and Cells in Bronchoalveolar Lavage Fluid (BALF)

Seventy-two hours later, mice were sacrificed after anesthesia by sodium pentobarbital (intraperitoneally, 40 mg/kg). The chests of mice were opened and the lungs were resected with the tracheotomy tube in place. The left main bronchus was ligatured, and the right lung was lavaged three times with 1.0 mL PBS and obtained approximately 0.8 ml of BALF. The cell counting was conducted by using a Neubauer Chamber as previously described [16]. The other BALF was centrifuged (1500×g, 10 min, 4 °C), and the supernatant was used for the determination of the total protein level with ELISA kits (Dakewe, Shenzhen, China).

MPO Activity Assay

Lung tissues were homogenized in 1 mL of 0.5% hexadecyltrimethylammonium bromide. MPO activity was measured using an MPO determination kit following the manufacturer's instructions (NanjingJiancheng Corp., Nanjing, China).

TNF-α, IL-6, IL-18, and IL-1β in BALF

The supernatants of BALF were harvested after centrifugation and stored at −80 °C until analysis. TNF-a, IL-6, IL-18, and IL-1β levels were measured by ELISA kits according to the manufacturer's instructions (Dakewe, Shenzhen, China).

Immunofluorescence

The sections were stained with rabbit polyclonal antibody against Gsdmd (1:200) overnight at 4 °C, followed by a mixture of goat anti-rabbit Cy5.5-conjugated secondary antibodies (Abcam; Cambridge, UK) for 2 h at room temperature. Then, the sections were incubated with a DAPI staining solution at room temperature for 10 min. Images were acquired with a confocal fluorescence microscope (RX51, Olympus Optical Co. Ltd., Tokyo, Japan).

Western Blot Analysis

Lung tissues were homogenized in protease inhibitor cocktail containing RIPA buffer, and a solution was obtained. Protein samples (30 μg) were loaded and electrophoresed on 10% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 5% skim milk, and incubated with primary antibodies overnight at 4 °C. NLRP3, ASC, Caspase1, Cleaved-Caspase1, IL-1β, IL-18, and Gsdmd antibodies were used (Abcam, Cambridge, UK). Then, the membranes were incubated with rabbit HRP-conjugated secondary antibody at room temperature for 2 h, and the protein bands were visualized by using chemiluminescent peroxidase substrate (Millipore, Boston, MA, USA). The densitometric analysis was quantified using ImageJ software (National Institutes of Health, USA).

Statistical Analysis

Data are expressed as the means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The results were analyzed by analysis of variance (ANOVA) with Tukey's *post hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

DHM Protects Mice against CLP-Induced ALI and Lung Edema

To determine the protective effects of DHM on CLP-induced ALI, lung tissue was histologically examined with H&E staining. Hemorrhage, pulmonary edema, alveolar wall thickening, and neutrophil infiltration were increased in the CLP group compared with the sham group (Fig. 1a).

However, these histopathological changes were attenuated by DHM treatment. The DHM treatment also reduced the histological lung injury grade (Fig. 1b), and the most effective DHM concentration was 150 mg/kg. In addition, the lung W/D weight ratio, a well-known hallmark of lung edema, was significantly increased in the CLP group, indicating a result similar to the histological grading (Fig. 1c). These results suggest that DHM treatment significantly alleviated CLP-induced ALI. The 150 mg/kg DHM dose was selected for further experiments.

DHM Reduces Inflammatory Infiltration and MPO Activity in Lung Tissue

To investigate the effect of DHM on lung inflammation, the number of inflammatory cells in BALF was quantified. The number of total cells, macrophages, and neutrophils in BALF was significantly increased in the CLP group compared with the sham group, which was effectively decreased by DHM treatment (Fig. 2a–c). Lung MPO activity is an indicator of neutrophil infiltration; thus, we evaluated MPO activity in the lung. Consistent with the number of neutrophils in BALF, MPO protein activity was also notably increased in the lung tissue of the CLP group and was substantially inhibited by DHM treatment (Fig. 2d). These results demonstrate that DHM attenuated inflammatory cell infiltration in CLP-induced ALI.

DHM Inhibits Lung Inflammation in CLP-Induced ALI

To further assess the effect of DHM on lung inflammation in the CLP model, TNF-α, IL-6, IL-18, and IL-1β levels in BALF were measured by ELISA. TNF-α, IL-6, IL-18, and IL-1β levels were notably increased in CLP-induced ALI and were reduced by DHM treatment (Fig. 3a–d). These findings indicate that CLP results in acute lung inflammation and the anti-inflammatory effect of DHM.

