Original Paper

Effect of Liuweibuqi Capsules in Pulmonary Alveolar Epithelial Cells and COPD Through JAK/STAT Pathway

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Key Words

Chronic obstructive pulmonary disease (COPD) • Liuweibuqi capsule • Inflammatory cytokines • Apoptosis • Cell viability

Abstract

Background/Aims: To evaluate the effect of Liuweibuqi capsules on chronic obstructive pulmonary disease (COPD) through the JAK/STAT pathway. **Methods:** Lung function was measured with a spirometer. Changes in lung histology were observed using H&E staining. Cigarette smoke extract combined with lipopolysaccharide (CSE+LPS) was used to establish the cellular COPD model. Cytokine levels were determined using ELISA, and changes in the JAK/STAT pathway were evaluated using western blotting. The CCK8 method and flow cytometry were used to measure cell viability and apoptosis, respectively. **Results:** Liuweibuqi capsules reduced the damage in the lung tissues and the loss of lung function in the COPD rats. Additionally, the levels of interleukin (IL)-1 β , interferon γ (IFN γ), IL-6, tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- β 1 were higher, whereas IL-10 was lower in the model control (MC) and CSE+LPS groups than in the normal group. The phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 were higher and the levels of SOCS1 and SOCS3 were lower in the MC group and CSE+LPS group compared with the normal group. After Liuweibuqi capsule treatment, the expression of inflammatory cytokines and elements of the JAK/STAT pathway were lower. In addition, over-expression of STAT3 blocked the effects of the Liuweibuqi capsules on the release of inflammatory cytokines, cell viability and apoptosis. **Conclusion:** Our findings suggested that Liuweibuqi capsule might effectively ameliorate the progression of COPD via the JAK/STAT pathway.

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by persistent airflow limitation that is associated with an enhanced chronic inflammatory response to noxious gases or particles in the airways and the lungs [1]. COPD is a major cause of morbidity and

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mortality worldwide [2]. Many studies have confirmed that the major risk factor of COPD is exposure to cigarette smoke (CS) [3]. The pathogenesis of COPD involves multiple detrimental processes, including inflammation, cellular apoptosis, alterations in alveolar maintenance, oxidative stress, and abnormal cellular repair [4, 5]. Currently, better medications and more aggressive intervention strategies are required to manage COPD.

Liuweibuqi (LWBQ) capsules are a Traditional Chinese Medicine (TCM) that include Renshen (*Radix Ginseng*), Huangqi (*Radix Astragali Mongolici*), Yuzhu (*Rhizoma Polygonati odorati*), Yizhi (*Fructus Alpiniae Oxyphyllae*), Chenpi (*Pericarpium Citri Reticulatae*) and Rougui (*Cortex Cinnamomi Cassiae*) [6]. Astragalus polysaccharides can reduce the inflammatory response [7]. Our previous studies have demonstrated that Liuweibuqi capsules can reduce the release of the inflammatory cytokines in a COPD rat model [6].

Numerous pathways are considered to play roles in COPD pathogenesis. The Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway is involved in COPD [8]. As a key component of the JAK/STAT pathway, STAT3 plays important roles in COPD [9]. Interleukin 6 (IL-6) activates intracellular signaling of STAT3 in epithelial and immune cells [10]. Once activated, STAT3 induces the expression of numerous pro-inflammatory genes in the lung [11]. Thus, the development of JAK/STAT inhibitors is considered to be a potential treatment for COPD.

Suppressors of cytokine signaling (SOCS) molecules are a family of proteins that act as negative regulators of cytokine signaling pathways [12]. The members of the SOCS family, SOCS-1 and SOCS-3, were identified as negative feedback regulators of the JAK/STAT pathway [13]. It has also been demonstrated that SOCS molecules can potentially inhibit the JAK/STAT pathway in various inflammatory diseases [14, 15]. In addition, the reduction of cytokine signaling through the action of SOCS may also play a critical role in the pathophysiology of COPD [16].

