Feibi decoction-medicated serum inhibits lipopolysaccharide-induced inflammation in RAW264.7 cells and BMDMs

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Abstract. Feibi decoction (FBD) is a traditional Chinese herbal medicine and has been clinically used in the treatment of pulmonary fibrosis (PF), which is characterized by diffuse interstitial inflammation and exaggerated collagen accumulation. However, the potential mechanisms remain to be elucidated. The present study aimed to investigate the effect of FBD-medicated serum (FBDS) on lipopolysaccharide (LPS)-induced inflammation in macrophages. In RAW264.7 macrophages and bone marrow-derived macrophages (BMDMs), FBDS treatment significantly inhibited the production of pro-inflammatory cytokines induced by LPS. In addition, it was indicated that FBDS treatment suppressed the activation of NF-κB and Smad2/Smad3 following LPS treatment. Furthermore, FBDS treatment decreased the expression of transforming growth factor-β1 and chitinase-3-like protein 1. In conclusion, the results demonstrated that treatment with FBDS inhibited LPS-induced inflammation in RAW264.7 and BMDM cells. These data may improve understanding of the effect of FBD on anti-inflammation and help determine the mechanisms underlying the alleviation of PF via FBD.

Introduction

Pulmonary fibrosis (PF) is characterized by increased fibroblasts proliferation, interstitial inflammation and the promotion of extracellular matrix synthesis and deposition (1). A number of possible treatments for pulmonary fibrosis have been investigated, but their effect in clinical trials is suboptimal (2). PF is a major therapeutic challenge for which novel therapeutic strategies are warranted, and inhibiting inflammation is increasingly regarded as one of the approaches.

Inflammation is the initial response following lung injury. Once activated, inflammatory cells, such as neutrophils and macrophages, accumulate in the lower airways and consequently release harmful amounts of reactive oxygen species and some proinflammatory cytokines and growth factors that regulate the proliferation and secretary activity of alveolar fibroblasts in the alveolar wall. The activated fibroblasts produce increasing amounts of matrix proteins, which distort the normal lung architecture and affect gas exchange. Therefore, inhibition of inflammation and oxidative stress represents a possible therapeutic strategy (3-7).

Traditional Chinese medicine has been widely used in numerous diseases and has shown notable curative effects (8-10). Feibi decoction (FBD) is a Chinese materia medica product extracted from 8 Chinese traditional medical herbs and widely used for treatment of patients with lung diseases, such as pulmonary fibrosis; however, the precise mechanisms remain to be elucidated. The present study investigated the effect of FBD-medicated serum (FBDS) on lipopolysaccharide (LPS)-induced inflammation in macrophages, and identified that FBDS significantly inhibited the pro-inflammatory cytokines expression in both RAW264.7 macrophages and bone marrow-derived macrophages (BMDMs). It was also observed that FBDS suppressed LPS-induced activation of NF-κB and Smad2/Smad3. Notably, it was identified that FBDS treatment decreased the expression of transforming growth factor (TGF)-β1 and chitinase-3-like protein 1 (CHI3L1). These findings may improve the function of FBD in the treatment of pulmonary fibrosis and expand current understanding of the mechanisms underlying Chinese traditional medicine.

Materials and methods

Animals. A total of 28 clean healthy Sprague-Dawley rats (all female; 8 weeks old), weighing 200-220 g, were provided by the Beijing University of Chinese Medicine and housed in a cage at 23±1˚C, 45-55% humidity with a 12-h light/dark cycle. Food and water were freely available. A total of 20 healthy C57BL/6J mice (female; 6-8 weeks old; 23.7±1.2 g) were

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obtained from Beijing University of Chinese Medicine to generate BMDMs. The mice were kept at room temperature (controlled at 25˚C) and at 50% humidity. with a 12-h light/dark cycle. The experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (11) and approved by the Animal Ethics Committee of the Scientific Investigation Board of Beijing university of Chinese Medicine.

