IL-10/IL-10 receptor 1 pathway promotes the viability and collagen synthesis of pulmonary fibroblasts originated from interstitial pneumonia tissues

HONG YE[1*], JIONGWEI PAN[1*], XIAOPING CAI[1*], ZHANGYONG YIN[1], LU LI[1], ENHUI GONG[1], CUNLAI XU[1], HAO ZHENG[1], ZHUO CAO[1], ENGUO CHEN[2] and JUNFENG QIAN[1]

[1]Respiratory Department, The Sixth Affiliated Hospital of Wenzhou Medical University/Lishui People's Hospital, Lishui, Zheijang 323000; [2]Department of Respiratory and Critical Care Medicine, Sir Run Run Shaw Hospital, Affiliated to Zhejiang University School of Medicine, Hangzhou, Zheijang 310016, P.R. China

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Abstract. Interstitial pneumonia is a pulmonary interstitial inflammatory and fibrosis disease with a variety of causes that causes respiratory disorders and threatens the lives of patients. The present study aimed to investigate the expression of interleukin (IL)-10 in peripheral blood of patients with interstitial pneumonia and its biological functions in pulmonary fibroblasts. A total of 42 patients with idiopathic pulmonary fibrosis (IPF) and 20 healthy subjects were included. ELISA was used to determine IL-10 concentration in serum from the patients and healthy subjects. Primary fibroblasts were isolated from lung tissue successfully and determined by morphology. The CCK-8 assay was performed to determine the effect of IL-10 expression on cell viability. Western blotting was used to determine COL1a1, COL1a2 and IL-10R1 protein expression. Flow cytometry was used for cell cycle analysis and to determine the number of IL-10$^+$ cells. Expression of IL-10 in serum from IPF patients was higher compared to that from healthy subjects. IL-10 promoted the viability and collagen synthesis and secretion of MRC-5 cells and primary pulmonary fibroblasts. IL-10 and IL-10 receptor (R) 1 served regulatory roles in the viability and collagen synthesis of MRC-5 cells. The ratio of peripheral mononuclear lymphocytes with positive expression of IL-10 was elevated in peripheral blood from patients with IPF. The present study demonstrated that IL-10 expression in peripheral blood of patients with IPF is increased significantly compared with healthy subjects. Activation of the IL-10/IL-10R1 signaling pathway promoted the viability and collagen synthesis and secretion of pulmonary fibroblasts, leading to pulmonary fibrosis. The present study provided experimental basis for further understanding the development mechanism of pulmonary fibrosis.

Correspondence to: Dr Junfeng Qian or Dr Zhuo Cao, Respiratory Department, The Sixth Affiliated Hospital of Wenzhou Medical University/Lishui People's Hospital, 15 Dazhong Street, Lishui, Zhejiang 323000, P.R. China
E-mail: 63631790@qq.com
E-mail: caozhuo001@126.com

*Contributed equally

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Introduction

Interstitial pulmonary diseases are a group of diseases caused mainly by pathological changes in the alveolar wall (1). The basic pathological changes of interstitial pulmonary diseases include diffuse lung parenchyma, alveolitis and interstitial fibrosis, and their clinical manifestations comprise active dyspnea, diffuse shadow in X-ray chest film, restrictive ventilation disorder, decreased diffusion function and hypoxemia (1,2). Interstitial pulmonary diseases have a variety of subtypes, among which idiopathic pulmonary fibrosis (IPF) is the most harmful one (3). IPF is a chronic, progressive and fatal interstitial pulmonary disease, and there is no effective treatment due to its unknown pathogenesis (4,5). The incidence rate of IPF varies according to different regions, different populations and different occupations (6). In developed countries, the incidence rate is as low as three in 100,000 (6). In developing countries, its incidence is slightly higher. Overall, the incidence rate of males is higher than that of females (6). It is believed that repeated pulmonary epithelial cell injury and myofibroblast activation are the direct causes of pulmonary fibrosis (7,8). A large number of fibrosis-promoting cytokines produced in the process of injury repair can induce fibroblasts to differentiate, proliferate, migrate and invade through the autocrine or paracrine tissues (9). This results in continuous deposition of extracellular matrix (ECM), hinders normal injury repair and ultimately leads to the destruction of lung structure and the occurrence of fibrosis (9,10).

Abnormal proliferation of fibroblasts and increase of ECM are the basic pathological features of interstitial pulmonary diseases, indicating that fibroblasts serve. Important biological roles in the diseases (11,12). Fibroblasts are the

main cell components in lung connective tissues, differentiated from mesenchymal cells with a spindle flat star shape and protuberances (13,14). Fibroblasts have large cell bodies and their cytoplasm has weak basophilia (15). Under normal conditions, the main functions of fibroblasts include sustaining normal lung morphology, synthesizing and releasing ECM, participating in gas exchange in lung tissues and maintaining normal physiological functions of pulmonary epithelial and endothelial cells such as secretion (16). However, fibroblasts demonstrate abnormal proliferation and differentiation and secrete a large amount of ECM in interstitial pneumonia (17).

