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Paeonol ameliorates lipopolysaccharides-induced acute lung injury by regulating TLR4/MyD88/ NF-κB signaling pathway

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Paeonol has been found to hold analgesic, antipyretic and anti-allergic activities. Here, we investigated the protective effect of paeonol on acute lung injury (ALI) induced by lipopolysaccharides (LPS) and explored the underlying mechanisms on TLR4/MyD88/NF-κB signaling pathway. C57BL/6 mice were randomly divided into control (normal saline, NS, 0.2 mL/d), LPS (NS, 0.2 mL/d), LPS + dexamethasone (DXMS) (5 mg/kg/d), LPS + paeonol (50, 25, 12.5 mg/kg/d) groups. The results of the lung tissue scores scale and HE staining showed that paeonol could attenuate the infiltration of inflammatory cells and the thickening of alveolar wall significantly. The result of W/D ratio showed that paeonol could also prevent pulmonary edema, as well as inhibit significantly the levels of TNF-α, IL-1β and IL-6 in serum and proteins expression and mRNA. In addition, paeonol can also downregulate the expression or phosphorylation of TLR4, MyD88 and NF-κB. In general, our findings showed that the protective effect of paeonol on LPS-induced ALI by regulating TLR4/MyD88/NF-κB signaling pathway. This study provides evidence for the application of paeonol in treating ALI.

1. Introduction

Acute lung injury (ALI) is an inflammatory lung disease caused by a variety of direct and indirect traumatic factors (Li et al. 2016; Wu et al. 2018). Direct damage to the lung tissue may be caused by pneumonia and laceration of lungs, while indirect damage is due to diseases of other organs such as acute pancreatitis. Its clinical treatment is difficult due to high mortality rate (Sadowitz et al. 2011). Modern medical research has shown that LPS can induce the occurrence of ALI. LPS is an ingredient of endotoxin in the outer membrane of Gram-negative bacteria with strong biological activity, leading to severe inflammatory response (Jiang et al. 2017). LPS stimulates the secretions of proinflammatory mediators, leading to pulmonary edema and diffuse alveolar damage (Gutbier et al. 2018; Ding et al. 2018).

Toll-like receptor (TLR) 4 is a membrane-spanning protein of the TLR family and plays a significant role in the nonspecific immune response system (Wu et al. 2017). TLR4 acts as a trigger for the systemic inflammatory response. Myeloid differentiation factor 88 (MyD88) is a key linker in TLR signaling pathway and plays an important role in the transmission of upstream information and the development of diseases. Furthermore, signal transduction activation of the TLR4 receptor leads to the increase of phosphorylation of NF-κB (Gao et al. 2018). The component of LPS can be recognized by TLR4. The activation of TLR4/MyD88/NF-κB signaling pathways is associated with the inflammation injury of ALI (Zhang et al. 2017; Zhao et al. 2016).

Paeonol (Fig. 1A) is a natural compound isolated from *Cortex Moutan*. It has antipyretic, anti-inflammatory and other pharmacological activities. Paeonol is a low molecular weight phenolic compound with poor solubility in water. It has been found to hold an apparent inhibitory effect on carrageen, 5-hydroxytryptamine (5-HT), xylene and endotoxin-induced inflammation (Liu et al. 2014; Chu et al. 2013). Accumulating studies showed that paeonol had regulation effect on the expression of inflammatory cytokines, and could attenuate the degree of lung injury (Pan et al. 2009;

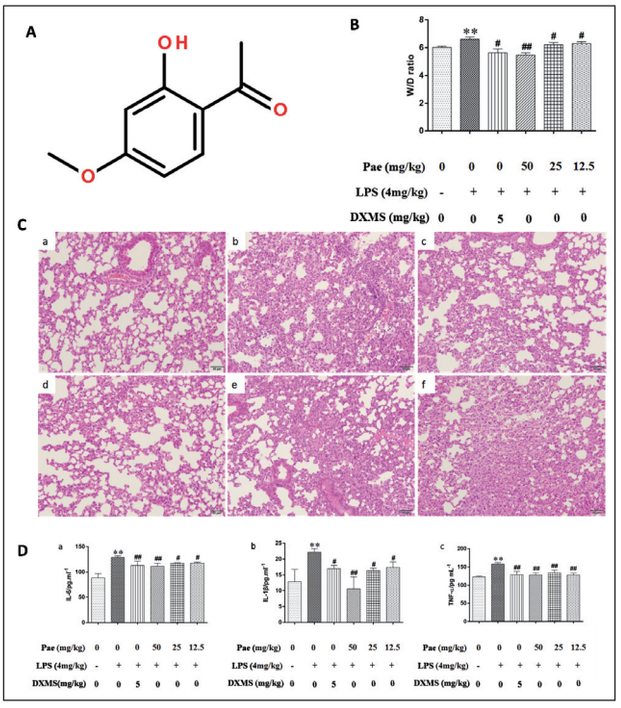

Fig. 1: Paeonol reduced lung injury and down-regulated the expression of peripheral inflammatory cytokines in mice. The structural formula of paeonol (A). Paeonol pretreatment reduces the lung W/D ratio in mice with LPS-induced ALI (B). Histological assessment for the effects of paeonol on LPS-induced ALI (C). a, b, c, d, e, and f represent negative control group, LPS group, LPS + DXMS group and LPS+paeonol group (50, 25, 12.5 mg/kg) (H&E staining, magnification, x200). Paeonol pretreatment reduces the expression of TNF-α, IL-1β and IL-6 of serum in mice with LPS-induced ALI (D). The data are presented as means ± SD (n=6 mice per group). *$p < 0.05$, **$p < 0.01$, vs. Control group; #$p < 0.05$, ##$p < 0.01$ vs. LPS group.