Effects of DHM on NLRP3, ASC, Caspase-1, IL-18, and IL-1β Protein Expression in Lung Tissue

To further determine if pyroptotic protein expression levels are downregulated by DHM, NLRP3, ASC, caspase-1, IL-18, and IL-1β protein expression in the lung tissue were detected by immunoblotting. The protein expression levels of NLRP3, ASC, caspase-1 p20, IL-18, and IL-1β were markedly increased in the CLP-challenged mice compared with the sham mice. These protein expression levels were significantly decreased in the DHM group compared with the CLP group (Fig. 4). These data

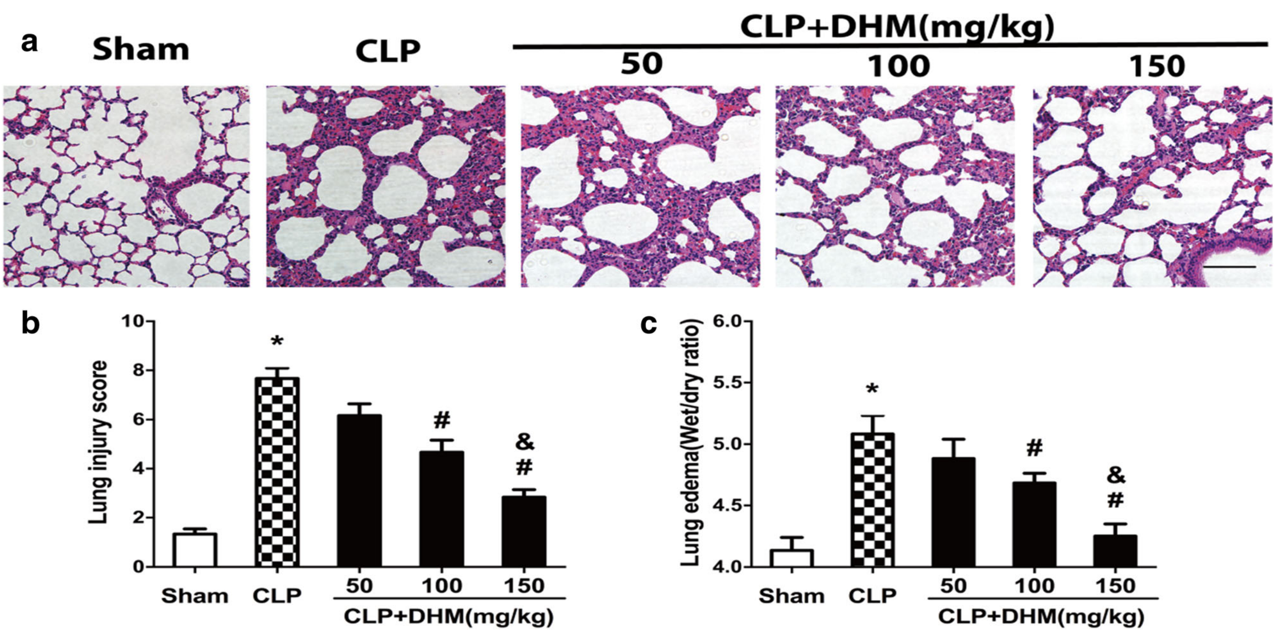

Fig. 1. DHM ameliorates pulmonary inflammation and pulmonary edema in CLP-induced ALI. **a** After 72 h interventions, representative pictures of lung tissue sections stained with H&E. The scale bar = 50 μm. **b** Histological scoring of ALI after CLP. **c** Lung edema. Data are presented as the means ± SEM, $n = 8$/group. $^{*}P < 0.05$ *versus* the sham group; $^{\#}P < 0.05$ *versus* the CLP group; $^{\&}$ *versus* the CLP + DHM (100 mg/kg) group. DHM = dihydromyricetin, CLP = cecal ligation and puncture, ALI = acute lung injury.

demonstrate that DHM inhibited NLRP3 inflammasome activation.

