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Protective effect of forsythoside B against lipopolysaccharide-induced acute lung injury by attenuating the TLR4/NF-κB pathway

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ABSTRACT

Acute lung injury (ALI), which is mainly triggered by infection, pneumonia, vasculitis, and sepsis, has no specific and effective therapy except for primary supportive treatment or bedside care. Excessive inflammation caused by innate immune cells is the major characteristic of ALI. Forsythoside B, a phenylethanoside compound, possesses good antioxidant and anti-bacterial properties *in vivo* and *in vitro*. In this study, the therapeutic potential of forsythoside B and its mechanism of action were investigated in a lipopolysaccharide (LPS)-induced ALI mouse model. The results showed that LPS-induced edema exudation and lung pathological changes in mice were significantly suppressed by forsythoside B pre-treatment. Furthermore, it also attenuated lung inflammation caused by LPS stimulation, evidenced by decreased inflammatory cell infiltration and down-regulated expression of cytokines, chemokines, and inducible enzymes. The anti-inflammation property of forsythoside B was confirmed *in vitro* using LPS-stimulated RAW 264.7 macrophages. Moreover, it alleviated LPS-induced inflammation by inhibiting the activation of TLR4/NF-κB signaling pathway *in vivo* and *in vitro*. In conclusion, the results demonstrated that forsythoside B protects against LPS-induced ALI by attenuating inflammatory cell infiltration and suppressing TLR4/NF-κB-mediated lung inflammation. Therefore, it might be a potential therapeutic agent for ALI caused by sepsis.

1. Introduction

Acute lung injury (ALI), which is characterized by hypoxic respiratory dysfunction, diffused pulmonary interstitium, and alveolar edema, is caused by factors such as infection, pneumonia, vasculitis, and severe sepsis [1,2]. Currently, there is no specific and effective treatment for septic ALI. Supportive therapies and bedside care, such as mechanical ventilation and corticosteroid administration, are the available treatment options; however, the survival benefit is limited [3,4], and the associated mortality rate is up to 38% [5,6].

Excessive inflammatory cell infiltration and inflammation are the main pathogenesis of ALI. Excessive infiltration contributes to pathological damage to the pulmonary endothelium, increase in capillary permeability, protein-rich cellular exudation, and pulmonary hyaline membrane formation [7,8]. Moreover, the pathological features are closely related to the cytokine cascades mediated by pro-inflammatory cytokines that are secreted by excessive infiltration [9]. In addition, the cytokine cascades aggravate the recruitment of neutrophils and monocyte/macrophages, contributing to tissue damage by production of reactive oxygen species (ROS) and nitrites [10,11]. Therefore, inhibiting the excessive inflammatory cell transmigration or inflammation might be an efficient approach to treat ALI [12].

Forsythoside B (FTS-B), a phenylethanoside distributed in several plants, has neuroprotective [13], antibacterial [14], anti-oxidant [15], and cardioprotective properties [16]. In addition, it has been reported that the anti-inflammatory effect of FTS-B occurs due to reduced serum lipopolysaccharide (LPS) activity and phosphorylated NF-κB expression [17]. Therefore, we hypothesized that FTS-B might a potential

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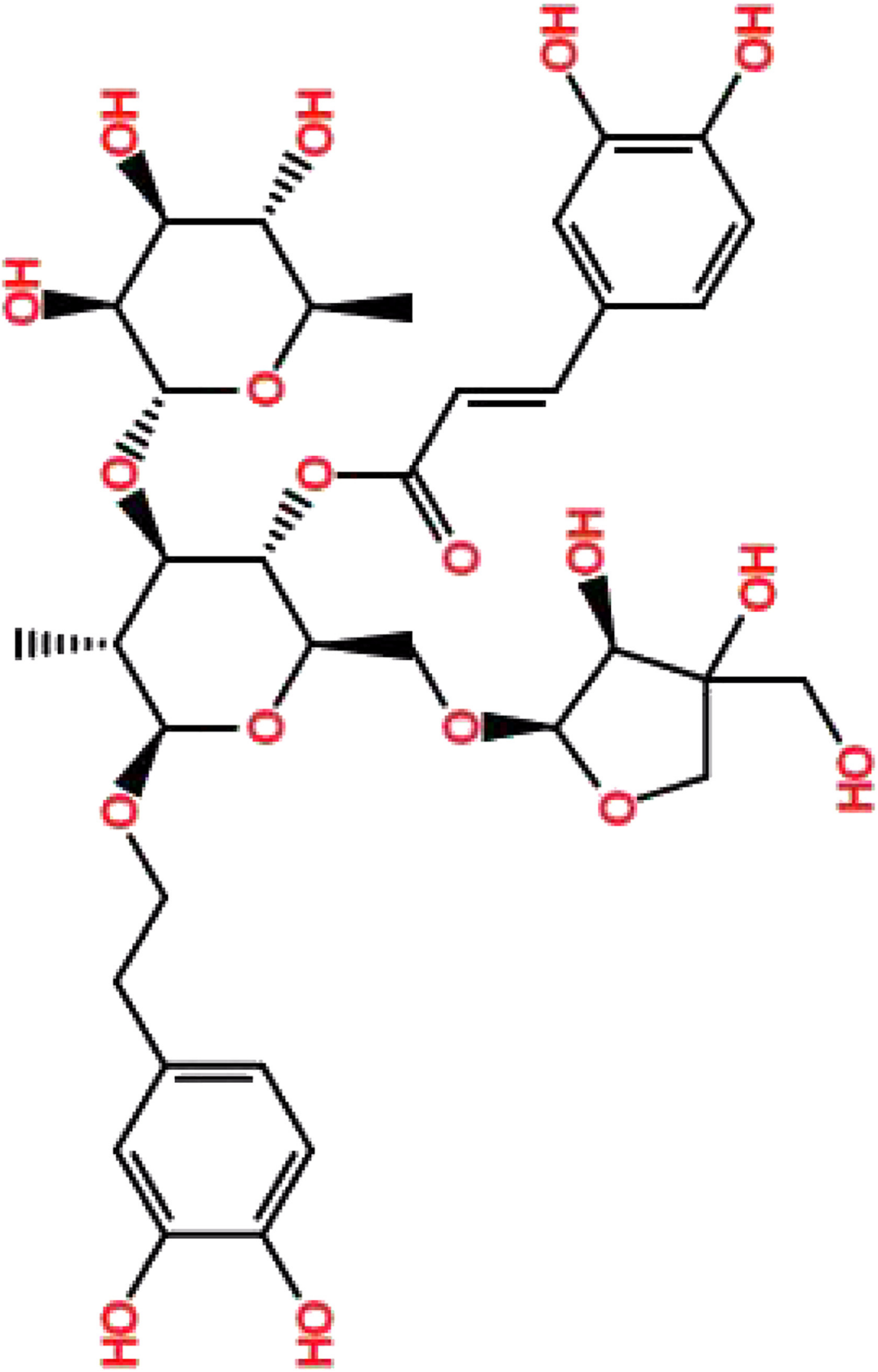

Fig. 1. Chemical structure of FTS-B.

