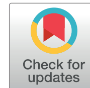

BIOMOLECULES & THERAPEUTICS

Original Article
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Deficiency of Sphingosine-1-Phosphate Receptor 2 ($S1P_2$) Attenuates Bleomycin-Induced Pulmonary Fibrosis

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Abstract

Sphingosine 1-phosphate (S1P) levels are often found to be elevated in serum, bronchoalveolar lavage, and lung tissue of idiopathic pulmonary fibrosis patients and experimental mouse models. Although the roles of sphingosine kinase 1 and S1P receptors have been implicated in fibrosis, the underlying mechanism of fibrosis via Sphingosine 1-phosphate receptor 2 ($S1P_2$) has not been fully investigated. Therefore, in this study, the roles of $S1P_2$ in lung inflammation and fibrosis was investigated by means of a bleomycin-induced lung fibrosis model and lung epithelial cells. Bleomycin was found to induce lung inflammation on day 7 and fibrosis on day 28 of treatment. On the 7th day after bleomycin administration, $S1P_2$ deficient mice exhibited significantly less pulmonary inflammation, including cell infiltration and pro-inflammatory cytokine induction, than the wild type mice. On the 28th day after bleomycin treatment, severe inflammation and fibrosis were observed in lung tissues from wild type mice, while lung tissues from $S1P_2$ deficient mice showed less inflammation and fibrosis. Increase in TGF-β1-induced extracellular matrix accumulation and epithelial-mesenchymal transition were inhibited by JTE-013, a $S1P_2$ antagonist, in A549 lung epithelial cells. Taken together, pro-inflammatory and pro-fibrotic functions of $S1P_2$ were elucidated using a bleomycin-induced fibrosis model. Notably, $S1P_2$ was found to mediate epithelial-mesenchymal transition in fibrotic responses. Therefore, the results of this study indicate that $S1P_2$ could be a promising therapeutic target for the treatment of pulmonary fibrosis.

Key Words: Fibrosis, Inflammation, Lung, Sphingosine 1-phosphate, $S1P_2$

INTRODUCTION

Pulmonary fibrosis is a lung disease that occurs when lung tissue is damaged and scarred, and presents a pathological feature of most chronic inflammatory diseases. Thickening stiffness of the tissue hampers proper functioning of the lungs; the stiffened lung tissue makes breathing difficult and reduces oxygen supply to the blood. If highly progressive, the fibrotic process eventually leads to organ malfunction and death (Wynn and Ramalingam, 2012). Scarring associated with pulmonary fibrosis can be caused by a multitude of factors; autoimmune diseases, chronic inflammation, environmental pollutants, infections, and radiation exposure all contribute towards pulmonary fibrosis. However, in most cases, doctors are unable to identify the exact causative factors responsible for the development of pulmonary fibrosis. In such cases, the condition is termed idiopathic pulmonary fibrosis (IPF) (Bourke, 2006). Often, lung damage caused by pulmonary fibrosis is not completely curable. However, medication and therapies help to ease symptoms and improve the quality of life; lung transplant might prove to be beneficial in some patients.

Numerous dysregulated mechanisms can promote fibrosis. At the cellular level, a seminal step involves the accumulation/activation of fibroblasts and myofibroblasts in the lung, with the subsequent release of pro-fibrotic factors and extracellular matrix proteins, including proteoglycans and collagens (Leask and Abraham, 2004). This excessive lung scarring compromises the structural integrity of alveoli, increases lung stiffness, decreases lung volumes, and ultimately leads to severe impairment of gas exchange (Berend, 2014). At the molecular level, elevated levels of growth factors, including transforming growth factor (TGF)-β and connective tissue growth factor (CTGF) are considered major, sometimes sufficient, inducers of fibrosis (Sonnylal *et al*., 2010). However, the mechanisms regulating lung fibrosis are not completely understood and it is difficult to predict whether new experimental interventions will alleviate or exacerbate fibrosis.

Epithelial-mesenchymal transition (EMT) has become

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Table 1. Primer sequences

Gene	Primer sequence (5'→3')	Annealing temperature (°C)	Cycles
mouse α-SMA	F : CTGACAGAGGCACCACTGAA R : GAAGGAATAGCCACGCTCAG	60	25
mouse Collα1	F : CACCCTCAAGAGCCTGAGTC R : GTTCGGGCTGATGTACCAGT	60	25
mouse Collα2	F : TGGCCCATCTGGTAAAGAAG R : ACCTTTGCCACCTTGAACAC	60	25
mouse CollIIα1	F : GTCCACGAGGTGACAAAGGT R : GTGCCCACTTGTTGGATCT	60	25
mouse fibronectin	F : ACCACCCAGAACTACGATGC R : ACGTGTCGTTCACATTG	60	25
mouse GAPDH	F : TTCACCACCATGGAGAAGGC R : GGCATGGACTGTGGTCATGA	60	27
mouse IL-1β	F : GGAGAAGCTGTGGCAGCTA R : GCTGATGTACCAGTTGGGGA	57	22
mouse IL-6	F : CCGGAGAGGAGACTTCACAG R : TGGTCTTGGTCCTTAGCCAC	57	24
mouse MCP-1	F : AGGTCCCTGTCATGCTTCTG R : TCTGGACCCATTCCTTCTTG	55	30
mouse N-cadherin	F : GGGACAGGAACACTGCAAAT R : CGGTTGATGGTCCAGTTTCT	60	25
mouse TGF-β	F : GCCCTGGATACCAACTATTGC R : AGCTGCACTTGCAGGAGCG	55	30
mouse TNFα	F : GACCCTCACACTCAGATCAT R : TTGAAGAGAACCTGGGAGTA	57	30
human Collα1	F : AGACTTTGGTGTGGGTCAGG R : CAGGACCAGGAGAAGAGTGC	60	25
human E-cadherin	F : GGTTCAAGCTGCTGACCTTC R : AGCCAGTTGGCAGTGTCTCT	60	30
human fibronectin	F : CCAACCTACGGATGACTCGT R : TGGCACCGAGATATTCCTTC	60	25
human GAPDH	F : GAGTCAACGGATTTGGTCGT R : TTGATTTTGGAGGGATCTCG	52	27
human N-cadherin	F : GGACAGTTCCTGAGGGATCA R : GGATTGCCTTCCATGTCTGT	60	25
human ZEB1	F : GAGAAGCGGAAGAACGTGAC R : GCTTGACTTTCAGCCCTGTC	60	30

