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Activation of the NLRP3 inflammasome by HMGB1 through inhibition of the Nrf2/HO-1 pathway promotes bleomycin-induced pulmonary fibrosis after acute lung injury in rats

Ying Huang[a#], Aili Wang[a#], Sheng Jin[b], Fang Liu[c*], Fang Xu[a*]

[a]*Department of Respiratory and Critical Care Medicine, Wuhan No. 1 Hospital, Wuhan, Hubei Province, China*
[b]*Nephrology Department of Integrated Traditional Chinese and Western Medicine, Hubei No. 3 People's Hospital of Jianghan University, Wuhan 430033, Hubei Province, China*
[c]*Department of Respiratory Medicine, Hubei No. 3 People's Hospital of Jianghan University, Wuhan 430033, Hubei Province, China*

[#]Ying Huang and Aili Wang contributed equally to this work.

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acute lung injury;
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Abstract
Objective: Acute lung injury (ALI) is a common complication of critical diseases with high morbidity and mortality. This study explored the regulatory role and mechanism of high mobility histone box 1 protein (HMGB1) on pulmonary fibrosis (PF) after ALI in rats through nucleotide oligomerization domain-like receptor protein-3 (NLRP3) inflammasome.
Methods: PF rat models after ALI were established by induction of bleomycin. Degree of fibrosis was assessed by Masson staining and Ashcroft scoring. Hydroxyproline (Hyp) contents in lung tissues and rat lung tissue morphology were detected by enzyme-linked-immunosorbent serologic assay (ELISA) and hematoxylin and eosin staining. The levels of NLRP3, major proteins of NLRP3 inflammasome (NLRP3/ASC/caspase-1), and downstream inflammatory cytokines interleukin (IL)-1 and IL-18 were determined using immunohistochemistry, Western blotting analysis, and ELISA. The nuclear/cytoplasmic nuclear factor erythroid 2-related factor 2 (Nrf2) levels and HO-1 levels were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blotting analysis. Rats was injected with lentivirus carrying short hairpin (sh)-HMGB1 and zinc protoporphyria (ZNPP) (HO-1 inhibitor) to assess the effects of HMGB1 and HO-1 on PF and NLRP3 inflammasome activation.
Results: Bleomycin induced PF after ALI in rats, manifested as patchy fibrosis, atelectasis, and excessive expansion, and increased Aschcroft score and Hyp content. Bleomycin treatment enhanced levels of NLRP3, ASC, caspase-1, IL-1, and IL-18 in rat lung tissues, which promoted activation of NLRP3 inflammasome. HMGB1 was up-regulated in bleomycin-induced rats. HMGB1 knockdown partially reversed NLRP3 inflammasome activation and PF progression. HMGB1

***Corresponding author**: Fang Xu, Department of Respiratory and Critical Care Medicine, Wuhan No. 1 Hospital, 215 Zhongshan Avenue, Qiaokou District, Wuhan 430022, Hubei Province, China. Fang Liu, Department of Respiratory Medicine, Hubei No. 3 People's Hospital of Jianghan University, 26 Zhongshan Avenue, Qiaokou District, Wuhan 430033, Hubei Province, China. *Email addresses*: FangXu12301230@163.com; flco4891452@163.com

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knockdown promoted Nrf2 nuclear translocation and up-regulated HO-1. Suppression of HO-1 partially reversed inhibition of HMGB1 knockdown on NLRP3 inflammasome activation and PF.
Conclusion: HMGB1 can activate NLRP3 inflammasomes and promote PF by inhibiting the Nrf2/HO-1 pathway.

Introduction

Acute lung injury (ALI) is a respiratory disease characterized by acute inflammatory reactions in the airway and lung parenchyma, and its sudden onset and dangerous condition seriously endangers the life and health of patients.[1,2] Persistent and repetitive lung injury can provoke tissue and cellular responses that ultimately lead to pulmonary fibrosis (PF),[3] which is a fatal and incurable lung disease with a median survival of 2–5 years.[4] The pathological features of PF are dysfunctional repair of normal lung tissues after damage, excessive proliferation of fibroblasts, and massive collagen deposition, which jointly lead to decreased lung compliance, blocked gas exchange, and ultimately respiratory failure and death.[5] However, presently, there is no cure for PF and its occurrence is expanding worldwide.[6] Consequently, understanding its underlying molecular mechanisms is essential to identify and develop effective treatments.

Inflammasome is a multi-protein complex that promotes the secretion of mature and pro-inflammatory cytokines, such as interleukin (IL)-1ß and IL-18 via caspase-1 activation.[7] In particular, among various inflammasome complexes, nucleotide oligomerization domain-like receptor protein-3 (NLRP3) inflammasome has been reported to be associated with various human autoinflammatory and autoimmune diseases.[8] NLRP3 inflammasome is normally inactive in cells, but it is immediately activated and promotes liver and kidney fibrosis once cells are exposed to danger signals.[9,10] A previous study has suggested that levels of NLRP3, caspase-1, and IL-1ß are distinctly elevated in PF patients.[11] Moreover, activation of NLRP3 inflammasome has also been observed in bleomycin-induced PF.[12] Apparently, inhibition of NLRP3 inflammasome activation alleviates PF.[13] Nevertheless, the mechanism of NLRP3 inflammasome in PF after ALI remains to be studied.

High mobility histone box 1 protein (HMGB1) is an alarm protein released by immune cells in response to cellular activation or stress.[14] HMGB1 is widely expressed in a variety of tissues and cells and is involved in the pathological process of many lung diseases by binding to corresponding receptors and activating downstream pathways.[15] A large number of studies have shown that HMGB1 may be associated with fibrosis, including myocardial fibrosis, PF, and liver fibrosis.[15,16] In addition, HMGB1 expression affects the activity of nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream genes.[17,18] For instance, repression of HMGB1 can activate the Nrf2 pathway to ameliorate oxidative damage.[19] In fact, Nrf2 is an antioxidant regulator through an antioxidant response element, which plays a crucial role in cellular protection against oxidative stress.[20] It is involved in many lung diseases, including lung injury, chronic obstructive pulmonary disease (COPD), and PF.[21,22] However, the effect of HMGB1/Nrf2 on PF after ALI through NLRP3 inflammasome has not been reported. Therefore, this study explored the regulatory mechanism of HMGB1 on PF after ALI in rats through NLRP3 inflammasome.