Table 1
the information of primers.

Genes	Forward	Reverse
IL-1β	TGGGATAGGGCCTCTCTTGC	CCATGGAATCCGTGTCTTC
IL-6	AGCAACAACATAAGCGTCAT	TACTCGGCAAACCTAGTGCG
IFN-γ	ACGGCACAGTCATTGAAAGC	TCACCATCCTTTTGCCAGTTC
TNF-α	ATGGCCTCCCTCTCATCAGT	TGGTTTGCTACGACGTGGG
MCP-1	CCACTCACCTGCTGCTACTCAT	TGGTGATCCTCTTGTAGCTCTCC
MIP-2	CAGTTAGCCTTGCCTTTGTTCAG	CAGTGAGCTGCGCTGTCCAATG
COX-2	TCTCCAACCTCTCCTACTAC	GCACGTAGTCTTCGATCACT
iNOS	TGAGTTCCGAAGCAAGCCAA	AGACCTCAACAGAGCCCTCA
GADPH	GTTTTCAGGGATGAAGCGGC	TGGGATAGGGCCTCTCTTGC

compound against LPS-induced excessive inflammation and ALI *via* suppression of the excessive activation of NF-κB. In the present study, the anti-inflammatory effects and mechanism of action of FTS·B in LPS-induced excessive inflammatory response condition were explored *in vitro* and *in vivo*.

2. Materials and methods

2.1. Mice

SPF Balb/c mice (n = 180) of either sex, weighing 18.0–22.0 g (approximately 5–6 weeks), were purchased from the Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China). All animal experiments were approved by the Laboratory Animal Services Center at Guangzhou University of Chinese Medicine (Guangzhou, China) (approval ID: SCXK (Guangdong) 2013–0034) and performed according to the guidelines of Animal Welfare and Ethics of the Institutional Animal Care and Use Committee (IACUC). All experiments were carried out in a specific pathogen free (SPF) animal lab.

2.2. Materials

FTS·B (purity > 98%, chemical structure in Fig. 1) was purchased from the National Institute for Food and Drug Control (Beijing, China). It was endotoxin-free, as determined by the tachypleus amebocyte lysate assay. Dexamethasone sodium phosphate injection was purchased from Hubei Tianyao Pharmaceutical Co. Ltd. (Xiangyang, China). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (NY, USA). Lipopolysaccharide (from *Escherichia coli* O111:B4) and primers were purchased from Invitrogen (CA, USA). Thermo RevertAid First Strand cDNA Synthesis Kit and Pierce BCA Protein Assay Kit were procured from Thermo Scientific (MA, USA). FastStart Universal SYBR Green Master was purchased from Roche Applied Science (Mannheim, Germany). The antibodies against TLR4, MyD88, NF-κB/p65, and p-p65 were purchased from Abcam (Cambridge, England), and Iκκ, p-Iκκ, IκB, and p-IκB were purchased from Santa Cruz Biotechnology Inc. (CA, USA). Wright-Giemsa stain, Griess reagent kit, and myeloperoxidase (MPO) Determination Kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme linked immunosorbent assay (ELISA) kits were purchased from Wuhan Beinglay Biological Technology Co. Ltd. (Wuhan, China).

2.3. Cell viability assay and stimulation

RAW 264.7 mouse macrophages were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 humidified incubator at 37 °C. The safety concentration of FTS·B was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after 24 h of incubation of cells. Briefly, 20 μL of MTT (5 mg/mL) working solution was added to each well, and then incubated for 4 h. The culture medium was then removed and 150 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. Cell viability (% control) was determined relative to untreated cells. Subsequently, the cells were treated with safety concentration of FTS·B (40, 80, and 160 μg/mL) or DMEM 1 h before incubation with LPS (1 μg/mL), and then for another 10 h for real time-polymerase chain reaction (RT-PCR) and western blotting (WB), and the pro-inflammatory mediators were detected 24 h after LPS incubation.

2.4. Nitrite analysis

After 24 h of incubation of cells with LPS, the production of NO was assessed through the accumulation of nitrites (NO^{2-}) in the supernatants. Briefly, the nitrite concentration in 100 μL of the supernatant was measured by using a colorimetric reaction generated by the addition of 100 μL of Griess reagent, composed of equal volumes of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride and 1.32% sulfanilamide in 60% acetic acid. The absorbance of the samples was determined at 540 nm with a spectrophotometer and interpolated by using a standard curve of $NaNO_2$ (1 to 10 μM) to calculate the nitrite concentration.

2.5. Mouse model of LPS-induced ALI

The mice were acclimated in their cages for one week before the experiments. The temperature and relative humidity were 22 ± 2 °C and 50 ± 10%, respectively, with the mice maintained on a 12-h light/dark cycle. Food and water were provided *ad libitum*. Five groups of mice were studied simultaneously; these groups included the control group, the LPS group, the FTS·B (50 and 100 mg/kg) groups, and the dexamethasone sodium phosphate injection (DEX) group. Two hours before LPS instillation, the mice in the FTS·B and dexamethasone groups were intraperitoneally administered FTS·B (50 or 100 mg/kg)

