Article

Protostemonine effectively attenuates lipopolysaccharide-induced acute lung injury in mice

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Abstract

Protostemonine (PSN) is the main anti-inflammatory alkaloid extracted from the roots of *Stemona sessilifolia* (known as “Baibu” in traditional Chinese medicine). Here, we reported the inhibitory effects of PSN on lipopolysaccharide (LPS)-induced macrophage activation *in vitro* and LPS-induced acute lung injury in mice. Macrophage cell line RAW264.7 cells and mouse bone marrow-derived macrophages (BMDMs) were treated with PSN (1, 3, 10, 30 and 100 $\mu\text{mol/L}$) for 0.5 h and then challenged with LPS (0.1 $\mu\text{g/mL}$) for 24 h. Pretreatment with PSN significantly inhibited LPS-induced phosphorylation of MAPKs and AKT, iNOS expression and NO production in the macrophages. C57BL/6 mice were intratracheally injected with LPS (5 mg/kg) to induce acute lung injury (ALI). The mice were subsequently treated with PSN (10 mg/kg, ip) at 4 and 24 h after LPS challenge. PSN administration significantly attenuated LPS-induced inflammatory cell infiltration, reduced pro-inflammatory cytokine (TNF- α , IL-1 β and IL-6) production and eliminated LPS-mediated lung edema. Furthermore, PSN administration significantly inhibited LPS-induced pulmonary MPO activity. Meanwhile, LPS-induced phosphorylation of p38 MAPK, iNOS expression and NO production in the lungs were also suppressed. The results demonstrate that PSN effectively attenuates LPS-induced inflammatory responses *in vitro* and *in vivo*; the beneficial effects are associated with the decreased phosphorylation of MAPK and AKT and the reduced expression of pro-inflammatory mediators, such as iNOS, NO and cytokines. These data suggest that PSN may be a potential therapeutic agent in the treatment of ALI.

Keywords: protostemonine; *Stemona sessilifolia*; acute lung injury; lipopolysaccharides; macrophages; iNOS; NO; pro-inflammatory cytokine; MAPKs; AKT

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Introduction

Inflammation is a defensive reaction that protects the host from harmful stimuli, such as pathologic microbes, cell debris, irritants, or tissue injury, with a primary aim of initiating repair of damaged tissue^[1]. Various cells participate in and prompt the process of inflammation. In particular, macrophages play an important role in inflammatory responses through the production of pro-inflammatory mediators, including reactive oxygen species, nitric oxide (NO), growth factors, chemokines and cytokines such as tumor necrosis factor alpha (TNF- α), interleukin (IL)-1 β , and IL-6^[2–4]. Lipopolysaccharide (LPS), an endotoxin derived from gram-positive bacterial cell walls, has been defined as one of the most potent

activators of macrophages. LPS-activated macrophages generate cytokines and chemokines that subsequently lead to a transient immune activation. This breaks the balance of the intracellular reduction-oxidation state, leads to oxidative stress and induces an inflammatory response^[5–7].

MAP kinases are a group of serine/threonine protein kinases comprising three subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. These kinases have been well characterized as an important superfamily that plays the pivotal role in cell proliferation, inflammation, and apoptosis^[8] by inducing phosphorylation of intracellular signaling molecules. AKT, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase and is a critical substrate of phosphoinositide-3-kinase (PI3K). The PI3K/AKT signaling pathway can be activated by LPS through Toll-like receptor 4^[9].

The inducible nitric oxide synthase (iNOS) produces NO,

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which is essential for host innate immune responses to pathogens such as viruses, bacteria, fungi, and parasites^[10]. Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against pathogens (viruses, bacteria, fungi, protozoa) and tumor cells^[11]. However, excessive production of NO is related to the development of many diseases, especially inflammatory conditions, such as septic shock, rheumatoid arthritis (RA), and other autoimmune disorders^[12]. Thus, the excessive expression of iNOS is one of the features of inflammation.

