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Houttuynia cordata Thunb. polysaccharides ameliorates lipopolysaccharide-induced acute lung injury in mice

Yan-Yan Xua, Yun-Yi Zhanga, Ying-Ye Ou a, Xiao-Xiao Lu a, Ling-Yu Pan a, Hong Li a, Yan Lub, Dao-Feng Chenb, c

a Department of Pharmacognosy, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai, China
b Department of Pharmacognosy, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai, China

ABSTRACT

Ethnopharmacological relevance: Houttuynia cordata (HC) has been used as a folk therapy to treat pulmonary infections. This study aimed to determine the role and mechanism of action of polysaccharides isolated from HC (HCP) in lipopolysaccharide (LPS)-induced ALI in the mice.

Materials and methods: LPS was delivered by the intratracheal route to Balb/c mice 2 h before HCP (40, 80 and 160 mg/kg) administration.

Results: The number of total cells, protein and tumor necrosis factor-α (TNF-α) concentrations in bronchoalveolar lavage fluid, the wet/dry weight ratio (w/d) of lungs and pulmonary pathology of each mouse were analyzed, it was found that HCP significantly alleviated ALI induced by LPS. Moreover, in lungs of mice, it was found that the infiltration of inflammatory cells, the expression of Toll-like receptor 4 and complement deposition were significantly decreased by HCP treatment. In vitro assays showed that C5a, a complement activation product, induced significant macrophage migration and treatment with HCP prevented it. The in vitro results also proved that LPS increased nitric oxide and pro-inflammatory cytokines (TNF-α, interleukin-6, and interleukin-1β) production, and HCP antagonized these effects of LPS. It was also found that HCP alone augmented secretion of some pro-inflammatory cytokines.

Conclusion: These results indicate that HCP may alleviate LPS induced lung inflammatory injury, which may be associated with its inhibitory effect on the over activation of complement and macrophages. This suggests a potential role to treat ALI.

1. Introduction

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), characterized by acute lung inflammation and edema, account for high rates of morbidity and mortality in humans (Fulkerson et al., 1996; McMullen et al., 2012). Some reports showed that complement activation, which can cause inflammatory cells recruitment to the alveolar-capillary membrane, has been implicated in the pathogenesis of ALI (Li et al., 2012; Robbins et al., 1987). As one of the complement activation products, C5a is a strong chemoattractant that increases the infiltration of activated neutrophils and macrophages into the pulmonary vasculature, lung interstitium, and alveolar space, resulting in immune response and disruption of the alveolar epithelial barrier (Flierl et al., 2008; Guo and Ward, 2005). Intensive investigations are still needed to elucidate the pathogenic mechanism of ALI, which remains obscure and is generally believed to be associated with over-activation of complement system, the recruitment of leukocyte, and inflammation mediated by inflammatory mediators including nitric oxide (NO), TNF-α, interleukin (IL)-1β, IL-6 (Naito et al., 2013; Tsai et al., 2014; Xie et al., 2012).

Houttuynia cordata Thunb. (HC, Saururaceae family) is a perennial herb and time-honored traditional Chinese medicine (TCM). It has a thin stalk and heart-shaped leaf, and reaches an average length of 15–50 cm. It is affluently distributed in the central, southeast and southwest regions of China and is harvested in summer or autumn (Yang and Jiang, 2009; Meng et al., 2009). HC...
possessed a large range of pharmacological activities, such as antiviral, anti-oxidative effects (Hayashi et al., 1995). During severe acute respiratory syndrome (SARS) outbreak in late 2002 to mid-2003, a TCM formula was proposed to general public as a preventive measure by the State Administration of TCM of China. HC 2003, a TCM formula was proposed to general public as a preventive measure by the State Administration of TCM of China. HC was one of the six ingredients in this formula, owing to its abilities to diminish inflammation (Lau et al., 2008; Zhang and Chen, 2008). Some reports showed that polysaccharides from plants possessed the activities of modulating host responses and improving immunity (Graff et al., 2009; Holderness et al., 2011; Lee and Hong, 2011). It is reasonable to presume that polysaccharides extracted from HC may exhibit the inhibitory effects against ALI.

