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Original Article

Recombinant human insulin-like growth factor binding protein 3 attenuates lipopolysaccharide-induced acute lung injury in mice

Lili Kang[1,3], Xiaoying Li[1], Jianhong Liu[1], Yanfang Li[1], Sitao Li[2], Cuifen Zhao[3]

[1]Department of Neonatology, Qilu Children's Hospital, Shandong University, NO. 23976, Jingshi Road, Jinan 250022, Shandong Province, China; [2]Department of Neonatology, The Sixth Affiliated Hospital, Sun Yat-sen University, NO. 26, Erheng Road, Yuancun Village, Tianhe District, Guangzhou 510655, Guangdong Province, China; [3]Department of Pediatrics, Qilu Hospital, Shandong University, No. 107, Wen Hua Xi Road, Jinan 250012, Shandong Province, China

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Abstract: Acute lung injury (ALI) severely impairs gas exchange and results in high mortality. Insulin-like growth factor binding protein 3 (IGFBP-3) plays a crucial role in diverse lung diseases; however, the expression and function of IGFBP-3 in ALI remain unclear. In the present study, mice were injected with lipopolysaccharide to establish ALI, and IGF and IGFBP3 expression was measured using ELISA, western blotting, and immunohistochemical staining. Mice with ALI were then treated with recombinant human IGFBP-3 (rhIGFBP-3), and treatment was evaluated using survival analysis, histological staining analysis, and inflammatory cytokine expression in lung tissues and bronchoalveolar lavage fluid (BALF). The expression of NF-κB and VEGF was also measured using western blotting and ELISA in ALI mice. Our results demonstrated the upregulation of IGF expression in lung tissues and BALF of ALI mice, accompanied by downregulation of IGFBP-3. Administration of rhIGFBP-3 prolonged the survival time and attenuated LPS-induced lung injury. The expression of TNF-α, IL-6, IL-1β, and IFN-γ both in lung tissues and BALF decreased after rhIGFBP-3 treatment, whereas IL-10 expression increased. These results suggest that rhIGFBP-3 inhibits the expression of NF-κB and VEGF in lung tissues. Collectively, our study demonstrates a protective role of rhIGFBP-3 in ALI by regulation of lung inflammation.

Keywords: Acute lung injury, IGFBP-3, inflammation, NF-κB, VEGF

Introduction

Acute lung injury (ALI), which is defined by damage to the alveolar epithelium and endothelium [1], manifests as respiratory distress in approximately 7% of all infants, with the highest incidence in preterm babies (30%), followed by post-term babies (20%), and term babies (4%) [2]. A previous study reported that ALI causes more than 74,500 deaths per year in Europe and the United States [3]. Although improved survival rates of patients with ALI have been observed during the past two decades, largely owing to advances in supportive critical care medicine, the mortality rate of ALI still ranges from 25% to 45% [4, 5]. Since conventional treatment measures have not effectively improved the prognosis in patients with ALI [6], providing novel therapeutic strategies would be beneficial.

Systemic inflammation is the main characteristic of ALI. Animal models and clinical data support the concept that interleukin (IL)-1, IL-6, tumor necrosis factor (TNF), and interferon (IFN)-γ are typical cytokines involved in acute inflammation, which may be caused by septic shock or ischemia-reperfusion injury [7, 8]. Treatment with IL-6 RNAi, which may be associated with upregulation of IL-10, can protect against intestinal ischemia reperfusion-induced ALI in rats [9]. Inhibition of TNF-α by Colquhounia root tablet and prophylactic also attenuated ALI in rats [10, 11]. Thus, targeting the acute inflammatory response is an efficient strategy for ALI treatment.