Effects of DHM on Gsdmd Protein Expression and Cleavage

Pro-Gsdmd protein expression in the lung tissue was detected by immunofluorescence and immunoblotting. The cleaved Gsdmd p30 protein is the "executor" of pyroptosis. Gsdmd p30 was assessed with immunoblotting to determine whether Gsdmd cleavage was inhibited by DHM treatment. We observed that CLP markedly increased pro-Gsdmd and cleaved Gsdmd p30 protein levels. Moreover, both of these changes were inhibited by DHM treatment (Fig. 5a–d). These data demonstrate that DHM inhibited pyroptosis activation.

DISCUSSION

The CLP model, a reliable animal model of the human sepsis, is currently regarded as a widely used sepsis model [17]. In the present study, this model was established to evaluate the effects of DHM on sepsis-induced ALI in mice. We demonstrated that DHM treatment inhibited CLP-induced severe lung injury, with characteristic symptoms of ALI, including pulmonary edema, hemorrhage, and excessive inflammatory cell accumulation. Furthermore, our results also suggest that DHM significantly inhibited CLP-induced NLRP3 inflammasome activation and the expression of pyroptosis-related proteins in lung. These results indicated that DHM might act as a protective agent for treating CLP-induced ALI.

Inflammation has been identified as the major cause that leads to lung injury. [18] The typical pathological manifestations of acute lung injury are lung edema, inflammatory cell infiltration, and intrapulmonary hemorrhage [3]. Several researches have showed that DHM has protective effect against organ injury induced by various pathological factors [12, 14, 19]. According to our results, DHM treatment significantly reduced the number of inflammatory cells in BALF, W/D weight ratio, and ameliorated the pathologic changes in the lung as compared with CLP group.

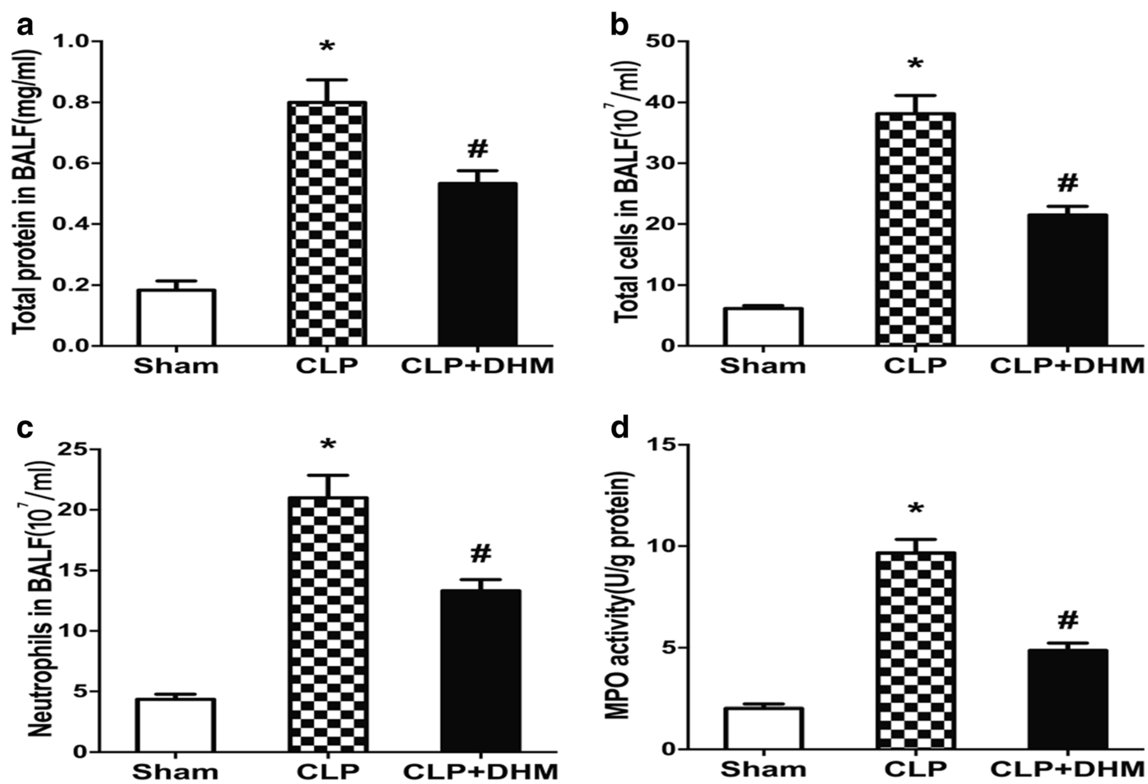

Fig. 2. DHM treatment reduced protein and inflammatory cell infiltration levels in BALF. **a** Protein and **b** albumin levels and the number of **c** total cells and **d** neutrophils in bronchoalveolar lavage fluid (BALF) at 24 h after the CLP operation were measured to assess pulmonary vascular leakage. Data are expressed as the means ± SEM, $n = 6$/group. $^{*}P < 0.05$ *vs.* the sham group; $^{\#}P < 0.05$ *vs.* the CLP group.