To assure our hypothesis and further make clear the possible mechanisms, LPS was used, a major cell wall component of bacteria and powerful initiators of inflammatory reaction to induce the model in mice. The model established through intratracheal instillation of LPS to mice provides a good experimental model of ALI/ARDS to mimic the in vivo model in mice. The model established through intratracheal instillation of LPS to mice was one of the six ingredients in this formula, owing to its abilities to diminish inflammation (Lau et al., 2008; Zhang and Chen, 2008). Some reports showed that polysaccharides from plants possessed the activities of modulating host responses and improving immunity (Graff et al., 2009; Holderness et al., 2011; Lee and Hong, 2011). It is reasonable to presume that polysaccharides extracted from HC may exhibit the inhibitory effects against ALI.

2. Materials and methods

2.1. Reagents

LPS (Escherichia coli 055:B5) and Polymyxin B (PB) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Dexamethasone Sodium Phosphate Injection (DEX, No. 1303040, used as the positive control in LPS-induced ALI model) was purchased from Chongqing Laimei Pharmaceutical Co. Ltd. (Chongqing, China). Cell culture medium (RPMI-1640) and endotoxin-free fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Dexamethasone (DEX, No.D4902, used in cultured peritoneal macrophages) was purchased from Sigma. Sterile sheep blood was purchased from Shunwei Biotechnology Co. Ltd. (Shanghai, China). Recombinant Mouse Complement Component C5a was purchased from R&D systems (Minneapolis, MN, USA). Mouse TNF-α, IL-6, IL-1β ELISA kits were purchased from Boatman Biotechnology Co. Ltd. (Shanghai, China). The enhanced BCA Protein Assay Kit (No. P0009) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Animals and ethics statement

Male BALB/c mice (5–8 weeks) were obtained from Slaccas-Shanghai Lab Animal Ltd. (SPF II Certificate; No. SCXK2012-0002) and housed in collective cages under a 12 h light/dark cycle, with free access to food and water.

All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health. All the study protocols were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University (approved identification: 2013-50). All the surgeries were performed under urethane anesthesia, and all efforts were made to minimize suffering.

2.3. Plant materials

The dried whole plants of HC were purchased from Huayu Materia Medica Co., Ltd. (Shanghai, China). Verification of identity was carried out by Dr. Yan Lu (Fudan University, Shanghai, China), and the voucher specimen (DFC-YXC-2006072601) was deposited at the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, PR China.

2.4. Preparation of HCP

The isolation and chromatographic studies of polysaccharides were accomplished similar as previously described by Cheng et al. (2010). The dried whole plants (1 kg) of HC were ground in a high-speed rotary cutting mill into fine particles and defatted with 15 L 95% ethanol for 24 h. The residue was dried in air and it weighed about 913 g, and was extracted in 27.39 L (almost 30 times residue weight) hot water in 90 °C. The extraction process was repeated 3 times and almost 75 L extracting solution was collected. The hot water extract was concentrated to 7.6 L and then 40.5 L 95% ethanol was added. After 24 h, it was centrifuged and the deposition was redissolved in 1.5 L water. The proteins existed in water solution were precipitated by adding 7% trichloroacetic acid at 4 °C. Then the supernatant was neutralized and was dialyzed in the running water for 3 days. The polysaccharides (15.3 g) were made as the supernatant was concentrated and then lyophilized for the following experiments.

