Inflammation, Vol. 42, No. 5, October 2019 (© 2019)
DOI: 10.1007/s10753-019-01047-5

ORIGINAL ARTICLE

Pseudoginsenoside-F11 Attenuates Lipopolysaccharide-Induced Acute Lung Injury by Suppressing Neutrophil Infiltration and Accelerating Neutrophil Clearance

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Abstract— Pseudoginsenoside-F11 (PF11), an ocotillol-type saponin, has been reported to have anti-inflammatory properties, but the effects of PF11 on acute lung inflammation were unknown. The present study aimed to investigate the protective effects and potential mechanisms of PF11 on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in male BALB/c mice. After being treated with PF11 (3, 10, and 30 mg/kg, intravenous) once a day for 3 consecutive days, the mice were challenged by intratracheal instillation of LPS, and then their lung tissues and bronchoalveolar lavage fluid (BALF) were collected for further analysis. The results showed that PF11 attenuated LPS-induced ALI, with alleviated histopathological damage, decreased lung wet/dry weight ratio, and reduced protein concentration and inflammatory cells number in BALF. Moreover, PF11 reversed the LPS-induced increases of mRNA expression and protein levels of interleukin-6, tumor necrosis factor-α, and interleukin-1β. Meanwhile, PF11 decreased LPS-induced myeloperoxidase activity and neutrophil infiltration in lung tissue by reducing the expression of macrophage inflammatory protein-2 and intercellular adhesion molecule-1, as well as enhanced neutrophil clearance by accelerating neutrophils apoptosis and their phagocytosis by alveolar macrophages. In conclusion, these results indicated that PF11 significantly attenuated LPS-induced ALI through suppressing neutrophil infiltration and accelerating neutrophil clearance, suggesting its potential in the treatment of ALI.

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KEY WORDS: pseudoginsenoside-F11; lipopolysaccharide; acute lung injury; neutrophil.

INTRODUCTION

Acute lung injury (ALI), especially its severe form acute respiratory distress syndrome (ARDS), is an inflammatory lung disease characterized by lung edema, endothelial and epithelial injury, and neutrophil infiltration [1, 2]. Although lung-protective ventilation and fluid-restrictive management have been used to treat ALI/ARDS, the current mortality is still higher than 40% [3]. Therefore, developing effective drugs or strategies to treat ALI is urgently necessary.

In response to the inflammatory response, neutrophils, as first innate immune cells, rapidly migrate to the site of inflammation [4, 5]. Excessive neutrophil infiltration and activation in the lungs have been identified as a pivotal event in the early stage of ALI [6, 7]. During this process, activated neutrophils impair the lung tissue by releasing multiple cytotoxic products, including proteinases, cytokines, reactive oxygen species, and cationic polypeptides [1]. Moreover, the life span of neutrophils is prolonged at inflammatory sites, resulting in delayed neutrophil apoptosis, prolonged release of cytotoxic products, and inflammatory response [8–10]. Therefore, enhancing neutrophil apoptosis and phagocytosis of apoptotic neutrophils by alveolar macrophages not only decreases the release of cytotoxic products and secondary lysis of apoptotic neutrophils but also induces the production of anti-inflammatory cytokines of macrophages [11, 12]. Based on the above, suppressing neutrophil infiltration and accelerating neutrophil clearance may be attractive therapeutic strategies for ALI.

Pseudoginsenoside-F11 (PF11), an ocotillol-type saponin, was isolated from the leaves of *Panax pseudoginseng* subsp. *Himalaicus* H_{ARA} (Himalayan Panax) [13]. Our previous studies have found that PF11 had wide-ranging neuroprotective properties, including its anti-neuroinflammatory and anti-oxidant effects in central nervous system [14–17]. Notably, *in vitro*, PF11 remarkably inhibited LPS-induced activation of microglial cells by inhibiting toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB) signaling pathway [18]. *In vivo*, PF11 significantly reduced the neuroinflammation response and the level of oxidative stress in the brain of D-galactose-induced cognitive impairment model mice by activating nuclear factor erythroid-related factor 2 (Nrf2)/anti-oxidative response elements (ARE) pathway and attenuating nod-like receptor protein 3 (NLRP3) inflammasome activation [19]. However, whether PF11 can inhibit neutrophilic inflammation in ALI remains unclear. Therefore, in the present study, we determined the effects of PF11 on LPS-induced ALI in mice and elucidated the potential mechanisms.

MATERIALS AND METHODS

Reagents

PF11 was provided by Department of Chemistry for Nature Products of Shenyang Pharmaceutical University (Shenyang, China) and was more than 98% pure as detected by HPLC. The chemical structure of PF11 is shown in Fig. 1. Lipopolysaccharide (LPS, from *Escherichia coli* 055:B5) was purchased from Sigma Co. Ltd. (St. Louis, USA). Dexamethasone (Dex) was obtained from Meilun Biotech Co., Ltd. (Dalian, China).

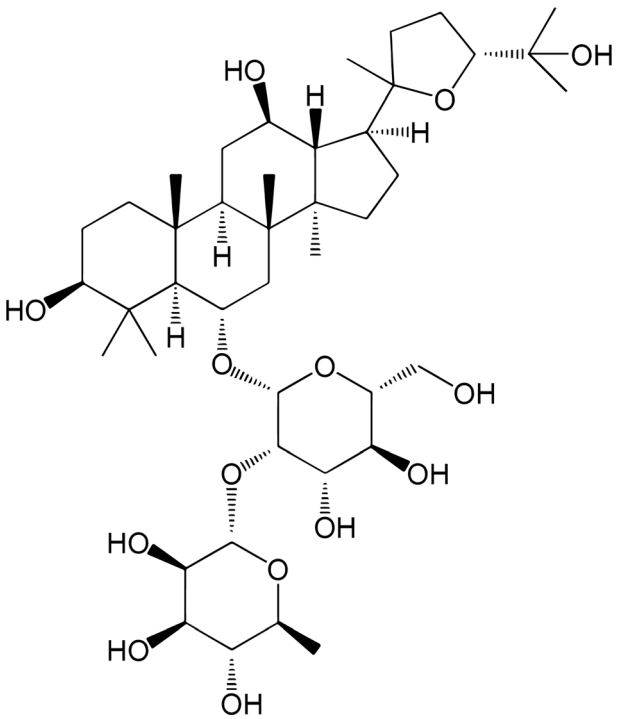

Fig. 1. Chemical structure of pseudoginsenoside-F11.