In the present studies, we sought to evaluate the effect of Liuweibuqi capsules on COPD and to determine whether their potential mechanism involved the JAK/STAT pathway.

Materials and Methods

Experimental animals

Male Sprague-Dawley (SD) rats (180-200 g) were purchased from the Experimental Animal Center of Anhui Province (Hefei, China). All animals were housed in specific pathogen-free (SPF) conditions and given access to water and food ad libitum. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital, Anhui Medical University. All experimental procedures that involved animals were approved according to the guidelines of the Care and Use of Laboratory Animals by the National Institute of Health, China.

Model establishment and grouping

The rats were randomly divided into seven groups with 10 rats in each group: normal control (NC) group, model control (MC) group, Jinshuibao (JSB) group, spleen aminopeptidase (PAT) group, low dose of Liuweibuqi (LWBQ low) group, mid dose of Liuweibuqi (LWBQ mid) group and high dose of Liuweibuqi (LWBQ high) group. With the exception of the NC group, all rats were anesthetized with 10% chloral hydrate and their tracheas were exposed to 200 µl of 1 mg/ml lipopolysaccharide (LPS, Sigma, St. Louis, MO, USA). Subsequently, the rats were placed in a chamber with smoke produced by the combustion of a mixture of 50 g sawdust and 0.682 g cigarette tobacco (Chuzhou, China, tar 13.5 mg/g and nicotine 0.48 mg/g). The rats were exposed to the smoke for 30 min per day for 28 d to establish the rat models [6].

Drug treatment

The drug treatments were initiated on day 28. The NC and MC groups were given 0.9% physiological saline (10 ml/kg). The JSB and PAT groups were respectively given Jinshuibao capsules (0.495 g/kg) and spleen aminopeptidase (0.33 mg/kg) daily for 30 d. The LWBQ groups were respectively given a low dose of Liuweibuqi capsules (0.2 g/kg), a mid dose of Liuweibuqi capsules (0.4 g/kg) and a high dose of Liuweibuqi capsules (0.8 g/kg) for 30 d. Subsequently, the rat body weights and respiratory rates were monitored. In

addition, we sampled 5 ml of their abdominal aortic blood. The blood was centrifuged at $1000 \times g$ for 5 min, and the serum was collected and stored at -80°C for later cytokine analysis.

Evaluation of pulmonary function

Pulmonary function was assessed on the basis of the FEV/FVC, which was calculated by the forced expiratory flow in 0.3 s ($\text{FEV}_{0.3}$) by the forced vital capacity (FVC), and multiplying by 100%. In addition, the peak expiratory flow (PEF) was assessed. These measurements were obtained using a pulmonary function test apparatus for small animals on the 30th d after the drug treatment was initiated.

Histological examination

After 30 d of drug treatment, the animals were sacrificed. The lung tissues were fixed with 10% formalin, embedded in paraffin, and cut into $5 \mu\text{m}$ tissue sections that were mounted on slides. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin (H&E) and observed using light microscopy (Olympus Inc., Tokyo, Japan).

Preparation of serum from the treated rats

Sera from the treated rats were prepared according to the previous protocols [17]. Briefly, 24 Sprague Dawley rats, aged between 6 and 8 weeks old and weighing between 180 and 220 g, were divided into 4 groups, with 6 rats in each group: normal control (NC) group, low dose of Liuweibuqi (LWBQ low) group, mid dose of Liuweibuqi (LWBQ mid) group and high dose of Liuweibuqi (LWBQ high) group. The rats in the Liuweibuqi capsule groups were administered 0.2 g/kg (low dose), 0.4 g/kg (mid dose) or 0.8 g/kg (high dose) of the Liuweibuqi capsules via gastric perfusion once a day for 3 days, whereas the NC group was treated with the same volume of 0.9% physiological saline. Blood was aseptically obtained from the abdominal aortas of the rats 1 h after the final administration, and the sera were isolated by centrifugation of the blood at $720 \times g$ for 20 min. Following 2 rounds of filtration using a $0.22 \mu\text{m}$ cellulose acetate membrane, the serum was sealed in vials, heated in a water bath at 56°C for 30 min, and then preserved at -80°C for future use.

