Juglanin suppresses fibrosis and inflammation response caused by LPS in acute lung injury

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Received November 27, 2016; Accepted February 5, 2018

DOI: 10.3892/ijmm.2018.3554

Abstract. Acute lung injury in children is a complication showing devastating disorders linked to fibrosis progression and inflammation response. Fibrosis and inflammation response are two markers for acute lung injury. Juglanin is a natural product mainly isolated from green walnut husks of *Juglans mandshurica*, which isconsidered as the functional composition among a series of compounds. It exhibited effective role in various diseases by inhibiting inflammation response. In our study, the protective effects and anti-inflammatory activity of juglanin were investigated in mice and lung cells treated by lipopolysaccharide (LPS) to reveal the possible mechanism by which juglanin attenuates acute lung injury. The mice were separated into four groups. The mouse model was established with 15 mg/kg LPS injection. Juglanin dramatically reduced the inflammation of cell infiltration. Compared to mice only treated with LPS, LPS-treated mice in the presence of juglanin developed less lung fibrosis with lower levels of α-smooth muscle-actin (α-SMA), collagen type I, collagen type III, and transforming growth factor-β1 (TGF-β1). Additionally, juglanin markedly downregulated inflammatory cytokine secretion and phosphorylated nuclear factor-κB (NF-κB) expression via inhibiting IKKα/IκBα signaling pathway. Our results indicate that juglanin has a protective role in LPS-triggered acute lung injury via suppression of fibrosis and inflammation response by NF-κB signaling pathways inactivation. Thus, juglanin may be a potential candidate as dietary supplement for acute lung injury for children in future.

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Key words: acute lung injury, juglanin, fibrosis, inflammation, nuclear factor-κB

Introduction

Acute lung injury (ALI) is pulmonary inflammation with severe disorders, resulting in diffuse alveolar damage, leading to hypoxemia, pulmonary edema and respiratory failure (1,2). The incidence of acute lung injury in children is high worldwide (3). ALI is associated with pediatric intensive care unit admissions, causing increased pediatric intensive care unit deaths (4). Previous studies in children and adults show strong relationship between the positive fluid balance and the worse outcomes, and death is involved, in patients with respiratory failure and/or ALI (5,6). Additionally, the percentage of fluid overload is linked with worse oxygenation, enhanced duration of mechanical ventilation, and upregulation of hospital length stay according to a previous study (7). Only a small number of children with acute lung injury was successfully treated to inhibit fluid overload (8,9). The mortality rate in patients with ALI is high due to the slow progress in understading the molecular mechanisms regarding the disease pathogenesis, as well as the poor therapeutic strategy. Further, lipopolysaccharide (LPS) results in symptoms in animal models, closely resembling ALI in human, highlighting strategies to investigate the pathogenesis of ALI (10,11). Thus, finding effective methods and exploring the molecular mechanism are necessary.

Juglanin is a natural compound belonging to flavonoids, it is extracted from crude '*Polygonum aviculare*', exhibiting inhibitory activity against the inflammation response as well as cancer growth (12-14). Flavonoids are common plant secondary metabolites, well known for having several biological activities *in vitro* and *in vivo* among which we can highlight their remarkable antioxidant activity (15,16). In addition, flavonoids also have interesting anti-inflammatory activities against several markers (17-19). For instance, quercetin and astragalin are two important flavonoids (20,21). Quercetin has been reported to have anti-inflammatory activity in various diseases (22-24). Thus, we supposed that juglanin may be also effective for suppressing inflammatory response in acute lung injury, and potentially provide a new therapeutic strategy for acute lung injury treatment. In addition, previous data also suggested that juglanin inhibits apoptosis and inflammatory response through TLR4-modulated MAPK/nuclear factor-κB (NF-κB) and JAK2/STAT3 signaling pathways respectively in rats with hepatitis (25). Also, juglanin could decrease the level of reactive oxygen species in senescent cells induced by

adriamycin treatment (26). Furthermore, juglanin displayed inhibitory activities against LPS-induced cytokine production in cells of macrophage RAW 264.7, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6 (27). In consideration of the valid function of juglanin in various disease treatments with little toxicity, we supposed that it may be utilized for patients of children with lung injury, which could be useful for the development of dietary supplements that alleviate lung injury for children.

In the present study, experimental mice were given LPS to induce acute lung injury with severe fibrosis and inflammation response. Then, juglanin at different concentrations were administered to mice. Following, the fibrosis and inflammation response *in vivo* were calculated through α-smooth muscle-actin (α-SMA), collagen type I and III as well as NF-κB-related signaling pathway to illustrate the role of juglanin in acute lung injury, supplying a new and effective treatment for children with acute lung injury in future.

Materials and methods

Animal treatment. Sixty male, 6-week-old C57BL6 mice, weight range 20-22 g, were purchased from experimental animals center of Nanjing Medical University of laboratory animal center (Nanjing, China). All the mice were carefully maintained at room temperature on a 12:12 h/light:dark cycle, 35% humidity, with free access to food and water in the cages. All animal experiments were performed to minimize animal suffering according to the Guide for the Care and Use of Laboratory Animals which was issued by the National Institutes of Health in 1996. This study was approved by the Ethics Committee on Animal Research at the Department of Pediatrics, Huai'an First People's Hospital, Nanjing Medical University, Nanjing, China. The mice were divided into 6 groups: the control group without any treatment (Con, n=10); LPS-treated group (15 mg/kg, LPS, n=10); 10 mg/kg juglanin-treated group after LPS teratment (JL, n=10); 20 mg/kg juglanin-treated group after LPS (JH, n=10); 10 mg/kg juglanin-treated group without LPS treatment (Con-JL, n=10); 20 mg/kg juglanin-treated group without LPS (Con-JH, n=10). After 10 days adaptation, mice from LPS group were treated by intraperitoneal injection with 15 mg/kg body weight LPS. The control group was administered with the same volume of saline. Then, the mice were consecutively administered with different concentrations of juglanin via oral gavage for 3 weeks. Juglanin, purchased from Yuanye Biotech (Shanghai, China), was dissolved in distilled water. Then, all experimental mice were sacrificed. Eyeball blood was collected and centrifuged at 12,000 x g for 15 min at 4˚C for following study, and the whole lung and liver tissues were carefully harvested on 4˚C glacial table, and then immediately frozen in liquid N_2, and stored at -80˚C for the following studies.

Cells culture and treatment. The human lung cell line, BEAS-2B, was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and human liver cell line L02, was obtained from KeyGen Biotech (Nanjing, China). The cells were maintained in a monolayer culture of 95% air and 5% CO_2 at 37˚C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 0.012% penicillin G, 0.027% streptomycin, 0.022% sodium pyruvate and 0.26% sodium bicarbonate. Juglanin stock solution was prepared in dimethyl sulfoxide (DMSO) and diluted to the desired final concentration (0, 1.25, 5, 10, 20, 40 and 80 μM) in culture medium for 24 h before use. The final concentration of DMSO did not exceed 0.1% (v/v).

Cell viability analysis. The lung and liver cells were planted in 96-well plates with a density of $5x10^3$ cells/well. Then, they were treated with different concentrations (0 to 80 μM) of juglanin for different times ranging from 0 to 72 h for cell viability analysis through Cell Counting kit-8 (CCK-8) (Zoman Biotechnology Co., Ltd., Beijing, China) to examine cell viability according to the instructions of the manufacturer.

Inflammatory cell counts of BALF. The samples from BALF were centrifuged at 3,000 rpm for 10 min at 4˚C to pellet the cells. Then, the cell pellets were re-suspended in phosphate-buffered saline (PBS) for the total cell number with a hemacytometer, and the cytospins were prepared for other differential cell number through Wright-Giemsa staining.

