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Protostemonine alleviates heat-killed methicillin-resistant *Staphylococcus aureus*-induced acute lung injury through MAPK and NF-κB signaling pathways

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ABSTRACT

Acute lung injury (ALI) and its most severe form acute respiratory distress syndrome (ARDS) caused by gram-positive bacteria threatens human life because effective treatments and medicines is unavailable. Protostemonine (PSN), an active alkaloid mainly isolated from the roots of *Stemona sesslifolia*, has anti-inflammatory effects on asthma and gram-negative bacteria-induced ALI. Here, we found that PSN exhibits anti-inflammatory effects and alleviates heat-killed methicillin-resistant *Staphylococcus aureus* (HKMRSA)-induced pneumonia. PSN treatment significantly attenuated HKMRSA-induced pathological injury, pulmonary neutrophil infiltration, tissue permeability and the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in murine ALI model. In addition, PSN decreased the content of TNF-α, IL-1β, IL-6 and the expression of iNOS, as well as the production of NO in HKMRSA-induced bone marrow derived macrophages (BMDMs). Furthermore, treatment with PSN suppressed the activation of MAPKs (e.g. p38 MAPK, JNK and ERK) and NF-κB. Collectively, our results suggest that PSN ameliorates gram-positive bacteria-induced ALI in mice by inhibition of the MAPK and NF-κB signaling pathways, and our studies suggest that PSN might be a novel candidate for treating ALI/ARDS.

1. Introduction

Gram-positive bacterial pathogens induced inflammation is a major contributor to the development of acute respiratory distress syndrome (ARDS) [1,2]. *Staphylococcus aureus* (*S. aureus*) is one of the most clinically relevant gram-positive bacterial pathogens that lead to rapid development of lung injury and ARDS [3,4]. Although antibiotics are provided to treat *S. aureus* infections, the pathogenesis associated with killed bacteria and the emergence of new antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA), result in a reoccurrence of pneumonia morbidity and mortality worldwide, and become a pressing public health issue [5–9]. Therefore, novel effective therapeutic strategies are urgently required.

Gram-positive bacterial can trigger inflammatory responses via Toll-like receptor 2 (TLR2)-mediated signal pathway [10,11]. By recognition of pathogen-associated molecular patterns (PAMPs), TLR2 can recruit myeloid differentiation factor 88 (MyD88) and then TLR2-MyD88 complex signaling complex associated with tumor necrosis factor receptor-associated factor (TRAF6), an E3 ubiquitin ligase [12]. Subsequently, the polyubiquitination of TRAF6 leads to phosphorylation of motigen-activated protein kinases (MAPKs) including p38, c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), as well as induces degradation of IκB and activates nuclear factor-κB (NF-κB) [13]. As a result, TLR2-mediated signaling promotes the releasing of nitric oxide (NO), the expression of inducible nitric oxide synthase (iNOS) and the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, ultimately account for lung tissue damage in pneumonia patients [9,14].

Chinese herbal has been widely used to treat diseases for centuries. *Stemona sessilifolia*, known as “Baibu” in traditional Chinese medicine, has been used as insecticide, and shown antitussive, antitumor and antibacterial activity [15,16]. Moreover, *Stemona sessilifolia* also

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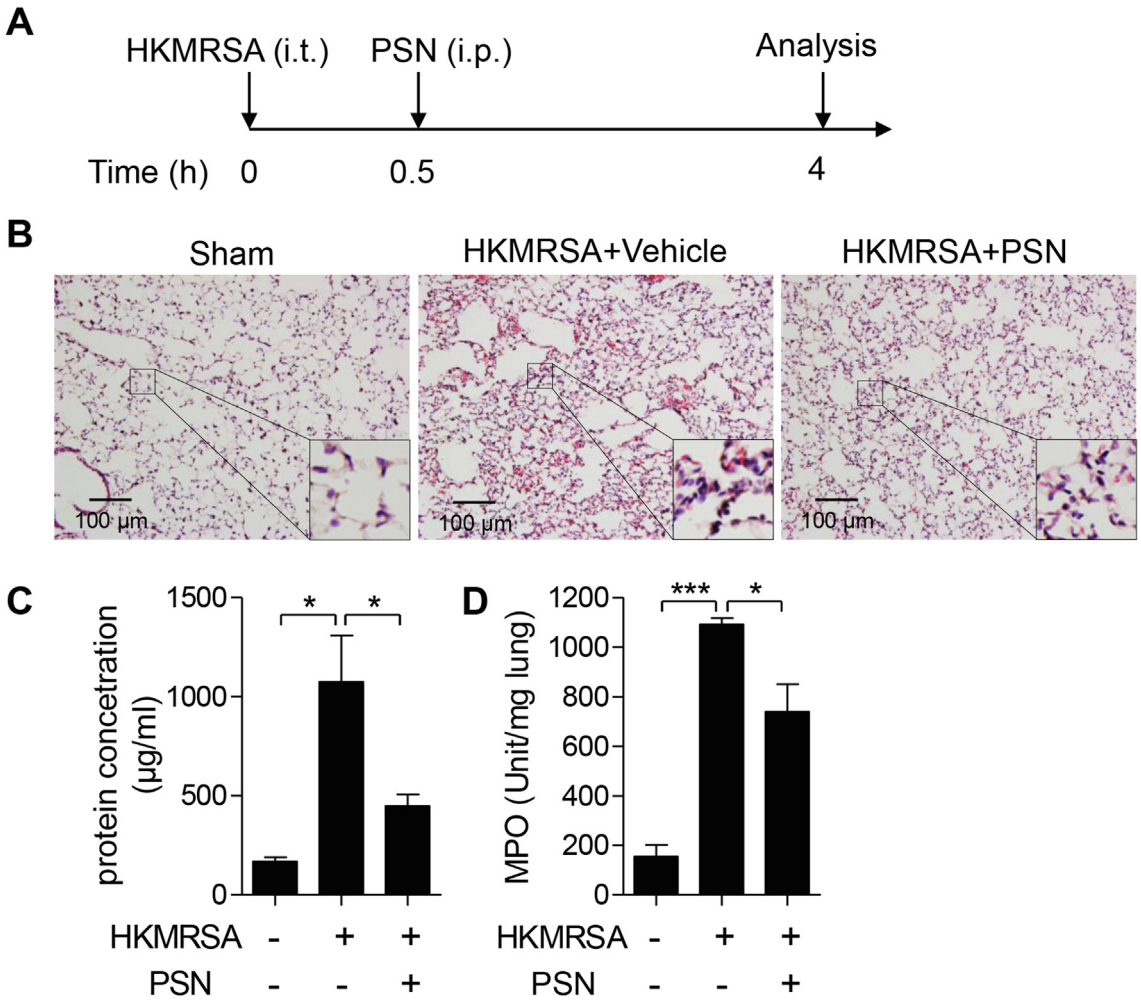

