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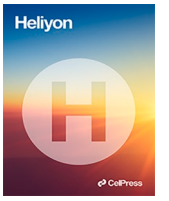

Imrecoxib attenuates bleomycin-induced pulmonary fibrosis in mice

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an incurable chronic progressive disease with a low survival rate and ineffective therapeutic options. We examined the effects of imrecoxib, a nonsteroidal anti-inflammatory drug, on experimental pulmonary fibrosis. The mouse IPF model was established by intratracheal instillation of bleomycin. From Day 0 to Day 13, the mice were orally administered imrecoxib (100 mg/kg) and pirfenidone (200 mg/kg) daily, and from Day 7 to Day 13, the mice were orally administered pirfenidone and imrecoxib daily. The tissues were dissected on the 14th day. Mouse body weight was measured, and histopathological examination and hydroxyproline content analysis confirmed that the administration of imrecoxib exerted a similar effect to pirfenidone. Compared with bleomycin-induced mice, imrecoxib-treated mice showed significantly reduced inflammatory factor expression (IL-1 and TNF-α) and inflammatory cell numbers (macrophages, lymphocytes, and neutrophils) in BALF (bronchoalveolar lavage fluid). Our experiment tested the ability of imrecoxib to inhibit the signal pathway involved in gene expression induced by TGF-β1 in the NIH-3T3 cell line in vitro. Western blotting showed that imrecoxib (20 μM and 40 μM) inhibited the expression of fibronectin, type I collagen and CTGF. In addition, imrecoxib reduced the levels of *p*-ERK1/2. The changes in the expression of related proteins in mouse lung tissue were similar to those in cells. In summary, our findings suggested that the administration of imrecoxib prevented and treated murine IPF by inhibiting inflammation and the TGF-β1-ERK1/2 signaling pathway.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is often associated with idiopathic interstitial pneumonia (IIP), is the most common form of IIP,

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occurs primarily in elderly individuals over 60 years of age, and has a 2.41-year median survival time [1]. Many causes of pulmonary fibrosis have been identified, such as genetic factors and environmental exposure; in addition, smoking can increase the risk of illness [2]. Pulmonary fibrosis is clinically characterized by progressive dyspnea with irritating dry cough; lung function is characterized by a restrictive ventilatory disorder, the condition continues to deteriorate, and eventually the patient may die due to respiratory failure. The initial stage of pulmonary fibrosis is the inflammatory phase, and the tissue undergoes abnormal repair as the inflammatory-immune response progresses. The pathological features generally include epithelial dysfunction, an inordinate increase in the number of fibroblasts and myofibroblasts, and excessive deposition of extracellular matrix (ECM) in the lungs [3].

In recent years, clinical drugs for IPF have included acetylcysteine, warfarin, pirfenidone, and nintedanib; however, patients with IPF generally exhibit poor responses to hormones and various drugs. Therefore, the discovery of potential targets and pharmacological mechanisms of pulmonary fibrosis and the development of safe drugs that effectively treat pulmonary fibrosis will be very important [4].

Imrecoxib is a highly selective COX-2 inhibitor that exerts anti-inflammatory effects and is often used to relieve the painful symptoms of osteoarthritis [5] (Fig. 1A). Since the early stage of pulmonary fibrosis is the inflammatory phase, we evaluated the pharmacodynamics of imrecoxib on inhibiting pulmonary fibrosis. The half-life of Imrecoxib in the blood of normal individuals is 8.20

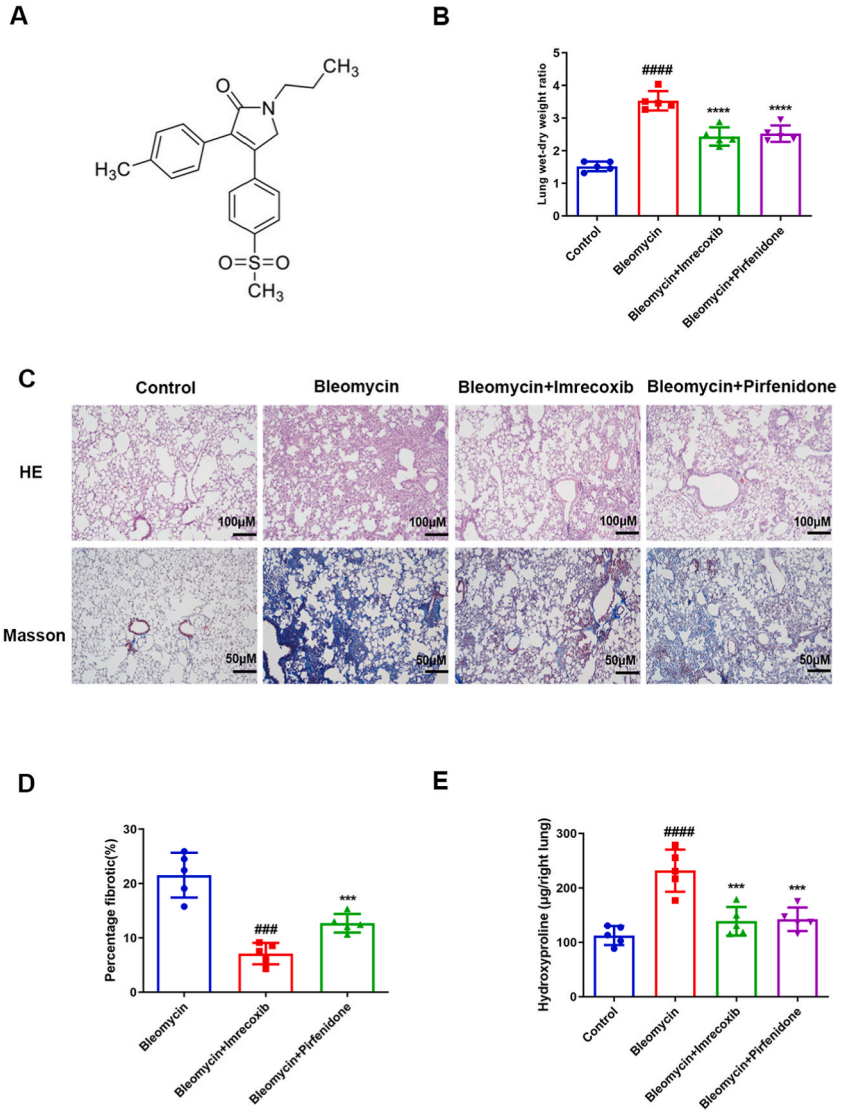

Fig. 1. Imrecoxib prevents bleomycin-induced pulmonary fibrosis in mice. (n = 5 in each experimental group.) **(A)** Structure of Imrecoxib. **(B)** Lung wet-dry weight ratio in the control group, bleomycin group, bleomycin + imrecoxib (100 mg/kg) and bleomycin + pirfenidone (200 mg/kg) group. **(C)** Photomicrographs of lung sections stained with hematoxylin–eosin (H&E) and Masson trichrome staining. **(D)** Lung fibrotic area analysis of the lung sections. The fibrotic area is presented as a percentage. **(E)** Hydroxyproline content on day14. $^{###}P < 0.001$ versus control group. $^{####}P < 0.0001$versus control group. ***P < 0.001 versus BLM group. ****P < 0.0001 versus BLM group.