Preparation of FBDS. The 28 Sprague-Dawley rats were randomly divided into FBD-low (n=7), FBD-moderate (n=7), FBD-high (n=7) and control (n=7) groups. Rats in the FBD groups received intragastric administration of FBD (low, 5 ml/kg; moderate, 10 ml/kg; high, 20 ml/kg) twice a day for 6 days. The control group received intragastric perfusion of physiological saline twice a day for 6 days. Then, 1 h following the last administration, rats were intraperitoneally anesthetized using 3% amobarbital (60 mg/kg) and blood was sampled from the abdominal aorta and centrifuged (4˚C; 3,500 x g; 10 min). Following anesthesia, the rats exhibited slow breathing and muscle relaxation, but no respiratory stagnation or mortality. The serum was aliquoted into 10 ml ampoules and preserved at -80˚C for future use.

Cell culture. Mouse macrophage cell line RAW264.7 (induced by leukemia virus) was obtained from the American Type Culture Collection. The cells were cultured at 37˚C under 5% CO_2 in DMEM supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin. BMDM generation was performed according to a previous study (12). Briefly, cells were prepared by flushing the bone marrow from femurs and tibias and then maintaining in DMEM medium containing 10% FBS and supplemented with 10 ng/ml macrophage colony stimulating factor (M-CSF; Peprotech, Inc.; cat. no. 315-02). Then, 4-5 days later, adherent cells were dissociated and cultured in DMEM supplemented with 10% FBS. BMDMs were identified by flow cytometry. The cells were stained for 20 min at 4˚C with 25 μg/ml of anti-F4/80 and anti-CD11b. FITC-anti-F4/80, APC-anti-CD11b were purchased from eBioscience (Thermo Fisher Scientific, Inc.). Data were collected using a FACSCanto (BD Biosciences) and analyzed using FlowJo version 10 software (FlowJo LLC). F4/80+CD11b+ cells were considered BMDMs.

Cell viability. Cell viability was assessed using Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). Briefly, 5,000 RAW264.7 cells or BMDMs per well were seeded in 96-well plates and treated with control rats serum or FBDS (10%) at 37˚C for 24 h, followed by LPS stimulation (100 ng/ml) for 8 h. Then the suspension was replaced with an equal volume of fresh culture medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% CCK-8, followed by 3 h incubation at 37˚C. The absorbance was determined at 450 nm on a micro-plate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative (RT-q) PCR. Following treatment, total RNA from RAW264.7 cells or BMDMs was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA concentration was detected by NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). mRNA (1 μg) was reverse transcribed using PrimeScript RT Master Mix (Perfect Real Time) kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The cDNA was denatured for 10 min at 95˚C. A LightCycler (ABI PRISM 7000; Applied Biosciences; Thermo Fisher Scientific, Inc.) and a SYBR RT-PCR kit (Takara Biotechnology Co., Ltd.) were used for RT-qPCR analysis. GAPDH was used as the internal control. Thermocycling conditions were 1 cycle (95˚C for 5 min) and 40 cycles (95˚C for 15 sec; 57˚C for 30 sec; 72˚C for 30 sec) and the $2^{-\Delta\Delta Cq}$ method was used to evaluate the relative quantities of each amplified product in the samples (13). For each qPCR analysis, three technical replicates were performed. Primer sequences used in qPCR are shown in Table I.

ELISA. Mouse tumor necrosis factor (TNF)α (cat. no. MTA00B) and interleukin (IL)-6 (cat. no. M6000B) ELISA kits (R&D Systems, Inc.) were used according to the manufacturer's instructions.

Western blot analysis. Western blot analysis was performed as described previously (14). The cells were homogenized, washed with PBS, and lysed in a RIPA buffer (Beyotime Institute of Biotechnology). The protein concentration of lysates was measured using Bio-Rad quantification assay (Bio-Rad Laboratories, Inc.). Proteins (20 μg/lane) were separated using 10% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The membrane was then blocked with 2.5% non-fat dry milk for 1 h at room temperature. The antibodies for p65 (1:500; cat. no. 8242), phosphorylated (p-)p65 (1:500; cat. no. 3033), IκB kinase (IKK)β (1:500; cat. no. 2678), p-IKKβ (1:500; cat. no. 2694) and β-actin (1:1,000; cat. no. 3700; endogenous control) were from Cell Signaling Technology, Inc. Smad2 (1:500; cat. no. ab40855), p-Smad2 (1:500; cat. no. ab53100), Smad3 (1:500; cat. no. ab40854), p-Smad3 (1:500; cat. no. ab52903), TGF-β1 (1:500; cat. no. ab92486) and CHI3L1 (1:500; cat. no. ab180569) were from Abcam. The primary antibodies were added and incubated overnight at 4˚C. Following incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP; 1:4,000; cat. no. sc2004; or goat anti-mouse IgG-HRP; 1:4,000; cat. no. sc2005; Santa Cruz Biotechnology, Inc.) at 25˚C for 2 h, the target protein was visualized by enhanced chemiluminescence (cat. no. 32106, Thermo Fisher Scientific, Inc.). Relative protein expression levels were determined by scanning densitometry (ChemiDoc XRS + Systems; Bio-Rad Laboratories, Inc.) and analyzed using Image Lab 5.0 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are presented as the mean ± standard deviation of three independent experiments. One-way analysis of variance (ANOVA) was performed to compare three or more groups. If the ANOVA analysis was significant, the Tukey's post hoc test was applied for comparison between each two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used in the present study.