Fig. 1. PSN ameliorates HKMRSA-induced acute lung injury. (A) The schematic timeline of HKMRSA-induced acute lung injury model. Mice (C57BL/6) were intratracheally (i.t.) injected with HKMRSA (2×10^8 CFUs/mouse) in 50 μL saline. PSN (20 mg/kg) or vehicle was intraperitoneally (i.p.) injected 0.5 h after HKMRSA challenge. After treated with HKMRSA for 4 h, mice were sacrificed and the lungs were lavaged and collected. (B) The right lung lobes were fixed and stained with hematoxylin-eosin (original magnification, ×200). (C) Total protein concentration in BAL fluid was measured to evaluate lung permeability. (D) Lung tissues were homogenized and the myeloperoxidase (MPO) assay was performed. Data shown as mean ± SE, n = 5 mice each group, $^*P < 0.05$.

exhibits effective therapeutic effects in pulmonary tuberculosis and bronchitis [17,18]. Protostemonine (PSN), an active alkaloid of *S. sessilifolia*, has been identified to have anti-inflammatory effects on LPS/GalN-induced liver injury [19]. Furthermore, our previous studies have been demonstrated that PSN not only protects against acute lung injury induced by LPS, but also has benefits on DRA-induced asthmatic inflammation [20,21]. However, to the best of our knowledge, there was no report about the therapeutic roles of PSN on gram-positive bacterial infection and pneumonia. Herein, we undertook the present study to evaluate the role of PSN on HKMRSA-induced inflammatory response and acute lung injury. In this study, we demonstrated that PSN treatment significantly alleviated HKMRSA-induced pathological damage, tissue permeability, neutrophil infiltration and cytokine secretion in mice. Moreover, PSN reduced HKMRSA-induced pro-inflammatory cytokine production, iNOS expression and NO production on macrophages by suppressing MAPK and NF-κB signaling pathways. These results imply that PSN may be a new agent for treating gram-positive bacteria induced by ALI/ARDS patients.

2. Materials and methods

2.1. Mice and ethics statement

Male C57BL/6 mice of 8–10 week of age were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China) and maintained at the Laboratory Animal Center of Jiangnan University (Wuxi, China). Mice were bred five per cage in a climate-controlled room (25 °C, 55% humidity, and 12-h light/darkness cycle) with specific pathogen free conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Materials and reagents

PSN (protostemonine, (5Z)-4-methoxy-3-methyl-5{(1S,3aR, 8S, 10aS, 10bR)-1-methyl-8-[(2S,4S)-4-methyl-5-oxotetrahydro-furan-2-yl] decahydro-2H-furo[3,2-c] pyrrolo[1,2-a]azepin-2-yli-dene}furan-2(5H)-one, $C_{23}H_{31}NO_6$; MW, 417.50; purity > 98%) was purchased from Baoji Herbest Bio-Tech Co, Ltd (Baoji, China). Primary antibodies against iNOS, p-p38 MAPK, p38 MAPK, p-ERK, ERK, p-JNK, JNK, p-p65, p65, IκBα and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). CD16/32 antibody, FITC-conjugated anti-Ly-6G antibody and PE-Cy7 conjugated anti-F4/80 antibody were

