

## Original Article

# Lugrandoside attenuates LPS-induced acute respiratory distress syndrome by anti-inflammation and anti-apoptosis in mice

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**Abstract:** This study aimed to investigate the protective effects and specific mechanisms of lugrandoside (LG) on lipopolysaccharides (LPS)-induced acute respiratory distress syndrome (ARDS). LG is a novel phenylpropanoid glycoside with many biological properties, isolated from the culinary leaves of *Digitalis lutea* L. and *Digitalis grandiflora* Miller. The primary indicators to assess the lung injury were infiltration of inflammatory cells; pulmonary edema; expression of proinflammatory cytokines, cyclo-oxygenase 2, and intracellular adhesion molecule 1; activation of nuclear factor- $\kappa$ B pathways; and cellular apoptosis. The results showed that LG evidently alleviated the inflammatory response, decreased the apoptosis of alveolar macrophages, and improved the lung injury in mice with LPS-induced ARDS. In conclusion, LG improved LPS-induced ARDS by anti-inflammation and anti-apoptosis and might be a promising pharmacological therapy for ARDS.

**Keywords:** Acute respiratory distress syndrome, anti-apoptosis, anti-inflammation, lipopolysaccharide, lugrandoside

## Introduction

Acute respiratory distress syndrome (ARDS), a severe clinical syndrome, is caused by multiple reasons that result in characteristic pathologic changes in lung tissues [1, 2]. In ARDS, the quintessential trait is the damage of the alveolar capillary endothelial cells and alveolar epithelial cells expressed by extensive pneumoedema and tiny pulmonary atelectasis [3-5].

ARDS has varied etiology. In 1992, it was summed up in two parts: factors with pulmonary and extrapulmonary origin, by the American Thoracic Society and the European Society of Intensive Care Medicine [6]. ARDS with pulmonary origin is characterized by direct injury to the lung, covering three aspects: chemical (aspiration of gastric content, smoke, water,

poison gas, oxygen toxicity, and so forth), physical (bruise and radiation-induced damage of lungs), and biological agents (severe, diffuse lung infection). ARDS with extrapulmonary origin is associated with systematic illness, including sepsis, shock, systematic inflammatory response syndrome, disseminated intravascular coagulation, trauma, severe pancreatitis, and drug intoxication [7-12].

In the last two decades, a number of researchers have sought to determine the specific pathogenesis of ARDS; however, it has not been fully elucidated till now [13, 14]. Recent evidence suggests that the primary cause of ARDS is the imbalance between inflammatory response and anti-inflammatory response and the uncontrolled systematic inflammatory response arising from activated cellular and humoral im-

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munity. The pathological changes include acute diffuse lesions on the alveolar capillary membrane, effusion of proteins in the alveolar space, and formation of the hyaline membrane [1, 11, 14, 15]. Moreover, a number of reactive oxygen species (ROS), including oxygen free radicals, are generated in ARDS. ROS react with various unsaturated fatty acids and cholesterol, resulting in severe cell apoptosis [16].

Lugrandoside (LG), a novel phenylpropanoid glycoside, is isolated from the culinary leaves of *Digitalis lutea* L. and *Digitalis grandiflora* Miller. [17, 18] Phenylpropanoid glycosides possess many biological properties, including antimicrobial, antitumor, immune-regulatory, and anti-inflammatory [19]. Previous studies have reported that phenylpropanoid glycosides of *Scrophularia ningpoensis* have a protective effect on the hepatic cells [20]. Agustín et al. explored specific free radical scavenger mechanisms and other pharmacological characteristics of phenylpropanoid glycosides [19]. However, sufficient evidence on the use of LG for treating ARDS was lacking, and the specific mechanism was unknown.

The present study mainly focused on whether LG improved the condition of ARDS, relieving inflammatory responses and reducing cell apoptosis.

### Materials and methods

#### *Animals*

Male BALB/c (21-25 g) mice were purchased from the Animal Lab Center of the Second Military University, housed in groups of six per standard cage, and held on a standard 12-h light/dark cycle, constant temperature, and humidity (20-24°C and 50%-60%, respectively) with free access to food and water. All procedures were conducted in accordance with the guidelines described in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 8023, revised 1978), and all the experiments were approved by the Animal Care and Use Committee of the Second Military Medical University (Shanghai, China).

#### *Development of ARDS model*

The mouse model of ARDS was induced using lipopolysaccharides (LPS). In brief, the mice

were anesthetized with an intraperitoneal injection of 0.75% pentobarbital solution at 10  $\mu$ L/g. After anesthesia and fixation, 50  $\mu$ L of LPS (4 mg/kg) was injected intratracheally using a MicroSprayer (Penn-Century, PA, USA) [21-23]. The mice were intraperitoneally injected with different doses of LG (10, 20, or 30 mg/kg) at 12 h before surgery and 1 h after LPS stimulation to assess the right dose of LG. Then, the right dose of LG was administered in the following experiments. After 6 h of LPS challenge, the mice were sacrificed for the next test.

#### *Cell culture*

NR8383, the alveolar macrophage cell line, was cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air. The cells were treated with LG for 12 h before stimulation with different doses of LPS (10, 20, or 30  $\mu$ g/mL). After LPS challenge for 6 h, the supernatants were collected for detecting the secretion of cytokines and chemokines, and the cellular proteins were extracted for subsequent protein studies.

