Polygonoatum sibiricum polysaccharides prevent LPS-induced acute lung injury by inhibiting inflammation via the TLR4/Myd88/NF-κB pathway

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Abstract. Inflammation plays an important role in cases of acute lung injury (ALI), and the Toll-like receptor 4/nuclear factor-κB (TLR4/NF-κB) pathway, which can be regulated by Polygonoatum sibiricum polysaccharides (PSPs), is closely related to the dynamics of lipopolysaccharide (LPS)-induced inflammation. Thus, we sought to evaluate whether or not PSPs prevent LPS-induced ALI by way of inhibiting inflammation via the TLR4/NF-κB pathway in rats. We established an ALI rat model by tracheal instillation of LPS, and by pre-injection of PSPs into rats to examine PSPs in the ALI rat model. We found that PSPs attenuated LPS-induced lung pathological changes in ALI rats, decreased LPS-induced myeloperoxidase (MOP) activity, and elevated malondialdehyde (MDA) levels in lung tissue. However, PSPs also decreased the LPS-induced increase in the neutrophil ratio, and decreased inflammatory factor levels in bronchoalveolar lavage fluid (BALF). Moreover, PSPs decreased LPS-induced increases in inflammatory factors measured by mRNA expression, and altered the levels of expression of TLR4, medullary differentiation protein 88 (Myd88), p-1KB-α/IKB-α and p-p65/p65 proteins in lung tissue. In vitro, PSPs also reduced apoptosis induced by LPS in BEAS-2B cells by suppressing inflammation through its effect of inhibiting the TLR4/NF-κB pathway. In conclusion, our results suggest that PSPs may be a potential drug for effective treatment of LPS-induced ALI, due to the ability to inhibit inflammation through effects exerted on the TLR4/Myd88/NF-κB pathway.

Introduction

Acute lung injury (ALI) is a type of a pulmonary inflammatory response syndrome and is mainly characterized by increased alveolar capillary permeability and alveolar epithelial damage. In the process of ALI, acute, persistent alveolar-capillary membrane damage results in large amounts of protein extravasations and induces the formation of a transparent membrane in the alveolar space (1). In addition, with an imbalance in ventilated blood flow, lung compliance typically declines, and resultant hypoxemia and dyspnea often occur in patients with ALI. ALI is a commonly encountered critical type of emergency in clinical practice, with a high mortality rate ranging from 50 to 70% (2,3). While the pathogenesis of ALI is not fully elucidated, previous research indicates that uncontrolled systemic inflammatory responses (SIRS) caused by varied factors can induce the onset and progression of ALI, especially as the lung is the foremost organ most susceptible to such types of inflammatory damage (4,5). Furthermore, it is certain that the imbalance between pro-inflammatory responses and anti-inflammatory responses is one of the main causes of the onset and progression of ALI (6,7).

Polygonoatum sibiricum polysaccharides (PSPs) mainly composed of four chemically distinct monosaccharides, were originally extracted from the dried rhizome of Polygonoatum, a genus of flowering plants. Recent modern pharmacological research suggests that PSPs have many biological activities including antioxidant, anti-aging, anti-fatigue, immune enhancement, antibacterial, and anti-inflammatory activities (8,9). In addition, previous research has indicated that PSPs can exert antitumor effects by inhibiting the transmission of the Toll-like receptor 4/mitogen-activated protein kinase/ nuclear factor-κB (TLR4-MAPK/NF-κB) signaling pathway (10). The TLR4 signaling pathway has also been found to be closely related to the dynamics underlying ALI (11,12). TLR4 is expressed in immune cells such as macrophages and neutro-
phils, and in non-immune cells such as alveolar endothelial cells and epithelial cells. Infiltration of alveolar inflammatory cells is the key to the inflammatory response, and endothelial cell damage is the basis of structural degradation of the alveolar basement membrane in ALI (13,14). Moreover, previous research has suggested that TLR4-mediated inflammation plays a key role in lipopolysaccharide (LPS)-induced ALI (15), and plays a key role in kidney injury (16).