HCP was a dark-brown powder and was soluble in water. The total carbohydrate and uronic acid contents were determined by the phenol-sulfuric acid and m-hydroxybiphenyl methods respectively, using d-galactose and d-galacturonic acid as respective standards. HCP comprises 77.21% of total carbohydrate and 36.69% of uronic acid. Furthermore, the total protein content was measured using Coomassie brilliant blue staining and the protein content in HCP was 6.17%. The monosaccharide composition of HCP was determined by gas chromatographic analysis, and HCP is mainly composed of Glc, Gal, Ara and Rha in the ratio of 3.40:2.14:1.17:1.00, along with trace of Man and Xyl. High-performance gel permeation chromatography (HPGPC) analysis showed that HCP contains one major polysaccharide with several minor ones (Fig. 1).

The content of LPS in HCP was 5.5 ng/100 μg (55 ppm), assayed by the limulus amebocyte lysate reagent method, which has been widely used (Chang and Sack, 2001; Ryder et al., 1976). To examine the anti-inflammatory effects of HCP (1, 10, and 100 μg/ml) in some of the following experiments, PB, a well-characterized LPS inhibitor (Jacobs and Morrison, 1977), was selected to pretreat with HCP at 37 °C for 24 h to eliminate the possibility that the contamination of endotoxin in HCP may affect the experiment results (Wu et al., 2013).

2.5. In vivo treatment

2.5.1. ALI model establishment

The mice were randomly assigned to six groups (n = 8): Sham-operated animals group (sham group, n = 9), LPS alone group (model group), LPS + HCP treated group (HCP 40 mg/kg group, HCP 80 mg/kg group, HCP 160 mg/kg group) and LPS + DEX (4 mg/kg) treated group (DEX group). The doses of these drugs and LPS were based on our previous studies and preliminary experiments.

HCP was dissolved in normal saline (NS) and administered orally by gavage (p.o.). The model of ALI was employed as previously described method by Li et al. (2012). Mice were anesthetized with 20% urethane before intratracheal instillation (i.t.) of NS (sham
group, i.t.) or LPS (E. coli O55:B5, 3 mg/kg i.t.). HCP was administered intragastrically (i.g.) 2 h after LPS instillation. DEX at 4 mg/kg was given via tail vein (i.v.) 2 h after LPS exposure as a positive control.

2.5.2. Measurement of cell counts, TNF-α, and protein concentration in BALF

Twenty-four hour after LPS exposure, mice were euthanized. The right lungs were ligated, and the bronchoalveolar lavage fluid (BALF) was obtained on the left lung using 0.5 ml normal saline four times. After centrifugation (4 °C, 1000g, 10 min), the cell-free supernatants were harvested and stored at –80 °C for subsequent analysis, and the cell pellets were re-suspended in 200 μl NS for cell counts using a hemocytometer as previously described by Xie et al. (2012). The levels of TNF-α and protein in BALF were measured by mouse TNF-α ELISA kit and BCA protein assay kit respectively according to the manufacturer’s instructions.

2.5.3. Lung w/d ratio

To evaluate the severity of tissue edema, the lung w/d was calculated. After euthanasia of mice, the inferior lobe of right lungs were excised and measured immediately to obtain the “wet weight”, and then dried in an oven to constant weight at 50 °C for 72 h and weighed again to obtain the “dry weight”.

2.5.4. Hematoxylin and eosin (H&E) staining and immunohistochemistry

The superior lobe of right lung was removed and fixed immediately in 4% formaldehyde in 0.01 M phosphate buffer (pH 7.4) and used for histological evaluation. The tissue fixed in formalin was dehydrated and embedded in paraffin and sections (5 μm) were mounted on glass slide and stained with H&E to evaluate the severity of pneumonia.

Immunohistochemical staining was performed as previously described by Chiu et al. (2004) and Jiang et al. (2012). High-temperature antigen retrieval was performed by immersion of the deparaffinized slides in 10 mM trisodium citrate buffer (pH 6.0) in a water bath at 99 °C for 20 min. After quenching of endogenous peroxidase with 3% hydrogen peroxide in methanol (v/v) for 15 min, the non-specific binding sites of the sections were blocked with 5% BSA. The sections were then incubated with rabbit anti-C3d antibody (1:300) or murine monoclonal anti-TLR4 antibody (1:100, Abcam) at 4 °C overnight in a humidified chamber, and followed by incubation at 37 °C with HRP-conjugated goat anti-rabbit or anti-mouse IgG antibody for 30 min.