#### *Bronchoalveolar lavage*

After anesthesia with 0.75% pentobarbital solution, the skin of the neck was anatomized to expose the trachea on the fixed plate. Then, the catheter was inserted deep into the trachea. The lungs were lavaged with 1 mL of cold phosphate-buffered saline three times, ensuring the recovery of the fluid more than 85%. Also, the level of inflammatory factors was measured using the enzyme-linked immunosorbent assay (ELISA).

#### *Protein concentration in BALF*

The protein concentration was determined using a protein concentration assay kit according to the instructions after collecting the bronchoalveolar lavage fluid (BALF).

#### *Ratios of wet-to-dry weight of lung tissues*

The tissue samples were harvested and weighed immediately (wet weight) after LPS challenge for 6 h, and then placed in an oven at 70°C for 72 h until a settled dry weight was

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achieved. Ratios of wet-to-dry weight were then calculated to quantify the magnitude of pulmonary edema.

### *Detection of cellular apoptosis*

All cells in the BALF were stained with Annexin V and 7-aminoactinomycin D (AAD) to measure apoptosis using a commercial PE Annexin V Apoptosis Detection Kit I (BD559763). The cells were washed with ice-cold phosphate-buffered saline, resuspended in 400  $\mu$ L of binding buffer, and analyzed using a FACSCalibur flow cytometer.

### *Histological analysis of lung tissue*

The mice were fixed, and the lungs were exposed. A small piece of lung tissue was obtained, fixed with 4% paraformaldehyde for 24 h, and embedded in paraffin. The lung tissues were cut into 6- $\mu$ m sections and stained with hematoxylin-eosin (HE) after deparaffinization and dehydration. The infiltration of inflammatory cells could be seen obviously in the HE-stained slides.

### *Real-time quantitative polymerase chain reaction*

After the mice were sacrificed, the unilateral lung tissue was treated with 1 mL of TRIzol reagent (Life Technologies, CA, USA) to extract the total RNA. The lung tissues were fully ground in a grinding tube and kept at room temperature for 10 min, and then 200  $\mu$ L of chloroform was added to the tube containing the TRIzol. The mixture was kept at room temperature for 5 min after centrifugation at 12,000 rpm for 15 min at 4°C, and 600  $\mu$ L of supernatant was obtained. The supernatant was transferred to the new Eppendorf (EP) tubes. Next, 600  $\mu$ L of isopropanol was added and oscillated for 15 s. The supernatant was kept at -20°C for 30 min and removed after centrifugation at 12,000 rpm for 10 min at 4°C. Then, 70% alcohol was added to the EP tubes and vortexed two times. Finally, RNA was dried on a clean bench at room temperature and dissolved in diethyl pyrocarbonate (DEPC) water. The cDNA was synthesized with RNA using a cDNA synthesis kit, including reverse transcription (37°C, 15 min), inactivation of reverse transcriptase (85°C, 5 s), and preservation (4°C). The SYBR Premix Ex Taq™ II (RR820A, TAKARA BIO INC.,

Shiga, Japan) with an automated PCR instrument (7500 system) was used to detect cDNA gene expression.

The specific gene primer sequences used were as follows: 1.  $\beta$ -actin: forward primer: AGTG-TGACGTTGACATCCGT; reverse primer: GCAGC-TCAGTAACAGTCCGC; 2. Interleukin (IL)-1 $\beta$ : forward primer: CACTACAGGCTCCGAGATGA; reverse primer: CGTTGCTTGGTTCTCCTTGT; 3. IL-6: forward primer: TAGTCCTTCTACCCCAATTTCC; reverse primer: TTGGTCCTTAGCCACTCCTTC; 4. Tumor necrosis factor (TNF)- $\alpha$ : forward primer: AAGCCTGTAGCCCACGTCGTA; reverse primer: GGCACCACTAGTTGGTTGTCTTTG.

### *Enzyme-linked immunosorbent assay*

The specific ELISA kits were used to determine the concentration of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in BALF. BALF was reasonably diluted, and the standard solution was diluted from a high- to a low-concentration gradient. Then, 100  $\mu$ L of stop buffer was added according to the manufacturer's instructions, and the optical density was spectrophotometrically measured at 450 nm using a microplate reader in 30 min.