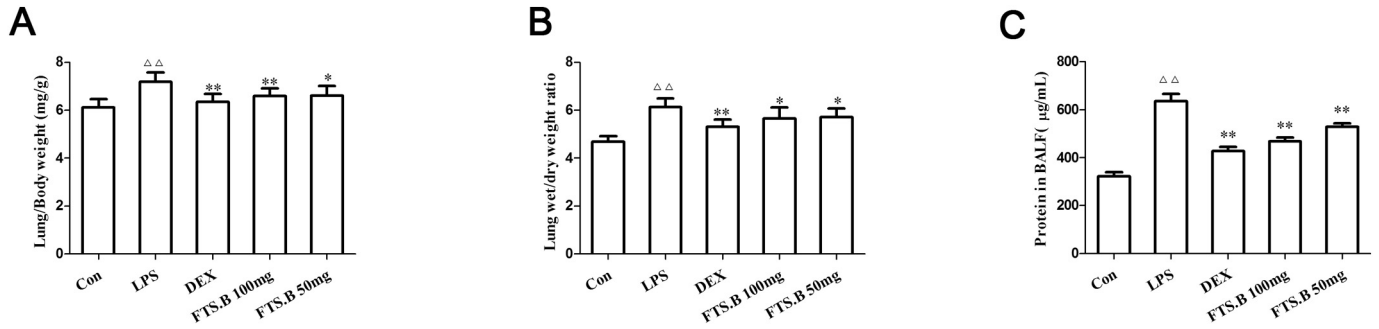

Fig. 2. Effects of FTS·B on lung exudation. The lung/Body weight (A), lung wet/dry weight ratio (B) and total protein concentration in BALF (C). Mice were administrated FTS·B (50 or 100 mg/kg) 2 h before intratracheal instillation of LPS, the edema were detected 24 h after LPS challenge. Data were analyzed by ANOVA, and values are expressed as the means ± S.D. △ $p < 0.05$, △△ $p < 0.01$ compared to control; *p < 0.05, **p < 0.01 compared to ALI mice (n = 10).

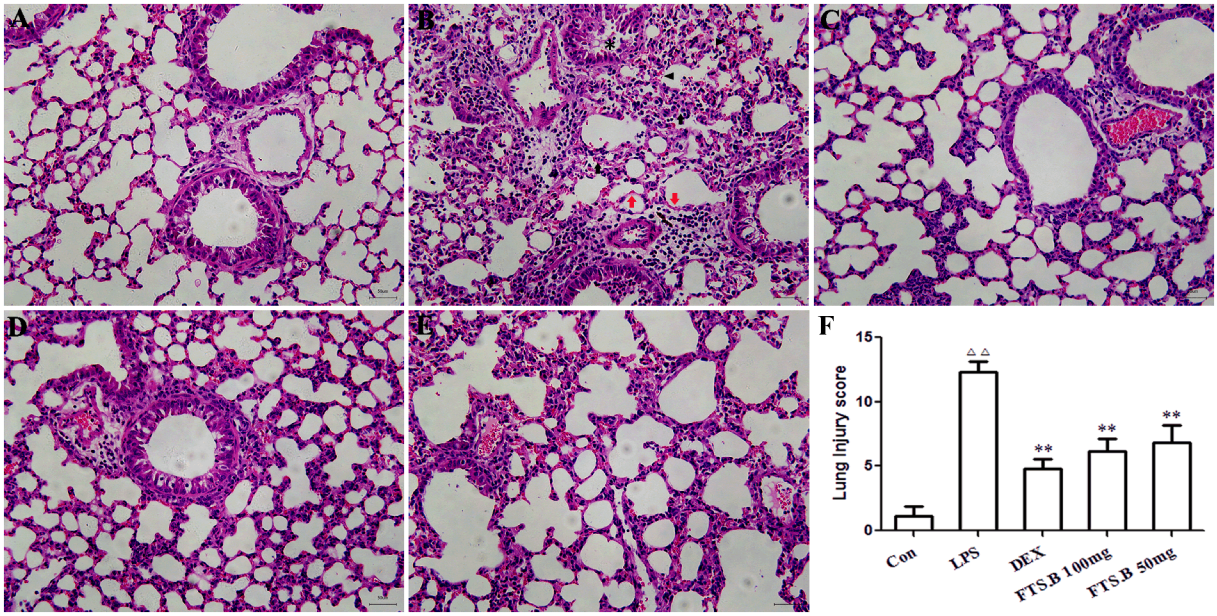

Fig. 3. Effects of FTS·B on lung histology. Control (A); LPS (B); DEX (C); FTS·B 100 mg/kg (D); FTS·B 50 mg/kg (E) and lung injury score (F). The images of histopathological changes of lung from each group (magnification 200×, arrow: inflammatory cells infiltration; arrowhead: hemorrhage; red arrow: hyaline membrane; asterisk: leakage; bar: 50 μm) 24 h after LPS challenge. Data were analyzed by rank-sum test in lung injury score. △ $p < 0.05$, △△ $p < 0.01$ compared to control; *$p < 0.05$, **$p < 0.01$compared to ALI mice ($n = 6$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and dexamethasone (2 mg/kg), respectively. The mice in the control and LPS groups were intraperitoneally administered equivalent volumes of sterile saline (0.2 mL/10 g). Then mice were anesthetized using ether and received intratracheal instillation of 10 μg of LPS (in 50 μL of PBS) or vehicle (control group). The mice were placed in a vertical position and rotated for 1 min to distribute the instillation in the lung. Mice were killed and the lungs were harvested 24 h post LPS instillation for further analysis.

2.6. Lung edema assessment

Twenty-four hours after LPS instillation, the mice were anesthetized with 1% pentobarbital sodium. The lungs were excised, blotted dry, weighed to obtain the wet weight, and then placed in an oven at 80 °C for 48 h to obtain the dry weight. The ratio of lung weight to body weight and wet lung weight to dry lung weight was calculated to assess tissue edema.

2.7. Cell counting in bronchial alveolar lavage fluid and MPO activity assay

The bronchial alveolar lavage fluid (BALF) was obtained 24 h after LPS instillation. Briefly, the lung was lavaged with 500 μL of PBS three times (total volume, 1.5 mL). The BALF samples were centrifuged at 300 ×*g* for 10 min at 4 °C and the supernatant was stored at −80 °C to determine the concentration of proteins using the BCA Protein Assay Kit. The cell pellet was washed with red blood cell lysis solution for 1 min, then centrifuged at 300 ×*g* for 10 min, and resuspended in 200 μL of PBS. The total cell count was determined using a hemocytometer; differential cell count was determined by Wright-Giemsa staining; and macrophages, neutrophils, and lymphocytes were quantified by counting 200 cells per slide at 400 × magnification. In addition, the activity of MPO in lung homogenate (lung was homogenized with 9-fold volume of sterile saline, and then centrifuged at 1500 ×*g* at 4 °C for 15 min) was determined using the ELISA kit.

2.8. Pro-inflammatory mediator measurements

Twenty-four hours after LPS challenge, the level of interleukin (IL)-1β, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) in RAW 264.7 cell culture medium or lung homogenate was determined using the ELISA kit, according to the manufacturer's instructions.