Primer name	Sequence (5'-3')
TNFα forward	GCCACCACGCTCTTCTGTCT
TNFα reverse	TGAGGGTCTGGGCCATAGAAC
IL-6 forward	ACAACCACGGCCTTCCCTAC
IL-6 reverse	CATTTCCACGATTTCCCAGA
IL-1β forward	ACCTTCCAGGATGAGGACATGA
IL-1β reverse	AACGTCACACACCAGCAGGTTA
IL-8 forward	GGGTCGTACTGCGTATCCTG
IL-8 reverse	AGACAAGGACGACAGCGAAG
TGF-β1 forward	CACTCCCGTGGCTTCTAGTG
TGF-β1 reverse	GGACTGGCGAGCCTTAGTTT
CHI3L1 forward	CCCCGTTCCTGCGTTCTTAT
CHI3L1 reverse	CAGGTGTTGGGCTATCTGGG
GAPDH forward	AATGACCCCTTCATTGAC
GAPDH reverse	TCCACGACGTACTCAGCGC

Results

FBDS reduces proinflammatory cytokine expression in LPS-stimulated RAW264.7 macrophages. To investigate the effect of FBD on lung inflammation, mouse macrophages RAW264.7 cells were incubated with saline or with FBDS (low, moderate or high dosage) followed by stimulation with LPS. Subsequently, the production of proinflammatory cytokines was examined. At first, the cell viability of RAW264.7 cells following FBDS treatment was examined and it was identified that RAW264.7 viability was not affected by FBDS treatment (Fig. S1A). As shown in Fig. 1A, treatment with FBDS significantly decreased the mRNA levels of pro-inflammatory cytokines induced by LPS stimulation (TNFα, IL-6, IL-1β and IL-8) compared with the group treated with LPS and control serum in a dose-dependent manner. In addition, consistent with the mRNA data, the ELISA data indicated that protein expression levels of TNFα and IL-6 were also decreased by FBDS incubation compared with the group treated with LPS and control serum (Fig. 1B).

FBDS reduces proinflammatory cytokine expression in LPS-stimulated BMDMs. To confirm the results in Fig. 1, BMDMs were used and the experiments repeated. The cell viability of BMDMs following FBDS treatment and identification of BMDMs was confirmed (Fig. S1A and B). As shown in Fig. 2 and consistent with the expression data in Fig. 1, it was identified that FBDS treatment also decreased the production of proinflammatory cytokines in both mRNA and protein levels in BMDMs compared with the group treated with LPS and control serum.

FBDS inhibits the activation of NF-κB signaling pathway. NF-κB represents a paradigm for signal transduction and proinflammatory cytokine production (15,16). Therefore, the present study examined whether FBDS treatment regulated LPS-induced proinflammatory cytokine production via the NF-κB signaling pathway. Western blotting demonstrated that LPS-induced

