Th17 cells and IL-17 promote the skin and lung inflammation and fibrosis process in a bleomycin-induced murine model of systemic sclerosis

L. Lei[1], C. Zhao[1], F. Qin[1], Z.-Y. He[2], X. Wang[1], X.-N. Zhong[2]

[1]*Department of Rheumatology and Clinical Immunology;*
[2]*Department of Respiratory Medicine, the First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Province, China.*

Ling Lei, MD, PhD
Cheng Zhao, MS
Fang Qin, MS
Zhi-Yi He, MD, PhD
Xu Wang, BS
Xiao-Ning Zhong, MD, PhD

Please address correspondence to:
Dr Xiao-Ning Zhong,
Department of Respiratory Medicine, the First Affiliated Hospital of Guangxi Medical University,
530021 Nanning,
Guangxi Province, China.
E-mail: xnzhong101@sina.com

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ABSTRACT

Objective. *Systemic sclerosis (SSc) is characterised by fibrosis of the skin and internal organs, such as the lungs. Enhanced Th17 responses are associated with skin fibrosis in patients with SSc, however, whether they are associated with lung fibrosis has not been clarified. This study aimed to investigate the potential association of Th17 responses with the skin and pulmonary fibrosis as well as the potential mechanisms in a mouse bleomycin (BLM) model of SSc.*

Methods. *BALB/c mice were injected subcutaneously with phosphate buffered saline (PBS) (control) or BLM for 28 days and the skin and pulmonary inflammation and fibrosis were characterised by histology. The percentages of circulating, skin and pulmonary infiltrating Th17 cells and the contents of collagen in mice were analysed. The levels of RORγt, IL-17A, IL-6 and TGF-β1 mRNA transcripts in the skin and lungs were determined by quantitative RT-PCR and the levels of serum IL-17A, IL-6 and TGF-β1 were determined by ELISA. Furthermore, the effect of rIL-17A on the proliferation of pulmonary fibroblasts and their cytokine expression was analysed. The potential association of Th17 responses with the severity of skin and lung fibrosis was analysed.*

Results. *In comparison with the control mice, significantly increased skin and pulmonary inflammation and fibrosis and higher levels of hydroxyproline were detected in the BLM mice. Significantly higher frequency of circulating, skin and lung infiltrating Th17 cells and higher levels of serum, skin and lung IL-17A, TGF-β1, IL-6 and RORγt were detected in the BLM mice. The concentrations of serum IL-17A were correlated positively with the percentages of Th17 cells and the contents of skin hydroxyproline in the BLM mice. The levels of IL-17A expression were positively correlated with the skin and lung inflammatory scores as well as the skin fibrosis in the BLM mice. In addition, IL-17A significantly enhanced pulmonary fibroblast proliferation and their type I collagen, TGF-β and IL-6 expression in vitro, which were attenuated by treatment with anti-IL-17A.*

Conclusion. *Our results indicate that Th17 cells participate in the pathogenesis of skin and lung fibrosis by enhancing fibroblast proliferation and cytokine production in a mouse BLM model of SSc.*

Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterised by excessive extracellular matrix (ECM) deposition in the skin, lung, and other visceral organs. Although the pathogenesis of SSc remains unclear, pro-inflammatory autoimmunity and high levels of pro-inflammatory cytokines have contributed to microvascular damage, inflammation and fibrosis (1). However, the details of inflammatory factors and their roles in the pathogenesis of SSc have not been clarified. Further understanding of their function may open new avenues to realise the pathogenesis of SSc.

Th17 cells play a critical role in the induction of autoimmune tissue injury (2). Naïve $CD4^+$ T cells can be activated and differentiate into Th17 cells in the presence of IL-6 and TGF-β1. Th17 cell development is dependent on transcription factor of RORγt and is positively regulated by IL-22 and IL-23. Functionally, Th17 cells produce IL-17A and other cytokines, and promote inflammation, contributing to the development and progression of autoimmune diseases (3). However, the role of Th17 cells and their pro-inflammatory cytokines in the development and progression of SSc remains controversial. Previous stud-

ies have shown high frequency of Th17 cells and high levels of serum IL-17A in patients with SSc (4-6). Our previous studies and those of others have indicated that Th17 cells contribute to the development of skin fibrosis and the pathogenesis of SSc through producing IL-17A in an animal model of SSc (7-9). In contrast, another study shows that IL-17A has a direct negative-regulatory role in the development of dermal fibrosis in humans (10). Particularly, whether Th17 cells and IL-17A regulate the pulmonary fibrosis of SSc has not been clarified.

In this study, we used a mouse bleomycin (BLM) model of SSc to examine the potential association of Th17 responses with the skin and lung fibrosis. Furthermore, we investigated the effect of IL-17A on the proliferation and cytokine and collagen expression in primarily cultured pulmonary fibroblasts *in vitro*.

Materials and methods

Animals and SSc induction.

Female BALB/c mice (8 weeks old; weight 20–25g) were purchased from the Guangxi Medical University Laboratory Animal Center (Nanning, China), and housed in a specific pathogen-free facility with free access to food and water *ad libitum*. All animal experimental protocols were reviewed and approved by the Laboratory Animal Ethics Committee of Guangxi Medical University.

The mouse model of SSc was induced, as previously described (11, 12). Briefly, individual mice were injected subcutaneously with vehicle PBS (100 μl) or 100 μg BLM (Nippon Kayaku, Tokyo, Japan) daily for 4 weeks (n=12 per group).