Immunohistochemical staining of polymorphonuclear leukocytes (PMNs) and macrophages were performed in accordance with the manufacturer’s instructions. Deparaffinized sections were treated with 20 μg/ml of proteinase K for 15 min at 37 °C before antigen retrieval. After quenching of endogenous peroxidase and then blocking for 30 min with normal rabbit serum (Vector), the sections were incubated at 4 °C overnight in a humidified chamber with rat monoclonal Ly-6C/Ly-6C (Gr-1) (1:100, clone: RB6-8C5; BioLegend) to PMNs or rat monoclonal F4/80 antibody (1:100, clone: CI:A3-1; AbD Serotec) to lung macrophages. The next day, the sections were incubated for 30 min at 37 °C with a rabbit anti-rat secondary antibody labeled with biotin and then with HRP-conjugated ABC Reagent (VECTASTAIN® Elite ABC-Peroxidase Kit, Cat. No. PK-6104, Vector Laboratories, Burlingame, CA).

Slices were visualized using chromogenic substrate solution DAB and counterstained with hematoxylin, and then observed under a microscope.

2.6. In vitro effect of HCP

2.6.1. Isolation of peritoneal macrophages

Male BALB/c mice were intraperitoneally injected with 1 ml of 5% sodium thioglycollate medium. Mice were sacrificed 4 days after injection and the peritoneal cavities were flushed with 5 ml of chilled RPMI 1640 medium and the peritoneal lavage fluids were centrifuged at 80g for 10 min at 4 °C to isolate peritoneal macrophages (Cheng et al., 2010; Wu et al., 2013).

All the procedures described here were carried out in aseptic conditions, and the materials were previously sterilized and pyrogen-free.

2.6.2. Complementary activity through the classical pathway

The in vitro anti-complement activity of the HCP was examined according to a previously described method by Xie et al. (2012). To prepare sensitized erythrocytes (EAs), the 2% sheep erythrocytes were pre-incubated with rabbit anti-sheep erythrocyte antibody (1:1000) in equal volumes. The samples were diluted in isotonic veronal-buffered saline (VBS2−) (containing 0.5 mM Mg2+ and 0.15 mM Ca2+). Guinea pig serum was used as the source of complement described by Xie et al. (2012). According to our preliminary experiment, the 1:60 diluted Guinea pig serum was able to give submaximal lysis in the absence of complement inhibitors, and was chosen as the complement source. As a positive control, Suramin (300 μg/ml) was able to inhibit 100% of the lysis.

The tested samples (200 μl) were mixed with 200 μl of Guinea pig serum, and then, 200 μl of EAs was added. The mixture was incubated at 37 °C for 30 min. The different assay controls were incubated in the same conditions: (1) vehicle control: 200 μl EAs in 400 μl VBS2−; (2) 100% lysis: 200 μl EAs in 400 μl ultrapure water; (3) 0% lysis: 100 μl 2% non-sensitized sheep erythrocytes and 200 μl Guinea pig serum in 300 μl VBS2−; and (4) samples background: 200 μl of each dilution of HCP sample in 400 μl VBS2−. The mixtures were centrifuged (1000g, 10 min) immediately at 4 °C after incubation, and the optical density of the supernatant was measured at 405 nm on a well scanner (Labsystems Dragon). Results were
indicated in percentage of hemolytic inhibition. Inhibition of lysis (%) = 100−100× (ODsample−ODbackground)/OD0% lysis−ODbackground lysis.  