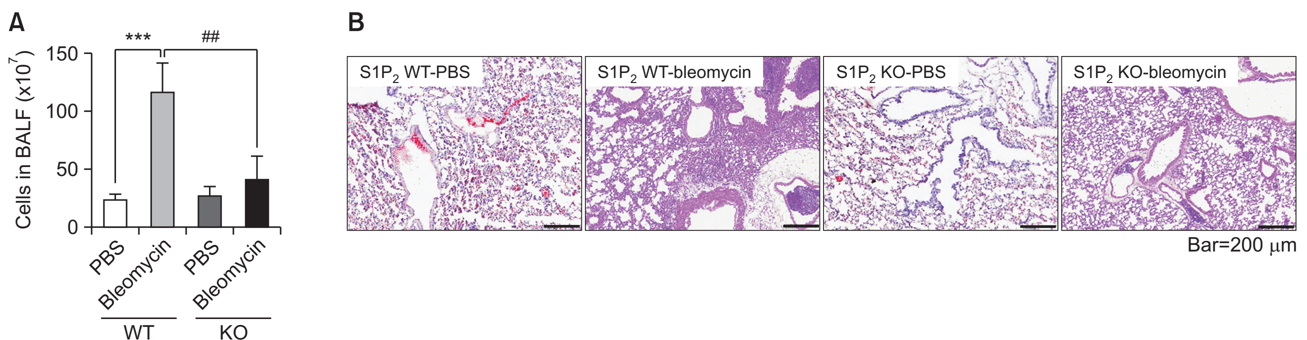

Fig. 1. Effect of $S1P_2$ deficiency on bleomycin-induced lung inflammation. Bleomycin or saline was administered intratracheally to $S1P_2$ WT and KO mice. Seven days after bleomycin instillation, cells in BALF were counted by flow cytometry. (A) Total cell counts in the BALF of PBS-treated $S1P_2$ WT mice ($S1P_2$ WT-PBS), bleomycin-treated $S1P_2$ WT mice ($S1P_2$ WT-bleomycin), PBS-treated $S1P_2$ KO mice ($S1P_2$ KO-PBS), and bleomycin-treated $S1P_2$ KO mice ($S1P_2$ KO-bleomycin). (B) Histological analysis of lung tissues from each group was performed by H&E staining. On the 7th day after treatment, lung tissues of bleomycin-treated WT mice showed lung inflammation, but lung tissues from KO mice exhibited a lower inflammatory response compared to the WT mice. Results are presented as mean ± SE (n=6). Statistical significance: ***p<0.001 vs. PBS-treated $S1P_2$ WT mice and $^{\#\#}p$<0.01 vs. bleomycin-treated $S1P_2$ WT mice.