Cell isolation and culture

Primary cultures of rat alveolar epithelial cells were performed as previously described [18]. Briefly, SD rats (6 weeks) were euthanized by exsanguination under deep anesthesia (pentobarbital 100 mg/kg), and the lungs were removed. The lungs were then perfused with a perfusion solution (2.65 mM phosphate buffer pH 7.4, 135 mM NaCl, 4.86 mM glucose, 1.9 mM CaCl_2 , 5.3 mM KCl, 1.3 mM MgSO_4 , 10 mM HEPES) via the pulmonary artery until they appeared white. After that, the lungs were digested at 37°C with elastase that was injected into trachea. Subsequently, the lung tissue was minced and filtered through 140- and $30\text{-}\mu\text{m}$ nylon mesh filters. The filtered cells were centrifuged, and the cell pellet was resuspended into DMEM medium (Gibco, Invitrogen) and incubated at 37°C for 1 h. The unattached cells were collected and seeded on 24-well plates at 2×10^6 cells/well. Then, the alveolar epithelial cells were cultured in DMEM containing 10% fetal bovine serum (FBS, Gibco, Invitrogen) at 37°C with 5% CO_2 in a humidified incubator.

Cell grouping and treatment

Cigarette smoke extract (CSE) was prepared as previously reported [19]. Briefly, CSE was prepared by bubbling the smoke from two cigarettes into 20 ml of serum-free DMEM, which was then filtered with a $0.2 \mu\text{m}$ filter to sterilize the mixture. An optical density of 0.65 (320 nm) was considered to represent 100% CSE and was diluted in serum-free DMEM to 2% CSE. The alveolar epithelial cells were then divided into five groups, including normal control (NC) group, CSE+LPS group, low dose of Liuweibuqi (LWBQ low) group, mid dose of Liuweibuqi (LWBQ mid) group and high dose of Liuweibuqi (LWBQ high) group. With the exception of the NC group, the cells were stimulated with CSE in combination with LPS ($0.1 \mu\text{g/ml}$) for 24 h. After that, the media were removed, and cells were incubated with 10% blank serum or 10% LWBQ-treated serum for 24 h. At the end of the incubation period, the cells were harvested and stored at -80°C until further use.

Cell transfection

A STAT3/pcDNA3.1 expression vector was constructed according to previous studies [20]. The sequence of the STAT3 siRNA was previously published and shown to be effective in previous tests [21]. Alveolar

epithelial cells (5×10^6 cells) were transfected with 2 μ g of pCDNA3.1-STAT3 or pCDNA3.1 or transfected with 3 nmol/l siRNA against STAT3 or control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 48 h, the cells were collected for measurements.

Cell viability assay

The Cell Counting Kit-8 (CCK8) method was used to measure the cell viability. Cells were seeded in 96-well plates at a density of 1×10^4 cells/ml. The cell viability was assessed using the CCK8 reagent (Dojindo Laboratories, Japan) according to the manufacturer's protocols. The absorbance at 450 nm was read on a microplate reader.

Assessment of apoptosis by flow cytometry (FCM)

Cell apoptosis was detected using an Annexin V/propidium iodide (PI) apoptosis kit (BioVision, USA). Briefly, 4×10^5 cells were added to each tube. Subsequently, 5 μ l of Annexin V-fluorescein isothiocyanate and 10 μ l of PI were added. After mixing, the tube was incubated at 37°C for 15 min in the dark. Analysis was performed using a FACSCalibur flow cytometer.

The apoptotic rate was calculated as the percentage of the Annexin V-positive cells. Annexin V-positive and PI-negative (Annexin V⁺/PI⁻) cells were considered to be at the early apoptotic stage, whereas those positive for both Annexin V and PI (Annexin V⁺/PI⁺) cells were considered to be at the late apoptotic stage.