Chemical index measurement. The serum tumor necrosis factor-α (TNF-α) (cat. no. MTA00B), interleukin-1β (IL-1β) (cat. no. MLB00C), IL-4 (cat. no. M400B), IL-18 (cat. no. 7625), IL-6 (cat. no. M600B), and IL-17 (cat. no. M1700) (R&D Systems, Minneapolis, MN, USA) were measured according to the manufacturer's protocol using an enzyme-linked immunosorbent assay (ELISA) kit. Total serum and BAL IgE and IgA levels were determined by a mouse IgE (cat. no. DY1197; R&D Systems) and IgA (cat. no. F00295; Lengton Biological Technology, Shanghai, China) ELISA set. For the determination of cytokine levels, blood samples from the rats were obtained after sacrifice and stored at -80˚C until use. Lung tissue samples were isolated from the mice immediately after sacrifice, and was snap-frozen in liquid nitrogen and stored at -80˚C for later analysis. Lung tissue samples were collected were homogenized, and the proteins were extracted using hypotonic buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 μg/ml leupeptin, 1 mM Pefabloc SC, 50 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 4 mM benzamidine) to yield a homogenate. Then the final supernatants were obtained by centrifugation at 12,000 rpm for 20 min. Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as a standard. Next, the pro-inflammatory cytokine levels, including TNF-α, IL-1β, IL-18, IL-6, IL-4 and IL-17 were measured using the commercial ELISA kits, respectively, following manufacturer's instruction.

Immunohistochemical analysis. Formalin-fixed, paraffin-embedded mouse lung tissue samples were processed and then stained based on routine protocol of hematoxylin and eosin (H&E) (28). The stained slides, sectioned at 4 μM, were then observed with the light microscope and the digital micrographs of slides, which were taken for analyzing. The

Table I. Primary antibodies for western blot analysis.

Primary antibodies	Dilution ratio	Corporation
Rabbit anti-NF-κB	1:1,000	Abcam, ab207297
Rabbit anti-p-NF-κB	1:1,000	Abcam, ab86299
Rabbit anti-IκBα	1:2,000	Abcam, ab7212
Rabbit anti-IL-1β	1:1,000	Abcam, ab9722
Rabbit anti-p-IKKα	1:500	Abcam, ab38515
Rabbit anti-α-SMA	1:1,000	Abcam, ab5694
Rabbit anti-collagen type I	1:1,000	Abcam, ab34710
Rabbit anti-TGF-β1	1:1,000	Cell Signaling Technology, ab3711
Collagen type III	1:1,000	Cell Signaling Technology, ab7778
GAPDH	1:1,000	Cell Signaling Technology, ab5174

NF-κB, nuclear factor-κB; IL-1β, interleukin-1β; α-SMA, α-smooth muscle-actin; TGF-β1, transforming growth factor-β1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

inflammation score was determined by a pathologist as mild, moderate, or severe and degree of inflammation annotated as inflammatory score from 0 to 4 (minimum, 0; maximum, 4; 0, no injury; 1, injury up to 25% of the field; 2, injury up to 50% of the field; 3, injury up to 75% of the field; and 4, diffuse injury) was calculated as an index of the degree of lung injury for each sample described.

For periodic acid-Schiff (PAS) staining methods, the lung tissue samples were fixed in 95% alcohol for 10 min, prior to being washed, dried, stained by 1% periodic acid for 15 min and then washed by chilled PBS and dried twice. The tissue samples were then stained using Schiff reagent for 1 h, washed and dried, and stained subsequently with hematoxylin for 5 min, washed and dried. Finally, the tissue samples were observed with light microscopy. Wright-Giemsa staining was also carried out, following standard instructions. For the analysis of collagen deposition, Sirius red staining was performed by Shanghai Zhenda Biotechnology, Co., Ltd. (Sanghai, China).

For immunohistochemistry analysis, the fresh lung tissue samples were fixed in formalin for 48 h. Then the tissue block was put into paraffin and next cut into slides for the desired thickness in a microtome, and was then fixed into a slide. After washing, the samples were prepared for blocking and incubating with antibody α-SMA (1:200) and transforming growth factor-β1 (TGF-β1) (1:200), which were diluted in 5% horse serum with chilled PBS at 4˚C overnight. Isotype-matched IgG was used instead of primary antibody as a negative control of the staining. Sections were then incubated with diluted streptavidin-peroxidase HRP at room temperature with a staining kit, following the manufacturer's instructions. The sections were then stained with hematoxylin for 5 min and mounted and observed with a phase-contrast microscope.

Fluorescence imaging. Tissue samples were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde in PBS for 15 min. Cells were permeabilised for 5 min with 0.1% Triton X-100. For p-NF-κB staining, 50 μg/ml mouse anti-caspase-3 (1:200) antibodies were employed followed by staining with 2 μg/ml Alexa Fluor 488 goat anti-mouse secondary antibodies. DAPI (Sigma-Aldrich) was used in this part of the studied. Images were acquired by confocal laser scanning via epifluorescence microscopy (Sunny Biotech Co., Ltd., Shanghai, China). After observation and obtaining the representative images, ImageJ software, focused on biological-image analysis, was used to calculate the fluorescent intensity, representing the number of positive cells (29).

Western blot assays. The lung tissue samples and lung cells were homogenized into 10% (wt/vol) hypotonic buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 μg/ml leupeptin, 1 mM pefabloc SC, 50 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 4 mM benzamidine) to yield a homogenate. The final supernatants were obtained by centrifugation at 12,000 rpm for 20 min. Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific) with bovine serum albumin as a standard. The total protein extract was also used for western blot assays. Equal amounts (30 μM) of total protein of tissues were subjected to 10 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting using the following primary polyclonal antibodies: rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-1β, P-NF-κB, NF-κB, p-IKKα, IκBα, α-SMA, TGF-β1, collagen type I and collagen type III. Immunoreactive bands were visualized by ECL immunoblot detection system (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to Kodak (Eastman Kodak Co., Rochester, NY, USA) X-ray film. Each protein expression level was defined as grey value (version 4.2b, Mac OS X, ImageJ; National Institutes of Health, Bethesda, MD, USA) and standardized to housekeeping gene (GAPDH) and expressed as a fold of control. The specific information of primary antibodies used here is listed in Table I.

RT-qPCR analysis. Total RNA from tissue samples and cultured cells was isolated through the mirVana miRNA isolation kit (Ambion, Foster City, CA, USA) based on the manufacturer's instruction. Then the cDNA was synthesized from total RNA with the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was conducted using the Applied Biosystems 7500 sequence detection system with iQ™ SYBR-Green SuperMix (Bio-Rad Laboratories, Hercules, CA, USA) containing 5 ng cDNA and 10 pM of each primer. The data were normalized to the geometric mean of housekeeping gene GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method. Fold changes in mRNA levels of target gene relative to the endogenous GAPDH control were calculated. In brief, the cycle threshold (Ct) values of each target gene was subtracted from Ct values of housekeeping gene cyclophilin (ΔCt). The target gene ΔΔCt was calculated as ΔCt of target gene minus ΔCt of control. The fold change in mRNA expression was evaluated as $2^{-\Delta\Delta Ct}$. All sequences involved in this study are shown as follows: sense primers of TGF-β1, 5'-GAA GGT GAG AGG ATG G-3' and antisense primers of TGF-β1, 5'-AGA CCT AGA CTA GGC CAA GT-3'; sense primers of collagen