Wang et al. 2014; Hsieh et al. 2006). It has been reported that the paeonol had a protective effect on LPS-induced ALI (Gao et al. 2018). However, the underlying molecular mechanism for this action remains incompletely understood. This study focuses on the protection of paeonol against ALI induced by LPS. In addition, the TLR4/MyD88/NF-κB signaling pathway was investigated to reveal its possible mechanism.

2. Investigations and results

2.1. Paeonol alleviates lung wet/dry weight ratio in lung tissues

Pulmonary edema is reported to be one of the major features of LPS-induced ALI (Qiushi et al. 2015). To evaluate LPS-induced changes in pulmonary edema, the lung W/D ratio in the lung tissues was analyzed. The W/D ratio in the lung tissues was significantly increased after LPS stimulation when compared with the control group ($p < 0.01$). However, paeonol (50, 25, 12.5 mg/kg) or DXMS could reduce lung W/D ratio remarkably ($p < 0.05$ or $p < 0.01$) (Fig. 1B). Therefore, the results indicated that paeonol could alleviate the symptoms of pulmonary edema caused by ALI.

2.2. Paeonol ameliorates LPS-induced lung histopathological changes

To assess the alveolar wall and neutrophil infiltration in lung tissue of LPS-induced acute lung injury, HE staining was performed in lung tissue. The lung tissues from the control mice exhibited normal structure without histopathological changes (Fig. 1C(a)). The stimulation of LPS could cause severe lung destruction, manifesting as pulmonary edema, extensive infiltration of inflammatory cells, thickening of the alveolar walls (Fig. 1C(b)). However, the lung destruction in the LPS + DXMS group and the paeonol groups (50, 25 mg/kg) was relieved (Fig. 1C(c-e)). Interestingly, the inflammatory cell infiltration and alveolar wall thickening in the paeonol groups (50, 25 mg/kg) were reduced effectively when compared with LPS group (Fig. 1C(d, e)). In addition, the histopathological scores caused by LPS was enhanced by the pretreatment of paeonol groups (50, 25 mg/kg) or positive control DXMS (Table). Thus, paeonol pretreatment effectively alleviated the destruction of lung structure induced by ALI.

Table: Effect of paeonol on histopathological scores in LPS-induced ALI

Groups	Dose	Scores
Control	0.2 mL/d	0.33 ± 0.52
LPS	0.2 mL/d	2.67 ± 0.52**
DXMS	5 mg/kg/d	0.67 ± 0.52##
LPS + paeonol	50 mg/kg/d	0.83 ± 0.41##
	25 mg/kg/d	1.50 ± 0.55##
	12.5 mg/kg/d	2.33 ± 0.52

The data are presented as the means ± SD (n=6 mice per group). *$p < 0.05$, **$p < 0.01$, vs. Control group; #$p < 0.05$, ##$p < 0.01$, vs. LPS group.

2.3. Paeonol reduces LPS-induced the levels of TNF-α, IL-1β and IL-6

As shown in Fig. 1D(a-c), the levels of TNF-α, IL-1β, IL-6 in serum were significantly increased by LPS stimulation, compared

