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# Propylene glycol alginate sodium sulphate attenuates LPS-induced acute lung injury in a mouse model

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**Abstract**

Propylene glycol alginate sodium sulphate, a sulphated polysaccharide, has been used to treat hyperlipidaemia and ischaemia–reperfusion injury of liver. This study aimed to investigate the effect of propylene glycol alginate sodium sulphate on LPS-induced acute lung injury. Propylene glycol alginate sodium sulphate was injected intraperitoneally into male C57BL/6 mice with or without LPS administration. Survival rates were calculated. Serum, bronchoalveolar lavage fluid and lung tissues were collected to determine lung histology, wet/dry ratio, Evans blue albumin permeability, protein levels, the counts of immune cells and the levels of inflammatory cytokines and chemokines. Serum alanine aminotransferase, aspartate transaminase, creatinine and blood urea nitrogen levels were also measured. Additionally, NF-κB signalling was detected in the lung. Propylene glycol alginate sodium sulphate treatment significantly improved the survival of mice suffering from LPS. Lung histological injury, wet/dry ratio, Evans blue albumin permeability, neutrophils and the inflammatory cytokines and chemokines were significantly reduced by propylene glycol alginate sodium sulphate treatment. NF-κB signalling was significantly inhibited by propylene glycol alginate sodium sulphate in the lung of mice subjected to LPS. Furthermore, serum alanine aminotransferase, aspartate transaminase, creatinine and blood urea nitrogen levels were also significantly decreased after propylene glycol alginate sodium sulphate administration. This study suggests that NF-κB signalling and inhibition of pro-inflammatory cytokines, chemokines and neutrophil accumulation may be involved in the process of acute lung injury attenuation by propylene glycol alginate sodium sulphate.

**Keywords**

Propylene glycol alginate sodium sulphate, acute lung injury, neutrophil, NF-κB

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## Introduction

The lung is directly exposed to the external environment and contains abundant blood vessels. Due to these structural characteristics, the lung is threatened by potentially harmful agents, such as viruses, bacteria and fungi, as well as some host-derived mediators. All these exogenous and endogenous triggers can give rise to acute lung injury (ALI).[1,2] Propylene glycol alginate sodium sulphate (PSS) is a heparinoid compound isolated from brown algae, which has been used in the treatment of hyperlipidaemia, ischaemic cardiocerebrovascular diseases and ischaemia–reperfusion injury of the heart or liver.[3–6] Pharmacological interventions have been widely used in animal studies to uncover protective functions against ALI, including arctiin, bumetanide and RGD peptides.[7–9] However, it is unclear whether PSS has any favourable roles in a model of ALI.

Circumscribed inflammatory reactions, especially in the lung, rapidly diffuse into the whole body, following invasion, such as endogenous danger-associated molecular patterns (DAMPs) or PAMPs. Detrimental mediators initiate immune cells, including monocytes, macrophages and neutrophils, in the bloodstream, liver and other tissues or organs, which in turn secrete

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chemokines and inflammatory cytokines.[10] These excessive and persistent inflammatory cytokines can cause systemic inflammatory response syndrome (SIRS), which finally leads to multiple organ failure, such as ALI or even its severe form, acute respiratory distress syndrome.[10–12] In addition, large amounts of trans-epithelial neutrophils have a positive effect on ALI. Excessive and prolonged neutrophil activation cause great damage to the basement membrane and the alveolar–capillary barrier.[13–15] Consequently, methods to control the excessive inflammatory response and neutrophil influx may help to suppress SIRS or even ALI.

PSS belongs to heparinoid, which has anti-inflammatory and anti-coagulant functions.[16] As a result, this kind of agent is likely to attenuate ALI caused by excessive inflammatory responses. Accordingly, we focused on the potential role of PSS as a modulator of inflammatory cytokines in a LPS-induced sepsis mouse model of ALI.

## Material and methods

### Reagents

PSS was purchased from Dalian Tianyu Pharmaceuticals Co. Ltd (Dalian, PR China). ELISA kits for IL-6, TNF-α, IL-1β and IL-10 measurements were obtained from R&D Systems (Minneapolis, MN). Microplate test kits for ALT and AST were purchased from the Nanjing Jiancheng Bioengineering Institute (Jiancheng Biotech, Nanjing, PR China). The fluorescein-conjugated mAbs (Ly6G and CD11b) and the isotype controls were purchased from BD Pharmingen (San Diego, CA). Anti-p-IKK, IKKβ, p-P65, P65, p-IκBα, IκBα and β-actin were purchased from Cell Signaling Technology (Danvers, MA). PCR assay kits and quantitative RT-PCR kits were purchased from Takara Biotechnology (Dalian, PR China).