± 2.70 h. The typical dose for adults is 0.1 g 2 times per day for 8 weeks [5].

Tracheal injection of bleomycin (BLM) is the most common method used to induce pulmonary fibrosis in animals. BLM induces lung injury in two stages: the first is the inflammatory phase, and the second is pulmonary fibrosis [6]. A variety of cytokines, including transforming growth factor beta1 (TGF-β1) and connective tissue growth factor (CTGF), participate in the development of bleomycin-induced pneumonia and fibrosis [7]. This study was designed to investigate whether imrecoxib ameliorated IPF in a mouse model by exerting anti-inflammatory effects and to explore whether the antifibrotic effect of imrecoxib was related to the TGF-β1 signaling pathway.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice aged 6–8 weeks were purchased from Beijing Weishenghe Experimental Animal Technology Co., Ltd. and kept in standard cages with light/dark periods of 12 h. The animals were kept in a specific pathogen-free environment at Nankai University and treated strictly according to the plan approved by the Animal Protection and Use Committee of Nankai University (approval number: 20140008). We received the animal ethics certificate before the animal experiments. All animal care and experimental procedures conformed to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (license number: SYXK 2021–0001). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act (1986) and associated guidelines such as EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). We affirm that these guidelines have been followed in our research.

In previous research, sodium pentobarbital has often been used to anesthetize mice. The dose was 0.1–0.2 ml/10 g. The concentration was 0.3 %. The dose was converted to mg of drug per kg of animal body weight, resulting in 30–60 mg/kg. In our study, we chose 50 mg/kg. Then, we selected a suitable method of euthanasia and injected 3 times the dose of sodium pentobarbital (150 mg/kg) into the intraperitoneal cavity. To confirm death in the mice, we followed the guidelines of Iowa State University. According to the guidelines, we did not use the single phenomenon of breathing cessation as a basis for judging death. We continued to observe other death criteria in the mice within 10 min of breathing cessation, such as the continuous disappearance of corneal reflexes, continuous breathing cessation, and continuous disappearance of heartbeat. After waiting for these criteria to be met, we observed that the heartbeat had stopped for 60 s to finally confirm death.

2.2. Bleomycin-induced PF and treatment

The bleomycin model is the most commonly used IPF model in rodents. A single dose of bleomycin (2 U/kg, Nippon Kayaku Co. Ltd.) was intratracheally administered to anesthetized mice on Day 0. In our study, we believe that the mice suffered little pain during the experiment. According to the principles on the humane endpoints of animal experiments that were jointly formulated by the Animal Experiment Management Committees of various institutions, the humane endpoints were as follows: the respiratory function of mice will be affected by tracheal intubation. Occasionally, mice will have symptoms such as twitching and tremors. At this time, we immediately stopped the experiment and euthanized the mouse to ensure that the principle of humane endpoints during the experiment was consistently implemented.

The model mice were intragastrically administered 100 mg/kg imrecoxib for 7 or 14 consecutive days, based on previous research [8,9]. This study used pirfenidone (200 mg/kg) as the positive control. According to the surface area conversion method of the doses of mice and humans, the dose of pirfenidone in humans was approximately 1538.46 mg/day, and so the clinically recommended daily dose was 1200–1800 mg. The two doses showed a corresponding relationship, which indicated the correctness of our use of this dose for the animal experiments.

2.3. Determination of the dry–wet weight ratio of mouse lungs

The left lung was weighed (wet weight), placed in a 70 °C constant temperature drying oven, and baked for 2 days. Then, the lung wet–dry weight ratio (W/D) was calculated.

2.4. Analysis of the hydroxyproline content

After the mouse was dissected, the two largest lobes of the right lung were removed and dried. The samples were hydrolyzed with 6 M hydrochloric acid for 16 h at 120 °C. The pH of the sample after hydrolysis was adjusted to 7.2–7.4 with NaOH, and the sample volumes were adjusted to 10 ml. Then, 50 μl of the sample was transferred to a new Eppendorf tube. The samples were incubated with chloramine-T for 20 min and perchloric acid for 5 min. Then, the samples were incubated at 60 °C for 20 min. The optical density was measured at 577 nm using a microplate reader. The hydroxyproline content was calculated after the conversion [10].

2.5. Histopathological examination

The left lung was removed and fixed in 4 % formaldehyde. Then, the lung tissues were dehydrated and embedded. The embedded lungs were cut into sections (4 μm thickness) and stained with hematoxylin-eosin (H&E) and Masson's trichrome.

2.6. Determination of the number of living cells in BALF

PBS was used to wash the pellets in the tubes, and then the pellets were resuspended in 200 μL of PBS for total cell counts and differential cell counts. The total number of cells in the tube was measured by a hemocytometer. A smear of each suspension was stained with H&E for differential cell counts, and 500 cells were classified as macrophages, neutrophils or lymphocytes using standard morphological criteria.

2.7. Inflammatory factor analysis and inflammatory cell counts

Lung tissues were immersed in PBS, homogenized and centrifuged to obtain the supernatant. ELISA was used to determine the concentration of TGF-β1 in lung tissues according to the manufacturer's protocol (Bio Legend).

The levels of other inflammatory factors and the number of inflammatory cells were determined by collecting bronchoalveolar lavage fluid (BALF). BALF was obtained by endotracheal intubation and lavage with 0.8 mL of cold, sterile PBS 3 times. Then, the collected alveolar lavage fluid was centrifuged at 1000 RPM for 10 min. According to the manufacturer's protocol (BioLegend), the supernatant was collected, and IL-4 and IL-13 concentrations were determined by ELISA. After the pellets were resuspended in erythrocyte lysis buffer, they were centrifuged. The supernatant was discarded, the cells were resuspended in Eppendorf tubes with PBS, and total cell counts and differential cell counts were performed.

2.8. Cell culture

The NIH-3T3 cell line was purchased from ATCC (American Type Culture Collection). In a humid environment of 37 °C with 5 % CO_2, DMEM containing 10 % FBS and antibiotics was used to culture NIH-3T3 cells. This cell line is a mouse embryonic lung fibroblast line that commonly used for research. Therefore, we used this cell line in this study.

2.9. Immunohistochemical staining

For immunohistochemical staining, the sections were dewaxed, antigen repaired in citrate buffer solution, and heated in a microwave oven. Staining was then conducted according to the instructions of the immunohistochemistry kit (Maxim KIT-9170). Histological images were captured using an Olympus BX53 microscope.