Tissue processing

The mice were sacrificed on one day post the last BLM injection and their blood samples were collected. A portion of blood sample was used for preparation of peripheral blood mononuclear cells and another portion of blood sample was used for preparation of serum samples. Their shaved dorsal skin at the injection site and lung samples was collected for histology, hydroxyproline assay, and flow cytometric analysis. In addition, the lung samples from mice in each group were minced for primarily culture of fibroblasts.

Histologic examination of the skin and lung

The dissected skin and lung tissues were fixed and paraffin-embedded. The paraffin tissue sections (4 μm) were stained with haematoxylin and eosin (H&E) and Masson's trichrome (MaT). Dermal thickness from the dermal-epidermal interface to the intradermal fat in individual skin sections was measured using the Photoshop Elements 3.0 software package (Adobe Systems, USA) and data were analysed by image analysis systems of DMR+Q550 (Leica, Germany). The severity of skin and lung inflammation was determined by a semi-quantitative scoring system as previously described (0, none; 1, little; 2, mild; 3, moderate; 4, severe) (11, 13). The severity of pulmonary fibrosis was semi-quantitatively assessed (14). Briefly, the degrees of lung fibrosis in the left middle lobe were evaluated and a total of 5 fields (200 x magnification) selected randomly were graded on a scale of 0 (normal lung) to 8 (total fibrous obliteration of fields). All of the sections were scored independently by 2 investigators in a blinded manner. If the scores were inconsistent both investigators discussed the samples to reach an agreement.

Determination of hydroxyproline contents in the skin and lung tissues

Hydroxyproline is a major component of collagen and the amount of hydroxyproline is an indicator of collagen contents in tissues. Hydroxyproline can be oxidised by 4-(Dimethylamino) benzaldehyde (DMAB) to produce a colorimetric (560 nm) product, which is proportional to the hydroxyproline concentrations. Individual skin and lung tissue samples (20-30 mg each) were homogenised in saline and hydrolysed in 37% HCl at 120°C for 3 hrs. The concentrations of hydroxyproline were determined using the hydroxyproline assay kit, according to the manufacturers' instruction (Sigma). Briefly, the tissue samples were reacted in triplicate with the chloramine T/oxidation buffer mixture for 5 min and reacted with DMAB at 60° C for 90 min, followed by measuring the absorbance at 560 nm. The concentrations of hydroxyproline were determined, according to the standard curve established using different concentrations of hydroxyproline and expressed as mg of hydroxyproline per g of tissues.

Preparation of cell samples for flow cytometry

Peripheral blood mononuclear cells were prepared by Ficoll-Hypaque density-gradient centrifugation using the ficoll-hypaque solution (Pharmacia, Uppsala, Sweden). Furthermore, inflammatory infiltrates in the skin and lung tissues were isolated, as described previously (15, 16). Briefly, some skin and lung tissues were digested with 20 kU/mL DNase I, 175 U/mL type IV collagenase and 1 kU hyaluronidase (Sigma, St. Louis, USA) in RPMI 1640 medium containing 10 mM HEPES (Maixin-Bio, Fuzhou, China) at 37°C for 2 hrs (skin) and 45 min (lung), respectively. The digested skin and lung samples were filtered through nylon meshes (70 μm pore size). The skin and lung cells were subjected to Ficoll-Hypaque density-gradient centrifugation and RBC lysis to prepare the tissue inflammatory infiltrates. Approximately, 1.6-4×10^6 cells were typically obtained from a 1×2 cm^2 piece of skin, and 2-5×10^6 cells were obtained from a half of lung from individual mice.

Flow cytometry

The prepared PBMC, skin and lung infiltrates (10^6/tube) were stimulated with 25 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma) and 1 μg/ml ionomycin (Sigma) at 37° C for 1 h and incubated in the presence of 10 μg/ml brefeldin (BFA; Sigma) for another 4 hrs. The cells were stained with PE-Cy5-anti-CD4, fixed, permeabilised and stained with PE-anti-IL-17A. The percentages of $CD4^+IL\text{-}17A^+$ Th17 cells and $CD4^-IL\text{-}17A^+$ cells were determined by flow cytometry on a BD FACSCalibur flow cytometer, and the data were analysed using CellQuest software (BD sciences, San Jose, USA).

Real-time polymerase chain reaction (RT-PCR)

Total RNA from the prepared PBMC, skin and lung infiltrates was extracted using the Trizol reagent (Invitrogen, Carlsbad, USA) and reversely transcribed

into cDNA using a reverse transcription system with Oligo(dT) (Fermentas, USA). The relative levels of IL-17A, RORγt, TGF-β1, IL-6, type I collagen (COL1A2) mRNA transcripts to control β-actin were determined by quantitative RT-PCR using the SYBR Green qPCR Master Mix (Code DRR081A, Takala, China) and specific primers on an ABI Prism 7500 sequence detector (Applied Biosystems, Warrington, UK). The sequences of primers are shown in Table I. Data were analysed using the Dissociation Curves v. 1.0 software (PE Applied Biosystems), according to double standard curve method (17). In addition, the primarily cultured pulmonary fibroblasts were stimulated with, or without, 25 ng/ml rIL-17A in the presence or absence of 2 μg/ml neutralising anti-mouse IL-17A mAb or control IgG (R&D Systems, Minneapolis, USA) for 48 hrs and the relative levels of COL1A2 and TGF-β1 mRNA transcripts to control β-actin were also determined by quantitative RT-PCR.

