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Baccatin III ameliorates bleomycin-induced pulmonary fibrosis *via* suppression of TGF-β1 production and TGF-β1-induced fibroblast differentiation

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive and generally lethal lung disease with a high mortality rate. Current therapeutic drugs exhibit limited efficacy but severe adverse effects. Paclitaxel has been identified to exert both anti-inflammatory and anti-fibrosis activity. Baccatin III (BAC), an important precursor of paclitaxel, has been identified as exhibiting immunomodulatory activity with decisively lower toxicity. However, its effects on pulmonary fibrosis remain unknown.

In this study, the role of BAC in bleomycin (BLM)-induced pulmonary fibrosis and inflammation in mice was investigated in addition to elucidation of its mechanism of action. Our results demonstrated that administration of BAC in a dose-dependent manner reduced inflammatory infiltration, secretion of the pro-fibrotic mediator TGF-β1 and deposition of collagen and other components of the extracellular matrix (ECM), including alpha smooth muscle actin (α-SMA) and fibronectin. Administration of BAC to treat isolated macrophages stimulated with IL-13, known to activate macrophages, the principal source of TGF-β1, resulted in markedly reduced TGF-β1 expression from macrophages. The AKT/STAT6 signaling pathway was shown to be involved in this process. In addition, we have provided *in vitro* evidence that BAC inhibits TGF-β1-induced fibroblast differentiation *via* the Smad2/3 signaling pathway. Furthermore, intratracheal injection of rTGF-β1 significantly exacerbated the degree of fibrosis which was down-regulated by treatment with BAC. Taken together, our data suggest that BAC exerts a protective effect against lung fibrosis and may serve as a potential therapeutic strategy for IPF.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and often fatal lung disease characterized by the accumulation of myofibroblasts and deposition of extracellular matrix (ECM), with median survival of < 3 years after diagnosis [1]. A number of clinical anti-fibrotic drugs are available, such as nintedanib and pirfenidone, which may reduce the decline of lung function in certain cases. However, these drugs have been shown to be ineffective in improving lung function and furthermore, they display serious systemic adverse effects [2,3]. Therefore, discovery of potential drug candidates with improved efficacy and reduced side-effects is urgently required.

The pathogenesis of IPF is believed to be related to the early inflammatory response initiated by aberrant wound healing [4]. Alternative macrophage activation results in cells (M2-phenotype) implicated in this process [5]. There is compelling evidence for this during the process of fibrosis as they produce large quantities of pro-fibrotic cytokines such as IL-10 and TGF-β1, which can directly activate fibroblasts [6,7]. IL-13 has been identified as an M2 activator that performs important roles in tissue remodeling and a fibrotic response both *in vitro* and *in vivo* [8]. The binding of IL-13 to its receptor complex in macrophages causes the transduction of signals by the activation of the major downstream pathways JAK1/STAT6, PI3K/AKT and p38 MAPK [9–11], which have been identified as being critical in the development of IPF. In addition, NF-κB also performs a vital role in inflammation and injury repair in pulmonary fibrosis progression [12,13].

TGF-β1, which is found to be elevated in tissue samples from both IPF patients and animal models of the disease, has been identified as a molecule that performs a vital role in the progression of IPF [14,15]. It can strongly induce the differentiation of fibroblasts into myofibroblasts, leading to increased collagen synthesis and other components of the ECM, including alpha smooth muscle actin (α-SMA) and fibronectin

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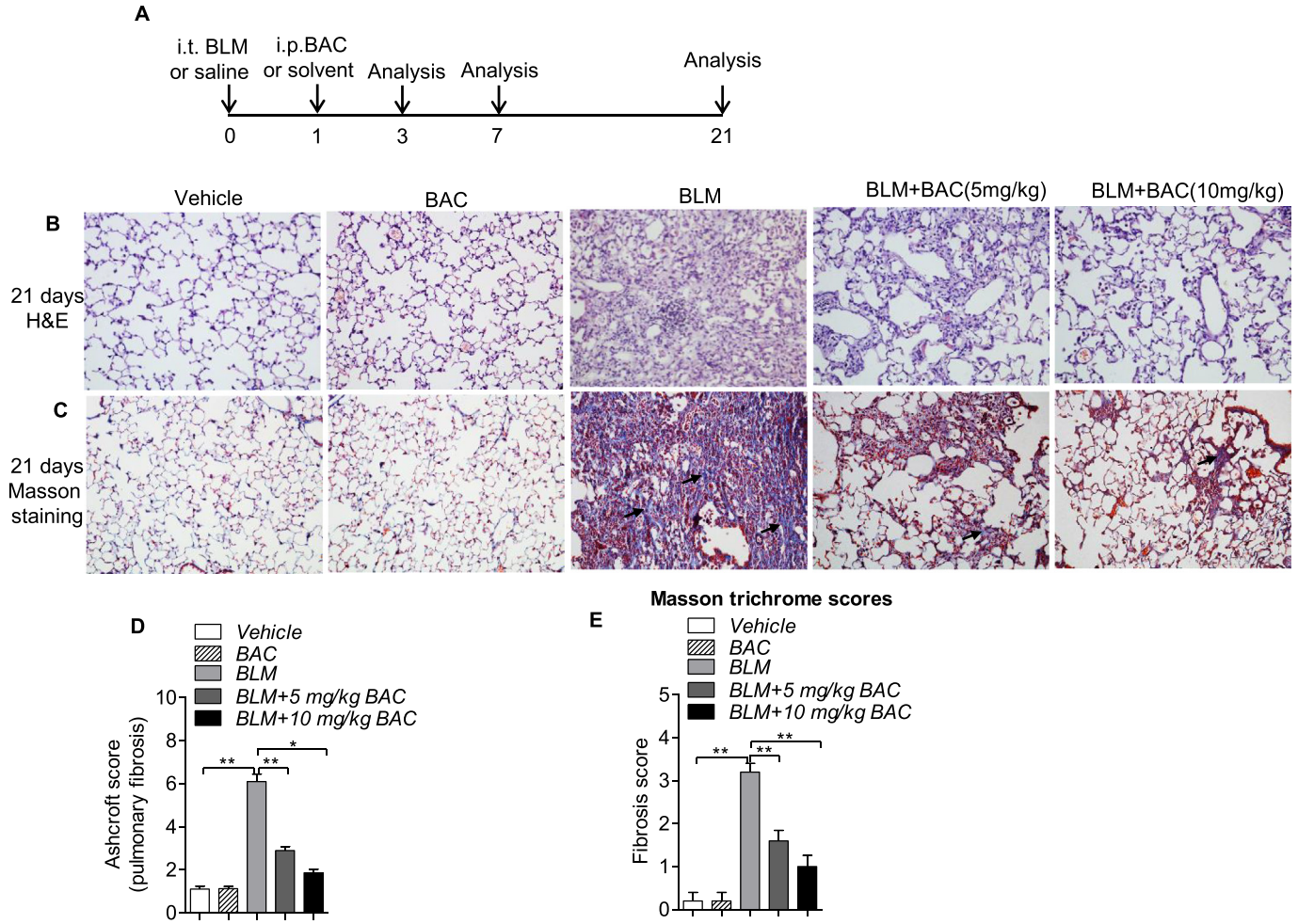

Fig. 1. Administration of BAC ameliorated BLM-induced pulmonary fibrosis in mice. (A) The schematic timeline demonstrates the process of establishing the BLM model and administration of BAC. C57BL/6 mice were i.t. injected with saline or BLM (1.4 U/kg) followed by i.p. injection of BAC at the dosage indicated, (B) H&E staining and (C) Masson's trichrome staining were performed in lung tissue sections from the five groups indicated on day 21 (original magnification ×200) (D) Ashcroft score was evaluated for the five groups indicated on day 21. (E) Masson's trichrome fibrosis score was evaluated for the five groups indicated on day 21. Data represent means ± SEM, n = 6–10, *P < 0.05, **P < 0.01, ***P < 0.001.