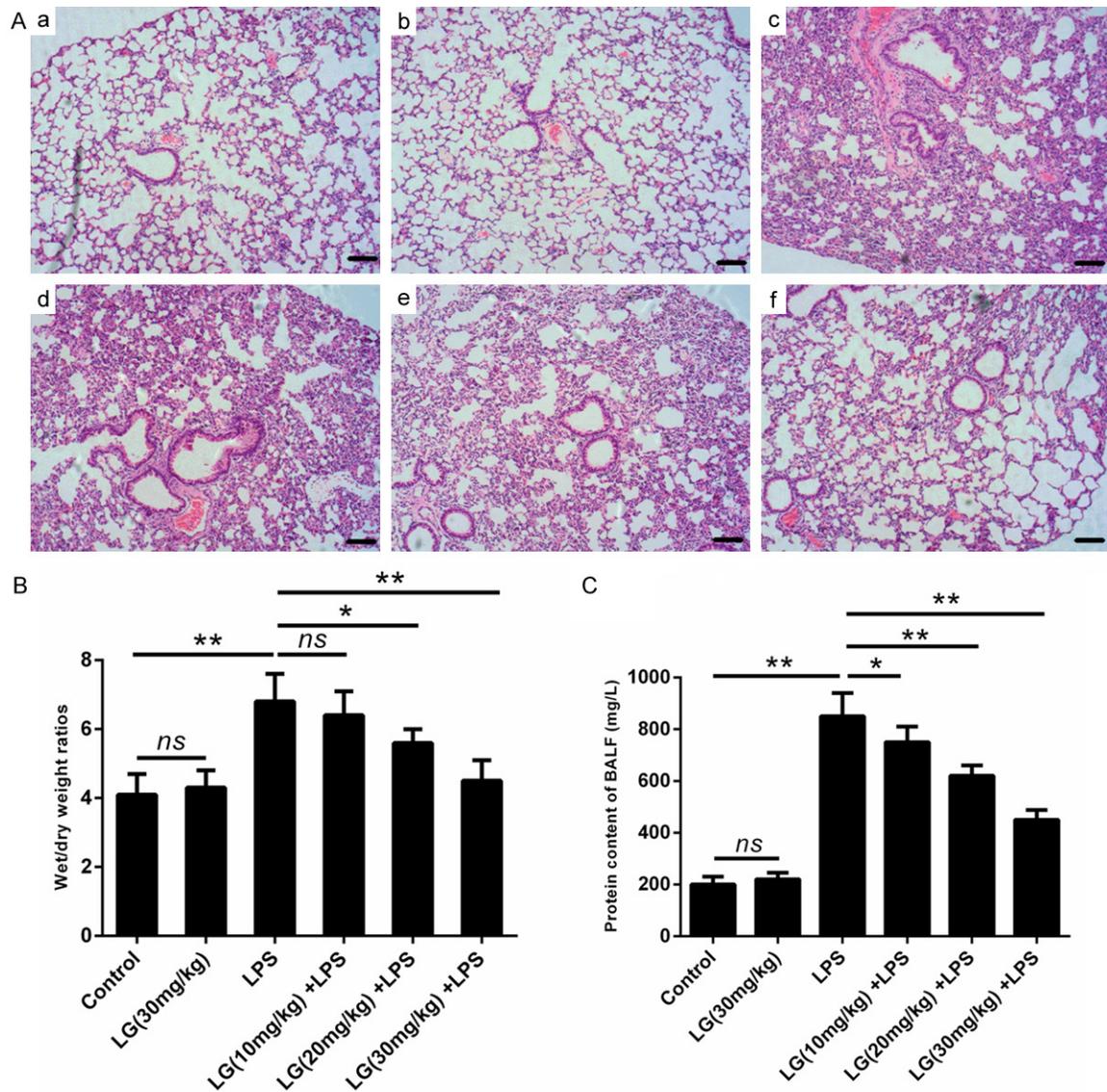
### *Western blotting analysis*

The cells were collected, and total proteins were extracted. The protein concentration in the supernatant was determined using the Enhanced Bicinchoninic Acid Protein Assay Kit (P0010, Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of protein were separated by 12% sodium dodecyl sulfate, transferred electrophoretically onto a nitrocellulose membrane, and incubated with the first antibody overnight at 4°C. The membrane was washed three times thoroughly and incubated with secondary antibody for 2 h at room temperature. Protein bands were visualized using the 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium alkaline phosphatase color development kit.

### *Statistical analysis*

All the calculations were conducted using SPSS 19.0 software (IBM, NY, USA), and data charts were made using Prism 6.0 (GraphPad Software, Inc., San Diego, USA). All the data were expressed as mean  $\pm$  standard deviation. Statistical analyses were performed using one-way

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**Figure 1.** Lugrandoside alleviated the lung injury of mice with ARDS. A. HE staining revealed pathological changes in the lung tissue. Magnification: 10 $\times$ . (a) Control group; (b) LG (30 mg/kg) group; (c) LPS group; (d) LG (10 mg/kg) + LPS group; (e) LG (20 mg/kg) + LPS group; (f) LG (30 mg/kg) + LPS group. B. The ratio of wet-to-dry weight was an indicator of pulmonary edema. C. The protein content in BALF of each group ( $n = 6$ ). Data are presented as mean  $\pm$  standard deviation. ns,  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

analysis of variance, and pairwise comparison was conducted using the Student-Newman-Keuls test. A  $P$  value of  $< 0.05$  was considered to indicate a statistically significant difference.