Insulin-like growth factor binding protein 3 (IGFBP-3) is the most abundant circulating IGFBP and modulates the bioactivity of IGFs [12]. Evidence from various studies has demonstrated the regulatory role of IGFBP3 in cancer progression through regulation of apoptosis, metastasis, and immune microenvironment [13, 14]. Furthermore, IGFBP-3 also plays a crucial role in lung diseases. A recent clinical trial indicated that treatment with recombinant human insulin-like growth factor 1 complexed with its binding protein (rhIGF-1/rhIGFBP-3) did not affect development of retinopathy of prematurity, but did decrease the occurrence of severe bronchopulmonary dysplasia, with a nonsignificant decrease in grades 3-4 intraventricular hemorrhage [15]. In experimental BPD, postnatal rhIGF-1/BP-3 treatment preserves lung growth and prevents pulmonary hypertension [16]. However, the expression and function of IGFBP3 in ALIare still unclear. Hence, in the present study, we investigated the potential therapeutic effect of targeting IGFBP3 in ALI in mice.

Materials and methods

Animals and treatment

BALB/c mice (6-8 weeks, female, 20 g body weight) were purchased from the HFKbio (Beijing, China). The mice received a standard diet and were housed in a room with controlled temperature. Forty-five mice were randomly divided into three groups and each group contains fifteen mice: control group: 50 μl PBS was instilled intranasally (i.n.); LPS group: 10 μg LPS in 50 μl PBS was instilled intranasally; LPS+rhIGFBP-3 group: 10 μg LPS in 50 μl PBS was instilled intranasally and 10 μg rhIGFBP-3 in 50 μl PBS was instilled intranasally. All animal experimental procedures were approved by the Committee of Animal Experimentation of the Shandong University.

Histological staining

The harvested lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 4-μm sections, and stained with haematoxylin and eosin (Beyotime, Beijing, China). Morphologic changes in lung tissues were evaluated by light microscopy in a blinded fashion based on the evaluation criterion described as previous study. The IGFBP-3 expression in lung tissues was determined according to the instructions of immunohistochemical staining kit (ZS-bio, Beijing, China). Primary antibody against IGFBP-3 (Cat. No. 25864, CST, MA, USA) was used for immunohistochemical staining (1:100 dilution).

BALF collection

At 12 hours post LPS injection, the mice were anesthetized by pentobarbital. Then the lungs were intratracheally instilled with 0.8 ml cold PBS. The BALF samples were centrifuged and the supernatants were used for further cytokine determination.

Protein extraction

At 12 hours post LPS injection, the mice were anesthetized by pentobarbital. Then the lung tissues were collected. About 100 mg lung tissues were grinded in liquid nitrogen, and 1 ml RIPA lysis buffer (Beyotime, Beijing, China) containing 1% proteinase inhibitor cocktail (Beyotime, Beijing, China) was added for protein extraction at 4°C for 30 minutes. After centrifugation for 12,000 g at 4°C for 15 minutes, the supernatants were collected for protein concentration determination following the instructions of BCA kit (Beyotime, Beijing, China). The extracted proteins were stored at -80°C for further analysis.

ELISA

The expression of inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-10 and IFN-γ) in the BALF and lung tissues was measured by ELISA kits in accordance with the manufacturer's instructions (Neobioscience, Shenzhen, China).

Western blotting

The proteins (20 μg/lane) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, MA, USA). Following blocking in 5% non-fat milk solution at room temperature for 1 h, membranes were probed with anti-NF-κB p65, p50, p52 and GAPDH primary antibodies at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies (Beyotime, Beijing, China) at room temperature for 1 h, the bands were detected with an enhanced chemiluminescence substrate

(Merck Millipore, MA, USA). The relative expression was normalized to GAPDH.

Myeloperoxidase (MPO) assay

At 12 hours post LPS injection, the lung tissues were collected for the determination of MPO activity. The MPO activity were determined following instructions of the MPO determination kit (Nanjing Jiancheng Bioengineering institute, Nanjing, China)

Statistical analysis

Statistical analysis was performed with GraphPad software version 5.01 (Prism, La Jolla, CA, USA). Comparison between groups was carried out using one-way ANOVA, followed by Mann-Whitney U test. Differences were judged to be significant when the *p* value was less than 0.05. All data are presented as the mean ± SD.