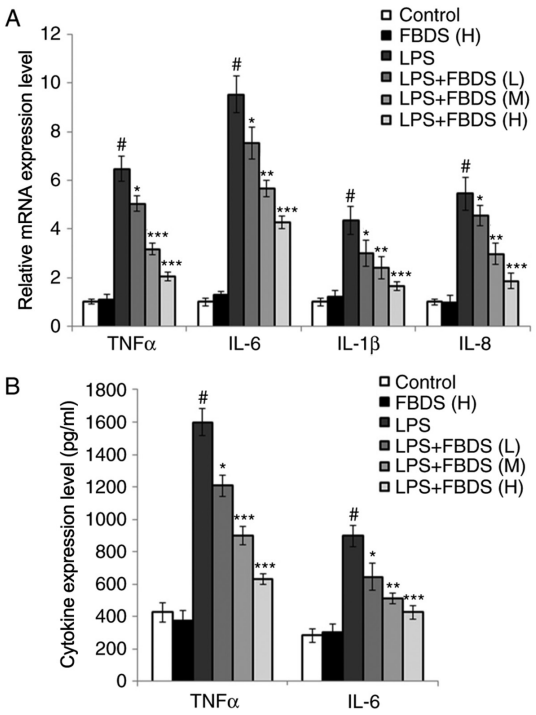

Figure 1. FBDS reduces proinflammatory cytokine expression in LPS-stimulated RAW264.7 macrophages. RAW264.7 cells were incubated with blank serum or FBDS (10%) for 24 h, followed by LPS stimulation (100 ng/ml) for 8 h. (A) The relative mRNA levels of TNFα, IL-6, IL-1β and IL-8. (B) The protein levels of TNFα and IL-6. Data are representative of three independent experiments (mean ± standard deviation). #P<0.05 vs. control group; *P<0.05, **P<0.01, ***P<0.001 vs. LPS group. FBDS, Feibi decoction-medicated serum; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin.

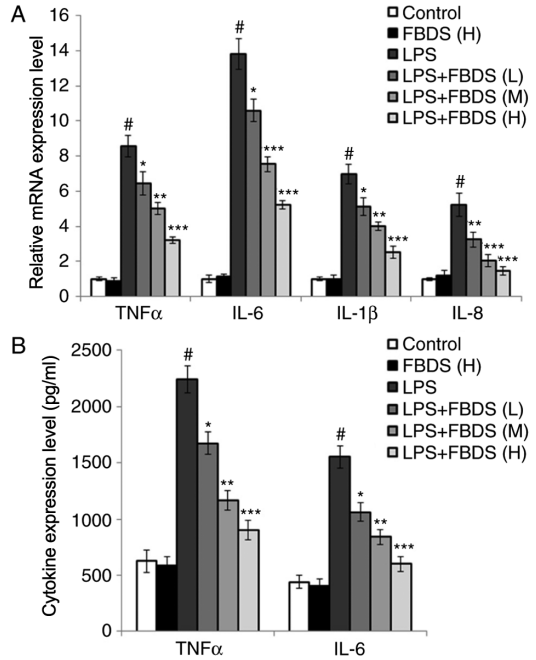

Figure 2. FBDS reduces pro-inflammatory cytokines expression in LPS-stimulated (BMDMs). BMDMs were incubated with blank serum or FBDS (10%) for 24 h, followed by LPS stimulation (100 ng/ml) for 8 h. (A) The relative mRNA levels of TNFα, IL-6, IL-1β and IL-8. (B) The protein levels of TNFα and IL-6. Data are representative of three independent experiments (mean ± standard deviation). #P<0.05 vs. control group; *P<0.05, **P<0.01, ***P<0.001 vs. LPS group. FBDS, Feibi decoction-medicated serum; BMDMs, bone marrow derived macrophages; LPS, lipopolysaccharide.

