The effects of interleukin-35 and interleukin-10 on pulmonary inflammation and fibrosis in a bleomycin-induced systemic sclerosis mouse model

Y. Huang[1,2], W. Zeng[1], X. Liao[1], F. Qin[1], X. Ma[1], J. Pan[1], G. Li[2], K. Tang[2], L. Lei[1]

[1]Department of Rheumatology and Immunology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Province, China; [2]Department of Rheumatology and Immunology, The Fourth Affiliated Hospital of Guangxi Medical University, Liuzhou, Guangxi Province, China.

Abstract

Objective

We investigated the impact of IL-35 and IL-10 on the immune response and pulmonary fibrosis using a bleomycin (BLM)-induced SSc mouse model.

Methods

BLM was administered subcutaneously to Balb/c mice and either mouse recombinant (rm)IL-35, rmIL-10 or neutralising antibody of IL-35 and IL-10 was injected intraperitoneally after BLM administration. Lung fibrosis was assessed by the pathological alterations, hydroxyproline content, and the collagen I and α-SMA mRNA expression. The expression of immune cells and their related factors were respectively measured by flow cytometry and ELISA. Western blot was used to measure STAT3 pathway expression.

Results

Compared with controls, BLM exposure induced increased Ashcroft ratings, hydroxyproline and lung collagen I and α-SMA expression, which was lessened by rmIL-35 or rmIL-10 intervention, while it did not change after blocking IL-35 and IL-10. BLM exposure increased IL-4 and IL-17A expression in bronchoalveolar lavage (BAL) supernatant, which was downregulated by rmIL-35 or rmIL-10 administration. Compared with the BLM group, the RmIL-35 and rmIL-10 group both downregulated Th2/nTreg and Th17/nTreg percentage, while increased Treg cell proportion in the spleen. Moreover, the spleen iTr35 cell ratio was negatively correlated with BAL supernatant IL-17A and IL-4 levels and lung collagen I and α-SMA expression. Further pathway analysis revealed that rmIL-35 administration decreased the phosphorylation of STAT3 compared with the BLM group.

Conclusion

Our findings suggest that IL-35 and IL-10 might alleviate pulmonary inflammation and fibrosis via upregulating the proportion of Treg cells and reducing BAL supernatant IL-17A and IL-4 levels in a bleomycin-induced SSc mouse model.

Key words

interleukin-35, interleukin-10, systemic sclerosis, interstitial lung disease, regulatory T cells

Yali Huang, MD
Wen Zeng, MD, PhD
Xiaoling Liao, MD
Fang Qin, MD
Xuejia Ma, MS
Jie Pan, MD, PhD
Guangting Li, MD
Kaijiang Tang, MS
Ling Lei, MD, PhD

Please address correspondence to:
Ling Lei
Department of Rheumatology and Immunology,
The First Affiliated Hospital of Guangxi Medical University,
Nanning 530000, Guangxi, China.
E-mail: leiling1972@aliyun.com

Received on July 22, 2024; accepted in revised form on November 18, 2024.

Funding: this study was supported in part by grants from The National Natural Science Foundation of China (Regional Science Foundation Project, no. 82060300), the Guangxi Natural Science Foundation (no. 2023GXNSFDA026061), and the Guangxi Medical and Health Technology Development and Promotion Project (no. S2022075).

Competing interests: none declared.

Introduction

Systemic sclerosis (scleroderma, SSc) is an autoimmune disease characterised by inflammation, vascular abnormalities, fibrosis of the skin and internal organs (1, 2). Presently, interstitial lung disease (ILD) remains a major contributing cause of both morbidity and mortality of SSc with an incidence of approximately 40% and a 10-year mortality of up to 40% (3). Data from the European Scleroderma Preliminaries and Exploration Gathering (EUSTAR) suggest that pulmonary fibrosis is attributed to 35% of disease-specific mortality (4).

The mechanisms involved in scleroderma-associated interstitial lung disease (SSc-ILD) remain poorly understood, but there is no doubted that inflammation and immune activation are part of pathogenic events in SSc-ILD. Evidences show that one important factor in the pathophysiology of SSc-ILD is excessive activation of $CD4^+$ T cells, especially follicular T helper cells (Tfh) (5). Our previous studies have pointed to a decreased T helper 1 (Th1)/ T helper 2 (Th2) cell ratio, as well as T helper 17 (Th17)/regulatory T (Treg) cell imbalances in patients with SSc (6). Interleukin-4 (IL-4) and interleukin-13 (IL-13) could modulate fibroblasts to myofibroblasts, leading to extracellular matrix(ECM) accumulation (7). The role of interleukin-17A (IL-17A) in fibrosis remains controversial, with some studies emphasising a profibrotic (8), and others an antifibrotic role (9). Therefore, it is presumed that the immune imbalance among T cell subsets is crucial for the progression of SSc-ILD.