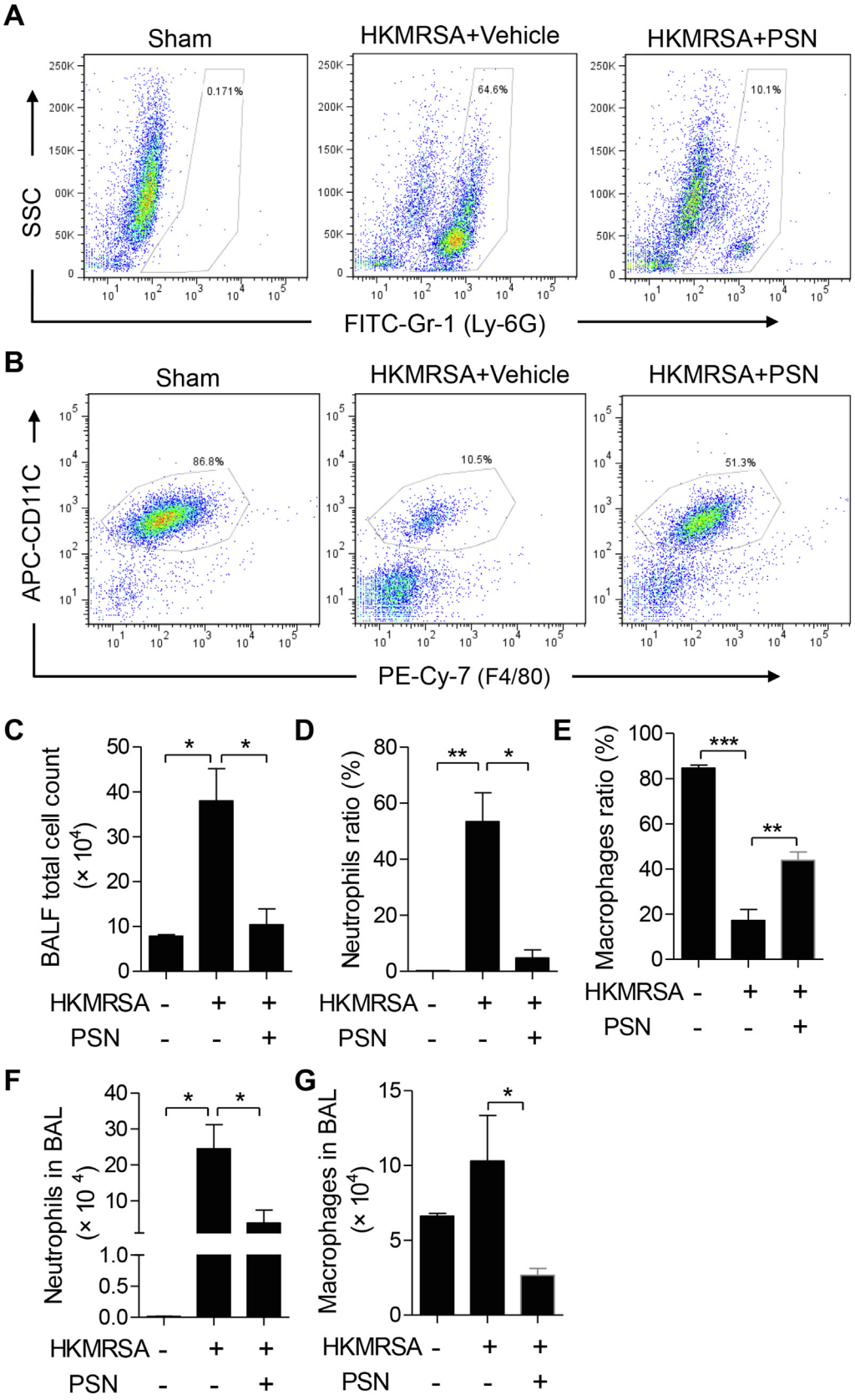

Fig. 2. PSN attenuates HKMRSA-induced inflammatory cell accumulation in lung tissue. (A) Infiltrated inflammatory cells in BAL fluid were stained with FITC-conjugated anti-Gr-1 (Ly-6G) antibody and analyzed by flow cytometry. (B) Resident alveolar macrophages in BAL fluid were stained with PE-Cy-7-conjugated anti-F4/80 and APC-conjugated anti-CD11C antibody and analyzed by flow cytometry. (C) Total cells in BAL fluid were counted. (D, E) The ratio of neutrophils (D) and alveolar macrophages (E) in BAL fluid were analyzed. (F, G) The number of neutrophils (F) and alveolar macrophages (G) in BAL fluid were calculated. Values represent mean ± SE, n = 5, $^{*}P < 0.05$.

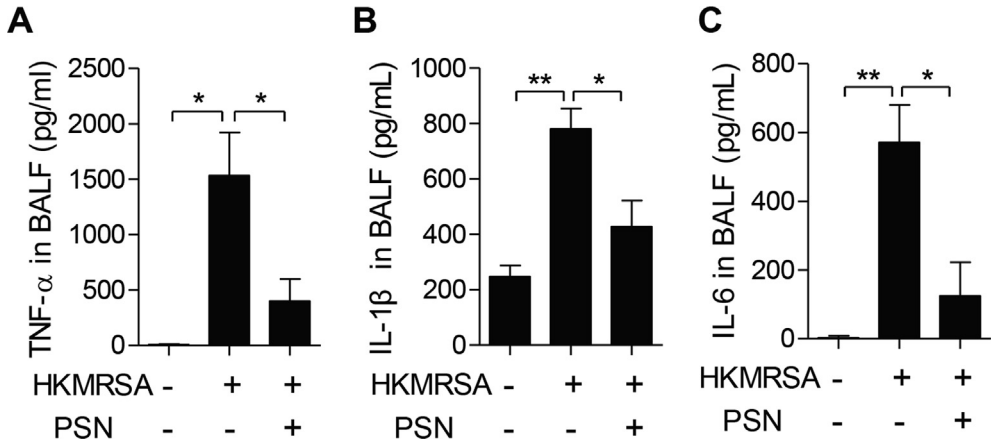

Fig. 3. PSN reduces pro-inflammatory cytokine expression in mice treated with HKMRSA. (A-C) After treatment with HKMRSA for 4 h, BAL fluid was collected and the expression of TNF-α (A), IL-1β (B) and IL-6 (C) was measured by ELISA kits.

