Anti-inflammatory effect of salusin-β knockdown on LPS-activated alveolar macrophages via NF-κB inhibition and HO-1 activation

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Abstract. Inflammation of alveolar macrophages is the primary pathological factor leading to acute lung injury (ALI), and NF-κB activation and HO-1 inhibition are widely involved in inflammation. Salusin-β has been reported to contribute to the progression of the inflammatory response, but whether salusin-β could regulate inflammation in lipopolysaccharide (LPS)-induced ALI remains unknown. The present study aimed to investigate the role of salusin-β in LPS-induced ALI and to uncover the potential underlying mechanisms. Sprague-Dawley rats were subjected to LPS administration, and then pathological manifestations of lung tissues, inflammatory cytokines levels in bronchoalveolar lavage fluid (BALF) and expression of salusin-β in macrophages of lung tissues were assessed. NR8383 cells with or without salusin-β knockdown were treated with LPS, and then the concentration of inflammatory cytokines, and the expression of high mobility group box-1 (HMGB1), NF-κB signaling molecules and heme oxygenase-1 (HO-1) levels were detected. The results showed that LPS caused injury of lung tissues, increased the levels of proinflammatory cytokines in BALF, and led to higher expression of salusin-β or macrophages in lung tissues of rats. *In vitro* experiments, LPS also upregulated salusin-β expression in NR8383 cells. Knockdown of salusin-β using short hairpin (sh)RNA inhibited the LPS-induced generation of inflammatory cytokines. LPS also enhanced HMGB1, phosphorylated (p)-IκB and p-p65 expression, but reduced HO-1 expression in both lung tissues and NR8383 cells, which were instead inhibited by the transfection of sh-salusin-β. In addition, knockdown of HO-1 using shRNA reversed the inhibitory effect of sh-salusin-β on the LPS-induced generation of inflammatory cytokines, activation of NF-κB signaling and inactivation of HO-1. In conclusion, this study suggested that knockdown of salusin-β may inhibit LPS-induced inflammation in alveolar macrophages by blocking NF-κB signaling and upregulating HO-1 expression.

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Introduction

Acute lung injury (ALI) is a serious disease with diffuse alveolar injury, which has a high morbidity and mortality rate in patients in intensive care (1). Uncontrolled acute inflammatory response and excessive secretion of proinflammatory cytokines are considered to be two of the primary pathological factors leading to ALI (2). Previous studies have confirmed that macrophages, neutrophils, lymphocytes, lung epithelial fibroblasts and platelets are associated with the occurrence and development of ALI (3,4). In addition, the inflammatory cytokines produced by these cells form a complex signaling network, which is activated by various external stimuli and can regulate all stages of the inflammatory response in ALI (5). Therefore, preventing the release of inflammatory cytokines is of great significance for treating ALI. As a component of Gram-negative bacterial cell walls, lipopolysaccharide (LPS) is one of the most potent activators for regulating the gene expression of inflammatory cytokines, and is commonly utilized to induce ALI in animals models and cell lines (6,7).

Salusin-β is a 20-amino acid peptide that is translated from an alternatively spliced mRNA of prosalusin (TOR2A), which encodes proteins of the torsion dystonia family (8). The first 18 amino acids of human salusin-β have high homology with the N-terminal sequence of rat salusin (9). TOR2A is widely expressed in the small intestine, stomach and lung, and salusin-β can be detected in macrophages of the hematopoietic and immune systems (9,10).

Over the past decade, salusin-β has been extensively reported to be associated with inflammatory-related diseases (11,12). For example, Xu *et al* (13) found that salusin-β was predominantly expressed in pulmonary macrophages and contributed to vascular inflammation associated with pulmonary arterial hypertension in rats. Salusin-β was also demonstrated to lead to inflammation in diabetic cardiomyopathy (DCM), and its knockdown could attenuate cardiac

dysfunction, oxidative stress and inflammation in DCM (14). Li *et al* (15) suggested the potential beneficial effects of salusin-β blockade in essential hypertension via downregulation of inflammatory molecules and oxidative stress. However, whether salusin-β could inhibit the inflammatory response in LPS-induced alveolar macrophages remains unknown.

Various molecular pathways participate in the occurrence or development of ALI. Among them, the high mobility group box-1 (HMGB1) protein and HMGB1-mediated NF-κB activation play important roles in LPS-induced ALI (16,17). Heme oxygenase-1 (HO-1) is a stress-response proteins, and can be induced by stimulants, such as proinflammatory cytokines, heat shock and oxidants (18). The activation of HO-1 has been demonstrated to be required for antioxidant and anti-inflammatory actions in LPS-induced ALI (19,20).