### Animals

Male wild type mice (C57BL/6; 8–12 wk old) were purchased from Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, PR China). All mice were fed in a laminar-flow, specific pathogen-free atmosphere at Jilin University. Animal protocols were approved by the Ethics Committee of the University of Jilin, and the experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals.

### Cell culture

Murine lung epithelial 12 cells (MLE) were obtained from ATCC (CRL-2110; American Type Culture Collection, Manassas, VA) and cultured in accordance with the manufacturer's instructions. MLE cells were cultured in DMEM-F12 (Gibco) with 10% FBS (DMEM-F12-10). MLE cells were incubated with PSS (10 μM) for 1 h prior to LPS (1 μg/ml) stimulation in an *in vitro* study. One h later, cells were harvested for testing NF-κB signalling proteins by Western blot. The total lung cells were obtained from the lung of mice according to a protocol described before.[17] Lung cells were then harvested for flow cytometry.

### Experimental design

PSS (50 mg/kg) was dissolved in sterile DMSO solution and stored at 4°C, protecting it from light until used. Twenty-five mg/kg or 50 mg/kg of PSS were injected i.p. into the mice according to a previous study.[3] The mice were randomly divided into four groups and anaesthetised by i.p. administration of 100 mg/kg ketamine and 10 mg/kg xylazine. Lung injury was induced by i.p. injection of LPS at a dose of 5 mg/kg body mass, which also leads to sepsis in mice.[18] The Sham + DMSO groups were injected i.p. with DMSO (100 μl) after PBS (50 μl) treatment. The LPS + DMSO groups were treated i.p. with sterile DMSO (100 μl) following LPS injection (50 μl). The Sham + PSS groups were injected i.p. with PSS (100 μl) after PBS treatment. The LPS + PSS groups were treated i.p. with sterile PSS following LPS injection. Twenty-four h later, mice were euthanized after anaesthesia. Subsequently, BALF, serum, lung, liver and kidney samples from these groups were collected for further detection.

### Histological examination

The appropriate lung, liver and kidney of each model were fixed with 4% formalin, dehydrated and embedded in paraffin. Serial 4 μm sections were cut and stained with hematoxylin and eosin (HE) for evaluation. Six high-power fields from each group (×200 magnification) were determined by light microscopy.

### Quantitative RT-PCR

Total RNA was extracted from approximately 100 mg of frozen lung or liver tissue using TRIzol reagent (Takara, Shiga, Japan), and then first-strand cDNA was synthesised by using a Reverse Transcription System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All primers were synthesised by Sangon Biotech (Shanghai, PR China), and the sequence was as follows: *IL-6* (F: 5′-TAGTCCTTCC TACCCCAATTTCC-3′; R: 5′-TTGGTCCTTAGCCA CTCCTTC-3′), *TNF-α* (F: 5′-TAGCAAACCACCA AGTG-3′; R: 5′-ACAAGGTACAACCCATCG), *IL-1β* (F: 5′-GAAATGCCACCTTTTGACAGTG-3′;

R: 5′-TGGATGCTCTCATCAGGACAG-3′), *IL-10* (F: 5′-CTTACTGACTGGCATGAGGATCA-3′; R: 5′-GCAGCTCTAGGAGCATGTGG-3′), *CXCL-1* (F: 5′-CTGGGATTCACCTCAAGAACATC-3′; R: 5′-CAGGGTCAAGGCAAGCCTC-3′), *CXCL-2* (F: 5′-CCAACCACCAGGCTACAGG-3′; R: 5′-GCGTC ACACTCAAGCTCTG-3′), *β-actin* (F: 5′-GGCTGT ATTCCCCTCCATCG-3′; R: 5′-CCAGTTGGTAA CAATGCCATGT-3′).

### Western blot analysis

Western blotting for NF-κB proteins was performed as standard protocol.[19] After running the gel, proteins were transferred to nitrocellulose filter membranes. Membranes were blocked by 5% skimmed milk for 1 h and incubated with primary Abs against p-IKK, IKKβ, p-P65, P65, p-IκBα and IκBα proteins overnight. Then, blots were incubated with secondary Ab (1:2000) for 1 h. After washing the blots, a gel documentation machine (LI-COR Odyssey; LI-COR, Lincoln, NE) was used to analysis the blots.

