

SPP1 promotes the polarization of M2 macrophages through the Jak2/Stat3 signaling pathway and accelerates the progression of idiopathic pulmonary fibrosis

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Abstract. Idiopathic pulmonary fibrosis (IPF) is a fatal pulmonary disease that requires further investigation to understand its pathogenesis. The present study demonstrated that secreted phosphoprotein 1 (SPP1) was aberrantly highly expressed in the lung tissue of patients with IPF and was significantly positively associated with macrophage and T-cell activity. Cell localization studies revealed that SPP1 was primarily overexpressed in macrophages, rather than in T cells. Functionally, knocking down SPP1 expression *in vitro* inhibited the secretion of fibrosis-related factors and M2 polarization in macrophages. Furthermore, knocking down SPP1 expression inhibited the macrophage-induced epithelial-to-mesenchymal transition in both epithelial and fibroblastic cells. Treatment with SPP1 inhibitors *in vivo* enhanced lung function and ameliorated pulmonary fibrosis. Mechanistically, SPP1 appears to promote macrophage M2 polarization by regulating the JAK/STAT3 signaling pathway both *in vitro* and *in vivo*. In summary, the present study found that SPP1 promotes M2 polarization of macrophages through the JAK2/STAT3 signaling pathway, thereby accelerating the progression of IPF. Inhibition of SPP1

expression *in vivo* can effectively alleviate the development of IPF, indicating that SPP1 in macrophages may be a potential therapeutic target for IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease that is primarily characterized by marked infiltration of macrophages and abnormal deposition of the extracellular matrix (ECM) (1,2). This disease severely impacts the quality of life of patients and has a high mortality rate, with a 5-year survival rate usually between 20-30% (3,4). Although pirfenidone and nintedanib have been approved for the treatment of IPF, their efficacy in improving patient prognosis is limited (5,6). In order to improve the survival rate and quality of life for patients with IPF, it is necessary to conduct further research into the pathogenesis of the disease and explore more effective treatment options and therapeutic targets.

Macrophages, immune cells with both phagocytic and bactericidal capabilities, can transition from an M1 to an M2 state in response to stimulation or alterations in their microenvironment (7,8). M1 macrophages are closely associated with inflammation and tissue damage, whereas M2 macrophages contribute to tissue repair and fibrosis by releasing pro-fibrotic factors (9-11). Given these insights, developing therapeutic strategies targeting M2 macrophages holds promise for achieving significant breakthroughs in the treatment of IPF (12,13).

Secreted phosphoprotein 1 (SPP1), also known as osteopontin, is a multifunctional secreted phosphoprotein (14,15). It was previously revealed that high expression of SPP1 in lung cancer is commonly associated with the severity of the tumor and poor patient prognosis (16). In terms of its function, SPP1 is primarily synthesized by both activated macrophages and

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epithelial cells. It plays a key role in regulating cell chemotaxis and adhesion, as well as impacting cell proliferation and migration. Ultimately, it contributes significantly to the development of fibrosis in the liver and kidneys (17-20). Studies reported that SPP1 can promote epithelial-mesenchymal transition (EMT) in epithelial cells, thereby promoting lung fibrosis (21). Furthermore, it was recently suggested that inhibiting the expression of the SPP1 gene in club cells can alleviate the progression of IPF and it also points out that macrophages may be a key source of SPP1 in IPF (22); however, the aforementioned studies did not continue to delve into the specific functions of SPP1 in macrophages and its mechanisms of action. On this basis, in the present study it was aimed to further investigate the function of SPP1 in macrophages and its mechanism of action in IPF.

The current study comprehensively employed RNA sequencing (RNA-seq) data and clinical information from lung tissues of patients with IPF to meticulously analyze the expression of SPP1 in these patients and its association with disease prognosis. Utilizing single-cell sequencing technology, the present study further elucidated the specific cellular localization and expression patterns of SPP1 within lung tissue. *In vitro* experiments investigated the effects of SPP1 on macrophage M2 polarization and its role in the EMT process of epithelial and fibroblastic cells. Concurrently, through *in vivo* animal experiments, the present study explored the potential intervention mechanism of SPP1 inhibitors on the pathological process of IPF. The present results enhanced the comprehension of the mechanisms by which SPP1 operates in the pathological process of IPF, establishing a scientific basis for the investigation of potential future treatment pathways.

Materials and methods

Download and analysis of data sets. The present study utilized the Gene Expression Omnibus (GEO) database, specifically the GSE70866 dataset (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70866>), which is based on the GPL14550 platform and includes RNA-seq data for 20 healthy control samples and 112 patients with IPF (23). The Limma package within R software version 4.0.2 (<https://www.r-project.org/>) was employed for comparative analysis to elucidate differences in SPP1 expression between healthy individuals and patients with IPF. Moreover, Kaplan-Meier survival curve analysis was conducted to explore the relationship between SPP1 expression levels and survival rates in patients with IPF. To gain a deeper understanding of SPP1's expression pattern at the cellular level, the GSE132771 single-cell RNA-seq dataset (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132771>) was retrieved from the GEO database and SPP1 cell expression localization analysis was conducted on three IPF samples (24). Concurrently, single-sample gene set enrichment analysis was applied to assess immune cell gene sets associated with SPP1 expression, aiming to reveal the connection between SPP1 and immune cell infiltration.

Cell isolation and cultivation. Initially, 10 ml of venous blood was drawn from healthy volunteers (Table SI) and transferred into a centrifuge tube. An equal volume of PBS solution (Biosharp Life Sciences; cat. no. BL601A) was

added for dilution and after gentle mixing, 5 ml of Ficoll Pague PLUS solution (Cytiva; cat. no. 17144002) was drawn into a new centrifuge tube. Then, the diluted blood was gently added to the top layer of Ficoll. After 15 min of centrifugation at 4°C and 300 x g, the middle white layer represents peripheral blood mononuclear cells (PBMCs). Subsequently, the PBMCs were transferred to another 15 ml centrifuge tube with 10 ml of PBS solution and centrifuged at 4°C and 1,000 x g for 10 min and the process was repeated twice. After discarding the supernatant, the cells were resuspended in complete RPMI-1640 medium (Thermo Fisher Scientific, Inc.; cat. no. 11875093) supplemented with 10% fetal bovine serum (Beyotime Institute of Biotechnology; cat. no. C0235) and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology; cat. no. C0222) and cultured for 7 days under 100 ng/ml macrophage colony-stimulating factor (M-CSF) (MedChemExpress; cat. no. HY-P7050A) to induce differentiation of PBMCs into macrophage-like cells (PBMC-m). THP-1 cells (cat. no. SCSP-567) were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in complete RPMI-1640 medium (90% RPMI-1640 + 10% fetal bovine serum + 1% penicillin/streptomycin). THP-1 cells are treated with 100 nM PMA (MedChemExpress; cat. no. HY-18739) for 48 h to induce macrophage differentiation. BEAS-2B and WI-38 cells (cat. nos. GNHu27 and SCSP-521, respectively) were likewise obtained from the Cell Bank of the Chinese Academy of Sciences, with BEAS-2B being cultured in complete DMEM medium (Thermo Fisher Scientific, Inc.; cat. no. 11965092) (90% DMEM + 10% fetal bovine serum + 1% penicillin/streptomycin). WI-38 cells were grown in complete MEM medium (Thermo Fisher Scientific, Inc.; cat. no. 11095080) (90% MEM + 10% fetal bovine serum + 1% penicillin/streptomycin) and all cells were maintained in an incubator at 37°C with 5% CO₂. Venous blood was collected from all volunteers after obtaining their consent, and an informed consent document was signed by all participants. The human tissue samples were sourced from the Oncology Hospital of the Huainan Dongfang Hospital Group (Huainan, China) in May 2023.