Interleukin-10 (IL-10), secreted by an array of cells including T cells, B cells, macrophages and dendritic cells (10), could modulate the differentiation and function of $CD4^+$ T cells (11). Martinez *et al*. revealed an up-regulation of IL-10 mRNA expression in alveolar macrophages and reduced levels of IL-10 protein in the bronchoalveolar lavage (BAL) of patients with ILD (12). IL-10 has also been demonstrated to play a protective role in bleomycin (BLM)-induced mouse models of lung fibrosis (13). However, opposite results were emerged when using pulmonary fibroblasts (14).

Interleukin-35 (IL-35), a heterodimer cytokine composed of p35 and Ebi3, is predominantly derived from Treg cells (15). Potent immunosuppressive properties of IL-35 could reduce the proliferation of effector T cells (Teff), alter the ratio of Th17 cells to Tregs, and influence Th2/Th17 responses (16). Evidence suggests that IL-35 is a significant mediator in fibrosis. Serum levels of IL-35 have been reported to be increased in SSc patients and associated with the severity of fibrosis in the skin and lungs (17, 18). Some experiments point to an anti-fibrotic role of Ebi3 (19). Nevertheless, another study observed contrasting results when using recombinant IL-35 protein (20).

The underlying mechanisms of in SSc-ILD are not completely understood. Moreover, the role of IL-35 and IL-10 in SSc-ILD and their mutual effect has been only partially studied (21, 22), whether IL-35 added to IL-10 further affects fibrosis responses remains unknown. To this aim, we conducted a murine experiment to assess the role of IL-35 and IL-10 in SSc-ILD development.

Materials and methods

Animals and animal experiments setup

Forty-two female Balb/c mice (aged 6–8 weeks, weighing 25–30g) were kept in Guangxi Medical University Laboratory Animal Center (Nanning, China). The experiment was carried out in conformity with the guideline for animal testing and approved by the Animal Management and Ethics Committee of Guangxi Medical University.

Seven groups of six mice each were randomly assigned: (1) PBS group (PBS) mice were subcutaneously injected with the identical amount of PBS; (2) BLM group (BLM) mice were subcutaneously administered with BLM (Macklin, China, cat. no. B802467) 100 μg per day daily for 3 weeks, as previously described (23); (3-4) BLM + rmIL-35 group (BLM + rmIL-35) and BLM + rmIL-10 group (BLM + rmIL-10) mice were respectively intraperitoneally treated with mouse recombinant IL-35 (Chimerigen, USA, no. CHIMF-11135) or mouse recombinant IL-10 (Pepro-