Materials and Methods

Establishment of bleomycin-induced PF rat model after ALI

A total of 72 male Wistar HsdCpb rats (10-week old, weighing 280–300 g) were purchased from Charles River (Beijing, China). Rats were anesthetized with 4% isoflurane inhalation at room temperature in a Plexiglas chamber. Rats were intubated with an 18G plastic catheter, and 1-U bleomycin, dissolved in 330-µL 0.9% NaCl, was infused into the lung with a microatomizer (PennCentury, Wyndmoor, PA, USA). Another group of rats was randomly assigned to the healthy control group that did not receive bleomycin infusion. Rats were anesthetized with 1% pentobarbital sodium (50 mg/kg) on the 7th day after bleomycin infusion, and their lungs were removed for subsequent experiments. After sampling, the rats were euthanized by narcotic overdose.

Lentivirus injection and animal grouping

Lentiviral vectors of short hairpin (sh)-HMGB1 and its short hairpin negative control (sh-NC) shRNA were designed and synthesized by GenePharma (Shanghai, China). The constructed vectors were diluted to a total volume of 300 µL containing 4×10^7 transduction unit (TU) and injected to animals through the tail vein. Rats were intraperitoneally injected with 30-mg/kg znic protoporphyrin (ZnPP; Sigma-Aldrich, MO, USA), and an equal dose of dimethyl sulfoxide (DMSO) as control.

Rats were grouped as follows: sham group, bleomycin (bleomycin-treated group), bleomycin + sh-HMGB1 (bleomycin was administered 48 h after injection of shRNA-HMGB1), bleomycin + sh-NC (bleomycin treatment was performed 48 h after injection of shRNA negative control), bleomycin + sh-HMGB1 + ZNPP (bleomycin was administered 48 hours after injection of shRNA-HMGB1 and HO-1 inhibitor ZNPP), and bleomycin + sh-HMGB1 + DMSO (bleomycin was administered 48 hours after injecting shRNA-HMGB1 and DMSO). Among the 12 rats in each group, six were used for histological staining and remaining for tissue homogenization.

Hematoxylin and eosin (H&E) staining

The lung was perfused with 4% paraformaldehyde (PFA) at a pressure of 25 cmH_2O through the left main bronchus at room temperature, and was immersed in PFA solution overnight after tracheal ligation. The lung tissue was then embedded in paraffin and sequentially sliced into 4-μm thick sections. Then H&E staining was performed for 2 min, and sections were observed under an optical microscope to determine the degree of alveolar inflammation.

Assessment of pulmonary fibrosis

The above-prepared paraffin sections of lung tissues were subjected to Masson staining for 8 min at room temperature, and the degree of PF was observed under an optical microscope. As described previously by Ashcroft scoring, two independent and blinded observers scored at least five high-power fields per section from at least three sections per mouse to evaluate severity of fibrosis.[23,24]

The biochemical detection of hydroxyproline (Hyp), performed after measuring Hyp content, reflected the collagen content in tissues.[25] Approximately 100 mg of lung tissue was homogenized. The Hyp level of the lung tissue was evaluated using a Hyp enzyme-linked-immunosorbent serologic assay (ELISA) kit (A030-3-1; Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Lung tissue was hydrolyzed at 100°C for 20 min and the pH value was adjusted to the pre-set value. The mixture was incubated at 60°C for 15 min and centrifuged at room temperature at 2813 × *g* for 10 min after cooling. The absorbance of each sample at 550 nm was determined and calculated as Hyp (μg)/mg lung wet weight.

Immunohistochemical staining

Paraffin-fixed sections were treated with xylene and graded ethanol for removal of paraffin and rehydration. Lung sections were microwave-heated (750 W, three cycles of 5-min) in citrate buffer (10-mM sodium citrate, pH = 6.0) for antigen extraction. Endogenous peroxidase was quenched with 3% hydrogen peroxide (H_2O_2) for 15 min at room temperature. Immunohistochemical staining was performed with a primary antibody against NLRP3 prior to incubation in EnVision Detection Systems (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The bounded antibodies were observed with diaminobenzidine. All slides were counterstained with hematoxylin, dehydrated with gradient ethanol, cleared with xylene, and fixed with glycerin gelatin. The sections were observed using an optical microscope.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

After homogenization of lung tissue, total RNA was extracted from lung tissue using the TRIzol reagent (ComWin Biotech Co. Ltd., Beijing, China). The quality and quantity of RNA was measured at 260 nm and 280 nm using a NanoDrop-2000 ultra-micro spectrophotometer (NanoDrop; Thermo Fisher, Wilmington, DE, USA). Subsequently, 1 mg of RNA was reverse-transcribed into complementary (c)DNA using the SuperRT cDNA Synthesis kits (ComWin Biotech) according to the manufacturer's protocol, and qPCR was performed using SYBR Green qPCR SuperMix (Thermo Fisher) in CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA). The reaction conditions were as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 30 s and at 60°C for 30 s, and finally extended for 5 min at 72°C. The relative expression of genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using the $2^{-\Delta\Delta Cq}$ method.[26] The primers of RT-qPCR are shown in Table 1.

Nuclear and cytoplasmic fractionation assay

Proteins were extracted from the cytoplasm and nucleus using a nuclear fractionation kit (Beyotime, Shanghai, China). After washing in phosphate-buffered solution (PBS), lung tissue homogenate was added with 200-mL protein extract A/protease inhibitor buffer and precooled on ice for 15 min. Cells were incubated with 10-mL cytoplasmic protein extractant at 4°C and centrifuged at 12,000 × *g* for 10 min to completely separate nuclear precipitation from the cytoplasm. The nuclei were suspended in 50-mL nucleoprotein extract, mixed on ice for 30 min, and centrifuged at 12,000 × *g* at 4°C for 10 min. The supernatant was served as nuclear extract.