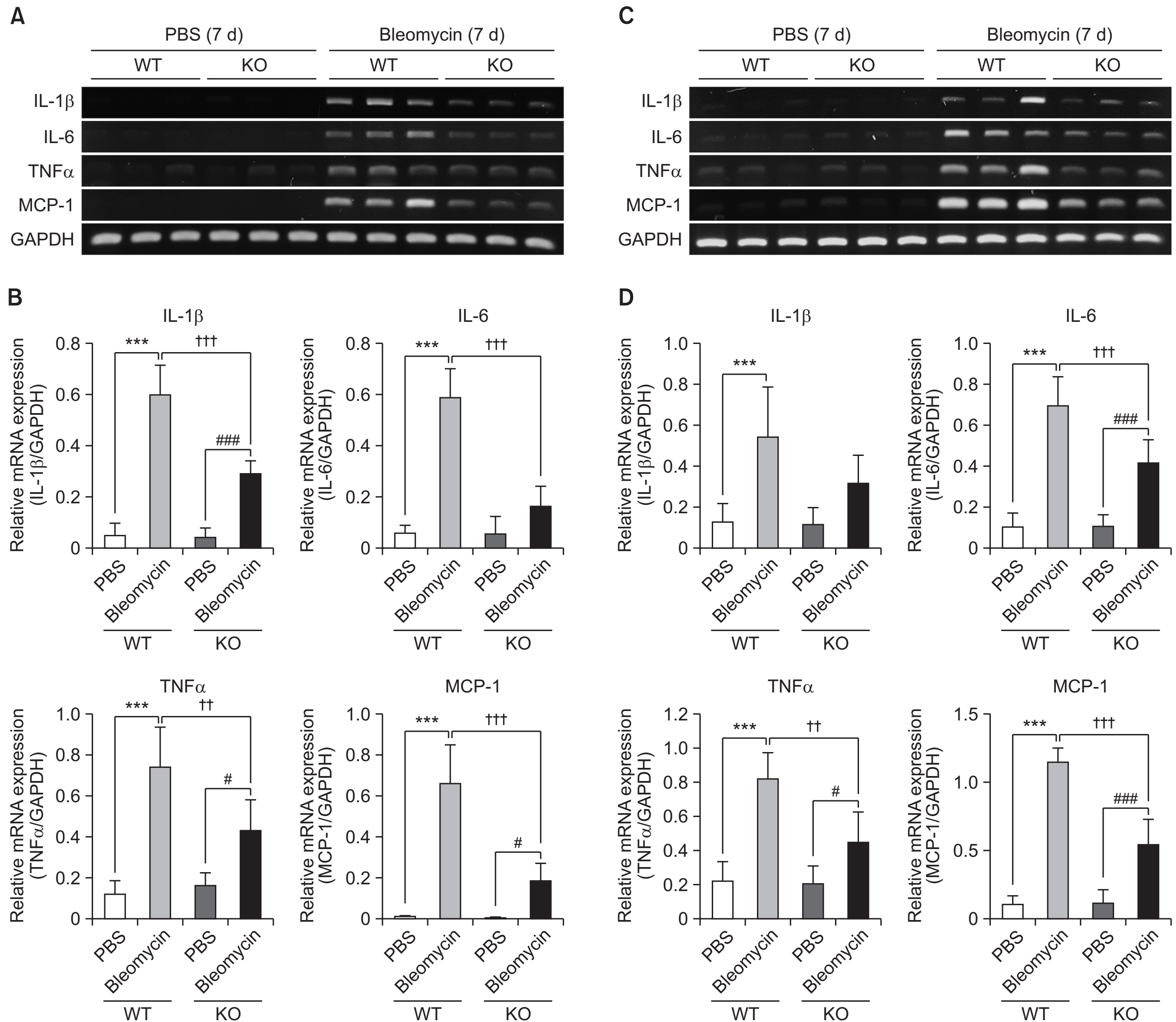

Fig. 2. Suppressive effect of $S1P_2$ deficiency on mRNA expression of pro-inflammatory cytokines in BALF or lung tissues. (A, B) RT-PCR analysis of pro-inflammatory cytokines (IL-1β, IL-6, TNFα, and MCP-1) was performed using mRNA in BALF-associated cells on the 7th day after bleomycin treatment. (C, D) mRNA expression of pro-inflammatory cytokines was checked by RT-PCR using mRNAs isolated from lung tissues. mRNA levels were expressed as ratios with GAPDH mRNA levels. The values shown are mean ± SE (n=6). Statistical significance: $^{***}p$<0.001 vs. PBS-treated $S1P_2$ WT mice, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 vs. PBS-treated $S1P_2$ KO mice, and $^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001 vs. bleomycin-treated $S1P_2$ WT mice.

widely accepted as a mechanism by which injured epithelial cells transform into mesenchymal cells that contribute to the development of fibrosis (Kage and Borok, 2012). EMT is one of several proposed mechanisms through which fibroblasts and myofibroblasts are generated (Kalluri and Neilson, 2003). There are two main observations that support the contribution of EMT to pulmonary fibrosis: results of lineage tracing studies in mouse models and co-expression of epithelial and mesenchymal markers in human lung samples with IPF (Willis *et al*., 2005; Willis and Borok, 2007; Masszi *et al*., 2010). Advances have been made in elucidating the causes and mechanisms of EMT, potentially leading to new treatment options, although contribution of EMT to lung fibrosis *in vivo* remains controversial.

Sphingosine 1-phosphate (S1P) is a specific ligand for five G protein-coupled receptors, $S1P_{1\text{-}5}$ (Park and Im, 2017). S1P and S1P signaling may contribute to the development and progression of IPF and pulmonary fibrosis in animal models (Dhami *et al*., 2010). S1P levels were found to be elevated in bronchoalveolar lavage fluid (BALF) of IPF patients compared to controls, and correlated with poor lung prognosis in IPF patients (Milara *et al*., 2012). Elevated S1P levels in a murine model of bleomycin-induced pulmonary fibrosis resulted from enhanced sphingosine kinase 1 (Dhami *et al*., 2010). In IPF patients, increased sphingosine kinase 1 levels contributed to EMT through $S1P_2$ and $S1P_3$ in alveolar type II cells (Milara *et al*., 2012). Buildup of extracellular matrix proteins (ECM), a characteristic feature of fibrosis, has also been attributed to

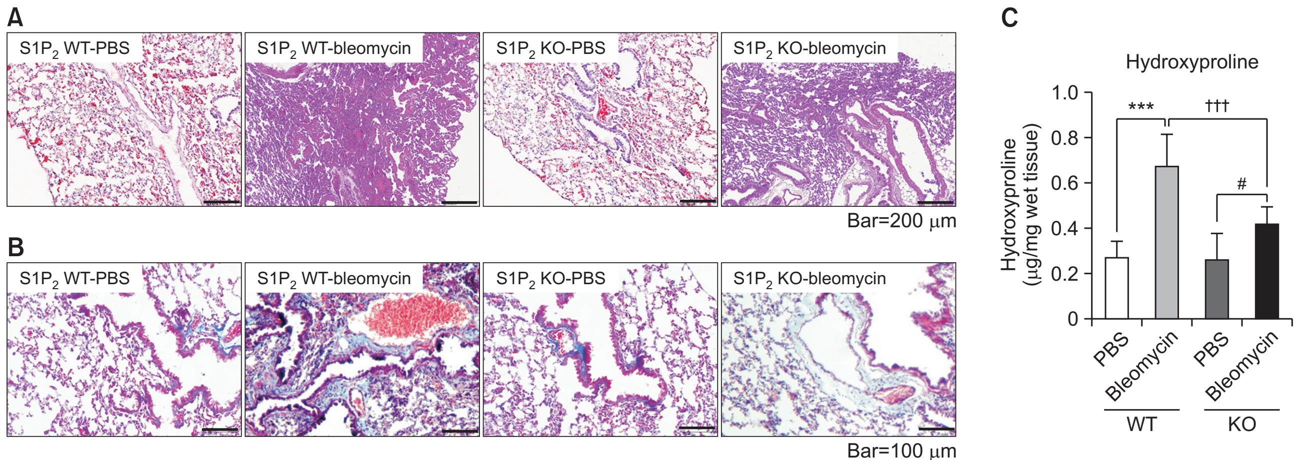

Fig. 3. Inhibitory effect of $S1P_2$ deficiency on fibrosis in lung. Bleomycin or saline was administered intratracheally to $S1P_2$ WT and KO mice. Twenty-eight days after bleomycin instillation, histological analysis in lungs was performed by H&E staining (A) and Masson's trichrome staining (B). The amounts of hydroxyproline in lung tissues from PBS- or bleomycin-treated mice were analyzed as quantitation of collagen (C). The values shown are mean ± SE (n = 6). Statistical significance: ***p<0.001 vs. PBS-treated $S1P_2$ WT mice, $^{\#}p$<0.05 vs. PBS-treated $S1P_2$ KO mice, and $^{\dagger\dagger\dagger}p$<0.001 vs. bleomycin-treated $S1P_2$ WT mice.