Results

Downregulation of IGFBP-3 in lung tissues of ALI mice

To investigate the expression of IGFBP-3 in lung tissues of ALI mice, LPS was administered to mice by i.v. injection for the establishment of ALI. At 24 h post LPS injection, the lung tissues and bronchoalveolar lavage fluid (BALF) were collected for further analysis. As shown in **Figure 1A**, collapsed alveoli and inflammatory cells were observed in the lung tissues of mice with LPS injection, accompanied by higher injury score. ELISA analysis indicated that IGF expression is significantly upregulated in the lung tissues and BALF of ALI mice (**Figure 1B** and **1C**). Western blotting results suggest that IGFBP-3 is downregulated in lung tissues of ALI mice, whereas IGFBP-2 expression is not changed (**Figure 1D**). Furthermore, immunohistochemical (IHC) staining also confirms the downregulation of IGFBP-3 in the lung tissues of ALI mice (**Figure 1E**). These results demonstrate the downregulation of IGFBP-3 in lung tissues of ALI mice.

rhIGFBP-3 prolongs the survival of ALI mice

To determine the potential function of IGFBP-3 in ALI, mice were treated with rhIGFBP-3 2 h prior to LPS injection. At 24 h post rhIGFBP-3 injection, the tissues were collected for IGFBP-3 determination using IHC staining. As shown in **Figure 2A**, higher expression of IGFBP-3 was detected in the lung tissues of ALI mice with rhIGFBP-3 treatment, compared with normal mice. **Figure 2B** shows that treatment with rhIGFBP-3 dramatically reduced the death rate and prolonged the survival time of ALI mice. Hematoxylin and eosin staining also demonstrated reduced lung injury in ALI mice treated with rhIGFBP-3 (**Figure 2C**). Collectively, these results suggest that rhIGFBP-3 prolongs the survival of ALI mice through attenuating lung injury.

rhIGFBP-3 regulates the expression of inflammatory cytokines in lung tissues and BALF of ALI mice

Inflammatory cytokines play a crucial role in the pathogenesis of ALI; thus, lung tissues and BALF were collected for analysis of the expression of these markers. As shown in **Figure 3A**, treatment with rhIGFBP-3 significantly inhibited the expression of MPO in lung tissues of ALI mice. Furthermore, rhIGFBP-3 reduced the expression of pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β, and IFN-γ, and increased the expression of IL-10 in lung tissues of ALI mice (**Figure 3B-F**). The same changes in cytokine expression occurred in BALF of ALI mice treated with rhIGFBP-3 (**Figure 4**). These results indicate that IGFBP-3 regulates the expression of inflammatory cytokines in ALI mice.

rhIGFBP-3 inhibits the expression of NF-κB and VEGF in lung tissues of ALI mice

To investigate the mechanism of IGFBP-3 on ALI, we examined the expression of NF-κB in lung tissues of ALI mice. The results indicate that treatment with rhIGFBP-3 inhibits the expression of NF-κB p65, p50, and p52 in lung tissues of ALI mice (**Figure 5A**), and also reduces VEGF expression in lung tissues and BALF (**Figure 5B** and **5C**). Collectively, these results suggest that IGFBP-3 inhibits the expression of NF-κB and VEGF during the pathogenetic process of ALI.

Discussion

In the present study, we demonstrated the downregulation of IGFBP-3 in lung tissues of