Western blotting (WB) analysis

After homogenization of lung tissues, the radioimmunoprecipitation assay lysis solution (Beyotime) containing protease inhibitor cocktail (Sigma-Aldrich) was added and mixed, followed by ice lysis for 30 min, centrifuged at 15,000 × *g* for 20 min, and collection of supernatant. The bicinchoninic acid (Pierce, IL, USA) was used to detect protein concentration. Extracted proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVD) membranes. The membranes were placed in 5% skimmed milk prepared by tris-buffered saline tween (TBST) and blocked for 1 h under shaking conditions. Then

Table 1 Primer sequences.

Genes	Forward 5′-3′	Reverse 5′-3′
HMGB1	AAAGCGGACAAGGCCCGT TAT	AAGAGGAAGAAGGCCGAA GGAG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

the membranes were added with the following primary antibodies and incubated overnight at 4°C: NLRP3 (1:1000, ab263899; Abcam), apoptosis-associated speck-like protein (ASC) (1:1000, ab283684; Abcam), caspase-1 (4 μg/mL, ab286125; Abcam), HMGB1 (1:2000, ab227168; Abcam), Nrf2 (1:1000, ab92946; Abcam), and heme oxygenase-1 (HO-1) (1:1000, ab68477; Abcam). The membranes were then washed with TBST (for three times, for 5 min each) and incubated with peroxidase-labeled secondary antibody Immunoglobulin G (IgG; 1:2000, ab205718; Abcam) for 1 h. Finally, the bands were visualized using the enhanced chemiluminescence kits (Pierce). *GAPDH* (1:10,000, ab9485; Abcam) and proliferating cell nuclear antigen (1:10,000, ab92552; Abcam) acted as internal references, with the Image J software used for quantification.

ELISA

The contents of IL-1β and IL-18 in lung tissues were detected by ELISA. The lung tissue was grounded at 0°C and centrifuged at 16,000 × *g* for 20 min. Levels of both IL-1β and IL-18 in lung tissues were determined using the IL-1β ELISA kit (RTDL00552; Genie, London, UK) and IL-18 ELISA kit (RTDL00548; Genie).

Statistical analysis

All data were analyzed and plotted using the SPSS 22.0 statistical software (IBM Corp. Armonk, NY, USA) and Graph Prism 8.0 (GraphPad Software Inc., CA, USA). Measurement data were expressed as mean ± standard deviation (SD). First, the normality test was performed to check whether the data conformed to normal distribution, followed by homogeneity of variance test. Student's *t*-test was used for data comparison between two groups; one-way analysis of variance (ANOVA) was used for data comparison between multiple groups; and Tukey's test was used for the *post hoc* test. P-value was obtained from bilateral tests, and $P < 0.05$ was considered as statistically significant.

Ethics statement

All animal experiments followed the standards established by the animal experiment committee of our hospital and approved by the Ethics Committee of Wuhan No. 1 Hospital.

Results

Bleomycin induced PF after ALI in rats and promoted NLRP3 inflammasome activation

In order to evaluate changes in the lung of rats after bleomycin perfusion, we assessed the degree of PF in rats on the 7th day of bleomycin treatment. Compared with the sham group, patchy fibrosis, atelectasis, and overexpansion were observed in the lungs of rats treated with bleomycin, and the Aschcroft scores was significantly increased (Figure 1A; $P < 0.001$). To biochemically quantify fibrosis, we further measured the content of Hyp in the lung tissue of rats in each group. It was found that the Hyp content in lung tissues of bleomycin-treated rats was significantly elevated (Figure 1B; $P < 0.001$). In addition, changes of pulmonary inflammation induced by bleomycin were detected by H&E staining. The results obviously showed thickened alveolar septum and dense interstitial infiltration of inflammatory cells in the bleomycin group (Figure 1C). Previously, NLRP3 inflammasome was inactive in normal cells and was activated once cells were exposed to danger signals, thereby promoting liver and kidney fibrosis.[9,10] Therefore, the expression of NLRP3 in lung tissues was measured by immunohistochemical staining, which manifested that bleomycin treatment increased the expression of NLRP3 in rats (Figure 1D). The NLRP3 inflammasome was mainly composed of NLRP3, ASC, and caspase-1,[27] and the expression of these proteins was further determined by Western blotting analysis. Expressions of NLRP3, ASC, and cleaved casp-1/pro-casp-1 in the lung tissues of rats treated with bleomycin were markedly increased (Figure 1E; all P-values <0.001). Additionally, levels of inflammatory cytokines IL-1β and IL-18 mediated by NLRP3 inflammasomes were measured using ELISA (Figure 1F), which elicited that bleomycin treatment visibly increased the levels of inflammatory cytokines in the lung tissue of rats ($P < 0.001$). Altogether, bleomycin can induce PF after ALI in rats and facilitate NLRP3 inflammasome activation.

Knockdown of HMGB1 partially reversed bleomycin-induced NLRP3 inflammasome activation and PF

Studies have shown that HMGB1 may be associated with fibrosis, including myocardial fibrosis, renal fibrosis, and liver fibrosis.[28-30] To ascertain whether HMGB1 plays a role in the process of PF, we first detected the expression of HMGB1 by RT-qPCR and Western Blotting analysis and down-regulated HMGB1 levels in rats by injecting lentivirus carrying sh-HMGB1. HMGB1 was highly expressed in the lung tissues of rats in the bleomycin group, while the HMGB1 expression was clearly lowered after the rats were infected with lentivirus packaged with sh-HMGB1 ($P < 0.001$; Figures 2A and B). Subsequently, Masson staining, Ashcroft scoring, and Hyp content detection were used to measure the effect of HMGB1 knockdown on PF, which uncovered that, bleomycin-induced PF after ALIin rats was partially reversed after HMGB1 knockdown (Figures 2C and D; $P < 0.001$). In addition, H&E staining revealed that knockdown of HMGB1 partially reversed thickening of the alveolar septum and infiltration of inflammatory cells (Figure 2E). Moreover, immunohistochemical staining and Western blotting analysis demonstrated that knockdown of HMGB1 partially reversed the promotion effect of bleomycin on NLRP3 inflammasome activation (Figures 2F and G; $P < 0.001$). ELISA showed that levels of IL-1β and IL-18 in the bleomycin + sh-HMGB1 group were reduced, compared to those in the bleomycin + sh-NC group (Figure 2H; $P < 0.001$). These results suggested that knockdown of HMGB1 partially reversed PF and NLRP3 inflammasome activation.