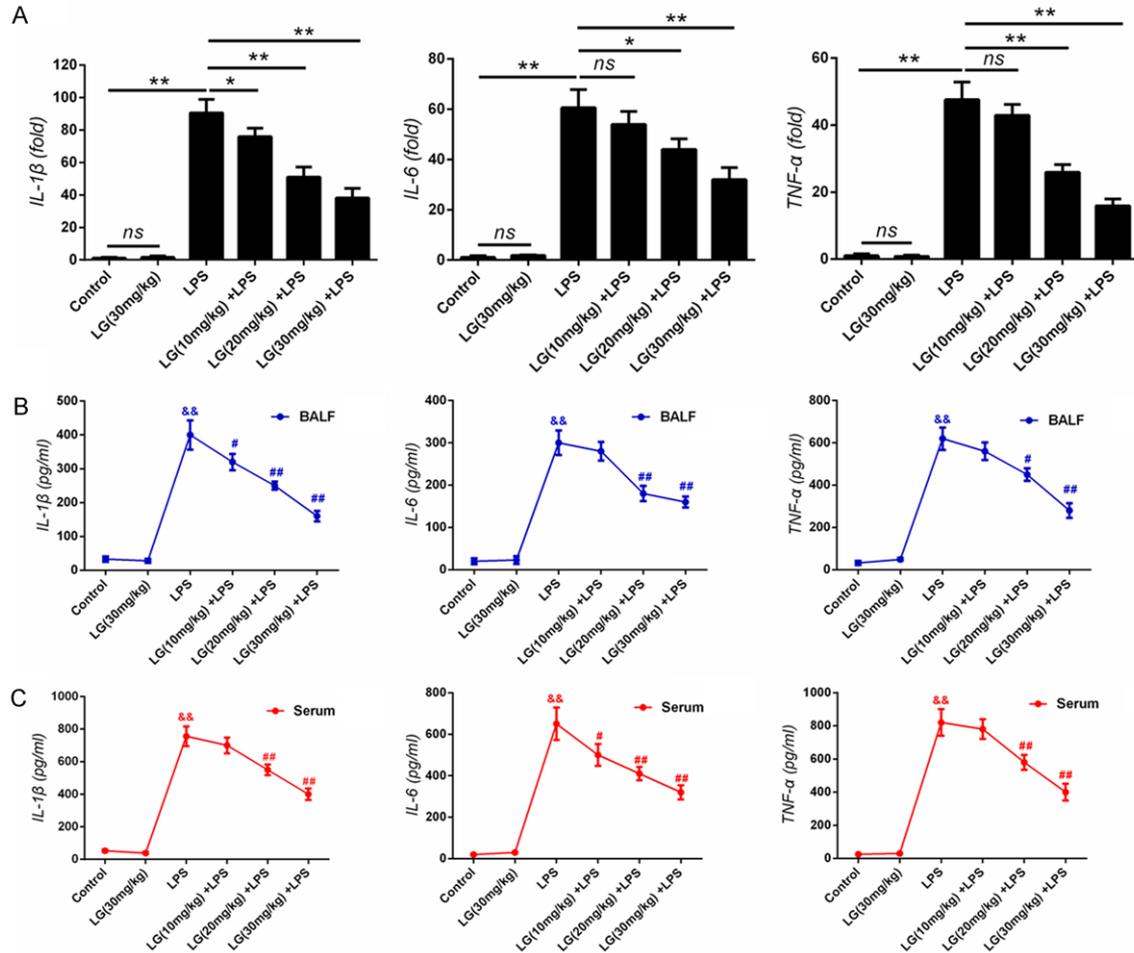
### Results

#### *LG had a protective effect on the LPS-induced ARDS in mice*

The mice were injected with three different doses (10, 20, or 30 mg/kg) to find the appro-

prate dose of LG. Obvious pulmonary edema and infiltration of inflammatory cells in the lung were observed in the single LPS group (**Figure 1A**); however, LG treatment significantly alleviated lung injury in a dose-dependent manner. The severity of pulmonary edema was also shown as the ratios of wet-to-dry weight (**Figure 1B**). The ratio of wet-to-dry weight increased apparently after LPS challenge, and lung edema was dose-dependently attenuated largely in the LG + LPS group. The protein content in BALF

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**Figure 2.** Lugrandoside inhibited the inflammatory response of LPS-induced ARDS in mice. A. The gene expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the lung tissue was determined by real-time quantitative polymerase chain reaction. B. The expression levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in BALF were measured by ELISA. C. The expression levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in serum were measured by ELISA. Data are presented as mean  $\pm$  standard deviation. ns,  $P > 0.05$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; && $P < 0.01$  compared with the control group; # $P < 0.05$  compared with the LPS group; ## $P < 0.01$  compared with the LPS group.

of the LPS group significantly increased compared with the control group, and LG exhibited an evidently protective effect on the LPS-induced ARDS (Figure 1C), corresponding to the aforementioned results.