S1P signaling via $S1P_2$ or $S1P_3$ in agonist-based studies on human lung fibroblasts (Sobel *et al*., 2013). Furthermore, $S1P_3$ knockout mice showed decreased inflammation and fibrosis in bleomycin-induced murine model of fibrosis (Murakami *et al*., 2014). However, underlying mechanism of fibrosis via $S1P_2$ has not been fully investigated. Therefore, in this study, we investigated the role of $S1P_2$ in lung inflammation and fibrosis, by using bleomycin-induced lung fibrosis model and lung epithelial cells.

MATERIALS AND METHODS

Materials

JTE-013 was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and recombinant human TGF-β1 was obtained from Peprotech, (London, UK). Bleomycin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Three $S1P_2$ heterozygous mice were kindly provided by Richard Proia at NIH (Kono *et al*., 2007a). They had been backcrossed to Balb/c mice for 8 generations. $S1P_2$ wild-type (WT) and knock out (KO) mice were housed in a Laboratory Animal facility in Pusan National University, and provided unrestricted amounts of food and water. The animal protocol used in this study was reviewed and approved by the Pusan National University–Institutional Animal Care Committee (PNU–IACUC) with respect to procedure ethicality and scientific care (Approval Number PNU-2016-1131).

Induction of lung inflammation and fibrosis in Balb/c mice

Eight-week old Balb/c mice were divided into four groups (n=6), that is, a PBS-treated control $S1P_2$ WT group, a bleomycin-treated $S1P_2$ WT group, a PBS-treated control $S1P_2$ KO group, and a bleomycin-treated $S1P_2$ KO group. Mice were anesthetized with Avertin (tribromoethanol, 200 mg/kg body weight) i.p. injection. Lung inflammation was induced by administration of 50 μl of bleomycin (50 mg/kg body weight) by intratracheal instillation. Control group mice received the same volume of sterile saline. On day 7 after bleomycin administration, mice were sacrificed, and lung and BALF were collected for succeeding experiments. For lung fibrosis, 50 μl of bleomycin (20 mg/kg body weight) was administrated the same way as described above. On day 28 after bleomycin administration, mice were sacrificed and lung tissues were examined.

Histological analysis of the lung and cell counting in BALF for fibrosis

After sacrificing the mice on experimental day 7 or 28, left lung tissues were fixed in 10% formalin, embedded in paraffin and sectioned (4 μm). Sections were stained with hematoxylin and eosin (H&E) or by Masson's trichrome method. For H&E staining, sections were washed in running tap water for 5 min, counterstained with hematoxylin solution for 90 s, washed in running tap water, dehydrated, and coverslipped with Permount. To confirm collagen production by Masson's trichrome staining, sections were deparaffinized, hydrated with water. Sections were stained in Bouin's solution for 1 min using microwave and then allowed to stand for 15 min at room temperature. After washing in running tap water, sections were placed in hematoxylin for 10 min, rinsed in running tap water, and stained in Biebrich scarlet for 5 min. Thereafter, sections were stained in phosphotungstic/phosphomolybdic acid for 15 min, transferred directly into aniline blue for 5 min, dehydrated and coverslipped. Flow cytometry was used to obtain total cell counts in BALF samples.

Hydroxyproline assay

Hydroxyproline content of the lungs was determined as follows, according to the manufacturer's instruction (BioVision, Milpitas, CA, USA): in brief, lung tissue homogenates in H_2O were hydrolyzed with concentrated HCl at 120°C for 3 h and the supernatants were collected after centrifuging the hydrolyzed homogenates at 10,000×g for 3 minutes. Followed by a