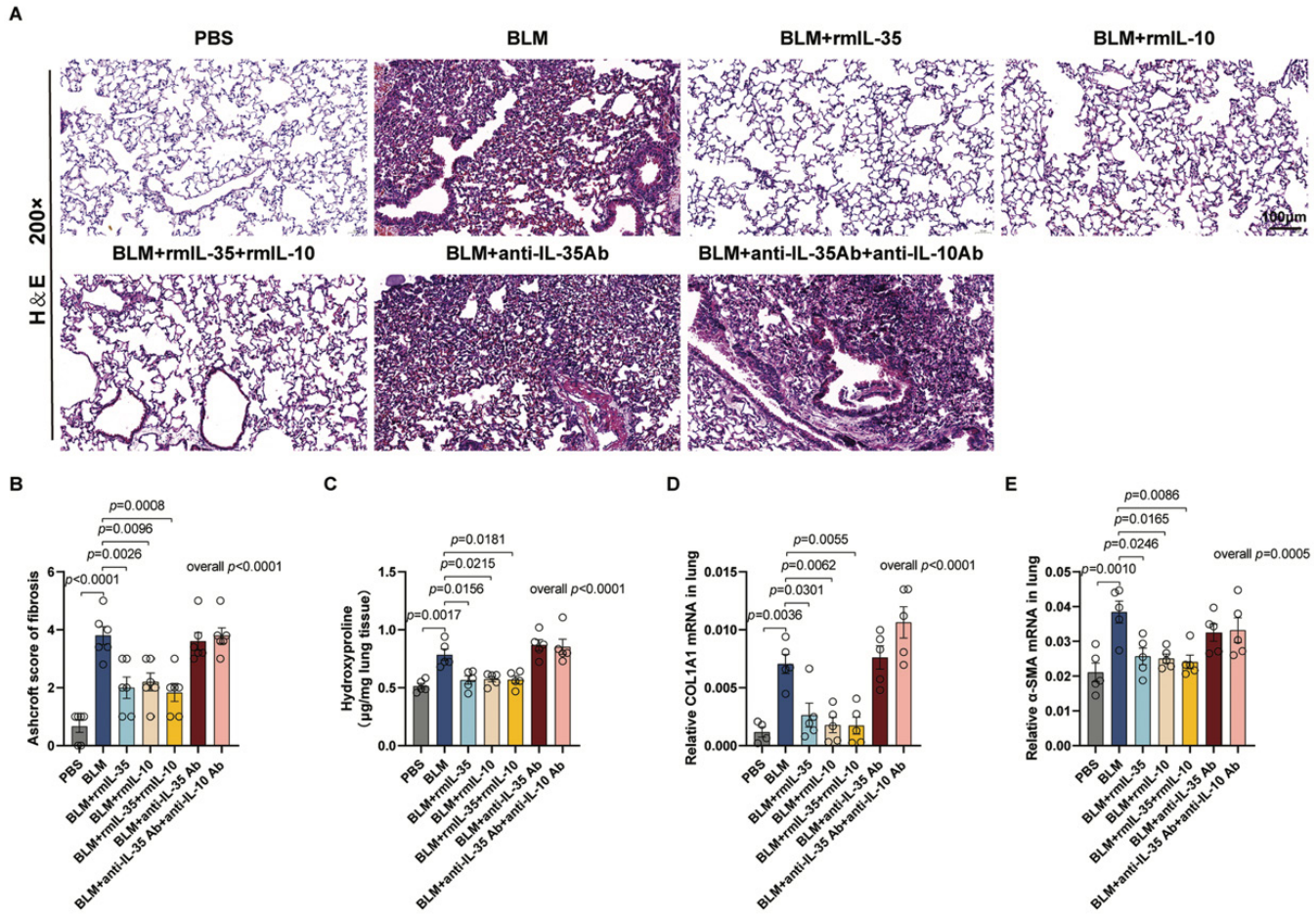

Fig. 1. Effects of IL-35 and IL-10 on the severity of pulmonary fibrosis after BLM induction.
A. Representative images for H&E (200× magnification). **B.** The Ashcroft scores for the degree of lung fibrosis. **C.** Quantification of lung hydroxyproline (measured value specific lung wet weight). **D-E.** RT-PCR analysis of Collagen I and α-SMA expression in the lung tissue.
Data represent the mean ± SEM.

Tech, USA, no. 210-10) 0.75 μg per day on 4, 7, 10, 13,16 and 19 days following BLM injection. (5) BLM + rmIL-35 + rmIL-10 group (BLM + rmIL-35 + rmIL-10) mice were intraperitoneally treated with mouse recombinant IL-35 and mouse recombinant IL-10 as previously mentioned (6). BLM + neutralising anti-IL-35 Ab group (BLM + anti-IL-35 Ab) mice were intraperitoneally treated with mouse anti-IL-35 Ab (R&D Systems, USA, no. MAB6688) 25 μg per day on 3, 6,9,12 and 15 days following BLM injection. (7) BLM + neutralising anti-IL-35 Ab + neutralising anti-IL-10 Ab group (BLM + anti-IL-35 Ab + anti-IL-10 Ab) mice were intraperitoneally treated with mouse anti-IL-35 Ab (R&D Systems, USA, no. MAB6688) and mouse anti-IL-10 Ab (Invitrogen, USA, no. 16-7101-85) as group (6). On day 21, all the mice were mercy killed.

Histopathological assessment

The dissected lung tissues were fixed and embedded, and eventually cut into slices for haematoxylin and eosin (H&E) staining. We randomly select 5 fields (200 x magnification) for each lung specimen to assess the severity of pulmonary fibrosis using Ashcroft scoring method (24) under the blind supervision of two pathologists.

Hydroxyproline assay

The hydroxyproline content was quantified with the kit (Solarbio, China, no. BC0255) as instructed by the manufacturer to determine the collagen deposition in lung tissue.