The rat alveolar macrophage cell line, NR8383 is a homogenous and expandable source of alveolar macrophage-like cells, which has been shown to express functional characteristics of alveolar macrophages, including properties of phagocytes, production of proinflammatory cytokines and oxidative stress (21). In the present study, LPS was used to induce ALI in rats and alveolar macrophage inflammation in NR8383 cells to investigate whether salusin-β was involved in LPS-induced lung inflammation, as well as to uncover the potential underlying mechanisms.

Materials and methods

Animals and protocols. A total of 15 specific pathogen-free male Sprague-Dawley rats (age, 7-8 weeks; weight, 240-280 g) were purchased from Guangdong Medical Laboratory Animal Center and housed at room temperature under a controlled 12/12 h light/dark cycle. All rats received food and water *ad libitum*. All procedures were performed in accordance with the Care and Use Guide of Laboratory Animals of the National Institutes of Health and with the approval of the ethics committee of Fujian Medical University Union Hospital (approval no. IACUC-2018 1212-09; Fuzhou, China). Rats were randomly assigned to two groups (n=5 rats/group): Control and LPS. LPS was intratracheally administered to rats as described previously (22). After anesthetization by intraperitoneal (i.p.) injection of 3% sodium pentobarbital, the LPS-treated rats received 50 mg/kg LPS (Sigma-Aldrich; Merck KGaA) by intratracheal instillation, while control animals were instilled intratracheally with 200 μl normal saline instead of LPS. At 24 h after LPS instillation, rats were anesthetized for subsequent experiments, and then subjected to cervical dislocation of the spine immediately.

Collection of broncho alveolar lavage fluid (BALF). BALF (n=5 rats/group) was collected at 24 h post-LPS treatment. Before lavage, rats were anesthetized by i.p. injection of sodium pentobarbital (3%, 10 ml/kg). After exposing the chest cavity and intubating the trachea, the lungs were washed with 2 ml normal saline three times, and the flushing fluid was collected. The collected solution was centrifuged at 1,500 x g for 10 min at 4˚C. The supernatant was collected and stored at -80˚C for further analysis.

Histological staining. At 24 h after LPS instillation, rats were anesthetized by injection of sodium pentobarbital (i.p., 3%, 10 ml/kg). The left lung tissues were isolated and fixed with 4% paraformaldehyde at 4˚C for 24 h, embedded in paraffin, and then cut into 5-μm thick sections. After staining with hematoxylin (5 min) and eosin (20 sec) at room temperature, the lung tissue sections were observed under an optical microscope for pathological examination (magnification, x400).

Cell culture and treatment. The rat alveolar macrophage cell line NR8383 (American Type Culture Collection) was maintained in F12 medium (Thermo Fisher Scientific, Inc.) supplemented with 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin, 100 U/ml penicillin and 2 mmol L-glutamine (Beyotime Institute of Biotechnology) at 37˚C in a humid atmosphere of 5% CO_2. The medium was discarded and replaced by fresh medium every 3 days until the NR8383 cells reached 60% confluence. Before passaging, adherent cells were harvested, and then centrifuged (100 x g, 4˚C, 5 min) and transferred to new microplates.

For induction of ALI *in vitro*, the cells were stimulated with or without 1 μg/ml LPS for various times (6, 12, 24 and 48 h) at 37˚C. Short hairpin (sh)RNA targeting salusin-β and HO-1 together with shRNA control were designed and synthesized by Shanghai GenePharma Co., Ltd. Then, 20 μg pcDNA 3.1 plasmids (Thermo Fisher Scientific, Inc.) and 20 nM shRNAs were transfected into cells at 70-80% confluence using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, as described previously (23). At 48 h post-transfection, cells were selected for subsequent experiments.

ELISA. The concentrations of TNF-α (cat. no. ab236712), IL-1β (cat. no. ab255730), IL-6 (cat. no. ab234570) and monocyte chemotactic protein 1 (MCP-1; cat. no. ab219045) in the BALF or cell culture medium were determined using specific ELISA kits (Abcam), according to the manufacturer's instructions.

Western blotting. NR8383 cells were lysed, total protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the total protein concentration was determined with a Bradford assay (Bio-Rad Laboratories, Inc.). Equal quantities of protein (20 μg) in each sample were separated via 10% SDS-PAGE, and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk at room temperature for 2 h and incubated with primary antibodies against salusin-β (1:200; cat. no. B-010-68; Phoenix Pharmaceuticals, Inc.), CD68 (1:1,000; cat. no. ab125212; Abcam), HO-1 (1:2,000; cat. no. ab189491; Abcam), HMGB1 (1:800; cat. no. ab18256; Abcam), IκBα (1:500; cat. no. ab76429; Abcam), p-IκBα (1:10,000; cat. no. ab133462; Abcam), p65 (1:1,000; cat. no. ab16502; Abcam), p-p65 (1:1,000; cat. no. ab76302; Abcam) and GAPDH (1:5,000; cat. no. ab8245; Abcam) overnight at 4˚C. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; cat. no. ab6721; Abcam) and goat anti-mouse IgG secondary antibodies (1:5,000; cat. no. ab6789; Abcam) were used for detection (room temperature, 2 h). The protein bands were visualized with an Enhanced Chemiluminescence Detection kit (Thermo Fisher Scientific, Inc.). Protein expression levels were semi-quantified using Image-Pro Plus software version 6.0 (Roper Technologies, Inc.).