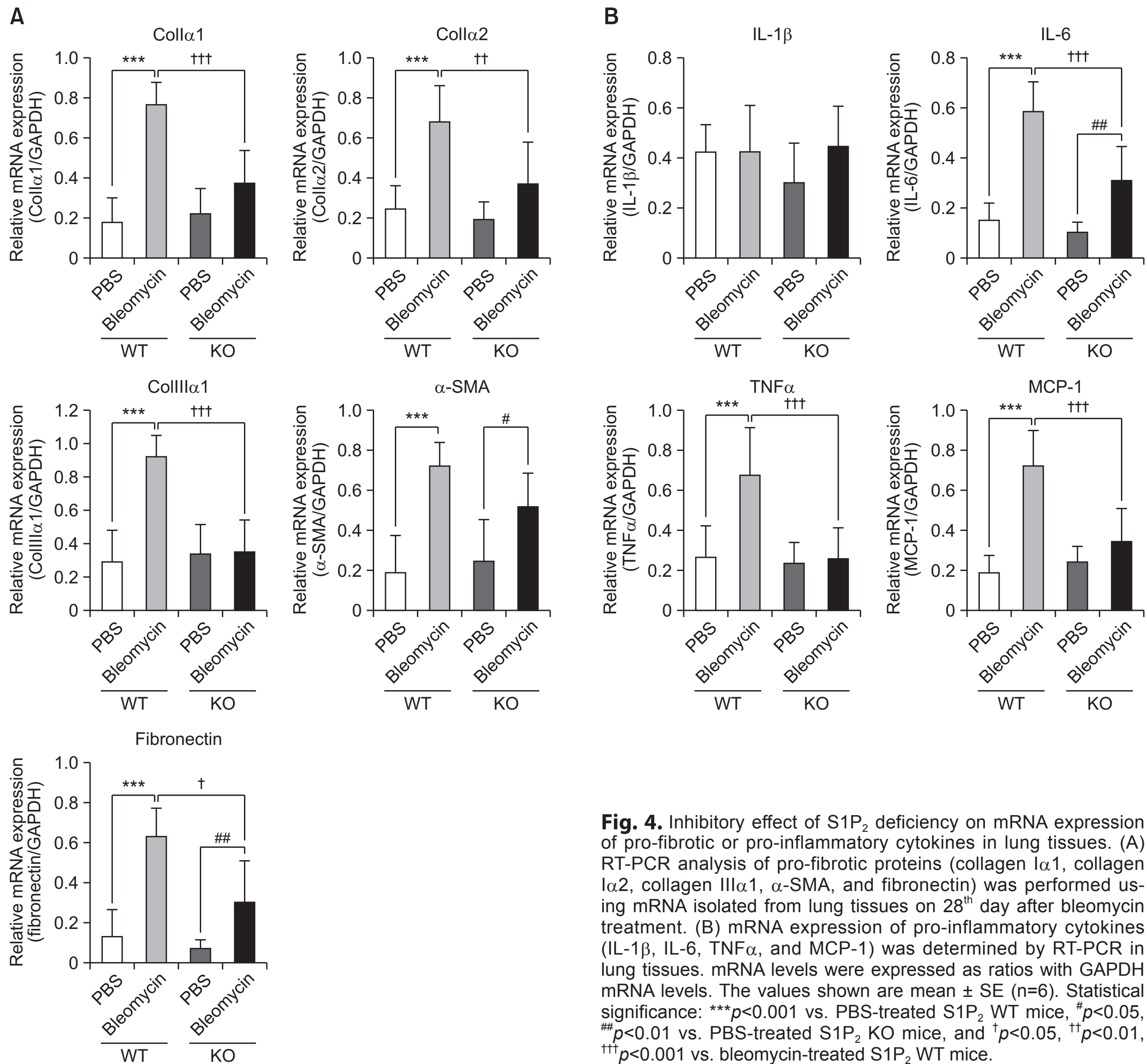

Fig. 4. Inhibitory effect of $S1P_2$ deficiency on mRNA expression of pro-fibrotic or pro-inflammatory cytokines in lung tissues. (A) RT-PCR analysis of pro-fibrotic proteins (collagen Iα1, collagen Iα2, collagen IIIα1, α-SMA, and fibronectin) was performed using mRNA isolated from lung tissues on 28th day after bleomycin treatment. (B) mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6, TNFα, and MCP-1) was determined by RT-PCR in lung tissues. mRNA levels were expressed as ratios with GAPDH mRNA levels. The values shown are mean ± SE (n=6). Statistical significance: $^{***}p<0.001$ vs. PBS-treated $S1P_2$ WT mice, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. PBS-treated $S1P_2$ KO mice, and $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs. bleomycin-treated $S1P_2$ WT mice.

drying step, 10 μl of the supernatants were loaded onto a 96-well plate, and the samples were assayed for hydroxyproline at 550 nm. The data were expressed as μg of hydroxyproline/mg wet tissue.

Western blot

Lung tissues were lysed in RIPA lysis buffer with protease inhibitors. Protein concentration was determined using a BCA protein assay. Proteins (30 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose paper. After transfer, the nitrocellulose membrane was incubated with specific primary antibodies, recognizing β-actin or α-smooth muscle actin (α-SMA), followed by incubation with an HRP-conjugated secondary antibody. Signals were developed using an enhanced chemiluminescence system.

Reverse transcriptase-PCR

To assess the expression of fibrotic markers by reverse transcription-PCR (RT-PCR), first strand cDNA was synthesized from total RNA, isolated from BALF-associatedcells and lung tissues, using Trizol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). Synthesized cDNA products, primers for each gene, and Promega Go-Taq DNA polymerase (USA) were used for PCR. Specific primers were summarized in Table 1. PCR was performed over 27-33 amplification cycles of denaturation at 95oC for 30 s, annealing at 49-57°C for 30 s, and elongation at 72°C for 30 s in an Eppendorf Mastercycler PCR machine (Hamburg, Germany). Aliquots (7 μl) were electrophoresed through 1.2% agarose gels and stained with StaySafe™ Nucleic Acid Gel Stain (Real Biotech Corporation, Banqiao, Taiwan).

Statistics

Results are expressed as mean ± standard error (SE) of the indicated number of individual values. Statistical significance of differences were determined by analysis of variance (ANOVA) with Turkey's post hoc test, and statistical significance was accepted for *p*-values <0.05. Analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Effect of $S1P_2$ deficiency on bleomycin-induced lung inflammation

Bleomycin is an effective chemotherapy drug, used to treat cancer, although it may cause inflammation of the lung that can result in lung scarring (Dhami *et al*., 2010). Based on this observation, bleomycin has been used to induce pulmonary fibrosis in mice. The bleomycin-induced fibrosis model indicates significant characteristics of human IPF, including an increased inflammatory response, accompanied by epithelial cell injury, basement membrane damage, and interstitial and intra-alveolar fibrosis. By utilizing the bleomycin-mediated fibrosis model, we investigated whether $S1P_2$ mediates pro-inflammatory and pro-fibrotic responses.