Animals

BALB/c mice (male, 8–10 weeks, weighing 18–21 g) were supplied by the Animal Centre of Shenyang Pharmaceutical University (Shenyang, China). Mice were housed in a temperature (21–24 °C), relative humidity (50–60%), and light (12-h dark-light cycle) controlled room and given standard chow and tap water *ad libitum*. All experimental procedures were approved by the Ethics Committee of Shenyang Pharmaceutical University, and followed the guidelines for the Care and Use of Laboratory Animals.

Experimental Procedures

ALI was induced in male BALB/c mice by intratracheal instillation of LPS, as previously described [20]. Briefly, mice were anesthetized by injecting pentobarbital sodium (50 mg/kg) intraperitoneally and then challenged intratracheally with either saline (50 μl) or LPS (3 mg/kg in 50 μl of saline). Mice were randomly classified into 7 groups ($n = 6$): control group, PF11 (30 mg/kg) group, LPS group, PF11 (3, 10, and 30 mg/kg) + LPS group, and Dex (1 mg/kg) + LPS group. Mice in PF11 (3, 10, and 30 mg/kg) + LPS group were intravenously administered PF11 once a day for 3 consecutive days while the mice in Dex + LPS group received Dex with the same way of PF11. PF11 (3, 10, and 30 mg/kg) and Dex were dissolved in saline. The last administration of PF11 or Dex was 0.5 h before LPS instillation. During this time, control group and LPS group were given saline. Six hours after LPS or saline administration, mice were euthanized, and then the bronchoalveolar lavage fluid (BALF) and lungs were collected.

Histological Analysis

For histological analysis, the left lung was isolated and fixed in 4% paraformaldehyde overnight at 4 °C followed by dehydration in ascending series of alcohol and then embedded in paraffin. Paraffin-embedded lung tissue was cut into 5-μm-thick slices, stained with hematoxylin and eosin, and then observed by light microscopy. Scoring of lung injury was done as previously described [21].

Lung W/D Weight Ratio

To assess the degree of tissue edema, the wet/dry (W/D) ratio was computed. The excised lungs were rinsed with saline, blotted, and the wet weight was recorded. They were then heated in a thermostatic incubator at 80 °C for 48 h to remove moisture, and subsequently, the dry weight was recorded and the W/D ratio was determined.

BALF Analysis

Six hours after LPS or saline challenge, mice were euthanized for collecting their BALF as previously described [22]. Briefly, BALF was obtained by cannulating the trachea with an intravenous infusion needle and flushing the lung three times with 0.5 ml ice-cold saline each time. The BALF samples were centrifuged (500×g at 4 °C) for 10 min, and the cell-free supernatants were maintained at − 80 °C for subsequent analysis. The pellets were resuspended in ice-cold PBS, and then, total cell count was carried out using a hemocytometer. Neutrophil count was performed on Wright-Giemsa stained smears. Protein concentration and levels of mouse macrophage inflammatory protein-2 (MIP-2), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) in BALF were quantified by BCA protein assay kit (Beyotime Institute of Biotechnology, China) and specific enzyme-linked immunosorbent assay (ELISA) kits (Boster Biological Technology Co., Ltd., China) respectively following standard protocols.

MPO Assay

Myeloperoxidase (MPO) activity, a clear index of neutrophil accumulation, was assayed using MPO test kits (Jiancheng Bioengineering Institute, China). Lung tissues were collected, weighed and homogenized in PBS, and subsequently processed following the manufacturer's instructions. Changes in optical density were detected at 460 nm. Data were expressed as units per gram of tissue.

Immunohistochemistry

Paraffin-embedded lung sections (thickness, 5 μm) were dewaxed with xylene, rehydrated in descending series of alcohol, and heated in sodium citrate buffer. Subsequently, the lung sections were incubated in 3% hydrogen peroxide, blocked with 5% bovine serum albumin, and incubated with anti-mouse Ly-6G antibody (1:100, eBioscience, USA) overnight at 4 °C. Then, horseradish peroxidase conjugated goat anti-rat IgG antibody was added for 30 min at 37 °C. Finally, the lung sections were stained with diaminobenzidine and then counterstained with hematoxylin.

RNA Isolation and Real-Time PCR

Total RNA was isolated from lung tissue with TRIzol (Life, USA), and then its purity was quantified by using a BioPhotometer plus (Eppendorf, Germany). cDNA was generated by using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Japan). Quantitative real-time

PCR analysis for IL-6 (specific primers: forward 5′-TCTATACCACTTCACAAGTCGGA-3′, reverse 5′-GAATTGCCATTGCACAACTCTTT-3′), TNF-α (specific primers: forward 5′-CCTGTAGCCCACGTCGTAG-3′, reverse 5′-GGGAGTAGACAAGGTACAACCC-3′), IL-1β (specific primers: forward 5′-GCAACTGTTCCTGAACTCAACT-3′, reverse 5′-ATCTTTTGGGGTCCGTCAACT-3′), intercellular adhesion molecule-1 (ICAM-1) (specific primers: forward 5′-AACAGAATGGTAGACAGCAT-3′, reverse 5′-TCCACCGAGTCCTCTTAG-3′), MIP-2 (specific primers: forward 5′-CCAAGGGTTGACTTCAAGAAC-3′, reverse 5′-AGCGAGGCACATCAGGTACG-3′), and β-actin (specific primers: forward 5′-GTGACGTTGACATCCGTAAAGACC-3′, reverse 5′-ATCTGCTGGAAGGTGGACAGTGAG-3′) were conducted using SYBR Premix Ex Taq™ (TaKaRa, Japan) on a BioRad CFX Connect™ Real-Time System (BioRad, USA). Relative gene expressions of IL-6, TNF-α, IL-1β, ICAM-1, and MIP-2 were calculated by the $2^{-\Delta\Delta CT}$ method, using the β-actin to normalize values.