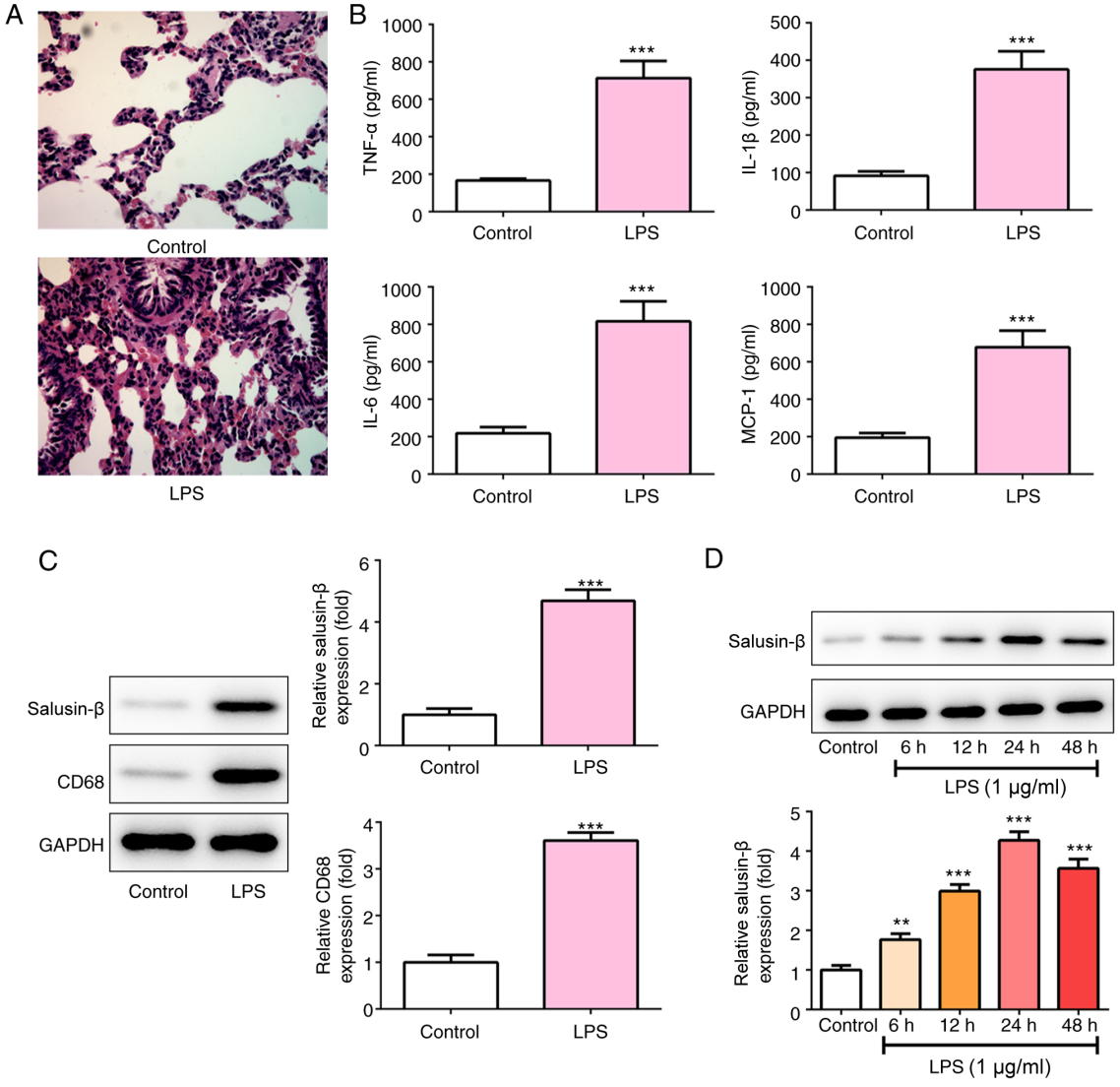

Figure 1. Salusin-β is upregulated in LPS-induced acute lung injury. (A) Representative hematoxylin and eosin staining of lung tissues of rats in the LPS or control group. (B) Concentrations of TNF-α, IL-1β, IL-6 and MCP-1 in the bronchoalveolar lavage fluid of rats in the LPS or control group (n=5). (C) Protein expression of salusin-β and CD68 in lung tissues of rats in the LPS or control group (n=5). (D) Protein expression of salusin-β in NR8383 cells treated with or without 1 μg/ml LPS for 6, 12, 24 and 48 h (n=3). **P<0.01 and ***P<0.001 vs. control. LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to analyze the expression of genes. Total RNA was isolated from cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). The PrimeScript RT Master Mix kit (Takara Biotechnology, Co., Ltd.) was utilized to synthesize cDNA according to the manufacturer's instructions. Subsequently, qPCR was performed with SYBR-Green PCR Master Mix (Roche Diagnostics) on an ABI Quantitative PCR 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used were as follows: Salusin-β forward, 5'-TCACTTCTCTCCTATCATCCACTCC-3' and reverse, 5'-GGCAGCTTGTCCATCTCATCG-3'; HO-1 forward, 5'-GTCCCAGGATTTGTCCGAGG-3' and reverse, 5'-GGAGGCCATCACCAGCTTAAA-3'; and GAPDH forward, 5'-GTGGAGTCTACTGGCGTCTT-3' and reverse, 5'-TGCTGACAATCTTGAGGGA-3'. PCR reaction conditions were as follows: 95˚C for 2 min, followed by 40 cycles of 95˚C for 20 sec and 65˚C for 40 sec. Expression levels of target genes were normalized to endogenous control GAPDH using the $2^{-\Delta\Delta Cq}$ method (24).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). All experiments were repeated at least three times and data are expressed as the mean ± standard deviation. One-way ANOVA followed by Tukey's post hoc test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Salusin-β is upregulated in lung tissues of LPS-induced rats and in the LPS-treated rat alveolar macrophage cell line NR8383. First, to determine whether Salusin-β plays a role in LPS-induced ALI, rats were treated with LPS via intratracheal instillation, which is a previously published method to induce ALI in animals (25,26). The lung tissues and BALF of rats with ALI were collected at 24 h after treatment with or without LPS. As shown in Fig. 1A, in the control group, the lung tissue structure was complete,