Small interfering (si)RNA transfection. PBMC-m and THP-1 cells were initially plated in separate culture dishes. Once the cell growth density reached 60-80%, the transfection was performed. In total, 50 pmol of siRNA was diluted in 200 µl of serum-free RPMI-1640 medium. Additionally, 4 µl of Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc.; cat. no. 11668500) was diluted in 200 µl of RPMI-1640 medium. The reagents were mixed well and then allowed to stand for 15 min. The mixture was then added to the culture dishes that had already been seeded with cells and the dishes were gently rocked to ensure even distribution of the complex. After 4-6 h of incubation in an incubator at 37°C and 5% CO₂, the medium was changed to complete RPMI-1640 medium (90% RPMI 1640 + 10% fetal bovine serum + 1% penicillin/streptomycin) for continued culture. The relevant siRNA sequences used in the experiment were as follows: Negative control siRNA (si_NC; sense: 5'-UUCUCCGAACGUGUCACGUTT-3'); and SPP1 specific siRNA (si_SPP1; sense: 5'-CUGUGCCAUACC AGUUA AATT-3').

Cell coculture and wound scratch assay. Bleomycin (BLM; MedChemExpress; cat. no. HY-17565A) was initially dissolved in DMSO (MedChemExpress; cat. no. HY-Y0320) to create a 10 mM stock solution, while Stattic (MedChemExpress; cat. no. HY-13818) was dissolved in DMSO to form a 5 mM stock solution. Initially, the supernatant was transferred from PBMC-m and THP-1 cells that were treated with 5 μ M BLM, 5 μ M STAT3 inhibitor (Stattic) and si_SPP1 into centrifuge tubes. Then, the supernatant was collected and transferred to a centrifuge tube. After centrifugal filtration, the supernatant was mixed with serum-free medium in a 1:1 ratio to prepare the conditioned medium (CM), which was stored at 4°C. BEAS-2B and WI-38 cells with a density of 80-90% were counted after trypsin (Thermo Fisher Scientific, Inc.; cat. no. 25200072) digestion, and then added to DMEM/MEM complete medium. The concentration of BEAS-2B and WI-38 cells was $\sim 5 \times 10^5$ cells/ml. The culture-dish was placed into the incubator at 37°C and 5% CO₂ for cultivation; once the cells were confluent, 10- μ l pipette tip was used to draw three straight lines along a ruler at the bottom of the dish. Washing was performed twice with PBS solution (Bioshore Life Sciences; cat. no. BL601A), then the pre-prepared CM was aspirated and added to the culture dish for further cultivation. The scratch area of the cells was recorded by capturing images at 0, 24 and 48 h. Finally, the migration and healing of the cells was assessed using ImageJ 1.52a software (National Institutes of Health).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Based on a previous protocol, total RNA was extracted from the collected mouse lung tissues and cells (25) and the RNA concentration was determined using a spectrophotometer. The RNA purity was considered acceptable when the optical density ratio at 260 and 280 nm (OD₂₆₀/OD₂₈₀) was between 1.8 and 2.0. The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.; cat. no. K1622) was used to perform reverse transcription of RNA according to the manufacturer's protocol, followed by gene amplification using the Genious 2X SYBR Green Fast qPCR Mix (ABclonal Biotech Co., Ltd.; cat. no. RK21204). The thermocycling protocol was as follows: 95°C for a duration of 3 min, then 95°C for 5 sec, 60°C for 32 sec, and 72°C for 40 sec; this cycling process was repeated 43 times. To standardize the results, the 2^{- $\Delta\Delta C_q$} method was applied, with GAPDH serving as the internal control gene (23). The primers used in the experiment were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. The specific primer sequences are detailed in Table SII.

Western blot (WB) analysis. Based on a previous protocol (25), proteins were extracted from the collected cells, and their concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc.; cat. no. A55864) as per the manufacturer's protocol. Subsequently, the protein samples (50 μ g per lane) were subjected to electrophoresis using a 10% SDS-PAGE gel and transferred to a 0.2- μ m PVDF membrane (MilliporeSigma; cat. no. ISEQ00010). After blocking the membrane with 5% skim milk at room temperature for 2 h, it was incubated with the corresponding primary antibodies at 4°C overnight. On the following day, the membrane was incubated with a diluted secondary antibody solution for 2 h. Subsequently, the protein bands were

visualized using a chemiluminescent agent (MilliporeSigma; cat. no. WBKLS100) and an imaging system (Cytiva; Amersham™ ImageQuant™ 800; cat. no. 29399481), and analysis was performed with ImageJ 1.52a software. The antibodies used in the experiment included: SPP1 (Proteintech Group, Inc.; cat. no. 22952-1-AP; 1:2,000), COL3A1 (Proteintech Group, Inc.; cat. no. 68320-1-Ig; 1:5,000), E-cadherin (Cell Signaling Technology, Inc.; cat. no. 3195T; 1:1,000), N-cadherin (Cell Signaling Technology, Inc.; cat. no. 4061T; 1:1,000), Vimentin (Cell Signaling Technology, Inc.; cat. no. 5741T; 1:1,000), phosphorylated (p)-JAK2 (ABclonal Biotech Co., Ltd.; cat. no. AP0531; 1:1,000), p-STAT3 (ABclonal Biotech Co., Ltd.; cat. no. AP0705; 1:1,000), JAK2 (ABclonal Biotech Co., Ltd.; cat. no. A11497; 1:1,000), STAT3 (ABclonal Biotech Co., Ltd.; cat. no. A1192; 1:1,000), GAPDH (ABclonal Biotech Co., Ltd.; cat. no. AC002; 1:5,000) and HRP-conjugated secondary antibody (ABclonal Biotech Co., Ltd.; cat. no. AS063; 1:10,000). Densitometric analysis was performed using ImageJ 1.52a software (National Institutes of Health).