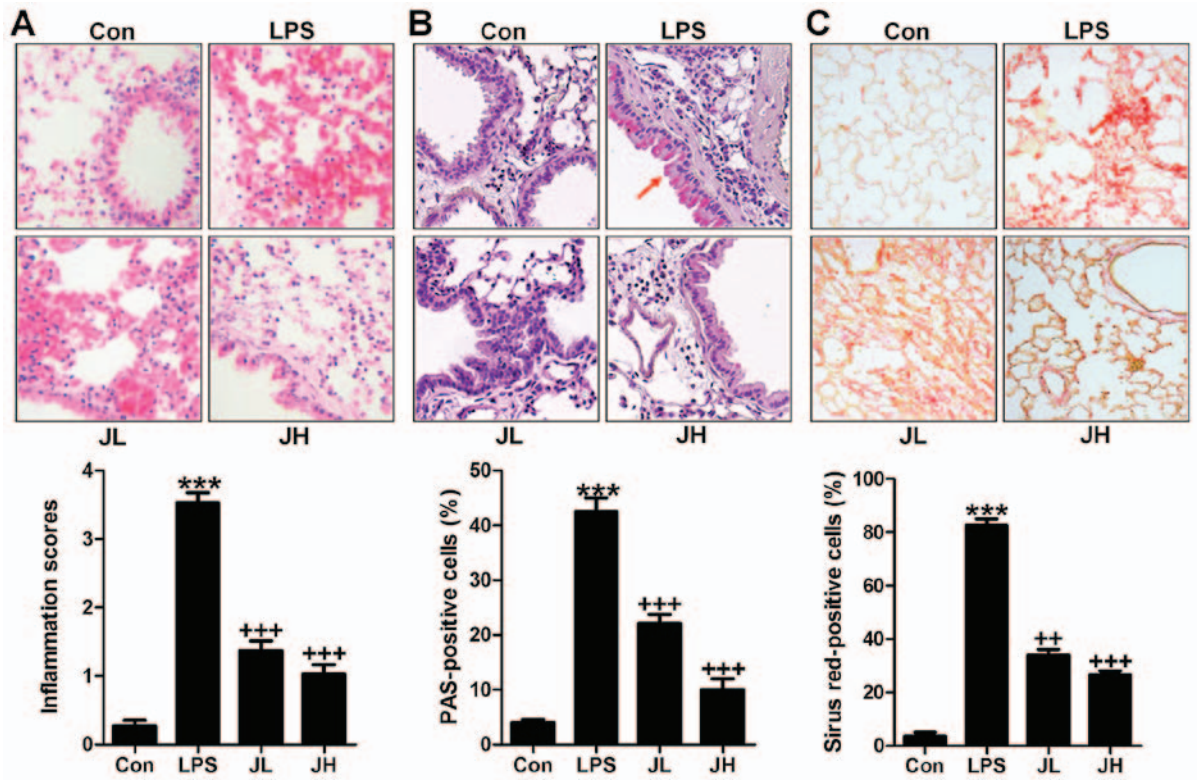

Figure 1. Lipopolysaccharide (LPS)-induced acute lung injury in mice is ameliorated for juglanin administration. (A) Upper, representative images of lung injury by hematoxylin and eosin (H&E) staining. Lower, the quantification of inflammatory response following H&E staining. (B) Upper, PAS staining was used to observe goblet cells in LPS-induced mice with acute lung injury. Lower, the quantification of PAS positive cells. (C) Upper, Sirius red analysis was used to calculate fibrosis levels in LPS-induced acute lung injury. Lower, the quantification of Sirius red positive cells is shown. The data are shown as mean ± SEM (n=10). $^{*}p<0.05$ and $^{**}p<0.001$ vs. the control (Con). $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. LPS-induced mice (LPS).

type I, 5'-AAA GGT AAC AAG ACG TGG-3' and antisense primers of collagen type I, 5'-TCG TAT ACT GTC AGG GAG AGA T-3'; sense primers of collagen type III 5'-GAA GGC AGA GAG AAG GTA G-3' and antisense primers of collagen type III, 5'-AGA CGG AAT GGA GAA GAC ACA T-3'; sense primers of α-SMA, 5'-AGC TAG TGA ACA ACA GTG CG-3' and antisense primers of α-SMA, 5'-TAA GCT CAG AGG CTA TCG TAT-3'; sense primers of GAPDH, 5'-AAT CAT AAC GCG AGG CCG GA-3' and antisense primers of GAPDH, 5'-CCA TAT ACA GTC ACC CGA CAC AC-3'.

Data analysis. Data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA) by ANOVA with Dunnet's least significant difference post-hoc tests. A p-value <0.05 was considered significant.

Results

LPS-induced acute lung injury in mice is ameliorated after juglanin administration. LPS induction is well known to cause injury in different organs, including liver, renal and heart, even the lung, and has been investigated before (30). In this study, preliminary experiment was performed to verify that the inflammatory-mouse model was set up correctly through evaluating the number of macrophages, neutrophils and lymphocytes, as well as the total cells in BALF, and H&E staining. The data indicated that after 15 mg/kg LPS treatment for 6 h, the number of macrophages, neutrophils and lymphocytes, as well as the total BALF was significantly higher than that in the control group, which is a hallmark for ALI. In addition, H&E staining analysis also indicated that LPS induced lung injury in mice compared to the Con group. After the pre-experiments mentioned above, we considered that ALI models were successfully established (data not shown). In order to prove whether juglanin has potential value on airway inflammation in children, LPS was also involved to cause acute lung injury in mice. In this regard, H&E staining was carried out to observe the injured condition of lung tissue samples. We found that after LPS treatment in mice, the lung tissue sample showed higher inflammation score compared to the Con group with significant difference (Fig. 1A). After, juglanin treatment, the inflammatory response was attenuated. Additionally, PAS analysis also indicated that the PAS positive cell levels were significantly increased with LPS exposure, while decreased by juglanin administration in a dose-dependent manner (Fig. 1B). Further, Sirius red assays illustrated that the fibrosis levels were more severe in LPS-treated lung tissue samples from mice. Of note, juglanin treatment indicated that the Sirius red positive cells were highly reduced ameliorating fibrosis progression (Fig. 1C). The data above suggested that LPS indeed resulted in severe lung injury, and juglanin showed protective role against acute lung injury caused by LPS in mice.

Juglanin reduces inflammatory cell infiltrate in LPS-induced mice with acute lung injury. In this regard, the role of juglanin

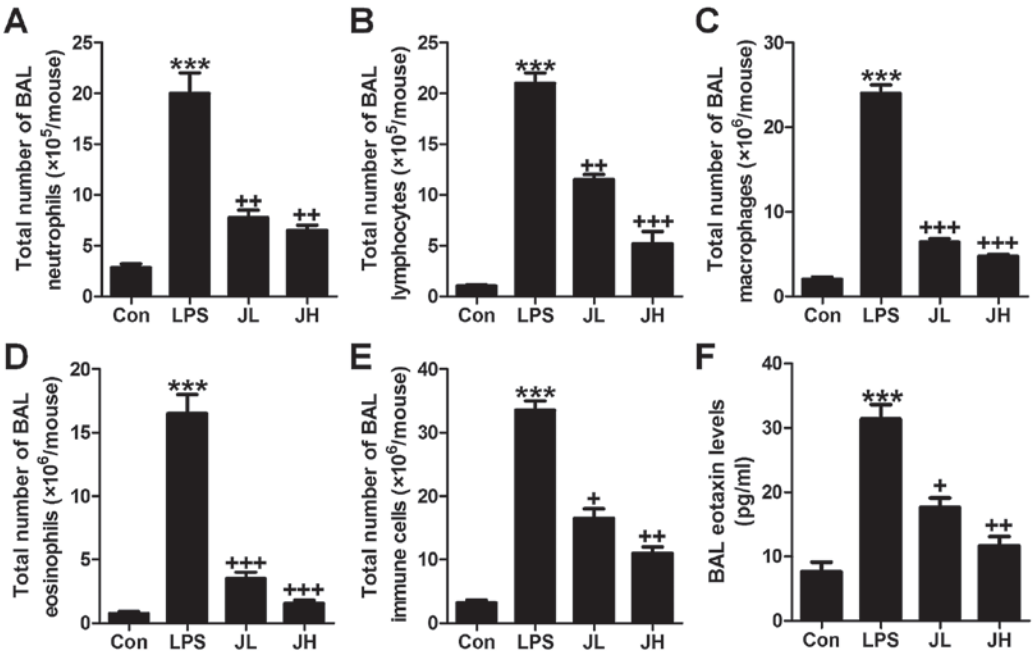

Figure 2. Juglanin reduces inflammatory cell infiltrate in lipopolysaccharide (LPS)-induced mice with acute lung injury. The number of cells in inflammation infiltration was determined. (A) Total neutrophils, (B) total lymphocytes, (C) total macrophages, (D) total eosinolhils and (E) total cells. (F) Eotaxin levels in bronchoalveolar lavages (BAL) were measured. The data are shown as mean ± SEM (n=10). $^{*}p<0.05$ and $^{**}p<0.001$ vs. the control (Con); $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. LPS-induced mice (LPS).