2.10. Western blotting

NIH-3T3 cells were grown to 85 % confluence, then the serum was removed to starve the cells for 24 h. Then, TGF-β1 (5 ng/ml) and imrecoxib (20 μM and 40 μM) were used to stimulate NIH-3T3 cells. The cells and their culture medium were separately collected. ERK1/2 phosphorylation was detected in cell extracts by Western blot analysis 30 min after TGF-β1 treatment. The levels of fibronectin in the cell culture medium or the expression of CTGF in cell extracts were determined by Western blot analysis 24 h after treatment with TGF-β1. The primary antibodies used in this study were specific for ERK1/2, fibronectin, type I collagen, CTGF, beta actin (Affinity) and *p*-ERK1/2 (Santa Cruz) [ERK1/2 antibody (source: rabbit; clonality: polyclonal), Affinity, Cat. #: AF0155; fibronectin antibody (source: rabbit; clonality: polyclonal), Affinity, Cat. #: AF5335; type I collagen antibody (source: rabbit; clonality: polyclonal), Affinity, Cat. #: AF7001; CTGF antibody (source: rabbit; clonality: polyclonal), Affinity, Cat. #: DF7091; beta actin antibody (source: rabbit; clonality: polyclonal), Affinity, Cat. #: AF7018; and *p*-ERK1/2 antibody (source: mouse; clonality: polyclonal), Santa Cruz, sc-7383].

In this study, all primary antibodies were diluted with antibody diluent at a ratio of 1:1000 and incubated overnight at 4 °C, and the secondary antibodies were diluted at a ratio of 1:10,000 and incubated at room temperature for 2 h.

2.11. Statistical analysis

The results are presented as the average ± SD and were calculated by PRISM version 7.0 software. Student's *t*-test was used to evaluate differences between the experimental group and the control group. Unpaired Student's *t*-test was used in our study. One-way ANOVA followed Bonferroni's correction was used to determine significant differences among groups. Grayscale analysis of the Western blot results was performed by ImageJ software and showed that the P value was less than 0.05 ($p < 0.05$). Statistical analysis of pulmonary fibrosis areas was performed by Image-Pro Plus software.

3. Results

1. Imrecoxib prevents bleomycin-induced pulmonary fibrosis in mice

We treated C57BL/6 J mice with imrecoxib on Days 0–14 after bleomycin injury to determine the effect of imrecoxib administration on preventing lung fibrosis in vivo. The results showed that imrecoxib significantly reduced the lung wet−dry weight ratio (Fig. 1B). The pathological analysis of lung tissue from bleomycin-induced mice showed atrophic and narrowed alveoli, marked alveolar septal thickening and extensive interstitial inflammatory cell infiltration (Fig. 1C). However, in drug-treated mice, there was reduced inflammatory cell infiltration, and the fibrotic features were attenuated. The efficacy of imrecoxib administration was similar to that of pirfenidone administration (Fig. 1C). The degree of deposition and distribution range of collagen in lung tissue were observed by Masson trichrome staining. Compared with that in the model group, collagen deposition in the imrecoxib-treated group decreased significantly (Fig. 1C). Statistical analyses of the percent of the fibrotic area revealed that imrecoxib-treated mice had fewer fibrotic areas than mice in the model group (Fig. 1D).

Collagen content is increased during the progression of pulmonary fibrosis. Since hydroxyproline is a unique amino acid in collagen, the hydroxyproline content was measured to determine the degree of collagen deposition. The levels of hydroxyproline in the lungs of PF mice were determined to evaluate the effect of imrecoxib on collagen accumulation. Compared with that in the control group, bleomycin administration significantly increased hydroxyproline levels, while hydroxyproline levels in the imrecoxib-treated group were decreased (Fig. 1E). Thus, imrecoxib significantly inhibited collagen deposition. In addition, imrecoxib significantly decreased the pathological scores of lung tissues (Fig. 5A).

2. Imrecoxib attenuates bleomycin-induced lung inflammation in mice

We collected BALF from each group of experimental mice and performed total and differential inflammatory cell counts in BALF to examine the anti-inflammatory effect of imrecoxib. We placed the BALF solution on a glass slide and then performed HE staining. Total cell counts were significantly increased in bleomycin-induced mice compared to normal mice, and the total number of cells in imrecoxib-treated mice was significantly lower than that in bleomycin-induced mice (Fig. 2A and B).

Compared with those in normal mice, the proportions of macrophages, lymphocytes and neutrophils in the BALF of mice injured by bleomycin were increased. The numbers of these cells in BALF were significantly reduced after treatment with imrecoxib (Fig. 2C–E). Based on these results, imrecoxib reduced pulmonary inflammation in bleomycin-induced mice.

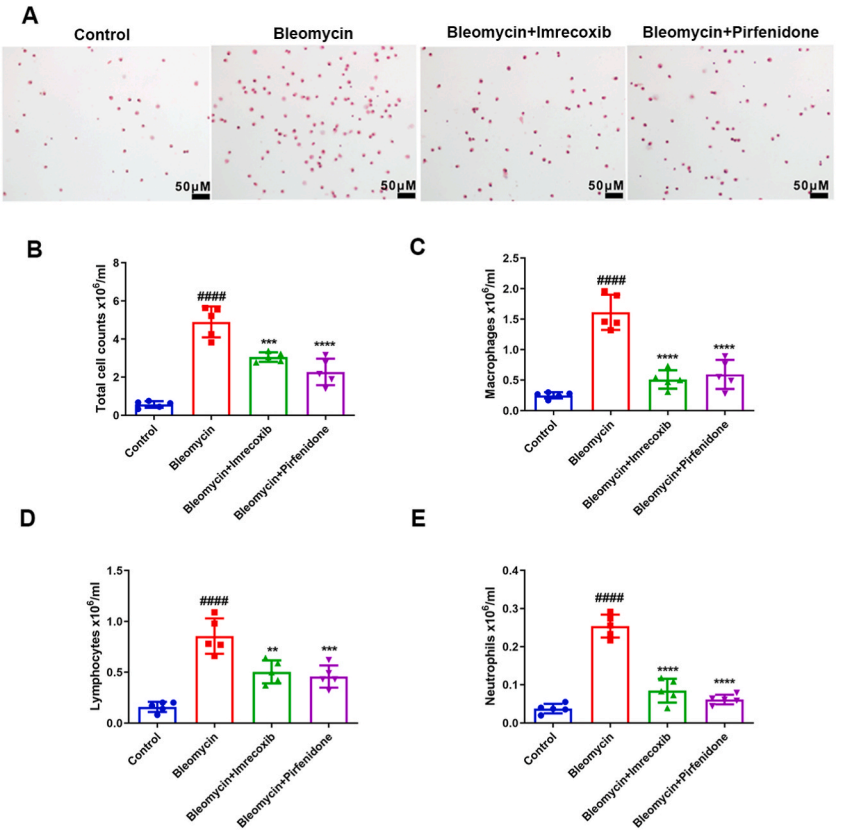

Fig. 2. Imrecoxib attenuates bleomycin-induced lung inflammation in mice. (n = 5 in each experimental group.) **(A)** H&E stained inflammatory cells in the control group, bleomycin group, bleomycin + imrecoxib (100 mg/kg) and bleomycin + pirfenidone (200 mg/kg) group. **(B)** Total cell counts on day 14. **(C)** Macrophage cell counts on day 14. **(D)** Lymphocyte cell counts on day 14. **(E)** Neutrophil cell counts on day 14. $^{\#\#\#\#}P < 0.0001$ versus control group. $^{**}P < 0.01$ versus BLM group. $^{***}P < 0.001$ versus BLM group. $^{****}P < 0.0001$ versus BLM group.