[16]. Thus, pharmacological agents that inhibit the production and function of TGF-β1 represent a promising therapeutic strategy for IPF.

Baccatin III (BAC) is the precursor of the semisynthesis of paclitaxel, a natural product from the Pacific yew tree and related species. Paclitaxel, a well-known anticancer drug, has also been found to be effective in treating human diseases other than cancer, including renal and hepatic inflammation and fibrosis through inhibition of the production of proinflammatory cytokines and TGF-β signaling [17]. BAC is decisively less cytotoxic than paclitaxel by a factor of 100–1000 while it exerts immunomodulatory activity with a substantially lower dose in numerous cell types [18,19], indicating that low-toxicity BAC may be an attractive pharmacological agent. However, it remains unknown whether BAC has anti-fibrotic or anti-inflammatory effects in lung tissue. The present study was conducted to investigate the anti-fibrotic function and underlying mechanism of action of BAC.

2. Methods

2.1. Reagent and antibodies

Baccatin III (CAS No.: 27548-93-2, purity > 99%) was purchased from TargetMol Co, Ltd. (Shanghai, China), Bleomycin was purchased from BioTang (Lexington, MA, USA). Primary antibodies against p-AKT (S473), T-AKT, p-STAT6 (Tyr641), STAT6, p-p38 MAPK (Thr180/Tyr182), p38 MAPK and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Recombinant TGF-β1 was purchased from PeproTech, USA.

2.2. Animal

Male C57BL/6 mice (8–10 weeks) were obtained from Nanjing Model Animal Research Center of Nanjing University of China (license No. SCXK (Su) 2017-0052) and housed in specific pathogen-free (SPF) animal facility were housed in specific pathogen-free conditions with temperature and light controlled conditions at the Laboratory Animal Center of Jiangnan University. The procedures involving mice were approved by the Instructional Animal Care and Use Committee (IACUC).

2.3. BLM-induced pulmonary fibrosis

Mice were randomly divided into 5 groups: vehicle group, BAC group (10 mg/kg), BLM group, BLM + BAC (5 mg/kg) group, BLM + BAC (10 mg/kg) group. BLM was dissolved in saline and BAC was dissolved in a vehicle (Saline:ethanol:polyoxyethylene hydrogenated castor oil = 8:1:1). After general anesthesia, mice were intratracheally (i.t.) injected with BLM (BioTang; 1.4 U/kg) or saline on day 0 and intraperitoneally (i.p.) administrated with indicated dosage

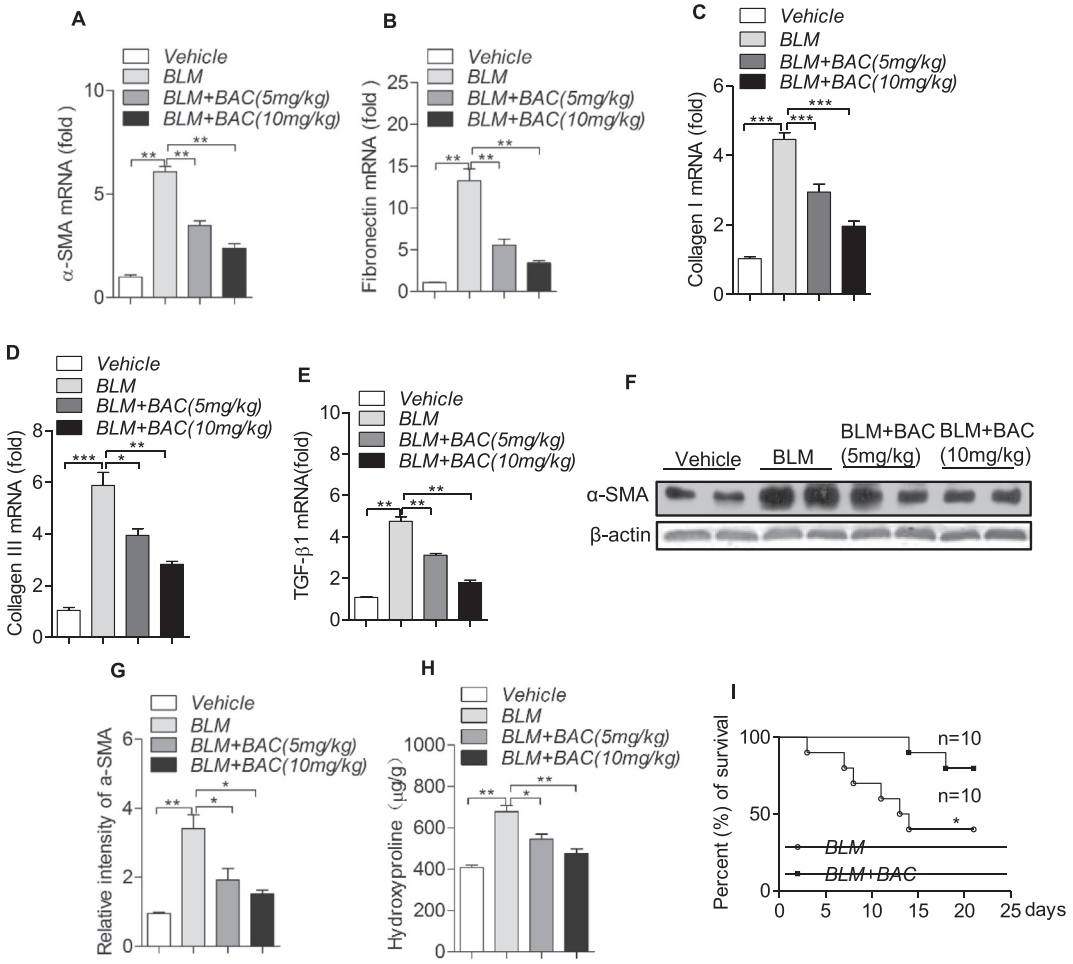

Fig. 2. Administration of BAC reduced fibrotic gene expression and collagen deposition, and increased survival rate in BLM-stimulated mice.
Lung tissues of the indicated groups on day 21 were collected respectively, the mRNA levels of (A) α-SMA, (B) fibronectin, (C) collagen I, (D) collagen III and (E) TGF-β1 were quantified by qRT-PCR. (F) The protein expression level of α-SMA was evaluated by Western blot analysis, β-actin as a loading control. (G) The quantitative analysis of α-SMA. (H) Hydroxyproline content was determined using a hydroxyproline assay kit. (I) C57BL/6 mice were i.t. injected with BLM (1.5 U/kg) on day 0, followed by i.p. injection with BAC (10 mg/kg) or vehicle on day 1 and then monitored for 21 days. The survival curve was recorded. Data represent means ± SEM, n = 6–10, *P < 0.05, **P < 0.01, ***P < 0.001.

of BAC or the solvent as a control for one time on day 1. Lungs were collected for further analysis at day 3, 7, 21 after the BLM exposure.