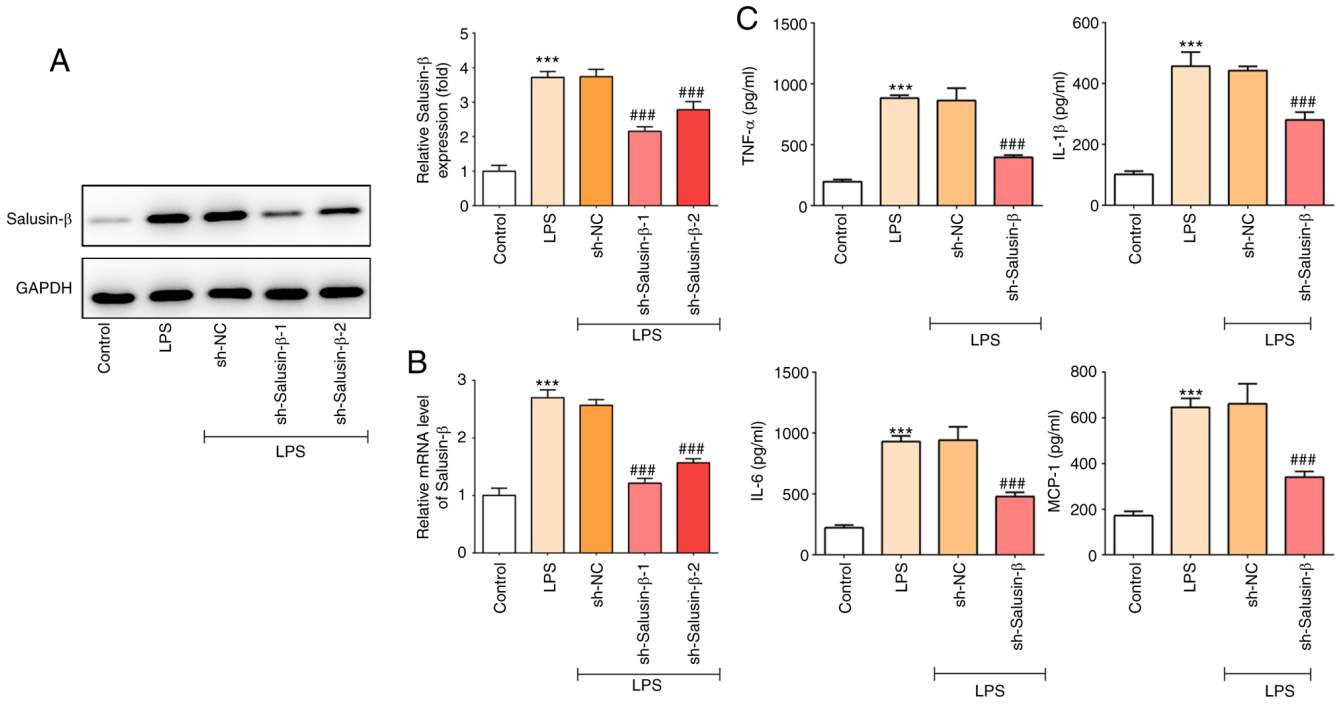

Figure 2. Effect of salusin-β knockdown on LPS-induced release of inflammatory cytokines. (A) Protein expression of salusin-β in NR8383 cells transfected with shRNA-salusin-β-1/2 or sh-NC (n=3). (B) mRNA expression of salusin-β in NR8383 cells transfected with shRNA-salusin-β-1/2 or sh-NC (n=3). (C) Concentrations of TNF-α, IL-1β, IL-6 and MCP-1 in the culture medium of LPS-induced NR8383 cells with or without salusin-β knockdown (n=3). ***P<0.001 vs. control; ###P<0.001 vs. sh-NC. NC, negative control; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; shRNA, short hairpin RNA.

the alveolar cavity was clear, the alveolar wall was not congested, and there was no inflammatory cell infiltration in the lung interstitium. By contrast, in the LPS group, the alveolar wall was diffusely thickened, and part of the alveolar wall was destroyed with obvious inflammatory cell infiltration, alveolar hemorrhage and structural damage. At the same time, the concentration of inflammatory cytokines, including TNF-α, IL-1β, IL-6 and MCP-1 in the BALF of the LPS group was significantly increased to nearly 4-fold of that of the control group (Fig. 1B). These results confirmed the induction of ALI in rats. CD68 is a marker of macrophages, and the results shown in Fig. 1C revealed that the lung tissues of rats in the LPS group expressed significantly higher levels of salusin-β and CD68. Additionally, NR8383 cells were exposed to 1 μg/ml LPS for 6, 12, 24 or 48 h, and the expression of salusin-β was measured. LPS also significantly promoted salusin-β expression in NR8383 cells (Fig. 1D). Considering that the expression of salusin-β reached the highest level (4.275±0.2177-fold of control) at 24 h post-LPS treatment, cells were exposed to LPS for 24 h in the subsequent experiments.