Flow Cytometry Analysis

Neutrophil apoptosis assay was carried out as previously described [23, 24]. Briefly, BALF cells were collected and stained with PerCP-Cyanine5.5-conjugated anti-mouse CD11b antibody (0.25 μg/10^6 cells, eBioscience, USA) and APC-conjugated anti-mouse Ly-6G antibody (0.5 μg/10^6 cells, eBioscience, USA) at 4 °C for 0.5 h. Subsequently, cells were washed and labeled with FITC-conjugated anti-annexin-V antibody (KeyGen Biotech Co., Ltd., China) following manufacturer's instructions. Neutrophils were identified as $CD11b^+Ly6G^+$ populations and then $Ly6G^+$anti-annexin-V^+ populations were defined as apoptotic neutrophils. Phagocytosis assay was also carried out as previously described [24, 25]. Briefly, BALF cells were blocked with anti-mouse CD16/32 antibody (0.5 μg/10^6 cells, eBioscience, USA) on ice for 10 min, stained with FITC-conjugated anti-mouse F4/80 antibody (0.5 μg/10^6 cells, eBioscience, USA) at 4 °C for 0.5 h, and permeabilized with 0.1% Triton X-100, and then labeled with APC-conjugated anti-mouse Ly-6G antibody (0.5 μg/10^6 cells, eBioscience, USA) at 4 °C for 0.5 h. The population of macrophages containing neutrophils was identified as $F4/80^+Ly6G^+$ cells.

Statistical Analysis

Data were expressed as means ± standard errors and analyzed by using SPSS 17.0 software. Except for lung injury scores, data were analyzed through one-way analysis of variance followed by Tukey's post-hoc test. For lung injury scores, statistical analysis was assessed with Kruskal-Wallis test followed by the Mann-Whitney U test. $P < 0.05$ signified a statistical difference.

RESULTS

Effects of PF11 on Lung Histopathological Changes

Histological analysis revealed lung tissues sections from the control and PF11 alone group presented clear visible alveolar structure with scarce histological change (Fig. 2a, b), while those lung tissues sections from LPS group showed significant pathological alterations, including inflammatory cell infiltration, alveolar wall thickening, alveolar congestion and hemorrhage (Fig. 2c). Compared with the LPS group, these histopathological damage were effectively alleviated in PF11 (10 and 30 mg/kg) + LPS and Dex + LPS groups, but no significant alleviation in histopathological damage was observed in PF11 (3 mg/kg) + LPS group (Fig. 2d–g). Moreover, the LPS-induced increase in lung injury score was significantly decreased by PF11 (10 and 30 mg/kg) or Dex (Fig. 2h).

Effects of PF11 on Alveolar-Capillary Barrier

Effects of PF11 on alveolar-capillary barrier were inferred from lung W/D ratio and protein concentration in BALF after LPS administration. As shown in Fig. 2i and j, LPS-challenged mice showed higher W/D ratio and BALF protein concentration than those in controls. However, the LPS-induced increase in W/D ratio was significantly inhibited by PF11 (30 mg/kg) or Dex (Fig. 2i). Moreover, the LPS-induced increase in BALF protein concentration was significantly reduced by PF11 (10 and 30 mg/kg) or Dex (Fig. 2j). These results indicated that PF11 could effectively protect against LPS-induced alveolar-capillary barrier dysfunction.

Effects of PF11 on Cytokine Expression in the Lung

The anti-inflammatory effects of PF11 were determined by cytokines expression in lung after LPS administration. As shown in Fig. 3a–c, the mice received LPS administration showed higher protein levels of IL-6, TNF-α, and IL-1β than those in controls. In contrast, PF11 (10 and 30 mg/kg) or Dex significantly decreased the LPS-induced elevated protein levels of these inflammatory cytokines in BALF. Additionally, PF11 (30 mg/kg) or Dex also