### Alveolar–capillary permeability

Alveolar–capillary permeability was estimated with Evans blue albumin (EBA) based on a study description.[9] EBA was administrated through the vena jugularis externa 1 h before sacrificing all models, and then the lung tissues were reserved for further research.

### Wet/dry ratio

Lung oedema was measured by tissue wet/dry (W/D) ratio. After dissection, right lung samples were weighed and then placed in a drying oven at 67°C until a constant mass was obtained.

### Flow cytometry

For cell death analysis, total lung cells were digested with 0.05% Trypsin-EDTA (Gibco, Thermo Fisher Scientific, Waltham, MA), washed with PBS and co-stained with annexin V/PI (V13242; Invitrogen) followed by flow cytometry. Cells were labelled with the fluorochrome-conjugated primary Abs to CD11b and Ly6G for 30 min. Then, cells were fixed with fixation solution (eBioscience, Inc., San Diego, CA). Cells were gated on CD11b and Ly6G-positive expression, which were identified as neutrophils. Unstained and fluorescene-conjugated isotype controls were induced. Samples were acquired on a flow cytometry analyser (LSR II; BD Biosciences, San Jose, CA), and data were analysed with DIVA software (BD Biosciences).

### Statistical analysis

Results are expressed as means ± *SD* of independent experiments. Group comparisons were performed using one-way ANOVA with Tukey's post hoc test. Survival curves were measured using the Mantel–Cox test. A *P*-value of $<0.05$ was considered statistically significant. All statistical analyses were carried out using GraphPad Prism v6.0 (GraphPad Software, Inc., San Diego, CA).

## Results

### PSS improves survival in LPS-induced septic mice

First, we investigated whether the systemic treatment of PSS could increase survival in LPS-induced septic mice. Mice that received PSS i.p. (25 mg/kg or 50 mg/kg) once daily for 5 d followed by i.p. injection of LPS (5 mg/kg) showed a significantly higher survival compared to mice in the LPS+DMSO groups (Figure 1a). Interestingly, mice that were injected i.p. with PSS (50 mg/kg but not 25 mg/kg) at 0 and 12 h following i.p. LPS administration also showed a higher survival rate (Figure 1b). Subsequently, PSS (50 mg/kg i.p.) post treatment was used in septic mice. We found that PSS post treatment significantly attenuated the secretion of pro-inflammatory cytokines (Figure 1c–e), whereas it enhanced anti-inflammatory cytokine release (Figure 1f) in serum from LPS-induced septic mice. Furthermore, PSS post treatment remarkably ameliorated liver and kidney damage caused by LPS administration (online Supplemental Figures S1 and S2). PSS post treatment improved the survival of septic mice by down-regulating excessive pro-inflammatory cytokine secretion and attenuating multi-organ damage.

### PSS attenuates LPS-induced ALI in mice

Next, we explored whether PSS (50 mg/kg i.p.) post treatment could ameliorate lung injury induced by LPS. PSS significantly attenuated lung injury, as shown by lung histological detection (Figure 2a), lung W/D ratio (Figure 2b), alveolar capillary permeability (Figure 2c) and total protein (Figure 2d) and cellular counts (Figure 2e) obtained in bronchoalveolar lavage fluid (BALF). Additionally, the LPS+PSS groups had significantly lower neutrophil infiltration (Figure 2f) but not macrophages (Figure 2g) in BALF compared to the LPS+DMSO groups. Inflammatory cytokines were also detected in BALF and lung sections. We found PSS post treatment significantly attenuated the secretion of pro-inflammatory cytokines in BALF (Figure 2h) and lung sections (Figure 2j) from LPS-induced septic mice, whereas it enhanced the release