Knockdown of salusin-β inhibits the LPS-induced release of inflammatory cytokines. Next, to further explore the role of salusin-β in LPS-induced ALI, salusin-β was knocked down using shRNA, as shRNA-salusin-β-1 showed the highest knock down effect, it was selected for subsequent experiments (Fig. 2A and B). As shown in Fig. 2C, knockdown of salusin-β significantly inhibited the concentration of inflammatory cytokines, including TNF-α, IL-1β, IL-6 and MCP-1, compared with the increase caused by LPS treatment, indicating the inhibitory effect of salusin-β knockdown on LPS-induced inflammation in alveolar macrophage cells.

Knockdown of salusin-β prevents LPS-induced activation of NF-κB and inhibition of HO-1. The present study then aimed to investigate the potential underlying mechanism involved in the action of salusin-β. The protein expression of HMGB1, phosphorylated (p)-IκB α, p-p65 and HO-1 in the lung tissues of rats was determined. The results from Fig. 3A show that, compared with that of control rats, the expression levels of HMGB1, p-IκBα and p-p65 were significantly upregulated, whereas that of HO-1 was downregulated (0.393±0.0662-fold of control), in rats that were subjected to LPS treatment. Consistently, LPS treatment also increased HMGB1, p-IκBα and p-p65 expression, but reduced HO-1 expression, in NR8383 cells (Fig. 3B). However, the knockdown of salusin-β partially recovered the LPS-induced expression changes of these proteins (Fig. 3B), suggesting that the knockdown of salusin-β could prevent the LPS-induced activation of NF-κB and inhibition of HO-1.

Knockdown of HO-1 weakens the inhibitory effect of shRNA-salusin-β on LPS-induced inflammation and NF-κB activation. Finally, to further confirm the aforementioned findings, the expression of HO-1 was silenced, and shRNA-HO-1-1 was utilized to knock down the expression of HO-1 in subsequent experiments, which was based on its higher efficacy (Fig. 4A and B). As shown in Fig. 4C, compared with cells that had been transfected with shRNA-salusin-β, cells that were subjected to co-treatment with shRNA-HO-1 produced a relatively higher (P<0.05) concentration of inflammatory