Cytokine analysis

The levels of several cytokines (IFN γ , IL-1 β , TNF- α , IL-4, IL-6, IL-10, IL-13 and TGF- β 1) in the sera or in the culture medium of the alveolar epithelial cells were measured using enzyme-linked immunosorbent assays (ELISA) with the appropriate kits (CUSABIO, Wuhan, China) according to the manufacturer's instructions.

Western blot analysis

Total protein was extracted from the cells and tissues in RIPA lysis buffer as described previously [22]. Briefly, forty micrograms of protein were loaded per lane and separated using 10% SDS-PAGE gel electrophoresis and then transferred to PVDF membranes. Subsequently, the membranes were incubated with primary antibodies against phospho-JAK1 (PY1022/1023), JAK1, phospho-JAK2 (PY1007/1008), JAK2, phospho-STAT1 (Tyr701), STAT1, phospho-STAT3 (Tyr705p), STAT3, phospho-STAT5 (Tyr694), STAT5, SOCS1 or SOCS3 at a 1:1000 dilution overnight at 4°C. The membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:2000; Boster, Wuhan, China) for 1 h at room temperature. Finally, the blots were analyzed using chemiluminescence detection (ECL, Amersham).

Statistical analysis

The statistical program SPSS16.0 was used to perform the statistical analyses. The data are expressed as the means $\pm$ standard deviation (SD). Student's *t*-test was used to analyze two groups and one-way analysis of variance (ANOVA) was used to examine multiple groups. *P* < 0.05 was considered statistically significant.

Results

LWBQ capsules improved the pulmonary function in rat COPD model

As shown in Fig. 1A, the body weights in the MC group were significantly less than those of the NC group. In addition, the respiratory rates of the MC group were significantly higher than those of the NC group (Fig. 1B). After the drug treatments, the body weights and respiratory frequencies of the rats were improved compared with the MC group. Moreover, the values for the LWBQ groups were better than those in the other treatment groups (Fig. 1A and 1B).

Compared with the NC group, the lung function parameters including FEV_{0.3}/FVC and PEF were significantly lower in the MC group. The FEV_{0.3}/FVC and PEF were significantly higher in the mid LWBQ and high LWBQ groups than in the MC group (Fig. 1C-1D).

Fig. 1. Comparison of body weights and lung function parameters in rats and morphological changes in lung tissues. (A) Body weight (g; n=10). (B) Respiratory rate (Hz; n=10). (C) The ratio of forced expiratory volume at 0.3 s and forced vital capacity ($FEV_{0.3}/FVC$; n=10). (D) Peak expiratory flow (ml/s; n=10). (E) Morphological characterization of airway epithelial layer and alveolar septa, and inflammatory cell infiltration into the COPD lungs by H&E staining, magnification $\times 200$ (n=3) and quantitative analysis of the numbers of infiltrated cells (n=3). NC: normal control; MC: model control; LWBQ: Liuweibuqi capsule; JSB: Jinshuibao capsule; PAT: spleen aminopeptidase. *** $P < 0.001$ compared with the NC group, # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ compared with the MC group.

LWBQ capsules improved lung histological condition in rat COPD model

The histological analysis of the lung tissues from the COPD rats under the various treatments was performed using H&E staining. The alveolar spaces were larger and the numbers of the infiltrated inflammatory cells were greater in the COPD lungs than that in normal lungs. In addition, some alveoli broke and fused into bullae (Fig. 1E). The various treatments helped to ameliorate the typical pathological features of COPD. In the LWBQ group, there was little inflammatory cell infiltration, less destruction of alveolar septa, and more complete columnar epithelial cells. More importantly, the lung histological conditions in the LWBQ group were better than those in the other treatment groups including the JSB and PAT groups (Fig. 1E). Furthermore, the infiltrated cell numbers were significantly increased in the MC group, and this could be remarkably reversed by the LWBQ capsules.