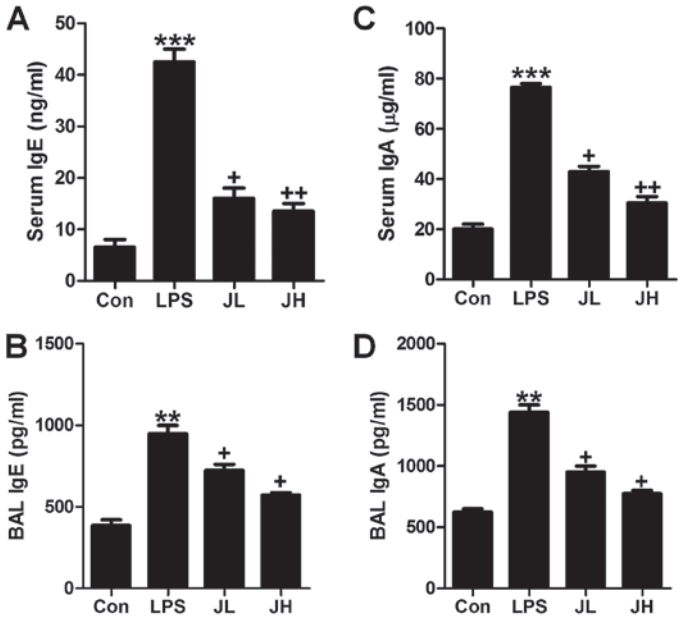

Figure 3. Juglanin downregulates IgE and IgA in serum and bronchoalveolar lavages (BAL) in lipopolysaccharide (LPS)-induced mice. (A) Serum IgE and (B) BAL IgE levels were calculated in juglanin-treated mice after LPS induction. (C) Serum IgA and (D) BAL IgA levels were measured in juglanin-treated mice after LPS induction. The data are shown as mean ± SEM (n=10). $^{*}p<0.05$ and $^{**}p<0.001$ vs. the control (Con); $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. LPS-induced mice (LPS).

in inflammation regulation was explored. First of all, the bronchoalveolar lavage (BAL) was conducted and investigated. As shown in Fig. 2, LPS treatment contributed to acceleration of neutrophils (Fig. 2A), lymphocytes (Fig. 2B), macrophages (Fig. 2C), and eosinophils (Fig. 2D), and elevation of the total number of BAL eventually (Fig. 2E). Juglanin significantly reduced the total number of neutrophils (Fig. 2A), lymphocytes (Fig. 2B), macrophages (Fig. 2C), and eosinophils (Fig. 2D), as well as the total number of immune cells in BAL (Fig. 2E). The whole process was in a dose-dependent manner. Furthermore, in the lung tissue samples after LPS induction, eotaxin level in BAL was apparently downregulated, which was attenuated by juglanin treatment (Fig. 2F). In conclusion, the data above indicated that juglanin has a potential role in controlling inflammation and cell infiltration in mice with acute lung injury induced by LPS.

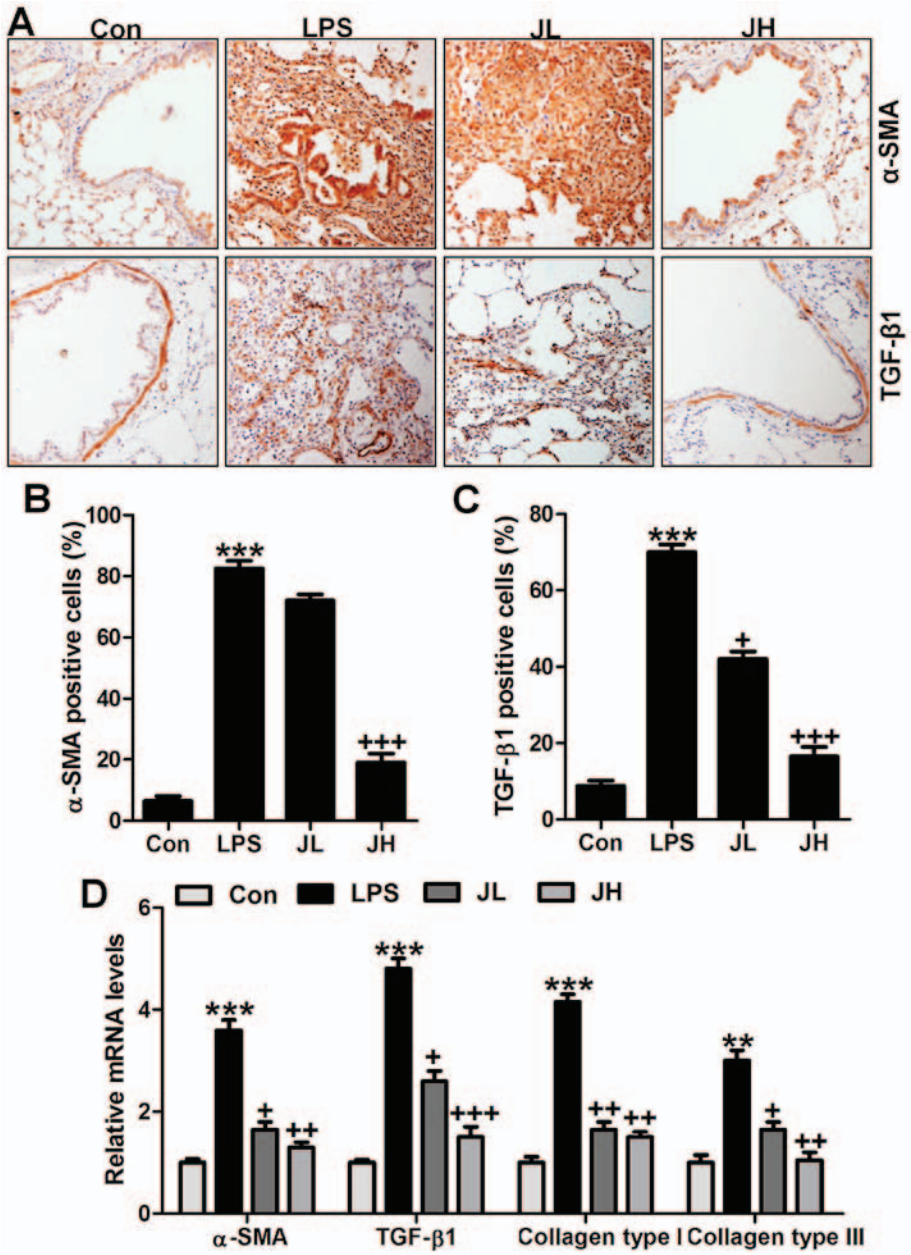

Figure 4. Juglanin-ameliorated fibrosis progression in mice induced by lipopolysaccharide (LPS). (A) Immnohistochemical analysis of fibrosis markers of α-smooth muscle-actin (α-SMA) and transforming growth factor-β1 (TGF-β1). The quantification of (B) α-SMA, and (C) TGF-β1 was exhibited following immunohistochemical analysis. n=6. (D) RT-qPCR assays were conducted to explore mRNA levels of α-SMA, TGF-β1, collagen type I and collagen type III. The data are shown as mean ± SEM (n=10). $^{*}p<0.05$ and $^{**}p<0.001$ vs. the control (Con); $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. LPS-induced mice (LPS).