TLR is a transmembrane transduction receptor for LPS signaling from extracellular to intracellular space. Furthermore, TLR4 can directly bind to LPS, and binds to lipopolysaccharide-binding protein (LBPs)-LPS-cluster of differentiation 14 (CD14), thereby activating the NF-κB signaling pathway through its impact upon dynamics underlying mediatory differentiation protein 88 (Myd88). This leads to the synthesis and release of various inflammatory mediators, and finally initiates and amplifies the inflammatory response (17,18). However, whether or not PSPs are able to prevent LPS-induced ALI by inhibiting inflammation via the TLR4/NF-κB pathway is unclear. Therefore, in the present study, we sought to examine and answer the above question.

## Materials and methods

### Animals and treatment.

We used Wistar rats (5-6 weeks of age; weight, 130-180 g) to establish animal-based models for the present research. In this study, total of 50 rats (male: female=1:1) were all purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Rats were fed daily, housed at 22±2˚C, and we used a 12 h light/dark cycle at our experimental center. Rats were randomly assigned to five treatment groups, including: the Control group, LPS group, L-PSP group, M-PSP group, and H-PSP group. The LPS group was composed of the ALI rats model without PSP treatment. The ALI rat model was composed of rats that were first intraperitoneally injected with normal saline. After 30 min, we used 1 mg/kg lipopolysaccharide (LPS, purity >95%, cat. no. L2880, Sigma-Aldrich; Merck KGaA) which was intraperitoneally injected. After 16 h, lipopolysaccharide (3 mg/kg) was instilled into the trachea, and 1 h later, the rats were intraperitoneally injected with normal saline. In total, 2 h prior to the establishment of the ALI model, rats in the L-PSP group, the M-PSP group, and the H-PSP group were injected intraperitoneally with PSPs (20 mg/kg for L-PSP, 40 mg/kg for M-PSP, 80 mg/kg for H-PSP). PSP was purchased from Ci Yuan Biotechnology Co., Ltd., and the purity was >98%. Saline was used to dissolve the PSPs. In addition, all rats in this study were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium before sacrifice.

All animal protocols were reviewed and approved by The Animal Care and Use Committee of The First Affiliated Hospital of Nanchang University (Nanchang, Jiangxi, China) and conformed to standard guidelines from The National Institution of Health (19).

### Hematoxylin and eosin (H&E) staining.

Frozen slices of rat lung were slightly dried, stained with hematoxylin for 3 min at room temperature, stained with eosin for 10 sec at room temperature, dehydrated using an ascending ethanol gradient, made transparent and then sealed with neutral gum. Histopathological changes were observed under light microscopy (magnification, x100; CKX41; Olympus Corporation) and imaged for assessment.

### Lung wet/dry (W/D) weight, myeloperoxidase (MOP) and malondialdehyde levels.

Rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium before euthanasia, before the rats were euthanized by CO₂ as previously described (20). Rats were sacrificed to obtain lung tissues, which were drained and measured for wet weight. Afterwards, lung tissues were incubated at 80˚C for 4 h and then tissues were weighed to obtain measures of dry weight. Measures of activity of MOP and the levels of malondialdehyde (MDA) were measured as previously described (21,22).

### Inflammatory cytokines and cell counting assay.

Rats were sacrificed and bronchoalveolar lavage fluid (BALF) was collected. Neutrophils and total cell numbers in BALF samples were counted as previously described (21,22). Rat tumor necrosis factor (TNF)α (cat. no. ab100785, Abcam), interleukin (IL)‑1β (cat. no. ab100768, Abcam), IL-6 (cat. no. ab100772, Abcam) and IL-8 (cat. no. SBJ-R0033, Nanjing Biological) ELISA kits were used to detect the levels of inflammatory cytokines in BALF samples.