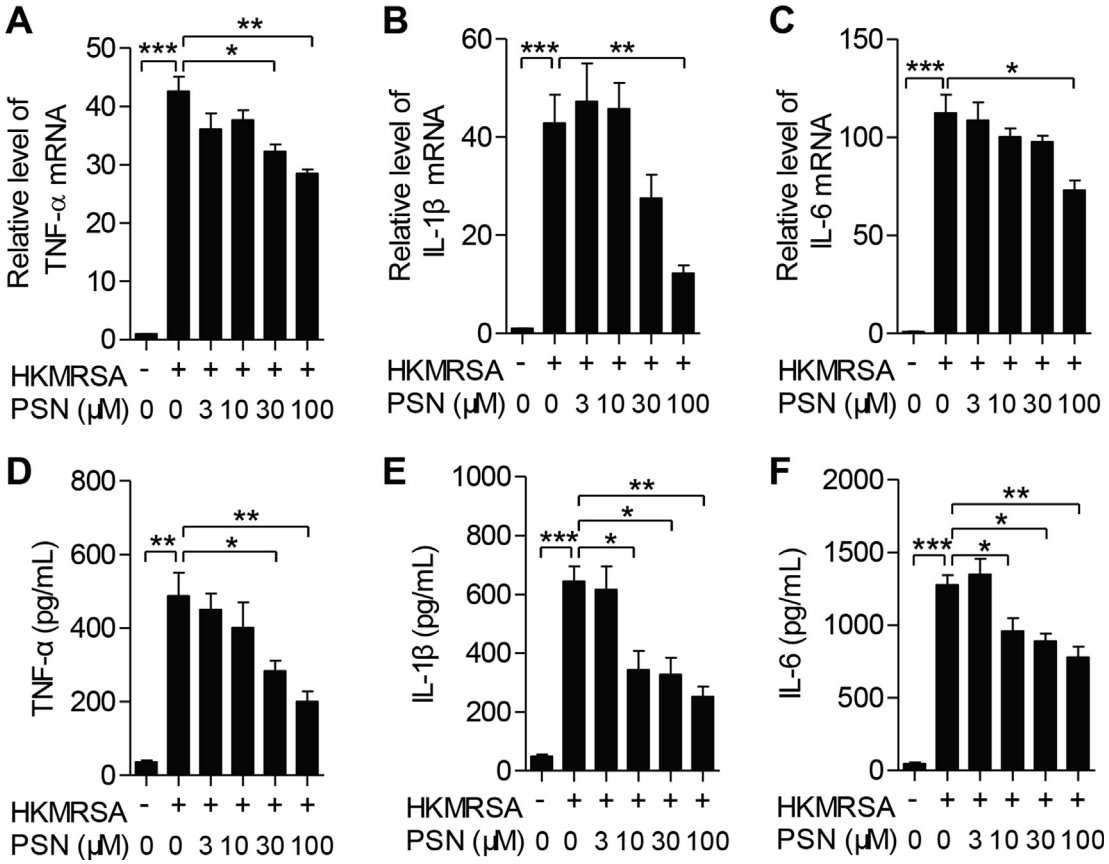

Fig. 4. PSN decreases pro-inflammatory cytokines production in HKMRSA-challenged BMDMs. BMDMs were pre-treated with PSN (0, 3, 10, 30 and 100 μM) for 0.5 h before stimulated with HKMRSA (MOI = 200) for 4 h. (A-C) BMDMs were collected and the mRNA level of TNF-α (A), IL-1β (B) and IL-6 (C) were determined by qRT-PCR. (D-F) The culture supernatants were collected for evaluation of TNF-α (D), IL-1β (E) and IL-6 (F) production by ELISA kits. Data shown as mean ± SE, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

obtained from BD Biosciences (San Jose, CA, USA). APC-conjugated anti-CD11C antibody was purchased from Biolegend (San Diego, CA, USA).

2.3. Preparation of MRSA suspension and heat-killed bacteria

Single colony of MRSA (ATCC43300) was picked and transferred into liquid Luria-Bertani (LB) medium and cultured at 37 °C with shaking (200 rpm/min) overnight. Then the culture was diluted 100-fold in liquid LB medium and grown at 37 °C with shaking (200 rpm/min). When the bacteria reached the mid-log phase (O.D. 600 nm) of grown, the culture was centrifuged at 1000 rpm at 4 °C for 30 min. After washed twice with PBS, the pellet was re-suspended by sterile normal saline solution. The bacterial concentration was acquired by serially diluting the bacteria in sterile normal saline solution, followed by plating on LB-agar and confirmed by counting colonies on LB-agar. HKMRSA was prepared by boiling the bacterial suspension for 1 h, and then stored at −80 °C.

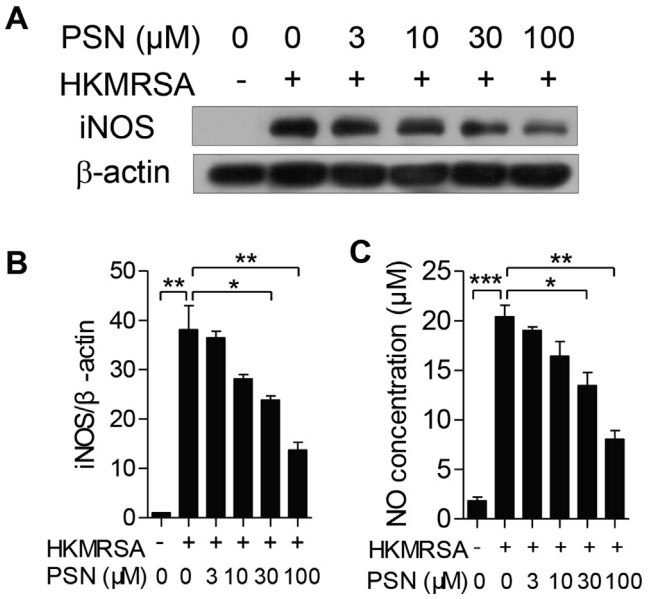

Fig. 5. PSN inhibits iNOS expression and NO production in BMDMs. BMDMs were pre-incubated with PSN (0, 3, 10, 30 and 100 μM) 0.5 h before stimulation with HKMRSA (MOI = 200) for another 24 h. (A) Cell lysates were collected to detect the expression of iNOS, β-actin was used as loading control. (B) The ratio of iNOS/β-actin was analyzed by ImageJ software. (C) Culture supernatant was collected for NO detection. Data shown as mean ± SE, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$.

2.4. HKMRSA-induced acute lung injury

Mice were randomly separated into three groups: Sham group, HKMRSA + Vehicle group and HKMRSA + PSN group [21]. PSN was dissolved in vehicle (polyoxyethylene castor oil: ethanol: saline = 1:1:8). After being anesthetized by pentobarbital, mice were administrated an intratracheal (*i.t.*) injection of HKMRSA (2×10^8 CFUs/mouse) in 50 μL saline. In the HKMRSA + PSN group, PSN (20 mg/kg) was intraperitoneal (*i.p.*) injection 0.5 h after HKMRSA challenge. Mice were euthanized and lung samples were collected for morphological and biochemical evaluations at 4 h after HKMRSA administration.

2.5. Acquisition and analysis of BALF

Lungs tissue were lavaged three times with 0.6 mL 0.5 μM EDTA (dissolved in DPBS) and BAL fluid was centrifuged at 4 °C. The cell-free supernatant was harvested for total protein analysis use BCA kit (Beyotime, Shanghai, China) and the remaining cells were collected for detection of neutrophil infiltration by flow cytometry.