A complex cytokine network mediates the inflammatory response and plays important roles in ALI/ARDS [3]. In the present study, our results revealed that CLP-induced ALI increased the number of inflammatory cells in BALF and pro-inflammatory cytokine levels, which were significantly inhibited by DHM treatment. Neutrophil lung infiltration is an early step in the inflammatory process of ALI [4]. In addition, MPO activity is an effective measure of neutrophil infiltration into tissues [20]. In this study, we found that DHM treatment significantly alleviated neutrophil infiltration and inhibited MPO activity. Furthermore, the CLP-induced lung histopathological changes were also inhibited by DHM. Above all, these results suggest that DHM has protective effects against CLP-induced ALI.

The NLRP3 inflammasome, a protein complex composed of NLRP3, caspase-1, and ASC, is involved in sensing microbes and a variety of endogenous danger signals, including bacterial toxins, hyaluronan, fibrillary amyloid-β peptide, and DAMP including ATP [21]. NLRP3 inflammasome, a key sensor in the pyroptosis signaling pathway, has been shown to be activated in ALI models [22, 23]. Grailer et al. have demonstrated that one potential mechanism of inflammatory propagation during experimental ALI is activation of the NLRP3 inflammasome, which involves extracellular histones. Furthermore, Wu et al. have demonstrated that NLRP-3-denpendent pyroptosis of alveolar macrophage is associated with ALI/ARDS in mice. Meanwhile, and Ac-YVAD-CMK, a specific caspase-1 inhibitor, can prevent lung injury by inhibiting alveolar macrophage-induced pyroptosis. In addition, a previous study demonstrated that inhibition of NLRP3 inflammasome could attenuate LPS-induced ALI [24]. Based on these studies, the inhibition of NLRP3 inflammasome activation could have significance for attenuating pyroptosis and thereby improving ALI.