Cytokines serve important roles in the occurrence and development of interstitial pneumonia (18). For example, TGF-β is the strongest fibrogenic cytokine that can stimulate the proliferation of pulmonary fibroblasts and make them transform into myofibroblasts, hence it is an important target for the inhibition of pulmonary fibrosis (19). Studies on cytokines that are involved in pulmonary fibrosis have important clinical implications (20). Interleukin (IL)-10 is a type of inflammatory cytokine that is involved in tumor development and progression, interstitial pulmonary diseases and autoimmune diseases (21,22). IL-10 is secreted mainly by lymphocytes, macrophages and mast cells and it can inhibit macrophages and Th1 cells and enhance the biological function of B cells (23). In addition, IL-10 reduces the production of inflammatory cytokines, such as IL-2 and IFN-γ, thus inhibiting inflammatory responses (24). It has also been reported that IL-10 participates in the occurrence and development of pulmonary fibrosis (25). However, the role of IL-10 in pulmonary fibroblasts remains unclear. The present study aimed to investigate the regulatory relationship between IL-10 and lung fibroblasts at a cellular and molecular level and to provide experimental basis for clinical targeted intervention in interstitial pneumonia.

Materials and methods

Subjects. A total of 42 patients with IPF (31 males and 11 females; age range, 53-71 years; mean age, 62.4±7.3 years) were diagnosed at Lishui People's Hospital (Lishui, China) between July 012 and July 2018 according to IPF non-traumatic diagnostic criteria formulated by ATS/ERS/JRS/ALAT in 2011 (26). The exclusion criteria for the patients were: i) Not having chronic obstructive pulmonary disease; ii) having collagen angiopathy complicated with interstitial pulmonary diseases; iii) having unstable angina pectoris; iv) having interstitial pulmonary diseases caused by occupational and environmental exposures; and v) having interstitial pulmonary diseases and other serious diseases caused by drugs or known causes. The inclusion criterion was IPF patients diagnosed in Lishui People's Hospital (Lishui, China) who had no history of cancer, diabetes, hypertension, autoimmune diseases or chronic medication. Among the 42 patients, 38 were smokers and 4 were non-smokers. Twenty healthy subjects were included as control group (age range, 33-45 years; 15 males and 5 females).

From all subjects, 10 ml blood was drawn from the elbow vein. Of the 10 ml blood, 8 ml was used for separating serum after centrifugation at 1,500 x g and 4˚C for 10 min, and 2 ml was used to obtain peripheral mononuclear lymphocytes for flow cytometry. The serum and peripheral mononuclear lymphocytes were stored at -80˚C until use. All procedures performed in the current study were approved by the Ethics Committee of Wenzhou Medical University (Lishui, China). Written informed consent was obtained from all patients or their families.

Cells and transfection. Lung tissues were collected during lobectomy and cut into 1 mm^3 size pieces. Then, the tissues were cultured in DMEM supplemented with 20% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. Every 2 days, the tissues were observed and the medium was replenished. Subsequently, ~3 weeks later, primary fibroblasts crawled out of tissue mass and continued growing. On reaching 70-80% confluency, the fibroblasts were passaged at a volume ratio of 1:3 (cell suspension vs. medium) and cultured in DMEM medium supplemented with 10% fetal bovine serum at 37˚C and 5% CO_2.

MRC-5 cells ($2x10^5$; Cell Bank of the Chinese Academy of Sciences, Shanghai, China) in logarithmic growth phase were seeded into 24-well plates, and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. When cells reached 70% confluency, they were transfected with the small-interfering RNA of IL-10RA1 (siR-IL-10RA1; 5'-GGTCTACAGCAT CGAGTAT-3'; Hanbio Biotechnology Co., Ltd.) or siR-NC (non-targeting siRNA sequences; Hanbio Biotechnology Co., Ltd.) using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 1.5 μl siR-IL-10RA1 or siR-NC (50 pmol/μl; Hanbio Biotechnology Co., Ltd.) was mixed with 50 μl Opti Mem medium (Thermo Fisher Scientific, Inc.) in a vial. In another vial, 1 μl Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.) was mixed with 50 μl Opti Mem medium. After standing for 5 min, the two vials were combined for further incubation at room temperature for another 20 min. Then, the mixtures were added onto cells in thesiR-IL-10RA1 and siR-NC groups. Next, 6 h later, the medium was replaced with RPMI-1640 medium containing 10% fetal bovine serum. After culturing at 37˚C for 48 h, the cells were collected for subsequent experimentation. To examine the effect of IL-10 on fibroblasts, MRC-5 cells were cultured with the serum of IPF patients (serum group) or the serum of IPF patients containing IL-10 antibody (serum+IL-10 antibody group) at 37˚C and 5% CO_2 for 24 h. Untreated MRC-5 cells were used as negative control (NC) group.

The present study included eight cases of primary cell culture and five cases were successful. Cellular experiments were performed on the five successful cases.

Enzyme-linked immunosorbent assay (ELISA). HumanIL-10 ELISA kit (abs510005-96T; Absin Bioscience Inc.) was used to determine the concentration of IL-10 in serum. In microplates, standards (100 μl) and samples (100 μl serum) were added into predefined wells, while blank wells were left empty. In the wells for standards and samples, horseradish peroxidase-labelled conjugates (100 μl) were added before sealing the plates for incubation at 37˚C for 1 h. After washing the plates 5 times, substrates A (50 μl) and B (50 μl) were added into each well. After incubation at 37˚C for 15 min, stop

solution (50 μl) was added into each well, and absorbance of each well was measured at 450 nm within 15 min.