Juglanin downregulates lgE and lgA in serum and BAL in LPS-induced mice. In order to further investigate if juglanin could ameliorate LPS-induced acute lung injury, lgE and lgA levels in serum and BAL were calculated. Accroding to a previous study, lgE and lgA high expression were markers contributing to various diseases (31). As shown in Fig. 3A and B, the lgE levels in serum and BAL were significantly upregulated in LPS-treatment. Juglanin administration reversed lgE expression levels in a dose-dependent manner. Consistent with lgE alteration, lgA levels in serum and BAL were also found to be highly expressed for LPS-induction, and were inhibited in serum and BAL (Fig. 3C and D). The results above indicated that juglanin has a role in suppressing lgE and lgA expression levels to improve acute lung injury induced by LPS in mice.

Juglanin-ameliorated fibrosis progression in mice induced by LPS. Lung fibrosis is known as a significant injury induced by LPS (32). In our study, to estimate the extent of fibrosis, we calculated α-SMA, TGF-β1, collagen type I and collagen type III through different analysis. As shown in Fig. 4A, α-SMA and TGF-β1 were significantly upregulated in LPS treatment in line with previous studies (33). Of note, juglanin administration significantly downregulated α-SMA and TGF-β1 levels in LPS-treated mice through immunohistochemical analysis (Fig. 4B and C). IgG-isotype control staining showed no positive signal by immunohistochemical analysis. Similarly, α-SMA and TGF-β1 mRNA levels, as well as collagen type I and collagen type III gene levels were also upregulated with significant difference compared to the Con ones after LPS treatment. Interestingly, juglanin at two

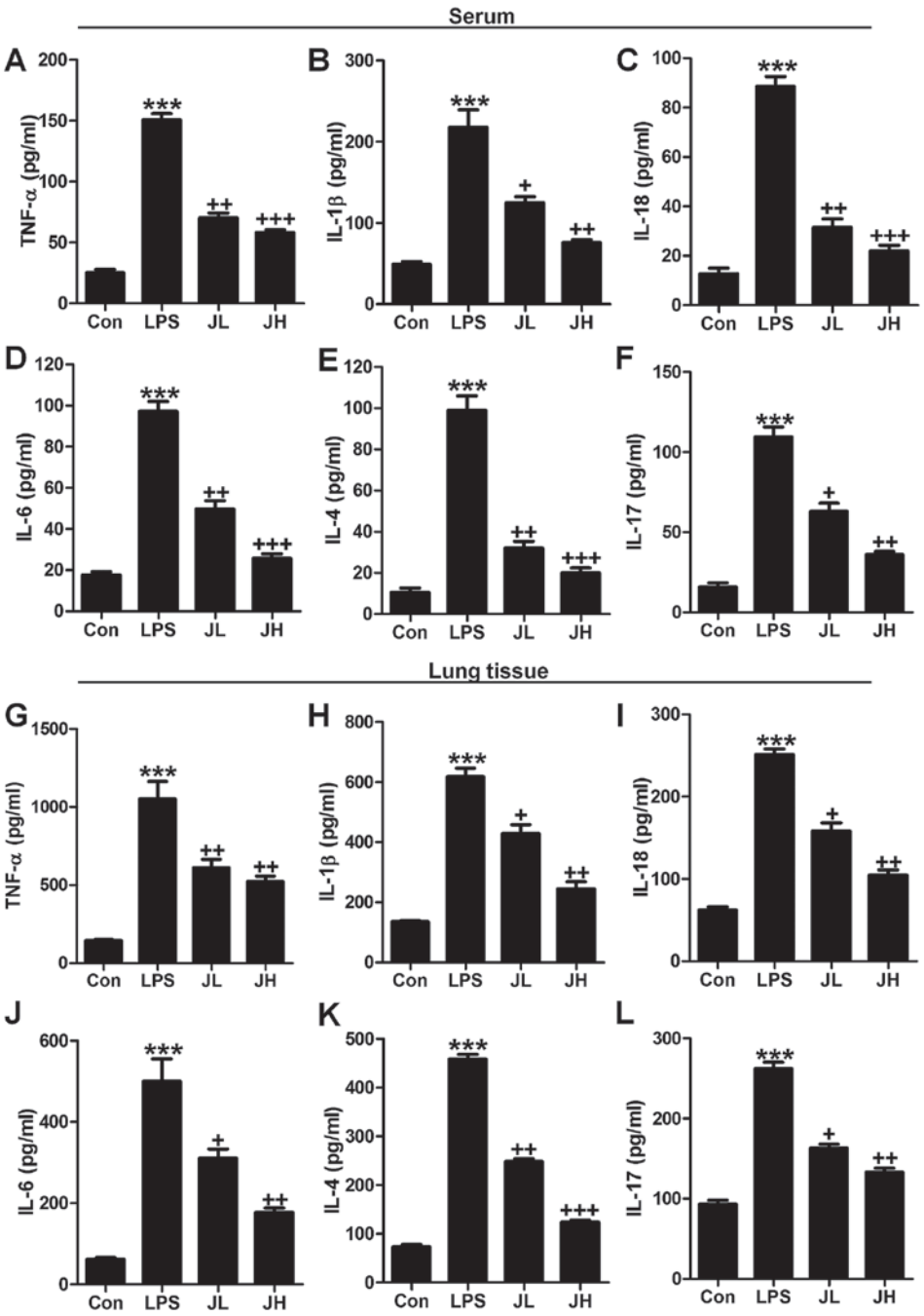

Figure 5. Juglanin improves pro-inflammatory cytokine releases caused by lipopolysaccharide (LPS). Serum pro-inflammatory cytokines were measured, including (A) TNF-α, (B) interleukin-1β (IL-1β), (C) IL-18, (D) IL-6, (E) IL-4 and (F) IL-17. Pro-inflammatory cytokines of (G) TNF-α, (H) IL-1β, (I) IL-18, (J) IL-6, (K) IL-4 and (L) IL-17 in lung tissue samples induced by LPS were measured after juglanin administration. The data are shown as mean ± SEM (n=10). $^{*}p<0.05$ and $^{**}p<0.001$ vs. the control (Con); $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. LPS-induced mice (LPS).

concentrations reduced α-SMA and TGF-β1, collagen type I and III apparently compared to the LPS alone-treated group through RT-qPCR analysis (Fig. 4D). Taken together, the results in this part indicated that fibrosis was induced in mice in LPS exposure, and juglanin exhibited suppressive role in fibrosis progression in acute lung injury in mice after LPS induction.

Juglanin improves pro-inflammatory cytokine release caused by LPS. Inflammation response is known as a major reason to cause tissue injury in different organs after LPS treatment, such as liver and renal injury (34). Also, previous studies reported that LPS induction resulted in lung injury, which was also attributed to inflammatory response (35). As further confirmation, serum and lung tissue samples of pro-inflammatory cytokines of TNF-α, IL-1β, IL-18, IL-6, IL-4 and IL-17 were determined. As shown in Fig. 5, we found that pro-inflammatory cytokines of TNF-α (Fig. 5A), IL-1β (Fig. 5B), IL-18 (Fig. 5C), IL-6 (Fig. 5D), IL-4 (Fig. 5E) and IL-17 (Fig. 5F) were significantly upregulated in LPS treatment, which was in line with previous studies (36). Of note, after juglanin administration, these pro-inflammatory cytokines were highly downregulated compared to the LPS ones. In addition, the pro-inflammatory cytokines in lung