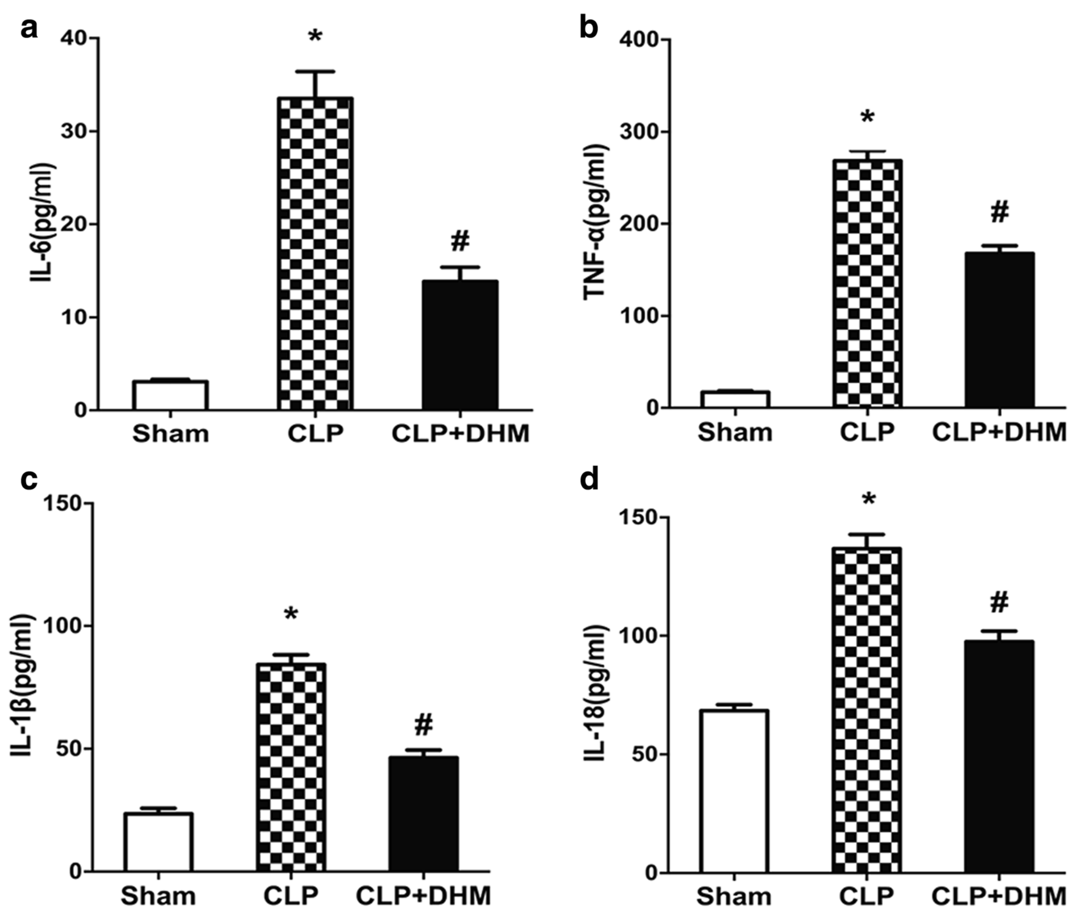

Fig. 3. Inflammatory cytokine levels were detected in the BALF and serum samples. **a** IL-6, **b** TNF-α, **c** IL-1β, and **d** IL-18 levels was measured by ELISA in BALF. Data are expressed as the means ± SEM ($n = 10$). $^{*}P < 0.05$ *vs.* the sham group; $^{\#}P < 0.05$ *vs.* the CLP group.

The precise mechanisms by which IL-1β and IL-18 reach the extracellular space have been debated for decades, but pyroptosis is increasingly regarded as a mechanism for promoting the passive release of these highly inflammatory cytokines [25]. NLRP3 inflammasome activation leads to caspase-1 activation, which proteolytically cleaves pro-IL-1β and pro-IL-18 into their mature, active forms and induces pyroptosis partially through the cleavage of gasdermin D (Gsdmd) [25, 26]. IL-1β and IL-18, as products of cell pyroptosis, are important inflammatory cytokines that involved in the development of ALI. IL-1β and IL-18 activate inflammatory cells and facilitate the release of other inflammatory cytokines, which trigger inflammatory cascades and amplify the inflammatory response [18, 27]. In addition, IL-1β can cause lung edema by increasing alveolar epithelial and vascular endothelial permeability [28, 29]. High IL-18 levels are associated with morbidity and mortality in ARDS patients [30]. IL-1β- and IL-18-neutralizing antibodies have been shown to prevent ALI in different models [22, 27]. Our results revealed that IL-1β and IL-18 levels were increased in BALF and serum after CLP and were significantly suppressed by DHM treatment.

Gsdmd, a critical target of caspase-1, has been identified as a key mediator of pore formation in cells undergoing pyroptosis [25]. The release of large quantities of IL-1β and IL-18 requires N-gasdermin D (the cleaved form of Gsdmd), which is the "executioners" of pyroptosis. Overexpression of cleaved Gsdmd-NT alone induced pyroptosis dependently of caspase-1 *in vitro* [31]. To