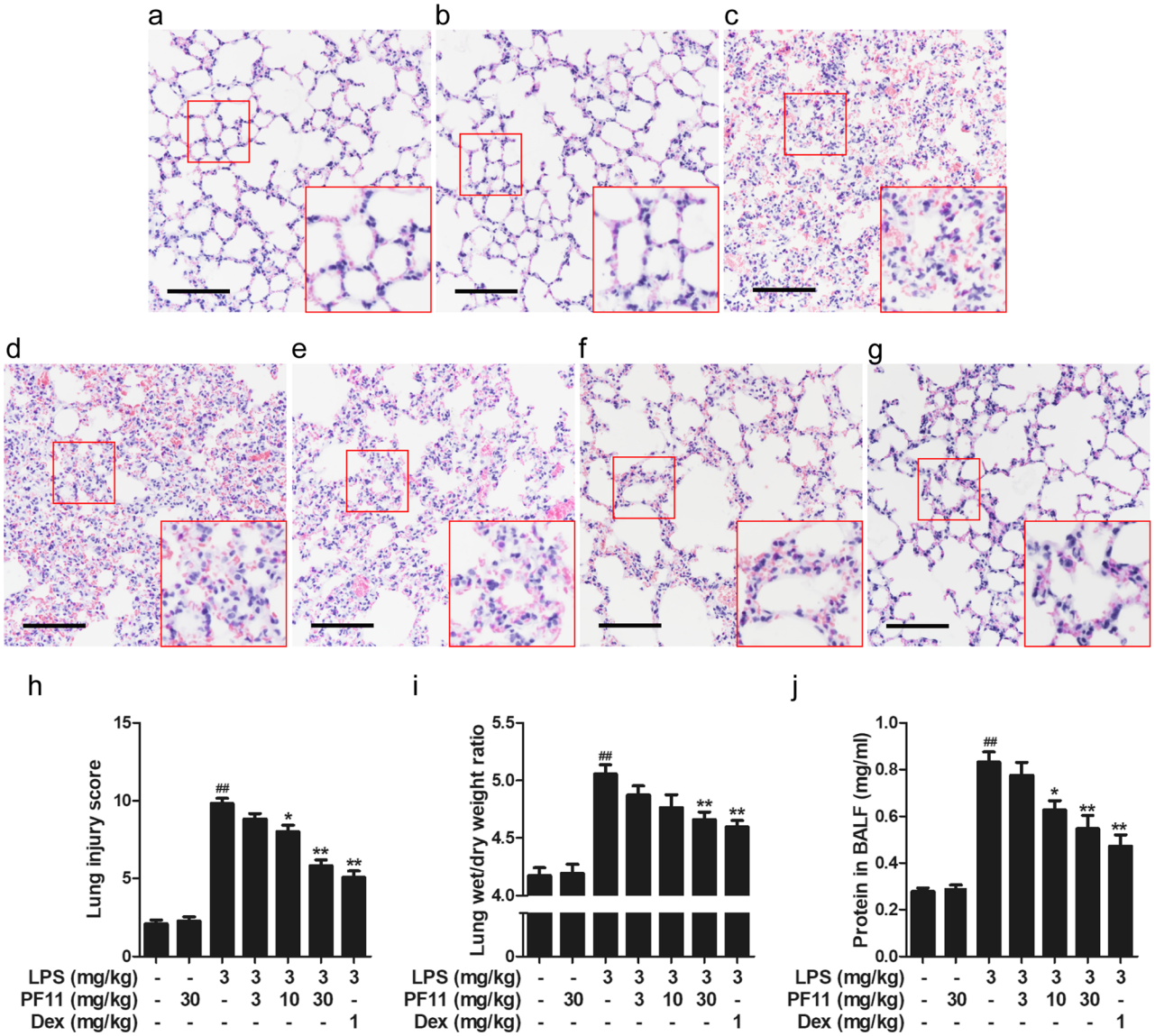

Fig. 2. Effects of pseudoginsenoside-F11 (PF11) on lipopolysaccharide (LPS)-induced histopathological changes and alveolar-capillary barrier dysfunction. Thirty minutes after PF11 or dexamethasone (Dex) pretreatment on day 3, mice were anesthetized, and then 3 mg/kg LPS was instilled intratracheally. Lung tissues were processed for histological evaluation at 6 h after LPS challenge. **a**–**g** Representative images of hematoxylin and eosin stained lung sections from seven experimental groups (magnification × 200, × 400, scale bar = 100 μm). **a** Control group. **b** PF11 30 mg/kg group. **c** LPS group. **d** PF11 3 mg/kg + LPS group. **e** PF11 10 mg/kg + LPS group. **f** PF11 30 mg/kg + LPS group. **g** Dex 1 mg/kg + LPS group. **h** Lung injury score. **i** Lung wet/dry weight ratio. **j** Bronchoalveolar lavage fluid protein concentration. Data are means ± standard errors ($n = 6$ per group). $^{\#\#}P < 0.01$ *versus* the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* LPS group.

significantly suppressed the LPS-induced upregulation of gene expression of these inflammatory cytokines in lung tissues (Fig. 3d–f). These results indicated that PF11 could effectively relieve LPS-induced inflammatory response.

Effects of PF11 on Neutrophil Infiltration and MPO Activity

Effects of PF11 on neutrophil infiltration were measured by Ly-6G^{+} cells and MPO activity in lung tissue and cell counts in BALF after LPS administration.