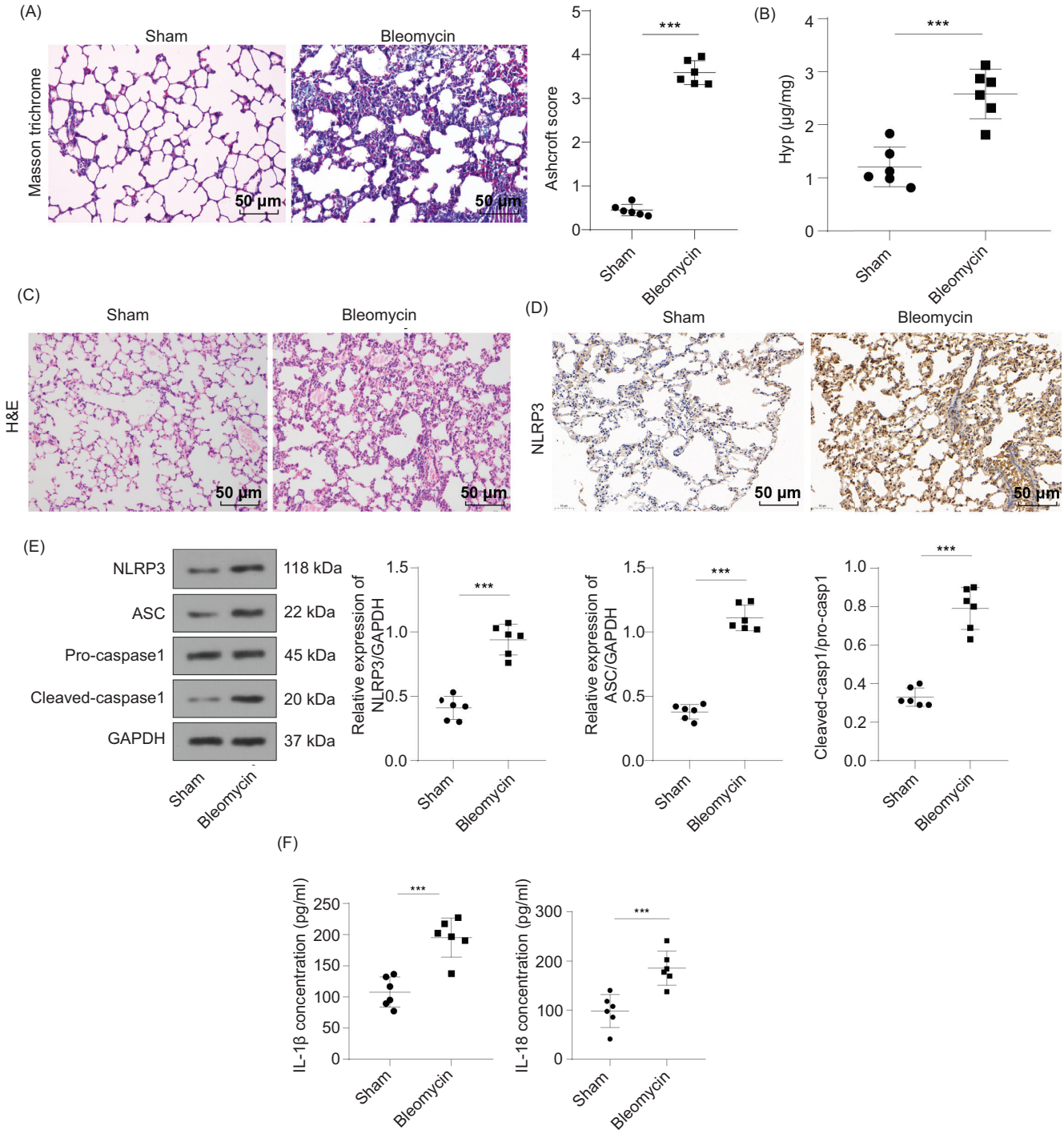

Figure 1 Bleomycin induced PF after ALI in rats and promotes NLRP3 inflammasome activation. The effect of bleomycin on PF in rats and NLRP3 inflammasome activation was examined. (A) Masson staining was used to examine the morphology of lung tissues, and Ashcroft score was performed; (B) Hyp content in rat lung tissues was detected by ELISA; (C) H&E staining was used to examine the morphology of lung tissues; (D) The expression of NLRP3 was determined by immunohistochemistry; (E) The levels of NLRP3, ASC, and cleaved casp-1/pro-casp-1 were detected by Western blotting analysis; (F) ELISA was used to detect the levels of inflammatory cytokines IL-1β and IL-18. *N* = 6, differences between the two groups were compared using Student's *t*-test, ***P < 0.001.