Immunofluorescence staining analysis. Based on a previous protocol (25), the cells that were fixed in formalin solution and the paraffin-embedded tissues from both healthy and patients with IPF, were incubated with 5% BSA (Wuhan Servicebio Technology Co., Ltd.; cat. no. GC305010-25g) at room temperature for 1 h. Then, they were incubated with the corresponding antibody overnight at 4°C. After adding the corresponding fluorescent secondary antibody working solution, the DAPI working solution (5 μ g/ml) (Thermo Fisher Scientific, Inc.; cat. no. 62248) was dropwise added. Finally, the specimens were observed and images were collected under a fluorescence microscope (Leica Microsystems, Inc.; cat. no. DMI3000 B). The antibodies employed in the experiment included: SPP1 (Proteintech Group, Inc.; cat. no. 22952-1-AP; 1:200), FAM13A (Proteintech Group, Inc.; cat. no. 55401-1-AP; 1:100), p-JAK2 (ABclonal Biotech Co., Ltd.; cat. no. AP0531; 1:100), p-STAT3 (ABclonal Biotech Co., Ltd.; cat. no. AP0705; 1:200), F4/80 (Proteintech Group, Inc.; cat. no. 29414-1-AP; 1:500), CD68 (ABclonal Biotech Co., Ltd.; cat. no. A23205; 1:200), CD206 (Proteintech Group, Inc.; cat. no. 60143-1-Ig; 1:500) and CD3 (Proteintech Group, Inc.; cat. no. 60181-1-Ig; 1:500).

Construction of animal models. All mice utilized in the present study were procured from Henan Skobes Biotechnology Co., Ltd., consisting of 15 SPF-grade 8-10-week-old male C57BL/6J mice, with body weights ranging from 18-22 g. These mice were housed in the animal facility of the School of Medicine at Anhui University of Science and Technology. All animal experimental protocols were granted approval by the Animal Ethics Committee at Anhui University of Science and Technology (approval no. NO. HX-001; Huainan, China). The mice were maintained under controlled conditions (temperature 21 $\pm$ 1°C, humidity 50 $\pm$ 16% and 12-h light/dark cycle), with a daily supply of food and water, and body weight was measured once the mice were acclimated to the environment. The mice were randomly divided into three groups to construct animal models: PBS control group (n=5); BLM-induced IPF disease group (n=5); and BLM + SPP1 inhibitor (MedChemExpress; cat. no. HY-146064) treatment group (n=5). For the PBS control group, mice were given 25 μ l

of 0.5% sodium carboxymethyl cellulose (CMC-Na; Beijing Solarbio Science & Technology Co., Ltd.; cat. no. IS9000) solution dissolved in sterile PBS via intraperitoneal injection every 3 days. For the establishment of the IPF disease group mouse model, isoflurane (RWD; cat. no. R510-22-10) was first introduced into the induction chamber of the anesthetic machine, with a concentration set at 3-4%. After waiting for 1 min until the induction chamber was filled with isoflurane, the mice were placed into the box for anesthesia. After ~2 min, the maintenance concentration was adjusted to 1-1.5% and the mice were then removed and administered 5 mg/kg of BLM via nasal drops to induce a pulmonary fibrosis model, once every 3 days. After three rounds of BLM stimulation, this was discontinued and the mice were administered every three days via intraperitoneal injection with 25 μ l of 0.5% CMC-Na solution. In the treatment group, mice were subjected to BLM stimulation and simultaneously received 25 μ l of SPP1 inhibitor solution (5 mg/kg) through intraperitoneal injection at 3-day intervals. The SPP1 inhibitor was dissolved by incorporating it with 0.5% CMC-Na solution. Prior to the end of the animal model experiments, all mice participating did not fulfill the conditions necessitating humane euthanasia. Specific criteria for the humane euthanasia of animals were as follows: i) Animals are unable to walk on their limbs, completely losing their mobility; ii) dyspnea is observed, characterized by the discharge of saliva or foam from the mouth and nose; iii) urine and feces output is reduced, with diarrhea or incontinence; iv) respiratory and pulse rates are abnormal (either excessively high or low). These criteria should be comprehensively assessed by professional veterinarians or researchers according to specific circumstances to ensure the welfare of the animals and the ethics of the experiment. Upon completion of the 28-day animal model period, the mice were subjected to an induction concentration of 3-4% isoflurane, within an environment maintained at 1-1.5% concentration, after which they were rendered unconscious to perform euthanasia through cervical dislocation. Once it was determined that the animal was motionless, without breathing or heartbeat, and the pupils were dilated, the death of the animal was confirmed after an additional 2-3 min of observation, and lung tissues were collected for subsequent pathological examination.

H&E, Masson and Sirius red staining analysis. Based on a previous study (25), mouse lung tissue samples were initially fixed in a 10% formalin solution at room temperature for 48 h, followed by paraffin embedding and sectioning. The 5 μ m-thick sections were stained with H&E and then observed under a light microscope. Subsequently, the collagen deposits were examined using Sirius Red and Masson's trichrome staining solutions. Finally, all pathological images were analyzed using ImageJ 1.52a software.

Statistical analysis. The present study utilized GraphPad Prism 9 (Dotmatics) software for data processing and analysis, with data represented as the mean $\pm$ standard deviation. When the sample size was ≤ 50 , the Shapiro-Wilk test was applied to evaluate the distribution of the data; when the sample size was > 50 , the Kolmogorov-Smirnov test was applied, and a $P > 0.05$ indicated a normal distribution. After normality testing, the two-tailed unpaired Student's t-test was used to compare

between two groups, whereas one-way ANOVA was used for comparisons among multiple groups under a single variable and multi-way ANOVA was used for comparisons among multiple groups under multiple variables, followed by Tukey's post hoc test to analyze significant differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

High expression of SPP1 is negatively associated with the prognosis of patients with IPF. To explore further the expression pattern SPP1 in patients with IPF and its relationship with prognosis, the present study examined the RNA-seq data from the GEO database. The results revealed that SPP1 gene expression is increased in the lung tissue of patients with IPF (Fig. 1A and B), and this upregulation correlates significantly with poor prognosis (Fig. 1C). Single-cell RNA-seq analysis demonstrated that SPP1 is primarily overexpressed in macrophages and monocytes within the lung tissue of patients with IPF (Fig. 1D). Additional analysis revealed a significant positive association between SPP1 expression and immune cell expression, particularly in macrophages and T cells, in the lungs of patients with IPF (Fig. 1E). These findings suggested a potential role for SPP1 in macrophage function within IPF.

SPP1 promotes macrophage polarization towards the M2 phenotype. Given the significant positive correlation between SPP1 expression and macrophages and T cells, the present study utilized immunofluorescence to assess SPP1 expression in macrophages and T cells of lung tissues from normal controls and patient with IPF. The results showed increased SPP1 expression in macrophages (Fig. 2A and B). To investigate the impact of SPP1 on macrophage function, PBMC-m and THP-1 cells were stimulated with 5 μ M BLM for 12 h and then transfected with si_SPP1 and si_NC. After 24 h, the expression levels of SPP1 and inflammation/fibrosis-related factors were measured through RT-qPCR. The results revealed that BLM stimulation increased the expression of inflammation and fibrosis-related factors in macrophages, and knockdown of SPP1 significantly reduced the expression levels of IL-10 and TGF- β , while the expression of IL-1 α , IL-1 β and TNF- α remained unchanged (Fig. 2C and D). To verify transfection efficiency, under conditions without BLM stimulation, PBMC and THP-1 cells were transfected with si-NC and si-SPP1, respectively. Subsequently, RT-qPCR technology was used to measure the expression levels of SPP1 mRNA. The experimental results identified that, in the absence of BLM stimulation, there was no significant difference in the expression levels of SPP1 in PBMC cells and THP-1 cells transfected with si-NC and si-SPP1 (Fig. S1A). Previous studies have reported that M1 macrophages primarily secrete pro-inflammatory factors such as IL-1 α , IL-1 β and TNF- α , while M2 macrophages primarily secrete factors promoting tissue repair, including IL-4, IL-10 and TGF- β (26-28); the present immunofluorescence analysis revealed a significant increase in the expression of M2 macrophage marker CD206 in PBMC-m and THP-1 cells following BLM stimulation. Moreover, the knockdown of SPP1 decreased CD206 expression (Fig. 2E and F). These findings suggested that