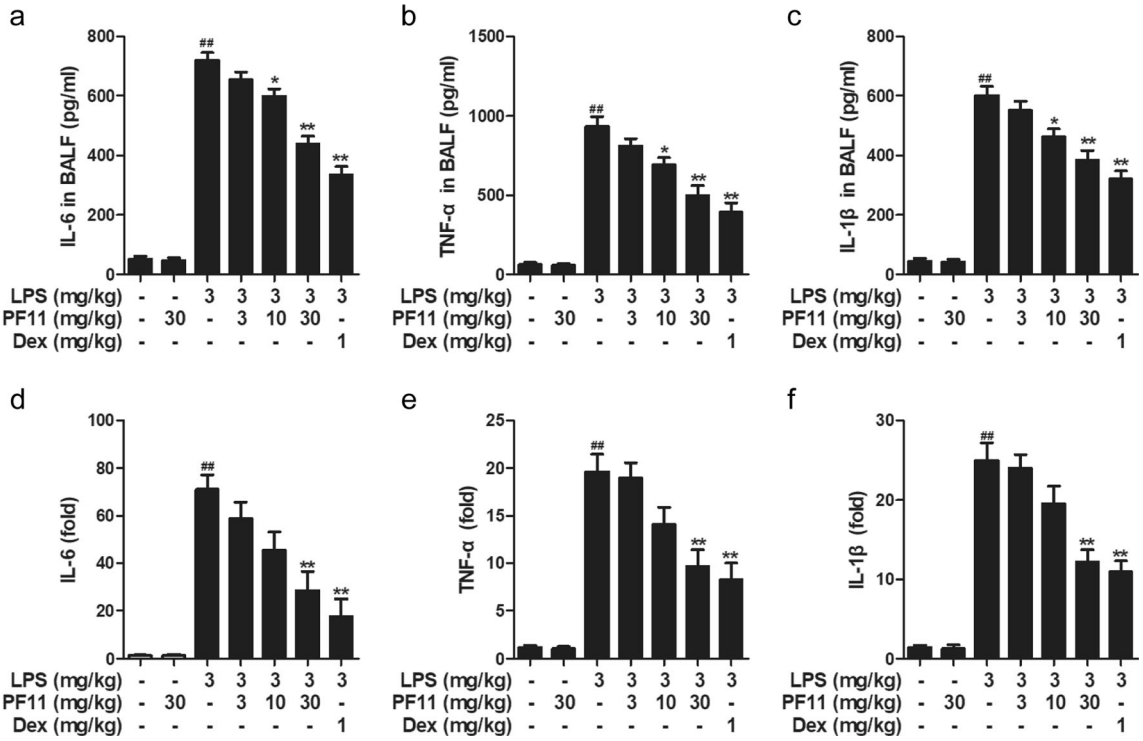

Fig. 3. Effects of pseudoginsenoside-F11 (PF11) on cytokines expression in lung. Thirty minutes after PF11 or dexamethasone (Dex) pretreatment on day 3, mice were anesthetized, and then 3 mg/kg lipopolysaccharide (LPS) was instilled intratracheally. Bronchoalveolar lavage fluid (BALF) and lung tissues were collected at 6 h after LPS challenge. Levels of IL-6 (**a**), TNF-α (**b**) and IL-1β (**c**) in BALF. IL-6 (**d**), TNF-α (**e**), and IL-1β (**f**) mRNA expression in lung tissues. β-Actin was used as an internal control. Data are means ± standard errors ($n = 6$ per group). $^{\#\#}P < 0.01$ *versus* the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* LPS group.

As shown in Fig. 4a and b, lung tissue sections from the control and PF11 alone group presented few Ly-6G$^+$ cells. In contrast, tissues sections from LPS group showed substantial increase of Ly-6G$^+$ cells (Fig. 4c). Compared with the LPS group, PF11 (10 and 30 mg/kg) or Dex significantly decreased the LPS-induced increase of Ly-6G$^+$ cells, but no significant change was observed in PF11 (3 mg/kg) + LPS group (Fig. 4d–g). Additionally, PF11 (10 and 30 mg/kg) or Dex also significantly decreased the LPS-induced increase of percentage of Ly-6G$^+$ cells (Fig. 4h). Moreover, as shown in Fig. 4i and j, LPS-challenged mice showed significant increase of total cell number and neutrophil number in BALF than those in controls. Notably, the LPS-induced increase of total cell number and neutrophil number was significantly reduced by PF11 (10 and 30 mg/kg) or Dex. Consistent with the immunohistochemical analysis and cell counts, PF11 (10 and 30 mg/kg) or Dex also significantly reduced the LPS-induced increase of MPO activity (Fig. 4k).

To illuminate the underlying mechanisms of PF11 on neutrophil infiltration, we detected the protein level of MIP-2 in BALF and mRNA expression of MIP-2 and ICAM-1 in lung tissue. As shown in Fig. 4l–n, LPS-challenged mice showed higher MIP-2 level and mRNA expression of MIP-2 and ICAM-1 than those in controls. However, LPS-induced raise in MIP-2 level was significantly reduced by PF11 (10 and 30 mg/kg) or Dex. Moreover, PF11 (30 mg/kg) or Dex significantly reduced the LPS-induced increase in mRNA expression of MIP-2 and ICAM-1. These results indicated that PF11 could effectively inhibit LPS-induced neutrophil infiltration.

Effects of PF11 on Neutrophil Clearance

Effects of PF11 on neutrophil clearance were inferred from percentage of apoptotic neutrophils and percentage of macrophages containing apoptotic neutrophil in BALF after LPS administration. As shown in Fig. 5a and b, LPS-challenged mice showed substantial inhibition of neutrophil apoptosis in BALF, which was significantly