2.6.3. Chemotaxis assay

After centrifugation, the macrophages were resuspended with RPMI 1640 medium containing 0.1% BSA. Cell migration was assessed using a 24-well 8-mm pore chemotaxis chamber (Chiou et al., 2004) (Transwell, Corning Costar). The cells (1 × 10^5 cells/ chamber) were plated in the upper chamber of Transwell inserts (Corning) while C5a (50 ng/ml) was added in the lower compartment as a stimulus without or with HCP (1, 10, and 100 μg/ml).

After incubation for 4 h at 37 °C in a humidified atmosphere with 5% CO₂, the inserts were removed, and the inner surface was rinsed and wiped with a little lump of cotton to remove non-migrated cells. The cells on the outside of the inserts were formalin-fixed and then stained using crystal violet. The inserts were observed under a microscope. Cells in each insert were counted in 10 randomly selected fields at ×400 magnification to evaluate chemotaxis. Chemotaxis index (CI) = cells of sample/cells of control × 100%. The inhibition ratio of cell migration (IC50) = (cells of model group−cells of sample group)/cells of model group. The experiments were performed in triplicate. Spontaneous migration in 0.1% BSA−RPMI 1640 served as control and was designed as a basic migration for each experiment, and complement 5a-induced cell migration in 0.1% BSA−RPMI 1640 acted as model group.

2.6.4. Secretion of macrophages

The cells were resuspended with RPMI 1640 medium containing 10% heat-inactivated FBS and cultured in 96-well plates (1 × 10^6 cells/well). After incubation for 2 h at 37 °C in a humidified atmosphere with 5% CO₂, the cells were washed two times to remove the non-adherent cells. Cells (1 × 10^6 cells/ml) were incubated with or without LPS (1 μg/ml), while HCP was added in at the same time, for 24 h at 37 °C in a humidified atmosphere with 5% CO₂, and then, the supernatant of the culture media was collected to evaluate the levels of TNF-α, IL-6, IL-1β and IL-10 using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instruction.

The content of nitric oxide in the culture media was measured according to the colorimetric method described previously by Bakker et al. (2009), Joh and Kim (2010), using Griess reagent. The culture medium (60 μl) was combined with an equal volume of Griess reagent [mixture of equal volume of 1% sulfanilamide in 5% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H₂O] in a 96-well flat-bottom microtiter plate and incubated for 10 min at 37 °C. Then the optical densities were measured at 540 nm using a spectrophotometer and the concentration of nitrite was determined by a standard curve.

2.7. Statistical analysis

Data were expressed as means ± standard deviation (S.D.). One-way ANOVA was performed for multiple group comparisons. If data were found to have significant changes, post-hoc comparisons were performed using Fisher’s PLSD. P < 0.05 was considered to be statistically significant.

3. Results

3.1. In vivo HCP treatment

3.1.1. Effect of HCP on histopathological changes in lungs of ALI mice

Lung tissues from ALI model group exhibited widespread pulmonary alveolar wall thickness accompanied by edema, obvious infiltration of inflammatory cells, and severe hemorrhage in the alveolus. While in sham group, the lungs showed a normal structure and these lesions were not apparent. Treatment with HCP or DEX obviously ameliorated these injuries (Fig. 2A).

3.1.2. HCP inhibits total cell counts and TNF-α production in BALF of ALI mice

Twenty-four hour after LPS administration, total cells and TNF-α production in BALF were assessed. Dramatic increases were observed in total cells and TNF-α production after LPS instillation compared to those of sham group (P < 0.001, as shown in Fig. 2B and C).

Compared with vehicle treated ALI group, HCP (40, 80, and 160 mg/kg) and DEX (4 mg/kg) treated ALI group exhibited decreased total cells (P < 0.05) and TNF-α concentration (P < 0.01) in BALF of mice.

3.1.3. HCP suppresses protein concentration in BALF of ALI mice

The concentration of protein in BALF supernatant was analyzed. As shown in Fig. 2D, compared with sham group, the levels of protein were significantly elevated (P < 0.001) in model group. Treatments with HCP (40, 80, and 160 mg/kg) or DEX (4 mg/kg) markedly reduced the concentration of protein as compared with model group (P < 0.05).