### LG improved the inflammatory condition of LPS-induced ARDS

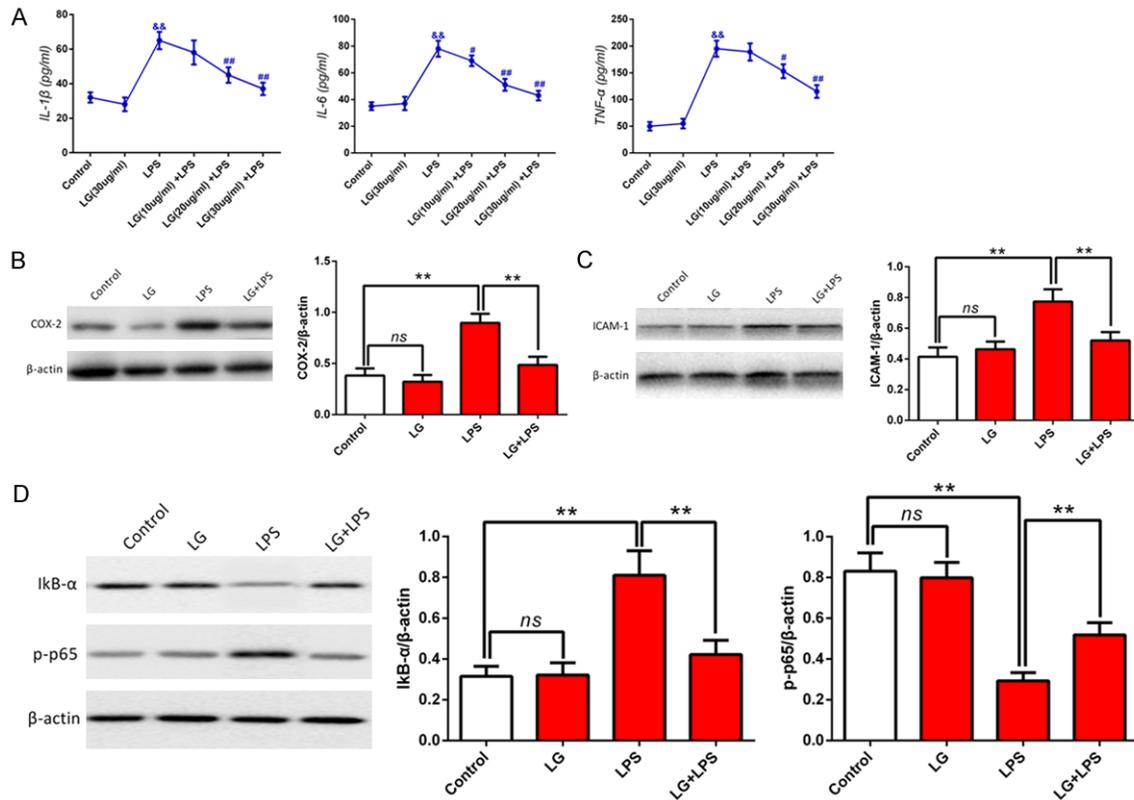
The total RNA of the lung was extracted to detect the increased fold of cytokines after 6-h LPS stimulation. The expression of proinflammatory factors was distinctly enhanced in the LPS group, but the increased folds induced by LPS were obviously inhibited in all the LG + LPS groups (Figure 2A) in a dose-dependent manner. ELISAs of BALF and serum were carried to

determine the expression levels of the proinflammatory factors to further verify the results. IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were highly expressed in BALF in the LPS group. Nevertheless, LG alleviated the LPS-related systemic inflammatory response, and the results suggested that the proper dose of lugrandoside was 30 mg/kg (Figure 2B). Moreover, the levels of proinflammatory cytokines in serum were consistent with the trend (Figure 2C).

### LG suppressed the expression of cyclo-oxygenase 2 and intracellular adhesion molecule 1 in the NR8383 cell line

After NR8383 cells were challenged with LPS for 6 h, the supernatants were collected to

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**Figure 3.** Lugrandoside suppressed the inflammatory reaction through inhibiting the nuclear translocation of NF-κB in NR8383. (A) The expression levels of IL-1β, IL-6, and TNF-α in supernatants were measured by ELISA. The COX-2 (B) and ICAM-1 (C) protein levels were determined by Western blotting. (D) The activation of NF-κB was assessed by Western blotting. The relative protein levels were determined after normalization with β-actin. LG group: LG (30 μg/mL); LG + LPS group: LG (30 μg/mL) + LPS. Data are presented as mean ± standard deviation. ns,  $P > 0.05$ ; \*\* $P < 0.01$ ; && $P < 0.01$  compared with the control group; # $P < 0.05$  compared with the LPS group; ## $P < 0.01$  compared with the LPS group.

determine the level of proinflammatory factors. The result showed that LPS promoted the release of cytokines and the trend was inhibited evidently after LG treatment, particularly in the LG (30 μg/mL) + LPS group (Figure 3A). Then, the study was divided into four groups: control, LG (30 μg/mL), LPS alone, and LG (30 μg/mL) + LPS groups. The expression of cyclooxygenase 2 (COX-2) and intracellular adhesion molecule 1 (ICAM-1) was increased evidently in the LPS group. Also, LG treatment significantly inhibited the expression level of proteins in the LG + LPS group (Figure 3B, 3C).