LWBQ capsules inhibited the release of inflammatory cytokines in rat COPD model

Compared with the NC group, the levels of IL-4 and IL-10 were lower and the IFN γ , IL-1 β , TNF- α , IL-6, IL-13 and TGF- β 1 levels were higher in the MC group. Compared with the MC group, the IL-4 and IL-10 levels were elevated whereas the IFN γ , IL-1 β , TNF- α , IL-6, IL-13 and TGF- β 1 levels were lower in the various drug treatment groups. In addition, the levels of IL-4 and IL-10 were significantly greater and the levels of IFN γ , IL-1 β , TNF- α , IL-6, IL-13 and TGF- β 1 were notably lower in the LWBQ high group than those in the MC group (Fig. 2A-2H).

Fig. 2. LWBQ capsules reduced the COPD-mediated release of inflammatory cytokines. The levels of (A) IFN γ , (B) IL-1 β , (C) IL-4, (D) TNF- α , (E) IL-6, (F) IL-10, (G) IL-13 and (H) TGF- β 1 in the sera under the various treatments (n=3). NC: normal control; MC: model control; LWBQ: Liuweibuqi capsule; JSB: Jinshuibao capsule; PAT: spleen aminopeptidase. **P<0.01 and ***P<0.001 compared with the NC group, #P<0.05, ##P<0.01 and ###P<0.001 compared with the MC group.

Fig. 3. LWBQ capsules inhibited the COPD-mediated activation of the JAK-STAT axis in rats. (A) Western blot analysis was performed to determine the protein levels of JAK1, p-JAK1, JAK2, p-JAK2, STAT1, p-STAT1, STAT3, p-STAT3, STAT5, p-STAT5, SOCS1 and SOCS3 in the various groups (n=3). (B) The relative phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 were calculated as the ratio of the band intensity of the phosphorylated form to the total levels (n=3). NC: normal control; MC: model control; LWBQ: Liuweibuqi capsule; JSB: Jinshuibao capsule; PAT: spleen aminopeptidase. *P<0.05 compared with the NC group, #P<0.05 compared with the MC group.

Fig. 4. Effect of sera from LWBQ-treated rats on the CSE+LPS-induced release of inflammatory cytokines, cell viability and cell apoptosis in alveolar epithelial cells. The levels of (A) IFN γ , (B) IL-1 β , (C) IL-6, (D) TNF- α , (E) IL-10, (F) TGF- β 1 in the culture medium of the alveolar epithelial cells under the various treatments (n=3). (G) The cell viability was measured using the CCK8 method (n=3). (H) SSC/FSC plots were used to select a population and exclude debris from the sample analysis. (I) The percentage of apoptotic cells was determined by flow cytometry (n=3). (J) Representative flow cytometric dot-plots. ***P<0.001 compared with the NC group, #P<0.05, ##P<0.01 and ###P<0.001 compared with the CSE+LPS group.

LWBQ capsules inhibited the activation of the JAK-STAT axis in rat COPD model

Next, we determined the effect of LWBQ capsules on the changes in the JAK/STAT signaling pathway. Western blot analysis revealed an increase in the phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 but a decrease in the SOCS1 and SOCS3 protein levels in the MC group compared to the NC group (Fig. 3A and 3B). However, the phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 were significantly lower and the levels of SOCS1 and SOCS3 were remarkably higher in the LWBQ group than those in the MC group (Fig. 3A and 3B). These results demonstrate that the LWBQ capsules reduce the JAK/STAT signaling in COPD rats.

Fig. 5. Effect of sera from LWBQ-treated rats on the CSE+LPS-induced up-regulation of the protein levels of elements of the JAK-STAT axis in alveolar epithelial cells. (A) Western blot analysis was performed to determine the protein levels of JAK1, p-JAK1, JAK2, p-JAK2, STAT1, p-STAT1, STAT3, p-STAT3, STAT5, p-STAT5, SOCS1 and SOCS3 in the various groups (n=3). (B) The relative phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 were calculated as the ratio of the band intensity of the phosphorylated form to the total level (n=3). *P<0.05 compared with the NC group, #P<0.05 compared with the CSE+LPS group.