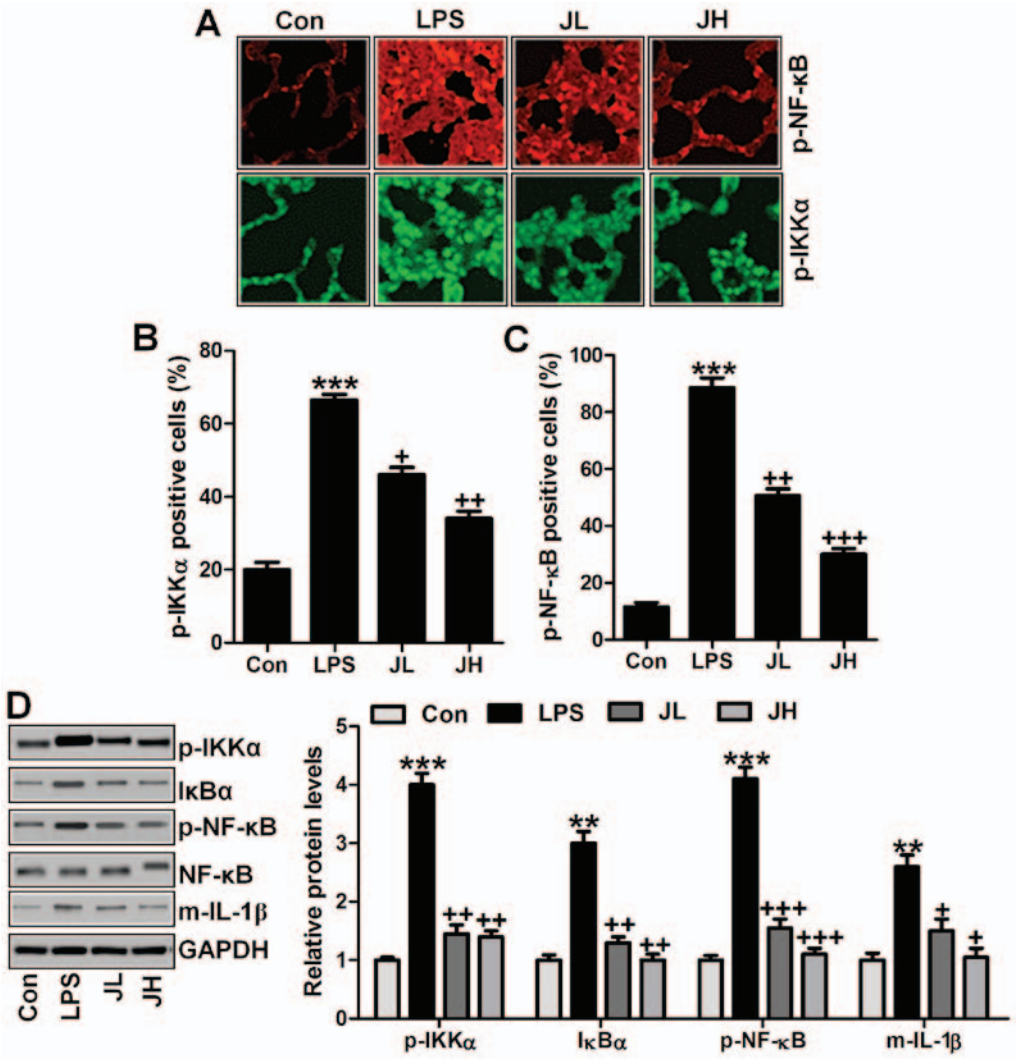

Figure 6. Nuclear factor-κB (NF-κB) signaling pathway was involved in juglanin-ameliorated inflammation response in mice with acute lung injury induced by lipopolysaccharide (LPS). (A) Immunofluorescence was included to explore phosphorylated NF-κB and IKKα expression levels. (B) The phosphorylated NF-κB and (C) IKKα levels were quantified following immunofluorescence analysis. (D) Western blot analysis was conducted to explore IKKα/NF-κB signaling pathway, including signals of IKKα phosphorylation, IκBα, NF-κB phosphorylation as well as the mature IL-1β, and the quantified levels are displayed in the right panel. The data are shown as mean ± SEM (n=10). *p<0.05 and **p<0.001 vs. the control (Con); $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 vs. LPS-induced mice (LPS).

tissue samples were also found to be upregulated with higher expression of TNF-α (Fig. 5G), IL-1β (Fig. 5H), IL-18 (Fig. 5I), IL-6 (Fig. 5J), IL-4 (Fig. 5K) and IL-17 (Fig. 5L), which were also reduced in juglanin treatment. The data above indicated that juglanin has a role in suppressing inflammation response in LPS-induced acute lung injury in mice.

NF-κB signaling pathway was involved in juglanin-ameliorated inflammation response in mice with acute lung injury induced by LPS. As mentioned above, inflammation response has been observed in LPS-induced mice with acute lung injury. Thus, here we attempted to explore how juglanin altered LPS-induced acute lung injury in mice. NF-κB signaling pathway is a key to modulate pro-inflammatory cytokine releases (37). Thus, in our study, this classic pathway was also investigated to prove whether NF-κB was involved in our study. As shown in Fig. 6A, immunofluorescent analysis was carried out to explore how phosphorylated NF-κB and IKKα are changed in LPS-treated mice with or without juglanin treatment. The data indicated that NF-κB and IKKα phosphorylation levels were dramatically upregulated in LPS treatment, which were then significantly downregulated for juglanin treatment in a dose-dependent manner (Fig. 6B and C). In addition, IgG-isotype control staining suggested that no positive signal was observed by immunofluorescent analysis. Furthermore, western blot analysis was used to prove that IKKα phosphorylated levels, IκBα expression, and NF-κB activity were found to be stimulated greatly by LPS treatment compared to the Con group, which was downregulated in the juglanin-treated groups, contributing to pro-inflammatory cytokine of IL-1β decrease (Fig. 6D). The data here indicated that juglanin could attenuate LPS-induced inflammation response through pro-inflammatory cytokine release via IKKα/NF-κB signaling pathway suppression.

The effects of juglanin on the toxicity in vitro and in vivo. We evidenced *in vivo* that juglanin showed suppressive role in acute lung injury progression in LPS-treated mice. As further confirmation *in vitro* studies were carried out. The cell viability was estimated for juglanin treatment in BEAS-2B

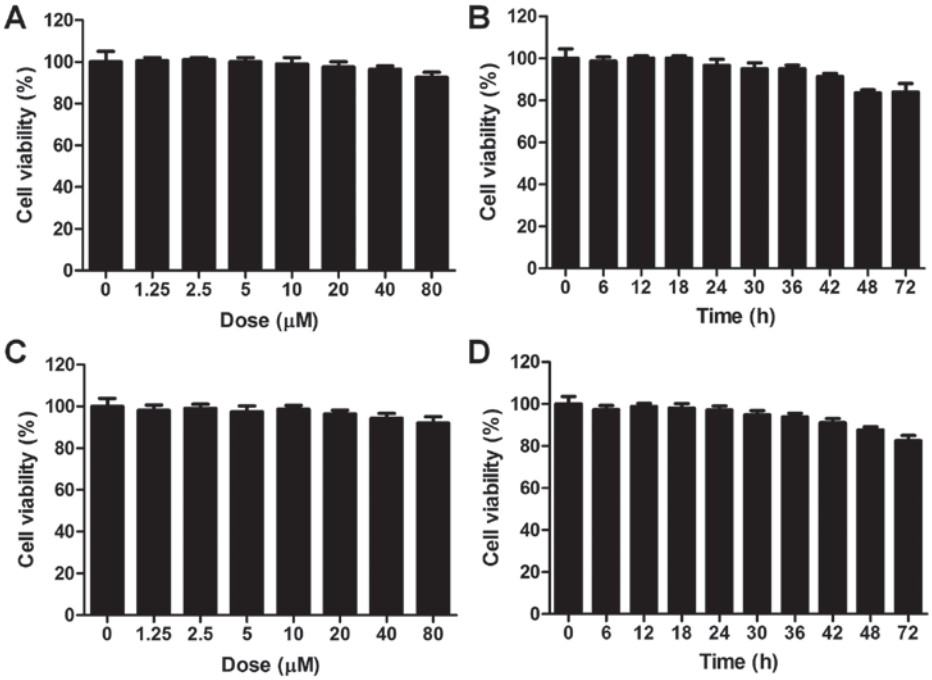

Figure 7. The effects of juglanin on the cell viability *in vitro*. (A) Cell counting kit-8 (CKK-8) assays were used to calculate normal lung cell BEAS-2B viability. The cells were treated with different concentrations (0, 1.25, 2.5, 5, 10, 20, 40 and 80 μM) of juglanin for 24 h, followed by CCK-8 analysis. (B) The BEAS-2B cells were treated with 80 μM juglanin for different times as indicated. Then, the cell viability was measured. (C) The liver normal cells, L02, were treated with different concentrations of juglanin ranging from 0 to 80 μM for 24 h. Then, CCK-8 analysis was conducted to calculate the cell viability. (D) The L02 cells were treated with 80 μM juglanin for various times (0 to 72 h). Then, the L02 cell viability was determined.