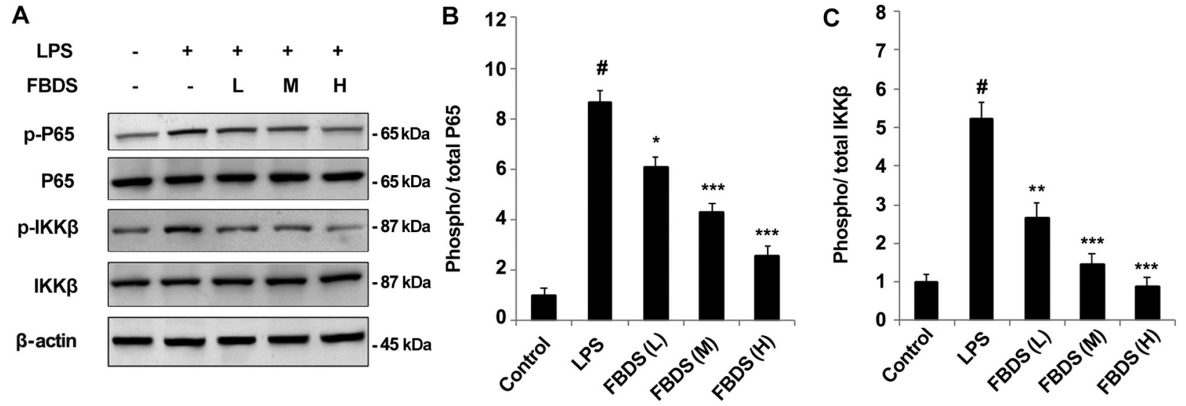

Figure 3. FBDS inhibits the activation of NF-κB signaling pathway. BMDMs were incubated with blank serum or FBDS (10%) for 24 h, followed by LPS stimulation (100 ng/ml) for 8 h. (A) Phosphorylation of p65 and IKKβ was examined by western blot assay. Quantification of protein levels of (B) p-p65 and (C) p-IKKβ in (A). Data are representative of three independent experiments (mean ± standard deviation). $^{\#}$P<0.05 vs. control group; *P<0.05, **P<0.01, ***P<0.001 vs. LPS group. FBDS, Feibi decoction-medicated serum; BMDMs, bone marrow derived macrophages; LPS, lipopolysaccharide; IKKβ, IκB kinase; p-, phosphorylated.

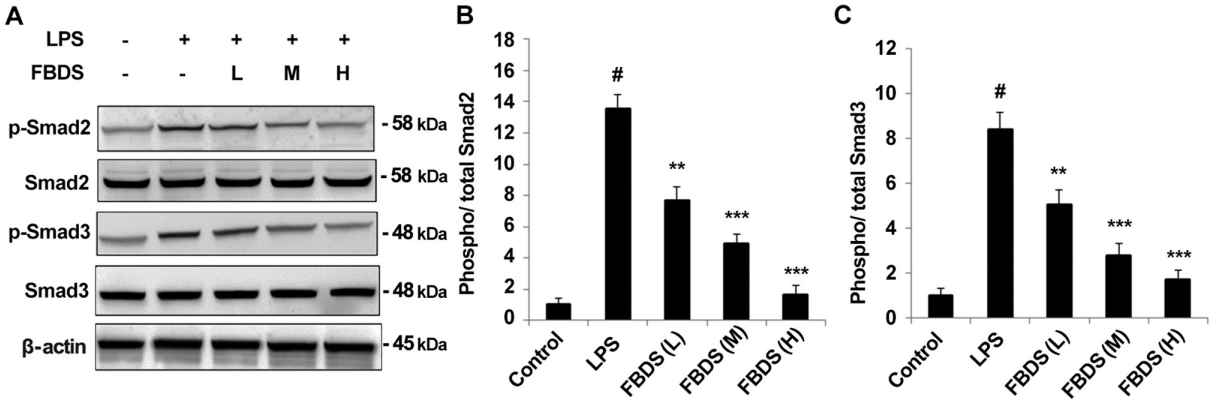

Figure 4. FBDS suppresses the activation of Smad2/Smad3. BMDMs were incubated with blank serum or FBDS (10%) for 24 h, followed by LPS stimulation (100 ng/ml) for 8 h. (A) Phosphorylation of Smad2 and Smad3 was examined by western blot assay. Quantification of protein levels of (B) p-Smad2 and (C) p-Smad3 in part (A). Data are representative of three independent experiments (mean ± standard deviation). $^{\#}$P<0.05 vs. control group; **P<0.01, ***P<0.001 vs. LPS group. FBDS, Feibi decoction-medicated serum; BMDMs, bone marrow derived macrophages; LPS, lipopolysaccharide; p-, phosphorylated.

phosphorylation of p65 and IKKβ were all suppressed by FBDS treatment in BMDMs (Fig. 3A) or RAW264.7 cells (Fig. S2A). Western blotting and quantification of protein levels of p-P65 and p-IKKβ are presented in Fig. 3.