Flow cytometry analysis

Splenic cell suspension was obtained from mice and activated for four hours prior to staining using the leukocyte activating agent (BD, USA, cat. no. 550583). The Fixable Viability Stain 510 (BD, USA, no. 564406) was used to stain the cells. Then cells were surface stained with anti-CD4-BB700 (BD, USA, no. 566408). For the intracellular cytokine analysis, cells were divided into group I and II. Group I was fixed and permeabilised with the Fixation/Permeabilisation Kit (BD, USA, no. 554714) and then stained with anti-IL-17A-PE (BD, USA, no. 561020) and anti-IL-4-APC (BD, USA, no. 554436). Group II was fixed and permeabilised using the Transcription Factor Buffer Set (BD, USA, no. 562574) and then stained with anti-Foxp3-BV421 (Invitrogen, USA, no. 404-5773-80), anti-Ebi3-APC (R&D Systems, USA, no. IC18341A), anti-IL-12p35-PE (Invitrogen, USA, no. MA523559) and anti-IL-10-FITC (Invitrogen, USA, no. 11-7101-81).
All stained cells were detected by a

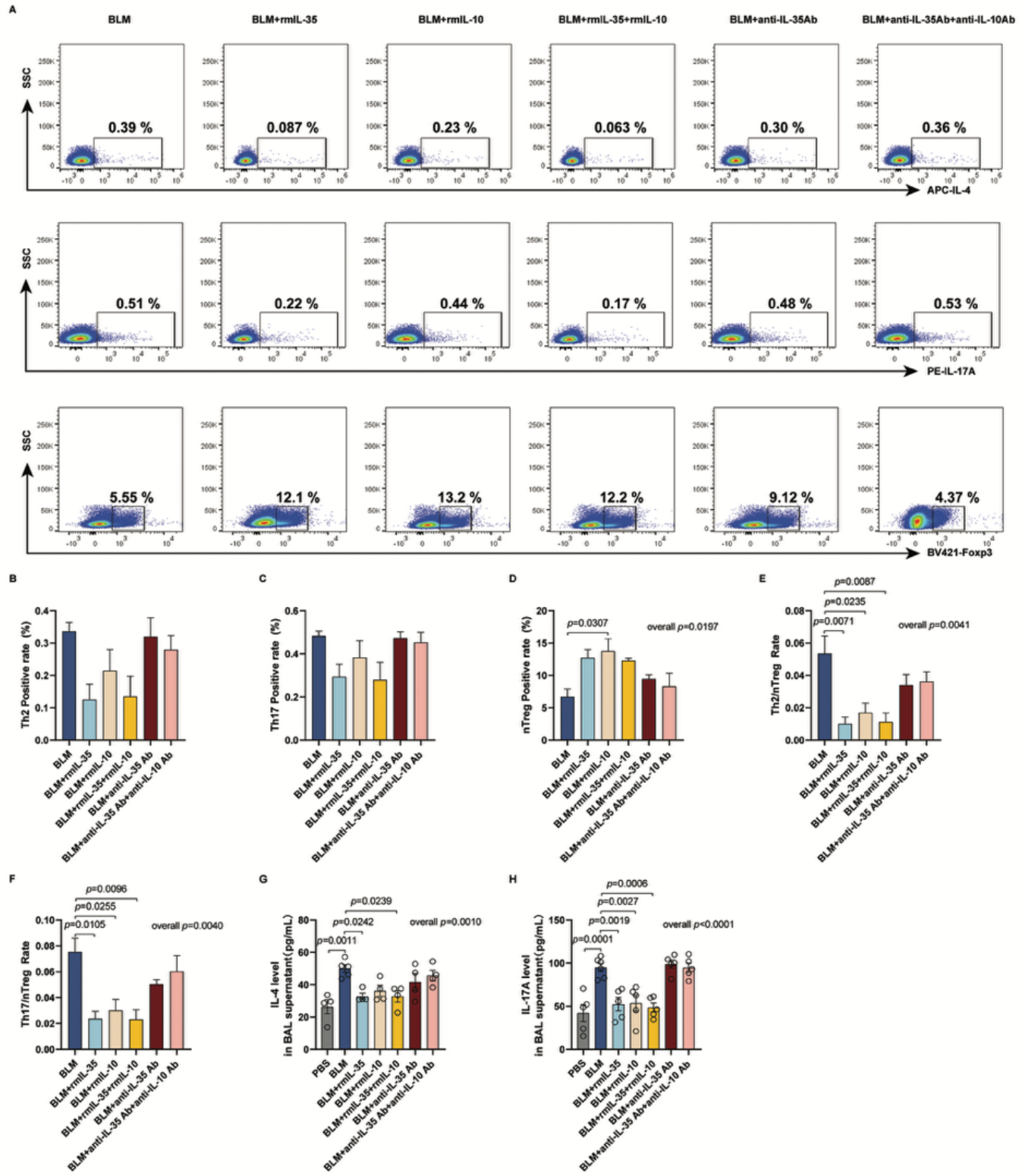

Fig. 2. Effects of IL-35 and IL-10 on Th2/Treg and Th17/Treg balance after BLM induction.
A. The proportions of Th2 (CD4+IL-4+), Th17 (CD4+IL-17A+) and nTreg (CD4+Foxp3+) cells in the mouse spleen were quantified by flow cytometry. **B-D.** Th2, Th17 and nTreg ratio between groups. **E-F.** The Th12/nTreg and Th17/nTreg ratio between groups. **G-H**. BAL supernatant fluid levels of IL-4 and IL-17A as determined by ELISA. Data represent the mean ± SEM.
Th17: T helper cell 17; Th2: T helper cell 2; nTreg: naturally occurring regulatory T cell.

FACSCelesta (BD, USA) and FlowJo software (version 10.8.1, BD, USA) was employed for analysis. Briefly, $CD4^+IL\text{-}17A^+$T cells were used to characterise Th17 cells. $CD4^+IL\text{-}4^+$T cells were used to characterise Th2 cells. Tregs that arise naturally (nTreg cells) were described as $CD4^+Foxp3^+$T cells.$CD4^+Foxp3^-Ebi3^+IL\text{-}12p35^+$T cells were used to

characterise inducible IL-35 producing regulatory T (iTr35) cells. While Type 1 regulatory T (Tr1) cells described as $CD4^+Foxp3^-IL\text{-}10^+$T cells.

ELISA

Bronchoalveolar lavage fluid (BALF) was obtained from each mouse and supernatant was separated via centrifugation. The levels of IL-35 (Elabscience, China, no. E-EL-M0733), IL-10 (Elabscience, China, no. E-HSEL-M0004), IL-17A (Elabscience, China, no. E-HSEL-M0005), and IL-4 (MULTI SCIENCE, China, no. EK204/2-96) in the supernatant samples were measured by ELISA in compliance with the manufacturer's guidelines.