Acute lung injury (ALI) is a life-threatening condition characterized by diffuse pulmonary interstitial and alveolar edema that leads to respiratory failure and death. Severe ALI may trigger acute respiratory distress syndrome (ARDS), the most severe form of ALI, and result in multiple organ failure with high mortality (approximately 30%-50%)^[13-15]. Unfortunately, there are no effective approaches to treat these acute inflammatory diseases and the pharmacotherapy for ALI is extremely limited. Therefore, it is vital to find effective therapies and drugs for ALI.

Chinese herbal medicine has a long history and is widely used for treating diseases^[16]. *Stemona sessilifolia*, known as “Baibu” in traditional Chinese medicine, belongs to the Stemonaceae family and is widely distributed in the East Asia^[17, 18]. The root of *S sessilifolia* has been recognized by the Pharmacopoeia of China (hereafter referred to as China Pharmacopoeia) as authentic sources of the herb Radix Stemonae since 2005^[19]. The extract of *S sessilifolia* roots was identified to have antitussive, antitumor and antibacterial activity. It has been used as the indigenous system of medicine for treating respiratory disorders, including pulmonary tuberculosis and bronchitis. Recently, it has also been used as an insecticide^[19-23]. The active components of *S sessilifolia* are a group of alkaloids, including oxymatrine, tetrandrine, palmatine, and protostemonine (PSN). These alkaloids are found to be protective in lipopolysaccharide/D-galactosamine-induced liver injury in mice^[24-26]. However, the precise components and effects in the acute lung injury have not yet been defined. In the present study, we determined the role of PSN (Figure 1A), one of the *S sessilifolia*-derived alkaloids, in LPS-induced inflammatory responses and ALI both *in vitro* and *in vivo*. LPS-induced MAPK and AKT signaling pathways and iNOS expression were detected in response to PSN treatment. Meanwhile, a LPS-induced ALI model was established and the effect of PSN on ALI was analyzed, including inflammatory cell infiltration, tissue injury and cytokines expression. Collectively, our results not only demonstrated that PSN can attenuate LPS-induced ALI but also identified a potential compound for treating ARDS.

Materials and methods

Material and reagents

PSN (protostemonine, (5Z)-4-methoxy-3-methyl-5{(1S,3aR,8S,10aS,10bR)-1-methyl-8-[(2S,4S)-4-methyl-5-oxotetrahydrofuran-2-yl]decahydro-2H-furo[3,2-c]pyrrolo[1,2-a]azepin-2-ylidene}furan-2(5H)-one, C₂₃H₃₁NO₆; MW, 417.50; purity >98%) was purchased from Baoji Herbest Bio-Tech Co, Ltd (Baoji,

China). A stock solution of PSN (for *in vitro* study) was prepared at a concentration of 100 mmol/L in dimethylsulfoxide (DMSO) (Sigma, St Louis, MO, USA) and stored at -20 °C. A bicinchoninic acid (BCA) protein assay kit was obtained from Pierce Biotechnology, Inc (Rockford, IL, USA). The ELISA kits for TNF- α and IL-6 were purchased from R&D Systems (Minneapolis, MN, USA). A Nitric Oxide Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China).

The primers used in this study were synthesized by HuaGene Biotech Co, Ltd (Shanghai, China). The primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), including antibodies against p-p38, p38 total, p-ERK, ERK, p-JNK, JNK, p-AKT (S-473), AKT, iNOS and β -actin. Other chemical reagents without special indication were obtained from Sigma (St Louis, MO, USA).

Cell culture and treatment

RAW264.7 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 media supplemented with antibiotics (100 units/mL of penicillin and 100 μ g/mL streptomycin) and 10% FBS (37 °C, 5% CO₂). BMDMs were isolated from male C57BL/6 mice, and cultured in DMEM media supplemented with antibiotics (100 units/mL of penicillin and 100 μ g/mL streptomycin), 10% FBS and 10% culture supernatant of L929 cell for 7 d (37 °C, 5% CO₂). Cells were treated with indicated concentrations of PSN according to experiment requirements; 0.1% DMSO was added into culture medium as the solvent control.