Reverse transcription-quantitative (RT-q) PCR. Transfected cells ($1x10^6$) were directly lysed with 1 ml TRIzol® reagent (Invitrogen; Thermo Fisher Scientific Inc.) at room temperature for 30 min. Total RNA was extracted using the phenol chloroform method. The concentration and quality of RNA was measured using ultraviolet spectrophotometry (NanoDrop ND2000; Thermo Fisher Scientific Inc.). The acceptable concentration was 50-150 ng/μl and the acceptable ratio of A260/A280 was between 1.8 and 2.0. Next, cDNA was obtained by reverse transcription from 1 μg RNA and stored at -20˚C. Reverse transcription of mRNA was performed at 50˚C for 45 min using TIANScript II cDNA First Strand Synthesis kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's manual. SuperReal PreMix (SYBR-Green) RT-qPCR kit (Tiangen Biotech Co., Ltd.) was used to detect mRNA expression of IL-10R1, using GAPDH as an internal reference gene. The sequences of IL-10R1 were: forward, 5'-TGAAAA CAAGAGCAAGGCCG-3' and reverse, 5'-ATCCCTCCGAGA CACTGGAA-3'. The sequences of GAPDH used were: forward, 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse, 5'-GGC TGTTGTCATACTTCTCATGG-3'. The reaction system (20 μl) was composed of 10 μl SYBR Premix EXTaq, 0.5 μl upstream primer, 0.5 μl downstream primer, 2 μl cDNA and 7 μl ddH_2O. The thermocycling conditions used were as follows: Initial denaturation at 95˚C for 10 min; denaturation at 95˚C for 1 min and annealing at 60˚C for 30 sec (40 cycles); elongation at 72˚C for 30 sec and were performed using the iQ5 instrument (Bio-Rad Laboratories Inc.). The $2^{-\Delta\Delta Cq}$ method (27) was used to calculate the relative expression of IL-10R1 mRNA against GAPDH. Each sample was tested in triplicate.

CCK-8 assay. MRC-5 cells were trypsinized and seeded into 96-well plates at a density of $2x10^3$/well. At 0, 24, 48 and 72 h, the medium was discarded, and the cells were washed with phosphate-buffered saline (PBS) twice, followed by addition of DMEM medium and 10 μl CCK-8 reaction reagent (5 g/l; Beyotime Institute of Biotechnology). After incubation at 37˚C and 5% CO_2 for 2 h, the absorbance of each well was measured at 490 nm for plotting cell viability curves.

Flow cytometry. Flow cytometry was used for cell cycle analysis. At 24 h after transfection or treatments with serum, $1x10^6$ MRC-5 cells of NC, serum and serum + IL-10 antibody groups or siR-NC and siR-IL-10R1 groups were washed twice with precooled PBS. The centrifugation was at 2,000 x g for 10 min and 4˚C. BD Cycletest Plus DNA Reagent kit (catalogue no. 340242; BD Biosciences) was used to perform the cell cycle analysis according to the manufacturer's instructions. The cells were permeabilized using the liquid provided in BD Cycletest Plus DNA Reagent kit (catalogue no. 340242; BD Biosciences). The staining buffer contained RNAse. The cells were incubated with 200 μl liquid A for 10 min and 150 μl liquid B for another 10 min. Then, the cells were incubated with 120 μl liquid C in the dark for 10 min before flow cytometry (FACSCalibur; BD Biosciences). The result was analyzed using ModFit software version 3.2 (Verity Software House Inc.).

For separating peripheral mononuclear lymphocytes, 3 ml sterile mononuclear cell separation solution was added into 15 ml tube before gently adding 2 ml peripheral blood on top of the solution. After centrifugation at 100 x g at room temperature for 20 min, the layer of peripheral mononuclear lymphocytes was gently aspirated and mixed with 10 ml sterile PBS, before centrifugation at 100 x g at room temperature for 5 min. Then, the supernatant was discarded and 5 ml PBS was added to resuspend the cells. After centrifugation at 100 x g at room temperature for 5 min, 250 μl BD Cytofix/Cytoperm reagent (BD Biosciences) was used to resuspend the cells, which were incubated at 4˚C for 20 min and permeabilized. Then, 1 ml PBS was added to stop perforation. After centrifugation at 100 x g at room temperature for 5 min, the cells were collected and stained with IL-10 antibody (1:3,000; cat. no. PI528; Beyotime Institute of Biotechnology) in the dark at room temperature for 30 min. Finally, the cells were examined by flow cytometry (FACSCalibur; BD Biosciences).The result was analyzed using ModFit software version 3.2 (Verity Software House Inc.).

Western blotting. After treatment for 48 h, the cells in NC, serum and serum+IL-10 antibody groups or siR-NC and siR-IL-10R1 groups were collected and washed with PBS twice. Then, the cells were lysed with 600 μl precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) for 5 min on ice. The mixture was centrifuged at 13,000 x g and 4˚C for 10 min. The supernatant was used to determine protein concentration using the bicinchoninic acid (BCA) protein concentration determination kit (RTP7102; Real-Times Biotechnology Co., Ltd.). Subsequently, the samples were mixed with 5X sodium dodecyl sulfate loading buffer before denaturation in a boiling water bath for 10 min. Subsequently, the protein samples (20 μg/lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 V. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (250 mA; 1 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-human IL-10R1 (1:1,000; abs136163), IL-10R2 (1:1,000; abs138477), collagen type I α1 chain (COL1a1; 1:1,000; abs118788), collagen type I α2 chain (COL1a2; 1:1,000; abs101119) or GAPDH (1:4,000; abs132004) polyclonal primary antibodies (all from Absin Bioscience Inc.) at 4˚C overnight. After extensive washing with PBS with 0.1% Tween-20 3 times (each wash for 15 min), the membranes were incubated with goat anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibody (1:4,000; abs20040; Absin, Shanghai, China) for 1 h at room temperature before washing with PBS with Tween 20 3 times (each wash for 15 min). Subsequently, membrane development was performed using the enhanced chemiluminescence detection kit (Abcam) for imaging. Image lab v.3.0 software (Bio-Rad Laboratories Inc.) was used to acquire and analyze imaging signals. GAPDH was used as the loading control.