3. Imrecoxib attenuates the expression of fibrogenic cytokines in bleomycin-induced mice and reduces fibronectin, type I collagen and CTGF expression in vivo.

We evaluated IL-4 and IL-13 protein levels and TGF-β1 levels in BALF to determine the functional effects of imrecoxib on inflammatory cytokines in mice with pulmonary fibrosis. IL-4 and IL-13 levels in the BALF of bleomycin-induced pulmonary fibrosis mice

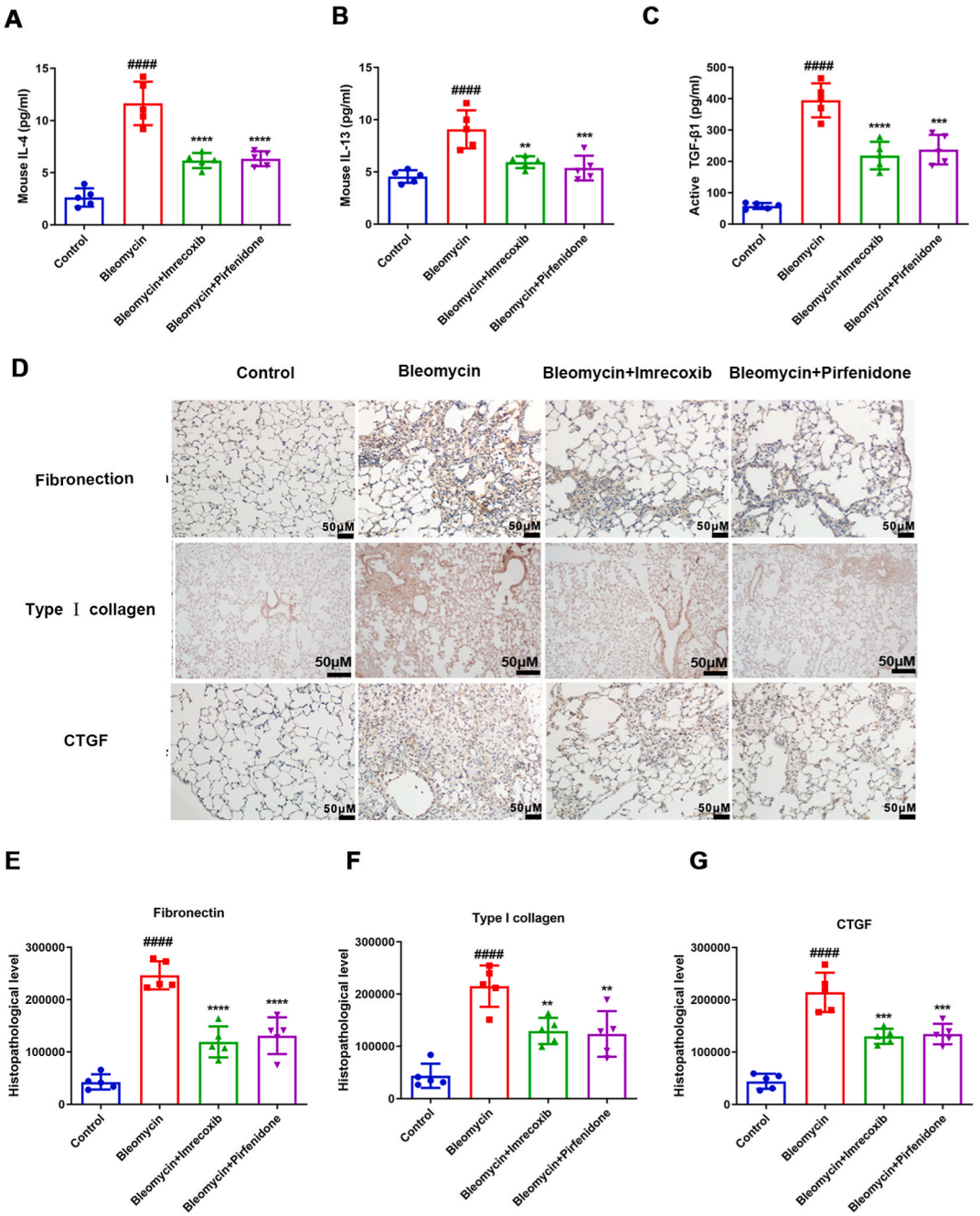

Fig. 3. Imrecoxib attenuates expression of fibrogenic cytokines in bleomycin-treated mice and reduced Fibronectin, Type I collagen and CTGF expression in vivo.
(A) IL-4 levels in BALF on day 14. **(B)** IL-13 levels in BALF on day 14. **(C)** TGF-β1 levels in lung homogenates on day 14. **(D)** Representative immunohistochemical staining of Fibronectin, Type I collagen and CTGF in lung tissues. **(E–G)** Expression levels of makers were evaluated by index of immunohistochemical staining. n = 5 per group. $^{\#\#\#\#}P < 0.0001$ versus control group. $^{**}P < 0.01$ versus BLM group. $^{***}P < 0.001$ versus BLM group. $^{****}P < 0.0001$ versus BLM group.

were higher than those in normal mice. Compared with those in the bleomycin group, significantly lower levels of IL-4 and IL-13 were detected in the imrecoxib-treated group, and the effects were similar to those of pirfenidone (Fig. 3A and B). Similarly, an analysis of active TGF-β1 levels showed higher active TGF-β1 concentrations in bleomycin-induced mice than in normal mice. Imrecoxib reduced the level of active TGF-β1 in mice with bleomycin-induced pulmonary fibrosis, and the effects were similar to those of pirfenidone (Fig. 3C).

To quantify the effect of imrecoxib on pulmonary fibrosis, we measured the expression levels of type I collagen, fibronectin and the profibrotic protein CTGF in the lung tissue of mice in the control group and bleomycin group by immunocytochemical staining. The expression levels of type I collagen, fibronectin, and CTGF in the lung tissue of the BLM group were significantly increased. However, mice treated with imrecoxib exhibited less and weaker positive staining than mice in the control group, indicating that imrecoxib significantly reduced the expression levels of these fibrotic markers (Fig. 3D–G). Thus, imrecoxib inhibited the expression of profibrotic proteins.

4. Imrecoxib attenuates the expression of fibrogenic and inflammatory cytokines in bleomycin-induced mice.

We further measured the transcription levels of IL-4, IL-13, and TGF-β in lung tissue by qPCR (Fig. 4A–C). The results were similar to those in BALF. Furthermore, to better determine the anti-inflammatory effect of imrecoxib, we measured the transcription levels of the classic inflammatory factors IL-1 and TNF-α in lung tissue. After treatment with imrecoxib, the transcription levels of related inflammatory factors in lung tissue were significantly reduced compared to those in the model group (Fig. 4D and E). Therefore, imrecoxib can reduce the transcription of inflammatory factors in lung tissue.