Cell viability assay

Cell viability was evaluated by MTT assay. RAW264.7 cells were planted into 96-well plates at a density of 4000 per well in a 37 °C, 5% CO₂ incubator overnight. Cells were then treated with different concentrations of PSN (1, 3, 10, 30, 100 μ mol/L) for 48 h and 10 μ L of MTT (5 mg/mL) was added to each well and incubated for 4 h. After 4 h, the supernatant was removed and the formation of formazan was resolved with 150 μ L/well of DMSO. The optical density was measured at 570 nm on a microplate reader. Concentrations were determined for three wells of each sample, and each experiment was performed in triplicate.

Animals

Male C57BL/6 mice (6-10 weeks old, 20 $\pm$ 3 g, specific pathogen free) were purchased from SLAC Laboratory Animal Corporation (Shanghai, China). Five mice were housed in one cage in a climate-controlled room (25 °C, 55% humidity and 12-h light/darkness cycle), fed a standard laboratory diet and acclimated for at least 2 weeks prior to the experiment. All experimental protocols described in this study were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University.

Mouse model building

Mice were randomly divided into 5 groups: control, LPS/vehicle (4 h and 24 h) groups, and LPS/PSN (4 h and 24 h) groups. Each group contained five mice. PSN was dissolved in a vehicle

Figure 1. PSN reduced phosphorylation of MAPKs and AKT in LPS-challenged RAW264.7 cells. (A) The chemical structure of PSN. (B and G) Western blot analysis of LPS-induced p-p38, p-ERK, p-JNK and p-AKT. RAW264.7 cells were pretreated with 30 μmol/L PSN or 0.1% DMSO for 30 min before stimulation with LPS (100 ng/mL) for indicated time. (H) The p-p38 and p-AKT were measured in RAW264.7 cells that were pretreated with PSN at 0, 1, 10 and 100 μmol/L for 30 min and then challenged with LPS (100 ng/mL) for 45 min. Whole cell extracts were prepared and the protein levels of p-p38, p-ERK, p-JNK and p-AKT were detected. β-Actin was used as loading control. Densitometric analysis of the ratios of p-p38/T-p38 (C, I), p-ERK/ERK (D), p-JNK/JNK (E), p-AKT/β-actin (G) and p-AKT/AKT (J) was performed using ImageJ software. Statistical analysis was performed using Student's *t*-test. *n*=3. **P*<0.05, ***P*<0.01.

(polyoxyethylene hydrogenated castor oil:ethanol:H₂O=1:1:8). Mice were administered an intratracheal injection of LPS (5 mg/kg) and subsequent intraperitoneal injection of vehicle or

PSN (10 mg/kg). For mice treated with LPS for 24 h, another injection with solvent or PSN was administered (12 h after LPS challenge). At the indicated time points (4 h and 24 h), mice

were euthanized and samples (bronchoalveolar lavage fluid and lung tissue samples) were collected.

Acquisition and analysis of BALF

The lungs were lavaged three times with 1 mL PBS and BAL fluid was centrifuged at 4 °C. The cell-free supernatant was harvested for total protein analysis using the BCA protein assay kit (Beyotime, Shanghai, China).

Histopathological Analysis

Lung tissues (left lobe) were fixed by 4% paraformaldehyde, embedded in paraffin and cut into 5- μ m-thick sections in a microtome (RM2235, Leica Biosystems, Wetzlar, Germany). Sections were stained with hematoxylin and eosin, and images were captured by a microscope (RX51, Olympus Optical Co Ltd, Tokyo, Japan). The degree of lung injury was assessed using a semiquantitative scoring method, with lung injury graded from 0 (normal) to 4 (severe) in four categories: interstitial inflammation, inflammatory cell infiltration, congestion, and edema. As previously reported^[27], the total lung injury score was calculated by adding up the individual scores of each category.