Immunohistochemistry. Sterilized cover glasses were placed in a 90 mm culture dish, and then the cells were seeded in the culture dish at a density of $2x10^4$/ml for cell climbing. On the following day, the cells attached on the cover slip were washed

with PBS for three times of 2 min. Then, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min and dried under room temperature for 5 min. After washing with PBS for 3 times of 2 min, the cells were incubated with 0.5% Triton X-100 (dissolved in DPBS) at room temperature for 20 min before washing again with PBS for 3 times of 2 min. After addition of 3% H_2O_2, the cells were incubated for 15 min at room temperature. Following washing with PBS for 3 times of 2 min, the cells were incubated with blocking serum at room temperature for 20 min. Then, primary vimentin antibody (abs131996; Absin, Shanghai, China) was added before incubation at 40˚C overnight. Subsequently, the cells were incubated with secondary antibody pv6001 at 37˚C for 30 min. After washing with PBS for 5 times of 2 min, 1 drop of diaminobenzidine (DAB) liquid was added to the section was dripped with. After 10 min, the section was washed with water for 5 min. After staining with hematoxylin at room temperature for 10 min, the section was washed with water for 5 min before being sealed in gum.

Statistical analysis. Results were analyzed using SPSS 18.0 (IBM Corp.). The data were expressed as means ± standard deviations. The number of biological replicates was three. Comparison between two groups was carried out using paired Student's t-tests. Multiple group comparisons were performed by one-way analysis of variance followed by a post hoc Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IL-10 promotes the viability and collagen synthesis of MRC-5 cells and primary pulmonary fibroblasts. To measure the levels of IL-10 in serum samples from 42 IPF patients and 20 healthy subjects, ELISA was performed. The data demonstrated that the level of IL-10 in serum from patients with (135.7±12.5 ng/μl) was significantly higher compared with that in normal subjects (21.6±5.8 ng/μl) ($P<0.05$; Fig. 1A). To examine the effect of IL-10 on fibroblasts, MRC-5 cells were cultured with the serum of patients with IPF for 24 h in the absence or presence of IL-10 antibody. The CCK-8 assay demonstrated that the absorbance of MRC-5 cells stimulated with serum from patients with IPF was significantly higher compared with that of MRC-5 cells treated with serum from healthy subjects at 24, 48 and 72 h ($P<0.05$ for all time points; Fig. 1B), while co-incubation with IL-10 antibody reduced the absorbance of MRC-5 cells to a level similar to that of MRC-5 cells treated with serum from healthy subjects ($P>0.05$; Fig. 1B). Flow cytometry demonstrated that, compared with MRC-5 cells that were not treated with serum (NC group), the transition from G_1 to S phase in MRC-5 cells treated with serum from IPF patients was accelerated ($P<0.05$; Fig. 1C), while treatment with IL-10 antibody reversed this to a level similar to NC group ($P>0.05$; Fig. 1C). Western blotting demonstrated that COL1a1 and COL1a2 protein expression in MRC-5 cells treated with serum from IPF patients was significantly higher compared with that in the MRC-5 cells of the negative control group ($P<0.05$; Fig. 1D), while treatment with IL-10 antibody reversed this to a level similar to the negative control group ($P>0.05$; Fig. 1D). To further confirm the effect of IL-10 on fibroblasts, primary fibroblasts were isolated and identified. Microscopy demonstrated that primary pulmonary fibroblasts were spindle-shaped (yellow-brown indicated positive signals), and immunohistochemistry detected the marker protein vimentin in the fibroblasts (Fig. 2A). The present study included 8 cases of primary cell culture and 5 cases were successful. Cellular experiments were performed on the 5 successful cases. Similar results were observed in primary fibroblasts compared to those from MRC-5 cells (Fig. 2B-D). The aforementioned results suggested that IL-10 promotes the viability and collagen synthesis of MRC-5 cells and primary pulmonary fibroblasts.

IL-10 and IL-10R1 play regulatory roles in the viability and collagen synthesis of MRC-5 cells. To investigate the mechanism by which IL-10 regulates MRC-5 cells, the mRNA and protein expression of IL-10 receptor IL-10R1. RT-qPCR and western blotting demonstrated that IL-10R1 mRNA and protein expression in MRC-5 cells treated with serum from patients with IPF was significantly higher compared with that in the negative control group ($P<0.05$; Fig. 3A and B), while addition of IL-10 antibody did not decrease the level of IL-10R1 mRNA and protein compared with serum group ($P>0.05$; Fig. 3A and B). After transfecting MRC-5 cells with siR-IL-10R1, the expression of IL-10R1 protein was decreased compared with the siR-NC group ($P<0.05$; Fig. 3C). The CCK-8 assay demonstrated that the viability of MRC-5 cells in the siR-IL-10R1 group was decreased compared with the siR-NC group ($P<0.05$ at all time points; Fig. 3D). Flow cytometry demonstrated that siR-IL-10R1 slowed down the transition from G_1 phase to S phase compared with that of siR-NC group ($P<0.05$; Fig. 3E). In addition, expression of COL1a1 and COL1a2 in siR-IL-10R1 group was lower compared with that in the siR-NC group (Fig. 3F). The aforementioned results suggested that IL-10 and IL-10R1 serve regulatory roles in the viability and collagen synthesis of MRC-5 cells.