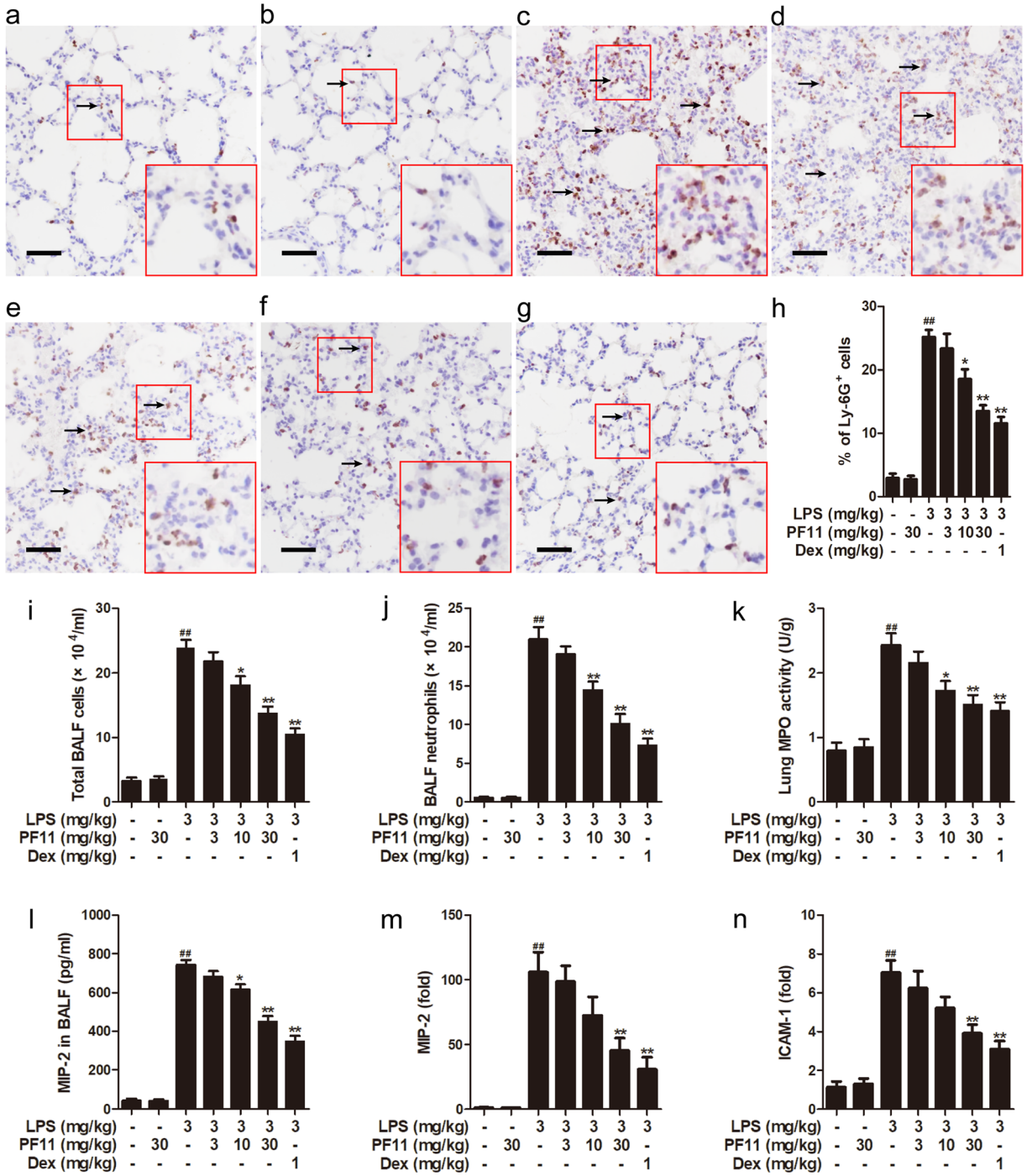

Fig. 4. Effects of pseudoginsenoside-F11 (PF11) on neutrophil infiltration. Thirty minutes after PF11 or dexamethasone (Dex) pretreatment on day 3, mice were anesthetized, and then 3 mg/kg lipopolysaccharide (LPS) was instilled intratracheally. Lung tissues and bronchoalveolar lavage fluid (BALF) were collected at 6 h after LPS challenge. **a**–**g** Representative images of anti-Ly-6G stained lung sections from seven experimental groups (magnification × 200, × 400, scale bar = 50 μm). **a** Control group. **b** PF11 30 mg/kg group. **c** LPS group. **d** PF11 3 mg/kg + LPS group. **e** PF11 10 mg/kg + LPS group. **f** PF11 30 mg/kg + LPS group. **g** Dex 1 mg/kg + LPS group. Arrow indicates Ly-6G positive staining. **h** the percentage of neutrophils in lung sections. Total cell number (**i**) and neutrophil number (**j**) in BALF. **k** MPO activity in lung tissues. **l** Level of MIP-2 in BALF. MIP-2 (**m**) and ICAM-1 (**n**) mRNA expressions in lung tissues. Data are means ± standard errors (n = 6 per group). $^{\#\#}P < 0.01$ *versus* the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* LPS group.