FBDS suppresses the activation of Smad2/Smad3. A previous study indicated that the Smad signaling pathways are closely associated with the genesis and development of pulmonary fibrosis in response to inflammatory stimulation (17). Therefore the effect of FBDS on Smad2/3 activation was examined. As shown in Fig. 4A, phosphorylation of Smad2/3 induced by LPS was significantly inhibited in FBDS pre-treated BMDMs in a dose-dependent manner compared with the group treated with LPS and control serum, and similar results were also observed in RAW264.7 cells (Fig. S2B). Quantification of protein levels of p-Smad2 and p-Smad3 are presented as Fig. 4B and C.

FBDS decreases the expression of TGF-β1 and CHI3L1. TGF-β is well known as the critical upstream ligand of Smad signaling pathways. It was observed that FBDS treatment significantly decreased the mRNA level of TGF-β1 (Fig. 5A), as well as the protein level of TGF-β1 (Fig. 5B and C). Previous studies suggested CHI3L1 as a novel biomarker of inflammation, and whether FBDS could also regulate the expression of CHI3L1 was investigated (1,2). Notably, it was identified that the mRNA level of CHI3L1 was also decreased by FBDS treatment compared with the group treated with LPS and control serum (Fig. 5A). The protein level of CHI3L1 was also suppressed in FBDS-treated BMDMs (Fig. 5B and D) compared with the group treated with LPS and control serum. Similar results were also observed in RAW264.7 cells (Fig. S2C).

Discussion

PF is a chronic, debilitating and often lethal lung disorder. Although the molecular mechanisms of PF are gradually becoming clear with numerous researchers' efforts, few effective drugs have been developed to reverse human PF or even halt the chronic progression to respiratory failure (8,18,19).