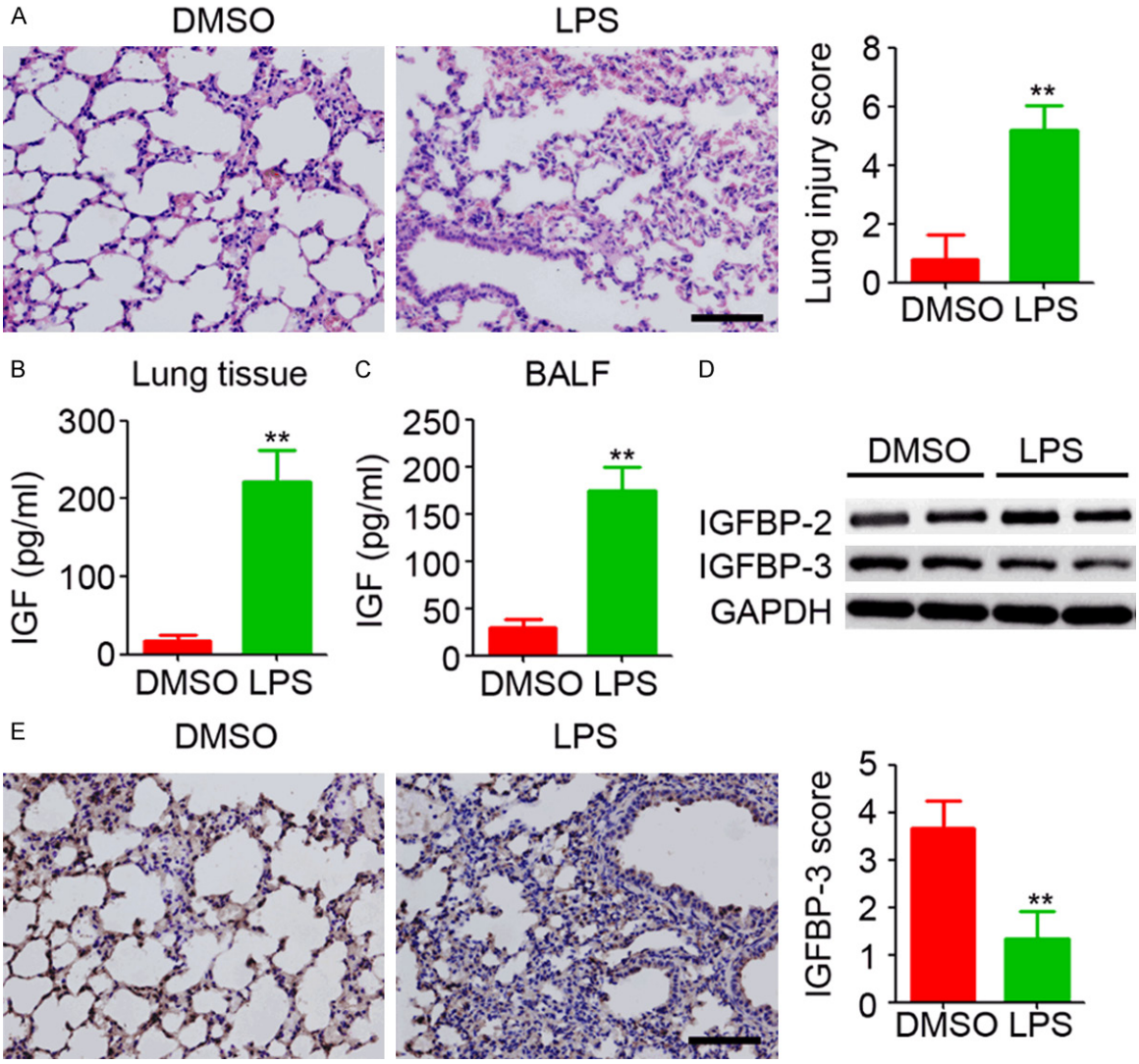

Figure 1. Expression of rhIGFBP-3 in lung tissues of ALI mice. A. H&E staining of lung tissues of mice after PBS and LPS injection. LPS, 10 μg in 50 μl PBS, i.n. injection. Scale bar = 100 μm. Analysis of lung injury score (n = 5, **P<0.01). B. ELISA analysis of IGF expression in lung tissues of mice after PBS and LPS injection (n = 3, **P<0.01). C. ELISA analysis of IGF expression in BALF of mice after PBS and LPS injection (n = 3, **P<0.01). D. Western blot analysis of IGFBP-2 and IGFBP-3 expression in lung tissues of mice after PBS and LPS injection. GAPDH was used as loading control. E. IHC staining of IGFBP-3 expression in lung tissues of mice after PBS and LPS injection. Scale bar = 100 μm. Analysis of IGFBP-3 score (n = 3, **P<0.01).

ALI mice. Administration of rhIGFBP-3 prolonged the survival time and attenuated the LPS-induced lung injury of ALI mice, via regulating the expression of inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-10, and IFN-γ) in lung tissues and BALF. Further results suggested the inhibitory role of rhIGFBP-3 in the expression of NF-κB and VEGF in lung tissues. Collectively, our findings demonstrate a protective role for rhIGFBP-3 in ALI through regulating lung inflammation.