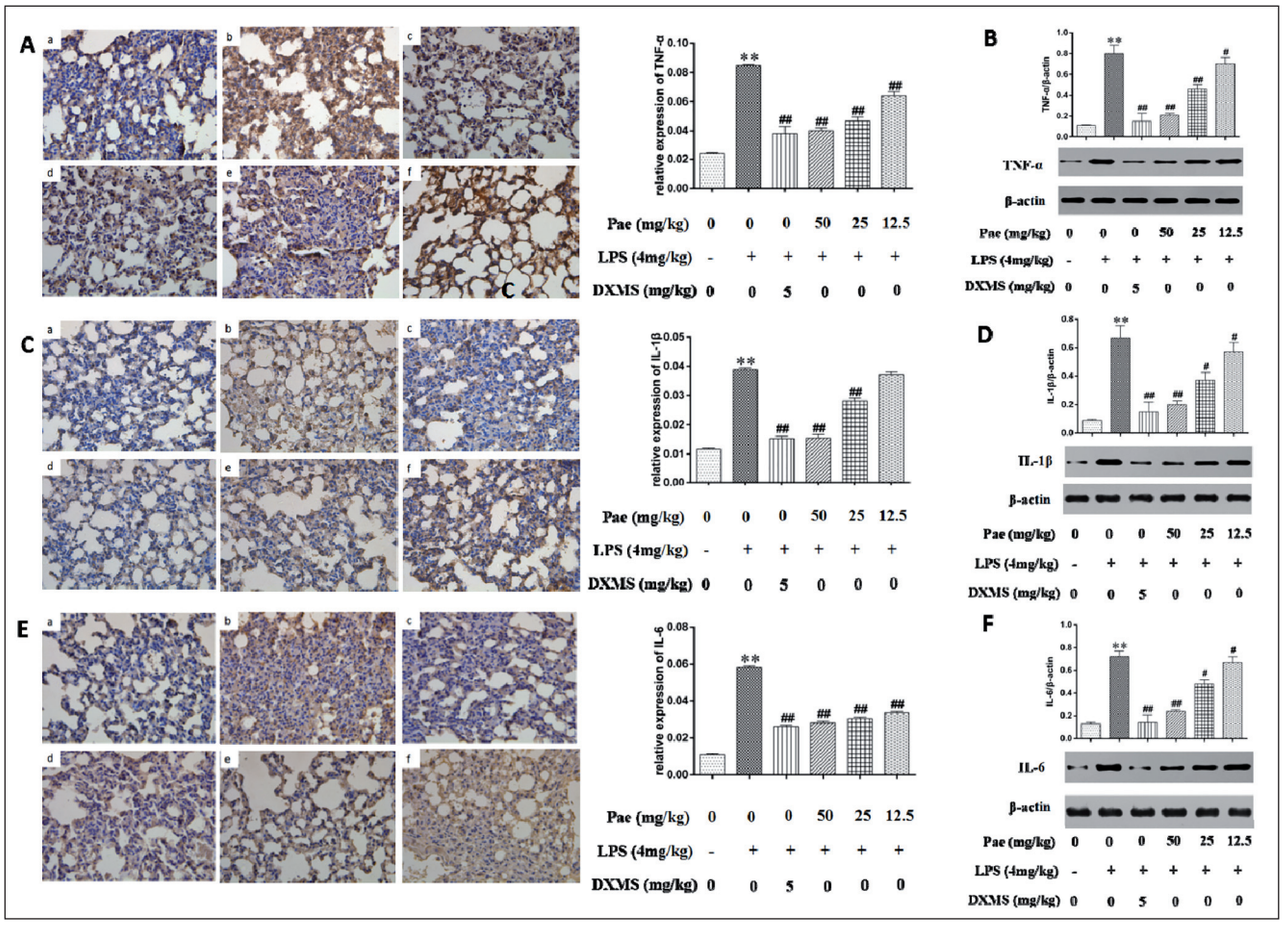

Fig. 2: Effect of paeonol on TNF-α, IL-1β and IL-6 levels in lung tissues. IHC was used for the determination of TNF-α (A), IL-1β (C) and IL-6 levels (E). a, b, c, d, e, and f represent control group, LPS group, LPS + DSMS group and LPS+paeonol group (50, 25, 12.5 mg/kg). WB was performed to examine the levels of TNF-α (B), IL-1β (D) and IL-6 (F). The data are presented as means ± SD (n=6 mice per group). *p < 0.05, **p < 0.01, vs. Control group; #p < 0.05, ##p < 0.01, vs. LPS group.