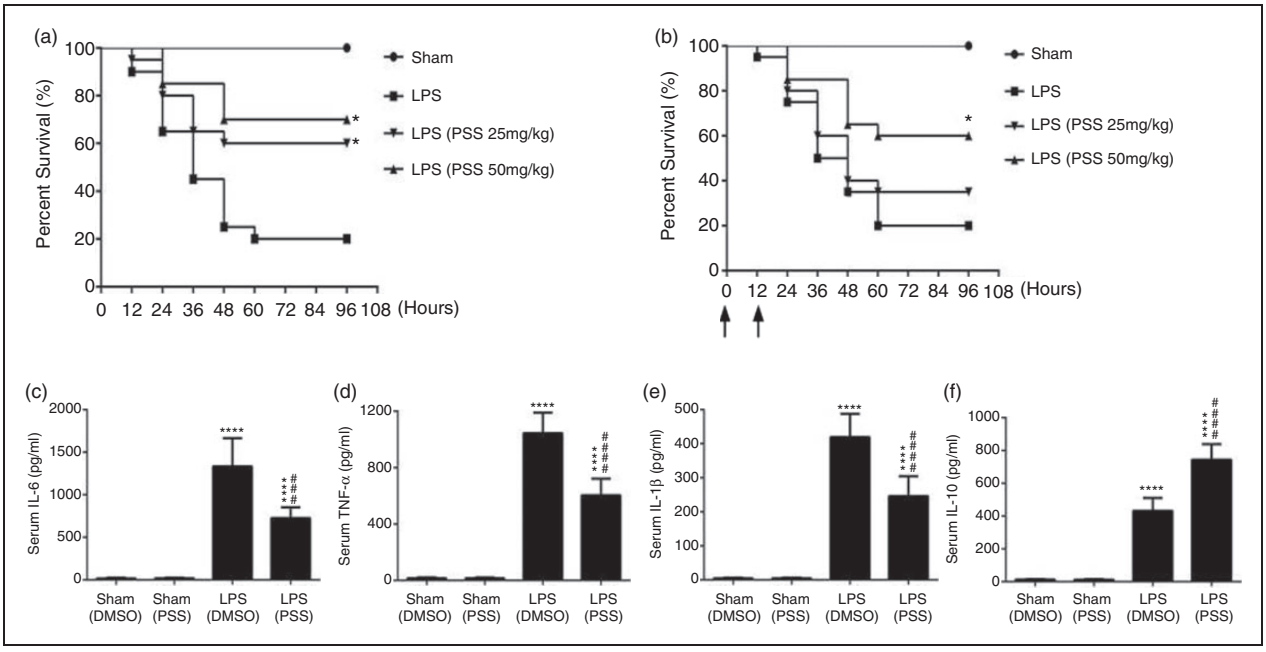


**Figure 1.** PSS improves survival in LPS-induced septic mice. (a) Mice received PSS (25, 50 mg/kg) or vehicle i.p. once daily for 5 d, followed by an i.p. injection of LPS (5 mg/kg; $n=20$ per group). (b) Mice were administered with PSS (25, 50 mg/kg) or vehicle i.p. at 0 (immediately) and 12 h after LPS injection ($n=20$ per group). Survival rates were calculated. Data were analysed using the Mantel–Cox test. $^{*}P < 0.05$ versus LPS groups. Mice were injected i.p. with PSS (50 mg/kg) or vehicle at 0 h (immediately) and 12 h after LPS administration. The secretion levels of (c) IL-6, (d) TNF-α, (e) IL-1β and (f) IL-10 were measured in serum of mice (sham or LPS, i.p., 24 h) with or without PSS treatment. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus LPS (DMSO). All the results are from at least three independent experiments; data represent means ± *SD*.

of anti-inflammatory cytokines (Figure 2i and k). Thus, PSS attenuates ALI in septic mice by decreasing the lung capillary permeability and excessive pro-inflammatory cytokine secretion.

### *PSS inhibits neutrophil accumulation in the lung of septic mice*

As neutrophil recruitment can be controlled by the chemokines CXCL-1 and CXCL-2 during tissue inflammation,[20] first we explored whether PSS could regulate CXCL-1 and CXCL-2 expression in the lung tissue and BALF. We found that PSS had no significant effect on CXCL-1 and CXCL-2 levels of non-septic mice, but it obviously down-regulated their gene transcriptions in lung tissues (Figure 3a and b) and decreased their secretions in BALF (Figure 3c and d) of LPS-induced septic mice. Then, neutrophils were detected by flow cytometry in lung tissues. We found that neutrophils were markedly accumulated in the lungs of septic mice compared to the lungs of non-septic mice. Interestingly, PSS treatment significantly reduced neutrophil accumulation in the lung of septic mice (Figure 3e). Consequently, PSS inhibits neutrophil accumulation in the lung via down-regulating excessive chemokine secretion.