### Reverse transcription-quantitative PCR.

TNF-α, IL-1β, L-6, and IL-8 levels in samples from rats were measured as previously described (23) by using fluorosence based real-time quantitative PCR (RT-qPCR) and gene-specific TaqMan primer/probe sets on a ABI Prism 7000 Sequence Detection System (Applied Biosystems). GAPDH mRNA transcription was used for internal loading control. PCR primer sets were composed as follows: TNF-α-F, 5'-CTGAACCTTCCGGGTATCGG-3' and TNF-α-R, 5'-GGCCTTTGTCACCTCGAATTGAGA-3'; IL-1β-F, 5'-GAAATGCCACCTTTGACATG-3' and IL-1β-R, 5'-TGAGTGCTCTCATCGAGGACAG-3'; IL-6-F, 5'-CTCTATACACCTCACAAGTGCGGA-3' and IL-6-R, 5'-GAATTTCCATTGCACAACACTTT-3'; IL-8-F, 5'-TCGAGACCAATTACTGCAACAG-3' and IL-8-R, 5'-CATTGCCCGGTGGAATTTCT-3'.

### Western blot analysis.

Protein levels were analyzed by using western blotting as previously described (23) and GAPDH protein transcription factor was used for an internal loading control. All antibodies were purchased from Abcam with the corresponding information as follows: anti-TLR4 (cat. no. ab22048, dilution 1:2,000), anti-MyD88 (cat. no. ab100785, dilution 1:3,000), anti-IkB-α (cat. no. ab32518, dilution 1:500), anti-IkB-β (cat. no. ab92700, dilution 1:500) anti-NF-κB p65 (acyetyl K310) (cat. no. ab19870, dilution 1:300), or anti-p65 phospho S636 (cat. no. ab86299, dilution 1:5,000), or anti-GAPDH (cat. no. ab9484, dilution 1:3,000).

### Flow cytometry detection of apoptosis.

Normal unafflicted human lung epithelial cells (BEAS-2B) (CRL-9609; ATCC) were cultured using DMEM (cat. no. 12491-15, Thermo Fisher Scientific, Inc.) at a temperature of 37˚C and in a constant atmosphere of 5% CO₂. In the LPS treatment group, only 24 h of LPS stimulation was permitted, but for the PSP (1.5 g/l)
treatment group, PSP was administered 1 h before LPS stimulation in the PSP+LPS group. BEAS-2B cells were collected 24 h post-LPS stimulation, and then the Annexin V FITC/PI kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used for flow cytometry to detect apoptosis. Beckman CytoFLEX Flow cytometry (Beckman Coulter, Inc.) was used to analyze levels of cell apoptosis.

Statistical analysis. We used SPSS v20.0 software (IBM Corp.) to analyze data. The Student's t-tests were used to compare differences between two treatment groups. One-way ANOVA was used to compare differences between multiple groups and the corresponding Tukey post-hoc test was used. P<0.05 was considered to indicate a statistically significant difference at which the null hypothesis of no differences among treatment groups was rejected.

Results

PSPs attenuate LPS-induced lung injury in ALI rats. We used H&E staining to detect pathological changes in lungs. As exemplified in Fig. 1, the alveolar structure of the control group was intact, the alveolar cavity was clearly visible, and there was no obvious exudation (Fig. 1A). However, we observed alveolar septal thickening, massive inflammatory cell infiltration in the interstitial and alveolar spaces, telangiectasia, hemorrhaging, alveolar insufficiency, or observed alveolar fusion in some areas (Fig. 1B). Additionally, the results indicated that PSPs significantly attenuated LPS-induced histopathological changes and that this effect occurred in a dose-dependent manner (Fig. 1C-E). In addition, data related to pathological changes in the lung tissues demonstrated that PSPs reduced LPS-induced increases in lung tissue wet/dry weight ratio (W/D) (Fig. 1F), reduced LPS-induced increases in MOP activity (Fig. 1G) and elevation in levels of MDA (Fig. 1H).