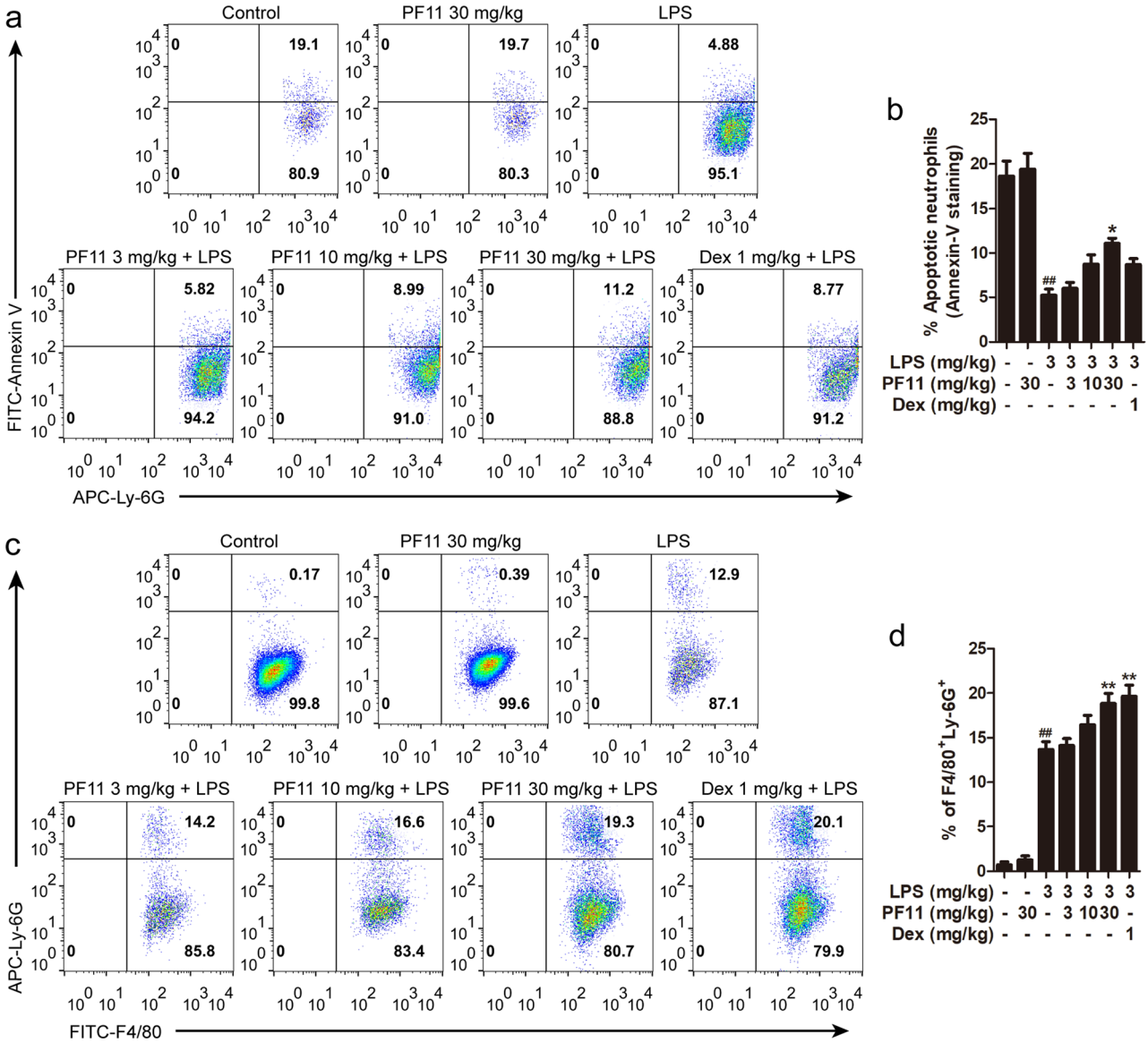

Fig. 5. Effects of pseudoginsenoside-F11 (PF11) on neutrophil clearance. Thirty minutes after PF11 or dexamethasone (Dex) pretreatment on day 3, mice were anesthetized, and then 3 mg/kg lipopolysaccharide (LPS) was instilled intratracheally. Bronchoalveolar lavage fluid (BALF) was collected at 6 h after LPS challenge. **a** Flow cytometric analysis of neutrophil apoptosis. **b** Percentage of apoptotic neutrophils in BALF. **c** Flow cytometric analysis of neutrophil phagocytosis by alveolar macrophages. **d** Percentage of macrophages containing apoptotic neutrophil in BALF. Numbers in each quadrant showed the percentage of cells within each quadrant. Data are means ± standard errors ($n = 6$ per group). $^{\#\#}P < 0.01$ *versus* the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* LPS group.

reversed by PF11 (30 mg/kg). Subsequently, we investigated whether PF11 promotes macrophage phagocytosis. Compared with the LPS group, PF11 (30 mg/kg) or Dex significantly increased the percentage of macrophages containing apoptotic neutrophil in BALF (Fig. 5c and d). These results indicated that PF11 could effectively enhance neutrophil clearance.

DISCUSSION

The present study indicated PF11 attenuated LPS-induced lung inflammatory injury, evidenced by alleviated histopathological damage, enhanced alveolar-capillary barrier integrity, reduced inflammatory response, and decreased neutrophil accumulation.

LPS, generated from Gram-negative bacteria, is known to elicit inflammatory response *in vivo* [26]. Importantly, the ALI mouse model induced by intratracheal administration of LPS is highly reproducible, easily titratable, and cause robust neutrophilic alveolitis and dysfunction of the alveolar-capillary barrier [27]. Moreover, the pathological features of this model are similar to the pathology observed in ALI patients [28]. In the present study, it was found that LPS could induce lung edema, inflammatory response and neutrophil infiltration, which was consistent with other reports [20, 29]. Thus, this model was used to explore the effects of PF11 on ALI. It was found that PF11 or Dex could reverse these changes induced by LPS.

As depicted in Fig. 6, once stimulated by LPS, alveolar macrophages are activated by toll-like receptor 4 (TLR4) and release inflammatory cytokines (IL-6, TNF-α, and IL-1β) and chemokine (MIP-2) [30]. Meanwhile, ICAM-1 is induced by LPS, TNF-α, and IL-1β in conditions of inflammation [31–33]. After adhesion and activation, numerous neutrophils migrate into the lung interstitium and alveolar airspace, release inflammatory mediators and cytokines, and initiate an inflammatory cascade, which can damage the alveolar-capillary barrier and result in protein-rich pulmonary edema [1, 34–36]. Based on the above, the benefits of decreasing neutrophil accumulation in ALI can be predicted. Thus, this study focused on neutrophil to explore the possible mechanisms of PF11 on LPS-induced ALI.

In ALI, numerous activated neutrophils damage the alveolar-capillary barrier, resulting in lung edema and alveolar hemorrhage [35, 36]. In the present study, lung W/D ratio was calculated to infer the extent of pulmonary edema and BALF protein concentration was measured to explore epithelial and endothelial permeability [37, 38], which were the common features of ALI/ARDS [39]. It was found that PF11 or Dex significantly protected against alveolar-capillary barrier dysfunction. Histopathological study also indicated that PF11 or Dex significantly inhibited LPS-induced alveolar hemorrhage and inflammatory cells infiltration in lung.

Dysregulated inflammation is a hallmark of ALI [40]. Previous studies showed that inflammatory cytokines (IL-6, TNF-α, and IL-1β) played a critical role in initiating, amplifying, and maintaining the inflammatory response in ALI [41]. During this process, inflammatory cells (mainly neutrophils) are recruited into the lung. Previous studies showed that neutrophil activation directly correlated with the levels of IL-6, TNF-α, and IL-1β [42, 43]. Furthermore, elevated levels of IL-6, TNF-α, and IL-1β in BALF are observed in patients with ARDS and are associated with poor outcome [44]. In this study, it was found that PF11 or Dex reversed the LPS-induced increases of mRNA expression and protein levels of these three cytokines. These results suggested that PF11 inhibited LPS-induced inflammatory response *via* suppressing inflammatory cell influx and cytokine secretion in BALF.

The activation and migration of neutrophils into the lung are crucial in the pathology of ALI/ARDS [45]. Neutrophilic infiltration of the alveolar spaces has long been observed in ARDS [45]. Furthermore, previous studies showed that the inhibition of neutrophil function attenuates neutrophil-mediated lung injury in patients with ARDS [46].