Various studies have demonstrated the deregulation of IGFBP-3 in lung diseases. A previous study indicated that IGFBP-3 in the lungs decreased during hyperoxia but significantly increased during hyperoxia plus treatment with retinoic acid [17]. In another study, IGFBP-3 significantly increased when fetal rat lung fibroblasts were exposed to IL-1β or TNF-α for 48 h [18]. In premature infants with and without bronchopulmonary dysplasia, the changes in serum IGF-1, IGFBP-2, and IGFBP-3 reflected the infants' nutritional status and demonstrated that the relationship between these proteins and nutritional intake differs [19]. The results of the present study suggest that IGFBP-3 is downregulated in the lungs of ALI

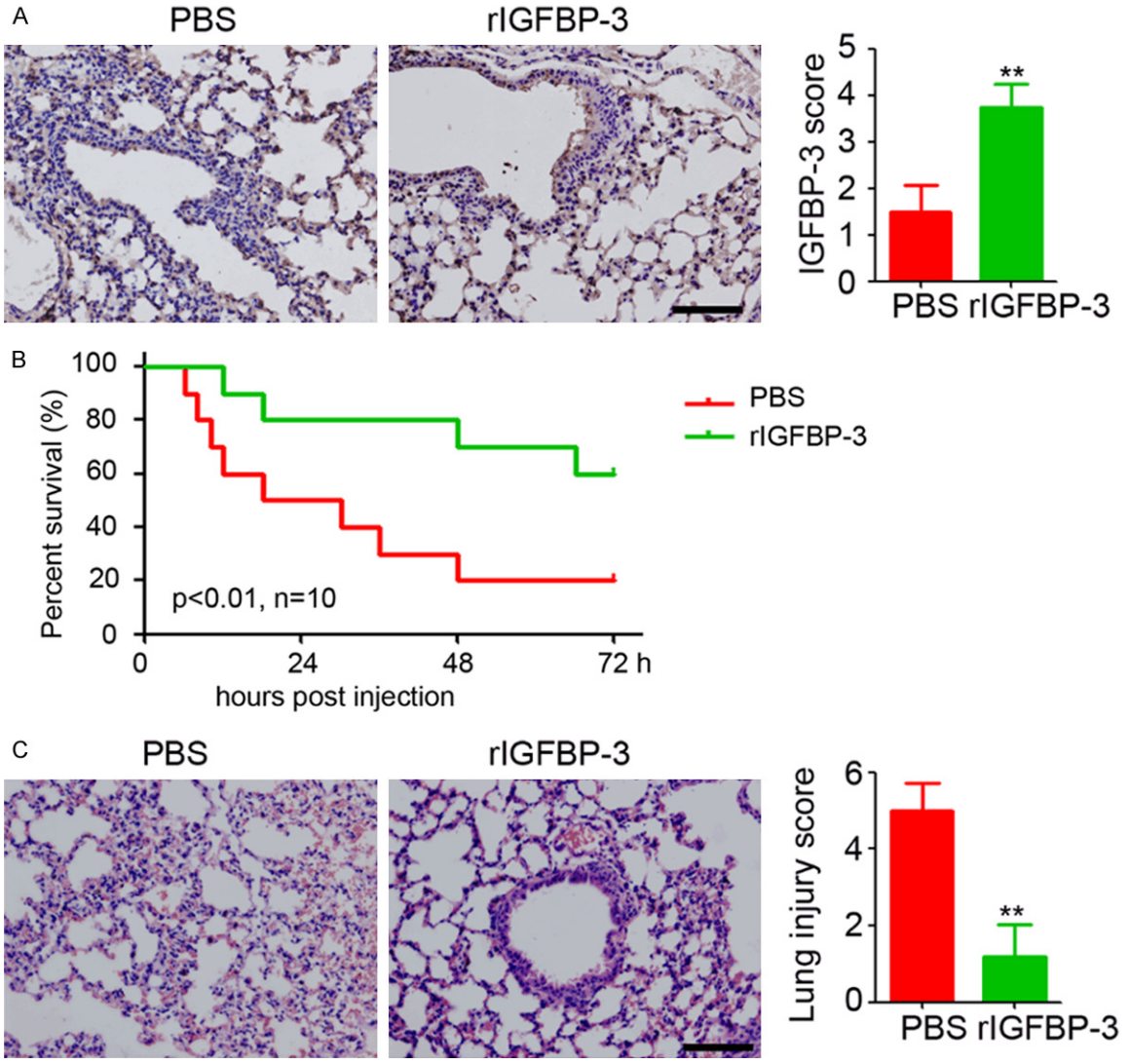

Figure 2. Effect of rhIGFBP-3 on ALI mice. A. IHC staining of IGFBP-3 expression in lung tissues of ALI mice after PBS and rhIGFBP-3 injection. Scale bar = 100 μm. Analysis of IGFBP-3 score (n = 3, **P<0.01). B. Log-rank (Mantel-Cox) Test analysis of survival of ALI mice after PBS and rhIGFBP-3 injection (n = 10, **P<0.01). C. H&E staining of lung tissues of ALI mice after PBS and rhIGFBP-3 injection. Scale bar = 100 μm. Analysis of lung injury score (n = 3, **P<0.01).

mice, accompanied by upregulation of IGF-1 in BALF and lung tissues. The reduction in IGFBP-3 during the pathogenetic process of ALI indicates that IGFBP-3 may play a functional role in ALI.

IGFBP-3 is well known as an inducer of apoptosis in various cell types, especially cancer cells. A phase II randomized controlled trial demonstrated the potential for therapeutic application of rhIGFBP-3 in severe bronchopulmonary dysplasia [15]. Addition of IGFBP-3 to aliquots of serum-free medium reversed the IGF-1-mediated acceleration of IGFBP-4 proteolysis and increased the accumulation of IGFBP-4 in conditioned medium, which participated in lung fibroblasts [18]. Administration of rhIGFBP-3 also preserved lung structure and prevented right ventricular hypertrophy after postnatal hyperoxia [16]. In the present study, we first employed rhIGFBP-3 for the treatment of ALI mice. The results confirmed the protective role of rhIGFBP-3 in ALI mice, evidenced by extended survival time and decreased lung injury. These results suggest that rhIGFBP-3 could be a novel therapeutic strategy for ALI.