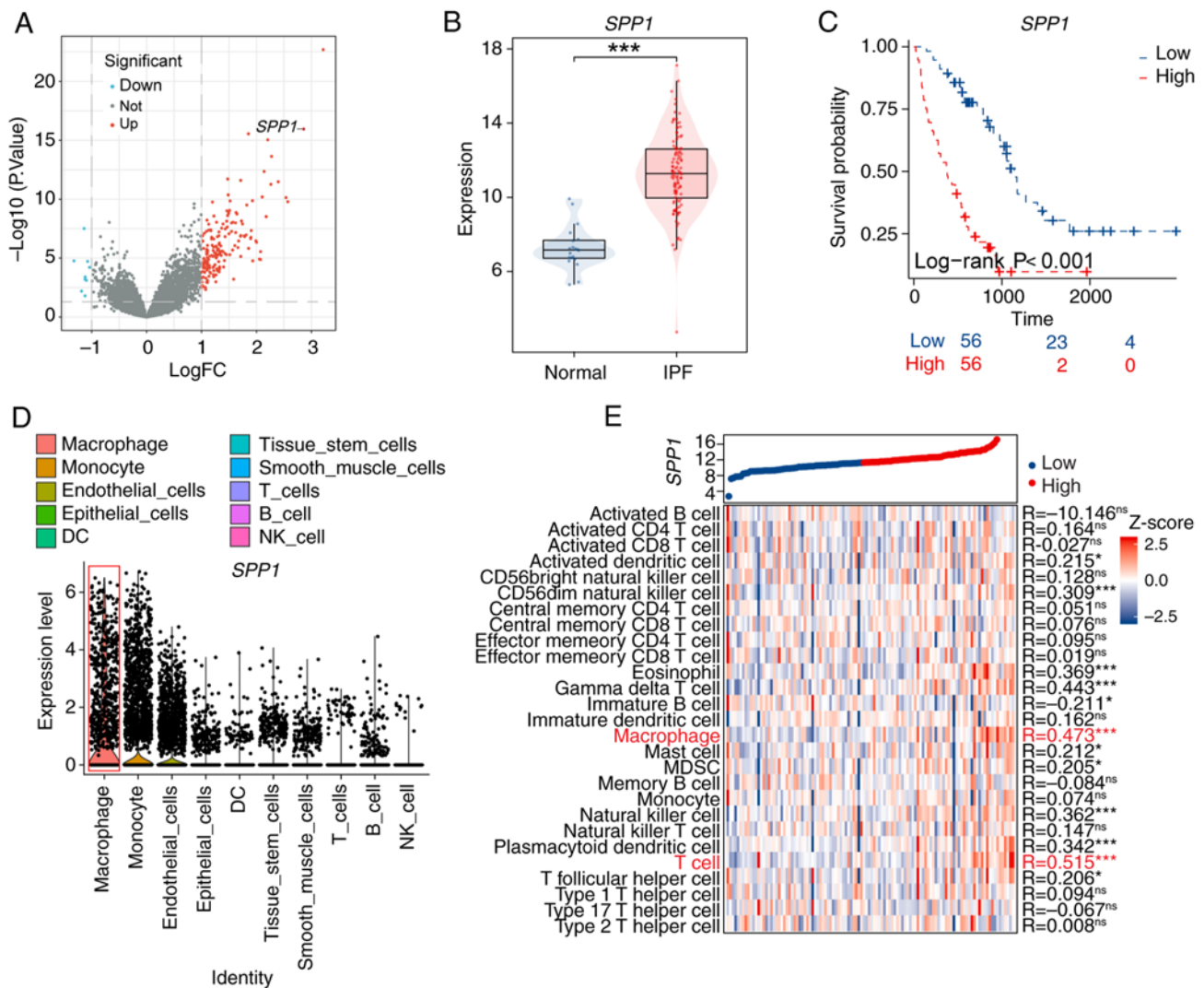


Figure 1. Expression and prognosis of SPP1 in patients with IPF. (A) Differentially expressed genes in normal and lung tissues of patients with IPF from the GSE70866 dataset, with red indicating upregulated genes and blue indicating downregulated genes. (B) Bar graph of SPP1 expression in normal subjects (n=20) and patients with IPF (n=112). (C) Analysis of the relationship between SPP1 gene expression and IPF patient prognosis. (D) Violin plot of SPP1 expression in lung tissue by cell enrichment. (E) Correlation analysis of SPP1 with immune cell expression in lung tissues of patients with IPF. Data are presented as the mean $\pm$ standard deviation. *P<0.05 and ***P<0.001. SPP1, secreted phosphoprotein 1; IPF, idiopathic pulmonary fibrosis; ns, not significant.

SPP1 knockdown inhibits BLM-induced M2 macrophage polarization.

SPP1 promotes the induction of EMT in epithelial and fibroblastic cells by macrophages. Previous studies by the authors revealed that high expression of SPP1 can promote macrophage polarization towards the M2 phenotype, which is known to secrete substantial levels of TGF- β and induce EMT in epithelial and fibroblastic cells, thereby promoting the development of pulmonary fibrosis (29,30). To investigate the role of SPP1 in macrophage-induced EMT, a cellular model where macrophages (PBMC-m and THP-1) induce EMT in epithelial (BEAS-2B) and fibroblastic (WI-38) cells was established in the present study. The supernatant was collected from macrophages treated with PBS, BLM and BLM + si_SPP1 for 24 h and formulated into conditioned media (PBS_CM, BLM_CM and BLM + si_SPP1_CM), which were used to stimulate epithelial (BEAS-2B) and fibroblastic (WI-38) cells, respectively. Scratch assay results showed that the supernatant

from macrophages stimulated with BLM (BLM_CM) significantly enhanced the migration of both epithelial (BEAS-2B) and fibroblastic (WI-38) cells. However, when SPP1 expression was knocked down in macrophages after BLM stimulation, their supernatant (BLM + si_SPP1_CM) significantly inhibited the migration of these cells (Fig. 3). WB analysis further confirmed that BLM_CM promoted the expression of COL3A1, N-cadherin and Vimentin proteins in epithelial (BEAS-2B) and fibroblastic (WI-38) cells while reducing the level of E-cadherin. By contrast, BLM + si_SPP1_CM suppressed these changes, suggesting that inhibition of SPP1 may help reverse the EMT process and may have a positive role in slowing down the fibrotic process.