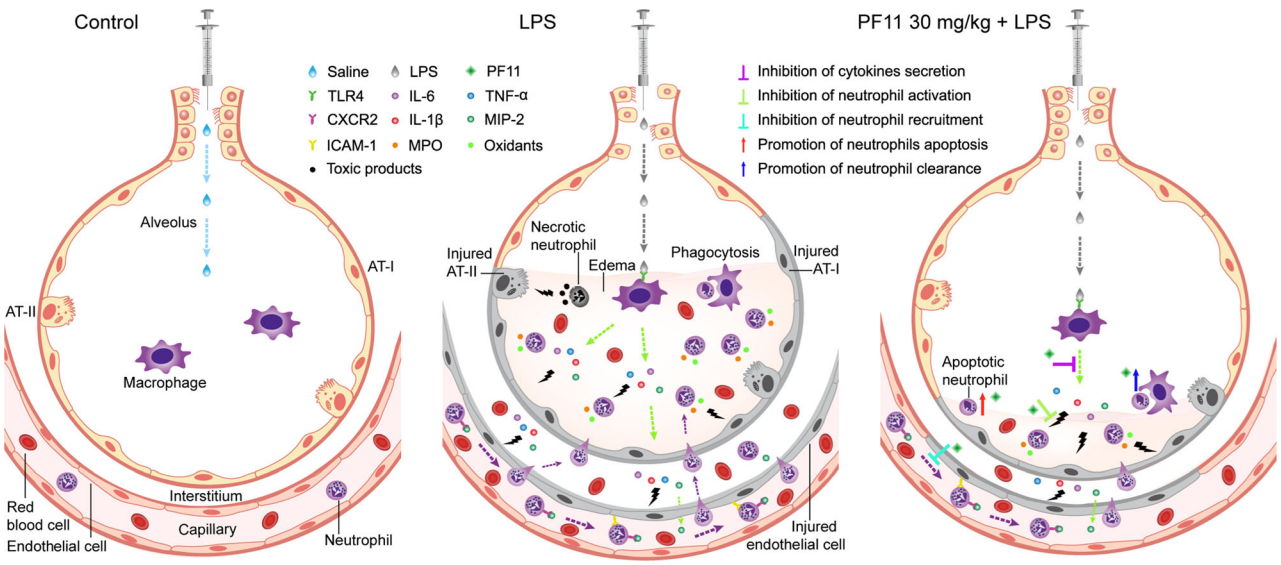

Fig. 6. Schematic diagram illustrate the protective effect of pseudoginsenoside-F11 (PF11) on lipopolysaccharide (LPS)-induced acute lung injury. AT-I alveolar type I cells, AT-II alveolar type II cells.

MPO is mainly synthesized and released *via* neutrophil degranulation, and its activity is considered as the marker of the accumulation of activated neutrophils in inflammation [47, 48]. In this study, the amassing of activated neutrophils was evaluated by determining MPO activity in lung tissues. It was found that PF11 or Dex not only attenuated the LPS-induced increase of percentage of Ly-6G$^+$ cells and MPO activity in lung tissues, but also decreased the LPS-induced increase of total cell number and neutrophil number in BALF. Herein, the underlying mechanisms of the effects of PF11 on neutrophil infiltration were further explored. MIP-2, a potent chemokine for neutrophils, is vital for the migration of neutrophils into the lung during ARDS [49]. Similarly, ICAM-1, an adhesion molecule, participates in neutrophil adhesion to and migration across the endothelium [50]. In this study, PF11 or Dex reduced LPS-induced expression of MIP-2 and ICAM-1, which effectively inhibited neutrophil infiltration and illustrated the underlying mechanisms.

Appropriate neutrophil clearance, as well as inhibition of further neutrophil recruitment, is critical for resolution of acute inflammation [51, 52]. Previous studies showed that apoptosis was critical for neutrophil functional shutdown and their phagocytosis by phagocytes [51, 53]. In addition, apoptotic neutrophils that are not engulfed by phagocytes undergo secondary necrosis, which is a major pathogenic event [54]. Thus, the effects of PF11 on neutrophil apoptosis and phagocytosis of apoptotic neutrophils by macrophages were further explored. It was found that PF11 significantly reversed the LPS-induced inhibition of neutrophil apoptosis. However, Dex had no effect on neutrophil apoptosis, which was consistent with a previous study [55]. In addition, PF11 or Dex significantly enhanced phagocytosis of apoptotic neutrophils by macrophages. Thus, PF11 appeared to act on both neutrophil apoptosis and phagocytosis of apoptotic neutrophils by macrophages in order to enhance neutrophil clearance.

Although the present study explored the possible mechanisms for the reduction of neutrophils number after PF11 treatment, it was still unclear how PF11 downregulated the expression of ICAM-1 and MIP-2 and accelerated neutrophils apoptosis as well as related phagocytosis. Previously, we have reported that PF11 exerted anti-neuroinflammatory activity in LPS-activated microglial cells partly through inhibiting TLR4/NF-κB signaling pathway [18]. Normally, NF-κB is located in the cytoplasm and bound by inhibitory proteins IκB [56]. After LPS exposure, the level of phosphorylated IκB protein (p-IκB) was elevated and then liberated NF-κB migrates to the nucleus, where it binds to specific promoter sites and triggers gene transcription [56–58]. NF-κB activation leads to the increase of inflammatory cytokines (including IL-6, TNF-α, and IL-1β) release [59], the upregulation of MIP-2 and ICAM-1 expression [60–62], as well as the inhibition of neutrophil apoptosis [63]. What is more, in LPS-induced ALI model, LPS can activate NF-κB pathway through TLR4 [64]. Also, TLR4 has been reported to inhibit neutrophil apoptosis directly by upregulating IL-8 mRNA after binding to LPS [11, 65]. Therefore, the inhibition of TLR4/NF-κB signaling pathway might inhibit inflammatory cytokines release, downregulate MIP-2 and ICAM-1 expression, and accelerate neutrophil apoptosis. Moreover, apoptotic neutrophils can express "eat-me" signals to trigger phagocytosis [66]. Thus, it can be assumed that the possible mechanism of PF11 on neutrophilic infiltration and neutrophil clearance may be associated with inhibiting TLR4/NF-κB signaling pathway, but the further studies are needed to verify this hypothesis.

CONCLUSION

In conclusion, the present study indicated that PF11 attenuated LPS-induced ALI through the prevention of neutrophils accumulation by decreasing neutrophilic infiltration, as well as enhancing neutrophils clearance, thus promoting the resolution of inflammation, suggesting its potential in the treatment of ALI.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethics Statement. All experimental procedures were approved by the Ethics Committee of Shenyang Pharmaceutical University, and followed the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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