2.9. Total RNA extraction and RT-PCR

Ten hours post LPS challenge, the total cellular RNA from cells was extracted and converted to cDNA. Gene expression in the synthesized cDNA was amplified using Roche Fast Start Universal SYBR Green Master. The polymerase chain reaction (PCR) was carried out under the following condition: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and final extension at 60 °C for 1 min. *GAPDH* gene was used as control. The sequence of primers used is presented in Table 1.

2.10. Western blotting

The expression of TLR4, MyD88, Iκκ, IκB, and p65 in LPS-challenged cells and mouse lung was determined by western blotting. Briefly, the sample was lysed in RIPA buffer and clarified by centrifugation at 10,000 ×*g* for 10 min at 4 °C. Total protein after denaturation was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, blocked with 5% skim milk, and sequentially incubated with anti-TLR4, MyD88, p65, Iκκ, mouse anti-IκB, and pIκB primary antibodies, followed by incubation with a secondary antibody. The area and integrated optical density (IOD) of the bands were analyzed using Image-Pro Plus 6.0 software.

2.11. Histopathology

The lungs of mice were harvested and fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned (thickness, 4 μm),

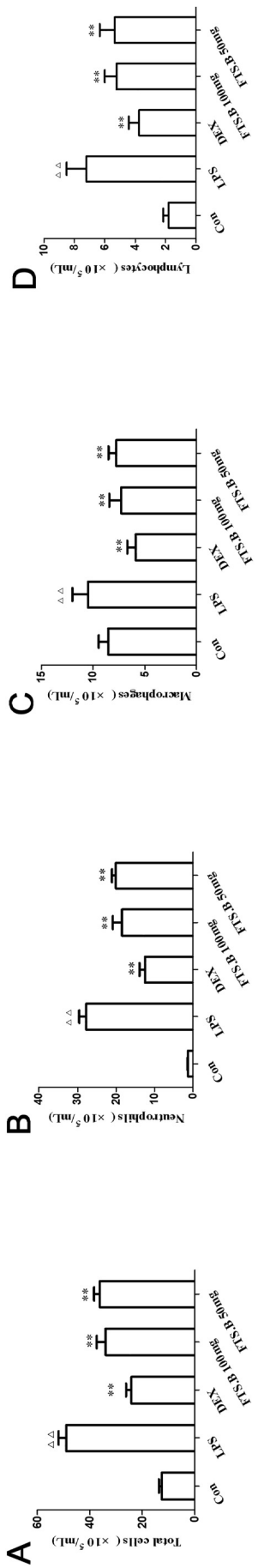

Fig. 4. Effects of FTS·B on cells infiltration. Total cells (A), neutrophils (B), macrophages (C) and lymphocytes (D) in BALF. Mice were administrated FTS·B (50 or 100 mg/kg) 2 h before intratracheal instillation of LPS, the BALF were collection and for leucocytes and differential were count 24 h after LPS challenge. Data were analyzed by ANOVA. Values are expressed as the means ± S.D. $\triangle p < 0.05$, $\triangle\triangle p < 0.01$ compared to control mice; $^{*}p < 0.05$, $^{**}p < 0.01$compared to ALI mice (n = 10).

and stained with hematoxylin and eosin (H&E). The lung injury was scored according to Smith scoring method by two pathologists [18]. The total lung injury score was the sum of lung edema, alveolar and interstitial inflammation, alveolar and interstitial hemorrhage, atelectasis, and hyaline membrane formation that were all scored from 1 to 4 points (extent < 25%: 1 point, 25–50%: 2 point, 50–75%: 3 point, > 75: 4 point), according to the standards of Smith scoring.

2.12. Statistical analyses

All results are expressed as mean ± standard deviation. Intergroup differences were evaluated by the one-way analysis of variance (ANOVA) or Dunnett's T3 when the data were not normally distributed. The rank-sum test was used to identify pathological damage. The results with *P*-values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effects of FTS·B on lung edema induced by LPS

The wet lung/body weight ratio, lung wet/dry weight ratio, and protein concentration in BALF were determined to evaluate edema and pulmonary vascular permeability induced by LPS. As shown in Fig. 2, the mice with LPS instillation alone presented a significant increase in lung/body weight ratio, lung wet/dry weight ratio, and protein concentrations when compared with those of the control mice. In contrast, the administration of FTS·B or dexamethasone significantly suppressed the LPS-induced lung edema, and protein-rich leakage when compared with those in the LPS group.

3.2. Effects of FTS·B on LPS-induced changes in lung histology

The histological changes in the lung were investigated by H&E staining. As shown in Fig. 3, the lung tissue of mice from the control group exhibited clear alveolar lobules and alveolar cavities, without leakage and cell infiltration in the alveolar spaces or the interstitium. However, the mice challenged with LPS exhibited alveolar septal wall thickening, hemorrhage, and protein leakage in the alveolar cavity, as well as increased number of neutrophil sequestration in the alveoli, interstitium, and around the pulmonary vessel. The pathological damages and lung injury score in dexamethasone- or FTS·B-treated mice were notably reduced, with mild inflammatory cell infiltration, hemorrhage, and protein leakage in the alveolar cavity.

3.3. Effects of FTS·B on cell infiltration in the lungs

To evaluate the effects of FTS·B on inflammatory cell infiltration, the total and differential cells in the BALF were counted 24 h after LPS challenge. The results revealed that the number of infiltrated cells, including macrophages, lymphocytes, and neutrophils, was significantly increased in the BALF from mice instilled with LPS. In contrast, the increased recruitment of macrophages, lymphocytes, and neutrophils in the BALF of FTS·B or dexamethasone treatment mice was significantly reduced (Fig. 4).