2.4. Lung histology, hydroxyproline (HYP) assay and MPO activity assay

Lungs were cannulated, inflated with 1 ml 4% paraformaldehyde under a constant pressure, removed from animals, and fixed in fresh 4% paraformaldehyde for 24 h at room temperature. Tissues were then embedded in paraffin, sliced (4 μm) and stained with hematoxylin and eosin (H&E) or Masson's trichrome (kit from NanJing Jiancheng bioengineering institute, China) according to introductions. The stained sections were evaluated under a light microscope. The morphological results were assessed blindly by two pathologists. The inflammatory scores, Ashcroft fibrosis scores and Masson trichrome scores were calculated according to the methods as previously described [20–22]. For HYP assay, lung samples were harvested from mice, weighed and subjected to collagen estimation according to the manufacturer's instructions of the commercial kits from Nanjing Jiancheng Bioengineering Institute.

Myeloperoxidase (MPO) activity of lung tissue was assessed using tetramethylbenzidine, as previously described [23].

2.5. Bronchoalveolar lavage fluid (BALF)

Mice were anesthetized and lungs were lavaged twice with 1 ml cold PBS. Bronchoalveolar lavage fluid (BALF) was collected and red blood cells were lysed, the supernatants were used for detecting protein concentration and pellets were for cell counts. Differential cell counts were determined with Wright-Giemsa staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) on cytospin slides according to the manufacturer's instructions.

2.6. Isolation and culture of primary mouse lung fibroblasts and macrophages

8–10 weeks old C57BL/6 were euthanized. The whole lungs were collected and cut into small pieces, minced, and then subjected to collagenase type IV and DNase I digestion (Sigma. USA) in DMEM with 1% penicillin-streptomycin for 30 min.

Mouse lung fibroblasts were obtained following the protocol as previously reported [24]. Simply, after centrifugation, the pellets containing tissue fragments and cells were washed and cultured in DMEM added with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2 for 7–14 days, then tissue fragments were discarded and cells were used for experiments. Interstitial Macrophages (IMs) from the lungs were obtained as previously described [25]. Simply, after filter and

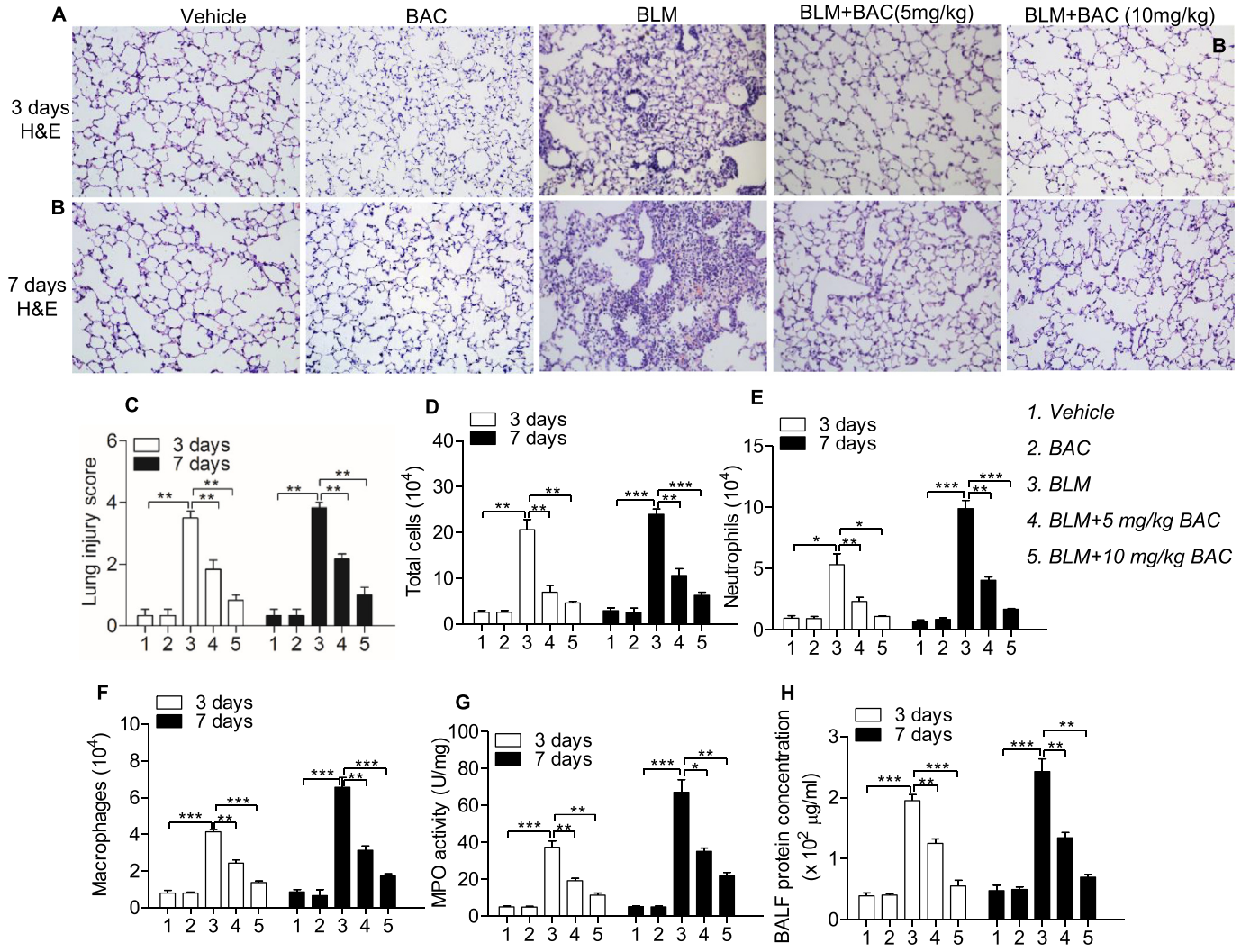

Fig. 3. Administration of BAC ameliorated BLM-induced inflammation in mice. H&E staining was performed in lung tissue sections from the five groups indicated on (A) day 3 and (B) day 7 (original magnification ×200). (C) Lung tissue injury was assessed by histological scores in all groups. (D) Total numbers of cells, (E) neutrophils and (F) macrophages in BALF. (G) MPO activity in mouse lung lobes. (H) Protein concentration of BALF was detected using a BCA protein assay kit. Data represent means ± SEM, n = 6–8, *P < 0.05, **P < 0.01, ***P < 0.001.

centrifugation, the cells were washed and cultured in DMEM added with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2 for 1 h, then the unattached cells were discarded and attached cells were used for experiments.

2.7. Cell viability assay (MTT assay)

Cells (both macrophages and fibroblasts) were seeded in 96-well plate at a density of 5×10^3/well, and then incubated with different dose of BAC for 48 h. MTT solution (5 mg/ml, 20 μl/well) was added to each well, followed by additional 4 h incubation. After extraction of culture supernatant, each well was added with 150 μl/well of DMSO. The absorbance was read at 570 nm on a microplate reader (BioTek Epoch, USA). The results are determined in percentages of the viable cells compared to the respective control.