### *PSS treatment reduces NF-κB expression in the lung of septic mice*

Studies have showed that NF-κB signalling can directly regulate chemokine secretion, which controls neutrophil accumulation in infected tissues. We investigated whether PSS could affect NF-κB signalling in mouse lung tissues. We found that PSS treatment significantly down-regulated NF-κB activities, as shown by p-IKK/IKK (Figure 4a and b), p-p65/p65 (Figure 4a and c) and p-IκBα/IkBα (Figure 4a and d) protein ratios in the lung tissues of LPS-mediated septic mice. Additionally, the role of PSS in regulating NF-κB signalling was also uncovered in an *in vitro study* (online Supplemental Figure S3). Thus, these results showed that PSS treatment inhibits NF-κB signal transduction by reducing the expressions of key mediators of this signalling.

## Discussion

In this study, we showed that LPS-induced lung inflammation and injury was ameliorated by PSS administered i.p. in mice. PSS prevented the increase of lung permeability and pro-inflammatory cytokine secretion in the lung of septic mice. Importantly, PSS improved the survival and also attenuated multi-organ injury in

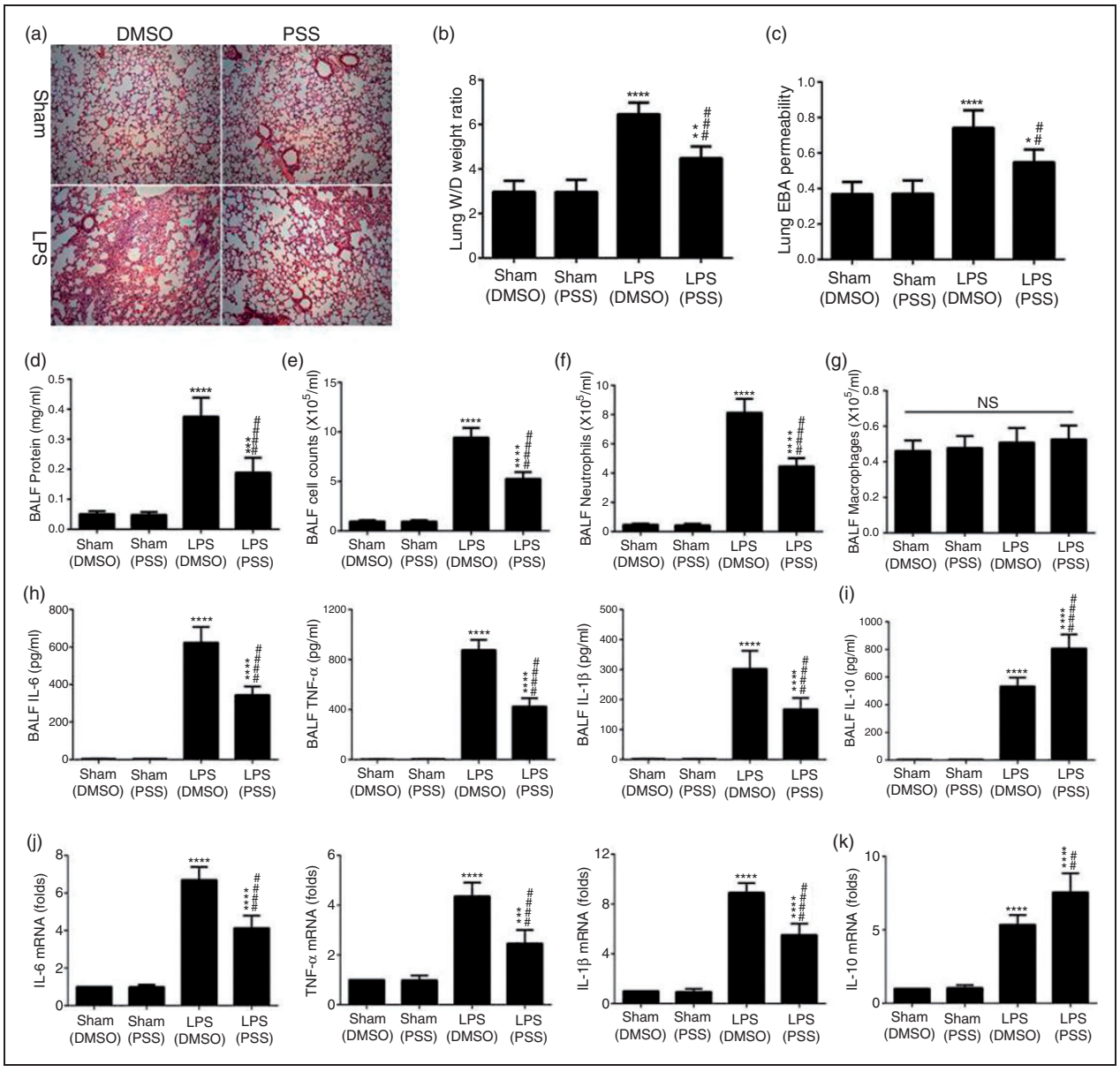


**Figure 2.** PSS attenuates acute lung injury in LPS-induced septic mice. Mice were injected i.p. with PSS (50 mg/kg) or vehicle at 0 h (immediately) and 12 h after LPS (5 mg/kg) injection. (a) Hematoxylin and eosin stained lung sections from four groups (×200 magnification). (b) Pulmonary wet-to-dry mass ratio and (c) EBA permeability, (d) proteins, (e) total cell counts, (f) neutrophils, (g) macrophages and secretion levels of (h) IL-6, TNF-α, IL-1β, and (i) IL-10 were measured in bronchoalveolar lavage fluid (BALF) of mice (sham or LPS, i.p., 24 h) with or without PSS treatment. Moreover, (j) *IL-6*, *TNF-α*, *IL-1β* and (k) *IL-10* mRNA levels were detected in the lung tissues of mice (sham or LPS, i.p., 24 h) with or without PSS treatment. $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus LPS (DMSO). All the results are from at least three independent experiments; data represent means±*SD*.

LPS-infected mice. Treatment with PSS obviously decreased chemokine expression and neutrophil accumulation in the lung of septic mice. Furthermore, we determined that PSS exerted a negative effect on NF-κB activity, suggesting it is responsible for regulating neutrophil accumulation in LPS-infected lung tissues, as NF-κB signalling can control chemokine secretion. Taken together, our findings display a therapeutic potential of PSS against sepsis and ALI.

The causes of ALI are still unknown, but acute excessive inflammatory response could be responsible for its development.[21,22] Several immune cells, including dendritic cells, macrophages and neutrophils, are involved in ALI, releasing pro-inflammatory mediators and chemokines.[23–25] Chemokines, such as CXCL-1, CXCL-2 and CXCL-8, are secreted by resident immune cells at the site of infection, and then neutrophils are rapidly recruited to the inflammation site