Western blotting analysis

Macrophages were planted on 6-well plates (1.0×10^6 cells per well) and incubated overnight (37 °C, 5% CO₂). Cells were then treated with different doses of PSN (0, 1, 3, 10, 30, and 100 μ mol/L) for 0.5 h before being stimulated with LPS (0.1 μ g/mL) for 24 h, or they were treated with 30 μ mol/L PSN for 0–8 h. Cells were collected with loading buffer (175 mmol/L Tris-HCl, 100 mmol/L DTT, 4.0% SDS, 7.5% glycerine and 0.2% bromophenol blue in ddH₂O). For *in vivo* analysis, lung tissues were collected and lysed in RIPA buffer (WEIBO Bio-Tech Co Ltd, Shanghai, China) plus 1 mmol/L PMSF. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were washed and incubated in 5% skim milk at room temperature for 1 h. The samples were then probed at 4 °C overnight with primary antibodies (1:1000 dilution) as described above, followed by incubation with secondary antibodies (KPL, Gaithersburg, MD, USA) for 1 h at room temperature. Quantification of Western blots was analyzed with ImageJ software (National Institute of Mental Health, Bethesda, MD, USA).

Measurement of cytokine production

Frozen lungs (right upper lobe) were homogenized and the supernatant was collected to measure the expression of cytokines TNF- α and IL-6 using ELISA assay kits. These concentrations were interpolated from the standard curves for recombinant TNF- α and IL-6.

RNA isolation, reverse transcription and quantitative PCR

RAW264.7 cells (5×10^5) were incubated with PSN (0, 1, 3, 10, 30, and 100 μ mol/L) and challenged with LPS for 4 h. The cells were collected, and total RNA was isolated using TRIzol

reagent (Invitrogen, Carlsbad, CA, USA). For *in vivo* analysis, frozen lungs (right upper lobe) were homogenized and total RNA was isolated. cDNA was prepared by ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and amplified by real-time PCR on a StepOne Plus system (Thermo Fisher Scientific, Waltham, MA, USA) with primer sets for IL-6 (forward, 5'-CCACCAAGAACGATAGTCAA-3'; reverse, 5'-TTCCACGATTTCCAG-3'), TNF- α (forward, 5'-TTCTCATTCTGCTTGTGG-3'; reverse, 5'-ACTTGGTGGTTTGCTACG-3'), IL-1 β (forward, 5'-CCAGCTTCAAATCTCACAGCAG-3'; reverse, 5'-CTTCTTTGGGTATTGCTTGGGATC-3'), and GAPDH (forward, 5'-TGCGACTTCAACAGCAACTC-3'; reverse, 5'-CTTGCTCAGTGTCTTGCTG-3').

MPO activity assay

The largest right lobes of lungs were collected and were homogenized in 50 mmol/L potassium phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB). After centrifugation, supernatant was discarded and pellets were resuspended in 0.5% HTAB. The pellet-HTAB was subjected to a freeze-thaw process three times. Supernatants were collected and were diluted in reaction solution containing 3, 3', 5, 5'-tetramethylbenzidine and H₂O₂. The mixture was incubated for 5 min at 25 °C and the change of absorbance at 655 nm was measured by the microplate reader (FlexStation 3, Molecular Devices, CA, USA). Protein concentrations of the supernatants were determined as described above. The MPO activity value was defined as the absorbance change per min per gram protein as previously described^[28].

Quantitative determination of nitrite levels

To assess LPS-induced inflammation, NO₂⁻ levels were determined using Griess reagent. Macrophages were planted on 12-well plates (5×10^5 cells per well) and incubated overnight (37 °C, 5% CO₂). Cells were then treated with different concentrations of PSN (0, 1, 3, 10, 30, and 100 μ mol/L) for 0.5 h before being stimulated with LPS (0.1 μ g/mL) for 24 h. Supernatants were collected and NO levels were measured using the Nitric Oxide Assay Kit (Beyotime, Shanghai, China), as previously described^[28].