3.4. Effects of FTS·B on pro-inflammatory mediators in the lungs and in RAW 264.7 cells

When compared with control mice, LPS-challenged mice exhibited increased levels of inflammatory cytokines (IL-1β, IL-6, IFN-γ, and TNF-α), chemokines (MCP-1 and MIP-2), and elevated activation of inducible enzymes COX-2 and nitric oxide synthase (iNOS) in the lungs (Fig. 5). As expected, FTS·B, especially the 100 mg/kg dose, or dexamethasone pre-treatment significantly suppressed these changes developed in ALI mice. FTS·B at a dose of 50 mg/kg had similar effects; however, the levels of MPO, IL-1β, and iNOS decreased without

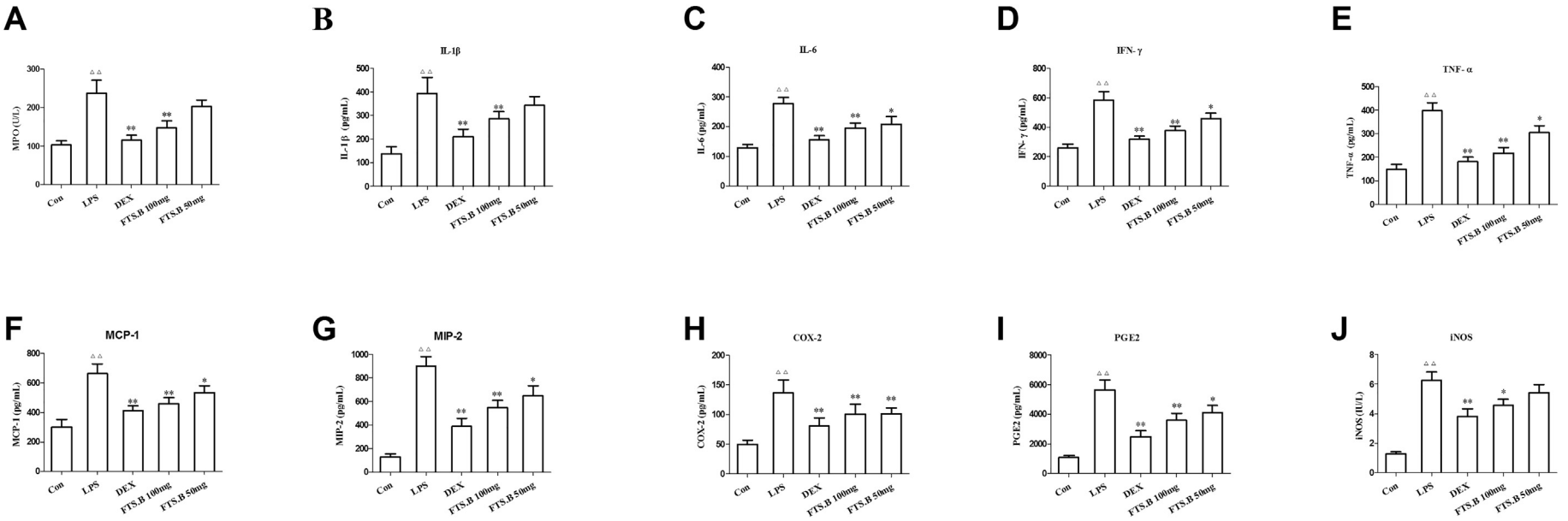

Fig. 5. Effects of FTS·B on inflammatory mediators in mice. The MPO activity (A), cytokines (B–E), chemokines (F and G), inducible enzyme (H and J) and PGE2 (I). Mice were administrated with FTS·B (50 or 100 mg/kg) 2 h before intratracheal instillation of LPS, the inflammatory mediators in lung were detected 24 h after LPS challenged by ELISA. Data were analyzed by ANOVA. Values are expressed as the means ± S.D. △ $p < 0.05$, △△ $p < 0.01$ compared to control mice; *$p < 0.05$, **$p < 0.01$compared to ALI mice (n = 10).

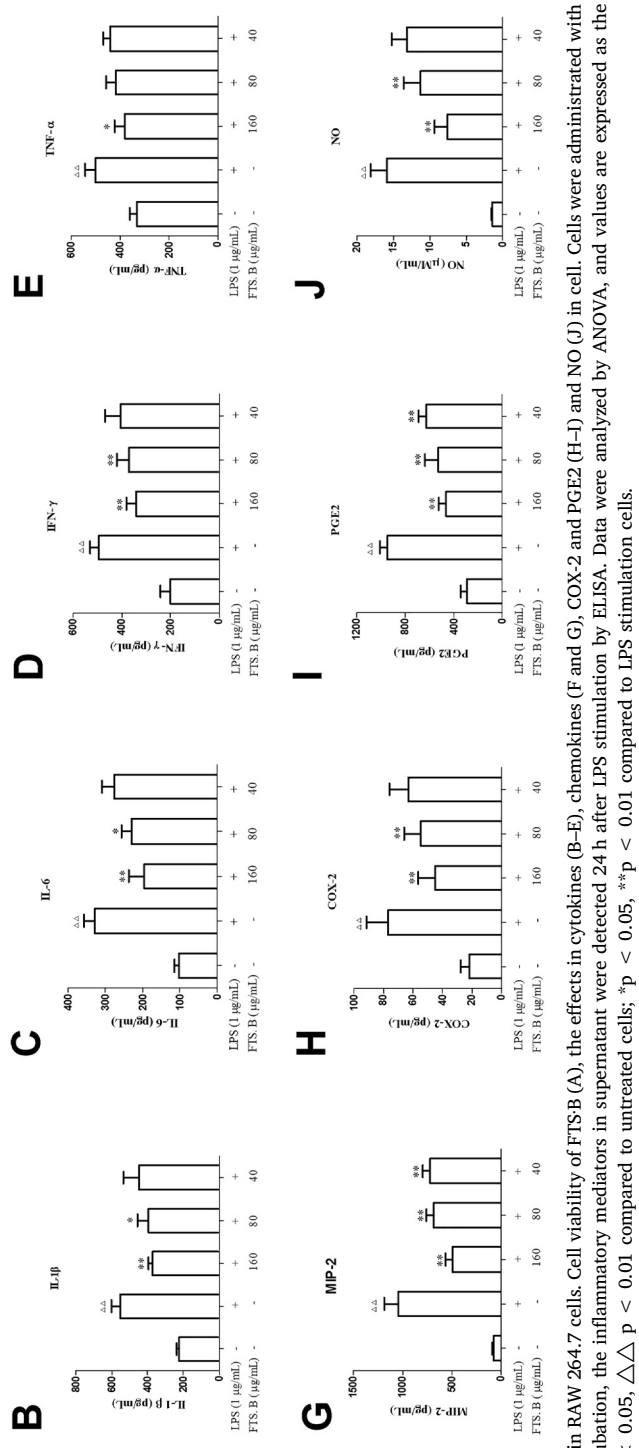

Fig. 6. FTS·B inhabits inflammatory mediators in RAW 264.7 cells. Cell viability of FTS·B (A), the effects in cytokines (B–E), chemokines (F and G), COX-2 and PGE2 (H–I) and NO (J) in cell. Cells were administrated with FTS·B (40, 80, 160 μg/mL) 1 h before LPS incubation, the inflammatory mediators in supernatant were detected 24 h after LPS stimulation by ELISA. Data were analyzed by ANOVA, and values are expressed as the means ± S.D. from three parallel test. △ $p < 0.05$, △△ $p < 0.01$ compared to untreated cells; *$p < 0.05$, **$p < 0.01$ compared to LPS stimulation cells.

statistically significance.