cells (Fig. 7A). The cells were treated with different concentrations of juglanin ranging from 0 to 80 μM for 24 h. The cells were collected for CCK-8 assays. No significant difference was observed after juglanin administration, even at the highest dose of juglanin. Also, 80 μM juglanin was used to treat cells for different times as indicated, and no difference was shown (Fig. 7B). In order to further ensure that juglanin was non-toxic on cells, liver normal L02 cells were used. As shown in Fig. 7C, L02 cells were treated with juglanin (0, 1.25, 2.5, 5, 10, 20, 40 and 80 μM) for 24 h. The cell viability was not changed between the groups. Also, after various times of treatment, no significant difference was observed among the groups with 80 μM treatment (Fig. 7D). Further, *in vivo*, the experimental mice without LPS induction were administered with juglanin at different concentrations. After sacrifice, the liver, renal and spleen tissue specimens were isolated for H&E staining. As shown in Fig. 7E, no significant difference was observed among the Con and the Con with juglanin treatment groups, indicating that juglanin induced little toxicity *in vivo*. The data above indicated that juglanin shows no toxicity on lung cells or liver cells. Thus, 40 and 80 μM juglalnin was used for the following studies *in vitro*.

Juglanin suppresses fibrosis development in lung cells in vitro. Fibrosis expression levels were further explored to prove that juglanin is useful for acute lung injury improvement by fibrosis inhibition. As shown in Fig. 8A and B, α-SMA was highly upregulated in 100 ng/ml LPS treatment, whereas downregulated by juglanin administration, which was in agreement with the results above *in vivo*. Next, western blot analysis further indiated that α-SMA, TGF-β1, collagen type I and collagen type III were dramatically upregulated in LPS treatment. Of note, juglanin administration showed extremely suppressive role for these fibrosis markers compared to the LPS alone-treated group (Fig. 8C and D). The data above illustrate that juglanin ameliorated LPS-induced fibrosis development in lung cells *in vitro*.

Juglanin inhibits inflammation response in lung cells induced by LPS in vitro. *In vivo* studies, we evidenced that inflammation response was induced for LPS. Thus, here we attempted to determine if juglanin could improve acute lung injury from inhibiting inflammatory response in lung cells of BEAS-2B. First, immunofluorescent analysis was carried out to calculate phosphorylated NF-κB levels. As shown in Fig. 9A and B, high intensity of phospho-NF-κB was observed in LPS-treated cells, while being downregulated in juglanin administration in a dose-dependent manner. Furthermore, western blot analysis indicated that phospho-IKKα, IκBα and phospho-NF-κB were dramatically upregulated in LPS-treated cells. Juglanin treatment at different concentrations could downregulate expression of these factors. In agreement with this, pro-inflammatory cytokines of IL-1β was apparently accelerated for LPS induction, which was reduced greatly for juglanin (Fig. 9C and D). Here, this study indicated that juglanin *in vivo* showed inhibitory role in inflammation response induced by LPS *in vitro* to attenuate lung injury.

Discussion

Acute lung injury in children has been reported previously (1,38). Currently, though treatments have been investigated and advanced, short-term morbidity as well as mortality still always occur in patients. Many patients are

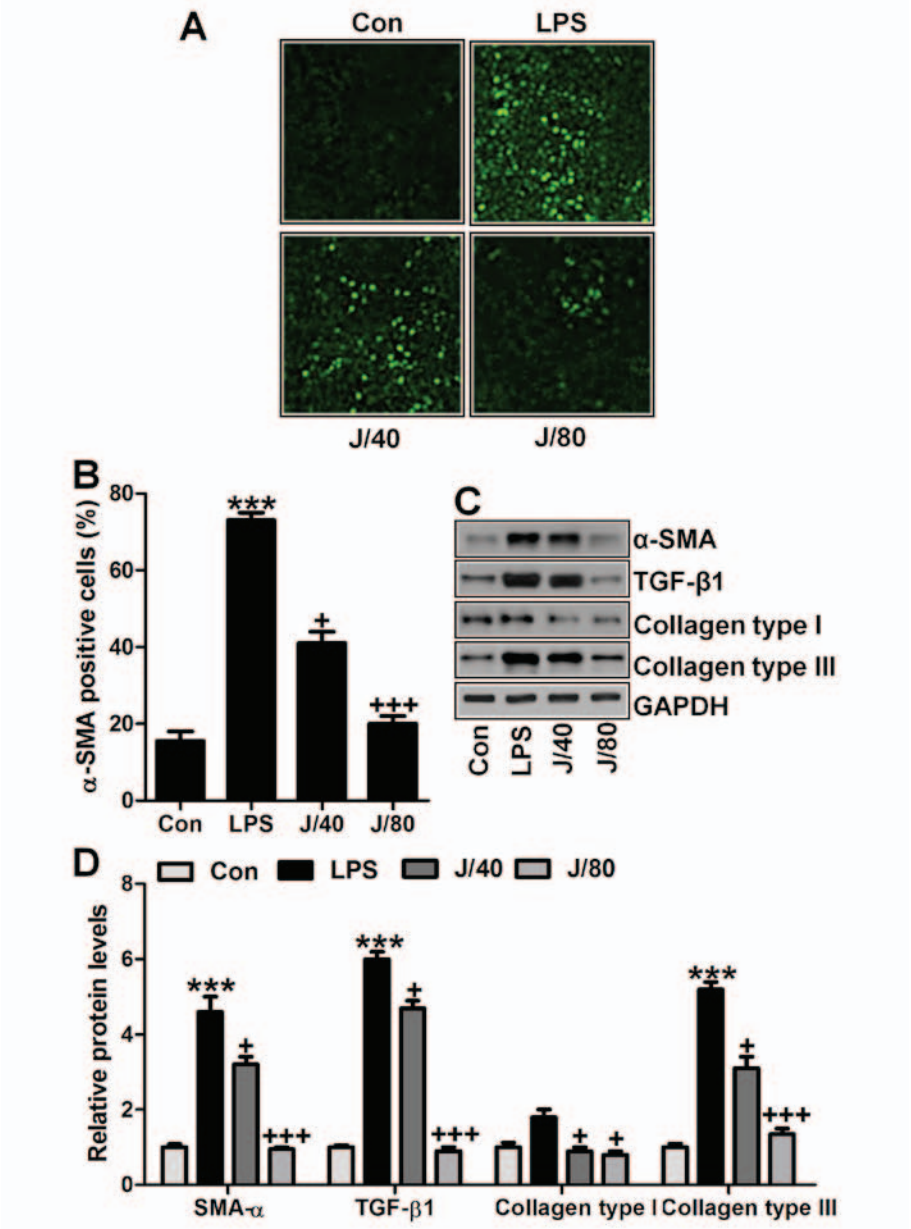

Figure 8. Juglanin suppressed fibrosis development in lung cells *in vitro*. (A) The lung cells of BEAS-2B were treated with 100 ng/ml lipopolysaccharide (LPS) for 24 h, followed by 40 or 80 mM juglanin administration for another 24 h. Then, immunofluorescent analysis was used to calculate α-smooth muscle-actin (α-SMA) positive cells. (B) The quantification of α-SMA was assessed after immunofluorescence assays. (C) Western blot analysis was used to determine α-SMA, transforming growth factor-β1 (TGF-β1), collagen type I and collagen type III expression levels in LPS-induced cells in the absence or presence of juglanin (40 or 80 μM). (D) The quantification of western blot analysis results is shown. The data are presented as mean ± SEM (n=6). *p<0.05 and **p<0.001 vs. the control (Con); $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 vs. LPS-induced mice (LPS).

unable to suppress lung injury due to severe tissue damage (39). Additionally, molecular mechanism still needs clarification on acute lung injury triggered by LPS, resembling the disease in human, leading to unusual metabolism (40,41). Accumulating evidence has suggested that LPS treatment could result in lung dysfunction with serious injury (42). Juglanin is a natural compound extracted from the crude '*Polygonum aviculare*', showing bioactivity against inflammation response, as well as cancer growth (43). Recently, the anti-inflammation and anti-oxidative activity of juglanin has been reported, suggesting that juglanin may possess effects on suppressing LPS-induced diseases (44). Therefore, in our present study, juglanin was investigated to clarify if it could be used as a therapeutic strategy in acute lung injury induced by LPS. This is the first time that juglanin was included in a study of acute lung injury from the aspects of fibrosis and inflammation regulation.