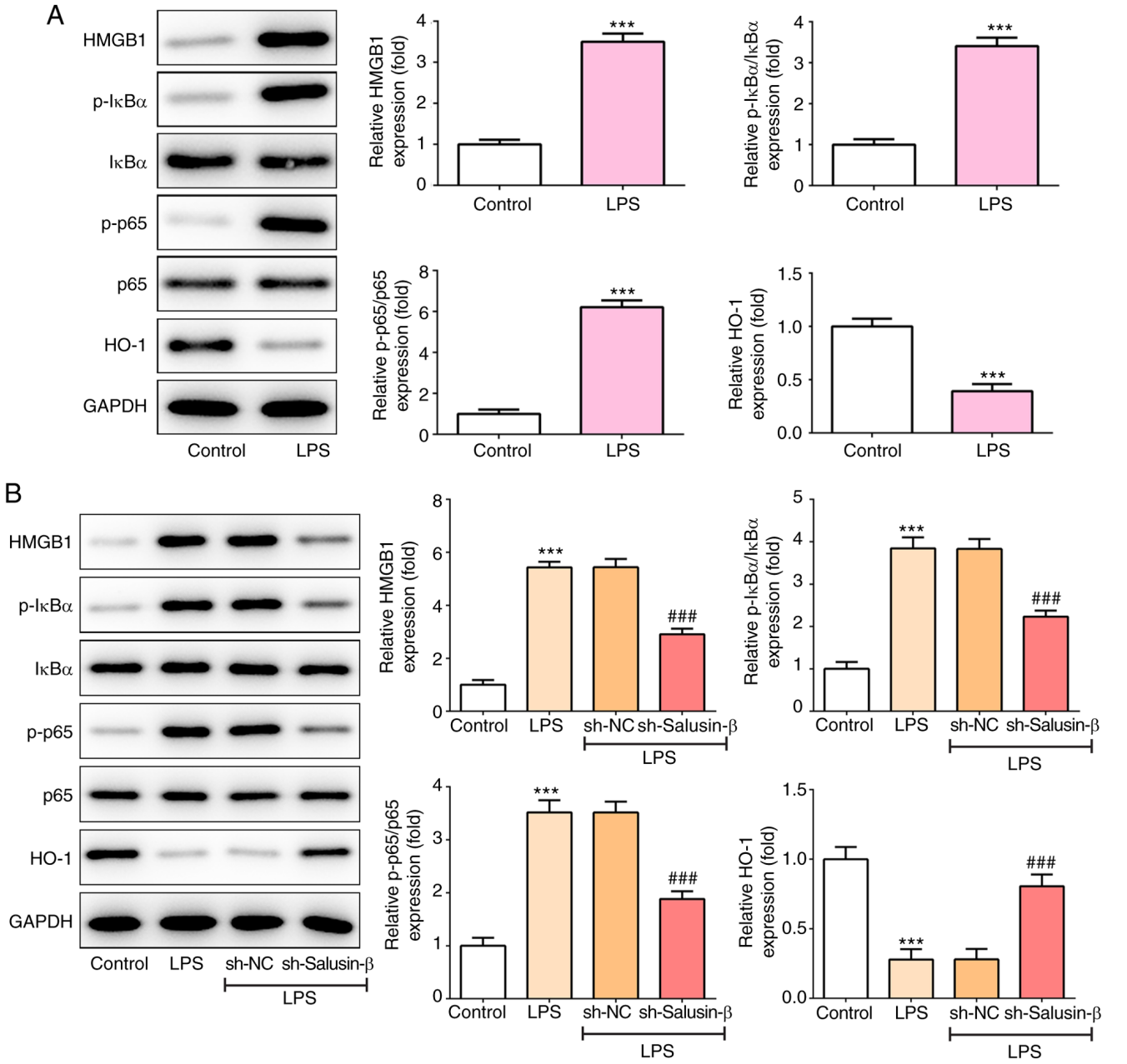

Figure 3. Effect of salusin-β knockdown on LPS-induced activation of NF-κB and inhibition of HO-1. (A) The protein expression of HMGB1, p-IκBα, p-p65 and HO-1 in lung tissues of rats in the LPS or control group (n=5). (B) The protein expression of HMGB1, p-IκBα, p-p65 and HO-1 in LPS-induced NR8383 cells with or without salusin-β knockdown (n=3). ***P<0.001 vs. control; ###P<0.001 vs. sh-NC. NC, negative control; p-, phosphorylated; HO-1, heme oxygenase-1; HMGB1, high mobility group box-1; LPS, lipopolysaccharide; shRNA, short hairpin RNA.

cytokines, including TNF-α, IL-1β, IL-6 and MCP-1, under LPS stimulation. In addition, shRNA-HO-1 also blocked the effect of shRNA-salusin-β on HMGB1, p-IκB, p-p65 and HO-1 expression (P<0.01; Fig. 4D). These results indicated that the inhibitory effect of shRNA-salusin-β on LPS-induced inflammation and NF-κB activation was dependent on the activation of HO-1.