Effect of serum from LWBQ-treated rats on cytokine production, cell viability and apoptosis of alveolar epithelial cells

Incubation of alveolar epithelial cells with CSE in combination with LPS for 24 h enhanced the release of IFN γ , IL-1 β , IL-6, TNF- α and TGF- β 1 but significantly reduced the release of IL-10 (Fig. 4A-4F). As expected, the serum from the LWBQ-treated rats (LWBQ serum) increased the release of IL-10 and decreased the release of IFN γ , IL-1 β , IL-6, TNF- α and TGF- β 1 from the alveolar epithelial cells in a dose-dependent manner (Fig. 4A-4F). In addition, the LWBQ serum dose-dependently reversed the CSE+LPS-induced down-regulation of cell viability (Fig. 4G). A SSC/FSC plot was used to select a population and exclude debris from sample analysis (Fig. 4H). The LWBQ serum decreased the CSE+LPS-induced increase in cell apoptosis (Fig. 4I and 4J).

Effect of LWBQ serum on the protein levels of JAK-STAT axis in alveolar epithelial cells

To analyze the signaling pathways involved in the CSE+LPS-induced cytokine production and cell viability, the expression of elements of the JAK-STAT axis was analyzed by western blotting. Stimulation of alveolar epithelial cells with the combination of CSE and LPS significantly increased the phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 but dramatically decreased the protein levels of SOCS1 and SOCS3 compared with those of the NC group (Fig. 5A and 5B). However, the LWBQ serum reduced the phosphorylation levels of JAK1, JAK2, STAT1, STAT3 and STAT5 and enhanced the levels of SOCS1 and SOCS3 in a dose-dependent manner compared with those of the CSE+LPS group (Fig. 5A and 5B). These results indicate that the LWBQ serum inhibited the activation of the JAK/STAT signaling that was induced by CSE+LPS in alveolar epithelial cells.

Over-expression of STAT3 blocked the effect of LWBQ serum

To further confirm the role of STAT3 in cytokine production, cell viability and apoptosis of alveolar epithelial cells, we constructed and transfected the recombinant pCDNA3.1-STAT3 plasmid into alveolar epithelial cells. Transfection of the pCDNA3.1-STAT3 plasmid significantly increased the STAT3 expression and phosphorylation in alveolar epithelial cells

Fig. 6. Effects of over-expression of STAT3 on the CSE+LPS-induced release of inflammatory cytokines, cell viability and apoptosis of alveolar epithelial cells. The cells were transfected with pCDNA3.1-STAT3 or pCDNA3.1 (vector). With the exception of the NC group, the cells were stimulated with CSE in combination with LPS (0.1 μg/ml) for 24 h. The cells were then incubated with 10% blank serum or 10% serum from LWBQ-treated rats (high dose) for 24 h. (A) Western blot analysis was performed to determine the protein levels of STAT3 and p-STAT3 in the various groups (n=3). (B) The relative phosphorylation levels of STAT3 were calculated as the ratio of phospho-STAT3 to total STAT3 (n=3). (C) IFNγ, (D) IL-1β, (E) IL-6, (F) TNF-α, (G) IL-10, (H) TGF-β1 levels in the culture medium of alveolar epithelial cells under the various treatment conditions (n=3). (I) The cell viability was measured using the CCK8 method (n=3). (J) The percentage of apoptotic cells was determined using flow cytometry (n=3). *P<0.05 and ***P<0.001 compared with the NC group, #P<0.05, ##P<0.01 and ###P<0.001 compared with the vector group, &P<0.05 and &&&P<0.001 compared with the vector+LWBQ group. NS, not significant (P > 0.05).