In our study, mice exposed to LPS were protected from acute lung injury due to juglanin administration. Mice exposed to LPS alone showed higher inflammation infiltration in BAL and lgE and lgA both in serum and in BAL. Neutrophils and macrophages were the main inflammatory cells in acute lung injury. They infiltrated into the lung tissues, releasing enzymes phagocytizing the pathogen (45,46). Moreover, these inflammatory cells were the fundamental source of inflammatory mediators *in vivo* (47). In LPS-induced inflammation, neutrophils and macrophages were activated. After activation, neutrophils and macrophages were recruited to the inflammation site (48). Lymphocytes have drawn increased

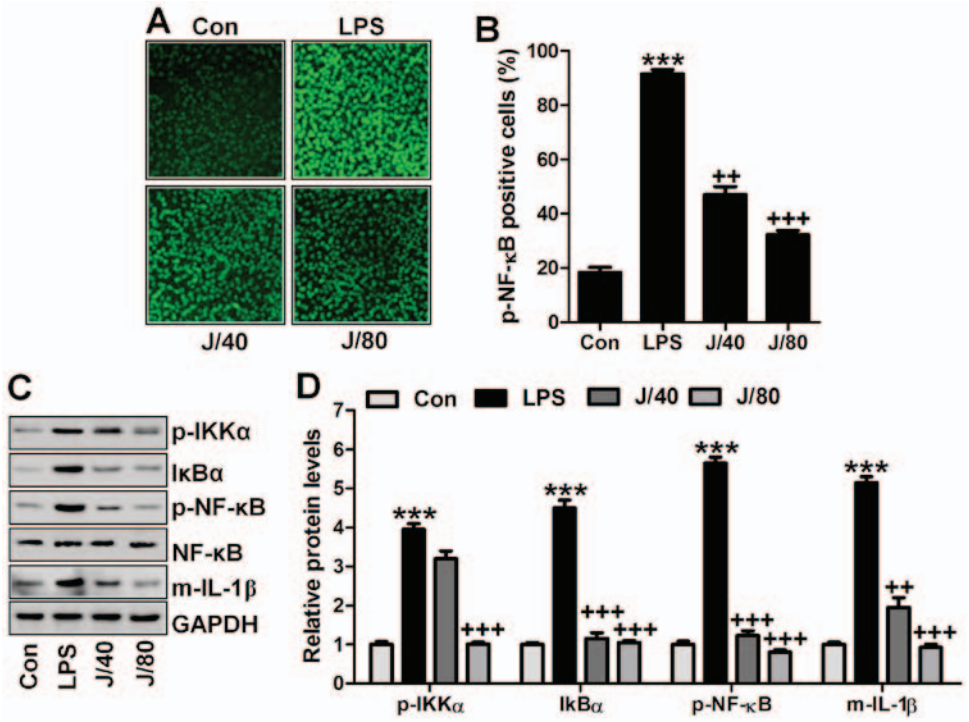

Figure 9. Juglanin inhibits inflammation response in lung cells induced by lipopolysaccharide (LPS) *in vitro*. The lung cells of BEAS-2B were treated with 100 ng/ml LPS for 24 h, and then 40 or 80 μM juglanin was administered to cells for another 24 h. (A) The phosphorylated nuclear factor-κB (NF-κB) levels were assessed via immunofluorescent analysis. (B) The quantification of NF-κB phosphorylation is displayed. (C) IKKα phosphorylation, IκBα, NF-κB phosphorylation, the mature interleukin-1β (IL-1β) protein levels were measured by immunoblotting analysis. (D) The quantification of phosphorylated IKKα/NF-κB, IκBα as well as IL-1β in mature formation. The data are represented as mean ± SEM (n=6). $^{*}p<0.05$ and $^{**}p<0.001$ vs. the control (Con); $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ vs. LPS-induced mice (LPS).

attention, and there is accumulating evidence indicating that lymphocytes play important roles in the development and progression of acute lung injury (49,50). It has been suggested that lymphocytes contribute to the progression of autoimmune and inflammatory diseases (51). Thus, these cells levels could be an indicator supporting the role of LPS on acute lung injury, and juglanin could reduce these cells to prevent inflammation response, revealing the anti-inflammatory effect of juglanin on lung injury treatment. Previous studies reported that abundant abnormal collagen accumulation in lung tissue samples was observed after examination of patients with lung injury (52-54). In addition, the collagen type III accumulation may occur in lung disease of acute injury (55). In addition, α-SMA expression is closely related to fibrosis accumulation (56). Further, LPS induction upregulated markers of fibrosis, such as α-SMA, collagen type I and collagen type III, as well as TGF-β1, which have been indicated as significant markers, inducing fibrosis development. TGF-β1 is known to modulate various cellular responses (57). Significantly, TGF-β1 is a key fibrotic cytokine, regulating organ fibrosis and dysfunction. During the progression of fibrosis, TGF-β1 stimulates a number of fibrogenic genes, such as α-SMA (58). In this study, we found that those markers were dramatically upregulated via western blot and RT-qPCR analysis, which was in line with previous studies (59). The results above indicated that LPS treatment, indeed, induced lung fibrosis development and progression in mice. Notably, juglanin has potential role in reducing fibrosis markers both *in vitro* and *in vivo*, improving fibrosis accumulation in the lung tissue samples of mice.

Inflammation is known to be involved in fibrosis development, accompanied with pro-inflammatory cytokine release, including IL-18, IL-6, IL-1β and TNF-α (60,61). NF-κB signaling pathway is of great importance in inflammation response through transfection activation (62). NF-κB phosphorylation could be activated following IκBα activity improvement (63,64). IKKα, as an up-stream signal of IκBα/NF-κB, was found to be upregulated in LPS-treated mice, activating IκBα and NF-κB expression. Pro-inflammatory cytokines of IL-6, IL-1β and TNF-α expression levels were also promoted subsequently, eventually contributing to inflammatory responses. Of note, juglanin exhibited anti-inflammatory role in LPS-induced acute lung injury, which was in agreement with previous studies. Similarly, *in vitro* juglanin downregulated pro-inflammatory cytokine release, as well as IKKα, and IκBα/NF-κB expression induced by LPS induction.

In conclusion, the data above suggested that LPS induced acute lung injury in mice with upregulation of α-SMA, TGF-β1 and collagen, which could be ameliorated by juglanin. Inflammatory response in mice and lung cells stimulated by LPS was also inhibited by juglanin through suppressing NF-κB signaling pathway. Significantly, our study suggested the possible role of juglanin in preventing acute lung injury by fibrosis formation and inflammation inhibition, which is of great value as useful assistant dietary supplements and for finding new and effective therapeutic strategy in acute lung injury for children in future.

Acknowledgements

The authors would like to thank the reviewers for their comments and help to promote this work.

Funding

No funding was received.

Availability of data and material

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZWD designed and performed the experiments. YFY wrote the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee on Animal Research at the Department of Pediatrics, Huai'an First People's Hospital, Nanjing Medical University, Nanjing, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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