On the 7th day after bleomycin instillation, the number of inflammatory cells in BALF of $S1P_2$ WT mice was found to be higher compared to PBS-treated mice. However, $S1P_2$ deficiency attenuated bleomycin-induced infiltration of cells in BALF, compared to WT mice (Fig. 1A). In the H&E-stained lung tissues, inflammation was apparent with slight progression of fibrosis in the WT mice, indicating infiltration of inflammatory cells, hyperplasia of the alveolar and bronchiolar epithelium, and interstitial collagen deposition. Lung tissues from $S1P_2$ KO mice were less inflamed than those from $S1P_2$ WT mice (Fig. 1B). In addition, the inflammatory responses were assessed by measurements of pro-inflammatory cytokines in lung tissue and BALF. The mRNA expression of pro-inflammatory cytokines, IL-1β, IL-6, TNFα, and monocyte chemoattractant protein-1 (MCP-1), in immune cells of BALF in bleomycin-treated $S1P_2$ WT mice was higher, as compared to the PBS-instilled $S1P_2$ WT mice (Fig. 2A, 2B). The elevated levels in bleomycin-treated $S1P_2$ WT mice was reduced in bleomycin-instilled $S1P_2$ KO mice (Fig. 2A, 2B). Furthermore, the mRNA levels of IL-1β, IL-6, TNFα, and MCP-1 were increased in lung tissues from the bleomycin-instilled WT mice. Those levels of inflammatory cytokines in lung tissues from the bleomycin-instilled KO mice were lower than WT mice (Fig. 2C, 2D).

Effect of $S1P_2$ deficiency on bleomycin-induced lung fibrosis

On the 28th day after treatment with bleomycin or saline, lung tissues from $S1P_2$ WT and KO mice were stained with H&E or Masson's trichrome staining, to measure histological changes during chronic lung fibrosis. Inflammation and fibrosis were apparent in the lung tissues from WT mice, while tissues from $S1P_2$ deficient mice exhibited less inflammation and fibrosis (Fig. 3A). When fibrosis was examined by Masson's trichrome staining of lung tissues, severe fibrosis was observed in lung tissues from bleomycin-instilled WT mice. However, in the lung of bleomycin-instilled $S1P_2$ KO mice, collagen deposition in fibrotic areas was suppressed (Fig. 3B). In addition, administration of bleomycin was found to increase the hydroxyproline content (a major component of collagen) in lung tissues from $S1P_2$ bleomycin-instilled WT mice, whereas the amount of hydroxyproline in lung tissues from bleomycin-instilled $S1P_2$ KO mice was less than those of $S1P_2$ WT mice (Fig. 3C).

Next, changes in the mRNA levels of pro-inflammatory cytokines as well as pro-fibrotic markers in lung tissues were measured in $S1P_2$ WT and KO mice. The mRNA expression of pro-fibrotic markers, collagen Iα1, collagen Iα2, collagen IIIα1, α-SMA, and fibronectin, in bleomycin-instilled lung tissues were markedly lower in $S1P_2$ deficient mice than in WT mice (Fig. 4A). Furthermore, the mRNA levels of IL-1β, IL-6, TNFα, and MCP-1 were elevated in lungs of bleomycin-treated WT mice, but these bleomycin-induced increases were suppressed in $S1P_2$ KO mice (Fig. 4B). In addition, the protein levels of α-SMA were increased in lung tissues of bleomycin-treated WT mice but not in bleomycin-treated KO mice (Fig. 5A, 5B).

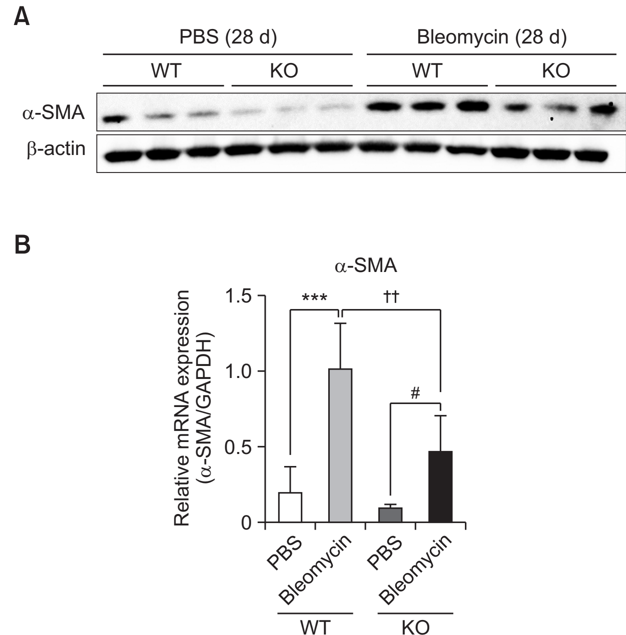

Fig. 5. Inhibitory effect of $S1P_2$ deficiency on level of pro-fibrotic protein in lung tissues. (A) Western blotting results of pro-fibrotic proteins (α-SMA), performed using protein isolated from lung tissues, on the 28th day after bleomycin treatment. (B) Protein levels were expressed as ratios with β-actin levels. The values shown are mean ± SE (n=6). Statistical significance: ****p*<0.001 vs. PBS-treated $S1P_2$ WT mice, $^{\#}p$<0.05 vs. PBS-treated $S1P_2$ KO mice, and $^{\dagger\dagger}p$<0.01 vs. bleomycin-treated $S1P_2$ WT mice.