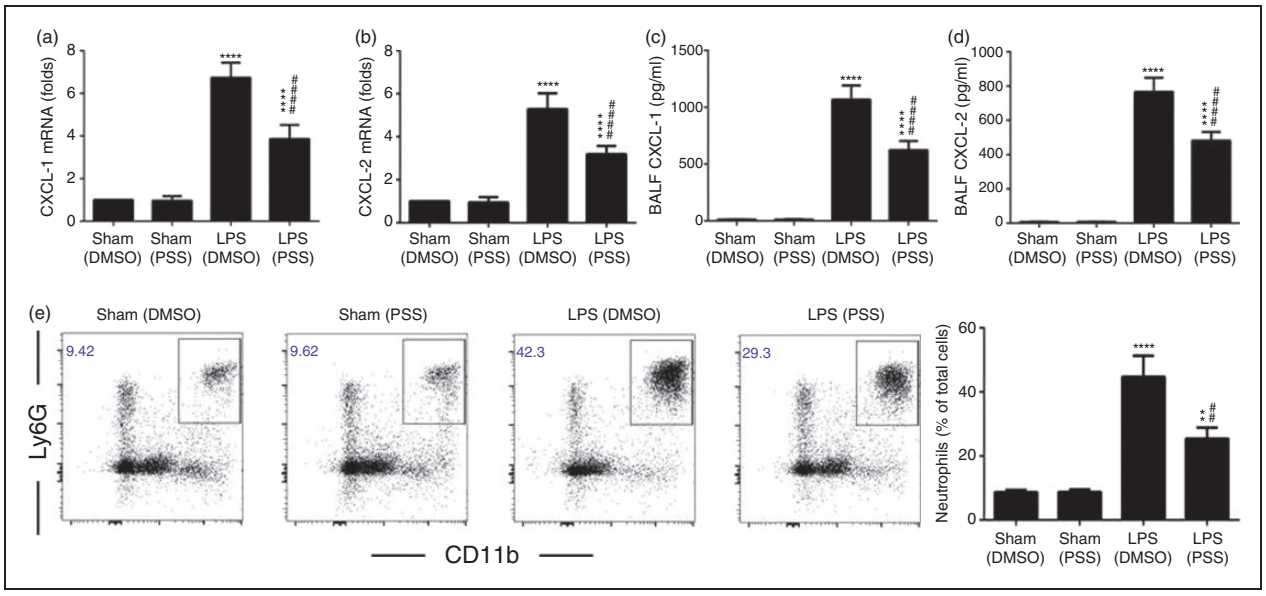


**Figure 3.** PSS reduces chemokine expression and neutrophil accumulation in the lung of septic mice. Mice were injected i.p. with PSS (50 mg/kg) or vehicle at 0 h (immediately) and 12 h after LPS administration or sham operation. (a) *CXCL-1* and (b) *CXCL-2* mRNA levels were measured by quantitative PCR in lung tissues of mice (sham or LPS, i.p., 24 h) with or without PSS treatment. (c) CXCL-1 and (d) CXCL-2 secretion was detected by ELISA in BALF from four groups. (e) Sham-operated mice and LPS-challenged mice were post treated with or without PSS for 24 h, and then sacrificed to isolate total lung cells. Expression of CD11b and Ly6G on lung neutrophils was detected by flow cytometry. $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus LPS (DMSO). Representative histograms and cell percentages are depicted. All the results are from at least three independent experiments; data represent means$\pm$*SD*.

following injury caused by DAMPs or PAMPs.[20,26,27] Once in the infected tissues, neutrophils are able to eliminate variable pathogens by phagocytosis, released enzymes and toxic oxygen species.[28] However, excessive neutrophil accumulation and activation, especially combined with their delay in clearance, can cause several diseases, such as asthma, chronic obstructive pulmonary disease and ALI.[29] Taking measures to inhibit excessive neutrophil accumulation in inflamed lungs may contribute to promoting inflammation retrogression and even attenuating ALI. Our study measured the expression of pro-inflammatory cytokines and chemokines as LPS-induced lung inflammatory responses and found that these increased cytokines were prevented with PSS treatment in LPS-mediated septic mice. We also showed excessive neutrophil accumulation was obviously attenuated by PSS in the lung of septic mice. These results observed in expression of inflammatory cytokines and neutrophils were in line with changes in lung permeability and histological morphology from lung tissue.

Bacterial pathogens and their products initiate the inflammatory response by induction of multiple inflammatory genes, which lead to the secretion of large numbers of inflammatory mediators, including cytokines, chemokines and reactive oxygen species.[30] This process contributes to the activation of inducible transcription factors, such as MAPKs and NF-κB.[31,32] NF-κB proteins play indispensable roles in immune and inflammatory responses, including the progression of LPS-induced ALI.[33,34] Several studies on animal models of ALI have demonstrated a critical role of NF-κB signalling, and the involvement of proteins such as p-IKK, IKKα/β, p-P65, P65, p-IκBα and IκBα in ALI have been uncovered.[35–37] In this mouse model of ALI, phosphorylation of IKK, P65 and IκBα were enhanced with LPS instillation, and PSS post treatment markedly decreased these three phosphorylated proteins. These results suggest that PSS post treatment protects against LPS-induced ALI by suppressing the activation of the NF-κB signalling pathway. PSS is a sulphated polysaccharide that involves β-D-mannuronic acid and α-L-guluronic acid, which is extracted from brown seaweeds.[38] It has been studied in many research fields, including anticoagulants, antihypertensives and functions of inhibiting liver ischaemia–reperfusion injury and reducing blood viscosity. However, whether PSS could have protective functions against ALI is still not clear. Our study is the first report to show the protective role of PSS in ameliorating ALI and the underlying molecular mechanism.