The suppressive effects of FTS·B on LPS-induced inflammatory response were further confirmed *in vitro*. Treatment with FTS·B significantly decreased the concentrations of pro-inflammatory cytokines and the level of activation of COX-2 and iNOS in LPS-stimulated RAW 264.7 cells (Fig. 6); however, the effects of FTS·B were not significant at all concentrations of FTS·B. As shown in Fig. 6, the concentrations of IL-1β, IL-6, IFN-γ, COX-2, and NO decreased significantly with 80 and 160 μg/mL of FTS·B, and only 160 μg/mL dose of FTS·B significantly decreased TNF-α level. Further research found that the mRNA levels of these inflammatory mediators were also suppressed in LPS-stimulated RAW 264.7 cells (Fig. 7). The 80 and 160 μg/mL doses of FTS·B significantly decreased the mRNA expression of these inflammatory genes, while the effects of 40 μg/mL dose of FTS·B were relatively weak and the decrease in MIP-2 and COX-2 was not statistically significant.

3.5. Effects of FTS·B on the activation of TLR4/NF-κB signaling pathway

To explore the signaling pathway through which FTS·B inhibited lung inflammation in LPS-induced ALI, western blotting was performed to evaluate the expression of proteins involved in the TLR4/NF-κB pathway. As expected, LPS administration significantly increased the expression of TLR4, MyD88, p-Iκκ, p-IκB, and p-p65 in the lung tissue. However, pre-treatment with dexamethasone and FTS·B significantly suppressed the increased expression of TLR4 and MyD88, as well as the phosphorylation of Iκκ, IκB, and p65 in the lung tissue (Fig. 8). As expected, similar trends were found after treatment with FTS·B in LPS-stimulated RAW 264.7 cells, in that the expression of TLR4, MyD88, p-Iκκ, p-IκB, and p-p65 was significantly decreased (Fig. 9).

4. Discussion

It have been demonstrated that FTS·B exerts antioxidant and antibacterial effects *in vitro* [14,15]. It also showed anti-inflammatory effects in LPS-challenged chicken, in rats that underwent caecal ligation and puncture, and rats that underwent myocardial ischemia-reperfusion. The mechanisms by which FTS·B exerted its anti-inflammatory effects were found to correlate with the effect of reducing the biological activity of serum LPS, reducing phosphorylated NF-κB expression, attenuating neutrophil infiltration, reducing MPO activity, and inhibiting the production of pro-inflammatory factors [16,17,19]. However, the effect of FTS·B on LPS-induced inflammation and ALI is not known; therefore, the present study was conducted. We demonstrated that administration of FTS·B can effectively protect against LPS-induced lung injury. In this study, lung edema, lung pathological changes, inflammatory cell infiltration, and MPO activity in the lung tissue were significantly attenuated in mice treated with FTS·B. Further, we also observed that FTS·B was able to decrease the level of PGE2, COX-2, nitrite, iNOS, and pro-inflammatory meditators in the lung tissue and in LPS-stimulated RAW 264.7 cells. The mechanisms by which FTS·B exerted its anti-inflammatory effect were found to correlate with inhibition of TLR4 expression and the phosphorylation of NF-κB.

LPS, an important constituent of gram-negative bacteria, is a major component of pathogens responsible for causing ALI. Intratracheal instillation of LPS is a well-known model of ALI, which is characterized by alveolar damage accompanied by infiltration of neutrophils and macrophages, hyaline membranes, and protein-rich edema in the alveolar space [1,20]. Although macrophages and neutrophils have beneficial actions in eliminating microbial infections and serving as an essential interface between innate and adaptive immunity during inflammatory responses, excessive recruitment, together with the release of cytokines and other pro-inflammatory mediators, contribute to increased vascular permeability, leakage of protein and water from capillaries, and formation of pulmonary hyaline membranes [21–23]. Moreover, alleviating excessive inflammatory cell transmigration and cytokine cascade contributes to reducing pathological damage and the severity of

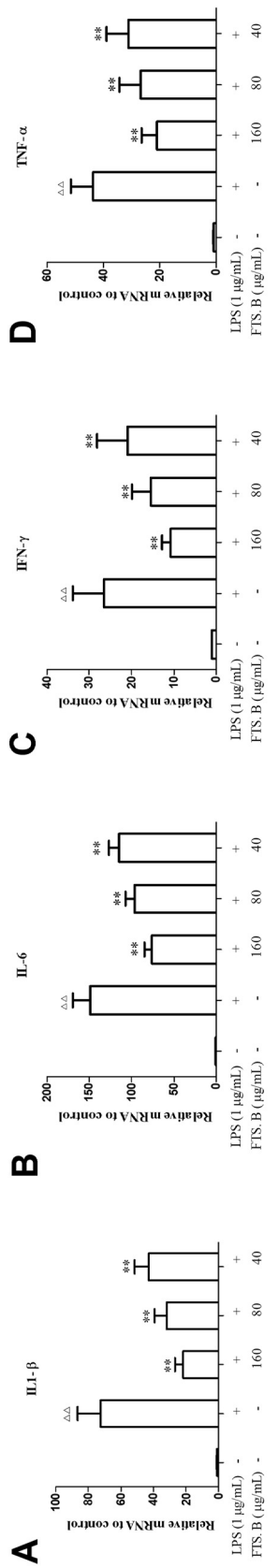

Fig. 7. Effects of FTS·B on LPS-induced inflammatory genes expression in RAW 264.7 cell. The transcription level of cytokines (A–D), chemokines (E–F) and inducible enzyme (G–H). Cells were administrated with FTS·B (40, 80, 160 μg/mL) 1 h before LPS incubation, the transcription were detected 10 h after LPS incubation. Data were analyzed by Dunnett's T3 test from three parallel test, and values are expressed as the means ± S.D. △ $p < 0.05$, △△ $p < 0.01$ compared to untreated cells; *$p < 0.05$, **$p < 0.01$ compared to LPS stimulation cells.