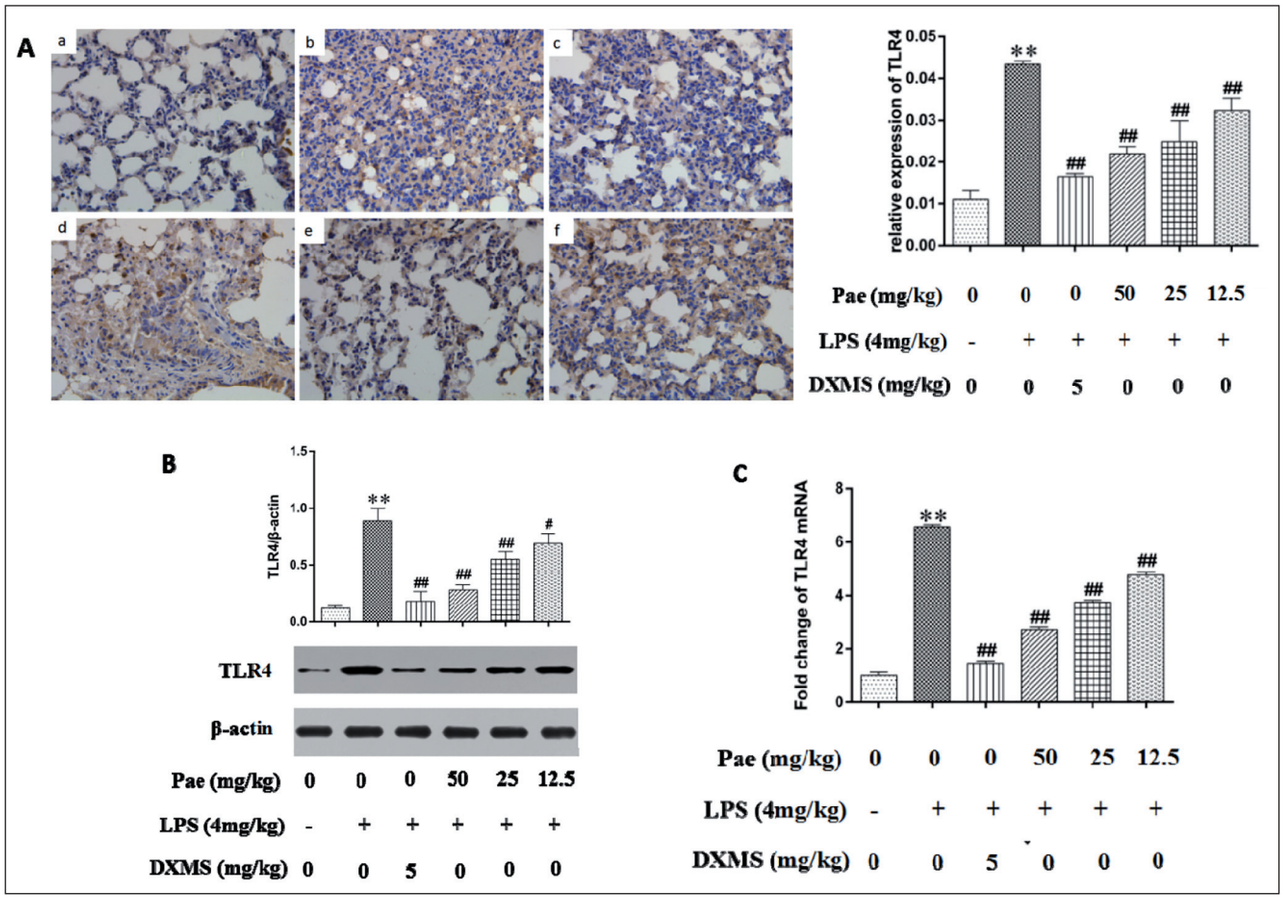

Fig. 3: Inhibition of paeonol on the TLR4 level in lung tissue. IHC was employed for the assay of the TLR4 level (A). a, b, c, d, e, and f represent negative control group, LPS group, LPS + DXMS group and LPS+paeonol group (50, 25, 12.5 mg/kg). Western blotting was performed to examine the level of TLR4 (B). The mRNA expression level of TLR4 protein by RT-PCR (C). The data are presented as means ± SD (n=6 mice per group).*$p < 0.05$, **$p < 0.01$, vs. Control group; #$p < 0.05$, ##$p < 0.01$, vs. LPS group.

with the control group ($p < 0.01$). However, the increased levels of inflammatory-related cytokines were downregulated by DXMS or paeonol groups (50, 25, 12.5 mg/kg). Furthermore,Paeonol inhibits LPS-induced the expression of TNF-α, IL-1β and IL-6 of lung tissue. Interestingly, we found that the dose of paeonol was negatively correlated with the expression of TNFα, IL-1β, and IL-6 in lung tissue of ALI mice (Fig. 2A-F).

2.4. Paeonol inhibits TLR4 expression induced by LPS

After being injected with LPS, the protein expression of TLR4 in lung tissue of mice was upregulated significantly by the stimulation of LPS when compared with the control group ($p < 0.01$). Interestingly, this enhancement level was downregulated markedly by the pretreatment of paeonol in IHC assay and Western blotting (Fig. 3A-B). It has to be noted that the increased level of TLR4 mRNA was also downregulated (Fig. 3C). The data indicated that paeonol might attenuate LPS-induced ALI by regulating TLR4 signaling.

2.5. Paeonol reduces MyD88 expression induced by LPS

We observed LPS could trigger the over-expression of MyD88 in lung tissue. As shown in Fig. 4A, B and C, the levels of MyD88 protein expression and mRNA was downregulated significantly, compared to LPS group ($p<0.05$ or $p<0.01$).

2.6. Paeonol downregulates nuclear phosphorylation NF-κB induced by LPS

NF-κB plays a key role in the inflammatory injury. As depicted in Fig. 5A, a positive deposition could be observed in lung tissues of LPS-induced mice while this deposition was reduced significantly by paeonol. In order to further evaluate the signal pathway, the nuclear phosphorylation level of NF-κB and mRNA expression were determined (Fig. 5B and 5C). The results show that the phosphorylation of NF-κB was inhibited markedly by paeonol, suggesting that its anti-inflammatory effect may be related to the inhibition of NF-κB nuclear phosphorylation.

3. Discussion

ALI has been considered as a major cause of high morbidity and mortality. The results from experimental reports show that the pathogenesis of LPS-induced ALI is similar to human's (Ding et al. 2017; Zhao et al. 2014). Acute inflammation is characterized by the secretion of pro-inflammatory cytokines, the expression of adhesion molecules, infiltration of neutrophils, and the production of numerous cytokines (Zhao et al. 2017). Paeonol extracted from *Paeonia suffruticosa* has been widely used in the clinical treatment of inflammatory diseases with anti-inflammation (Wang et al. 2014). In the present study, the preventive and protective function of paeonol on ALI of mice was evaluated and also the underlying mechanism was also explored. This study provides experimental evidence for the traditional use of paeonol.

To investigate the preventive and protective effects of paeonol on ALI, we examined the histopathological damage of ALI mice and found that paeonol can reduce inflammatory cell infiltration and capillary hemorrhage. Moreover, the result of the W/D ratio further demonstrated that paeonol could improve the pulmonary edema of ALI mice, providing experimental evidence for the pharmacological effects and molecular mechanisms.