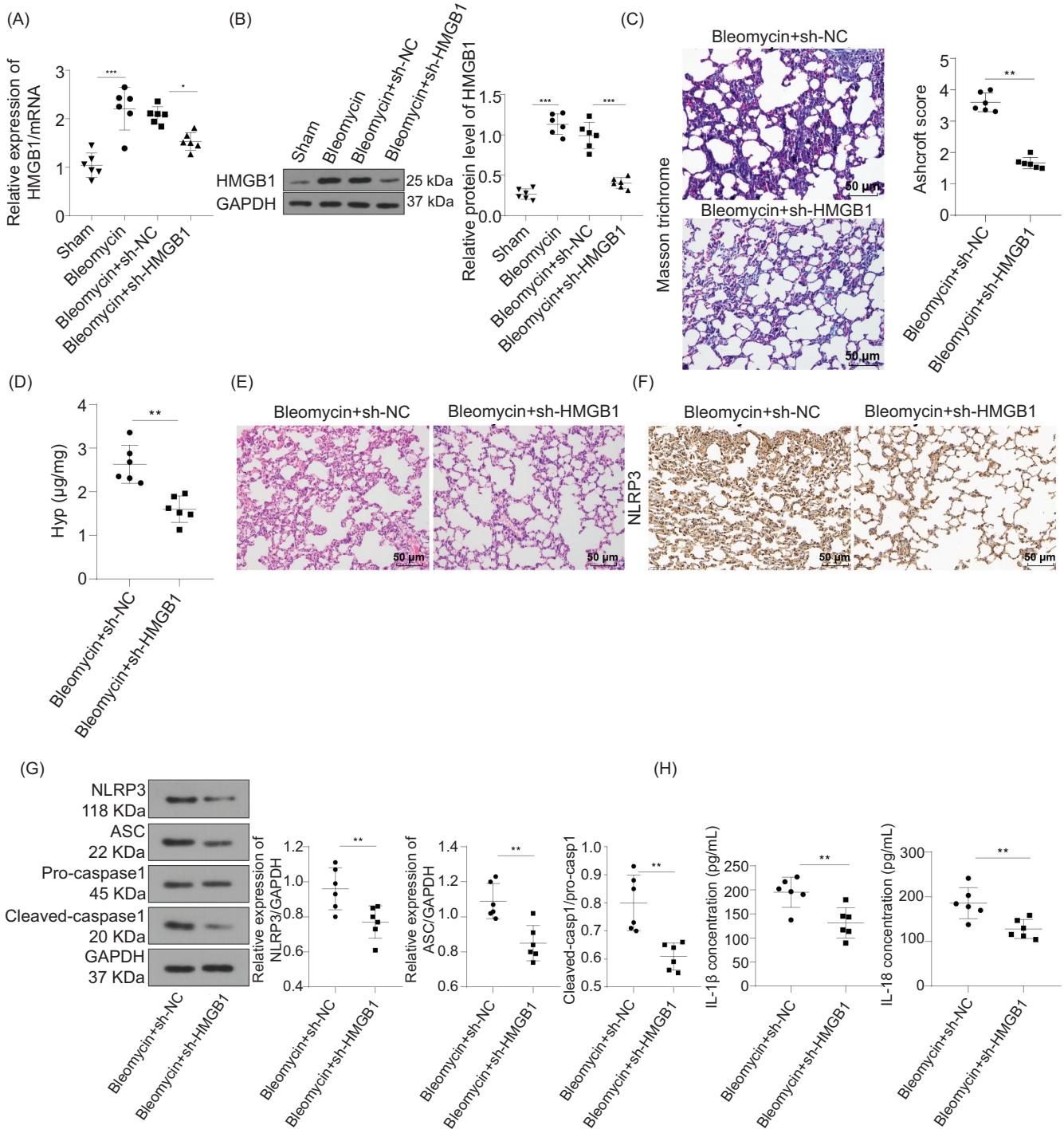

Figure 2 Knockdown of HMGB1 inhibited bleomycin-induced NLRP3 inflammasome activation and PF. The effect of HMGB1 knockdown on PF was observed. The expression of HMGB1 was detected by (A) RT-qPCR and (B) Western blotting analysis; (C) Masson staining was used to examine the morphology of lung tissue, and Ashcroft scoring was performed; (D) Hyp content in rat lung tissues was detected by ELISA; (E) H&E staining was used to examine the morphology of lung tissues; (F) The expression of NLRP3 was detected by immunohistochemistry; (G) The levels of NLRP3, ASC, and cleaved casp-1/pro-casp-1 were detected by Western blotting analysis; (H) ELISA was used to detect the levels of inflammatory cytokines IL-1ß and IL-18. *N* = 6, measurement data were expressed as mean ± SD. Student's *t*-test was used for data comparison between the two groups, one-way analysis of variance (ANOVA) was used for data comparison between multiple groups, and Tukey's test was used for the *post hoc* test. *P < 0.05, **P < 0.01, ***P < 0.001.

Inhibition of HMGB1 promoted Nrf2 nuclear translocation and HO-1 expression

There is evidence that HMGB1 can regulate the Nrf2/HO-1 pathway in macrophages.[31] Hence, we investigated whether HMGB1 affected NLRP3 inflammasome activation by regulating the Nrf2/HO-1 pathway in lung tissues. We discovered the nuclear and cytoplasmic components isolated from the lung tissue of rats by Western blotting analysis to confirm the level of Nrf2 in both cytoplasm and nucleus. Compared to the sham group, Nrf2 in the lung tissues of bleomycin-treated rats was obviously transferred to the cytoplasm, while the level of Nrf2 was up-regulated in the nucleus of rats after treatment with sh-HMGB1 (Figure 3A; $P < 0.001$). Moreover, the level of HO-1 in each group was detected by Western blotting analysis, which revealed that HO-1 level in the lung tissues of bleomycin-treated rats was lower than that in the sham group whereas HO-1 level was raised upon the inhibition of HMGB1 (Figure 3B; $P < 0.001$). Precisely, by inhibiting the activity of HMGB1, Nrf2 was promoted to enter the nucleus, and HO-1 expression was enhanced.