Effect of JTE-013 on TGF-β1-mediated EMT signaling in A549 cells

Repeated cycles of inflammation and repair initiate tissue damage and may progress to fibrotic changes, resulting in excessive matrix deposition and scar formation in tissues. In this process, TGF-β signaling is quite important and EMT is a critical phenomenon. To confirm the effect of $S1P_2$ on TGF-β1–induced EMT, $S1P_2$ antagonist, JTE-013 was used in A549 human alveolar basal epithelial cells. TGF-β1 (2 ng/ml, for 24

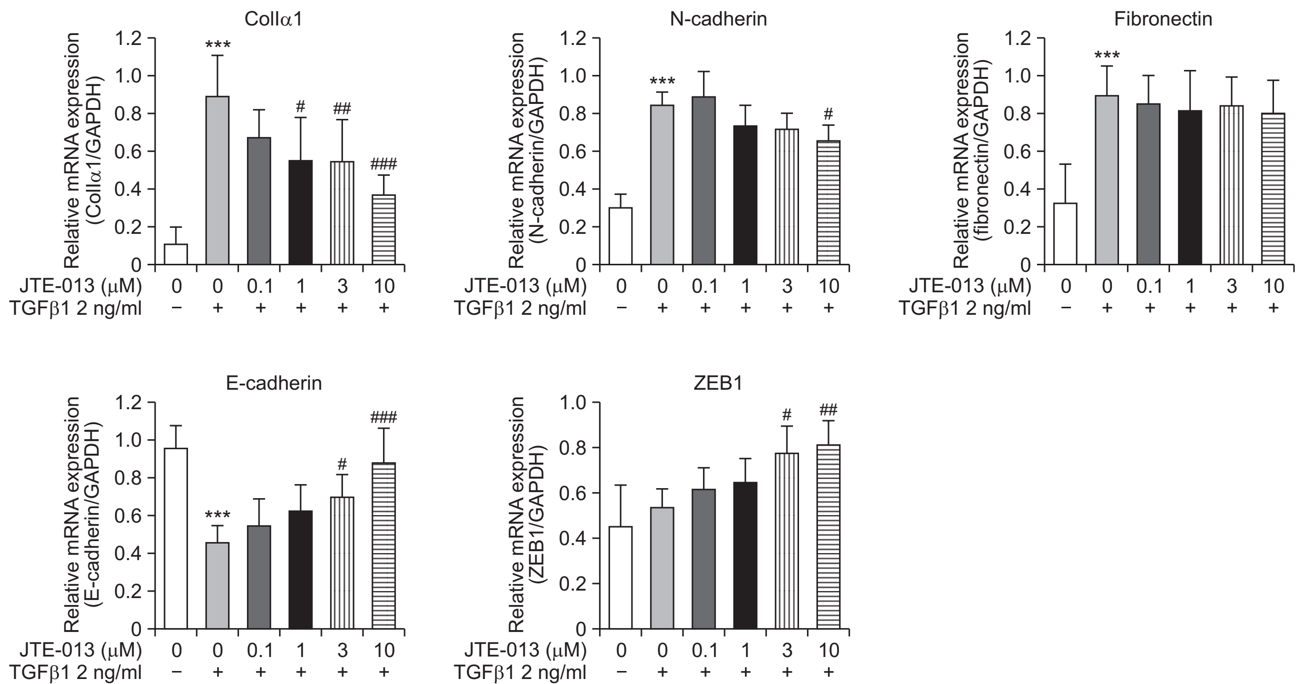

Fig. 6. Effect of JTE-013 on TGF-β1-induced ECM expression and EMT in A549 cells. Expression of ECM and EMT associated proteins (collagen Iα1, N-cadherin, fibronectin, E-cadherin, and ZEB1) confirmed in A549 cells by RT-PCR analysis. A549 cells were cultured for 24 h in media containing 0.5% FBS and then treated with JTE-013 and TGF-β1 for 24 h. mRNA levels were expressed as ratios with GAPDH mRNA levels. The values shown are mean ± SE (n=3). Statistical significance: ***p<0.001 vs. vehicle-treated cells and $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. TGF-β1-treated cells.

h) activated the expression levels of EMT and ECM accumulation markers including collagen Iα1, N-cadherin, and fibronectin, but decreased the mRNA expression levels of epithelial markers such as E-cadherin (Fig. 6). The mRNA expression of zinc finger E-box-binding homeobox 1 (ZEB1), which is an E-cadherin repressor, was not changed by TGF-β1. However, JTE-013 pretreatment attenuated the increased collagen Iα1 and N-cadherin expression (except fibronectin) and elevated the E-cadherin and ZEB1 expression in A549 cells (Fig. 6). Our results indicate that although activation of E-cadherin could be induced via suppression of ZEB1, increased expression of E-cadherin is probably mediated by other inducers or repressors, but not ZEB1. Rather, these results suggest that $S1P_2$ regulates TGF-β1–induced EMT and ECM accumulation, hence contributing to the anti-fibrotic activity *in vivo*.

Bleomycin instillation increased N-cadherin expression and suppressed E-cadherin in lung tissues, but these bleomycin-induced changes were suppressed in $S1P_2$ deficient mice (Fig. 7).

DISCUSSION

S1P and S1P receptors are known to be involved in many different physiological processes, and may contribute to the development and progression of IPF and pulmonary fibrosis in animal models. S1P levels were found to be elevated in the BALF of IPF patients that correlated with poor prognosis (Milara *et al*., 2012). Furthermore, S1P, S1P receptors, and sphingosine kinase 1 were up-regulated in the serum, bronchoalveolar lavage, and lung tissue of experimental mouse models of bleomycin-induced lung fibrosis, human liver fibrosis, and experimental models of cholestasis-induced liver fibrosis (Kono *et al*., 2007b; Li *et al*., 2009; Dhami *et al*., 2010; Li *et al*., 2011; Sobel *et al*., 2013). Long-term treatment with fingolimod (a well-known $S1P_1$ modulator) caused fibrosis of lungs, such as increased lung weight, hypertrophy of smooth muscle, hyperdistension of alveoli, and increased collagen deposition in mice (Shea *et al*., 2010). It has been reported that $S1P_2$ signaling contributes to the pathogenesis of liver fibrosis. S1P inhibited proliferation through $S1P_2$ in hepatocytes of rats, and $S1P_2$ deficiency inhibited accumulation of hepatic stellate cells and ameliorated CCl_4-induced liver fibrosis (Ikeda *et al*., 2009). Some studies also suggest a correlation between $S1P_2$ and lung inflammation and fibrosis, but the role and function of $S1P_2$ in the pathogenesis of lung diseases has been poorly understood.