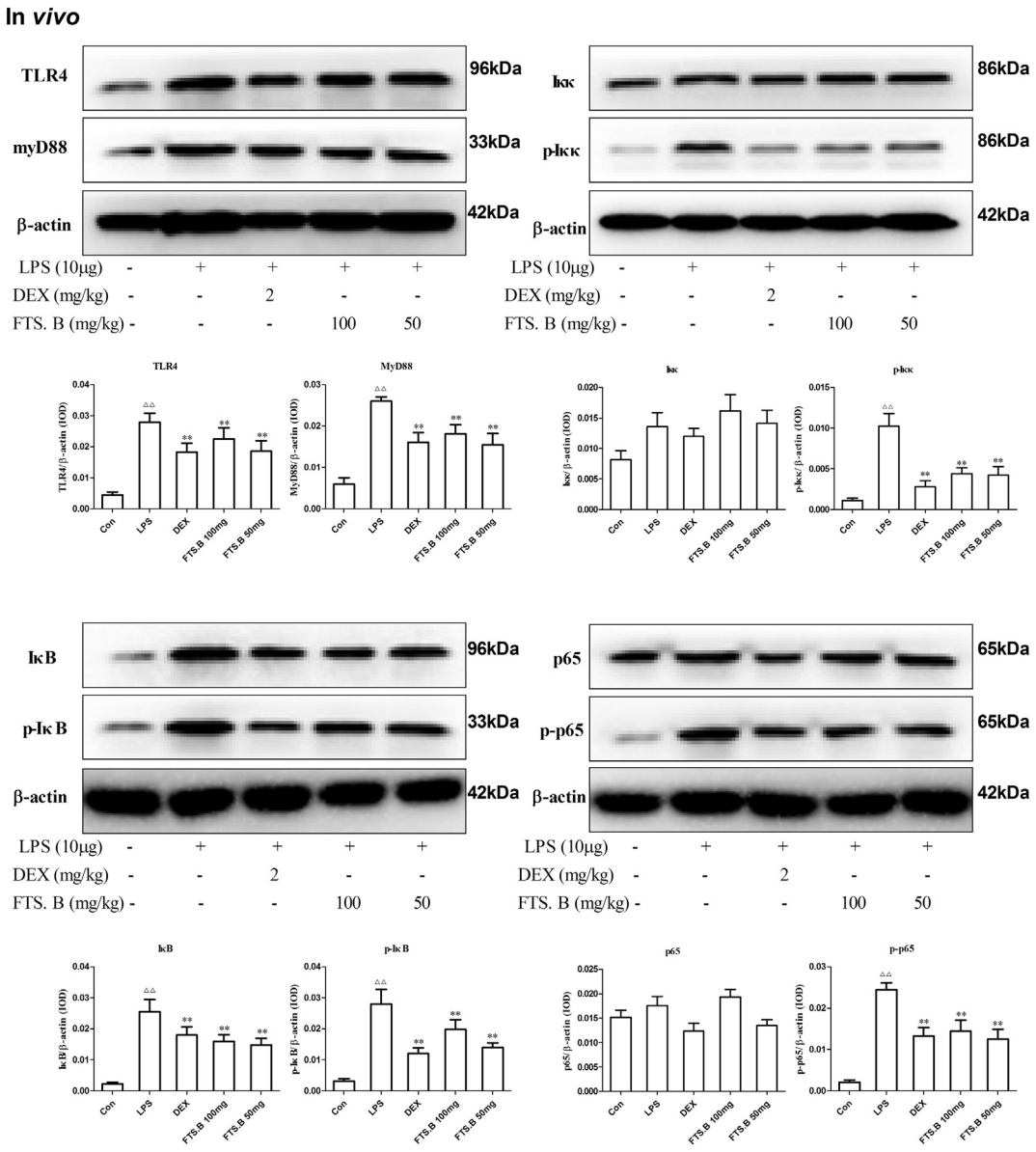

Fig. 8. FTS.B suppresses TLR4/NF-κB signaling activation in mice. Mice were administrated FTS.B (50 or 100 mg/kg) 2 h before intratracheal instillation of LPS. All mice were sacrificed 24 h after LPS challenge and lung tissues were harvested and analyzed by Western blot. Data were analyzed by ANOVA, and values are expressed as the means ± S.D. from three parallel test. △ $p < 0.05$, △△ $p < 0.01$ compared to control mice; *$p < 0.05$, **$p < 0.01$ compared to ALI mice.

ALI symptoms [24,25]. In the present study, the results showed that the number of macrophages and neutrophils in lung tissues are markedly elevated and accompanied by protein-rich edema after LPS challenge. In contrast, the effects of FTS·B on attenuating macrophage and neutrophil infiltration and MPO activity were found in mice treated with FTS·B. In addition, ratios of the lung weight to body weight and of lung wet to dry weight and protein concentration in the BALF were decreased in FTS·B treatment mice.

Pro-inflammatory cytokines play a critical role in the initiation of inflammatory pathogenesis in LPS-induced ALI. Alveolar macrophages and neutrophils are an important source of cytokines in ALI, and the cytokines or chemokines secreted by them, such as IL-1β, IL-6, IL-8, MCP-1, MIP-2, and TNF-α, in turn reinforcing the transmigration of macrophages and neutrophils, amplifying the inflammatory cascade, and aggravating lung injury [1,9,23,26]. The excessive infiltration stimulated by cytokines in turn contributes to excessive production of ROS, nitrites, and MPO, which exacerbates capillary permeability and hemorrhage, and extends the time of neutrophil sequestration [27,28]. In the present study, the levels of IL-1β, IL-6, IL-8, IFN-γ, TNF-α, MCP-1, and MIP-2 in lung tissues are markedly elevated along with an elevation in MPO and nitrite levels after administration of LPS *in vivo* or *in vitro*. In contrast, pretreatment with FTS·B dramatically decreased the levels of these cytokines and chemokines. These results reveal that the effects of FTS·B on LPS-induced ALI and inflammation are associated with inhibition of pro-inflammatory cytokines, and consistent with a decrease in macrophage and neutrophil accumulation in lung tissues.