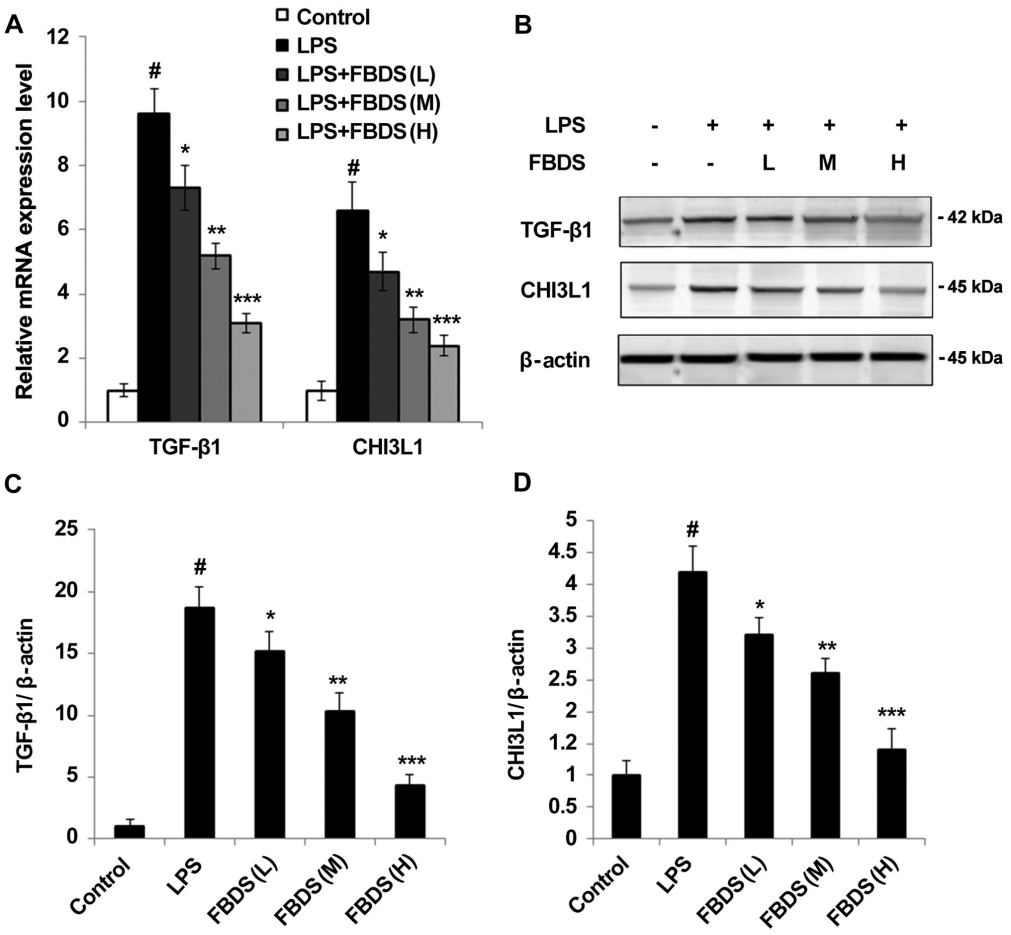

Figure 5. FBDS decreases the expression of TGF-β1 and CHI3L1. BMDMs were incubated with blank serum or FBDS (10%) for 24 h, followed by LPS stimulation (100 ng/ml) for 8 h. (A) The relative mRNA levels and (B) the protein levels of TGF-β1 and CHI3L1. Quantification of protein levels of (C) TGF-β1 and (D) CHI3L1 in B. Data are representative of three independent experiments (mean ± standard deviation). #P<0.05 vs. control group; *P<0.05, **P<0.01, ***P<0.001 vs. LPS group. FBDS, Feibi decoction-medicated serum; TGF, transforming growth factor; CHI3L1, chitinase-3-like protein 1; BMDMs, bone marrow derived macrophages; LPS, lipopolysaccharide.

Therefore, novel strategies are urgently required. Traditional Chinese medicine, which is the main component of the medical practice used for >5,000 years in China, has been demonstrated to be effective in the treatment of a diverse range human diseases (20-22). FBD has been widely used for treatment of patients with lung diseases such as cough and PF. For the first time, to the best of the authors' knowledge, it was identified in the present study that FBDS significantly inhibit proinflammatory cytokine production in macrophages, which suggested that FBD may also serve an anti-inflammatory role.

NF-κB, is reported as a central dimeric transcription factor, regulating the expression of genes responsible for innate and adaptive immunity, cell proliferation and apoptosis (15,16). Previous studies have also reported that NF-κB signaling participates in the regulation of PF (23,24). In present study, it was observed that FBDS treatment significantly alleviated the inflammatory cytokines production induced by LPS in a dose-dependent manner in RAW264.7 cells and BMDMs. In addition, it was identified that FBDS treatment individually did not affect the cell viability in RAW264.7 cells and BMDMs. Consistently, the inflammatory cytokines expression levels were not changed following LPS stimulation (data not shown), which indicated that FBDS may inhibit inflammation by affecting downstream signaling pathways stimulated by LPS, and NF-kB signaling pathway is the most important downstream pathway in response to the LPS stimulation. It was identified that FBDS suppressed the phosphorylation of P65 and IKKβ, indicating that FBD may inhibit the activation of NF-κB. Phosphorylation of Smad2 and Smad3 induces fibrosis and was hypothesized to be the main cause of PF (25). The effect of FBDS on LPS-induced phosphorylation of Smad2 and Smad3 was also detected, and it was identified that the levels of phosphorylation were significantly decreased by FBDS treatment; these data suggested that FBDS alleviated LPS-induced inflammation primarily through the NF-κB and Smad2/3 signaling pathways.

Notably, it was also identified that the mRNA and protein expression of TGF-β1, the critical upstream ligand of Smad signaling pathways, were significantly inhibited in FBDS-treated macrophages. In addition, the expression of CHI3L1, a novel biomarker of inflammation, was also inhibited at both mRNA and protein levels. However, the precise mechanisms by which FBD regulates the mRNA and protein expression of TGF-β1 and CHI3L1 requires further study. Although RAW264.7 cells and BMDMs were used to illustrate the effect of FBDS in inflammation, both of these cells are widely used in LPS-related inflammatory studies, so the results of the present study may also need to be repeated

in another different type of macrophage cell line to prove the robustness of the hypothesis.

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Availability of data and materials

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

Authors' contributions

WW, GL and YJ contributed to the conception and design of the study, data acquisition, analysis and revised the manuscript. ZL and XZ contributed to data collection and statistical analysis. HY and SL contributed to data collection, statistical analysis and manuscript preparation. XZ and YJ confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (26). The experiments were carried out according to the NIH Guide for the Care and Use of Laboratory Animals (26) and approved by the Animal Ethics Committee of the Scientific Investigation Board of Beijing university of Chinese medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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