2.8. Real-time PCR analysis

Total RNA was extracted from the lung tissues and cells using TRIzol reagent (Invitrogen), converted to complementary DNA by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, California, CA, USA). The mRNA levels were detected with the following primer pairs by Fast SYBR Green master mixed: αSMA (forward, *5′-GACGCTGAAGTATCCGATAGAACACG-3′*; reverse, *5′- CACCA TCTCCAGAGTCCAGCACAAT-3′*), fibronectin (forward, *5′- TCTGGGAA ATGGAAAAGGGGAATGG-3′*; reverse, *5′-CACTGAAGCAGGTTTCCTCG GTTGT-3′*), collagen I (forward, *5′- TGCCGTGACCTCAAGATGTG-3′*; reverse, *5′- CACAAGCGTGCTGTAGGTGA-3′*), collagen III (forward, *5′-ACGTAGATGAATTGGGATGCAG-3′*, reverse, *5′-GGGTTGGGGCAGT CTAGTC-3′*), TGF-β1 (forward, 5′- CAACAATTCCTGGCGTTACCT TGG-3′; reverse, 5′-GAAAGCCCTGTATTCCGTCTCCTT-3′), TNF-α (forward, 5′-TTCTCATTCCTGCTTGTGG-3′; reverse, 5′-ACTTGGTGGTTTG CTACG-3′); Arginase 1 (forward, 5′-GAACACGGCAGTGGCTTTAAC-3′; reverse, 5′-TGCTTAGCTCTGTCTGCTTTGC-3′), IL-10 (forward, 5′-CAA GGAGCATTTGAATTCCC-3′; reverse, 5′-GGCCTTGTAGACACCTTG GTC-3′), GAPDH (forward, *5′-* TGCGACTTCAACAGCAACTC-*3′*, reverse *5′- CTTGCTCAGTGTCCTTGCTG-3′*).

Relative gene amounts were calculated using ΔΔCt method normalized against GAPDH.

2.9. ELISA

Quantitative measurement of TGF-β1 in lung homogenates was performed by ELISA (R&D Systems), according to the manufacturers'

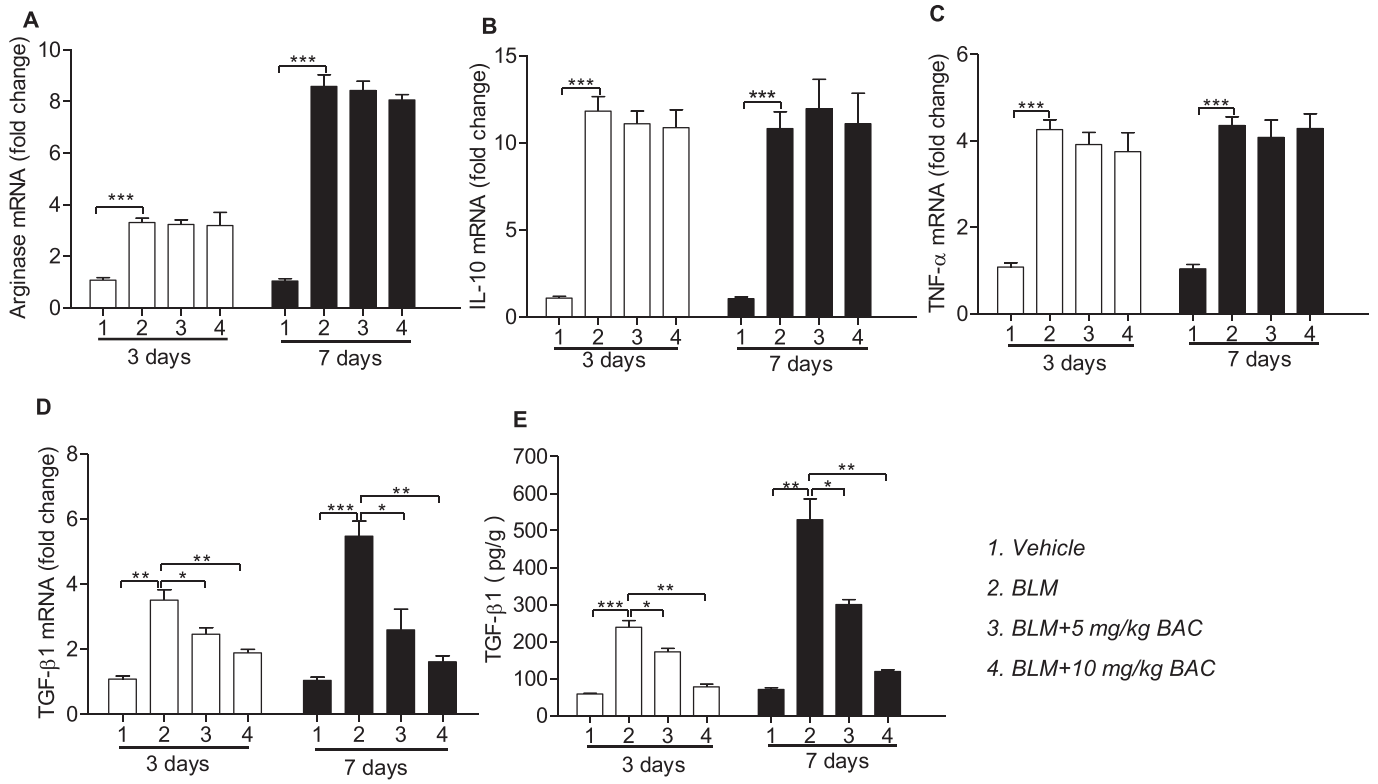

Fig. 4. Administration of BAC reduced the expression of TGF-β1 in BLM-induced mice. The mRNA expression levels of the inflammatory and pro-fibrotic genes. (A) Arginase, (B) IL-10, (C) TNF-α and (D) TGF-β1 in lung tissues on days 3 and 7 were quantified using qRT-PCR. (E) The protein expression levels of TGF-β1 in lung tissues on days 3 and 7 were measured using ELISA. Data represent means ± SEM, n = 6–8, *P < 0.05, **P < 0.01, ***P < 0.001.

instructions.

2.10. Western-blot analysis

The protein lysates were harvested using RIPA buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF) and the protein concentration was determined by a bicinchoninic acid (BCA) kit. Same Proteins (10–20 μg) of each sample were separated on 10%–12% SDS/PAGE gels at 200 V for 1 h and transferred onto PVDF membranes at 80–100 V for 1 h, blocked in TBS containing 5% skimmed milk and then incubated with the indicated primary antibodies at 4 °C overnight. After incubation with HRP-conjugated secondary antibodies, immunoreactive bands were detected with the ImmunoStar®LD (Thermo Fisher Scientific Inc.). The intensity was quantified by Image J software.

2.11. Statistical analysis

Data were expressed as means ± SEM obtained from experiments which were repeated at least three times. The unpaired Student's *t*-test and one-way ANOVA were used to determine statistically significant differences between groups. Kruskal–Wallis was used to determine statistically significant differences between groups for the inflammatory scores, Ashcroft fibrosis scores and Masson trichrome scores. *P < 0.05, **P < 0.01, and ***P < 0.001. P < 0.05 was considered to be of statistically significance.