The ratio of peripheral mononuclear lymphocytes with positive expression of IL-10 is elevated in peripheral blood from patients with IPF compared with healthy subjects. To detect the expression of IL-10 in peripheral mononuclear lymphocytes, flow cytometry was performed. The data demonstrated that the ratio of $IL\text{-}10^+$ peripheral mononuclear lymphocytes in patients with IPF was significantly higher compared with that in normal subjects ($P<0.05$; Fig. 4A and B). Peripheral mononuclear lymphocyte population itself looked much smaller in the normal subjects compared with the patients with IPF, possibly because of the proliferation of inflammatory cells caused by IPF inflammation. The aforementioned result indicated that the ratio of peripheral mononuclear lymphocytes with positive expression of IL-10 is elevated in peripheral blood from patients with IPF.

Discussion

Chronic airway inflammation, myofibroblast proliferation and ECM synthesis are the main mechanisms of pulmonary subepithelial fibrosis in patients with interstitial pneumonia (28,29). In recent years, pulmonary fibroblasts have become important targets in improving airway remodeling (30). Hence, the role

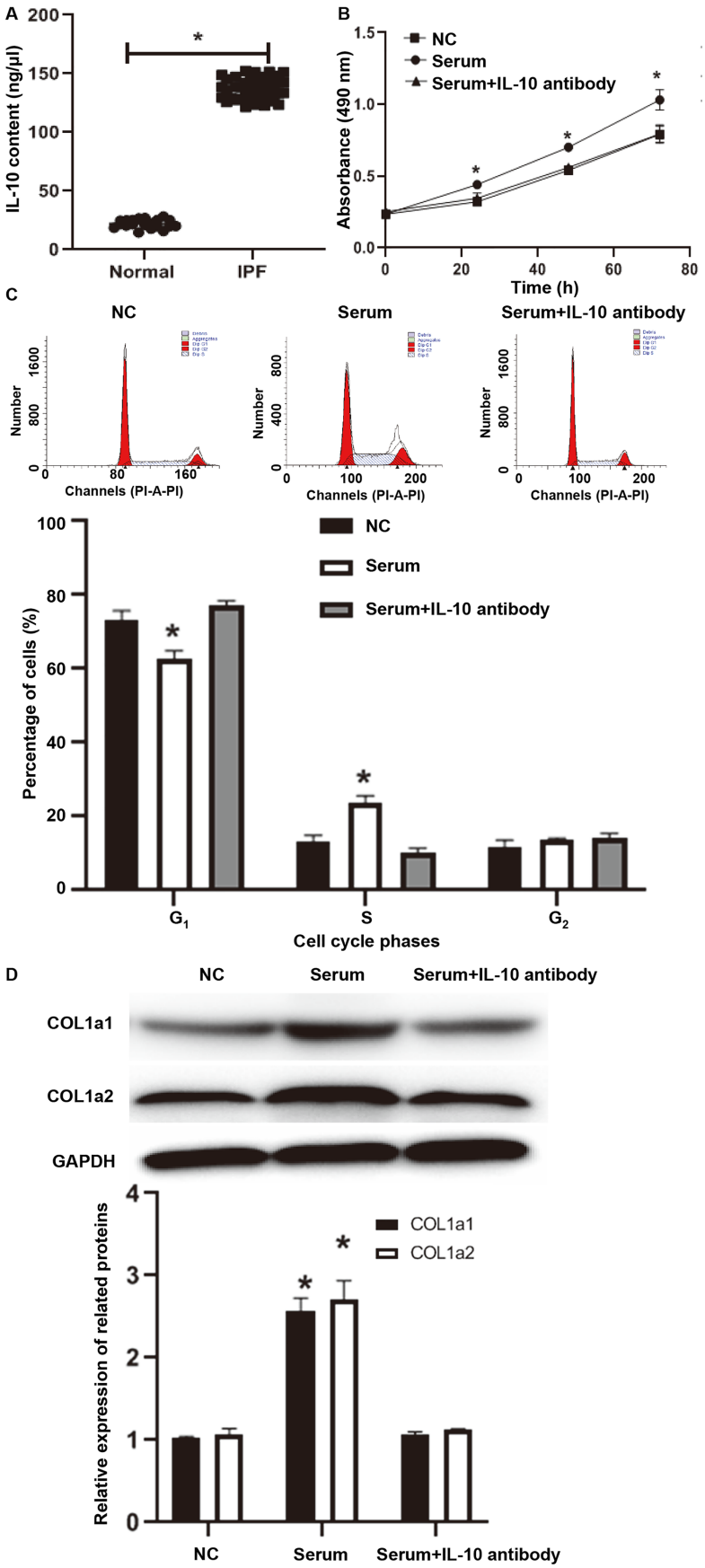

Figure 1. Expression of IL-10 in peripheral blood of patients with IPF and its effect on the biological function of MRC-5 cells. (A) Content of IL-10 in peripheral blood of IPF patients. ELISA was used to determine IL-10 content. *P<0.05. (B) Viability of MRC-5 cells after treatment with IPF serum in the absence or presence of IL-10 antibody. CCK-8 assay was used to determine viability. *P<0.05 compared with NC group. (C) Percentages of MRC-5 cells at G_1, S and G_2 phases of cell cycle. Flow cytometry was performed to investigate the cell cycle. *P<0.05 compared with the NC group. (D) Expression of COL1a1 and COL1a2 proteins in MRC-5 cells. Western blotting was used to determine protein expression. *P<0.05 compared with NC group. NC, negative control group (untreated MRC-5 cells); IPF, idiopathic pulmonary fibrosis; PI, propidium iodide; COL1a1, collagen type I α 1 chain; COL1a2, collagen type I α2 chain.