In the current study, the role of $S1P_2$ was demonstrated using $S1P_2$ deficient mice in bleomycin-induced pulmonary fibrosis model. First, pro-inflammatory and pro-fibrotic functions of $S1P_2$ were determined in the animal model. During the acute and chronic phase of lung injury, analysis of lung tissue and BALF showed that $S1P_2$ KO mice showed decreased inflammation and reduction in the number of total cells, compared to WT mice. These findings suggest that inactivation of $S1P_2$ may prevent bleomycin-induced acute inflammation and chronic fibrosis, by inhibiting inflammatory and fibrotic signaling. Second, $S1P_2$ regulates TGF-β1-induced EMT and ECM accumulation in lung epithelial cells, indicating that $S1P_2$–mediated blockade of ECM production and EMT (which are possible causes of IPF) could be a strategy to treat IPF.

IPF, the most common interstitial fibrotic pulmonary disease

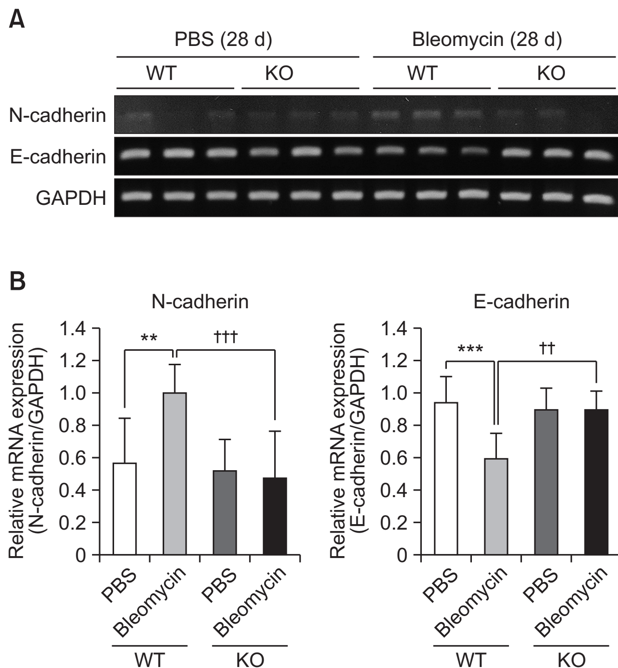

Fig. 7. Inhibitory effect of $S1P_2$ deficiency on EMT in lung tissues. (A) RT-PCR analysis of N-cadherin and E-cadherin was performed using mRNA isolated from lung tissues on 28th after bleomycin treatment. (B) mRNA levels were expressed as ratios with GAPDH mRNA levels. The values shown are mean ± SE (n=6). Statistical significance: $^{**}p$<0.01, $^{***}p$<0.001 vs. PBS-treated $S1P_2$ WT mice and $^{††}p$<0.01, $^{†††}p$<0.001 vs. bleomycin-treated $S1P_2$ WT mice.

is a chronic and progressive disease, caused by proliferation of fibroblasts and deposition of collagen and ECM proteins, resulting in failure of lung function. However, there is no effective treatment for preventing and curing the development of fibrosis in IPF. Several drugs for IPF have been tested, but currently, lung transplantation is the best available treatment option for improving the survival of IPF patients. There are several key fibrogenic pathways that can be exploited for potential therapeutic approaches, including myofibroblasts and the TGF-β signaling, pro-inflammatory pathways, and profibrotic type 2 immune response. A more multipronged and integrated anti-fibrotic strategy will probably emerge as the most successful way to treat the complex pathogenesis of IPF. In this study, we demonstrate that the blockade of $S1P_2$ attenuates TGF-β signaling and pro-inflammatory pathways triggered by bleomycin, suggesting that the inactivation of $S1P_2$ could be a promising therapeutic target for pulmonary fibrosis disorders, such as IPF.

Recently, the bleomycin-induced lung fibrosis mouse model has been applied for studying the S1P receptor deficient mice. Knock-out mice of $S1P_3$ attenuated inflammation and fibrosis via connective tissue growth factor expression (Murakami *et al*., 2014). Zhao *et al*. (2018) reported that $S1P_2$ facilitated pulmonary fibrosis through potentiating IL-13 pathway in macrophages. In their study, bleomycin was administrated repeatedly by intraperitoneal injection into C57BL/6J mice, in contrast to the direct intratracheal instillation of bleomycin into Balb/c mice performed in our study (Zhao *et al*., 2018). In the lung tissues from $S1P_2$ deficient mice, Zhao *et al*. (2018) noted reduced fibroblast accumulation and attenuation of fibronectin and collagen1α1 mRNA, supporting our observation. They suggested $S1P_2$ facilitated lung fibrosis through macrophages and IL-13 signaling (Zhao *et al*., 2018). Using *S1pr2*$^{LacZ/+}$ mice, they found $S1P_2$ was expressed in alveolar macrophages, vascular endothelial cells, and alveolar epithelial cells in the lung (Zhao *et al*., 2018). In their model, $S1P_2$ was found to be involved in the production of IL-13 and STAT-6 phosphorylation response in macrophages, upon bleomycin intraperitoneal administration. In our model, $S1P_2$ was found to play an important role in early inflammatory responses and late fibrotic ECM accumulation and EMT transition in lung epithelial cells, upon bleomycin intratracheal administration.

In summary, this study identifies $S1P_2$ as a molecular target in lung inflammation and fibrosis. The inhibition of $S1P_2$ attenuates fibrotic signaling and pro-inflammatory pathways triggered by bleomycin. Therefore, this study suggests that the inhibition of $S1P_2$ could be a therapeutic target for pulmonary fibrosis such as IPF.

CONFLICT OF INTEREST

Authors declare there is no conflict of interest.

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