3. Results

3.1. BAC administration ameliorated BLM-induced pulmonary fibrosis in mice

The anti-pulmonary fibrosis effect of *BAC* was tested in BLM-induced mice, the most common model of experimental lung fibrosis [26]. Mice challenged with BLM (1.4 U/kg) at day 0 were treated intraperitoneally with two doses of BAC (5 or 10 mg/kg) at day 1 (Fig. 1A). Twenty one days after challenge with BLM, histologic analysis using hematoxylin and eosin (H&E) and Masson's trichrome staining were performed to evaluate the pathological changes. The results revealed that challenge with BLM caused fibroblasts and myofibroblasts to aggregate and infiltrate into a large number of normal alveolar structures, in addition to enhancing collagen deposition in the lungs. The degree of fibrosis was evaluated by an Ashcroft analysis according to pathological changes and Masson's trichrome score according to collagen accumulation in the lungs. The results demonstrated that both Ashcroft and Masson's trichrome scores were markedly upregulated after administration of BLM. No further damage to the lung structure was observed after treatment with BAC for 21 days. Importantly, all BLM-induced fibrotic characteristics were clearly alleviated following treatment with BAC in a dose-dependent manner (Figs. 1B–E).

3.2. BAC reduced fibrotic gene expression and mortality in BLM-induced pulmonary fibrosis

To further quantify the effects of BAC on lung fibrosis, both mRNA and protein expressions were evaluated in addition to hydroxyproline content in the lung tissues of mice from the indicated groups on day 21. As shown in Fig. 2A–D, BLM treatment significantly enhanced the mRNA expression of the fibrosis markers α-SMA, fibronectin, collagen I, collagen III and TGF-β1 while administration with BAC substantially reduced their expression levels in a dose-dependent manner. Likewise, the increased protein level of fibrosis marker a-SMA in lung homogenates stimulated with BLM was significantly downregulated by BAC administration as demonstrated by Western blot analysis (Fig. 2E and F). The elevated level of BLM-induced hydroxyproline (Fig. 2G), a major component of collagen, decreased significantly after treatment

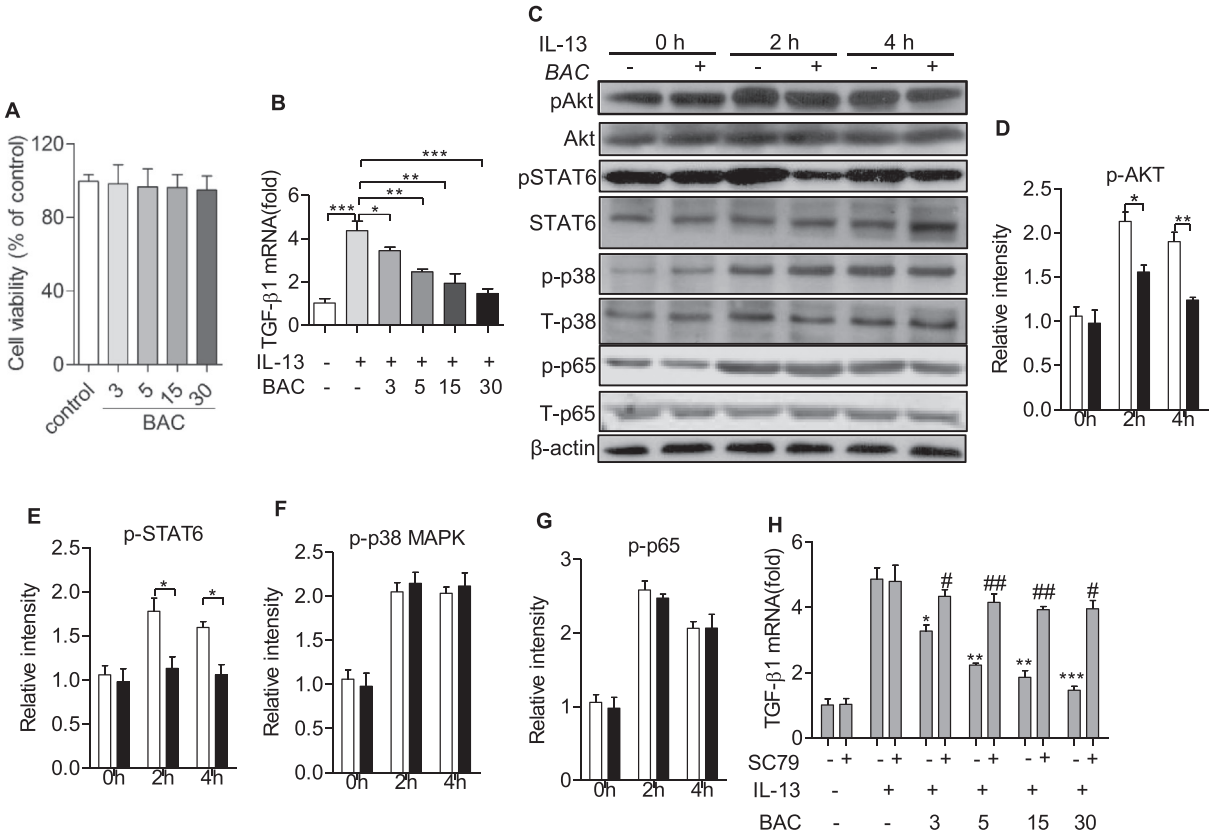

Fig. 5. BAC inhibited macrophage-derived TGF-β1 expression.
(A) Lung macrophages were treated with BAC at concentrations of 0, 3, 5, 10 and 30 μmol/l for 48 h, then cell viability was determined by MTT assay. (B) Lung macrophages were pre-incubated with BAC at concentrations of 0, 3, 5, 10 and 30 μmol/l for 30 mins, then stimulated with IL-13 (10 ng/ml) for 24 h. mRNA levels of TGF-β1 were quantified by qRT-PCR. (C) Lung macrophages were challenged with IL-13 (10 ng/ml) for 4 h. p-AKT (Ser473), p-STAT6 (Tyr641), p-p38 MAPK (Thr180/Tyr182), AKT, STAT6, p38 MAPK and p-NF-κB (p65) were detected by Western blot analysis, β-actin as a loading control. Statistical analysis of differences in the phosphorylation level of (D) AKT, (E) STAT6, (F) p38 MAPK and (G) p-p65 normalized to total protein levels. (H) Lung macrophages were pre-incubated with BAC at concentrations of 0, 3, 5, 10 and 30 μmol/l for 30 mins or together with SC79 (5 μg/ml), then stimulated with IL-13 (10 ng/ml) for 24 h. The mRNA levels of TGF-β1 were quantified by qRT-PCR. * compared with the group induced by IL-13 without BAC treatment; # compared with the corresponding group treated with IL-13 and BAC treatment. Data represent means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001; #P < 0.05, ##P < 0.01, ###P < 0.001.

with BAC. In addition, BAC treatment reduced the mortality caused by high doses of BLM challenge in comparison with mice which were treated with only the vehicle, using a survival assay for 21 days (Fig. 2H).

Collectively, these data suggest that administration of BAC reduced fibrotic markers expression and collagen production and improved their survival rates.