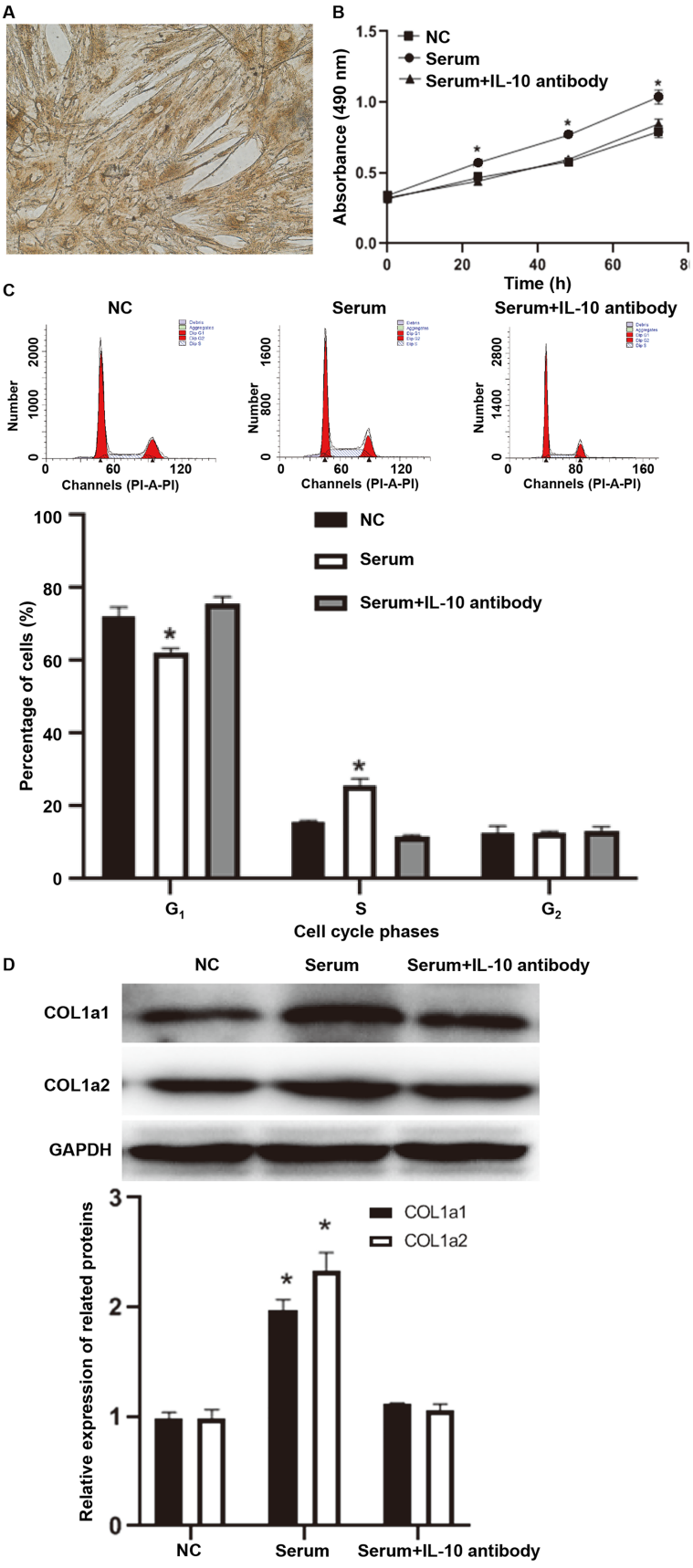

Figure 2. Microscopy of primary pulmonary fibroblasts and the effect of IL-10 on the biological function of the fibroblasts. (A) Immunohistochemistry detection of vimentin in primary pulmonary fibroblasts. Magnification, x100. (B) Viability of primary pulmonary fibroblasts after treatment with IPF serum in the absence or presence of IL-10 antibody. CCK-8 assay was used to determine viability. *P<0.05 compared with NC group. (C) Percentages of primary pulmonary fibroblasts at G_1, S and G_2 phases of cell cycle. Flow cytometry was performed to investigate the cell cycle. *P<0.05 compared with NC group. (D) Expression of COL1a1 and COL1a2 proteins in primary pulmonary fibroblasts. Western blotting was used to determine protein expression. *P<0.05 compared with NC group. NC, negative control group (untreated primary pulmonary fibroblasts); IPF, idiopathic pulmonary fibrosis; PI, propidium iodide; COL1a1, collagen type I α 1 chain; COL1a2, collagen type I α2 chain.