3.1.4. HCP Prevents the lung edema in ALI mice

The w/d were significantly elevated in vehicle treated model group compared with sham group (Fig. 2E). By comparison, such elevations were blunted by HCP (80 and 160 mg/kg) and DEX treatment (P < 0.05)

3.1.5. HCP reduces complement deposition

To determine whether HCP might affect ALI by reducing complement deposition in the lungs of mice, the lung tissue sections were stained to examine the presence of C3d. In agreement with our previous studies (Xie et al., 2012), immunohistochemistry showed very low levels of staining for complement deposition in the lungs of sham group (Fig. 3A). The model group exhibited intense immunostaining of C3d in lung tissue. Treatments with HCP (40, 80, and 160 mg/kg) and DEX (4 mg/kg) after instillation of LPS significantly inhibited C3d deposition in lung tissue.

3.1.6. HCP inhibits TLR4 expression

The effect of HCP on TLR4 expression in lungs of mice was evaluated. The results showed intense immunostaining for TLR4 expression in lung tissue of model group (Fig. 3B). Compared with model group, HCP (40, 80 and 160 mg/kg) and DEX treated ALI group exhibited relatively low levels of staining in lung tissue.

3.1.7. HCP suppresses PMNs and macrophages infiltration in lungs of mice

The effect of HCP on attenuating the infiltration of PMNs and macrophages in lung tissue of mice was evaluated. The results showed intense immunostaining for PMNs (Fig. 3C) and macrophages (Fig. 3D) infiltration in lungs of model group. Compared with model group, HCP (40, 80 and 160 mg/kg) and DEX treated ALI group exhibited relatively low levels of staining in lung tissue.

3.2. In vitro HCP treatment

3.2.1. HCP inhibits the activation of complement

The anti-complementary activities of HCP were evaluated and the polysaccharides significantly inhibited the hemolysis of sensitized sheep erythrocytes when incubated with Guinea pig serum (IC50 = 0.348 ± 0.012 mg/ml) (As shown in Fig. 4).
3.2.2. HCP prevents C5a induced chemotaxis on macrophage migration

The chemotactic effect on peritoneal macrophages was performed to evaluate whether HCP could inhibit macrophage migration. As shown in Fig. 5A, macrophages without C5a displayed no chemotaxis and low random migration (8.23 ± 1.26...
C5a (50 ng/ml) induced significant migration compared with spontaneous mobility (60.4 ± 5.1 cells/field, CI=739.10 ± 55.13%) as shown in Fig. 5B. Treatment with HCP (1, 10, and 100 μg/ml) caused a concentration-related inhibition of C5a-induced cell migration (by 28.5 ± 12.9%, 31.2 ± 8.9%, and 45.5 ± 9.8%).

3.2.3. HCP suppresses NO and pro-inflammatory cytokines release from LPS-stimulated macrophages

To clarify whether HCP could lessen the production of pro-inflammatory mediators, which have been demonstrated to be associated with the lung injury in ALI (Chua et al., 2007), the peritoneal macrophages were isolated and cultured. The influences of HCP on iNOS/NO, TNF-α, IL-6, IL-1β, and IL-10 production were also evaluated. As shown in Fig. 6, stimulation with LPS (1 μg/ml) significantly augmented the levels of NO, TNF-α, IL-6 and IL-1β (P < 0.001; Fig. 6). In contrast, HCP (10, 100 μg/ml) and DEX (10 μM) markedly suppressed levels of those inflammatory mediators after LPS administration (P < 0.001; Fig. 6A–D). The level of IL-10, an anti-inflammatory cytokine, was greatly elevated by LPS (P < 0.001; Fig. 6E). HCP (1, 10, and 100 μg/ml) further promoted the IL-10 production in LPS-stimulated cells (P < 0.001).