Similarly, these functions of PSPs were found to have occurred in a dose-dependent manner.

PSPs attenuate LPS-induced pulmonary inflammation in ALI rats. Excessive or uncontrolled inflammation is an underlying cause of ALI, and neutrophils play an important role in associated dynamics. Thus, we used BALF tests to assess measures of lung inflammation in the different treatment groups of rats, and found that PSPs were able to attenuate LPS-induced increases in neutrophil ratios (Fig. 2A) and attenuated levels of inflammatory factors in BALF (Fig. 2B-E). Moreover, PSPs were able to reduce LPS-induced increases in mRNA expression of inflammatory factors in lung tissues of the ALI-afflicted rats (Fig. 2F).

PSPs inhibit activation of the TLR4/NF-κB pathway in lung tissue of ALI rats. The TLR4/NF-κB pathway is known to be closely related to the dynamics of LPS-induced inflammation. Thus, we measured the expression levels of key proteins in the TLR4/NF-κB pathway by using western blotting. As shown in Fig. 3, LPS was able to induce elevated levels of TLR4, Myd88, p-IκB-α/IκB-α, and p-p65/p65 proteins in lung tissue of ALI afflicted rats. In contrast, the expression levels of TLR4, Myd88, p-IκB-α/IκB-α, and p-p65/p65 proteins in lung tissue of the H-PSP rat treatment group were significantly decreased when compared with the levels in the LPS treatment group (Fig. 3).

PSPs reduce LPS-induced apoptosis in human normal lung epithelial cells in vitro. We cultured normal unafflicted human lung epithelial cells (BEAS-2B cells) in vitro, added LPS to the culture medium, and detected apoptosis by using flow cytometry. As shown in Fig. 4A, LPS was able to significantly increase the proportion of cells that underwent apoptosis in the BEAS-2B cell treatment group, and PSPs
significantly reduced the proportion of apoptosis induced by LPS in the BEAS-2B cells. The mRNA expression levels of inflammatory factors were assessed using RT-qPCR, and the results indicated that PSPs could also reduce LPS-induced increases in mRNA expression levels of inflammatory factors in BEAS-2B cells in vitro (Fig. 4B). Furthermore, we measured the levels of expression of key proteins in the TLR4/NF-κB pathway by using western blotting and found that PSPs were also able to reduce LPS-induced increases in the expression levels of TLR4, Myd88, p-IκB-α/IκB-α, and p-p65/p65 proteins in BEAS-2B cells cultured in vitro (Fig. 4C and D).
Discussion

The main causes of acute lung injury (ALI) include infection, trauma, shock, and inhalation of toxic gases among others, and approximately 30 to 50% of cases are caused by serious infections. Endotoxin is the main pathogenic substance of bacteria, and the active ingredient of endotoxin is lipopolysaccharide (LPS), which is an important pathogenic factor of ALI (24,25). Therefore, in the present study, we sought to establish a rat model for assessment of factors related to ALI by using intraperitoneal injections of LPS in combination with tracheal instillation of LPS, and by treatments with pre-intraperitoneal injection of PSPs. The results indicated that PSPs attenuated LPS-induced lung pathological changes in ALI rats, and decreased LPS-induced increases in MOP activity as well as the elevated levels of MDA in lung tissue. The occurrence of endotoxin-induced ALI involves a variety of pathophysiological mechanisms, which can be divided into classifications of direct and indirect LPS injury (such as inflammatory theory, oxidative stress and apoptosis), among which inflammatory reactions have been the most extensively studied (26). Previous research has indicated that PSPs have antibacterial and anti-inflammatory effects (8,9). An experiment using the filter paper and cup method indicated that PSPs displayed antibacterial effects against *Micrococcus luteus* (29), *Saccharomyces cerevisiae* (30) and other similar bacterial strains. Previous research also indicated that PSPs were able to control inflammation caused by bacterial infections, and that the mechanism of these effects may involve reducing the level of inflammatory mediators in the serum; however, the details of any such mechanisms remain undescribed (8,9).