3.3. BAC administration ameliorated BLM-induced lung inflammatory response in mice

BLM causes lung damage that results in an inflammatory response, followed by fibrosis, a process similar to human pulmonary fibrosis, which also shares many of the same pathological characteristics. Hence, a large number of studies also evaluate anti-fibrotic properties of novel target drugs by investigating the therapeutic effect on stages of inflammation (within the first week after exposure to bleomycin) [27].

To investigate the effect of BAC on the BLM-induced pulmonary inflammatory response, H&E staining of lung slices was conducted to evaluate pathological changes. As shown in Fig. 3A and B, BLM challenge contributed to pulmonary edema, thickening of the alveolar wall and inflammatory cell infiltration. Conversely, treatment with BAC caused no damage to the histological structure and biochemical characteristics of the lung tissue and effectively reduced the degree of pathological lung injury 3 and 7 days after BLM challenge. To quantitatively evaluate the degree of lung injury, lung injury score analysis was performed. As shown in Fig. 3C, treatment with BAC significantly diminished pathology scores in a dose-dependent manner. Bleomycin-induced inflammatory cell infiltration into the lung tissue was ascertained by counting cells in bronchoalveolar lavage fluid (BALF). Cell counts of total cell number (Fig. 3D), neutrophils (Fig. 3E) and macrophages (Fig. 3F) in BALF were clearly attenuated by treatment with BAC. Myeloperoxidase (MPO) activity analysis was performed to determine neutrophil infiltration and pulmonary inflammatory injury. As shown in Fig. 3G, MPO activity decreased significantly following treatment with BAC. In addition, protein concentration in BALF, examined to evaluate lung alveolar microvascular permeability, experienced a considerable reduction after treatment with BAC (Fig. 3H). Taken together, these results suggest that BAC alleviated BLM-induced pulmonary inflammation.

3.4. BAC reduced TGF-β1 expression in lungs from BLM-stimulated mice

Numerous cytokines and chemokines have been identified as potential contributors to the initiation and progression of IPF. To explore the mechanism of action of BAC in regulating IPF, we evaluated the expression of many cytokines demonstrated to be central factors in the development of IPF in lung tissues 3 and 7 days after BLM challenge, with or without treatment with BAC. However, the mRNA levels of arginase, IL-10 and TNF-α did not change substantially (Fig. 4A–C), but BAC treatment did result in significantly lower mRNA levels of TGF-β1. Furthermore, high doses of BAC were associated with a more significant

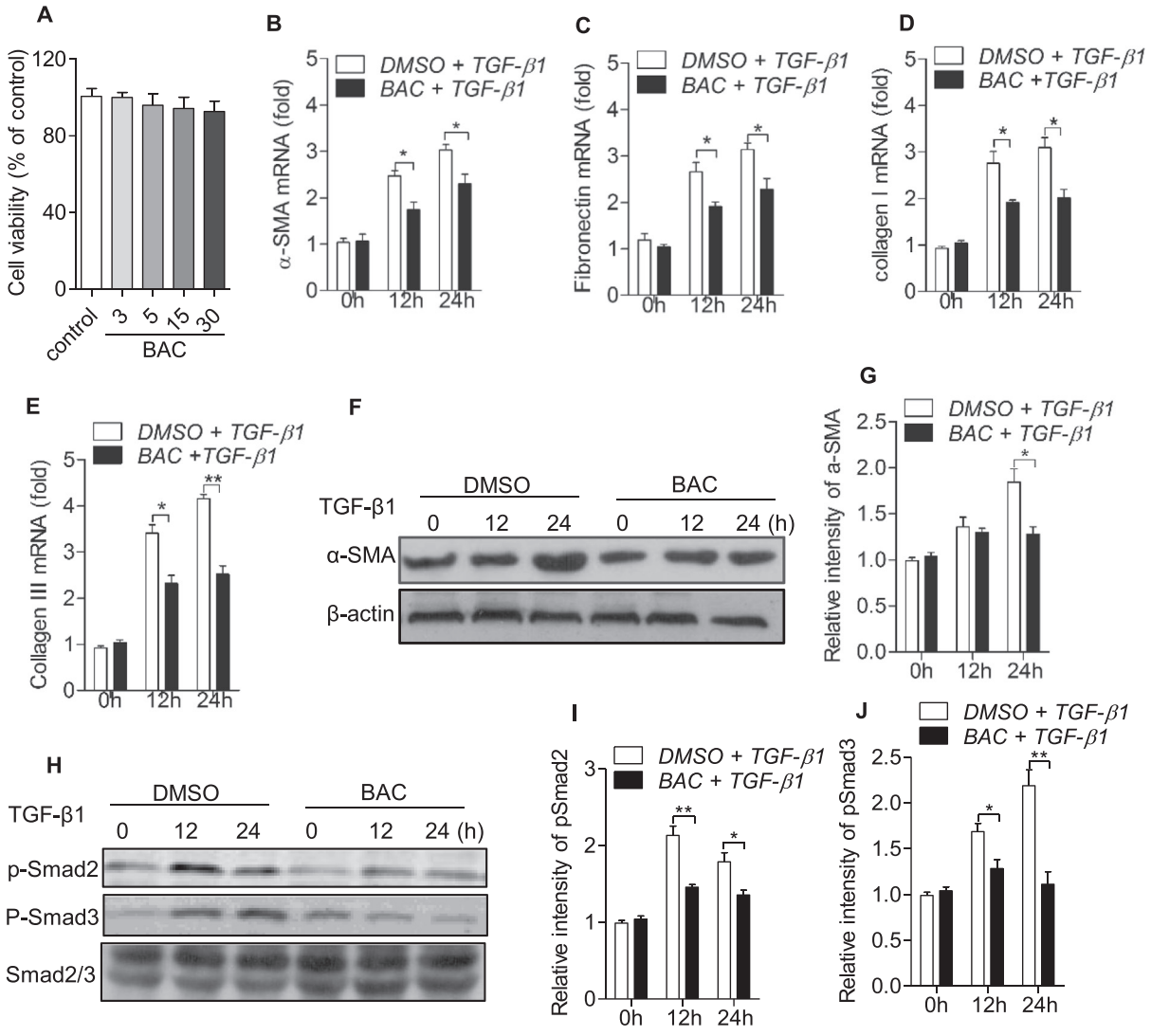

Fig. 6. BAC inhibited TGF-β1-induced pulmonary fibroblast differentiation.
(A) Lung fibroblasts were treated with BAC at concentrations of 0, 3, 5, 10 and 30 μmol/l for 48 h, then cell viability was determined by MTT assay. Pulmonary fibroblasts were pre-treated with BAC or DMSO as control for 0.5 h, then stimulated with TGF-β1 (10 ng/ml) for 24 h, the mRNA levels of (B) α-SMA, (C) fibronectin, (D) collagen I and (E) collagen III were quantified by qRT-PCR and normalized to GAPDH; (F) the protein levels of α-SMA were examined by Western blot analysis, β-actin as a loading control. (G) Quantitative analysis of α-SMA. (H) The protein expression of p-Smad2, p-Smad3, Smad2/3 was detected by Western blot analysis, β-actin as a loading control. Statistical analysis of differences in the phosphorylation level of (I) Smad2 and (J) Smad3 normalized to total protein levels. Data represent means ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001.

reduction (Fig. 4D). We further evaluated the effect of BAC on the protein expression of TGF-β1. Consistently, the protein levels of TGF-β1 in BLM-induced lungs were also substantially decreased by treatment with BAC (Fig. 4E). These findings suggest that BAC may alleviate BLM-induced fibrosis by mediating TGF-β1 expression during the process of the inflammatory response in mice.