Suppression of HO-1 partially reversed the inhibitory effect of HMGB1 silencing on NLRP3 inflammasome activation and PF

In order to verify whether HMGB1 can regulate NLRP3 inflammasome activation and PF through HO-1, HO-1 inhibitor ZNPP was used to inhibit HO-1 expression in rats in the bleomycin + sh-HMGB1 group. The results illustrated that compared to the biromycin + sh-HMGB1 + DMSO group, HO-1 protein level was decreased after down-regulating HO-1 (all $P < 0.05$; Figure 4A). Masson staining, Ashcroft scoring, and Hyp content detection displayed that relative to rats treated with DMSO, the PF of rats treated with HO-1 inhibitor was aggravated (all P-values <0.05; Figures 4B and C). Both H&E and Masson staining exhibited that the lung inflammatory cell infiltration and PF were aggravated in rats treated with HO-1 inhibitor, compared to rats treated with DMSO alone (Figure 4D). Immunohistochemical staining and Western blotting analysis demonstrated that the levels of NLRP3, ASC, and caspase-1 were significantly increased after adding HO-1 inhibitor (Figures 4E and F), suggesting that inhibition of HO-1 promoted NLRP3 inflammasome activation. In addition, ELISA unveiled that inhibition of HO-1 facilitated the production of inflammatory cytokines, compared to the control group ($P < 0.05$; Figure 4G). These results demonstrated that inhibition of HO-1 partially reversed the inhibitory effect of HMGB1 knockdown on NLRP3 inflammasome stimulation and PF.

Discussion

Acute lung injury is a pulmonary disease characterized by diffused alveolar damage that can lead to pulmonary tissue fibrosis.[3] PF is a fatal lung disease characterized by deposition of extracellular matrix.[32] Compared to persistent pulmonary dysfunction, pathological fibroproliferative response after ALI reduces the quality of life of severe patients.[33] At present, the pathogenesis of PF has not been determined. Abnormal NLRP3 expression has been reported to be associated with fibrosis formation.[34] This study revealed that HMGB1 activated NLRP3 inflammasomes by inhibiting the Nrf2/HO-1 pathway, thereby promoting PF after ALI in rats.

Bleomycin is a commonly used antitumor agent that induces interstitial PF in a dose-dependent manner.[35] Specifically, bleomycin can cause lung epithelial cell damage, inflammation, epithelial–mesenchymal transformation (EMT), and extracellular matrix production in rodent lungs, similar to idiopathic PF in humans.[6] Accordingly, we established rat models of PF by inducing rats with bleomycin. It has been reported that Aschcroft scoring can be adopted to evaluate and quantify lung pathological changes.[36] In parallel, the content of collagen in lung tissues can directly reflect the degree of PF in animals,[37] and the degree of collagen deposition can be reflected by Hyp content.[38] In the present study, the Aschcroft score and content of Hyp in the lung tissues of rats treated with bleomycin were elevated, accompanied by inflammatory cell infiltration, suggesting evident fibrosis in the lung tissue of rats. Recently, over-activation of NLRP3 inflammasome has been reported to facilitate lung inflammation and tissue damage.[39] Emerging evidence suggests that NLRP3 inflammasome promotes the maturation of caspase-1 and the release of important pro-inflammatory cytokines (IL-1β and IL-18), thereby playing a key role in a variety of lung diseases, including asthma, fibrotic lung disease, cystic fibrosis, and ALI.[11,40] Therefore, we measured NLRP3 level, proteins of NLRP3 inflammasomes (NLRP3/ASC/caspase-1), and content of inflammatory cytokines (IL-1β and IL-18) mediated by NLRP3 inflammasomes in lung tissues. As expected, bleomycin treatment elevated the expression of NLRP3, the protein levels of NLRP3 inflammasome, and the content of inflammatory factors in lung tissue of rats. Further, in accordance with our findings, bleomycin exposure has been shown to visibly up-regulate NLRP3 protein levels and accelerate the production of inflammatory cytokines, such as IL-1β and IL-18 in PF rats.[4,41] From the aforementioned findings, it was discovered that bleomycin induced PF after ALI in rats and accelerated NLRP3 inflammasome stimulation.

It has been documented that HMGB1 is a pro-inflammatory medium belonging to the alarmin family and is involved in the induction of airway inflammation and injury in patients with allergy, idiopathic PF, and respiratory viral infection.[42] On the other hand, HMGB1 expression was up-regulated in bleomycin-induced models, which significantly increased collagen deposition and thus mediated fibrosis.[43,44] In our current study, HMGB1 was highly expressed in bleomycin-treated rat lung tissues. Interestingly, knockdown of HMGB1 partially annulled bleomycin-induced PF after ALI, alveolar septal thickening, and inflammatory cell infiltration in rat lung tissue. In addition, knockdown of HMGB1 partially reversed the promoting effect of bleomycin on NLRP3 inflammasome activation, accompanied by a decrease in the contents of inflammatory cytokines. Consistently, inhibition of HMGB1 weakened lung injury in cystic fibrosis mice provoked by bacterial infection.[14] Moreover, inhibition of HMGB1 could improve intestinal inflammation in necrotizing enterocolitis