The massive increase of inflammatory mediators in circulating blood is the material basis of the systemic inflammatory response.

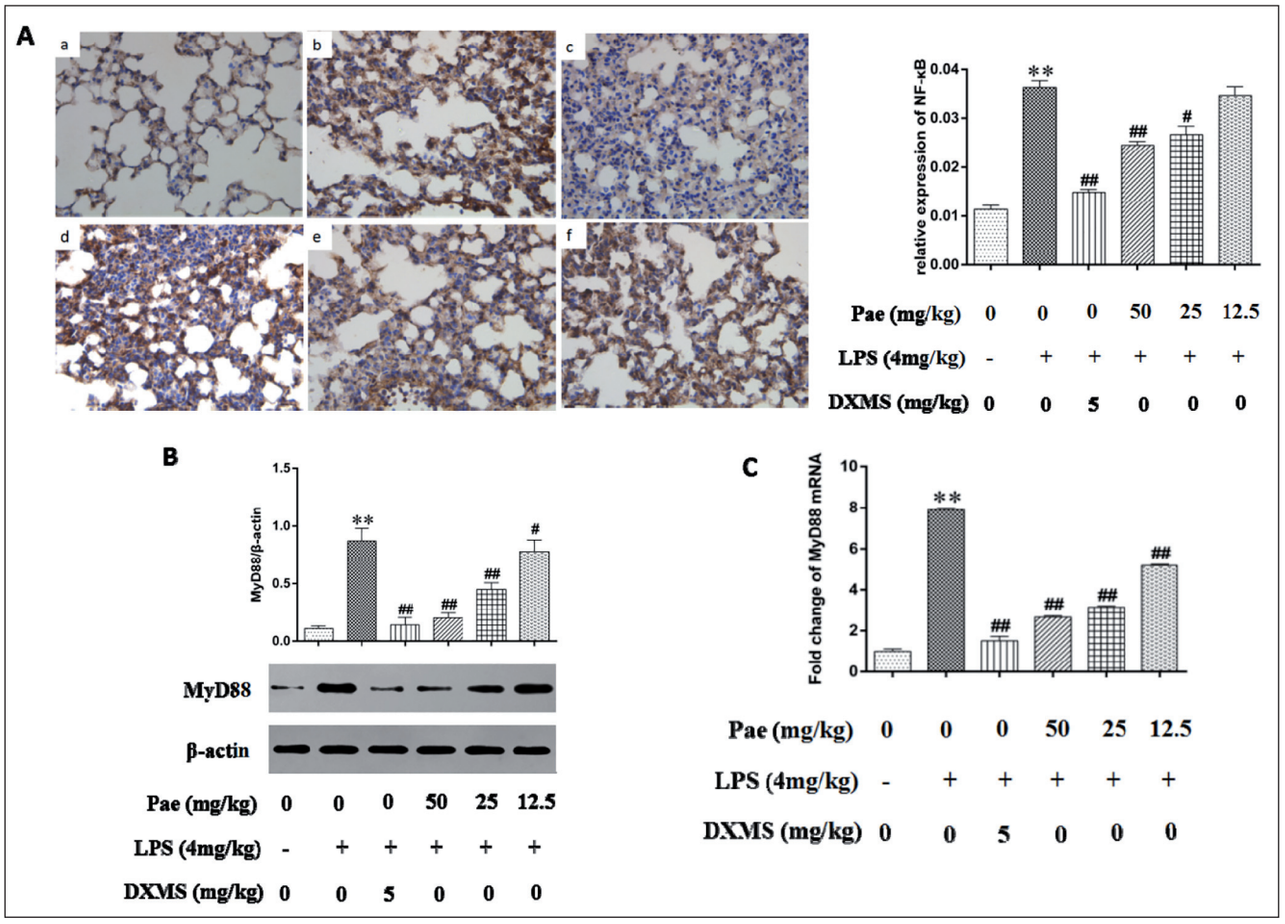

Fig. 4: Paeonol inhibits the expression level of MyD88 in lung tissue. The MyD88 level was analysed by IHC (A). Negative control group, LPS group, LPS + DXMS group and LPS+-paeonol group (50, 25, 12.5mg/kg) was represented by a, b, c, d, e, and f. The level of MyD88 was examined by western blotting (B). mRNA expression levels of MyD88 protein were detected by RT-PCR (C). The data are expressed as means ± SD (n=6 mice per group). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. LPS group.

Inflammatory cytokines not only act as effector molecules in the inflammatory response, but also release signaling molecules of the inflammatory response. Therefore, the level of inflammatory cytokines in the peripheral blood can reflect the entire body (Pogue et al. 2017; Leem et al. 2018). TNF-α, IL-1β, and IL-6 are pro-inflammatory factors, contributing to the development of ALI, which can enhance the infiltration and adhesion of inflammatory cells and stimulate macrophages, to release more pro-inflammatory cytokines (Pan et al. 2013; Dou et al. 2013; Jiang et al. 2017). To understand the anti-inflammatory effects of paeonol, we detected the contents of TNF-α, IL-1β, and IL-6 in the serum and expression level in lung tissues of mice. The results show that paeonol could reduce the levels of TNF-α, IL-1β, and IL-6 neither in serum or in tissues. These data indicate that the protective effect of paeonol on LPS-induced ALI might be related to the attenuation of inflammatory cytokines.