2.6. Myeloperoxidase activity assay

The lung tissues were collected and homogenized in 50 mM phosphate buffer (pH = 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB). After centrifugation, supernatant was discarded and pellets were resuspended in 0.5% HTAB, then treated with a freeze–thaw process three times. Supernatants were collected at 4 °C, and the protein concentration was measured by BCA kit. Subsequently, 3,3′,5,5′-tetramethylbenzidine and H_2O_2 were added to the supernatant, the change of absorbance at 655 nm was monitored by the microplate reader (FlexStation 3, Molecular Devices). Myeloperoxidase (MPO) activity was calculated as the absorbance change per min per gram protein of lung tissue as previously described [20].

2.7. Flow cytometry assay

BALF Cells incubated with Fc-blocking anti-mouse CD16/32 antibody and followed by incubation with FITC-conjugated anti-Ly-6G antibody, PE-Cy7 conjugated anti-F4/80 and APC-conjugated anti-CD11C antibodies to label the neutrophils (Ly-$6G^+$) and macrophages ($F4/80^+$ $CD11C^+$), respectively. Cells were collected on a flow cytometer (SD LSRFortessa, BD Biosciences, San Jose, CA, USA) and data were analyzed with FlowJo software as our previous study [22,23].

2.8. Enzyme-linked immunosorbent (ELISA) assay

The concentrations of TNF-α, IL-1β and IL-6 in BAL fluid and the supernatant of cell culture were detected by ELISA kits (R&D system, Minneapolis, MN, USA).

2.9. BMDMs preparation and treatment

Bone marrow cells in femoral and tibia were isolated from C57BL/6 male mice. Bone marrow cells were flushed with PBS and then cultured in DMEM (Hycolone, South Logan, UT, USA) with supplement 10% FBS (Hycolone, South Logan, UT, USA), 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA), and 10 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ, USA) for 6 days. Bone marrow derived macrophages (BMDMs) were incubated with various concentrations of PSN (0, 3, 10, 30 and 100 μM) for 0.5 h and stimulated with HKMRSA (multiplicity of infection, MOI = 200) according to the experiment requirements. Cells and culture supernatants were collected for mRNA and protein analysis.

2.10. RNA isolation, reverse transcription and quantitative PCR

Total cellular RNA was isolated from BMDMs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was prepared with ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) and amplified by SYBR Green qRT-PCR Master Mix Kit (Toyobo, Osaka, Japan) on a StepOne Plus system (Thermo Fisher Scientific, Waltham, MA, USA) with primer sets for TNF-α (forward,5′-CCTGTAGCCCACGTCGTAG-3′; reverse, 5′-GGGAGT AGACAAGGTACAACCC-3′), IL-1β (forward,5′-GAAATG CCACCTTTTG ACAGTG-3′; reverse, 5′-TGGATGCTCTCATCAGGACAG-3′), IL-6 (forward,5′-CTGCAAGAGACTTCCATCCAG-3′; reverse, 5′-AGTGGTATA GACAGGTCTGTTGG-3′).

2.11. Quantitative determination of nitrite levels

BMDMs were seeded in 6-well plates at 1.0×10^6 per well and incubated overnight (37 °C, 5% CO_2). Cells were treated with diverse doses of PSN (0, 3, 10, 30 and 100 μM) for 0.5 h, then stimulated with HKMRSA (MOI = 200) for 24 h. Medium supernatants were collected and NO levels were detected using Griess reagent (Beyotime, Shanghai, China), as previous described [24].

2.12. Western blotting analysis

BMDMs were plated on 6-well plates (1.0×10^6 cells/mL) and incubated overnight (37 °C, 5% CO_2). Cells were then pretreated with different doses of PSN (0, 3, 10, 30 and 100 μM) for 0.5 h before being challenged with HKMRSA (MOI = 200) for 0.5 h or 24 h. Cells were collected and equal amounts of proteins (quantified by BCA kit, Beyotime, Shanghai, China) from cell lysates were denatured followed by loaded onto 10% SDS/PAGE gels, transferred to nitrocellulose membranes (Millpore, Billerica, MA, USA) and blocked with 5% skim milk at room temperature for 2 h. Proteins were incubated with primary antibodies (1:1000 dilution) overnight at 4 °C as our previous study, followed by incubating with secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h at room