It has been confirmed that the activation of toll-like receptors (TLRs), especially TLR4, is pivotal in pathogen recognition, immunity activation, and inflammation [29–31]. As the receptor of LPS, TLR4 plays a principal role in the recognition of gram-negative bacteria. Previous research demonstrated that LPS signals *via* TLR4 and MyD88 to activate NF-κB [32,33], which eventually leads to the expression of many genes related to inflammation, such as IL-6, IL-1β, and TNF-α, and the infiltration of neutrophils and macrophages in LPS-induced ALI [34,35]. Meanwhile, attenuating the excessive activation of TLR4 contributes to alleviating inflammation and tissue damage [36,37]. In addition, NF-κB is also closely related to the expression of iNOS and COX-2 [38,39], which respectively associates to the production of NO

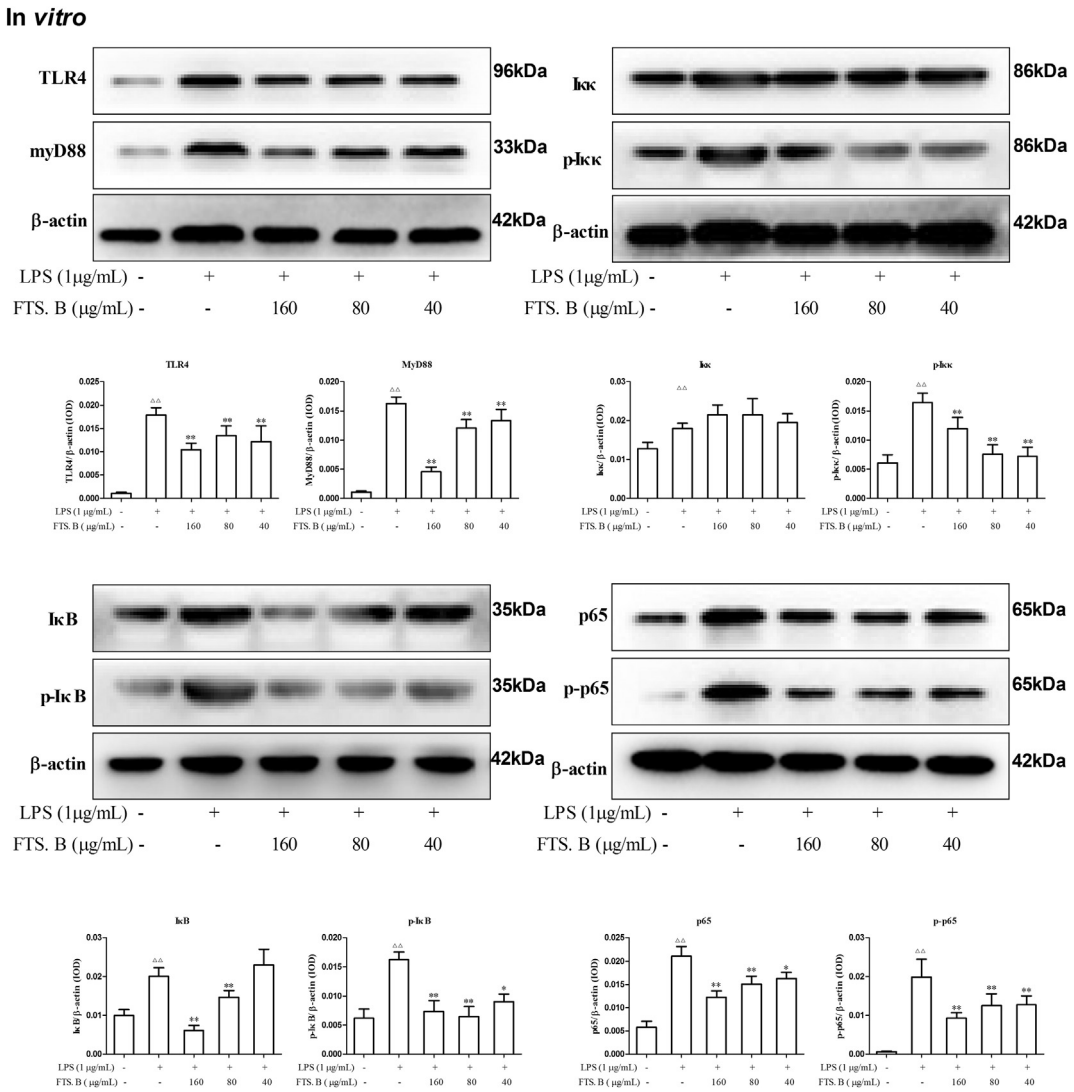

Fig. 9. FTS.B inactivation TLR4/NF-κB signaling in RAW 264.7 cells. The cells were pretreated with different concentrations of FTS.B (40, 80, 160 μg/mL) for 1 h and stimulated with LPS for 10 h. Then the protein expressions were assessed by Western blot. Data were analyzed by ANOVA, and values are expressed as the means ± S.D. from three parallel test. △ $p < 0.05$, △△ $p < 0.01$ compared to untreated cells; *$p < 0.05$, **$p < 0.01$ compared to LPS stimulation cells.

and PGE2 that leads to lung microvascular endothelial cell damage, vasodilatation, increasing vascular permeability, and facilitating neutrophil transmembrane transmigration [25,28,40], which is involved in protein-rich fluid exudation in alveoli and lung edema in ALI [41–43]. Therefore, inhibition of the activation of NF-κB might help mitigate inflammation and further decrease the damages that are caused by excessive inflammatory responses. Our data suggested that FTS·B significantly inhibits TLR4 and MyD88 and the phosphorylation of Iκκ, IκB, and p65 but does not influence their synthesis. This consequently decreased the levels of pro-inflammatory cytokines, which are responsible for LPS-induced ALI. Additionally, FTS·B effectively decreased the production of inflammatory cytokines in LPS-stimulated RAW 264.7 cells, which was consistent with the results of the inflammatory cytokines generation *in vivo*. Collectively, our *in vitro* experiment verified that FTS·B significantly downregulates the LPS-induced overexpression of inflammatory genes and exerts anti-inflammatory activity through the TLR4/NF-κB pathway.

In conclusion, pretreatment with FTS·B could attenuate the pulmonary histological changes and lung edema, reduce inflammatory cell infiltration in the lung, and inhibit the release of inflammatory cytokines. In addition, FTS·B significantly suppresses the expression of TLR4 and the phosphorylation of Iκκ, IκB, and p65 elevated by LPS stimulation *in vivo* and *in vitro*. These results revealed that FTS·B exhibited anti-inflammatory effect against LPS-induced inflammation and ALI through the TLR4/NF-κB pathway *in vivo*, suggesting that FTS·B could be a potential candidate for the treatment of ALI or excessive inflammation.

Conflicts of interest

The authors have no financial conflicts of interest.

Acknowledgments

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