Fig. 7. Silencing of STAT3 reduced the CSE+LPS-induced release of inflammatory cytokines, promoted cell viability and inhibited apoptosis of the alveolar epithelial cells. The cells were transfected with STAT3 siRNA or control siRNA (vector). With the exception of the NC group, the cells were stimulated with CSE in combination with LPS (0.1 μ g/ml) for 24 h. The cells were then incubated with 10% blank serum or 10% serum from rats that had been treated with LWBQ (high dose) for 24 h. (A) Western blot analysis was performed to determine the protein levels of STAT3 and p-STAT3 in the various groups (n=3). (B) The relative phosphorylation levels of STAT3 were calculated as the ratio of phospho-STAT3 to total STAT3 (n=3). (C) IFN γ , (D) IL-1 β , (E) IL-6, (F) TNF- α , (G) IL-10, (H) TGF- β 1 levels in the culture medium of the alveolar epithelial cells under the various treatment conditions (n=3). (I) The cell viability was measured using the CCK8 method (n=3). (J) The percentage of apoptotic cells was determined by flow cytometry (n=3). *P<0.05, **P<0.01 and ***P<0.001 compared with the NC group, #P<0.05 and ###P<0.001 compared with the vector group, \$P<0.05, \$\$P<0.01 and \$\$\$P<0.001 compared with the CSE+LPS group. NS, not significant (P > 0.05).

compared to the pCDNA3.1 control. Furthermore, over-expression of STAT3 abolished the LWBQ serum-induced down-regulation of STAT3 expression and phosphorylation (Fig. 6A and 6B). In addition, over-expression of STAT3 blocked the LWBQ serum-induced down-regulation of the IFN γ , IL-1 β , IL-6, TNF- α and TGF- β 1 levels and the up-regulation of the IL-10 levels (Fig. 6C-6H). Furthermore, the LWBQ serum significantly enhanced the cell viability and reduced the apoptosis of the alveolar epithelial cells. However, these effects were reversed by over-expression of STAT3 (Fig. 6I-6J). These results further indicate that LWBQ capsules may modulate the CSE+LPS-induced cytokine production, cell viability and apoptosis of alveolar epithelial cells through down-regulation of the expression of STAT3.

Knockdown of STAT3 reduced the release of inflammatory cytokines, promoted cell viability and inhibited cell apoptosis

Western blot analysis showed that the STAT3 siRNA dramatically decreased the STAT3 expression and phosphorylation in the cells (Fig. 7A and 7B), indicating that the STAT3 was effectively knocked down. In addition, LWBQ serum also significantly inhibited the expression of STAT3 and its phosphorylation (Fig. 7A and 7B). Moreover, knockdown of STAT3 significantly reduced the release of IFN γ , IL-1 β , IL-6, TNF- α and TGF- β 1 and enhanced the release of IL-10 from the alveolar epithelial cells that had been treated with CSE+LPS. Additionally, the levels of the inflammatory cytokines were similar in the STAT3 siRNA and LWBQ groups (Fig. 7C-7H). Furthermore, knockdown of STAT3 or the addition of LWBQ serum significantly prevented the CSE+LPS-induced decreases in cell viability and the increases in cell apoptosis (Fig. 7I-7J). These results demonstrate that knockdown of STAT3 reduced the release of inflammatory cytokines, promoted cell viability and inhibited cell apoptosis in a manner similar to that of the LWBQ capsules.