Fibroblast culture and with IL-17A stimulation

To isolate lung fibroblasts, the lung tissues from the BLM and control groups of mice were cut into small pieces, and cultured in DMEM medium (Invitrogen) containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37ºC in a 5% CO_2 humidified atmosphere for 1-2 weeks. The growing fibroblasts were trypsinised and passaged. The cells were used for immunohistochemical analysis of vimentin expression. The primarily cultured pulmonary fibroblasts ($1.2x10^4$/well) were cultured in 96-well plates overnight, and stimulated in triplicate with, or without, 25 ng/ml (an optimal concentration) of murine rIL-17A (R&D Systems, Minneapolis, MN) for 24 or 48 hrs in the presence or absence of 2 μg/ml of neutralising anti-mouse IL-17A mAb or control IgG. The concentration of IL-17A (25 ng/mL) was based on preliminary optimisation and the cell proliferation was determined by the BrdU-based ELISA assay using a colorimetric BrdU cell proliferation ELISA kit, according to the manufacturers' instruction (Roche, Applied Science, Indianapolis, USA). Briefly, after BrdU incorporation for 24 hrs, the cells were fixed and their DNA was denatured using FixNedat. The incorporated BrdU was detected by anti-BrdU and the immunocomplex was detected with HRP-conjugated secondary antibodies, followed by substrate reaction and detection in an ELISA plate reader.

Measurement of cytokines

The concentrations of IL-6 and TGF-β1 in the supernatants of cultured pulmonary fibroblasts (48 hrs) were measured by ELISA using specific kits (R&D Systems) according to the manufacturer's protocols. In addition, the concentrations of serum IL-17A, IL-6 and TGF-β1 in individual mice were measured by ELISA.

Statistical analysis

Data are expressed as the mean ± SD. The difference among groups was analysed by independent-samples *t*-test, repeated ANOVA, and Spearman or Pearson correlation using SPSS statistical software v. 16 (SPSS, Chicago, USA). A *p*-value of <0.05 was considered statistically significant.

Results

The skin and lung pathological manifestations

To induce the skin and lung fibrosis, BALB/c mice were randomised and injected subcutaneously with control PBS or BLM daily for 28 days and their skin (at injection site) and lung tissue sections were stained with H&E and Masson's trichrome. As shown in Figure 1A, the structural integrity of skin and lung tissues and no apparent inflammatory infiltrate and fibrosis were observed in the control mice. In contrast, a significant increase in dermal thickness, collagen deposition (which were stained blue, suggesting fibrosis), and many inflammatory infiltrates displayed in the skin and lung tissue sections of the BLM group of mice (Fig. 1A). Furthermore, the lung structure in the BLM group of mice displayed obvious destruction. Quantitative analysis revealed that the dermal thickness, skin and lung inflammatory scores and lung fibrosis scores in the BLM group of mice were significantly greater than that in the control group of mice

Table I. The sequences of primers and reaction conditions of RT-PCR.

Detection index	Forward primer	Reverse primer	Base pair (bp)	Reaction conditions
β-Actin	5′-ATCCACGAAACTACCTTCAA-3′	5′-CCAAATTGTATTGCAGATGTTCCAC-3′	200	94°C, 3min; (94°C, 20s;57.5°C, 30s)×40 cycles; 72°C, 31s
RORγt	5′-ACGGCCCTGGTTCTCATCA-3′	5′-CCAAATTGTATTGCAGATGTTCCAC-3′	79	94°C, 3min; (94°C, 15s; 58°C, 30s)×40 cycles; 72°C, 31s
IL-17A	5′-GGAAAGCTGGACCACCACA-3′	5′-CACACCCACCAGCATCTTCTC-3′	117	95°C, 15s; (95°C, 5s; 60°C, 34s)×40 cycles
TGF-β	5′-GTGTGGAGCAACATGTGGAACTCTA-3′	5/-TTGGTTCAGCCACTGCCGTA-3	143	95°C,15s, (95°CC, 5s, 62°C, 34s), 40 cycles
IL-6	5/-CCACTTCACAAGTCGGAGGCTTA -3/	5/-GCAAGTGCATCATCGTTGTTCATAC -3/	111	95 ˚C, 15s, (95°C, 5s, 62°C, 34s), 40 cycles
COL1A2	5′-CAGGGTATTGCTGGACAACGTG-3′	5/-GGACCTTGTTTGCCAGGTTCA-3/	107	95°C, 15s, (95°C, 5s, 56°C, 34s), 40 cycles