### LG decreased the activation of NF-κB in NR8383 cell line

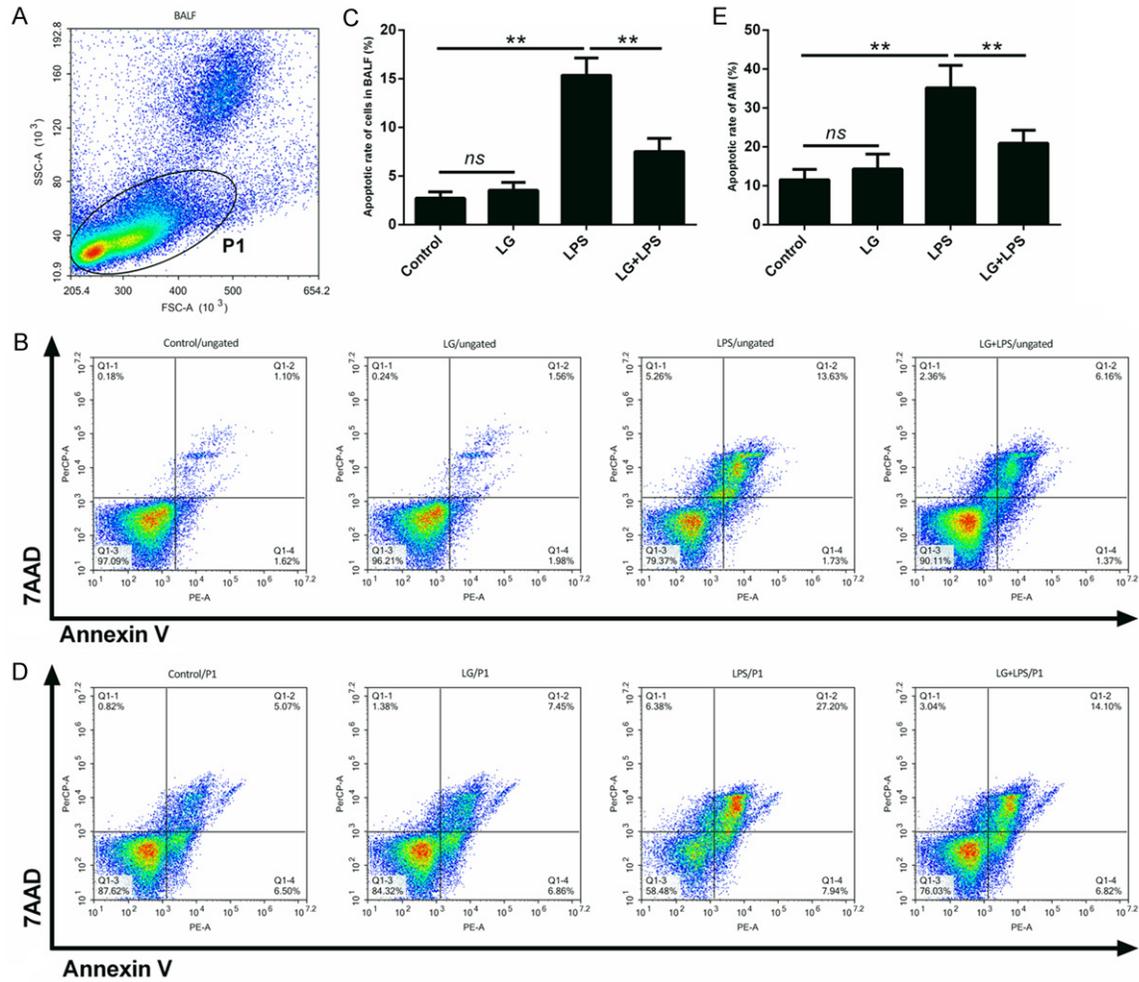
NF-κB activation mediated the pathophysiology of organ injury and inflammatory reaction. The LPS stimulation reduced the expression of

IκB-α and induced phosphorylation and nuclear translocation of NF-κB. However, the condition was reversed evidently in the LG + LPS group (Figure 3D).

### LG reduced the apoptosis of alveolar macrophages in mice with LPS-induced ARDS

The apparent apoptosis of cells in BALF was observed after LPS stimulation, which was partially alleviated with the administration of LG (Figure 4A-C). Previous studies have shown delayed apoptosis of neutrophils in BALF of mice with LPS-induced ARDS [24, 25]. Therefore, the specific apoptotic cells in BALF were further explored. After a series of screenings, the apoptosis of alveolar macrophages was detected covering a large proportion in BALF, and the condition was distinctly improved after LG administration (Figure 4D, 4E).

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**Figure 4.** Lugrandoside decreased the apoptosis of alveolar macrophages in mice with LPS-induced ARDS. A. The bivariate dot plot of cells in BALF displayed by forward scatter and side scatter characteristics. B. The apoptosis of all cells in BALF was determined by flow cytometry. C. The quantitative figure of apoptotic ratios of all cells in BALF. D. The apoptosis of alveolar macrophages was determined by flow cytometry. E. The quantitative figure of the apoptotic ratio of alveolar macrophages. Alveolar macrophages were gated by physical map and stained with Annexin V (PE) and 7-AAD (PerCP). LG group: LG (30 mg/kg); LG + LPS group: LG (30 mg/kg) + LPS. Data are presented as mean  $\pm$  standard deviation. ns,  $P > 0.05$ ;  $**P < 0.01$ .