Discussion

ALI is a common clinical critical illness, which is usually induced by infection, trauma and shock. In the early stage of ALI, diffuse alveolar injury and damage to the barrier function of alveolar epithelial cells can activate various intracellular signaling pathways involved in inflammatory responses, thus causing a cascade of inflammatory factors and ultimately leading to uncontrolled inflammation (27). The present study showed that LPS caused obvious pathological manifestations, including intra-alveolar hemorrhage, inter-alveolar septum thickening, inflammatory cell infiltration in lung tissues of rats, and increased production of inflammatory cytokines, including TNF-α, IL-1β, IL-6 and MCP-1, in BALF. Therefore, controlling the primary disease and preventing the inflammatory response is an effective strategy and method for preventing and treating ALI.

Salusin-β has been extensively reported to be upregulated in inflammatory tissues and to play a proinflammatory effect in various diseases (13,14). Consistent with previous

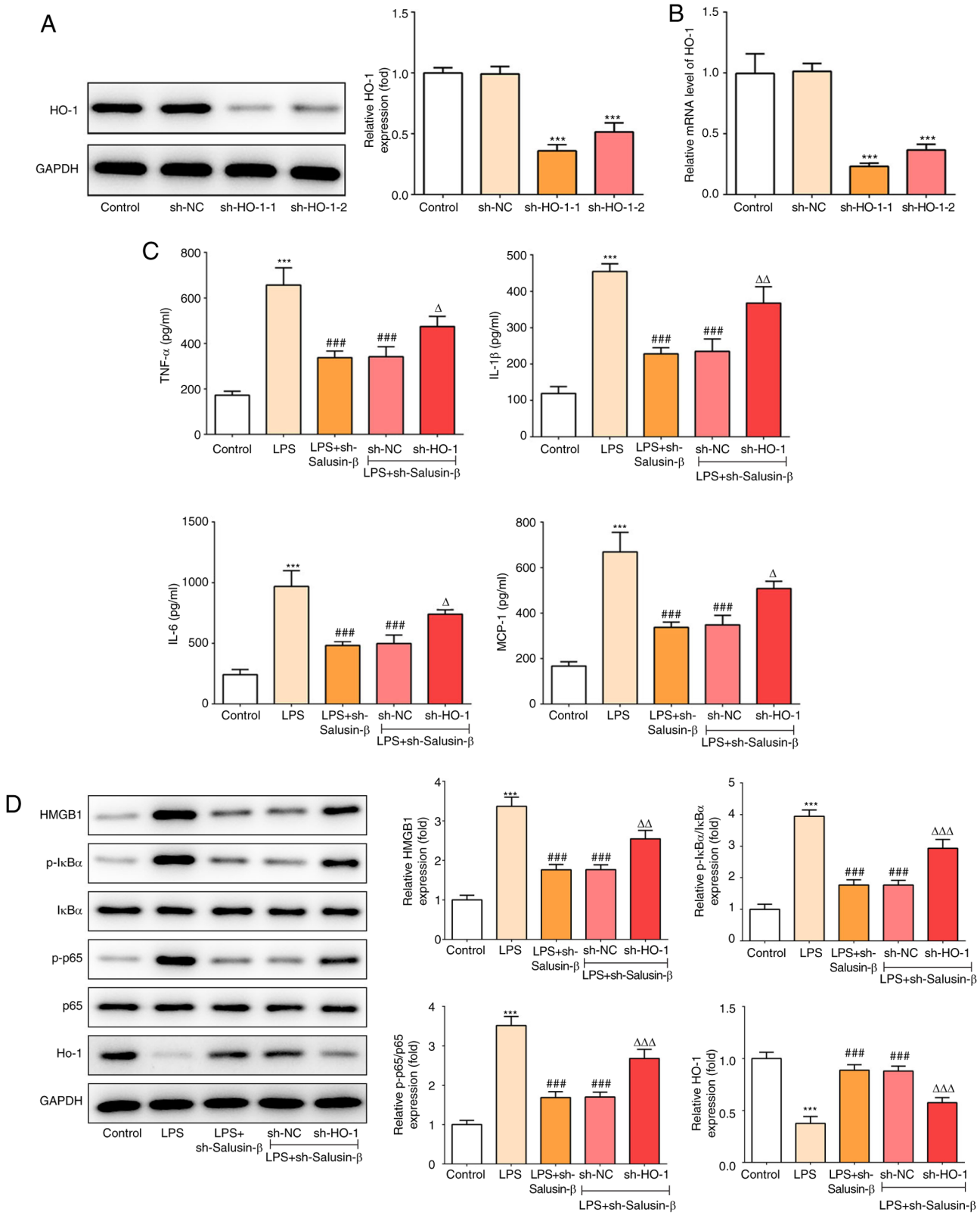

Figure 4. Effect of HO-1 knockdown on LPS-induced inflammation and NF-κB activation in the presence of shRNA-salusin-β. (A) Protein expression of HO-1 in NR8383 cells transfected with shRNA-HO-1-1/2 or sh-NC (n=3). (B) mRNA expression of HO-1 in NR8383 cells transfected with shRNA-HO-1-1/2 or sh-NC (n=3). (C) Concentrations of TNF-α, IL-1β, IL-6 and MCP-1 in the culture medium of LPS-induced NR8383 cells with or without HO-1 knockdown in the presence of shRNA-salusin-β (n=3). (D) Protein expression of HMGB1, p-IκBα, p-p65 and HO-1 in LPS-induced NR8383 cells with or without HO-1 knockdown in the presence of shRNA-salusin-β (n=3). ***P<0.001 vs. control; $^{###}$P<0.001 vs. LPS; ^{Δ}P<0.05, ^{ΔΔ}P<0.01, ^{ΔΔΔ}P<0.001 vs. LPS + sh-salusin-β + sh-NC. HO-1, heme oxygenase-1; p-, phosphorylated; NC, negative control; LPS, lipopolysaccharide; shRNA, short hairpin RNA; MCP-1, monocyte chemotactic protein 1.

studies (13,14,28), the present results revealed that salusin-β expression was increased in LPS-induced lung tissues compared with that of normal lung tissues of rats (nearly 5 times as much as the control group). Moreover, LPS treatment could

enhance salusin-β expression in a time-dependent manner in a rat alveolar macrophage cell line. Of note, the expression of salusin-β was highest at 24 h post-LPS treatment, which could be because the stimulation of LPS for 48 h caused damage to cells, leading to cell death, therefore the relative expression of salusin-β at 48 h post-treatment was lower than 24 h stimulation. These results indicated that salusin-β may also have a proinflammatory effect on LPS-induced lung injury. Therefore, in the present study, the expression of salusin-β was knocked down to observe the alterations in LPS-induced alveolar macrophage inflammation. The results demonstrated that knockdown of salusin-β significantly reduced the release of inflammatory cytokines, including TNF-α, IL-1β, IL-6 and MCP-1, in NR8383 cells, indicating that salusin-β silencing could exert an anti-inflammatory effect on LPS-induced lung injury.