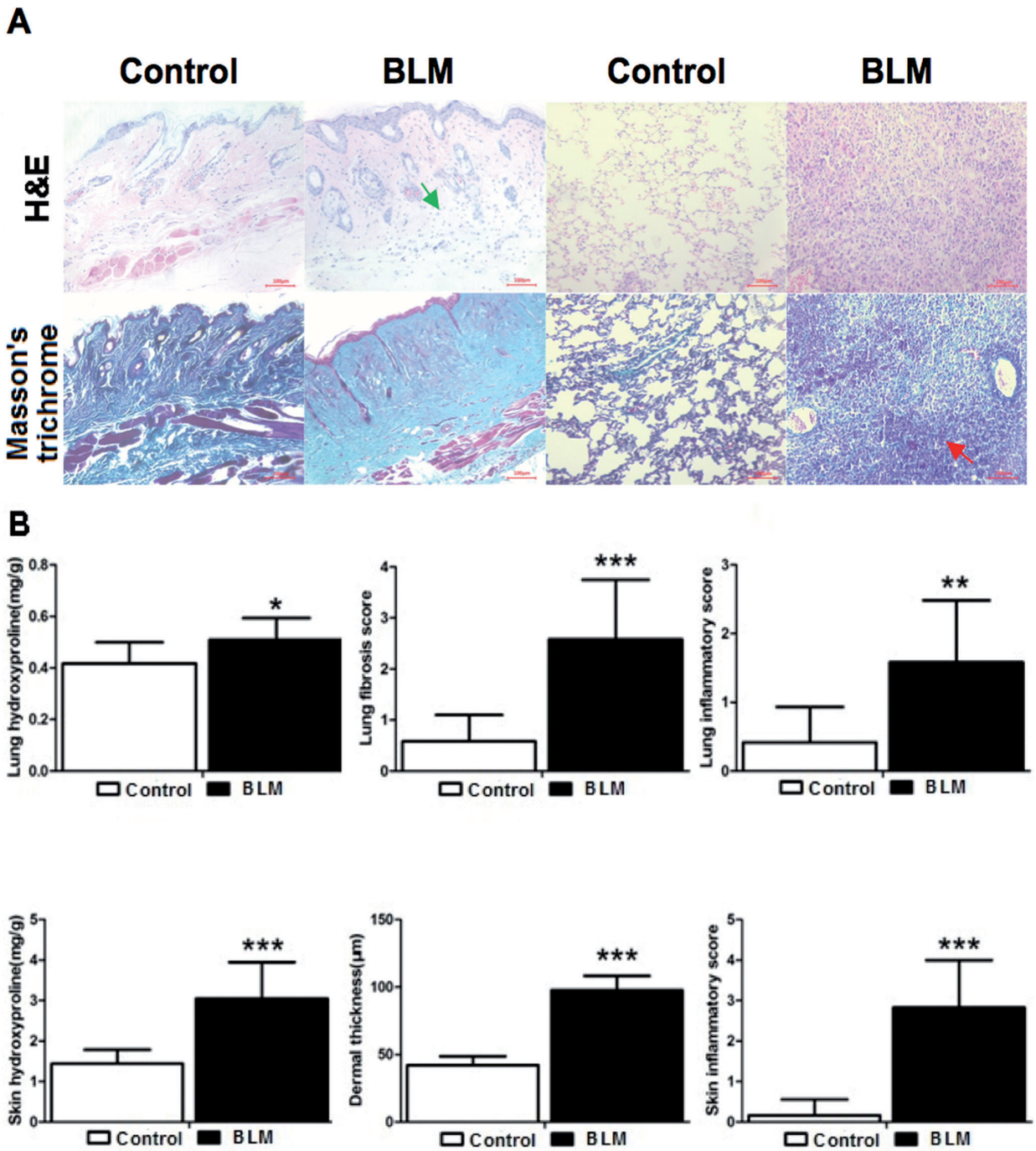

Fig. 1. The skin and lung inflammation and fibrosis in the BLM-injected mice. BALB/c mice were injected subcutaneously with PBS, or 100 μg BLM daily for 4 weeks. Their skin and longs were examined by H&E and Masson's trichrome staining. The contents of hydrooxyprolline and the degrees of inflammation and fibrosis in the skin and lungs of individual mice were analysed. Data are representative images (magnification × 200, scale bar=100 μm) or expressed as the mean ± SD of each group of mice (n=12). A. The H&E and Masson's trichrome staining. B. Quantitative analysis. The green arrow indicates inflammatory infiltrates. The red arrow indicates fibrous areas. *p<0.05; **p<0.01; or ***p<0.001 *vs*. the PBS control.

(p<0.01, p<0.001, Fig. 1B). Similarly, the contents of skin and lung hydroxyproline in the BLM group of mice were greater than that in the control group of mice (p<0.001 or p<0.05, Fig. 1B). Clearly, injection with BLM induced the skin and lung inflammation and fibrosis in BALB/c mice.

Increased frequency of Th17 cells in the peripheral blood, skin and lung infiltrates in the BLM group of mice

Previous studies suggest that Th17 cells may participate in the pathogenesis of SSc (5-8, 18). To understand the pathogenesis of BLM-induced skin and lung inflammation and fibrosis, the frequency of Th17 cells in peripheral blood, skin and lung infiltrates of the control and BLM groups of mice were characterised by flow cytometry. In comparison with that in the control mice, the percentages of $CD4^+IL\text{-}17A^+$ Th17 and $CD4^-IL\text{-}17A^+$ cells in the peripheral blood mononuclear cells, skin and lung