5. Imrecoxib inhibits bleomycin-induced pulmonary fibrosis in mice

Then, we examined the effect of imrecoxib on pulmonary fibrosis. Bleomycin was injected into the trachea on Day 0. Pirfenidone, imrecoxib or saline was orally administered to the mice daily from Day 0 to Day 13 and from Day 7 to Day 13. On the 14th day, lung tissues were obtained. Imrecoxib significantly decreased the pathological scores of lung tissues (Fig. 5A). Imrecoxib effectively delayed pulmonary fibrosis in mice, as shown by H&E and Masson's trichrome staining (Fig. 5B). The results showed that imrecoxib significantly reduced the lung wet−dry weight ratio and the percentage of fibrosis and decreased the hydroxyproline content in lung tissues

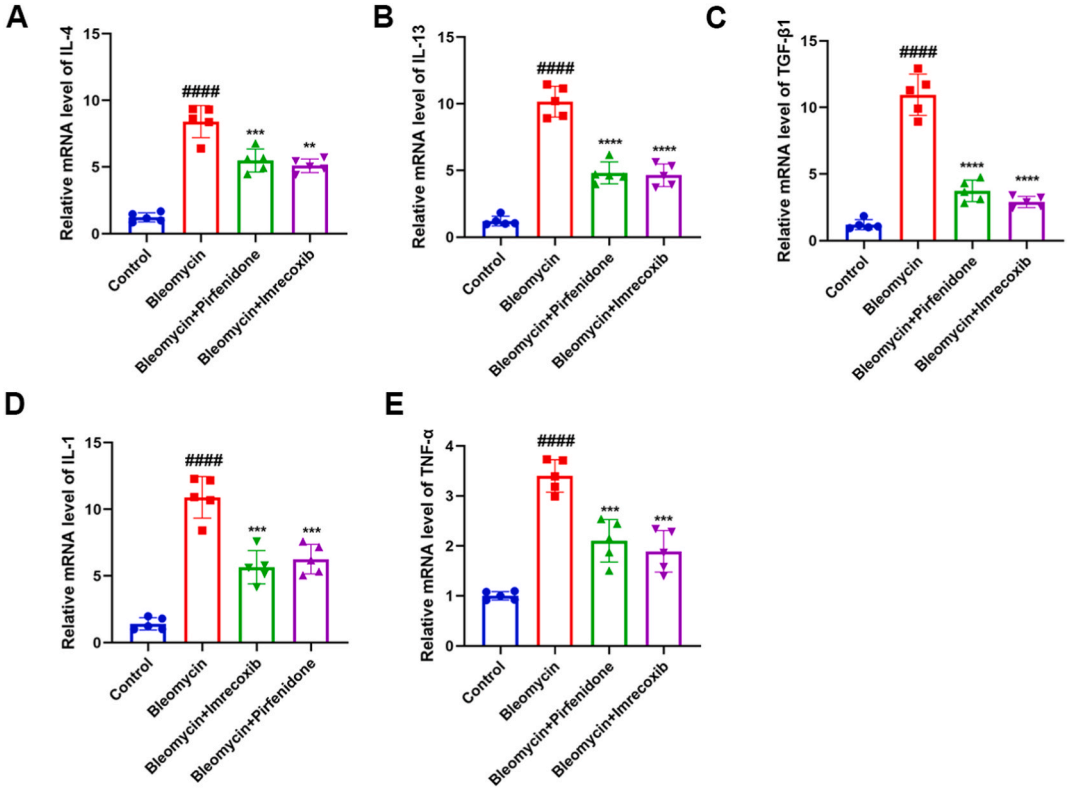

Fig. 4. Imrecoxib attenuates expression of fibrogenic and inflammatory cytokines in bleomycin-treated mice.
(A) The mRNA levels of IL-4 in lung on day 14. **(B)** The mRNA levels of IL-13 in BALF on day 14. **(C)** The mRNA levels of TGF-β1 in lung on day 14. **(D)** The mRNA levels of IL-1 in lung on day 14. **(E)** The mRNA levels of TNF-α in lung on day 14. n = 5 per group. $^{\#\#\#\#}P < 0.0001$ versus control group. $^{**}P < 0.01$ versus BLM group. $^{***}P < 0.001$ versus BLM group. $^{****}P < 0.0001$ versus BLM group.

A

Group	Ashcroft Score(n=5) Gavage for 14 days	Ashcroft Score(n=5) Gavage for 7 days
Control	0.11±0.24	0.18±0.31
Bleomycin	5.48±0.95####	6.55±1.08####
Bleomycin+Imrecoxib	3.09±0.77****	4.52±1.32***
Bleomycin+Pirfenidone	3.63±0.62***	4.64±1.05***

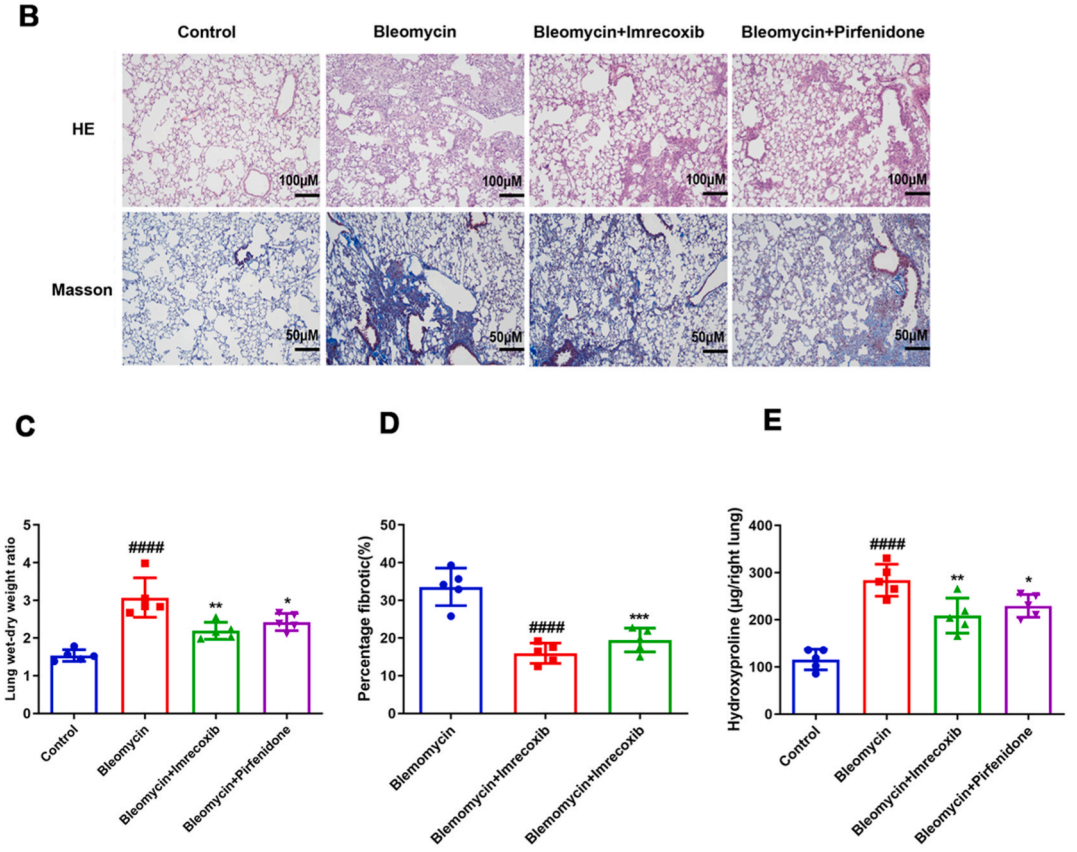

Fig. 5. Imrecoxib inhibits bleomycin-induced pulmonary fibrosis in mice
(A) Ashcroft score to quantify the degree of fibrosis. **(B)** Photomicrographs of lung sections stained with hematoxylin–eosin (H&E) and Masson trichrome staining. Imrecoxib (100 mg/kg) and PFD (200 mg/kg) were given orally once a day from day 7–13. **(C)** Changes in wet-dry weight ratio of mice lung in each group. **(D)** Percentages of fibrotic area in lung tissues. **(E)** Hydroxyproline contents in right lung tissues. $^{\#\#\#\#}P < 0.0001$ versus control group. $^{*}P < 0.05$versus BLM group. $^{**}P < 0.01$versus BLM group. $^{***}P < 0.001$versus BLM group. $^{****}P < 0.0001$ versus BLM group.