Discussion

Recent studies have demonstrated that systemic inflammation exerts vital roles in the development of COPD. Furthermore, inflammation of the airway and lungs is a predominant feature of COPD [23, 24]. There is a very long list of chemokines and cytokines that have been implicated in the pathogenesis of COPD [25, 26]. In COPD patients, the serum levels of several inflammatory mediators such as IFN γ , IL-1 β , IL-13 and IL-6 are increased [27-29]. Takanashi et al. reported that the level of IL-10 in sputum from COPD patients was decreased compared with that of healthy non-smokers [30]. Our results showed that the pro-inflammatory cytokines including IFN γ , IL-1 β , IL-6 and TGF- β 1 were higher and the anti-inflammatory cytokines including IL-4 and IL-10 were lower in the MC and CSE+LPS groups than those in the normal group, which was consistent with previous studies [6]. However, the Liuweibuqi capsules could improve COPD symptoms by up-regulating the expression of IL-4 and down-regulating the expression of IFN- γ [31]. Our studies also demonstrated that Liuweibuqi capsules decreased the levels of IFN γ , IL-1 β , IL-6 and TGF- β 1 and increased the levels of IL-4 and IL-10 in the COPD rats and the CSE+LPS-induced cells. Therefore, our results indicate that Liuweibuqi capsules can inhibit COPD-induced inflammatory response.

The production of chemokines and cytokines can be regulated by multiple signal transduction pathways including the JAK/STAT pathway [32]. We therefore used western blotting to examine the phosphorylation pattern of elements of the JAK-STAT axis in response to CSE+LPS. JAKs are essential mediators of the cellular signaling by cytokine receptors [33]. Four members of the JAK family: JAK1, JAK2, JAK3 and TYK2, and seven STAT proteins have been identified in mammalian cells [34]. LPS can directly or indirectly stimulate lung macrophages and neutrophils, thereby inducing the phosphorylation of JAK, which directly phosphorylates STAT proteins [35]. Victoni et al. found that CSE combined with LPS enhances the release and expression of several chemokines by A549 cells, which appears to be dependent on the ERK1/2 and JAK/STAT pathways [32]. Zhang et al. proved that the JAK-STAT1/3 pathway is activated in COPD patients [36]. Our previous studies demonstrated

that JAK1 and STAT3 mRNA and protein expression in lung tissue was higher in the MC group than in the NC group [6]. Compared with the MC group, JAK1, p-JAK1 and p-STAT3 protein expression was lower in the LWBQ group [6]. In this study, we found that LWBQ capsules reduced the COPD-induced up-regulation of JAK/STAT signaling *in vitro* and *in vivo*, which was in agreement with previous studies.

JAK/STAT signaling is often inhibited by negative regulators such as SOCS proteins, which themselves are STAT-target genes [37]. Springer observed a transcriptional down-regulation of SOCS3 in COPD patients [38]. Dong et al. proved that SOCS1 expression was significantly decreased in the lung tissues from COPD patients [39]. In our studies, we also found a significant reduction of SOCS1 and SOCS3 expression in COPD rats and CSE+LPS-induced cells. However, these effects were blocked by the LWBQ capsules, which suggested that the role of LWBQ capsules was associated with the SOCS pathway.

Although the JAK/STAT pathway is involved in the pathogenesis of COPD, only certain STATs are involved [8]. STAT3, in particular, has been further investigated as a target for COPD treatment. Over-expression of STAT3 in respiratory epithelial cells causes alveolar destruction, which suggests that it has a role in maintaining alveolar structure and function [40]. STAT3 induces the inflammatory response, which leads to aberrant expression of cytokines/chemokines and abnormal inflammatory cell infiltration [41]. Moreover, the expression of the STAT3 gene was up-regulated in the tissues from humans with COPD [42]. We speculated that the Liuweibuqi capsules improved COPD symptoms by regulating the JAK1/STAT3 signal transduction [6]. In the present studies, we found that over-expression of STAT3 abolished the effects of the Liuweibuqi capsules. However, knockdown of STAT3 produced effects similar to the Liuweibuqi capsules under the CSE+LPS treatment conditions. Therefore, our studies indicated that the effects of the Liuweibuqi capsules on COPD may be mediated by down-regulation of the expression of STAT3.

Conclusion

In summary, this study demonstrated that Liuweibuqi capsules inhibited the COPD-induced inflammatory response by regulating the JAK/STAT pathway. Thus, Liuweibuqi capsules could be a new therapeutic drug in COPD treatment.

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Disclosure Statement

The authors declare no conflicts of interest.

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