3.5. Administration of BAC suppressed TGF-β1 production in macrophages

Macrophages are known to be the principal cell type which secrete profibrotic mediators, including TGF-β1 and perform pivotal roles in the pathogenesis of fibrosis. The potential cytotoxic effects of BAC on macrophages were tested by MTT assay after incubation of lung macrophages with BAC for 48 h. The data demonstrated that BAC exhibited no cytotoxicity against macrophages (Fig. 5A). To evaluate whether BAC regulated the production of TGF-β1, lung macrophages were stimulated with IL-13, which is produced by various cell types such as epithelial cells and Th2 lymphocytes during wound healing. As shown in Fig. 5B, the production of TGF-β1 in response to IL-13 stimulation decreased after treatment with BAC in a dose dependent manner. To gain insight into the mechanisms underlying BAC inhibition of TGF-β1 production in macrophages, we examined the activity of AKT, p38 MAPK, STAT6 and NF-κB signaling molecules, which are critical for macrophage activation and TGF-β1 production. As shown in Fig. 5C–G, treatment with BAC significantly down-regulated elevated Akt and STAT6 phosphorylation in response to IL-13 stimulation, but did not affect the phosphorylation of p38 MAPK or NF-κB in macrophages. To determine whether Akt/STAT6 signaling participated in the reduced production of TGF-β1 resulting from treatment with BAC, macrophages were subsequently treated with a combination of BAC and SC79 (an activator of Akt). The reduction in BAC-mediated TGF-β1 was reversed through the activation of Akt (Fig. 5H). Together, these findings indicate that treatment with BAC suppresses TGF-β1 production by macrophages through the AKT/STAT6 signaling pathway.

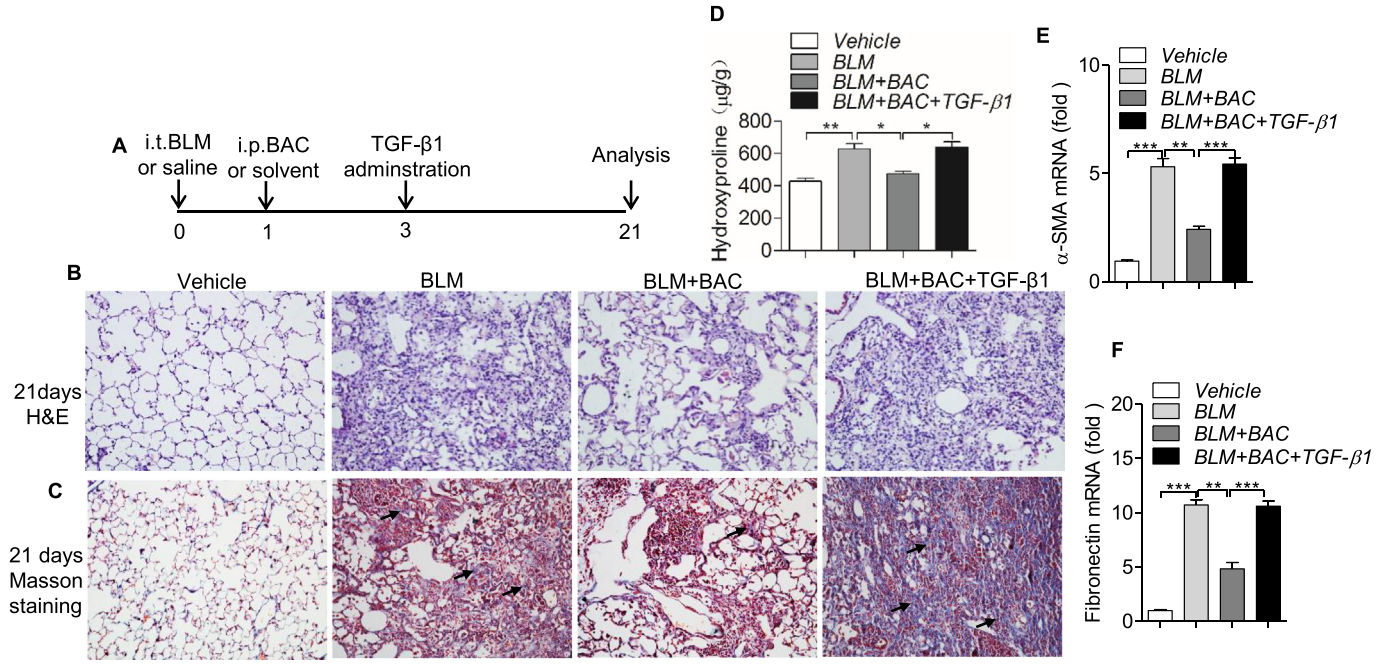

Fig. 7. TGF-β1 exacerbated BLM-induced pulmonary fibrosis treated with BAC.
(A) Schematic diagram of experimental procedure. Mice were injected intratracheally with saline or BLM (1.4 U/kg) on day 0, then treated intratracheally with BAC (5 mg/kg) or solvent on day 1, followed by intratracheal injection of recombinant TGF-β1 (1 μg in 50 μl of PBS) on day 3. After 21 days, mice were sacrificed for the analysis of pulmonary fibrosis. (B) Lung sections were stained with H&E. Original magnification ×200. (C) Masson's trichrome staining. Original magnification ×200. (D) Hydroxyproline concentration. (E and F) mRNA levels of α-SMA and fibronectin were quantified by qRT-PCR. Data represent means ± SEM, n = 6–8, *P < 0.05, **P < 0.01, ***P < 0.001.

3.6. Administration of BAC suppressed fibroblast differentiation into myofibroblasts in response to TGF-β1

The differentiation of fibroblasts into myofibroblasts is the principal method by which TGF-β1 contributes to pulmonary fibrosis. Having established the role of BAC in TGF-β1 production in macrophages, we next explored the effect of BAC on fibroblast differentiation in response to induction by TGF-β1. The experiments conducted on fibroblasts demonstrated that administration of BAC was not cytotoxic towards fibroblasts (Fig. 6A) and significantly reduced the mRNA expression of the fibrosis markers α-SMA, fibronectin, collagen I and collagen III in addition to the protein level of α-SMA (Fig. 6B–G). To elucidate the underlying mechanism of fibroblast differentiation, we explored the activity of the Smad signaling pathway, pivotal for fibroblast differentiation in response to TGF-β1. As shown in Fig. 6H–J, TGF-β1 stimulation significantly increased the levels of p-Smad2 and p-Smad3, while treatment with BAC significantly reduced this phosphorylation, resulting in attenuated activation of Smad signaling. These findings indicate that treatment with BAC suppresses TGF-β1-induced fibroblast differentiation *via* inhibition of the activity of the Smads signaling pathway.