TLR4 is a transmembrane receptor in the immune system, which recognizes pathogenic molecules and activates downstream conducting molecules. Some studies found that the expression of TLR4 in mice with ALI was significantly higher than that in the control group, which was in accordance with our results (Zhang et al. 2017; Dou et al. 2013; Zhu et al. 2012). MyD88 has been found to be a junction protein containing the TLR domain. TLR4 can activate MyD88 for intracellular signaling. In the present study, the expression of MyD88 in LPS group is significantly higher than that in the control group, indicating the activation of MyD88 contributes to ALI. NF-κB plays a key role in the regulation of inflammatory genes (Li et al. 2014). Some studies found that the activation of NF-κB could trigger or accelerate the pathogenesis of ALI (Jiang et al. 2017; Fu et al. 2012; Zhao et al. 2014), and was proved to cause the inflammatory response (Ding et al. 2017; Feng et al. 2012). The secretion of pro-inflammatory cytokines, the expression of adhesion molecules, the infiltration of neutrophils, as well as the production of a large number of cytokines can be observed in the pathological change of LPS-induced ALI (Luo et al. 2017).

To elucidate the mechanism of paeonol on LPS-induced ALI, we further determined the activation of TLR4/MyD88/NF-κB signaling pathway. Our findings show that the activation of TLR4/MyD88/NF-κB pathway results in the secretion of downstream inflammatory factors. Importantly, the expression levels of TLR4, MyD88, NF-κB caused by LPS were downregulated by paeonol. These results indicate that the protective effect of paeonol on ALI might be associated with the regulation on TLR4/MyD88/NF-κB pathway. In summary, our data revealed that paeonol attenuates LPS-induced ALI by regulating the TLR4/MyD88/NF-κB signaling pathway. The present study contributes to new insights on the beneficial effects of paeonol on ALI, which may help to develop effective candidates and targets.

4. Experimental

4.1. Reagents

Sodium carboxymethylcellulose (CMCNa) was purchased from Sinopharm Chemical Reagent Co., Ltd (Nanjing, China). Lipopolysaccharide was obtained from Sigma (USA). Dexamethasone sodium phosphate injection (DXMS) was ordered from Tianjin Kingyork Group Co., Ltd. (Tianjin, China). Paeonol (purity≥98%) was from Nanjing Jingzhu Biotechnology Co., Ltd (Nanjing, China). Mouse IL-1β, IL-6, and TNF-α ELISA kits were purchased from Nanjing JianCheng Bioengineering Institute (Nanjing, China). Antibodies for TNF-α and IL-6 were ordered from Absin Bioscience Inc (Shanghai, China). Antibodies for MyD88 and IL-1β were ordered from Cell Signaling Technology, Inc (China). Antibodies for TLR4 and NF-κB were ordered from Wuhan Boster Biological Technology., LTD (Wuhan, China). Secondary anti-horseradish peroxidase goat anti-rabbit IgG, goat anti-mouse IgG were from