(Fig. 5C–E). Therefore, imrecoxib significantly reduced BLM-induced pulmonary fibrosis in mice.

6. Imrecoxib suppresses bleomycin-induced lung inflammation in mice

To further determine the therapeutic effect of imrecoxib on the mouse model, we further evaluated the effect of intragastric imrecoxib administration on pulmonary inflammation in mice from Day 7 to Day 13. On the 14th day, H&E staining was performed and showed alveolar inflammatory cell infiltration in BALF, and imrecoxib effectively reduced alveolar damage (Fig. 6A). Compared with that in normal mice, the total cell count in the model group was significantly increased. Furthermore, the proportions of macrophages, lymphocytes, and neutrophils were significantly increased. Compared with that in the model group, imrecoxib reduced the number of total cells and macrophages in the lung (Fig. 6B and C). Imrecoxib significantly reduced the number of lymphocytes and neutrophils (Fig. 6D and E). These results prove that imrecoxib has a certain therapeutic effect on the mouse model.

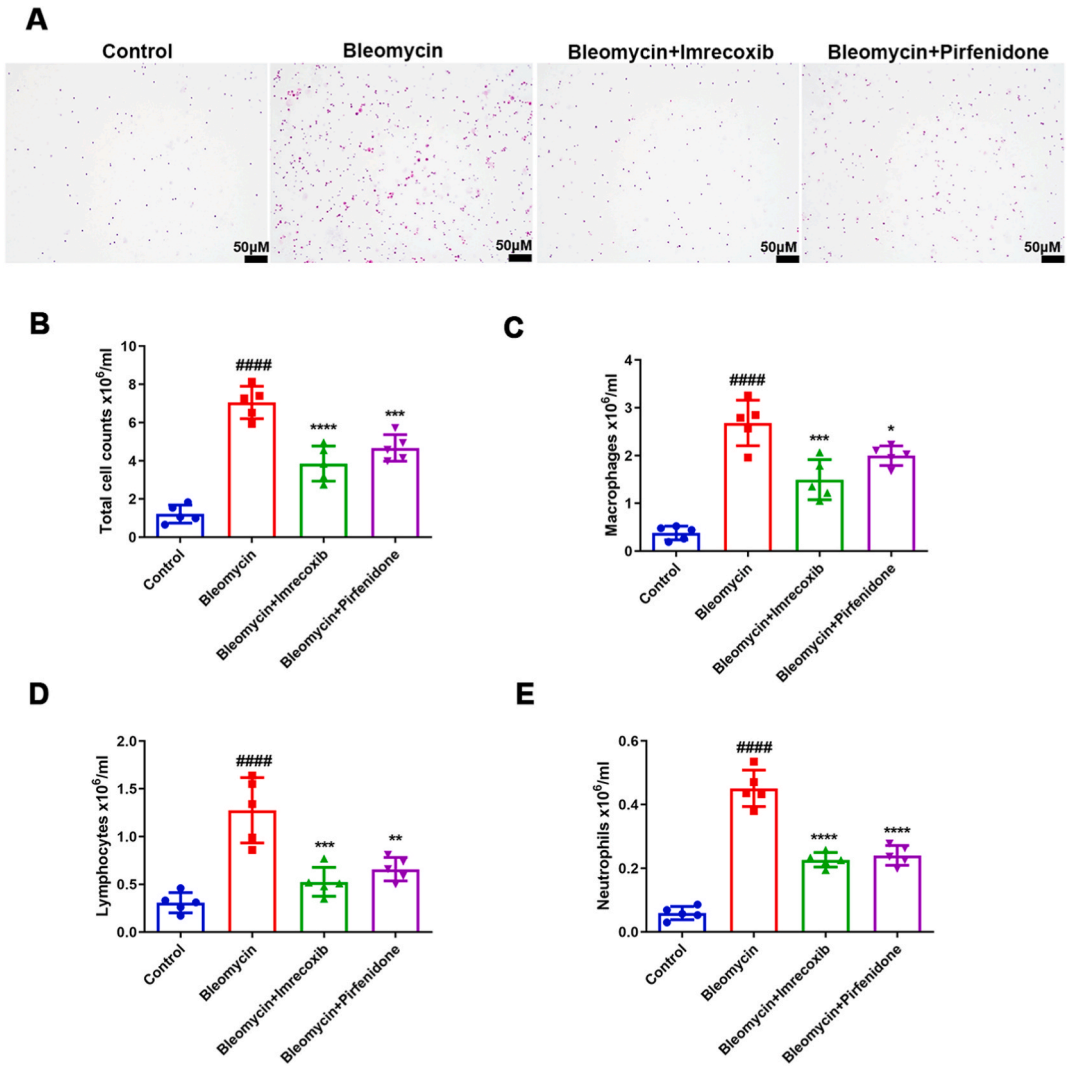

Fig. 6. Imrecoxib suppresses bleomycin-induced lung inflammation in mice
(A) Imrecoxib (100 mg/kg) and PFD (200 mg/kg) were given orally once a day from day 7–13 after BLM-treatment and lung tissue were harvested at day 14. H&E staining pictures of cells in BALF of each group. **(B)** Total cell counts on day 14. **(C)** Macrophage cell counts on day 14. **(D)** Lymphocyte cell counts on day 14. **(E)** Neutrophil cell counts on day 14.$^{\#\#\#\#}P < 0.0001$ versus control group. *P < 0.05 versus BLM group. **P < 0.01 versus BLM group. ***P < 0.001 versus BLM group. ****P < 0.0001 versus BLM group.

7. Imrecoxib inhibits the TGF-β1/ERK1/2 signaling pathway in TGF-β1-activated myofibroblasts

The main source of pulmonary collagen production in patients with IPF is myofibroblasts. Therefore, we established a TGF-β1-induced activated myofibroblast model and assessed the expression levels of CTGF, type I collagen and fibronectin in active myofibroblasts by western blotting. Imrecoxib (20 μM and 40 μM) significantly inhibited the protein expression of the fibronectin, type I collagen and CTGF in TGF-β1-activated myofibroblasts (Fig. 7A, C-E).