SPP1 induces macrophage M2 polarization through the JAK2/STAT3 pathway. To uncover the molecular mechanisms by which SPP1 regulates the polarization of M2 macrophages in IPF, the present study integrated previous research findings suggesting that SPP1 may function by influencing the

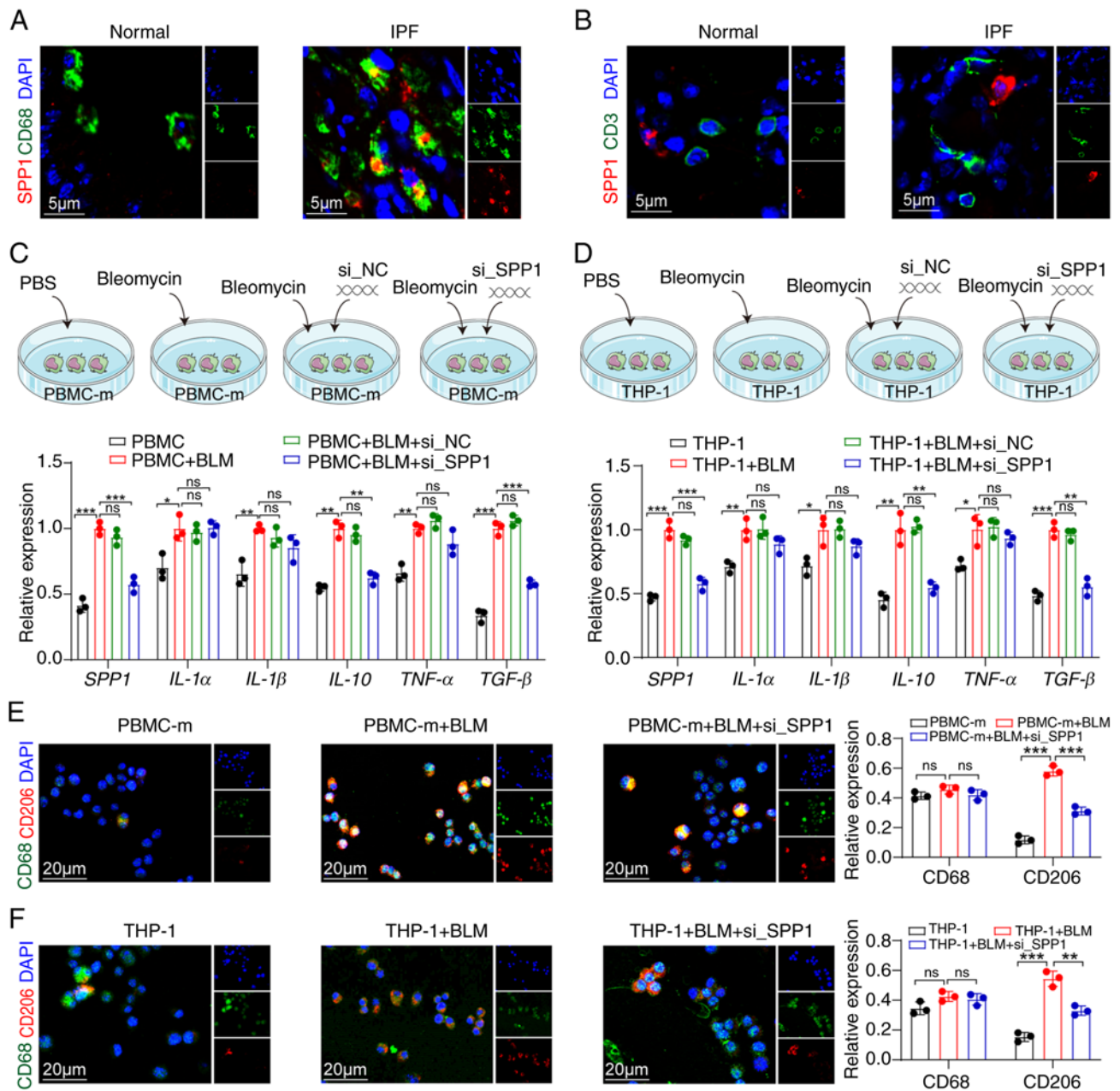


Figure 2. SPP1 promotes macrophage polarization towards the M2 phenotype. (A and B) Immunofluorescence analysis of SPP1 (red) expression in (A) macrophages (CD68, green) and (B) T cells (CD3, green) of normal and idiopathic pulmonary fibrosis patient lung tissues, with nuclear staining (DAPI, blue) (scale bar, 5 μ m). (C and D) Expression and analysis of inflammation and fibrosis-related genes in (C) PBMC-M and (D) THP-1 cells treated with PBS, BLM, BLM + si_NC, and BLM + si_SPP1 (experiments repeated three times). (E and F) Immunofluorescence analysis of CD68 and CD206 expression in (E) PBMC-M and (F) THP-1 cells treated with PBS, BLM, and BLM + si_SPP1, with macrophage marker (CD68, green), M2 macrophage marker (CD206, red) and nuclear staining (DAPI, blue) (scale bar, 20 μ m). Data are presented as the mean $\pm$ standard deviation. * P <0.05, ** P <0.01 and *** P <0.001. SPP1, secreted phosphoprotein 1; BLM, bleomycin; si-, small interfering; PBMC, peripheral blood mononuclear cells; ns, not significant.

activation of STAT3 (31,32). After transfecting si_SPP1 into PBMC-m and THP-1 cells stimulated by BLM, the protein expression levels of SPP1, STAT3 and p-STAT3 were determined. The results indicated that after downregulating the expression of SPP1, the expression levels of p-STAT3 and the ratio of p-STAT3 to STAT3 in macrophages were significantly reduced (Figs. 4A, B and S1B). JAK2 is a key kinase involved in STAT3 activation (33,34), and our detection showed that after downregulating SPP1 expression, the protein expression levels of p-JAK2 and the ratio of p-JAK2 to JAK2 in macrophages were also reduced (Figs. 4A, B and S1B), suggesting that SPP1

may influence the polarization of M2 macrophages through the JAK2/STAT3 signaling pathway. Cell scratch assays revealed that BEAS-2B cells cultured with BLM-stimulated macrophage supernatant (BLM_CM) exhibited enhanced cell migration, which was significantly inhibited after treatment with 5 μ M STAT3 inhibitor (stattic; Fig. 4C and D). Similarly, in the co-cultivation model with macrophages and fibroblast line WI-38, the STAT3 inhibitor significantly suppressed the migration of WI-38 cells (Fig. 4E and F). These findings collectively support the hypothesis that SPP1 promotes M2 macrophage polarization through the JAK2/STAT3 signaling