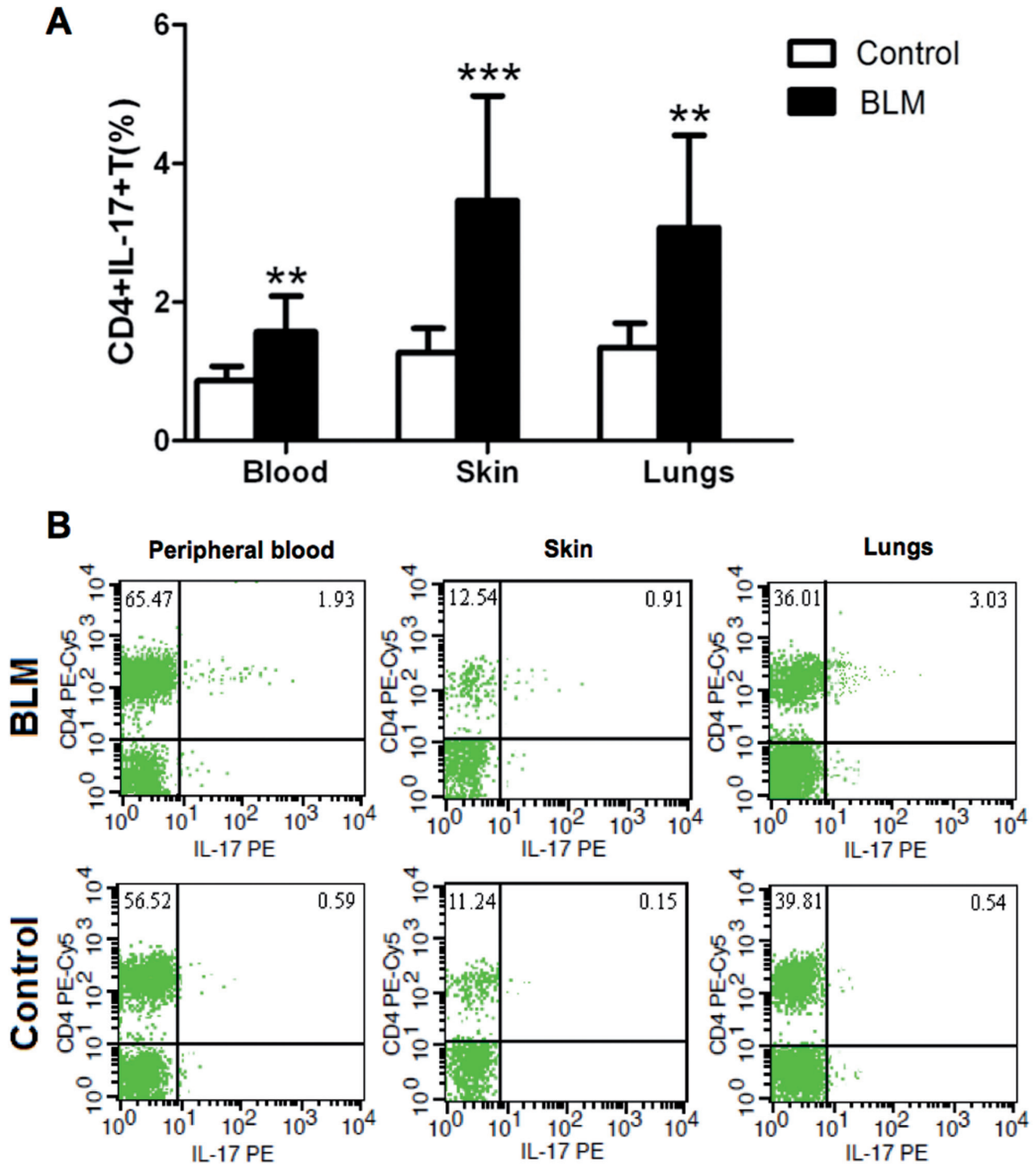

Fig. 2. Increased frequency of Th17 cells in the peripheral blood, skin and lungs of BLM-injected mice. After injection with PBS or BLM for 4 weeks, the mice were sacrificed and their peripheral blood mononuclear cells and skin and lung infiltrates were isolated. The percentages of $CD4^+IL\text{-}17A^+$ Th17 cells were determined by flow cytometry analysis. Data are representative FACS charts or expressed as the mean ± SD of each group (n=12) from two separate experiments. **A**. Quantitative analysis of the percentages of Th17 cells. **B**. Representative FACS charts. In comparison with that in the control mice, the percentages of $CD4^+IL\text{-}17A^+$ Th17 and $CD4^-IL\text{-}17A^+$ cells significantly increased in the peripheral blood mononuclear cells, skin and lung infiltrates in the BLM mice. $^{**}p<0.01$; $^{***}p<0.001$ *vs*. the PBS control.

infiltrates in the BLM mice significantly increased (Fig. 2). Hence, increased frequency of Th17 and $IL\text{-}17A^+$ cells is associated with the development of skin and lung inflammation and fibrosis.

Higher levels of Th17-related cytokine expression in the BLM group of mice

IL-17A, a potent pro-inflammatory cytokine, is produced by Th17 cells and has pleiotropic effects on both innate and adaptive immune responses. To understand the molecular pathogenesis of skin and lung fibrosis in the BLM mice, we measured the concentrations of serum IL-17A, TGF-β1 and IL-6 in the control and BLM groups of mice by ELISA. We found that the concentrations of serum IL-17A, TGF-β1 and IL-6 in the BLM group of mice were significantly higher than that in the control mice ($p<0.01$ or $p<0.001$; Fig. 3A). In paralleling, we characterised the relative levels of IL-17A, RORγt, TGF-β1 and IL-6 mRNA transcripts in the skin and lungs from the control and BLM groups of mice by quantitative RT-PCR.