Next, we investigated whether the antifibrotic effects of imrecoxib were associated with the inhibition of ERK1/2 activation. The immunoblot assay showed dose-dependent decreases in *p*-ERK levels in cells treated with imrecoxib (Fig. 7B and F).

8. Imrecoxib inhibits TGF-β1-induced fibronectin, type I collagen and CTGF expression and ERK1/2 activation in vivo.

To further verify the mechanism of imrecoxib in vivo, we examined the lung tissue of mice in each group. The expression levels of CTGF, collagen I and fibronectin were evaluated by Western blotting. Imrecoxib significantly inhibited the protein expression of fibronectin, collagen I and CTGF in lung tissue (Fig. 8A, C-E). We further investigated whether the antifibrotic effect of imrecoxib was related to the inhibition of ERK1/2 activation in vivo. The immunoblotting assay showed that imrecoxib significantly inhibited the phosphorylation of ERK (Fig. 8B and F).

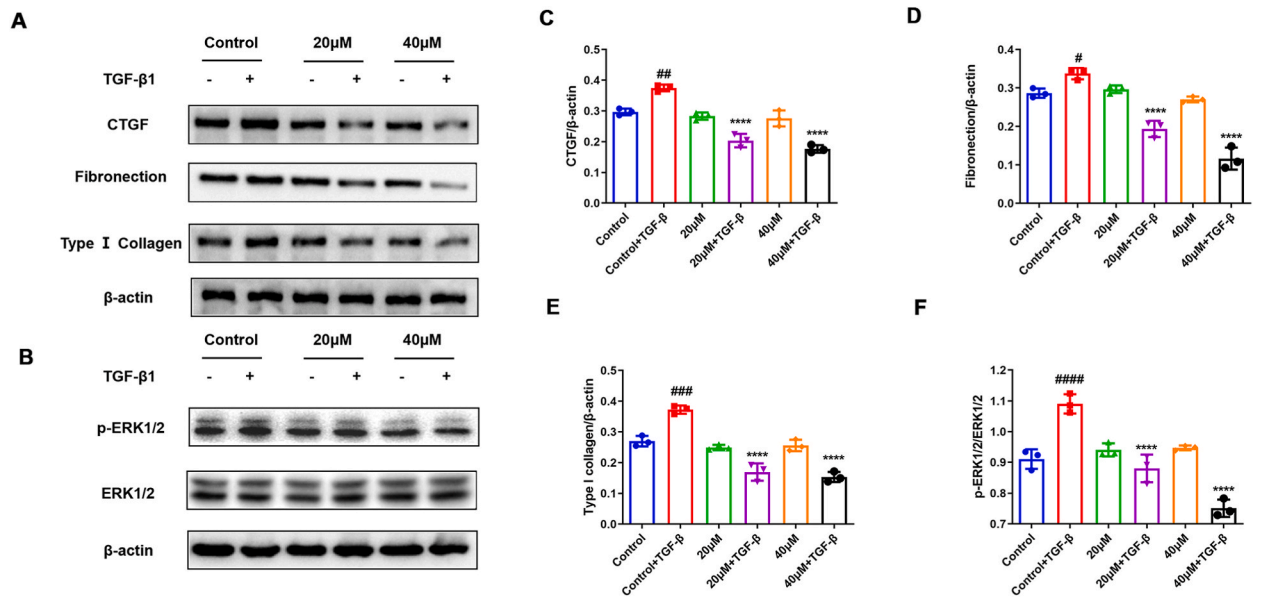

Fig. 7. Imrecoxib inhibites TGF-β1-induced fibronectin, Type I collagen and CTGF expression and ERK1/2 activation in vitro.
(A) Western blotting results of fibronectin, Type I collagen and CTGF in TGF-β1-stimulated NIH 3T3 cells. **(B)** Western blotting results of *p*-ERK1/2 in TGF-β1-stimulated NIH 3T3 cellls. **(C)** Gray analysis of CTGF expression. **(D)** Gray analysis of Fibronection expression. **(E)** Gray analysis of Type I collagen expression. **(F)** Gray analysis of *p*-ERK1/2 expression. $^{\#}P < 0.05$ versus control group. $^{\#\#}P < 0.01$ versus control group. $^{\#\#\#}P < 0.001$ versus control group. $^{\#\#\#\#}P < 0.0001$ versus control group. $^{****}P < 0.0001$ versus control + TGF-β1.

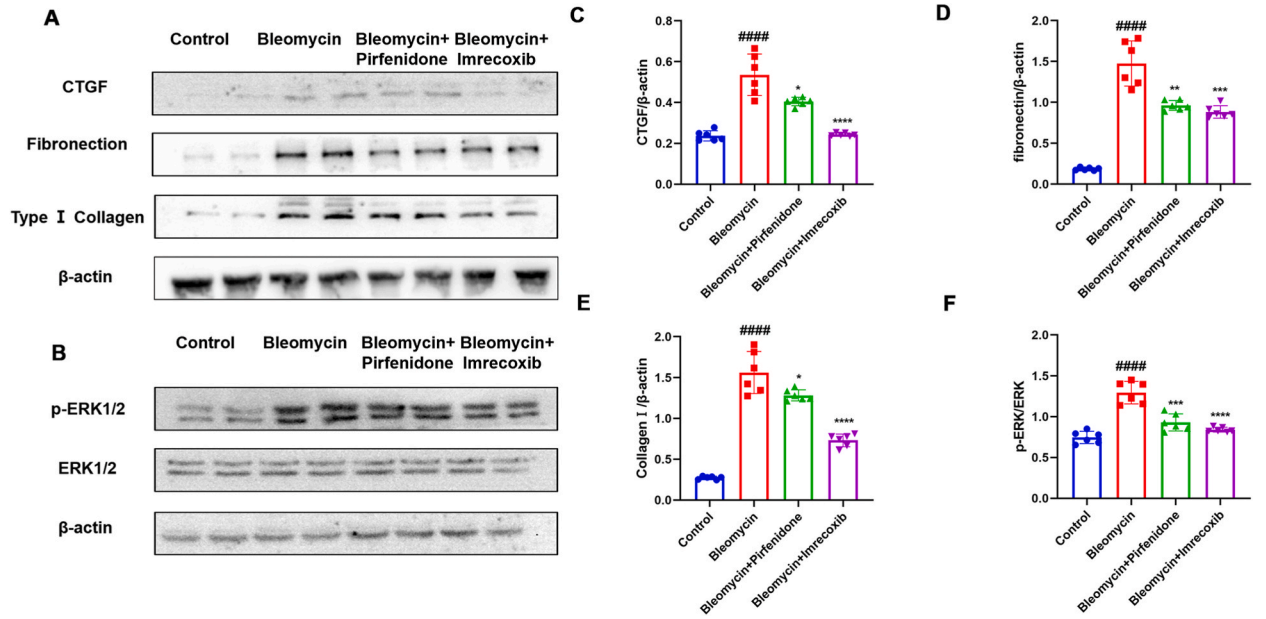

Fig. 8. Imrecoxib inhibites TGF-β1-induced fibronectin, Type I collagen and CTGF expression and ERK1/2 activation in vivo.
(A) Western blotting results of fibronectin, Type I collagen and CTGF in lung tissues. **(B)** Western blotting results of *p*-ERK1/2 in lung tissues. **(C)** Gray analysis of CTGF expression. **(D)** Gray analysis of Fibronection expression. **(E)** Gray analysis of Type I collagen expression. **(F)** Gray analysis of *p*-ERK1/2 expression. $^{\#\#\#\#}P < 0.0001$ versus control group. $^{*}P < 0.05$ versus BLM group. $^{**}P < 0.01$ versus BLM group. $^{***}P < 0.001$ versus BLM group. $^{****}P < 0.0001$ versus BLM group.