Statistical analysis

Data are presented as mean $\pm$ SD obtained from at least three independent tests. Student's *t*-test (paired comparison) was performed using Prism 5 (GraphPad, San Diego, CA, USA). *P*<0.05 was considered statistically significant.

Results

PSN attenuated the phosphorylated MAPKs and AKT induced by LPS in both RAW264.7 cells and BMDMs

To elucidate the effects of PSN on MAPK and AKT signaling pathways in macrophages, we measured the phosphorylation of p38 MAPK, ERK, JNK and AKT in the macrophage cell line RAW264.7 (Figure 1) and BMDMs (Figure 2). Macrophages were treated with 30 μ mol/L PSN or 0.1% DMSO for 0.5 h, followed by LPS (0.1 μ g/mL) challenge for 0–8 h. LPS mark-

Figure 2. PSN reduced the phosphorylation of MAPKs and AKT in LPS-challenged BMDMs. (A) Western blot analysis of LPS-induced p-p38, p-ERK, p-JNK and p-AKT expression. BMDMs were pretreated with 30 μ mol/L PSN or 0.1% DMSO for 30 min before stimulation of LPS (100 ng/mL). The expressions of p-p38, p-ERK, p-JNK and p-AKT were detected as above. Total p38, ERK, JNK and AKT antibodies were used as loading controls, respectively. (B–E) Densitometric analysis of the ratios of p-p38/T-p38, p-ERK/ERK, p-JNK/JNK, and p-AKT/AKT was performed using ImageJ software. Statistical analysis was performed using Student's t-test. $n=3$. * $P<0.05$, ** $P<0.01$.

edly increased the levels of p-p38, p-ERK, p-JNK and p-AKT that were significantly inhibited by the PSN treatment (Figure 1B-1G, 2A-2E). RAW264.7 cells were treated with PSN (0, 1, 10, 100 μ mol/L) for 0.5 h, followed by LPS (0.1 μ g/mL) challenge for 45 min. LPS-induced expression of p-p38 and p-AKT was inhibited by PSN in a dose-dependent manner (Figure 1H-1J). These results demonstrate that PSN attenuates LPS-induced activation of MAPKs and AKT in macrophages.

PSN inhibited the LPS-induced expression of pro-inflammatory mediators in both RAW264.7 cells and BMDMs

The potential cytotoxicity of PSN on macrophages was measured using an MTT assay after incubating RAW264.7 cells with PSN for 48 h. PSN displayed no cellular toxicity against RAW264.7 cells (Figure 3A). Pretreatment with PSN (0, 1, 3, 10, 30, and 100 μ mol/L) reduced the LPS-induced iNOS expression in a dose-dependent manner in both RAW264.7

Figure 3. PSN inhibited iNOS expression, NO and pro-inflammatory cytokine production in LPS-stimulated macrophages. (A) PSN had no effect on toxicity of macrophages. RAW264.7 cells were treated with PSN at 0, 1, 3, 10, 30 and 100 $\mu\text{mol/L}$ for 48 h. Cell viability was determined by MTT assay. (B and D) PSN decreased LPS-induced iNOS expression in RAW264.7 cells and BMDMs. Macrophages were pretreated with PSN at 0, 1, 3, 10, 30 and 100 $\mu\text{mol/L}$ for 30 min before stimulation with LPS (100 ng/mL). After 24 h incubation, cell lysates were collected for Western blot analysis and supernatant was collected for NO detection. β -Actin was used as loading control. (C and E) Densitometric analysis of the ratios of iNOS and β -actin was performed using ImageJ software. (F and G) PSN reduced the NO production in LPS-induced RAW264.7 cells and BMDMs. (H–J) PSN inhibited LPS-induced mRNA expression of pro-inflammatory cytokines. RAW264.7 cells were treated with PSN at indicated concentrations before LPS challenge for 4 h. Total RNAs were then isolated, and the mRNA levels of IL-6 (H), TNF- α (I) and IL-1 β (J) were quantified by quantitative real-time PCR. Statistical analysis was performed using Student's *t*-test. $n=3$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