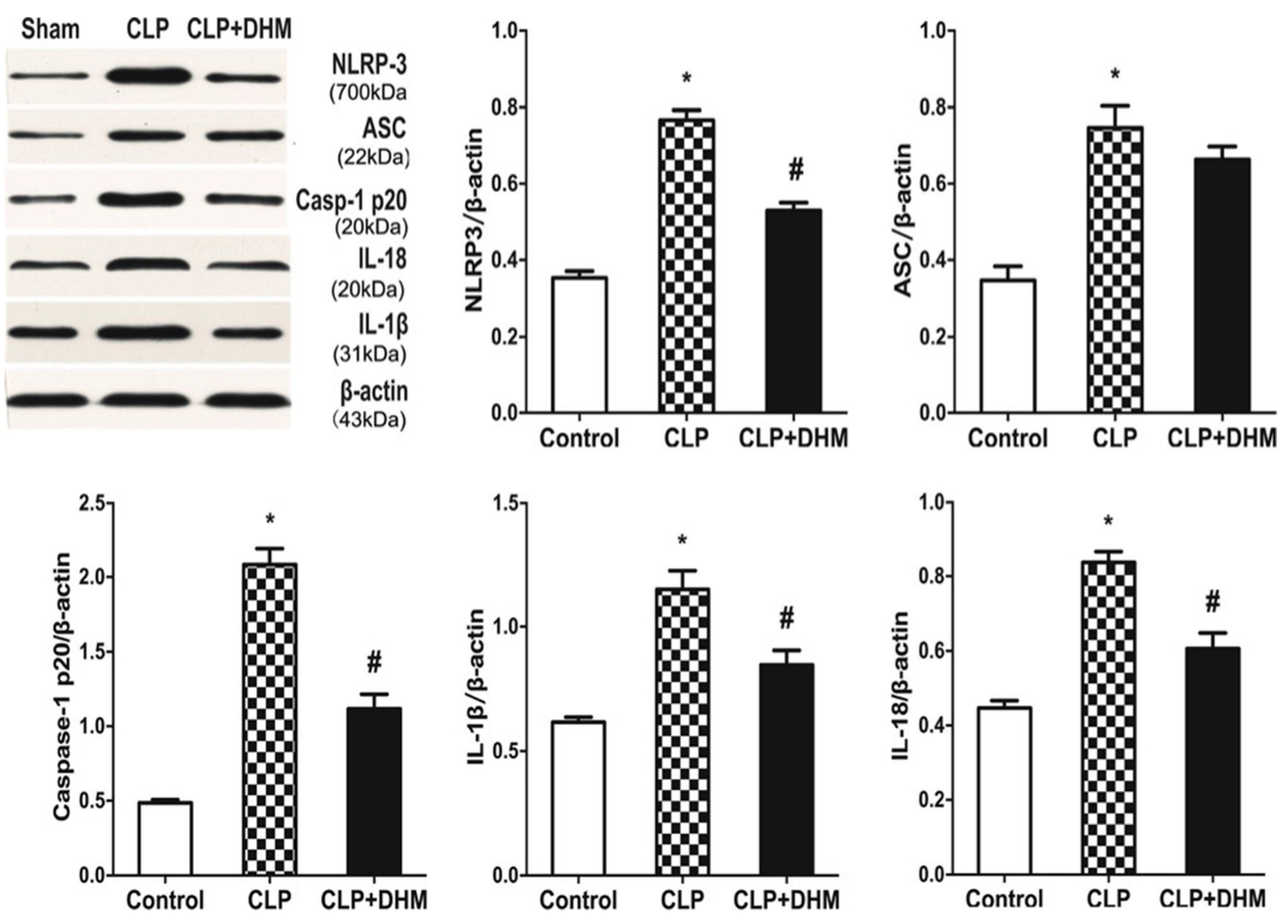

Fig. 4. Effects of DHM on pyroptosis-related protein levels in lung tissue. Representative Western blots showing the NLRP3, ASC, caspase-1 p20, IL-18, and IL-1β (an autoprocessed fragment of caspase-1) levels. Western blot analysis and densitometric quantification of NLRP3, ASC caspase-1 p20, IL-18, and IL-1β. The data are expressed as percentages of the control. The results are expressed as the means ± SEM, $n = 6$. $^{*}P < 0.05$ *vs.* the sham group, $^{\#}P < 0.05$ *vs.* the CLP group.

investigate the protective mechanism of DHM, NLRP3 inflammasome-dependent pyroptosis signaling pathways were evaluated. In the present study, CLP-induced NLRP3 inflammasome activation was inhibited, and the increased caspase-1 p20, Gsdmd p30, IL-1β, and IL-18 expression in lung tissues was also suppressed in the DHM-treated group.

In summary, our present study demonstrates that DHM exhibits protective effects in mice with CLP-induced ALI by inhibiting inflammatory reactions. This inhibitory effect was based on the suppression NLRP3 inflammasome-dependent pyroptosis signaling pathways. Therefore, DHM may serve as a new therapeutic approach in CLP-induced ALI.