The production of NO and cytokines in macrophages treated with HCP (1, 10, and 100 μg/ml) alone was investigated to determine whether HCP itself can affect the immunologic functions of macrophages. Substantial increases in NO, TNF-α, IL-6, IL-1β, and IL-10 were observed (P < 0.05) in the HCP (100 μg/ml) treatment group (Fig. 7 A–E).

To exclude the possibility that the results above were due to LPS contamination in the drug, HCP was pretreated with PB (30 μg/ml), a LPS inhibitor, at 37 °C for 24 h before being added to the cell cultures. As seen in Fig. 7, significantly lower levels of NO, IL-6, IL-1β, and IL-10 were observed (P < 0.05) in the HCP (100 μg/m) treatment group, compared with the HCP without PB group (P < 0.05).

4. Discussion and conclusion

4.1. Discussion

HC is used as a folk therapy against influenza, asthma, allergy,
cancer (Yang and Jiang, 2009), and also had the anti-inflammatory activity (Lee et al., 2013). In China, the traditional medicines are mostly prepared in a form of apozem. At present, it is becoming an increasing fascinating approach to use polysaccharides to treat various inflammatory diseases because polysaccharides are major components in the decoction and have remarkable therapeutic effects and relatively low toxicity (Cheng et al., 2010; Jiang et al., 2012).

In the present study, intratracheal instillation of LPS was used to produce the ALI model in mice to mimic the inflammatory response in patients (Xie et al., 2012). Instillation of 3 mg/kg LPS into the trachea of mice activated inflammatory cascades within two hours, causing lung injury characterized by accumulation of leukocyte, proteinaceous alveolar exudates, pulmonary edema and hemorrhagic spots.

The results of this study demonstrated the effectiveness of the isolated natural HCP in ameliorating experimental lung injury in mice. HCP, given orally, consistently ameliorated LPS-induced lung injury, with a direct reduction in lung w/d, total cell counts, inflammatory cells infiltration, protein and TNF-α concentration in BALF. Of interest, it was observed that HCP produced a marked decrease in TLR4 expression as seen in lung tissue. In addition, the present research clearly showed that HCP treatment consistently suppressed activation of complement both in vivo and in vitro.

In healthy individuals, the complement system defends the body against microbial pathogens as an effective innate immune mechanism of host. However, over activation of complement has also been involved in many forms of acute inflammatory, including ALI (Linton and Morgan, 1999). As a kind of complement protein split product, C5a exerts a broad spectrum of functions. C5a appears to contribute to promoting and perpetuating inflammatory reactions. It is also a potent chemoattractant and is implicated in the recruitment of many inflammatory cells like neutrophils and macrophages (Schmid et al., 1997). In our experiment, pre-incubation of HCP inhibited the hemolysis of sensitized sheep erythrocytes when complement was present. Besides, complement deposition was found in LPS-mediated ALI and relevantly treatment with HCP prevented the deposition. These effects indicate that HCP inhibit the activation of complement system in model mice, consequently, suppress the release of various protein split products including C5a, which may probably contribute to the heavy infiltration of macrophages and neutrophils in the lung.

Leukocyte infiltration is a hallmark of severe lung injury. According to Silva (2010), in the early stage of the lung injury, macrophages detect the pathogens and then recruit monocytes, which mature into inflammatory macrophages and neutrophils. However, the redundant recruitment could lead to the aggregation of macrophages and neutrophils in lungs of ALI mice. As discussed earlier, C5a is one of the most inflammatory peptides among the complement activation products, and has chemoattractant activity for monocytes and macrophages. In the experiments, the peritoneal macrophages were cultured and the chemoattractant effects in the presence of HCP were investigated. Results showed that C5a induced significant migration and treatment with HCP prevented it, which indicates that HCP could directly inhibit the migration, consequently, might decrease the influx of inflammatory cells in lungs of model mice, leading to the palliation of inflammation.