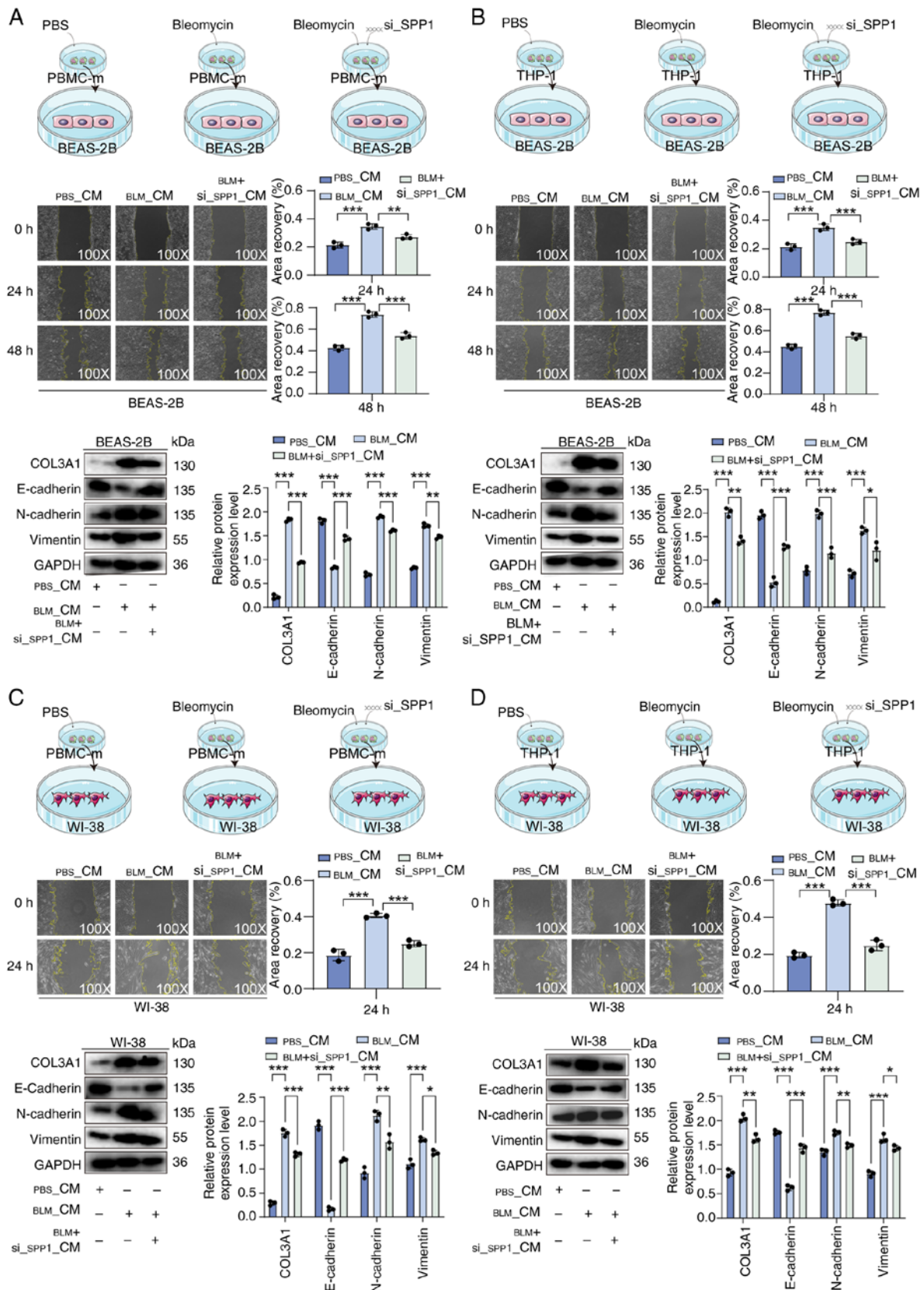


Figure 3. SPP1 promotes the induction of EMT in epithelial and fibroblastic cells by macrophages. (A and B) Schematic diagrams illustrating the indirect co-culture method of PBMC-m and THP-1 with BEAS-2B cells. After treating (A) PBMC-m and (B) THP-1 cells under various conditions, their supernatants were used to culture BEAS-2B cells. Cell scratch assays (magnification, x100) and assessments of EMT-related protein expression levels (E-cadherin, N-cadherin, Vimentin and COL3A1) were performed (with the experiment repeated three times). (C and D) Schematic diagrams respectively depict the indirect co-culture methods of PBMC-M and THP-1 with WI-38 cells. After treating (C) PBMC-m and (D) THP-1 cells under different conditions, their supernatants were utilized to culture WI-38 cells. Subsequent cellular scratch assays (magnification, x100) and assessments of EMT-related protein expression levels (E-cadherin, N-cadherin, Vimentin and COL3A1) were conducted. (with the experiment repeated three times). Data are presented as the mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. SPP1, secreted phosphoprotein 1; EMT, epithelial-to-mesenchymal transition; PBMC, peripheral blood mononuclear cells; ns, not significant.