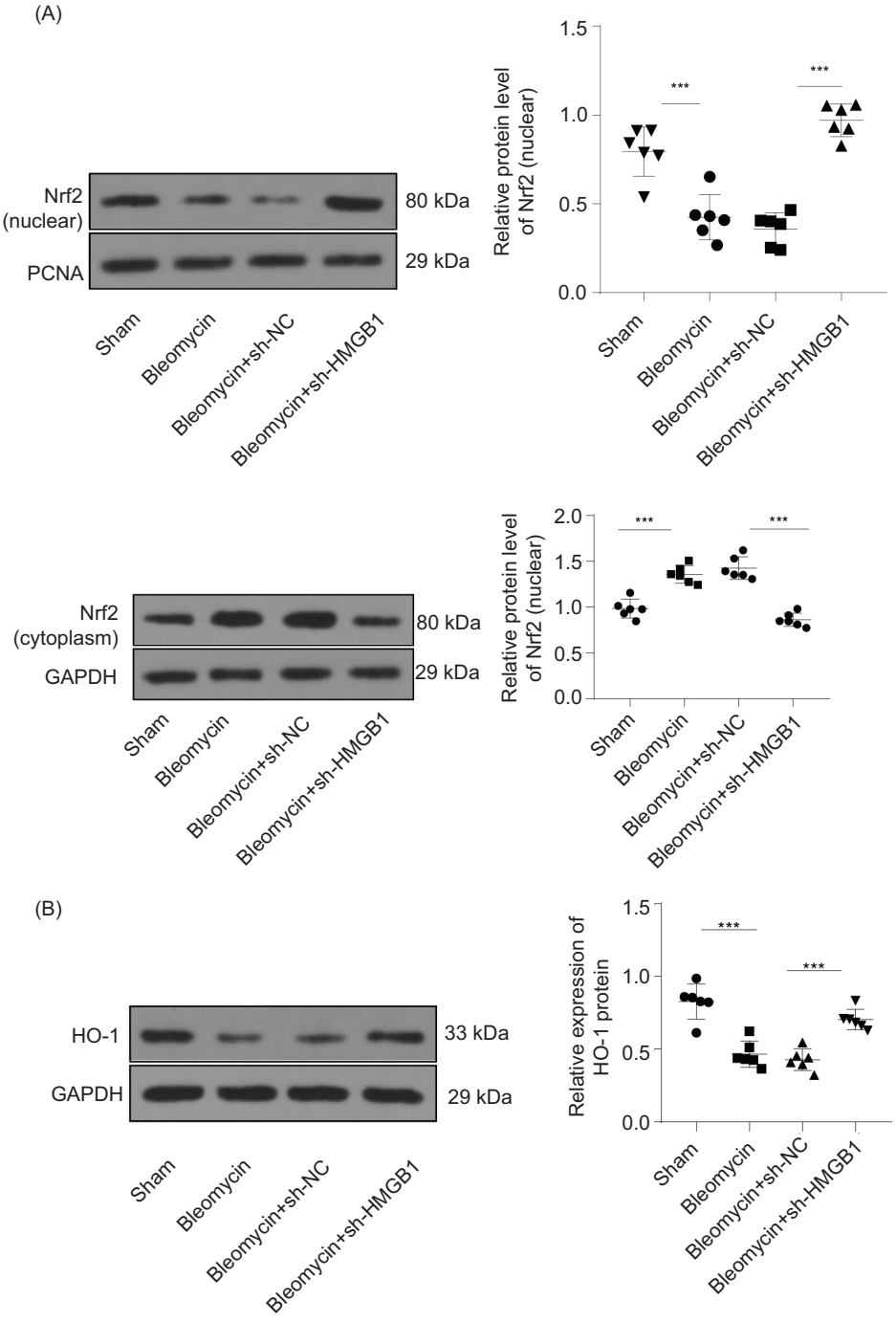

Figure 3 Inhibition of HMGB1 activity promoted Nrf2 nuclear translocation and enhanced HO-1 expression. The effect of inhibiting HMGB1 activity on Nrf2 nuclear import and HO-1 expression was detected. (A) Western blotting analysis was used to detect Nrf2 levels in the nucleus and cytoplasm of rats; (B) Western blotting analysis was used to detect the expression of HO-1. *N* = 6, measurement data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used for data comparison between multiple groups, and Tukey's test was used for *post hoc* test. ***P < 0.001.

rats by inhibiting NLRP3.[45] It is reasonable enough to conclude that knockdown of HMGB1 partially abrogated PF after ALI in rats and NLRP3 inflammasome activation.

Nrf2/HO-1 pathway, as the main regulatory pathway of intracellular defense against oxidative stress, has attracted extensive attention and is considered as an ideal target to reduce endothelial cell injury.[46] Under normal conditions, Nrf2 is fixed in the cytoplasm, and the activation of Nrf2 protein promotes the translocation of Nrf2 from the cytoplasm to the nucleus, thereby activating the transcription of antioxidant, cell protection, and anti-inflammatory genes.[47] In addition, HO-1, as one of the major downstream