Alveolar macrophages, a large mononuclear phagocytes located in the alveolar compartment of the lungs, play a critical role to defend the lungs against inflammatory conditions by producing and releasing a numerous of inflammatory mediators (Niesler et al., 2014; Zhao et al., 2006), including cytokines, chemokines, and complement factors. A pro-inflammatory micromilieu can be caused by these mediators (Sharma et al., 2007) which may function as chemoattractants for invading cells or stimulate local cells (Seitz et al., 2010). In addition to suppression of macrophage migration, the beneficial action of HCP in ameliorating ALI in mice is also likely associated with its ability to prevent the over-production of pro-inflammatory mediators in macrophages. High levels of inflammatory mediators such as TNF-α generated in pathophysiological conditions lead to the development of inflammatory responses and cytotoxicity, which may have direct role in mediating tissue damage (Giebel et al., 2007). The present study demonstrated that levels of NO, TNF-α, IL-1β and IL-6 from LPS-stimulated macrophages were clearly suppressed in the presence of HCP in vitro, as was consistent with the reduction in BALF TNF-α in LPS-induced ALI mice after HCP treatment. Interestingly, in vitro treatment with HCP markedly elevated IL-10 secretion, a relevant anti-inflammatory cytokine, in the macrophage culture supernatant after LPS administration.

The present study demonstrated that, HCP alone stimulated mild expression of inflammatory cytokines, including NO, TNF-α, IL-1β and IL-6. However, polysaccharides extracted from natural plant sources such as HC can be easily contaminated by LPS during the isolation process, which may interfere with biological evaluation studies (de Santana et al., 2012). To further investigate whether HCP-induced cytokines expression could be just a consequence of LPS contamination in polysaccharides, the critical experiments were repeated in the presence of PB, a drug that can bind to the lipid A portion of LPS to exclude cell activation by LPS (Coyne and Fenwick, 1993; Stokes et al., 1989). According to our previous study, 30 μg/ml PB is sufficient to inhibit up to 10 ng/ml LPS, and has no effect on cell viability. This study showed that treatment with PB suppressed but did not completely abolis HCP-

![Fig. 5. Effects of HCP on peritoneal macrophages migration in response to C5a.](image-url)
induced secretion of these inflammatory mediators. It is reasonable to suggest that it is HCP but not contaminated LPS in HCP that functions to modulate the secretion of macrophages. Taking the results above into account, HCP decreases the LPS-induced excessive production of NO and cytokines in inflammatory condition but enhances the immunologic functions in physiologic level. This phenomenon demonstrates that HCP may have biphasic actions.

As a pattern recognition receptor that responds to LPS, TLR4 is up-regulated in many inflammatory diseases induced by LPS (Deng et al., 2013). This study showed that HCP strikingly reduced the expression of TLR4 in lung tissue. This result demonstrated that HCP might inhibit the interaction between LPS and its receptor-TLR4 to restrict the inflammatory process in the lung.

4.2. Conclusion

HCP attenuated LPS-induced lung injury, as evidenced by an inhibition of lung w/d, protein level, TNF-α concentration, and inflammatory cells aggregation in BALF. In vivo, HCP decreased the expression of TLR4 and the deposition of complement in the lungs of ALI mice, and in vitro, HCP attenuated C5a-induced chemotaxis and LPS-induced secretion of inflammatory mediators on macrophages. These results suggested that the mechanism of HCP seems to be multifactorial to reduce inflammatory responses in lung. And the most important factor was probably associated with the inhibition of the over activation of complement and macrophages.
Con

Conflict of interest

The authors have declared that no competing interests exist.

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Fig. 7. Effects of HCP on NO and cytokines secretion. HCP was pretreated without (blue line) or with (red line) PB, and then added to the cell cultures. The culture media was assayed for (A) NO, (B) TNF-α, (C) IL-6, (D) IL-1β, and (E) IL-10. Data was presented as means ± S.D. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 compared with corresponding control (Con) group; #P < 0.05, ###P < 0.001 compared between groups with and without PB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