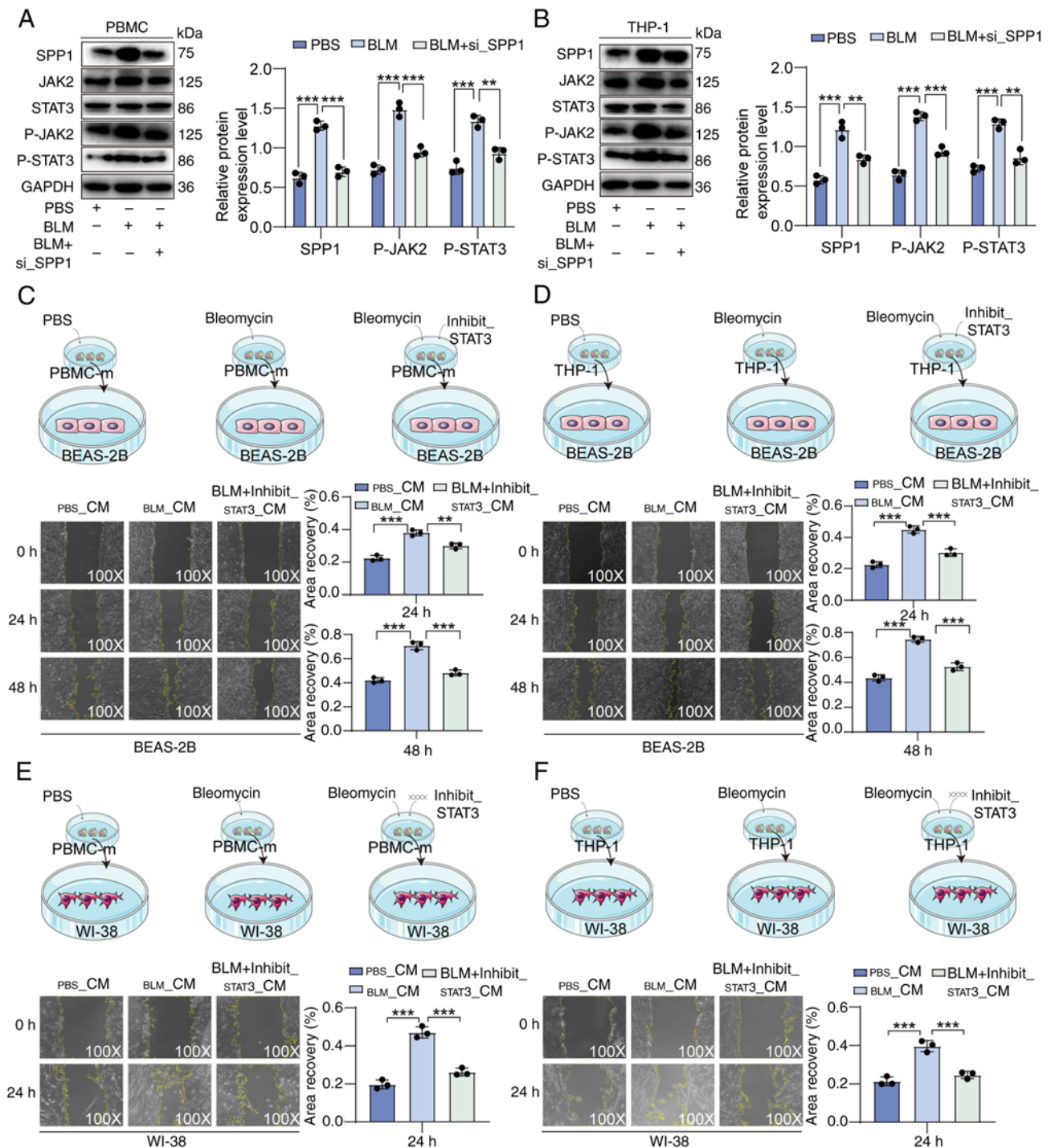


Figure 4. SPP1 induces macrophage M2 polarization through the JAK2/STAT3 pathway. (A and B) After treating PBMC-m and THP-1 cells with PBS, BLM, and BLM + si_SPP1 for 48 h, the protein expression of SPP1, JAK2, STAT3, p-JAK2 and p-STAT3 were detected (with the experiment repeated three times). (C-F) PBS, BLM and BLM + Stattic were used to treat PBMC-m and THP-1 cells, and the supernatants were collected and co-cultured with BEAS-2B and WI-38 cells, respectively, for cell scratch assays (magnification, x100) (with the experiment repeated three times). Data are presented as the mean $\pm$ standard deviation. ** $P < 0.01$ and *** $P < 0.001$. SPP1, secreted phosphoprotein 1; BLM, bleomycin. PBMC, peripheral blood mononuclear cells; p-, phosphorylated; ns, not significant.

pathway, thereby facilitating the progression of pulmonary fibrosis.

In vivo inhibition of SPP1 expression can effectively treat IPF in mice. To elucidate the role of SPP1 in IPF, three groups of mouse animal models were established: Control: IPF disease and an SPP1 inhibitor treatment group (Fig. 5A). Through

monitoring the body weight of the mice, it was found that the mice treated with SPP1 inhibitors gradually regained and stabilized their body weight starting from the fifth day of treatment (Fig. 5B). At the end of the experiment, the mice were sacrificed and their lung tissues were examined histologically (Fig. 5C). H&E staining, Masson's trichrome staining and Sirius Red staining revealed a significant reduction in fibrotic