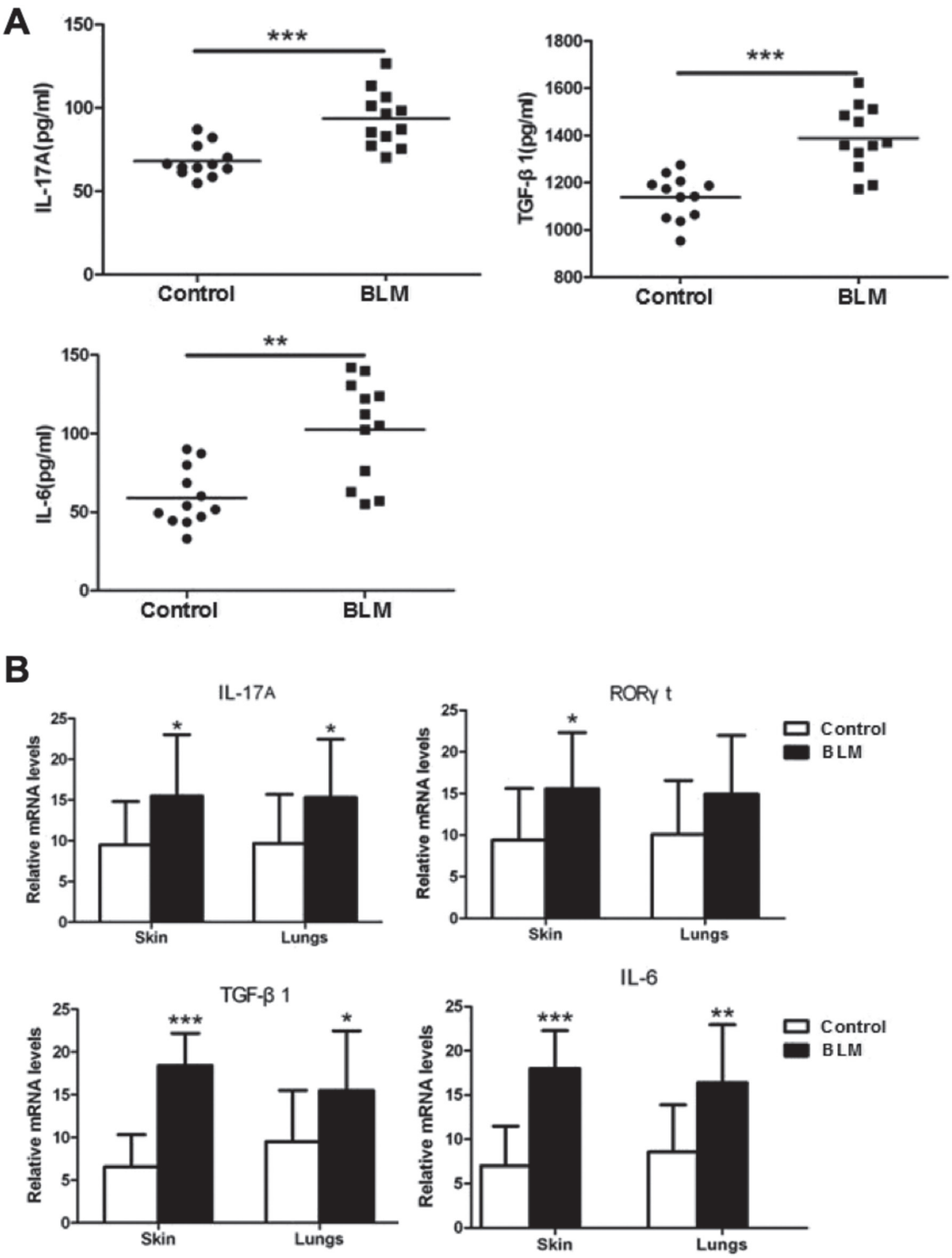

Fig. 3. Higher levels of Th17-related cytokine expression in the BLM group of mice. After injection with PBS or BLM for 4 weeks, the levels of serum IL-6, IL-17A and TGF-β1 in individual mice were determined by ELISA and the relative levels of IL-6, IL-17A, TGF-β1 and RORγt mRNA transcripts to the control β-actin in the skin and lungs of individual mice were determined by quantitative RT-PCR. Data are expressed as individual values or mean ± SD of each group (n=12) from two separate experiments. **A**. The levels of serum cytokines. **B**. The relative levels of Th17-related cytokine and RORγt expression in mice. *p<0.05; **p<0.01; or ***p<0.001 *vs*. the PBS control.

In comparison with that in the control mice, significantly increased levels of IL-17A, RORγt, TGF-β1 and IL-6 mRNA transcripts to the control β-actin were detected in the skin and lung of mice (p<0.05 or p<0.001, Fig. 3B).

Further analysis indicated that the percentages of circulating Th17 cells were positively correlated with the levels of serum IL-17A (r=0.620, p=0.031), and with the relative levels of IL-17A mRNA transcription in the skin