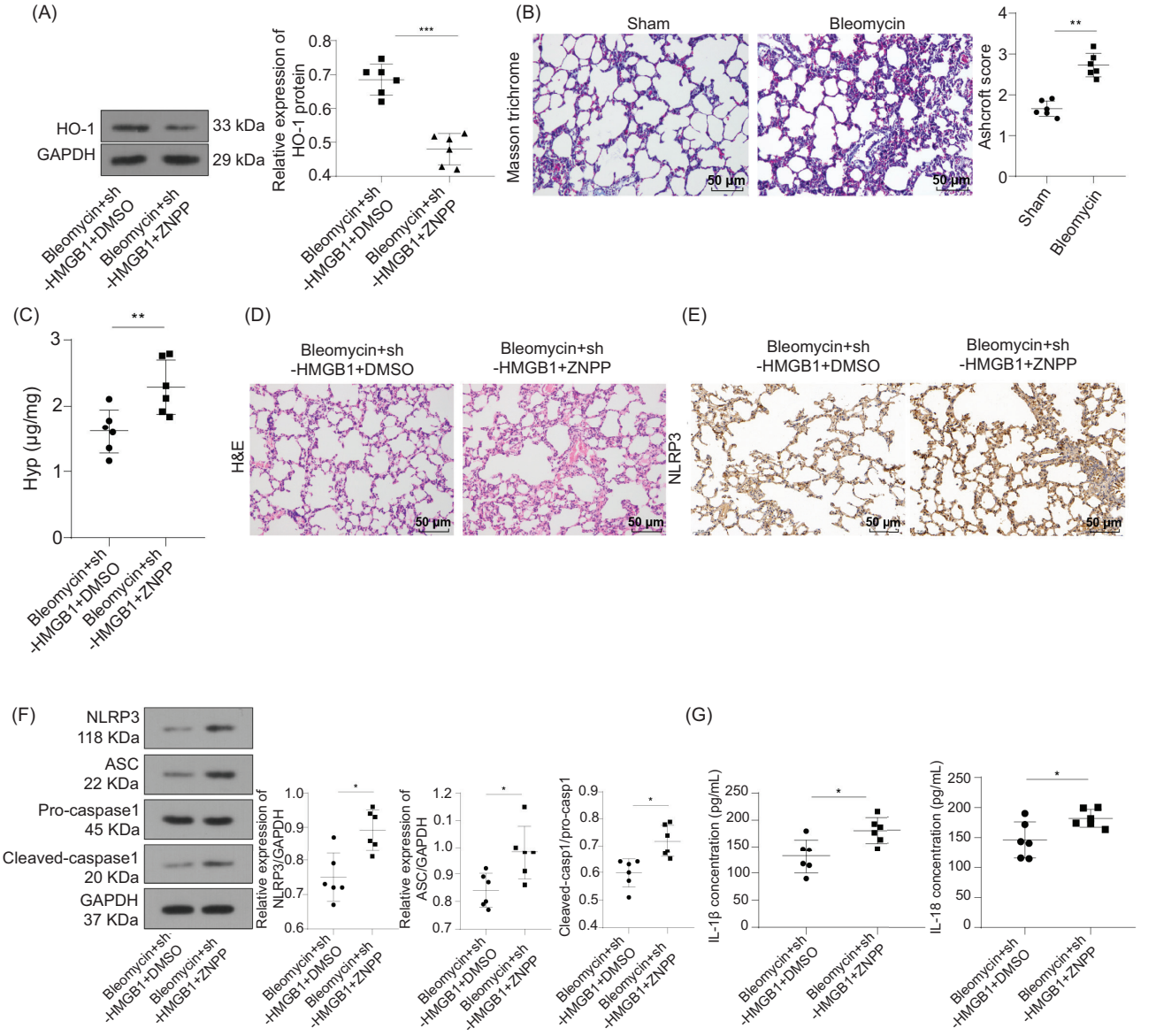

Figure 4 Inhibition of HO-1 partially reversed the inhibitory effect of HMGB1 knockdown on NLRP3 inflammasome activation and PF. To detect whether HMGB1 could regulate NLRP3 inflammasome activation and PF through HO-1 (A) Western blotting analysis was used to detect HO-1 expression; (B) Masson staining was used to examine the morphology of lung tissues, and Ashcroft scoring was performed; (C) Hyp content in rat lung tissues was detected by ELISA; (D) H&E staining were used to examine the morphology of lung tissues; (E) the expression of NLRP3 was detected by immunohistochemistry; (F) levels of NLRP3, ASC, and cleaved casp-1/pro-casp-1 were detected by Western blotting analysis; (G) ELISA was used to detect the levels of inflammatory cytokines IL-1ß and IL-18. *N* = 6, measurement data were expressed as mean ± SD. Student's *t*-test was used for data comparison between two groups, and Tukey's test was used for *post hoc* test. *P < 0.05, **P < 0.01, ***P < 0.001.

molecules of Nrf2, coordinates oxidative stress and inflammatory responses in a variety of diseases and pathological conditions.[48] Nrf2 and downstream antioxidant HO-1 were significantly decreased in the lung tissues of mice after administration of bleomycin,[49] consistent with our findings. Our results also uncovered that the levels of Nrf2 in the nucleus and HO-1 were enhanced after inhibition of HMGB1. Activating Nrf2 antioxidant pathway can suppress EMT during PF.[50] HMGB1 silencing is required for the protective role of Nrf2 in EMT-mediated PF.[18] Together, the aforementioned finding highlighted that inhibition of HMGB1 activity can promote Nrf2 nuclear translocation and enhance HO-1 expression.

The Nrf2/HO-1 pathway inhibited NF-κB-P65 phosphorylation, thereby inhibiting NLRP3 inflammasome activation.[51] Inhibition of Nrf2/HO-1 signaling leads to the activation of NLRP3 inflammasome in osteoarthritis.[52] In order to verify the influence of HO-1 on NLRP3 inflammasome, we silenced HMGB1 and HO-1 expressions in bleomycin-treated rats simultaneously. Not surprisingly, suppression of HO-1 expression aggravated pulmonary inflammatory cell infiltration and PF, promoted activation

of NLRP3 inflammasome, and encouraged the production of inflammatory cytokines. Consistently, the suppression effect of tubulointerstitial fibrosis and renal inflammation by intermedin, a peptide related to calcitonin gene-related peptide (CGRP), was blocked by HO-1 inhibitor ZnPP.[53] Meanwhile, HO-1 can degrade heme into biliverdin, CO, and free iron, and CO negatively regulates the activation of NLRP3 inflammasomes.[54] More importantly, inhibition of HO-1 blocked the inhibition of emodin on NLRP3 inflammasome and contributed to acute pancreatitis-associated lung injury in rats.[55] Altogether, inhibition of HO-1 partially annulled the inhibitory effect of HMGB1 knockdown on NLRP3 inflammasome activation and PF.

Conclusion

This study demonstrated that inhibition of HMGB1 could promote the entry of Nrf2 into the nucleus, enhance HO-1 levels, and inhibit NLRP3 inflammasomes, thus inhibiting PF and protection against lung injury. In addition, by inhibiting HO-1 in rats in the bleomycin + sh-HMGB1 group (adding HO-1 inhibitor ZNPP), we confirmed that HMGB1 knockdown enhanced HO-1 expression by promoting Nrf2 entry into the nucleus, thereby inhibiting bleomycin-induced NLRP3 inflammasome activation and PF. Here, the direct regulation effect of HMGB1 on HO-1 was confirmed. However, the relationship between HMGB1 and Nrf2 and between HO-1 and NLRP3 inflammasome has not been checked directly, which is a limitation of this study. Moreover, this study simply revealed that NLRP3 inflammasomes promoted PF after ALI in rats, and the regulatory mechanism of downstream factors affected by NLRP3 on PF has not been further studied. In the future, we need to further study the specific regulatory mechanism of NLRP3 inflammasomes in PF. In addition, the relationship between HMGB1 and Nrf2 and between HO-1 and NLRP3 inflammasome must be affirmed.

Competing Interests

The authors declared that they had no competing interests to assert.

Availability of Data and Materials

All the data generated or analyzed during this study are included in this published article.

Author Contributions

Ying Huang and Aili Wang contributed to the concept and study design. Sheng Jin contributed as a guarantor of integrity of the entire study. Fang Liu contributed to literature research. Ying Huang, Aili Wang, and Fang Xu contributed to experimental studies and data acquisition. Ying Huang, Aili Wang, and Sheng Jin contributed to manuscript preparation, and Fang Liu and Fang Xu contributed to manuscript editing and review. All authors read and approved the final manuscript.

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