4. Discussion

This study examined the driving effect of inflammation on fibroblasts, and so we chose the NIH-3T3 cell line for this research. Our results showed that the administration of imrecoxib prevented and treated bleomycin-induced pulmonary fibrosis in mice. First, the lung wet−dry weight ratio, hydroxyproline content, HE and Masson's trichrome staining indicated that imrecoxib attenuated bleomycin-induced pulmonary fibrosis. Second, IL-1 and TNF-α levels were measured and revealed that imrecoxib exerted an anti-inflammatory effect. Finally, IHC and in vitro studies showed that imrecoxib reduced the expression of fibronectin, type I collagen and CTGF in fibrotic lung tissues and NIH-3T3 cells stimulated with TGF-β1, suggesting that imrecoxib could exert an antifibrotic effect by inhibiting the TGF-β1/ERK1/2 signaling pathway.

In recent years, nintedanib and pirfenidone have been approved as treatments for IPF, which has changed the clinically unresolved and embarrassing situation of IPF, but these two drugs can only be used in patients with mild-to-moderate IPF. Both drugs induce side effects, such as gastrointestinal reactions, nervous system toxicity, and liver and kidney function damage. For example, nintedanib cannot be administered to patients with liver dysfunction, and pirfenidone cannot be administered to patients with renal dysfunction [11,12]. The cost of treatment is high, and thus, the clinical demand is far from being met.

Imrecoxib is a nonsteroidal anti-inflammatory drug (NSAID) and a COX-2 inhibitor that inhibits the production of inflammatory prostaglandins to inhibit inflammation. Because COX-1 expression is inhibited to a lesser extent, physiological protective functions are rarely affected. Based on this mechanism of action, imrecoxib exerts good anti-inflammatory and analgesic effects and induces only a few side effects. In recent years, imrecoxib has been widely used to treat arthritis. The use of imrecoxib in combination with other drugs has been reported to inhibit tumor growth and lymph node metastasis [13]. The cyclooxygenase (COX) family, which includes COX-1, COX-2 and COX-3, plays a key role in catalyzing the conversion of arachidonic acid to prostaglandin. COX-2 is a prostaglandin E2 (PGE2)-specific upstream enzyme that synthesizes PGE2. The role of COX-2 in pulmonary fibrosis remains controversial. Traditional wisdom suggests that COX-2 promotes inflammatory responses to pulmonary fibrosis in patients with IPF. In recent years, researchers have shown that COX-2 exerts a protective effect against pulmonary fibrosis [14–17]. In this study, we confirmed that COX-2 inhibitors significantly inhibited bleomycin-induced pulmonary fibrosis, which may be relevant to their anti-inflammatory effects.

The infiltration of inflammatory cells is a chronic and persistent process involved in the development of pulmonary fibrosis that is characterized by increased numbers of macrophages, lymphocytes and neutrophils. These inflammatory cells drive fibroblasts to differentiate into myofibroblasts, which then secrete ECM into the alveolar interstitial space to cause pulmonary fibrosis [18]. In fact, in many fibrotic diseases, a sustained inflammatory response is critical for activating the fibrotic wound healing process. A number of key factors trigger the activation, proliferation, and survival of myofibroblasts, including growth factors (CTGF and PDGF), matrix factors (mechanical stress and/or stiffness), and cytokines (IL-1, TNF, TGF-β1, and IL-13) [19]. Macrophages are divided into two phenotypes: M1 and M2. M2-type macrophages are induced by IL-4 and IL-13 and induce fibroblast differentiation by expressing arginase-1 (ARG1) and then acting as profibrotic factors in BLM-induced pulmonary fibrosis [20]. Our results showed that imrecoxib reduced the expression of IL-4, IL-13 and TGF-β1 in BALF, indicating that imrecoxib inhibited bleomycin-induced mouse lung fibrosis through its anti-inflammatory effects.

TGF-β1 and CTGF play crucial roles in fibrosis in various organs [21–25]. TGF-β1 is the profibrotic cytokine that is directly involved in pulmonary fibrosis because it promotes the proliferation of lung fibroblasts and accelerates the transformation of lung fibroblasts into myofibroblasts, leading to the differentiation of myofibroblasts and excessive secretion of ECM. As a downstream mediator of TGF-β1 signaling, CTGF reflects the profibrotic activity of TGF-β1 in fibroblasts and may also lead to collagen overproduction and lung deposition [26]. Based on our experimental results, imrecoxib (20 and 40 μM) significantly inhibited the expression of fibronectin and type I collagen in mouse embryonic fibroblasts stimulated with TGF-β1 by decreasing CTGF expression.

The TGF-β1 signal transduction pathway is divided into the Smad and non-Smad pathways, and the TGF-β1/Smad protein pathway is considered the classic pathway of TGF-β1 signaling. Non-Smad pathways include the PI3K/Akt, MAPK, Notch and other signaling pathways. The MAPK (mitogen-activated protein kinase) signaling pathway consists of four components: extracellular signal-related kinase (ERK1/2), Jun amino terminal kinase (JNK1/2/3), p38-MAPK and ERK5 [27]. The ERK (extracellular signal-regulated kinase) cascade is critical for the activation of processes such as cell proliferation, differentiation and migration. The level of *p*-ERK1/2 is increased in a mouse model of BLM-induced pulmonary fibrosis [28]. As shown in our Western blot results, imrecoxib (20 and 40 μM) dose-dependently reduced *p*-ERK1/2 levels in cells.

In conclusion, the administration of imrecoxib ameliorates inflammation and pathological changes in mice with BLM-induced pulmonary fibrosis and inhibits fibrosis by inhibiting fibronectin, type I collagen, CTGF and *p*-ERK1/2 expression. Our results suggest that imrecoxib may be a new candidate compound for the treatment for pulmonary fibrosis.

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Author contributions

Conceived and designed the study: Xiaoting Gu, Honggang Zhou and Yanping Zhang; cell experiments: Yang Miao, Jiahe Mao and Lingxin Meng; animal experiments: Yang Miao, Jianwei Zhang and Yue Yang; collection and assembly of data: Yue Yang, Jianwei

Zhang and Xiaohe Li; data analysis and interpretation: Xiaohe Li, Jingjing Gao; manuscript writing: Yang Miao, Jiahe Mao and Lingxin Meng; ad-ministrative support: Honggang Zhou, Cheng Yang and Yanping Zhang. All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

All animal care and experimental procedures complied with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (project code: SCXK 2021–0001, date of approval: 14 January 2021).

All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines such as EU Directive 2010/63/EU for animal experiments and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). We affirm that the above guidelines have always been followed in our research.

Not applicable.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20914.

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