3.7. TGF-β1 exacerbated BLM-induced pulmonary fibrosis that had been attenuated by treatment with BAC

To further ascertain the role of TGF-β1 in BAC-attenuated pulmonary fibrosis, recombinant TGF-β1 (rTGF-β1) was injected (i.t.) into BLM-induced mice that had been treated with BAC, and lung tissues harvested on day 21 (Fig. 7A). The results showed that administration of rTGF-β1 substantially enhanced BLM-induced lung tissue damage, as observed in H&E staining (Fig. 7B) and resulting in greater collagen deposition as observed in Masson's trichrome staining (Fig. 7C), which had been down-regulated by treatment with BAC. Consistently, hydroxyproline levels in the lungs of BLM-induced mice challenged with both rTGF-β1 and BAC were also elevated compared with mice without rTGF-β1 (Fig. 7D). The expression levels of α-SMA and fibronectin in whole lungs from BLM-induced mice with BAC treatment were also substantially elevated after receiving rTGF-β1 (Fig. 7F). Hence, these data further demonstrated that treatment with BAC attenuated IPF through TGF-β1.

Collectively, our data support the hypothesis that administration of BAC attenuates IPF by suppression of TGF-β1 production and function.

4. Discussion

BAC, a specific precursor of paclitaxel, was considered an inactive derivative of paclitaxel in the past because of its decisively weaker cytotoxic activity compared with paclitaxel [18,19]. Little is known about the anti-inflammatory and anti-fibrosis activity of BAC. In search of pharmacological agents that exert anti-fibrosis activity with lower toxicity, we found that BAC attenuated pulmonary fibrosis by reducing the accumulation of TGF-β1 and by suppression of fibroblast differentiation. As BAC has low cytotoxicity [28], it can be considered an attractive candidate for treatment of pulmonary fibrosis.

This study provided clear evidence that BAC exerts anti-fibrosis activity both *in vitro* and *in vivo*. BLM-induced inflammatory and fibrotic reactions in lungs are commonly used as a pre-clinical model that resembles IPF [29]. After BLM instillation, alveolus inflammation is prominent within the first week. The significant development of fibrosis can be observed both biochemically and histologically by day 21. Therefore, time points for investigation of our present animal model were set at days 3, 7 and 21 [30]. Our results showed substantial histopathological changes in the lungs, including collapsed alveolar space, inflammatory cell infiltration, fibroblast accumulation and synthesis of collagen in mice challenged with BLM. However, these pathological alterations were alleviated by treatment with BAC with the doses investigated. The levels of the major biomarkers of fibrosis, including hydroxyproline, α-SMA, fibronectin and collagens, decreased

significantly. Additionally, the major biomarkers of inflammation, the number of inflammatory cells, MPO activity and total protein concentration in BALF, also reduced markedly after administration of BAC. These results reveal that BAC is able to suppress both early and late fibrogenic process in pulmonary fibrosis.

During the early stages of exposure to BLM in the lungs, alveolar epithelial cells underwent DNA damage and triggered the infiltration of inflammatory cells to pulmonary loci, initiating fibrosis. Macrophages reportedly serve as a central contributor to this process *via* the production of cytokines such as TNF-α [31], IL-10 [32], arginase [33] and TGF-β [34], creating a fibrotic microenvironment and inducing the accumulation of ECM produced by myofibroblasts with higher levels of α-SMA and fibronectin [31,35]. Among the numerous fibrogenic factors, TGF-β is the most pivotal profibrotic molecule [36]. Increasing evidence has indicated that overexpression of TGF-β1 alone, one of the three TGF-β isoforms, is sufficient to induce fibrosis [37]. TGF-β1 can induce the recruitment of inflammatory cells that promote the inflammatory cascade [38]. In the present study, treatment with BAC significantly decreased the levels of TGF-β1 in lungs from BLM-challenged mice during the inflammation and fibrosis stage. Considering macrophages are the primary sources of TGF-β1, we examined the mRNA levels of TGF-β1 derived from macrophages in response to IL-13, a cytokine often abundant during wound healing and fibrosis. Consistently, BAC substantially reduced the expression of TGF-β1 in macrophages induced by IL-13. In order to investigate how BAC is able to regulate macrophage-produced TGF-β1, we examined the activation of the AKT, p38 MAPK, STAT6 and NF-κB signaling pathways, which have previously been shown to be important for macrophage activation and TGF-β1 production [39–42]. Our study revealed that BAC suppressed the activation of AKT and STAT6 but did not affect the p38 MAPK signaling pathway. The observation that the reduction in levels of BAC-mediated TGF-β1 was reversed by the Akt activator SC79 further confirmed that the Akt signaling pathway was closely associated with the role of BAC in reducing macrophage-derived TGF-β1 production. These results both *in vivo* and *in vitro* indicate that administration of BAC could reduce levels of the pro-fibrotic cytokine TGF-β1 in both BLM-stimulated lungs and macrophages in response to IL-13.

To determine whether Akt/STAT6 signaling participated in BAC-reduced TGF-β1 production, macrophages were subsequently treated with a combination of BAC and SC79 (an activator of Akt). The reduction in BAC-mediated TGF-β1 secretion was reversed through the activation of Akt (Fig. 5G). All these findings together indicate that treatment with BAC is able to suppress TGF-β1 production by macrophages through the AKT/STAT6 signaling pathway.

Another central role of TGF-β1 is the stimulation of fibroblast proliferation and migration and promotion of the uncontrolled differentiation of fibroblasts into myofibroblasts, resulting in excessive ECM deposition, including that of α-SMA, fibronectin and collagen I [43,44]. To examine whether BAC affects fibroblast differentiation, primary lung fibroblasts were stimulated with TGF-β1 with and without treatment with BAC. The results demonstrate that BAC treatment was able to substantially suppress TGF-β1-induced α-SMA protein expression, in addition to mRNA levels of α-SMA and fibronectin, two important markers of fibroblast differentiation. The mechanism of reduced fibroblast differentiation caused by BAC was found to be mediated through Smad2/3 phosphorylation, which has been widely identified as a pivotal mechanism in the development of pulmonary fibrosis [45,46]. These findings suggest that the suppression of TGF-β1-induced fibroblast differentiation by treatment with BAC through the Smad2/3 pathway was also responsible for its protective role against BLM-induced pulmonary fibrosis. To further confirm that BAC exerted anti-fibrosis activity through TGF-β1, BAC-treated mice were administered rTGF-β1 by i.t. injection. Results demonstrated that rTGF-β1 significantly exacerbated the fibrogenesis in lung tissue which had been decreased by treatment with BAC (Fig. 7). Collectively, these data strongly demonstrate that BAC performs an effective role in reducing BLM-induced fibrosis not only through suppression of TGF-β1 expression but also by preventing the process of TGF-β1-induced fibroblast differentiation.

Idiopathic pulmonary fibrosis (IPF) is a severe public health problem worldwide that seriously threatens the health of humans [47]. Due to its rapid progress, poor prognosis, high morbidity and mortality, IPF has been termed a refractory lung disease [48]. The effectiveness of drugs used in the treatment of IPF is still obscure and is always accompanied by severe side-effects [49,50]. This study is the first to demonstrate that BAC, the precursor of the semisynthesis of paclitaxel, could substantially ameliorate BLM-induced pulmonary fibrosis by targeting TGF-β1, the major contributor to pulmonary fibrosis. Because BAC is characterized by much reduced toxicity *in vitro* and *in vivo*, it is an attractive candidate for the treatment of pulmonary fibrosis.

Author contributions

Yun-juan Nie designed the experiments; Yun-juan Nie and Dan Zhang performed the experiments and analyzed the data; Feng Qian and Yaxian Wu prepared the manuscript.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

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