### LG altered the expression of apoptosis-related proteins in NR8383

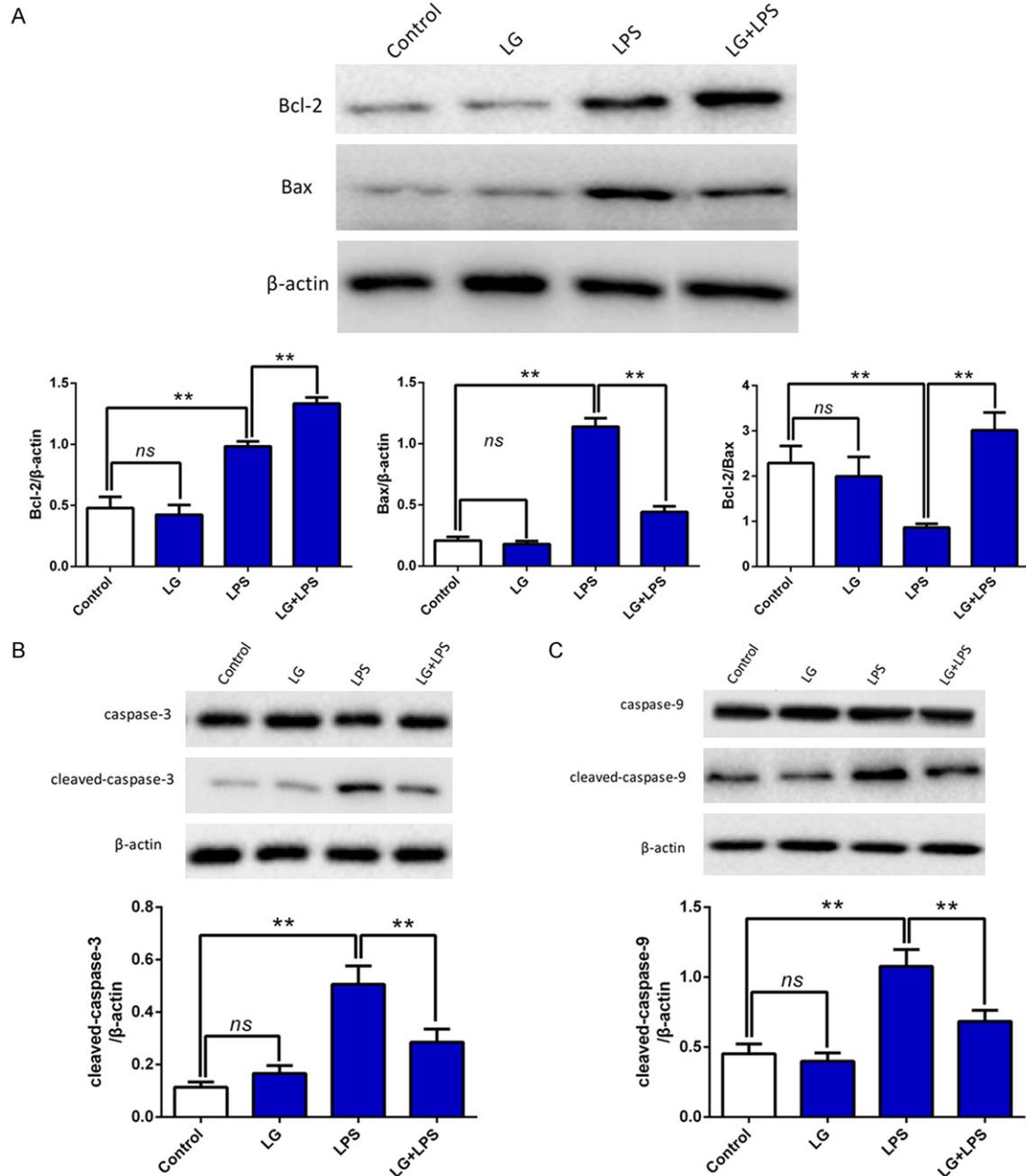
The expression of apoptosis-related proteins B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), caspase-3, and caspase-9 was measured to explore the exact mechanisms of anti-apoptosis. The expression of Bcl-2 increased and the expression of Bax and activated caspase-3 and caspase-9 was evidently promoted with LPS challenge. Also, the ratio of Bcl-2 to Bax decreased. The level of Bcl-2 was significantly elevated, and the expression of Bax and activated caspase-3 and caspase-9

was apparently suppressed with LG treatment. Hence, the ratio of Bcl-2 to Bax was elevated significantly (**Figure 5A-D**).

### Discussion

ARDS is one of the refractory complications in the clinic, especially after shock, trauma, severe infection, and surgery. It is induced by a multitude of factors. It is difficult to cure and still a serious challenge for modern medicine [26, 27]. Previous studies have reported that the primary pathogenesis of ARDS is the uncontrolled systematic inflammatory response syndrome induced by activated cellular and humor-

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**Figure 5.** Lugrandoside affected the expression of apoptosis-related proteins in NR8383. (A) The protein levels of Bcl-2 and Bax in NR8383 were detected by Western blotting. The relative protein levels were determined after normalization with  $\beta$ -actin. The ratio of Bcl-2 to Bax was calculated with the relative protein levels. The protein levels of activated (cleaved) caspase-3 (B) and caspase-9 (C) in NR8383 were determined by Western blotting. The relative protein levels were determined after normalization with  $\beta$ -actin. LG group: LG (30  $\mu$ g/mL); LG + LPS group: LG (30  $\mu$ g/mL) + LPS. Data are presented as mean  $\pm$  standard deviation. ns,  $P > 0.05$ ; \*\* $P < 0.01$ .

al immunity [28]. The major changes include the damage of the alveolar capillary endothelial cells and alveolar epithelial cells, pulmonary edema, reduction of  $\text{PaO}_2$ , and increased pulmonary vascular permeability, further resulting

in severe apoptosis [29]. Although some prospective treatments have been applied in patients with ARDS, few of these therapies showed specific therapeutic action on ARDS to evidently decrease the mortality [26, 30].