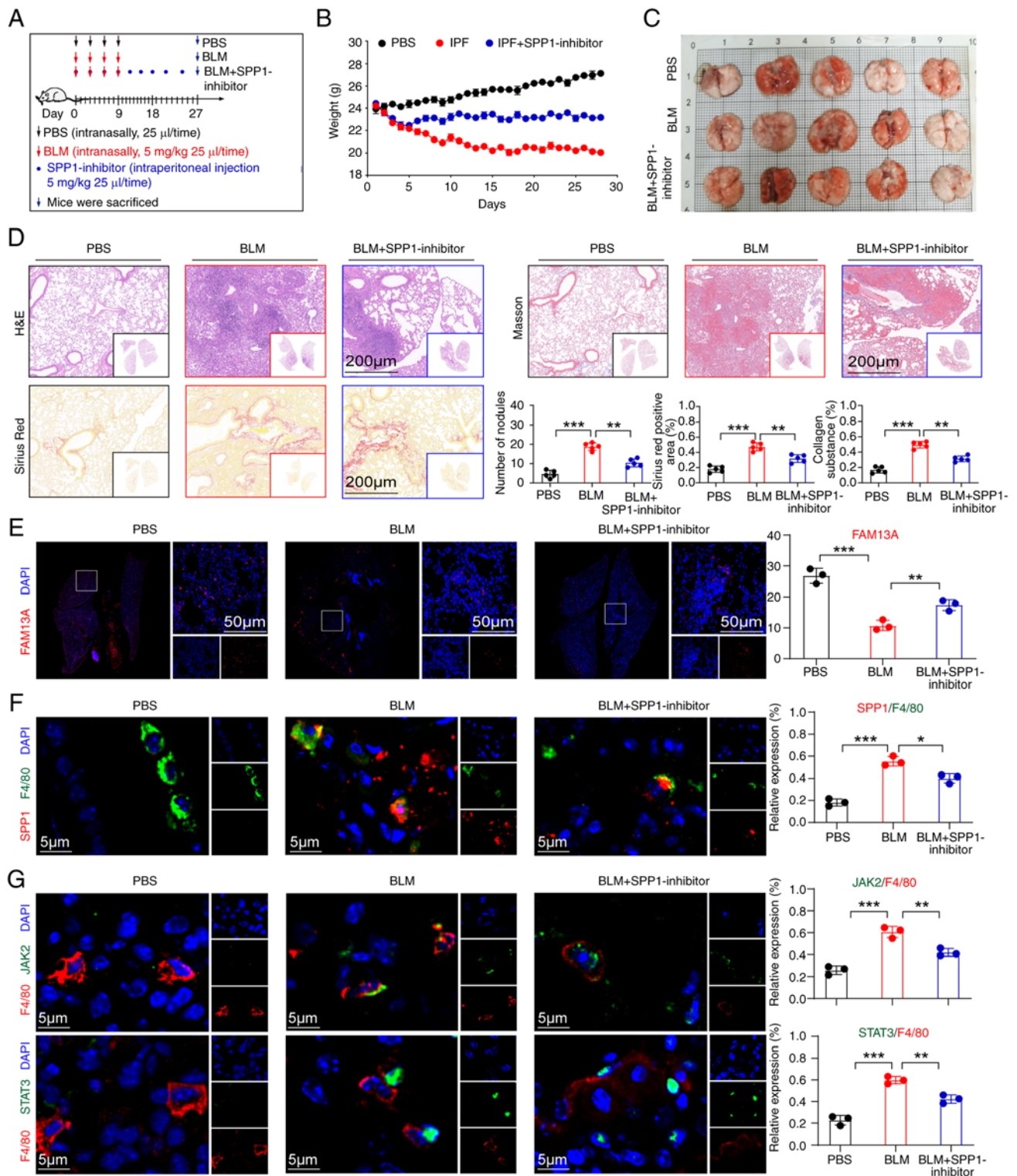


Figure 5. *In vivo* inhibition of SPP1 expression can effectively treat IPF in mice. (A) Schematic illustration of the SPP1 inhibitor treatment in an IPF mouse model. (B) Graphical representation of weight changes in three groups of mice. (C) Images of lung tissues from three groups of mice at the end of the experiment (scale bar, 1 cm). (D) Analysis of H&E, Masson and Sirius Red staining of lung tissues from three groups of mice (each dot represents one mouse; scale bar, 200 μ m). (E) Immunofluorescence detection of FAM13A expression in mouse lung tissues (red) (scale bar, 50 μ m). (F) Immunofluorescence analysis of SPP1 (red) expression and localization in lung tissues of normal, IPF and SPP1-inhibitor treated IPF mice, with macrophages (F4/80, green) and cell nuclei (DAPI, blue) (scale bar, 5 μ m). (G) Detection of p-JAK2 (green) and p-STAT3 (green) expression in lung tissues from normal, IPF and SPP1 inhibitor-treated IPF mice, with macrophages (F4/80, red), cell nuclei (DAPI, blue) (scale bar, 5 μ m). Data are presented as the mean $\pm$ standard deviation. * P <0.05, ** P <0.01 and *** P <0.001. SPP1, secreted phosphoprotein 1; IPF, idiopathic pulmonary fibrosis; p-, phosphorylated; BLM, bleomycin; ns, not significant.

areas and inflammatory cell infiltration in the lung tissues of the treated mice, along with a decrease in collagen fiber deposition (Figs. 5D and S1C). In addition, three lung tissue

specimens were randomly selected from each group of mice models to carry out RT-qPCR to examine the expression levels of genes associated with inflammation and fibrosis. The results

indicated that compared with the disease group, the levels of inflammatory genes (IL- α , IL- β and IL-6) and fibrotic genes (TGF- β , α -SMA and COL3A1) in the lung tissues of the treated mice were significantly reduced (Fig. S1D). In addition, immunofluorescence analysis revealed a significant increase in the expression level of FAM13A (a marker of pulmonary function) (35) in the treated group compared with the disease group (Fig. 5E). Further immunofluorescence detection revealed high expression of SPP1 in alveolar macrophages of IPF mice and that SPP1 inhibitor treatment significantly reduced SPP1 expression in macrophages (Fig. 5F). These results suggested that SPP1 expression is closely associated with the development of IPF. Moreover, assessing the expression levels of JAK2 and STAT3 in lung tissue macrophages of the three groups, it was found that the expression levels of JAK2 and STAT3 in the SPP1 inhibitor group were significantly decreased (Fig. 5G), further suggesting that SPP1 may promote the development of IPF by activating the JAK2/STAT3 signaling pathway.

Discussion

The present study comprehensively analyzed the crucial role of SPP1 in the development of IPF. The current findings revealed that SPP1 expression is increased in lung macrophages of patients with IPF, correlating negatively with their prognosis. Experiments confirmed that SPP1 predominantly promotes M2 macrophage polarization and accelerates fibrosis by activating the JAK2/STAT3 signaling pathway. The findings demonstrated that SPP1 has a crucial role in the progression of IPF, offering beneficial insights for the future study and therapy of IPF.

Although the precise pathogenic mechanisms of IPF have not been fully explained, research has indicated a strong connection between IPF and the repeated injury to alveolar epithelial cells. This includes abnormalities in epithelial repair and the disturbances in the interactions between epithelial and fibroblast cells. These factors collectively lead to the persistent activation of mesenchymal cells and excessive deposition of the ECM (36,37). In recent years, macrophages have become a research focus due to their dual roles in fibrotic remodeling, promoting fibrosis and anti-fibrosis (9). Recent studies observed infiltration of M2-type macrophages in patients with IPF and those with lung fibrosis caused by SARS-CoV-2, and have confirmed that inhibiting M2-type macrophage polarization can mitigate BLM-induced lung injury and fibrosis in model mice (34,38). Given the heterogeneity and plasticity of macrophages under healthy and disease states, elucidating their complex roles and interactions with other lung cells presents a significant challenge. In the current study, it was found through database analysis that high expression of SPP1 in patients with IPF is associated with poor prognosis and positively correlated with macrophage expression. Immunofluorescence localization demonstrated that SPP1 is predominantly located in macrophages in lung tissue of patients with IPF. After BLM stimulation, the expression of CD206 (a marker of M2-type macrophages) colocalizing with CD68 significantly increased, while the expression of cytokines such as IL-10 and TGF- β secreted by M2-type macrophages decreased following SPP1 knockdown, suggesting that SPP1 inhibition

significantly suppresses M2-type macrophage polarization, hinting at a potential key role of SPP1 in the progression of IPF. To further study the role of SPP1 in IPF, BLM-stimulated macrophage supernatant was used to co-culture with lung epithelial cells and it was found that BLM-stimulated macrophages promote EMT and collagen expression in lung epithelial cells, which can be reversed by inhibiting SPP1 in macrophages.

As the JAK2/STAT3 signaling pathway is a key pathway for macrophage polarization, its role in M2-type macrophage polarization and the progression of pulmonary fibrosis warrants further investigation (39-41). Previous studies showed that dihydroartemisinin can alleviate lung inflammation and fibrosis in IPF mice by inhibiting the JAK2/STAT3 signaling pathway (42). Consistent with this, the present study observed that BLM-stimulated macrophages not only enhanced SPP1 expression but also activated the JAK2/STAT3 signaling pathway. Treatment with inhibitors of this signaling pathway was able to suppress cell healing. It was hypothesized that SPP1 may promote M2-type polarization of macrophages induced by BLM through activation of the JAK2/STAT3 signaling pathway, thereby driving IPF progression. In the BLM-induced IPF model, the application of SPP1-specific inhibitors significantly reduced lung fibrosis and collagen deposition, further supporting the importance of SPP1 in the IPF process. Although SPP1 inhibitors have shown significant anti-fibrotic effects in animal models, future research is needed to explore therapeutic dosing strategies or consider their combination with existing treatments such as *Stephania tetrandra* (43).

In summary, given the critical role of macrophages in the pathological process of IPF, the current study has uncovered that SPP1 regulates M2-type polarization of macrophages via the JAK2/STAT3 signaling pathway, thereby promoting IPF progression. The molecular mechanisms underlying this process offer a scientific foundation for the clinical treatment of IPF in the future.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XLY designed cell and animal experiments, curated data, developed methodology, conducted formal analysis and wrote the original draft. ZQL curated data, conducted formal and software analysis, and developed methodology. JWZ, JQG, TH, YFL, YYL, YB and YRX collected the information and revised and finalized the manuscript. JW and DH proposed the preliminary idea and design for the present study and reviewed and revised the manuscript. XLY and DH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental protocols were granted approval by the Animal Ethics Committee at Anhui University of Science and Technology (approval no. HX-001; Huainan, China). Human studies were approved by the Medical Research Ethics Committee of Anhui University of Science and Technology (approval no. HX-001; Huainan, China). Venous blood was collected from all volunteers after obtaining their consent, and an informed consent document was signed by all participants. The human tissue samples were sourced from the Oncology Hospital of the Huainan Dongfang Hospital Group (Huainan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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