In summary, this study demonstrates that PSS, a heparinoid compound prepared from brown algae, reduces mortality and attenuates acute lung inflammatory injury in a LPS-induced sepsis model. Moreover, our observations also uncovered for the first time that

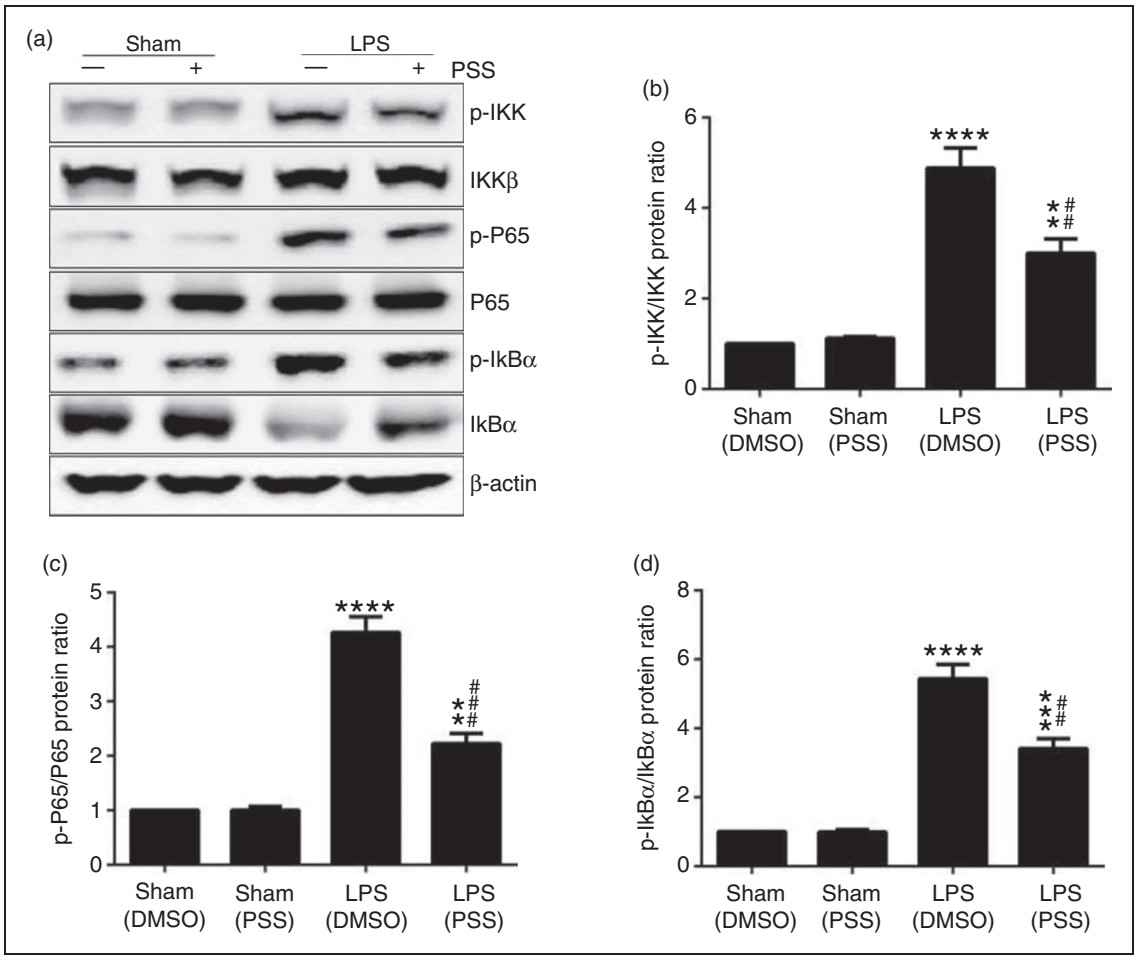


**Figure 4.** PSS inhibits NF-κB signalling in the lung from LPS-induced septic mice. Mice were injected i.p. with PSS (50 mg/kg) or vehicle at 0 h (immediately) and 12 h after LPS administration or sham operation. Lung sections were then harvested from mice (24 h following LPS treatment) for Western blotting. (a) NF-κB signalling proteins, including p-IKK, IKKβ, p-P65, P65, p-IκBα and IκBα, were detected in the lung tissues of mice (sham or LPS, i.p., 24 h) with or without PSS treatment. (b) The protein ratio of p-IKK with IKKβ, (c) the protein ratio of p-P65 with P65 and (d) the protein ratio of p-IκBα with IκBα were depicted with representative histograms. $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus LPS (DMSO). All the results are from at least three independent experiments; data represent means±*SD*.

the beneficial effects of PSS are associated with NF-κB signalling and reducing neutrophil accumulation. Thus, the conclusion of this study might contribute to providing a new perspective for PSS therapy in LPS-induced ALI.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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## Supplemental material

Supplemental material for this article is available online.

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