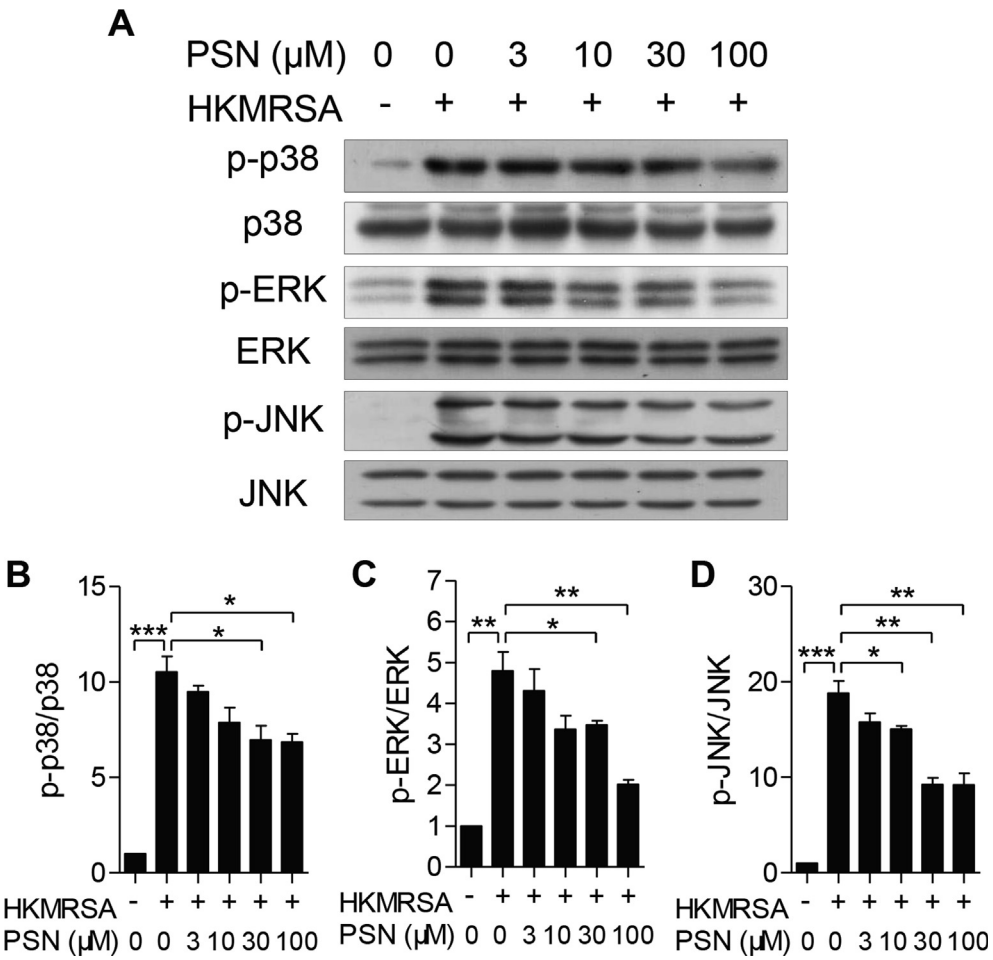

Fig. 6. PSN suppresses the phosphorylation of HKMRSA-induced MAPK activation in BMDMs. (A) BMDMs were treated with PSN (0, 3, 10, 30 and 100 μM) for 0.5 h, then stimulated with HKMRSA (MOI = 200) for another 0.5 h. (A) The protein expression levels of p-p38, p38, p-ERK, ERK, p-JNK and JNK were evaluated by western blotting. (B-D) The ratio of p-p38/p38 (B), p-ERK/ERK (C) and p-JNK/JNK (D) was performed by ImageJ software. Results shown as mean ± SE, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

temperature. The membrane was washed with TBST 3 times for each time 10 min before performing the enhanced chemiluminescence reaction. All subsequent procedures were performed in a dark room. The membrane was incubated with chemiluminescent substrate reagent (Thermo Fisher Scientific, Waltham, MA, USA) for 1 min, followed by wrapped with a plastic sheet and exposed to X-ray film (Fujifilm, Tokyo, Japan) at room temperature for 15 s to 2 min. Subsequently, the film was developed according to the instructions of the manufacturer and the data was collected with HP scanner (Palo alto, California, USA). Quantification of immunoblot was performed by Image J software (National institute of Mental Health, Bethesda, MD, USA).

2.13. Statistical analysis

Data were presented as the mean ± SEM of the results obtained from at least three independent experiments. One-way ANOVA and Student's *t*-test (paired comparison) were performed using Prism 5 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. PSN alleviated HKMRSA-induced acute lung injury

To evaluate the effects of PSN on gram-positive bacteria-induced acute lung injury, we established a mouse model of HKMRSA-induced ALI by intratracheal injection of HKMRSA (2×10^8 CFUs/mouse) and treated mice with PSN (20 mg/kg) by intraperitoneal injection 0.5 h after HKMRSA challenged (Fig. 1A). The pathological alteration of lung tissues was detected by H&E stain to determine the effect of PSN on HKMRSA-induced ALI in mice. As shown in Fig. 1B, HKMRSA caused severe lung damage, as indexed by inflammatory cell accumulation, alveolar histological structure destruction and increased red blood cells. In comparison, treatment with PSN markedly ameliorated the pathological changes of lung tissues, indicating that PSN protected against HKMRSA-induced lung injury. We also detected the protein concentration in BALF since it was considered as an important examination index of pulmonary edema. As shown in Fig. 1C, Protein concentration in BALF was significantly increased in HKMRSA challenged mice, whereas that was decreased in PSN treated mice. MPO activity of lung tissues is a specific marker of neutrophil. In this study, our results revealed that PSN treatment significantly reduced neutrophil infiltration caused by HKMRSA challenge (Fig. 1D). Taken together, these results indicated that PSN ameliorates HKMRSA-induced acute lung injury.

3.2. PSN reduced HKMRSA-induced pulmonary inflammatory cell infiltration

Total cells, neutrophils and alveolar macrophages in BAL fluid were counted and stained with anti-Ly6G-FITC (Fig. 2A), anti-F4/80-PE-Cy7 and anti-CD11C-APC, respectively (Fig. 2B). As shown in Fig. 2C, PSN markedly reduced total inflammatory cells infiltration which induced

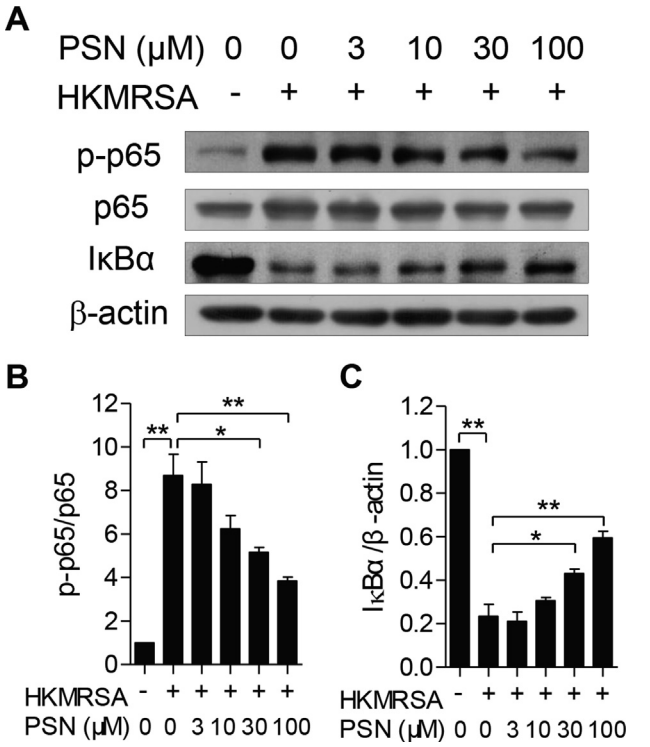

Fig. 7. PSN deactivates NF-κB signaling pathway in HKMRSA-induced BMDMs. (A) BMDMs were pre-treated with PSN (0, 3, 10, 30 and 100 μM) 0.5 h before stimulation with HKMRSA (MOI = 200) for 0.5 h. (A) The protein expression of p-p65, p65, IκBα were detected by western blot, β-actin was used as loading control. (B, C) The ratio of p-p65/p65 (B) and IκBα/β-actin (C) was calculated by ImageJ software. Data shown as mean ± SE, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$.