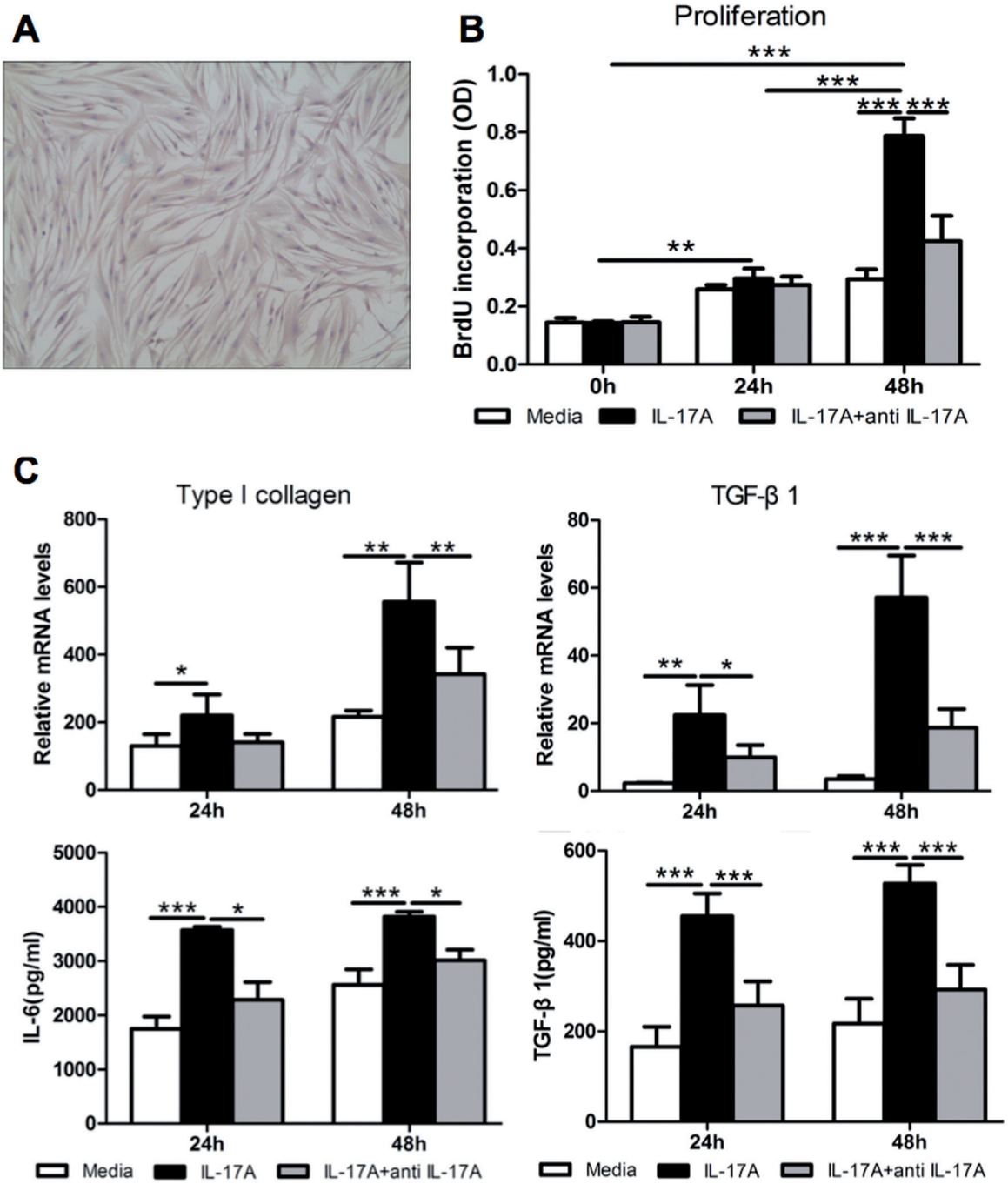

Fig. 4. IL-17A enhances the activity of pulmonary fibroblasts *in vitro*. After injection with BLM for 4 weeks, the lung tissues were dissected from the BLM group of mice and the prepared pulmonary fibroblasts were examined for vimentin expression by immunohistochemistry. Subsequently, the primarily cultured fibroblasts were treated with, or without, IL-17A for the indicated time periods in the presence or absence of anti-IL-17A or control IgG. The proliferation of pulmonary fibroblasts was determined by the BrdU-based ELISA. The relative levels of type I collagen and TGF-β1 mRNA transcripts to the control β-actin were determined by quantitative RT-PCR. The levels of IL-6 and TGF-β1 in the supernatants of cultured cells were determined by ELISA. Data are expressed as the mean ± SD of each group of cells from three separate experiments. Treatment with control IgG did not affect the proliferation of fibroblasts and their IL-6, TGF-β1 and type I collagen expression (data not shown). **A**. The immunohistochemical staining of vimentin in the cells (magnification x400). **B**. The proliferation of primarily cultured pulmonary fibroblasts. **C**. The levels of IL-6, TGF-β1 and type I collagen expression. *p<0.05;**p<0.01; or ***p<0.001 *vs*. the cells without IL-17A or anti-IL-17A treatment.

of the BLM group of mice (r=0.650, p=0.022). Furthermore, the concentrations of serum IL-17A were correlated positively with the skin and lung inflammatory scores (r=0.802, p=0.002 for the skin; r=0.578, p=0.049 for the lung), the contents of skin hydroxyproline (r=0.716, p=0.009) in the BLM group of mice. In addition, the levels of IL-17A mRNA transcripts in the skin were correlated positively with the skin hydroxyproline contents (r=0.707, p=0.010), and the levels of IL-17A mRNA transcripts in the lungs were positively correlated with the lung in-

flammatory scores (r=0.590, p=0.044) of the BLM group of mice. These data suggest that IL-17A is crucial for the pathogenesis of BLM-induced skin and lung inflammation and fibrosis in mice.

IL-17A enhances the proliferation of pulmonary fibroblasts in vitro

To test the impact of IL-17A on the function of fibroblasts, the primarily cultured pulmonary fibroblasts expressed high levels of vimentin (Fig. 4A). The cells were treated with, or without, rIL-17A in the presence or absence of neutralising anti-mouse IL-17A mAb or control IgG and their proliferation was analysed. As shown in Figure 4B, treatment with rIL-17A enhanced pulmonary fibroblast proliferation in a time-dependent manner. Treatment with anti-IL-17A markedly attenuated the IL-17A-enhanced pulmonary fibroblast proliferation. Further analysis revealed that treatment with rIL-17A significantly increased the levels of type I collagen and TGF-β1 mRNA transcription in fibroblasts and enhanced the levels of IL-6 and TGF-β1 secretion by fibroblasts *in vitro* (Fig. 4C). Treatment with anti-IL-17A markedly mitigated the IL-17A-elevated type I collagen and TGF-β1 mRNA levels in fibroblasts and IL-6 and TGF-β1 secretion by fibroblasts *in vitro* (Fig. 4C). Thus, IL-17A enhanced fibroblast proliferation and fibrosis-related gene expression *in vitro*.