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LG, as a widely used phenylpropanoid glycoside, possesses powerful anti-inflammatory and anti-apoptotic properties. Previous studies have illustrated that phenylpropanoid glycosides could significantly inhibit the growth of tumor cells and improve the level of blood sugar. Quite a few studies demonstrated the effects of analgesia, depressurization, and immunoregulation of phenylpropanoid glycosides [31-33]. However, the effect of LG in ARDS and the underlying mechanism are still not quite clear. Therefore, this study evaluated the efficacy of LG in ARDS.

LPS is an important component of the cell wall of Gram-negative bacteria. LPS can activate neutrophils, produce (ROS, oxidize membrane lipids, and produce oxygen free radicals through the activation of reduced nicotinamide adenine dinucleotide phosphate oxidase, inducing ARDS [34, 35]. Therefore, LPS plays an extremely important role in the development of ARDS.

The present study explored the protective effect of LG on ARDS induced by LPS and the specific mechanisms. The data revealed that LG significantly alleviated the inflammatory cell infiltration, decreased the ratios of wet-to-dry weight, and reduced the protein content in BALF. Also, lugrandoside administration suppressed the expression of proinflammatory factors, COX-2 and ICAM-1. LG treatment evidently decreased the apoptosis ratio of cells in BALF, especially the alveolar macrophages. Finally, LG inhibited the activation of NF- $\kappa$ B signaling pathway and reduced the expression of apoptosis-related proteins.

NF- $\kappa$ B, known as nuclear factor- $\kappa$ B, is found in almost all types of cells. The activating pathways of NF- $\kappa$ B are complicated, mediated by a multitude of signal transduction pathways in cells [36]. I $\kappa$ B, the inhibitor of NF- $\kappa$ B, holds the NF- $\kappa$ B complex (p65 and p50) in the cytoplasm under unstimulated conditions. When stimulating signals activate the inhibitor of nuclear factor kappa-B kinase (IKK), I $\kappa$ B- $\alpha$  is released from NF- $\kappa$ B and degraded. NF- $\kappa$ B is activated and translocated rapidly into the nucleus to combine with the inflammation-related genes. Transcription is activated to trigger the expression of cytokines, adhesion molecules, inflammatory reaction enzymes, and major histocompatibility complex [37, 38].

Previous studies have shown that the Fas/FasL pathway plays an important role in ARDS-related apoptosis [39]. The combination of Fas/FasL promotes the activation of procaspase-8 and a series of apoptotic proteases of caspase family, finally resulting in Fas-related apoptosis [40, 41]. Recent studies have highlighted the mitochondrion, rather than the nucleus, as the center of apoptosis-related regulation. Some studies even demonstrated that mitochondrion played a determinative effect on apoptosis; and the caspase family was involved in the apoptotic pathway regulated by mitochondrion [42, 43].

Caspase-8 could activate apoptosis induced by downstream caspase factors in two ways. First, when caspase-8 is sufficient in the death-inducing signal complex, caspase-3, caspase-6, and caspase-7 can be activated to cause cell lysis and apoptosis. However, when caspase-8 is insufficient, it catalyzes p15 in the mitochondrion and the integrity of the mitochondrial membrane is broken. Finally, activated procaspase-9 activates caspase-3 to cause apoptosis [44].

Bcl-2 family, including an anti-apoptotic gene (Bcl-2) and a pro-apoptotic gene (Bax), is also closely associated with apoptosis [45]. The mitochondrial permeability transition pore (mPTP) is the major route of the release of cytochrome C and apoptosis-inducing factor (AIF) in the mitochondrion. The mPTP is under the control of Bcl-2; therefore, the release of cytochrome C and AIF is prevented and apoptosis is avoided [46]. The sensitivity to apoptotic signals mainly depends on the process of competitive dimerization of Bcl-2/Bax in cells. When Bcl-2 is expressed at a high level, Bcl-2/Bcl-2 homodimer and Bcl-2/Bax heterodimer are created and the DNA cleavage activity of endonuclease is inhibited to suppress apoptosis. When Bax is overexpressed, Bax/Bax homodimer is formed to accelerate apoptosis. Finally, it has been demonstrated that the ratio of Bcl-2 to Bax is a crucial index in the process of apoptosis, indicating the severity of apoptosis [47].

The present results suggested that LG protected against ARDS via anti-inflammation and anti-apoptosis. LG decreased the expression of inflammatory cytokines through the suppressing NF- $\kappa$ B pathway. Also, it alleviated the apop-

tosis of alveolar macrophages; the specific mechanisms included the elevated expression of Bcl-2 and the reduced expression of Bax, caspase-3, and caspase-9.

### Conclusions

LG could significantly improve LPS-induced ARDS via decreasing the release of proinflammatory factors and the ratio of cellular apoptosis. Therefore, LG might be a promising pharmacological therapy for acute lung injury.

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### Disclosure of conflict of interest

None.

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