by HKMRSA. In addition, HKMRSA led to higher neutrophil ($Ly\text{-}6G^{+}$) infiltration of 53.35% ± 10.31% in BALF compared to 0.2007% ± 0.2090% of sham group. After treatment of PSN, the ratio of neutrophil accumulation was reduced to 4.752% ± 2.871% (Fig. 2D). Meanwhile, HKMRSA-induced neutrophil infiltration was decreased significantly after PSN treatment based on total BALF cell and the ratio of neutrophil infiltration (Fig. 2F). The percentage of alveolar macrophages ($F4/80^{+}$ $CD11C^{+}$) in BAL fluid were also detected by flow cytometry. As showed in Fig. 2E, the percentage of $F4/80^{+}$ $CD11C^{+}$ macrophages in lung was decreased after HKMRSA administrated from 84.60% ± 1.389% to 17.22% ± 4.880%, which was notably increased to 43.80% ± 3.722% after treated with PSN (20 mg/kg). However, HKMRSA challenge did not affect total resident alveolar macrophages, which was significantly reduced by PSN (20 mg/kg) treatment (Fig. 2G). Altogether, these data demonstrated that PSN reduces HKMRSA-induced pulmonary inflammatory cell infiltration, especially neutrophils.

3.3. PSN inhibited HKMRSA-induced pro-inflammatory cytokine production in mice

To elucidate the anti-inflammatory effect of PSN, we collected BALF at 4 h after HKMRSA treatment. ELISA assay was performed to evaluate the protein content of TNF-α, IL-1β and IL-6. The results revealed that the expression levels of TNF-α, IL-1β and IL-6 were reduced approximately 73.9%, 45.2% and 78.1% respectively by treatment with 20 mg/kg PSN (Fig. 3A-C).

3.4. PSN suppressed HKMRSA-induced pro-inflammatory cytokine expression in BMDMs

BMDMs were pretreated with PSN (0, 3, 10, 30 and 100 μM) for 0.5 h, and followed by HKMRSA challenged for 4 h. Then the cells and culture supernatants were accumulated to detect the mRNA levels and protein levels of TNF-α, IL-1β and IL-6 by qRT-PCR and ELISA, respectively. Data showed that HKMRSA stimulation dramatically initiated expression of TNF-α, IL-1β and IL-6 at both mRNA (Fig. 4A-C) and protein level (Fig. 4D-F). However, PSN significantly reduced pro-inflammatory cytokine expression induced by HKMRSA in BMDMs.

3.5. PSN inhibited iNOS expression and NO production in BMDMs

Since iNOS and NO were important inflammatory mediators during inflammatory response, we evaluated the effects of PSN on iNOS expression and NO production upon HKMRSA challenge. Western blotting displayed that PSN remarkably inhibited iNOS expression (Fig. 5A-B) in a dose-dependent manner in response to HKMRSA stimulation. The concentration of NO in the BMDMs supernatant showed a similar trend (Fig. 5C).

3.6. PSN decreased the phosphorylation of MAPKs induced by HKMRSA in BMDMs

TLR2 is a primary pattern recognition receptor that recognizes mainly gram-positive bacteria, participates in HKMRSA infection, which leads to activation of MAPK signaling pathway. To determine the effects of PSN on TLR2-mediated MAPK signaling pathway in macrophage, BMDMs were treated with PSN (0, 3, 10, 30 and 100 μM) for 0.5 h, then challenged by HKMRSA for another 0.5 h. The phosphorylation of p38, ERK and JNK were dramatically induced by HKMRSA, and significantly inhibited by PSN treatment (Fig. 6A-D). The results demonstrated that PSN inhibited HKMRSA-induced MAPK activation in macrophages.

3.7. PSN suppressed HKMRSA-induced NF-κB activation in BMDMs

NF-κB is an important downstream signaling pathway mediated by TLR2, and displays a critical role in inflammation. To demonstrate whether PSN alleviated HKMRSA-induced ALI by modulating NF-κB signaling pathway, the phosphorylation of NF-κB p65 and degradation of IκBα were evaluated by western blot. As shown in Fig. 7A-C, PSN markedly reduced the activity of NF-κB p65 and the degradation of IκBα, which indicated that PSN may relieve HKMRSA-induced ALI by inhibition NF-κB signaling pathway.

4. Discussion

Staphylococcus aureus (*S. aureus*), a gram-positive bacterium, is a major cause of both health care- and community-associated infections. With the increased prevalence of MRSA, it is urgently required to find novel effective therapeutic strategies [25,26]. In the present study, we investigated the anti-inflammatory effect of PSN on MRSA-induced inflammation both *in vivo* and *in vitro*. Our results revealed that administration of PSN significantly alleviated lung injury cause by MRSA infection in mice. Moreover, we found that PSN could markedly suppress the infiltration of pulmonary neutrophils, production of pro-inflammatory cytokines and releasing of NO. Furthermore, we showed that PSN not only could suppress the phosphorylation of p38 MAPK, ERK and JNK, but also blocked the NF-κB pathway via suppressing the degradation of IκBα and decreasing the phosphorylation of NF-κB. Taken together, our results suggested that PSN might be a promising therapeutic agent against MRSA-induced pulmonary inflammation.