Discussion

SSc usually manifests as the skin fibrosis, and may affect other organs, such as the lungs. In this study, we employed a mouse BLM model of SSc to examine the potential association of Th17 responses with the lung inflammation and fibrosis. We found that subcutaneous injection with BLM induced the skin and lung inflammation and fibrosis in mice, consistent with a previous report (11). Evidentially, significantly increased dermal thickness, collagen deposition, and many inflammatory infiltrates displayed in the skin and lung tissues of the BLM group of mice. All of these manifestations closely mimic the pathogenic features of human SSc and this model may provide a unique tool to study the pathogenesis of human SSc.

Th17 responses participate in the pathogenesis of autoimmune diseases. However, previous findings about the role of pro-inflammatory Th17 cells in the development and progression of pulmonary fibrosis are controversial (4, 18-21). In this study, we detected significantly higher frequency of circulating Th17 cells and higher levels of serum IL-17A in the BLM mice. Furthermore, we detected significantly higher percentages of Th17 cells in the skin and lung infiltrates and higher levels of IL-17A, IL-6 and TGF-β1 as well as RORγt expression in the skin and lung tissues of the BLM mice. More importantly, the percentages of circulating Th17 cells and the levels of serum IL-17A were correlated positively with the skin and lung inflammation scores and hydroxyproline contents in the BLM mice. Our data were consistent with previous observations in humans and rodents (4-9, 18, 22-24) and extended previous findings that Th17 cells are associated with the development of SSc-related interstitial lung disease. In addition, recent studies indicate that IL-17A is also critical for the development of BLM, silica, or IL-1 induced lung fibrosis (25-27). Th17 cells and IL-17A are observed around the vessels in the dermal and subcutaneous layers of SSc patients and may be involved in endothelial inflammation (28). Together, these findings support the notion that Th17 responses participate in the pathogenesis of skin and lung fibrosis. Hence, Th17 cells may be therapeutic targets for the design of new therapies for SSc and the percentages of circulating Th17 cells and the levels of serum IL-17 may be valuable for evaluating the disease severity of SSc.

TGFβ1, one of the major fibrogenic cytokines, can induce fibroblast cell activation and proliferation, stimulate ECM and collagen synthesis and deposition in various types of cells, and promote the fibrotic process (29). IL-6 is a pro-inflammatory cytokine and together with TGF-β, induces Th17 cell functional development (3). Furthermore, IL-6 plays an important role in chronic inflammation, autoimmunity, endothelial cell dysfunction, and fibrogenesis (30). Consequently, IL-17A and other mediators produced by Th17 cells can stimulate IL-6 production in fibroblasts (31). We detected significantly higher levels of serum TGF-β1 and IL-6 and higher levels of TGF-β1 and IL-6 mRNA transcripts in the skin and lungs of the BLM group of mice. Our data were consistent with previous observations that many inflammatory infiltrates, particularly for $CD4^+$ T cells and higher levels of Th17 and Th2 cytokines as well as TGF-β1 are detected in patients with SSC and are positively correlated with disease severity (22, 32). IL-6 is a pro-fibrogenic cytokine and can regulate the expression of vascular endothelial growth factor (VEGF), an important mediator of angiogenesis and fibrosis, which is elevated in patients with SSc (33). Indeed, treatment with Tocilizumab, an anti-IL-6 receptor antibody, can improve clinical symptoms in patients with SSc (34). TGF-β1 can activate the Smad, ERK/MAPK, and p38MAPK signalling to promote the trans differentiation of fibroblasts into myofibroblasts in the skin lesion of SSc patients (35). Th17 cells may enhance the pulmonary fibrogenesis through TGF-β1-dependent and -independent manners. These findings suggest that Th17 cells and their cytokines as well as TGF-β1 may be therapeutic targets for intervention of the fibro-proliferative lung diseases (26).

In paralleling, we found that rIL-17A enhanced the proliferation of primarily cultured pulmonary fibroblasts and increased their type I collagen, TGF-β1, and IL-6 production in a time-dependent manner. Treatment with neutralising anti-IL-17A markedly mitigated the IL-17A-elevated the levels of type I collagen and TGF-β1 mRNA transcripts in fibroblasts and the levels of IL-6 and TGF-β1 secretion by fibroblasts *in vitro*. Thus, IL-17A enhanced fibroblast proliferation and fibrosis-related gene expression *in vitro*. Our data were consistent with previous findings in the skin fibroblast and treatment with anti-IL-17 effectively blocks the production of IL-17-induced collagen in the skin fibroblast (9, 18). IL-17A deficient mice are resistant to BLM-induced skin fibrosis (9). In addition, our findings argued against the notion

that Th17 cells restrain collagen production in fibroblast (20) and IL-17A does not induce α-smooth muscle actin (α-SMA) expression in cultured fibroblasts from SSc patients (21). The disparity between our data and those of others may stem from different experimental conditions. We are interested in further investigating the regulatory effect of Th17-related mediators on the pulmonary fibrogenesis.

In summary, our data indicated that Th17 cells were important players in the pathogenic process of skin and pulmonary inflammatory and fibrotic lesions in mice. IL-17A enhanced the proliferation of primarily cultured pulmonary fibroblasts and their type I collagen, TGF-β1 and IL-6 production to promote the fibrosis in mice. Our findings suggest that Th17 cells may be therapeutic targets for intervention of SSc and may provide new insights into the pathogenesis of SSc.

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