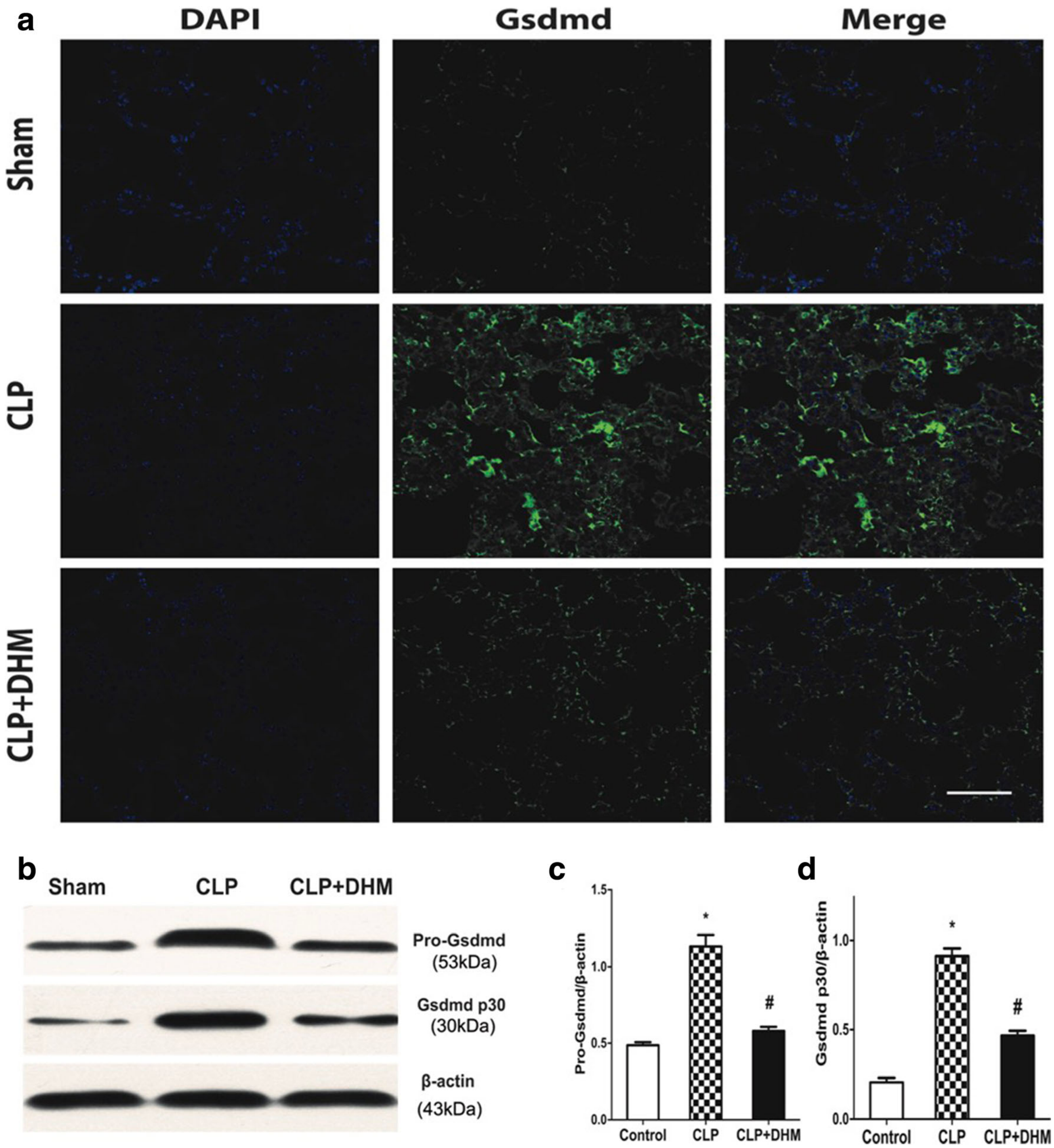

Fig. 5. Effects of DHM on Gsdmd expression. **a** Representative immunofluorescence images showing Gsdmd expression. The scale bar = 100 μm. **b** Representative Western blots showing Gsdmd and Gsdmd p30 (cleaved Gsdmd) protein levels. **c** Western blot analysis and **d** densitometric quantification of Gsdmd and Gsdmd p30 (cleaved Gsdmd) protein levels. $^{*}P < 0.05$ *vs.* the sham group; $^{\#}P < 0.05$ *vs.* the CLP group.

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AUTHORS' CONTRIBUTIONS

Zhan-fei Li and Xiang-jun Bai equally contributed to the design of the research and interpretation of the data. Yu-chang Wang, Qiang Zheng, Qin-xin Liu, Tao Liu, Xing-hua Liu and Xi-e XU performed the animal experiments and collected data. Yu-chang Wang and Wei Gao analyzed the data and cytokines. Molecular biological parts of the study were performed by Yu-chang Wang and Zhan-fei Li. Zhan-fei Li and Xiang-jun Bai supervised the project. All authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no conflict of interest.

Consent Statement. Not applicable. No individual personal data are included in the study.

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