Alkaloid is one of the most important active components isolated from herbal medicine, which has been applied to treat diseases. For

instance, alkaloids vincristine and camptothecin are widely used in the treatment of tumors. Ephedrine, an alkaloid extracted from ephedra, has been identified as an anti-asthmatic agent [27]. In our study, PSN, is a stemona alkaloid isolated from *S. sessilifolia*, and has been reported to attenuate inflammatory responses. For example, Zhuo et al. [19] demonstrated that PSN could suppress hepatic oxidative stress and inflammatory responses by upregulating HO-1 expression, and protected against LPS/GalN-induced mouse acute liver failure. Our previous study unveiled that PSN exhibited protective effects on LPS-induced ALI by suppressing iNOS expression, reducing pro-inflammatory cytokine production and inhibiting MAPK and PI3K/AKT signaling pathways [20]. Furthermore, we also demonstrated that PSN attenuated STAT6 phosphorylation, KLF4 and IRF4 expression induced by IL-4 in macrophages, which in turn repressed AAM polarization, and contributed to the therapeutic effect of PSN on DRA-induced asthmatic inflammation in mice [21]. Considering that most strain of *S. aureus* are already resistant to all antibiotics, we investigated the effects of PSN on HKMRSA-induced pulmonary inflammation. During lung injury, neutrophil is the earliest immune cell recruited to the site of injury. Thus, neutrophil infiltration is an important marker for acute inflammation of lung injury, inhibition of neutrophil infiltration can effectively attenuate inflammation [28,29]. For example, Zhu et al. [30] revealed that partial neutrophil depletion lead to diminished pulmonary inflammation and decreased host morbidity. Meanwhile, Calvente et al. [31] showed that mice undergoing neutrophil depletion during the resolution phase exhibited unresolved hepatic inflammation. As inflammatory response is frequently accompanied by excessive pro-inflammatory cytokines releasing, inhibiting the production of these cytokines (e.g. TNF-α, IL-1β and IL-6) production may attenuate inflammation [23,32]. Furthermore, the expression of iNOS and production of NO is essential for host defense induced by pathogens [33,34]. Excessive releasing of NO can promote cytokine production and mitochondrial dysfunction, resulting in accelerating of inflammation and tissue injury [35]. In addition, Weimann et al. [36] found that iNOS-deficient mice are more resistant to LPS-induced ALI than wide-type mice. In this study, we proved that PSN inhibits neutrophil accumulation, reduces pro-inflammatory cytokine production, decreases iNOS expression and NO production induced by HKMRSA. Moreover, PSN ameliorates histologic change as well as pulmonary permeability. Collectively, these findings suggested that PSN might be a potential agent for gram-positive bacterium infection.

During infection, neutrophils are the earliest immune cell recruited to the injury site, resulting in pro-inflammatory cytokine production, edema formation and alveoli basement membrane destruction. Inhibition of neutrophil infiltration can alleviate inflammation effectively. As one kind of tissue-resident innate immune cells, macrophage plays as the body's first line of defense against the invasions of foreign bacteria [37–39], and is involved in the initiation and maintenance of acute inflammation [40]. Along with the accumulation of neutrophil, activated macrophages can secrete pro-inflammatory cytokines and mediators (e.g. TNF-α, IL-1β, IL-6, iNOS and NO) [41–43], which can exacerbate inflammatory response and tissue injury. In the present study, PSN ameliorated HKMRSA-induced lung injury by attenuating neutrophil infiltration, macrophage activation and inflammatory mediators' production. However, in this study, we found that PSN might clear alveolar macrophages in murine model, which may result in decreased invasiveness as well as reduced inflammatory mediators releasing. Besides, neutrophil infiltration may be related to the increased permeability of endothelium and epithelium, which is the characteristic of lung damage in ALI [29,44]. The activation of them can also release pro-inflammatory mediators. Studies have reported that suppression of endothelium and epithelium activity could relieve pulmonary injury [45–47]. Further studies should be focus on whether PSN have protective effects on endothelium and epithelium.

Within the respiratory tract, macrophage is considered to be an important sentinel of innate immunity, and expresses pattern recognition receptors (PRPs) including TLRs, to recognize PAMPs [48]. Among TLRs, TLR2 may be identified as the most important receptor for gram-positive bacterial products [49]. TLR2 stimulation by gram-positive bacteria results in the activation of MAPK and NF-κB pathways [50]. Studies revealed that both the MAPK and NF-κB pathways play crucial role in the progression of inflammation and deterioration of tissue injuries [51]. Inhibition of the key MAPK members such as p38, JNK and ERK could ameliorate inflammatory response. IκBα is a known NF-κB inhibitor. Once IκBα is degraded, the NF-κB subunit p65 translocates from the cytoplasm to nucleus, then binds to the promoter of pro-inflammatory mediator genes, and triggers the transcription of these target genes, including iNOS, TNF-α, IL-1β and IL-6 [52]. Therefore, both the MAPKs and NF-κB have been investigated as potential targets for the development of anti-inflammatory agents [53,54]. Many natural products have been reported to have anti-inflammatory activities via inhibiting MAPKs and/or NF-κB activation. For example, costunolide was reported to exert a strong inhibitory effect on HKSA-induced septic lung injury through the MAPK pathway [9]. Both MAPK and NF-κB signaling have also been shown to be targeted by tabersonine, an alkaloid mainly isolated from *Catharanthus roseus*, and then attenuated inflammatory response and pulmonary injury induced by LPS [23]. Our previous study also demonstrated that PSN could attenuate LPS-induced macrophage activation and pulmonary injury via MAPK signaling pathway [20]. In this study, we found that PSN attenuates HKMRSA-induced inflammatory response and lung injury by inhibiting the activation of MAPK accompanied with NF-κB signaling pathway. These results indicated that PSN may target upstream molecules of these signaling pathways, which need further studies.

In conclusion, our current study showed the anti-inflammatory activities of PSN against HKMRSA-induced inflammatory responses both *in vivo* and *in vitro*. Treatment with PSN could significantly alleviate pulmonary pathological injury, neutrophil infiltration and pro-inflammatory mediators' production in HKMRSA-induced ALI by blocking MAPK and NF-κB signaling pathways. Taken together, our results indicated that PSN might be a potential drug candidate to treat HKMRSA-induced ALI/ARDS patients.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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