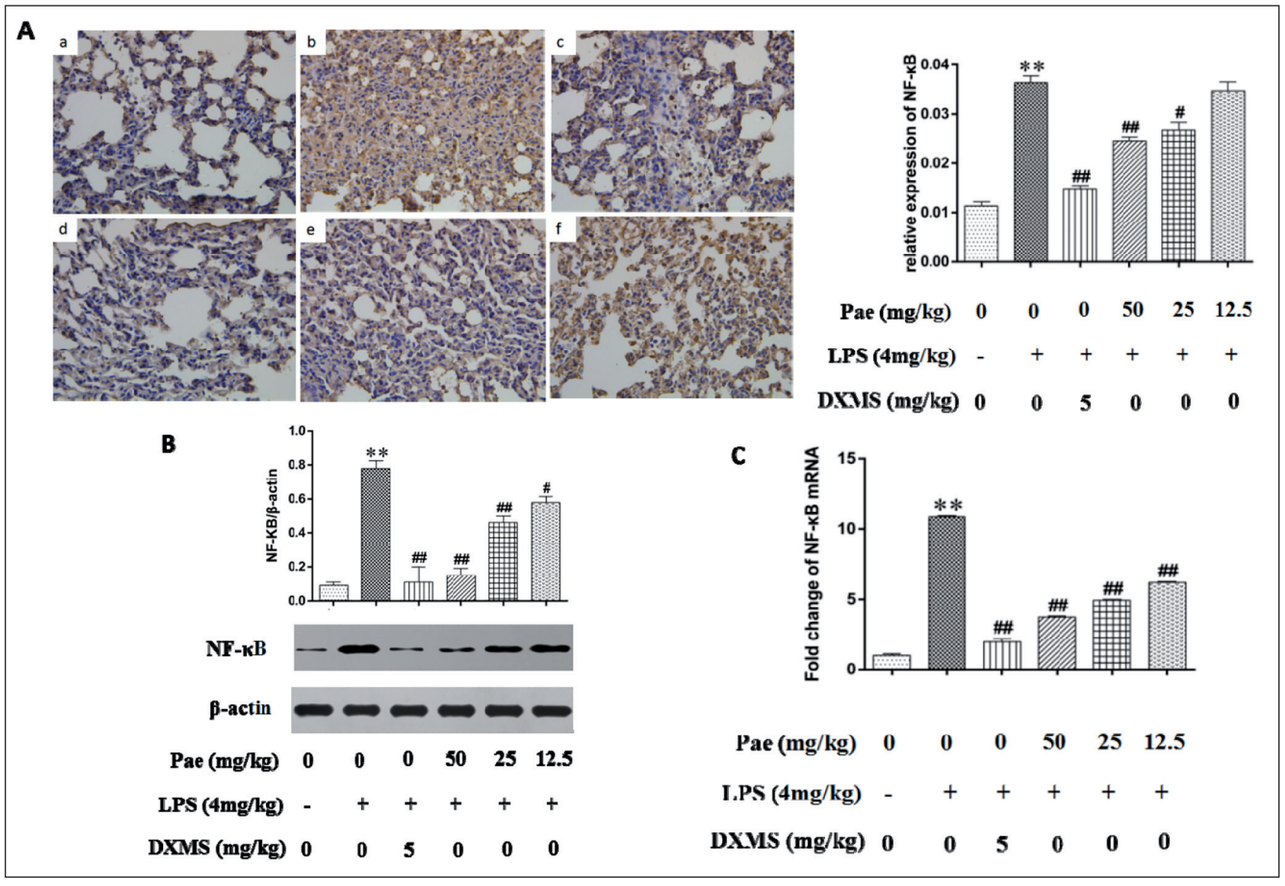

Fig. 5: The expression of MyD88 level in lung tissue was inhibited by paeonol. IHC was used to analyze the MyD88 level (B). a represents negative control group, b represents LPS group, c represents LPS + DXMS group, d, e, and f are LPS+paeonol group (50, 25, 12.5mg/kg). RT-PCR was used to detect the mRNA expression level of MyD88 protein (C). The data are indicated as means ± SD (n=6 mice per group). $^{*}p < 0.05$, $^{**}p < 0.01$, vs. Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. LPS group.

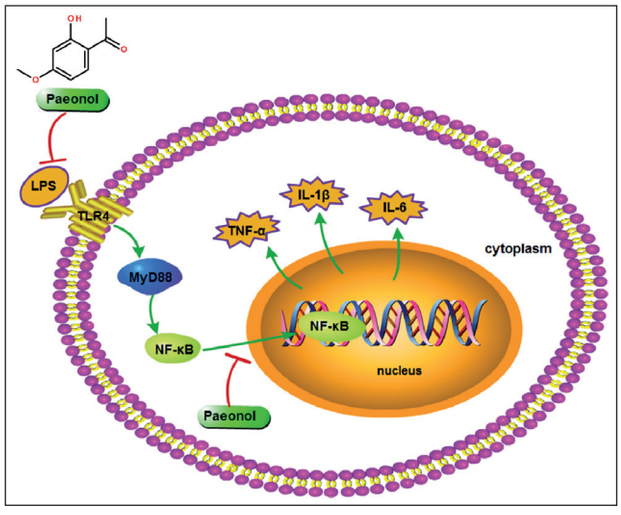

Fig. 6: Schematic diagram on the protection and mechanism of paeonol on LPS-induced inflammation, which is associated with the TLR4/MyD88/NF-κB signaling pathway.

Abcam. SDS-PAGE Gel Preparation kit from Beyotime Institute of Biotechnology (China). First Chain cDNA Synthesis kit was obtained from Vazyme Biotech Co., Ltd (Nanjing, China). All other reagents, solvents, and chemicals used were of analytical grade.

4.2. Animals

Male C57BL/6 mice weighing 20±2 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Nanjing Branch, China (license number: SCXK (Su) 2016-0003) in line with clean grade experimental animal standards. The mice were housed in the animal center, China Pharmaceutical University at room temperature (23±2 °C), relative humidity 45±10% and diurnal illumination of 12 h light/12 h dark (L/D) cycles, standard diet, and free access to water. Animal experiments conformed to the guidelines of the Animal Care and Use Committee of China Pharmaceutical University and were guided by the ethical committee of China Pharmaceutical University.

4.3. LPS-induced lung injury mice model and paeonol treatment

C57BL/6 mice were randomized and divided into control group (normal saline, NS, 0.2 mL/d), LPS group (NS, 0.2 mL/d), LPS + DXMS (5 mg/kg/d) (Pinheiro et al. 2018), and paeonol groups (50, 25, 12.5 mg/kg/d) with 12 mice each. Paeonol was resolved in 0.5% CMCNa. LPS + DXMS group mice were treated by intraperitoneal injection and other groups mice were administered by gavage every day for sustained one week. One hour after the last administration,a LPS-induced ALI animal model was established by intraperitoneal injection of LPS (4 mg/kg) (Fu et al. 2018) After being injected with LPS for 6 h, the blood from retro-orbital venous plexus of mice was taken for serum separation at 3000 r/min for 10 min. Finally, the supernatant was stored at -20°C for further experimentation.