cells (Figures 3B and 3C) and BMDMs (Figures 3D and 3E). Accordingly, the LPS-induced generation of nitric oxide (NO), the major product of iNOS, was dose-dependently inhibited by PSN (Figures 3F and 3G). Moreover, PSN pretreatment

reduced the production of pro-inflammatory cytokines including IL-6, TNF- α and IL-1 β (Figure 3H-3J) in LPS-induced RAW264.7 cells. These data suggest that PSN attenuates LPS-induced iNOS expression, NO secretion and pro-inflammatory cytokine production in macrophages.

PSN inhibited the lung edema and inflammatory cell infiltration in LPS-induced ALI

To determine whether PSN can attenuate endotoxin-induced pneumonia, we established the mouse model of LPS-induced ALI, in which intratracheal injection of LPS (5 mg/kg in PBS) into mice was followed by intraperitoneal administration of solvent (polyoxyethylene hydrogenated castor oil:ethanol:H₂O=1:1:8) or 10 mg/kg PSN (Figure 4A). A schematic illustration of the experimental design is shown in Figure 4A. The total protein concentration in BALF was increased approximately 10-fold after treatment with LPS for both 4 h (Figure 4B) and 24 h (Figure 4C). In response to PSN treatment for 4 h and 24 h, the protein concentration in BALF was reduced by approximately 58% and 32%, respectively (Figure 4B and 4C, $P<0.05$).

MPO activity of lung tissue is a specific marker of neutrophil infiltration and indicates the pulmonary inflammatory injury. Compared with increased MPO activity in LPS-induced lung tissue, the MPO activity was relieved with a decrease of approximately 46% and 63% by treatment with PSN for 4 h and 24 h, respectively (Figure 4D and 4E).

PSN ameliorated lung histopathological alteration in LPS-induced ALI

To assess the effects of PSN on LPS-induced acute lung injury, we detected the histological changes of lung tissue. As shown in Figures 5A-5E, mice treated with 5 mg/kg of LPS displayed severe ALI. In comparison, treatment with 10 mg/kg of PSN for 4 h (Figures 5B, 5C) and 24 h (Figures 5D, 5E) significantly attenuated LPS-induced lung injury, including decreased infiltration of inflammatory cells and thickening of the alveolar walls. The degree of lung injury was further determined by a semiquantitative scoring method^[27] (Figure 5F). These data indicate that PSN protects against LPS-induced lung injury.

PSN reduced pro-inflammatory mediator production and inhibited p38 MAPK activation in LPS-induced ALI

To further demonstrate the anti-inflammatory effect of PSN, we measured pro-inflammatory cytokine production in the lungs of ALI mice that were treated with LPS for 4 h. The analysis for mRNA expression showed that 10 mg/kg of PSN significantly inhibited the LPS-induced enhancement of IL-6, TNF- α and IL-1 β (Figures 6A-6C, $P<0.05$). The protein levels of IL-6 and TNF- α were also reduced by PSN treatment by approximately 48% and 57% (Figures 6D, 6E, $P<0.05$), respectively, by ELISA assay. In addition to pro-inflammatory cytokines, the LPS-induced expression of iNOS (Figures 7A and 7C) and NO release (Figure 7D) were also decreased by PSN treatment. In addition, PSN also inhibited the phosphorylation of p38 without affecting total p38 expression (Figures 7A

Figure 4. PSN attenuated inflammatory cell accumulation in lung tissues of ALI mice. (A) The schematic timeline of LPS-induced acute lung injury model. Mice were subjected to an intratracheal injection of LPS (5 mg/kg) and subsequent intraperitoneal injection of solvent or PSN (10 mg/kg). An additional injection of solvent or PSN was administered (12 h after LPS challenge) to mice that underwent a 24 h LPS treatment. (B, C) The total protein concentrations in BALF after LPS challenge for 4 h (B) and 24 h (C) were detected. (D, E) Myeloperoxidase (MPO) activity was determined according to the manufacturer's instructions 4 h (D) and 24 h (E) after LPS challenge. Values represent mean $\pm$ SD. $n=5$ mice each group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

and 7B). These *in vivo* data suggest that PSN alleviates LPS-induced inflammatory responses and inhibits the activation of p38 MAPK.