![Figure 4](https://example.com/image4)

Figure 4. Effect of PSPs on LPS-induced apoptosis of BEAS-2B cells *in vitro*. (A) Data of BEAS-2B cell apoptosis following the different treatments. (B) Effects of the different treatments on the mRNA expression of inflammatory factors in BEAS-2B cells. (C and D) Effects of the different treatments (control group, LPS group and LPS+PSP group) on the expression of proteins in the BEAS-2B cells; At least 3 independent repetitions per experiment were conducted; ***P<0.001 vs. the control group; ##P<0.01 and ###P<0.001 vs. the LPS group. PSPs, *Polygonatum sibiricum* polysaccharides; LPS, lipopolysaccharide.)
In theory, regarding the underlying dynamics of inflammatory effects upon ALI, LPS enters the body and is recognized by lipopolysaccharide-binding protein (LBP), and then binds to cluster of differentiation 14 (CD14) to form the LPS/LBP/CD14 complex, which can induce inflammation through multiple signal transduction pathways to ultimately release a large amount of inflammation factors. This release causes lung damage, of which TLR4/Myd88/NF-κB is one of the most critical pathways (31,32). Our results also indicated that PSPs decreased LPS-induced increases in mRNA expression of inflammatory factors, and of TLR4, Myd88, p-IκB-α/IκB-α and p-p65/p65 proteins in lung tissue. In vitro, we found that PSPs could reduce apoptosis induced by LPS in BEAS-2B cells by inhibiting inflammation via limiting the TLR4/Myd88/NF-κB pathway.

Toll-like receptor 4 (TLR4) is the first TLR member to have been discovered, and it is now widely accepted that TLR4 is a transmembrane transduction receptor for LPS signaling from extracellular to intracellular space (33,34). TLR4 directly binds to LPS and binds to LBP-LPS-CD14, which signals into the cell and initiates an inflammatory response that activates a series of downstream molecules whereby amplification of the inflammatory response occurs (34). The TLR4/NF-κB pathway is divided into a MyD88 (medullary differentiation protein 88)-independent pathway and a MyD88-dependent pathway. The MyD88-independent pathway is controlled by the MyD88 linker protein MAL (MyD88 linker protein), which interacts with MAL to induce activation of NF-κB (35,36). The MyD88-dependent pathway involves the interaction of TLR4 with MyD88, whereby MyD88 is activated and phosphorylates TLR4 with an IL-1-related enzyme (IRAK), thereby activating NF-κB (37). NF-κB is an important transcription factor that binds to the binding site of the target gene, initiates and regulates the expression of a series of inflammatory cytokines involved in the inflammatory response, participates in the inflammatory process of ALI, and mediates organ damage such as in the lungs (38). Previous research has shown that the IKK/IκB /NF-κB signaling pathway is an important pathway for the activation of NF-κB (39), in which it initiates signal transduction after LPS stimulation and activates NF-κB. Sustained activation of NF-κB promotes massive cell synthesis and release of cytokines including such as TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12 (40,41). These cytokines directly lead to lung injury, and can also activate other signaling pathways, as well as promote expression of inflammatory factors, ultimately leading to lung injury (42,43).

In conclusion, we found that PSPs were able to reduce measures of inflammation of lung tissue in ALI afflicted rats through its effects upon the TLR4/Myd88/NF-κB pathway. Thus, PSPs are a promising potential drug that can be used for the treatment of LPS-induced ALI.

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Availability of data and material

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

Authors' contributions

TYL and LLZ designed and performed this study, and wrote the article. TYL, SBC, BCH, JH, XH, LQ, YF and ZT carried out the data collection and analysis. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The Ethics Committee of The First Affiliated Hospital of Nanchang University (Nanchang, China) has reviewed and approved this study. All the experimental procedures were carried out according to the National Institutes of Health guidelines for the use of experimental animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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