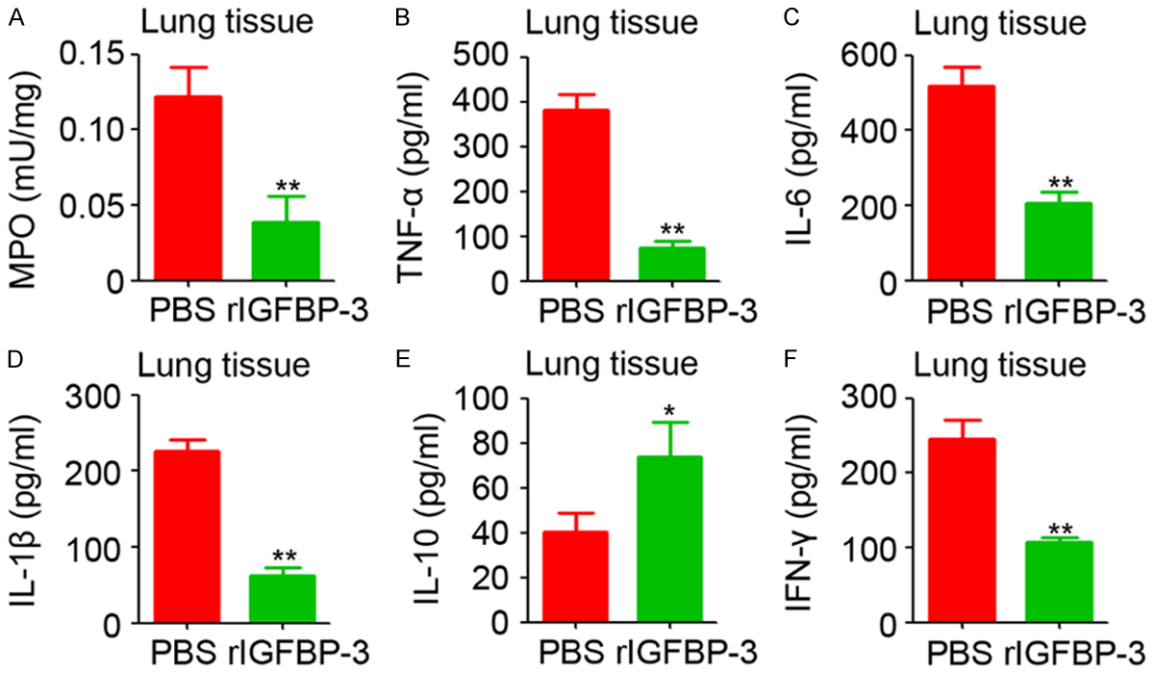

Figure 3. Expression of inflammatory cytokines in lung tissues of ALI mice. A. Expression of MPO in lung tissues of ALI mice after PBS and rhIGFBP-3 injection (n = 3, **P<0.01). B-F. ELISA analysis of TNF-α, IL-6, IL-1β, IL-10 and IFN-γ in lung tissues of ALI mice after PBS and rhIGFBP-3 injection (n = 3, **P<0.01).

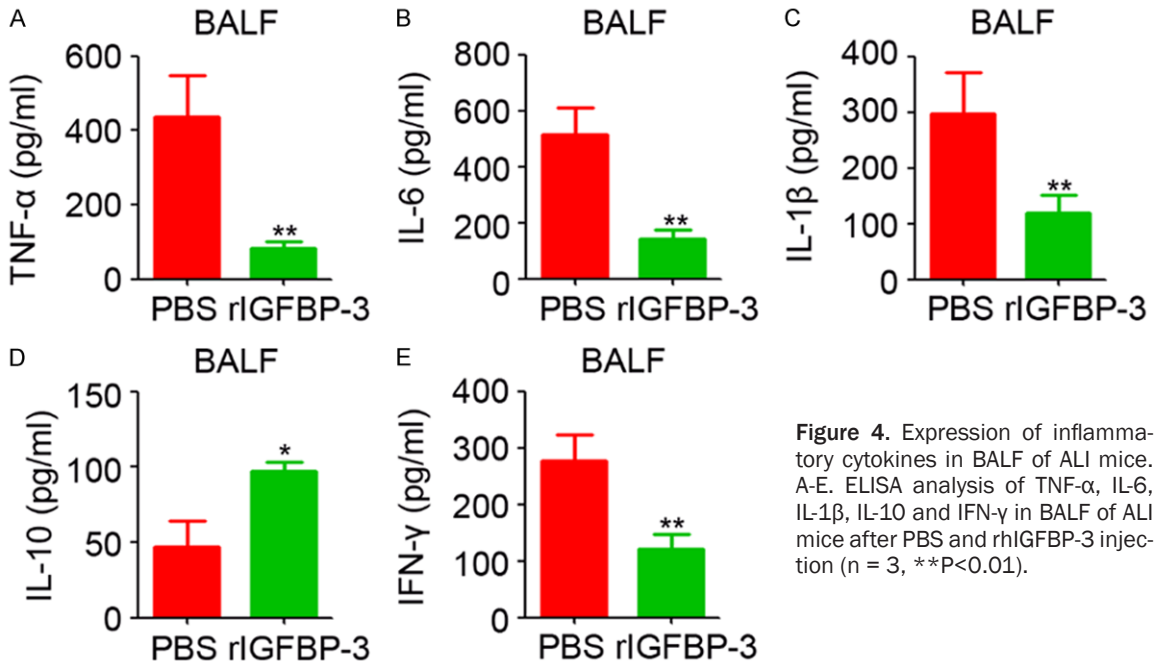

Figure 4. Expression of inflammatory cytokines in BALF of ALI mice. A-E. ELISA analysis of TNF-α, IL-6, IL-1β, IL-10 and IFN-γ in BALF of ALI mice after PBS and rhIGFBP-3 injection (n = 3, **P<0.01).