Discussion

Sepsis-induced ALI is a leading cause of mortality and morbidity worldwide^[29, 30]. ALI is part of a systemic inflammatory process, particularly systemic sepsis, which is closely associated with the excessive release of inflammatory mediators, extravasation of protein-rich fluid, and excessive inflammatory cell infiltration^[31-33]. It is well known that inflammatory

Figure 5. PSN ameliorated pathological changes in ALI mice. The right lobes were fixed and processed for histological evaluation. (A–E) After H&E staining, the representative changes of the lungs from mice of different groups were shown with original magnification (100×) and partial enlarged detail (400×). (F) The degree of lung injury was quantitatively evaluated according to the histological analysis. Values are mean±SD. $n=5$. * $P<0.05$, ** $P<0.01$.

responses promote lung injury following an endotoxin challenge. Once LPS, the bacteria-derived endotoxin, enters the bloodstream, it can elicit systemic inflammation that mimics many of the initial clinical features of ALI^[34]. In recent years, the mouse model of LPS-induced ALI has been widely used to

study the pathogenesis of ALI^[35–37].

Suppression of pro-inflammatory cytokine production provides a possibility of regulating inflammatory responses, while inhibiting TNF- α , IL-1 β and IL-6 production can alleviate LPS-induced inflammation^[38–43]. In recent decades, several

Figure 6. PSN decreased expression of IL-6, TNF- α and IL-1 β in mice treated with LPS for 4 h. After treatment with LPS and LPS/PSN for 4 h, mice were euthanized and the lung tissues were collected for homogenization. The total RNAs were isolated from lung homogenates, and the supernatant was stored. The mRNA levels of IL-6 (A), TNF- α (B), IL-1 β (C) and protein production of IL-6 (D) and TNF- α (E) were then quantified by real-time PCR and ELISA assays, respectively. Values represent mean $\pm$ SD. $n=3$. * $P<0.05$.

commercially successful anti-cytokine therapeutics have been developed, including adalimumab and etanercept, which target TNF- α signaling^[44]. However, these medicines also have the drawbacks of difficult production, high costs, and side effects. To date, there are no clinically available medicines or efficient treatments for ALI.

Herbal medicines have been widely used for treating various human diseases. However, traditional Chinese medicine treatment has been criticized for its unclear composition and toxicology. With the development of pharmacology research, numerous therapeutic components and/or analogs derived from herbal extracts have been identified, such as Taxol, camptothecin and flavoxate^[29]. Increasing attention has been aimed at the re-evaluation and development of traditional Chinese medicines (TCMs). Alkaloids are major active components isolated from the roots of *S sessilifolia*, which is often used in prescription inflammatory therapy as a TCM and as a Korean herbal formula^[16]. PSN, an alkaloid, can significantly inhibit the production of pro-inflammatory cytokines, including TNF- α , IL-1 β and IL-6, and attenuate acute liver injury by reduction of boosted malondialdehyde (MDA) and reactive oxygen species (ROS) levels^[45]. In this study, both *in vivo* and *in vitro* data showed that PSN inhibited pro-inflammatory cytokine expression and protected against LPS-induced ALI