4.4. Measurement of lung wet/dry weight (W/D) ratio in LPS-induced ALI mice

The total lung was isolated, and the body fluid coating the removed lung was wiped gently. The lung tissues were put on the tinfoil, and the weight of whole lung and tinfoil was recorded, and then placed into an oven at 80 °C for 72 h. Then the sample was taken out of the oven and weighed again with an electronic scale and recorded as dry lung weight (DLW). The DLW divided by the WLW denoted the lung wet/dry (W/D) weight ratio.

4.5. Assessment of histopathological changes in the lung

The half left lung of the remaining six mice in each group was removed under aseptic conditions. The embedded left lung tissue blocks were cut into 4 μm sections, then fixed for 24 h with 10% neutral formalin. The sections were deparaffinized, rehydrated, and treated by hematoxylin and eosin (H&E). The histopathological lesions of the lungs were observed under a microscope. Ten visible areas were selected at

random to observe histopathological changes. Normal presentation in each category was scored as 0 and the most severe damage in each category was scored as 3. If the alveolar space is filled with inflammatory cells (such as neutrophils and macrophages) and the alveolar wall is thickened, it is denoted as grade 2. Grade 1 reflects only a few inflammatory cells infiltrated or only alveolar wall thickened.

4.6. Determination of serum TNF-α, IL-1β, IL-6 in mice

The levels of TNF-α, IL-1β, and IL-6 in the blood samples were measured using ELISA kits, according to the manufacturer's protocols. Using the standard concentration value as the ordinate and the standard OD value as the abscissa, the standard curve was calculated by Graphpad prism statistical software. The sample concentration was calculated according to standard curve.

4.7. Immunohistochemistry (IHC) assay

Paraffin sections of lung tissues (4 μm) were obtained and deparaffinized. Sequentially, citrate buffer, water, and PBS were used to get antigen retrieval. After that, 3% H_2O_2-methanol solution was added to each slice and incubated at room temperature for 15 min to block endogenous peroxidase activity, and then primary antibodies labeled with anti-TLR4 (diluted in PBS at 1:250), anti-MyD88 (diluted in PBS at 1:100), anti-p-NF-κB (diluted in PBS at 1:250), anti-TNF-α (diluted in PBS at 1:250), anti-IL-1β (diluted in PBS at 1:600) and anti-IL-6 (diluted in PBS at 1:50) were used to detect the relative protein expression. Next, the HRP-labeled secondary antibody was added to the incubate for 30 min in a humidified chamber for blocking. The slices were treated in hematoxylin solution for re-staining. At last, the sections were dehydrated and sealed for ultimate histologic evaluation.

4.8. Western blot analysis

The lung tissue of mice was weighed, lysed and extracted by RIPA lysis buffer. In short, the protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to the polyvinylidene fluoride (PVDF) membrane. Next, cells were blocked with 5% BSA and incubated with the primary antibodies including TLR4 (1:200), MyD88 (1:1000), NF-κB (1:200), TNF-α (1:200), IL-1β (1:1000), IL-6 (1:1000) and β-actin (1:500 dilution, loading control) at 4 °C for overnight. Subsequently, the membrane was incubated with the peroxidase-conjugated secondary antibody with 1:3500 dilution at room temperature for 120 min. Finally, the visualization was developed by ECL-Plus reagent and analyzed by Image-Pro Plus 6.0 software.

4.9. Quantitative real-time RT-PCR

Total RNA was extracted from the lung tissue using TRIzol reagent, reverse-transcribed to cDNA with First chain cDNA Synthesis Kit and GAPDH was used as the reference. Primers were provided by GENEWIZ (Suzhou, China) and designed by NCBI software. The primer sequences were as follows:

MyD88: F, 5'-CGAGCTAATTGAGAAAAGGTGTCG-3';
R, 5'-GGATACTGGGAAAGTCCTTCTTCAT-3';
NF-κB: F, 5'-AAAGCCAGCTTCCGTGTTTG-3';
R, 5'-TGGCGTTTCCTTTGCACTTC-3';
TLR4: F, 5'-TTGGTGTGCTGTTGTCCTTG-3';
R, 5'-TGGTGTCAAGCAACCAAGTG-3';
GAPDH: F, 5'-TTCAACGGCACAGTCAAGG-3';
R, 5'-TGTTAGTGGGGTCTCGCTCC-3';

The PCR method was performed as follows: 95 °C for 5 min, 95 °C for 15 s, 60 °C for 20 s, 72 °C for 40 s for 40 cycles, 95 °C for 1 cycle 15 s and 60 °C for 1 min and 95 °C for 15 s. After the PCR reaction, quantitative real-time PCR analysis was done using the software.

4.10. Statistical analysis

All data were expressed as mean±standard deviation (SD) in this study, all experiments were repeated for three times. One-way ANOVA was used to evaluate differences between groups, followed by Dennett's post hoc test (SPSS 22.0 for Windows, SPSS Inc., USA). Data with $p < 0.05$ were considered statistically significant.

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Conflicts of interest: None declared.

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