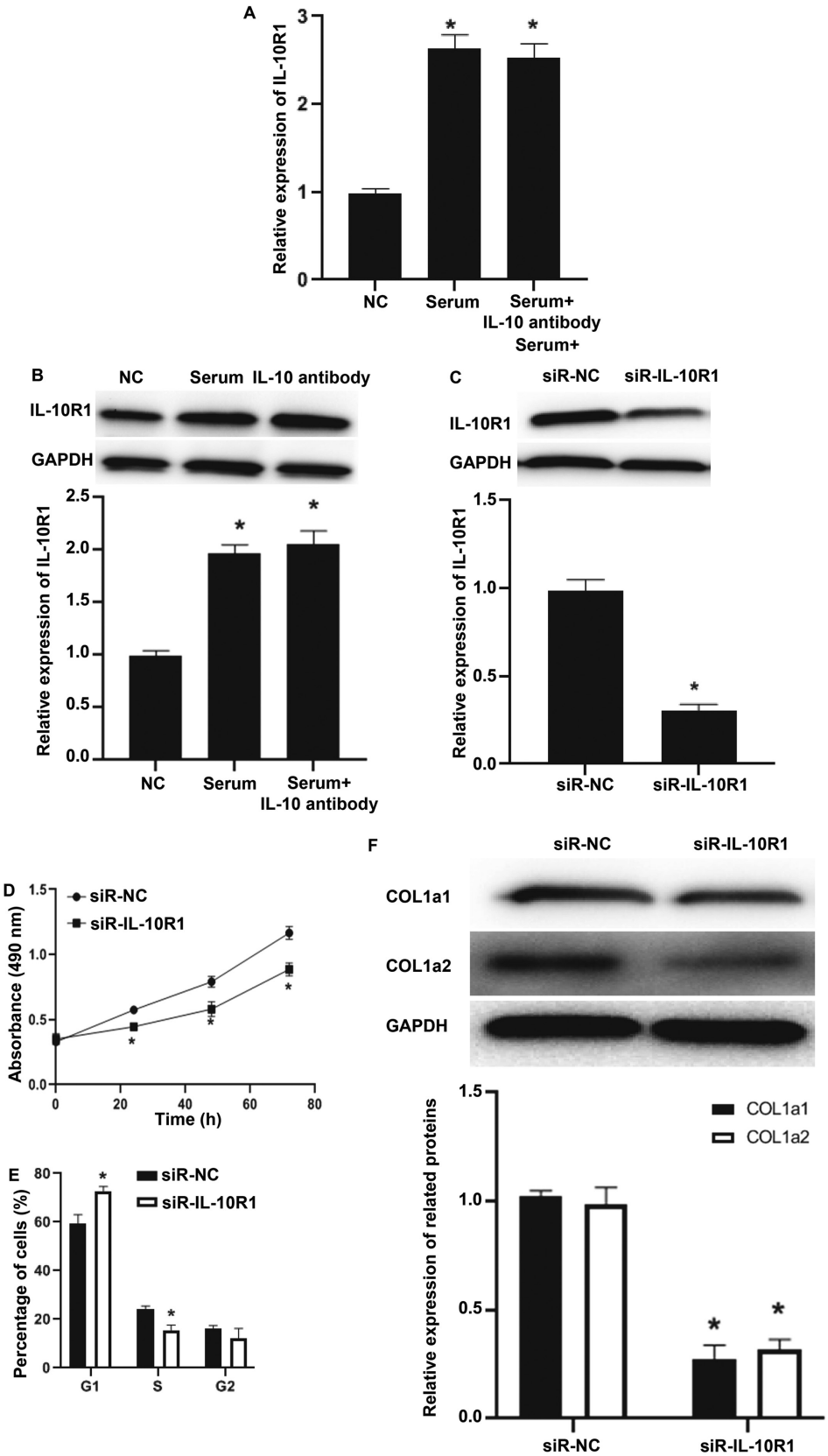

Figure 3. Regulation of the functions of MRC-5 cells by IL-10/IL-10R1. Expression of (A) IL-10R1 mRNA and (B) protein in MRC-5 cells in NC group (untreated cells), serum group (incubation with IPF serum), and serum+IL-10 antibody group (incubation with IPF serum and IL-10 antibody). Reverse transcription-quantitative PCR was used to determine mRNA expression, while Western blotting was employed to determine protein expression. *P<0.05 compared with NC group. (C) Expression of IL-10R1 protein in MRC-5 cells transfected with siR-negative control (siR-NC) or siR-IL-10R1. Western blotting was employed to determine protein expression. *P<0.05 compared with siR-NC group. (D) Viability of MRC-5 cells transfected with siR-NC or siR-IL-10R1. CCK-8 assay was employed to determine viability. *P<0.05 compared with siR-NC group. (E) Cell cycle of MRC-5 cells transfected with siR-NC or siR-IL-10R1. Flow cytometry was employed to determine cell cycle. *P<0.05 compared with siR-NC group. (F) Expression of COL1a1 and COL1a2 proteins in MRC-5 cells transfected with siR-NC or siR-IL-10R1. Western blotting was used to determine protein expression. *P<0.05 compared with siR-NC group. IL-10R1, IL-10 receptor 1; si, small interfering; NC, negative control; COL1a1, collagen type I α 1 chain; COL1a2, collagen type I α2 chain.

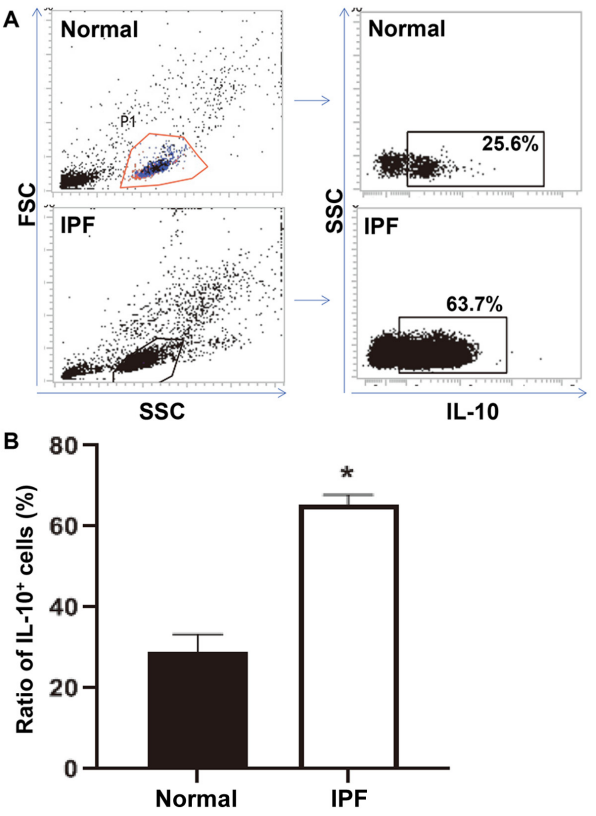

Figure 4. Ratio of mononuclear lymphocytes with positive expression of IL-10. (A) Flow cytometric analysis of IL-10$^+$ mononuclear lymphocytes. (B) Ratio of IL-10$^+$ mononuclear lymphocytes. Lymphocytes were isolated from healthy normal subjects and patients with IPF. Flow cytometry was used to detect IL-10$^+$ mononuclear lymphocytes. *P<0.05 compared with normal subjects. IPF, idiopathic pulmonary fibrosis.