Numerous studies have demonstrated that HO-1 and its products can exhibit antioxidant, anti-apoptotic and immunomodulatory functions in various models of cell and tissue injury (29,30). In addition, HO-1 has been demonstrated to significantly block the expression of the proinflammatory mediator HMGB1 and the pro-inflammatory NF-κB signaling pathway induced by LPS in animal models and cell lines, thus alleviating the pathogenesis of ALI (19,31). In accordance with the aforementioned findings, the present study also confirmed that, following LPS stimulation, HMGB1, p-IκBα and p-p65 expression increased, and HO-1 expression decreased. However, the present study found that the knockdown of salusin-β successfully inhibited the activation of NF-κB, but upregulated the expression of HO-1. These results revealed that the anti-inflammatory effect of salusin-β knockdown on LPS-induced lung cell injury may be dependent on inactivating NF-κB, while activating HO-1. Subsequently, HO-1 was also knocked down in the presence of salusin-β knockdown, and the results revealed that knockdown of HO-1 significantly blocked the inhibitory effect of salusin-β knockdown on the generation of inflammatory cytokines. At the same time, the LPS-induced expression of HMGB1, p-IκBα and p-p65, which was reduced by salusin-β knockdown, was restored by knockdown of HO-1. These data revealed that the loss of HO-1 could partially reverse the anti-inflammatory effect of salusin-β knockdown on LPS-induced lung cells, thus further confirming the findings that the anti-inflammatory effect of salusin-β knockdown on LPS-induced lung cell injury was dependent on HO-1 activation. However, the knockdown of HO-1 did not completely reverse the effects of salusin-β knockdown, which indicates that other downstream mediators or pathways are also involved, which need to be investigated in future studies.

To the best of our knowledge, the present study reported for the first time that salusin-β is involved in LPS-induced lung injury, and its knockdown can exert anti-inflammatory effects on LPS-induced lung injury, potentially via NF-κB inhibition and HO-1 activation. These findings provided a novel target and an improved understanding of the potential underlying mechanism of pathogenesis and a molecular therapeutic strategy for ALI.

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Availability of data and materials

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

Authors' contributions

PZ and SC contributed to the conception and design of the present study; SC and YH contributed to the acquisition of data; SC and JZ contributed to the analysis and interpretation of data; and SC and PZ drafted the article and revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of Fujian Medical University Union Hospital (approval no. IACUC-20 181212-09).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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