and inflammatory responses. During the early stage of lung injury, inflammatory cells, especially neutrophils and macrophages, are recruited into lungs and contribute to LPS-induced inflammatory responses^[46-48], including the production of nitric oxide (NO) and inflammatory cytokines (eg, TNF- α , IL-1 β , and IL-6)^[2, 3]. The expression of iNOS and NO are hallmarks of classically activated macrophages. LPS-induced NO release is of importance in host defense and invader-killing at inflammatory sites^[49]. However, excessive amounts of NO can promote cytokine and matrix metalloproteinase production, mitochondrial dysfunction and cell apoptosis, leading to the aggravation of inflammation and tissue injury^[50]. NO is generally produced by iNOS in macrophage activation^[51]. The iNOS-deficient mice are more resistant to LPS-induced ALI than wild-type mice^[52]. In the present study, PSN treatment significantly reduced the LPS-induced expression of iNOS in a murine macrophage cell line, RAW264.7 cells and primary BMDMs. These results suggest that PSN inhibits LPS-stimulated inflammatory responses by suppressing the expression of iNOS in macrophages.

Our data indicate that PSN can reduce the MAPKs and PI3K/AKT signaling pathways (Figures 1-3). Both pathways were triggered by TLR4, a recognition receptor of TLR4 implicated in cytokine expression^[5, 53-56]. Accumulating evidence

Figure 7. PSN decreased p38 phosphorylation, iNOS expression and NO production in the lungs of ALI mice. Sepsis-induced ALI were made by intratracheal injection of LPS (5 mg/kg) with or without PSN (10 mg/kg) administration for 4 h. Mice were then euthanized, and the lung tissues were collected. (A) Western blot analysis of p-p38 and iNOS protein expression in the lungs of control mice, ALI mice and PSN treated mice. (B and C) The ratio of p-p38/p38 and iNOS/β-actin were measured by densitometry. (D) The lung tissues were homogenized, and the supernatants were collected to detect the amounts of secreted NO. Data represent mean±SD. $n=3$. * $P<0.05$.

demonstrates that TLR4 plays a critical role in innate immune and inflammatory responses, in which MAPK and AKT signals are important for mediating inflammatory processes in response to infection^[57-62]. Compounds that can down-regulate MAPKs and AKT signaling are expected to be useful in protecting against inflammatory diseases. Because MAPKs and AKT-mediated signals also modulate cell proliferation and differentiation, most compounds against these two signals

display severe cytotoxic effects and inhibit cell proliferation. However, our data and those obtained in other studies demonstrated that PSN had no side effects *in vitro* or *in vivo*^[45]. Based on our data, PSN could protect against LPS-induced lung injury and inflammatory responses both *in vivo* and *in vitro* by inhibiting MAPK pathways and AKT signaling. Collectively, our study suggests that PSN might be a promising agent in the treatment of acute inflammatory diseases.

Conclusions

PSN, the main active alkaloid component of a traditional Chinese herb *S sessilifolia*, has anti-inflammatory activities against LPS-induced inflammatory responses both *in vitro* and *in vivo*. Treatment with PSN in a LPS-induced murine ALI model resulted in reduction of inflammatory cell infiltration, decreased expression of pro-inflammatory cytokines and alleviated pathologic changes in lung tissue. *In vitro* analysis demonstrated that PSN inhibited the expression of iNOS in both RAW264.7 and BMDM cells challenged with LPS. The results also demonstrated that PSN can modulate inflammatory processes by inhibiting MAPKs and PI3K/AKT signaling pathways. Furthermore, PSN had no cytotoxic effects *in vitro* or *in vivo*. Therefore, our results suggested that PSN protected against LPS-induced ALI by inhibiting iNOS expression and suppressing MAPK and PI3K/AKT signaling transduction in macrophages. PSN may serve as a potential agent in the treatment of sepsis-induced ALI.

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Author contribution

Feng QIAN and Ya-xian WU conceived of the study and drafted the manuscript; Hui-qiong HE participated in the design, established animal models and helped draft the manuscript; Yun-juan NIE performed the experiments and data analysis; Yun-he DING participated in the animal experiments; and Lei SUN was involved in discussion of the experiments. All authors read and approved the final manuscript.

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