In the pathogenesis of ALI, NF-κB is activated and promotes the expression of several pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β [20, 21]. Various studies have demonstrated that targeting NF-κB is an efficient strategy for the therapy of ALI [22, 23]. In gastric cancer cells, overexpression of IGFBP-3 resulted in significant inhibition of total and phosphorylated p65 NF-κB [24]. Addition of IGFBP-3 protein to cell cultures as well as enforced

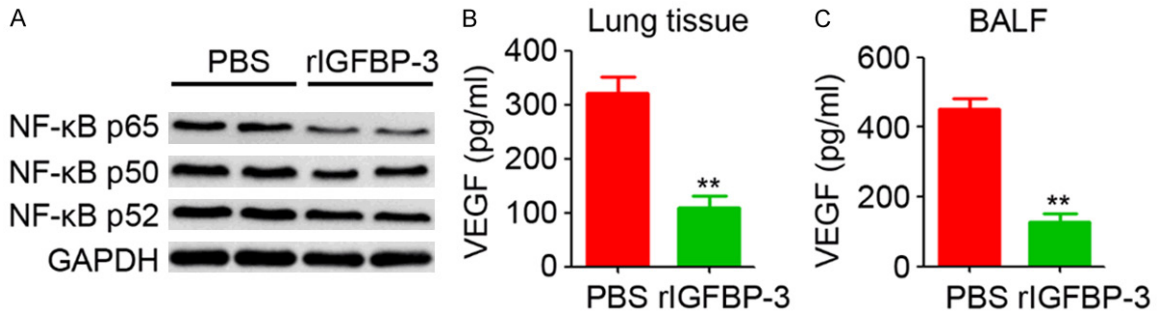

Figure 5. Expression of NF-κB and VEGF in lung tissues of ALI mice. A. Western blot analysis of NF-κB p65, p50 and p52 expression in lung tissues of ALI mice after PBS and rhIGFBP-3 injection. GAPDH was used as loading control. B. ELISA analysis of VEGF in lung tissues of ALI mice after PBS and rhIGFBP-3 injection (n = 3, **P<0.01). C. ELISA analysis of VEGF in BALF of ALI mice after PBS and rhIGFBP-3 injection (n = 3, **P<0.01).

expression of IGFBP-3 in colorectal carcinoma cells inhibited NF-κB activation [25]. In the present study, we confirmed the reduction of NF-κB activity in lung tissues of ALI mice treated with rhIGHBP-3. IL-6, TNF-α, and IL-1β, which are transcriptionally controlled by NF-κB [26, 27], were also decreased in the BALF and lung tissues of rhIGFBP-3-treated ALI mice. The upregulation of IL-10 and IFN-γ, which is a downstream effect of IL-6, TNF-α, and IL-1β reduction, could be mediated by the reduced infiltration of inflammatory cells in the lung tissues.

VEGF is involved in angiogenesis, but also functions as an immunomodulator in the tumor immune microenvironment and other diseases [28]. VEGF also has been implicated in the pathogenesis of ALI; thus, targeting VEGF could alleviate ALI [29]. Our results indicate that the expression of VEGF in the lungs is significantly inhibited by rhIGFBP-3 treatment, which is consistent with a previous study [30]. This result suggests that rhIGHBP-3 attenuates ALI by inhibiting VEGF-regulated lung inflammation.

Collectively, our study demonstrated that administration of rhIGFBP-3 attenuates LPS-induced ALI in mice, and this may be a novel therapeutic strategy for ALI. However, further investigations are needed to clarify the optimal time and dose of rhIGFBP-3 treatment in future.

Acknowledgements

All experimental procedures were approved by the Institutional Animal Care and Use Committees of Shandong University. Consent for publication is not applicable in this study, no individual person's data was used.

Disclosure of conflict of interest

None.

Address correspondence to: Cuifen Zhao, Department of Pediatrics, Qilu Hospital, Shandong University, No. 107, Wen Hua Xi Road, Jinan 250012, Shandong Province, China. Tel: +86-053-187182176; E-mail: cfzhao20@163.com

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