of pulmonary fibroblasts in airway remodeling in asthma has attracted increasing attention. IL-10 is a type of cytokine that has attracted a lot of attention in recent years (31). Numerous tissues and cells, such as T cells, B cells, macrophages, bronchial epithelial cells and tumor cells produce IL-10 (32,33). IL-10 is a multidirectional immunoregulatory factor and its main activity is immunosuppression (34). Studies have demonstrated that IL-10 has different biological functions in a number of other cells. For example, IL-10 induced the expression of E-selectin on the surface of vascular endothelial cells and regulated the barrier function of endothelial cells (35). Treatment targeting IL-10 inhibited the apoptosis of CD8$^+$ cells induced by dendritic cells (36). In addition, IL-10 serves important roles in lung injury and pneumonia. For example, the expression of IL-10 and TGF-β in biopsy tissues of patients with IPF was significantly increased compared with that from healthy subjects and involved in pulmonary fibrosis, indicating that they are important therapeutic targets (37). In addition, a study demonstrated that IL-10 delivered by hydrogel improved treatment of bleomycin-induced lung fibrosis in mice (25). Consistent with a previous report (38), the present study revealed that expression of IL-10 in peripheral blood of patients with IPF was significantly increased compared with normal subjects, suggesting that IL-10 may serve a role in pulmonary fibrosis. Pulmonary fibroblasts serve important roles in pulmonary fibrosis and secrete cytokines that are involved in the local inflammatory response and collagen secretion inducing matrix microenvironment remodeling (37). During the occurrence and development of interstitial pneumonia, abnormal proliferation of pulmonary fibroblasts occurs and the ability of collagen synthesis increases significantly (39). In the present study, primary pulmonary fibroblasts were cultured with the serum of patients with IPF which stimulated the viability and collagen synthesis of MRC-5 cells and primary pulmonary fibroblasts. Following addition of IL-10 antibody, the stimulation effect by IPF serum was reduced which suggested that IL-10 promoted the viability and collagen synthesis of lung fibroblasts.

IL-10 is a secretory cytokine that transmits extracellular signals mainly by binding to its membrane surface receptor IL-10R (40). IL-10R mainly consists of IL-10R1 and IL-10R2 subunits, and IL-10 first binds to IL-10R1 and then binds to IL-10R2 to activate downstream signaling pathways (41). IL-10/IL-10R pathway is involved in numerous pathological processes (42). For example, detection of IL-10 expression in serum may help predict the prognosis of patients with rheumatoid arthritis (43). In addition, IL-10 promoted drug resistance in non-small cell lung cancer (44). The present study demonstrated that the expression of IL-10R1, but not IL-10R2, in primary fibroblasts was increased significantly compared with untreated primary fibroblasts after treatment with serum from IPF patients. Serum from IPF patients could promote collagen secretion of fibroblasts, and IL-10 antibody was able to block this effect. Following interference with the expression of IL-10R1, the treatment effect of IPF serum on primary fibroblasts was significantly weakened in the present study. This suggested that IL-10/IL-10R1 promoted the viability and collagen secretion of primary fibroblasts. IL-10 can be secreted by a variety of cells, of which monocytes are the main cells (45). Using flow cytometry, the present study demonstrated that the number of IL-10-positive mononuclear lymphocytes in peripheral blood of patients with IPF was significantly higher compared with that in normal subjects, which suggested that mononuclear lymphocytes secreted IL-10 in patients with IPF and IL-10 promoted the viability and collagen secretion of lung fibroblasts leading to pulmonary fibrosis.

The present study had several limitations. In the current study, the heterogeneity of primary fibroblasts was very large and even the generational differences between fibroblasts from the same patient were very large. If cell sorting was used to select monoclonal cells, the heterogeneity could have been avoided to some extent, but diversity would have been lost at the same time. In addition, the present study only suggested that monocytes secrete IL-10 that acts on fibroblasts as there is infiltration by a lot of monocytes in interstitial pneumonia. In order to prove that IL-10 is derived from monocytes, more evidences required from future studies.

In conclusion, the present study demonstrated that the IL-10 level in peripheral blood of patients with IPF is increased significantly compared with normal subjects. Activation of the IL-10/IL-10R1 signaling pathway promotes the viability and collagen synthesis of pulmonary fibroblasts leading to pulmonary fibrosis. The present study explored the mechanism of IL-10 in the occurrence and development of interstitial pneumonia, and provided experimental basis for understanding the role of inflammatory factors in this disease.

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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

HY, JP, XC, ZC, EC and JQ contributed to the design of the study. HY, JP, XC, ZY, LL, EG, CX and HZ performed the experiments. HY and JP confirm the authenticity of all the raw data. HY, JP and XC analyzed the data. HY, JP, XC, ZC, EC and JQ interpreted results and drafted the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

All procedures performed in the current study were approved by the Ethics Committee of Wenzhou Medical University (Lishui, China) with approval no. IACUC-20180901-28. Written informed consent was obtained from all subjects or their families.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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