Quantitative real-time PCR

Total RNA was extracted from the lungs of mice using TRlzol reagent (Takara, China, no. 9109) according to the user manual. A Reverse Transcription Kit (Applied Biological Materials Inc., Canada, no. G492) was utilised to synthesise cDNA. Real-time PCR assays were performed on a ViiA™ 7 real-time PCR system (Applied Biosystems, USA) using the StarLighter SYBR Green qPCR kit (Beijing Qihengxing Biotechnology Inc., China, no. FS-Q1002). The mRNA expression levels were normalised to beta-actin. To determine each target's relative expression, the $2^{-\Delta ct}$ technique was employed. The primer sequences used were listed in the supplemental materials.

Western blot analysis

The lung tissues' total protein was extracted using RIPA lysis buffer (Beyotime, China, no. P0013B). The primary antibodies used in this study included p-STAT3 (1:500 dilution, CST, USA, no. 9131) and STAT3 (1:1000 dilution, Abcam, UK, no. ab68153). An internal control was provided by beta-actin (1:5000, Proteintech, China, no. 20536-1-AP).

Statistical analysis

The means ± SEMs are used to express the data. To conduct the statistical analysis, GraphPad Prism (v. 8.0, GraphPad Software Inc., USA) was used. Comparison between two groups were performed using the Student's t-test or the Mann Whitney U-test. Variables in more than two groups were compared by one-way ANOVA. The correlation coefficients were determined using Spearman's correlation analysis. *p*-values less than 0.05 were regarded as statistically significant.

Results

Inhibition of BLM-induced pulmonary fibrosis by IL-35 and IL-10

Compared with controls, BLM exposure induced increased pulmonary fibrosis, which was diminished remarkably by rmIL-35, rm-IL-10, or rmIL-35 combined with rm-IL-10 administration (Fig. 1A). Particularly, BLM exposure increased the degree of pulmonary fibrosis and the hydroxyproline levels compared with PBS treatment (Fig. 1B-C). The Ashcroft ratings were reduced in the rmIL-35, rm-IL-10, or rmIL-35 combined with rm-IL-10 groups than that in the BLM group (Fig. 1B). Furthermore, the hydroxyproline levels were much less in the rmIL-35 and/or rmIL-10 groups than that in the BLM group (Fig. 1C). However, there is no additive effect when rmIL-35 was added in combination with rmIL-10. We used RT-PCR analysis to measure fibrosis markers levels (collagen 1 and α-SMA) in the lung tissue in order to further evaluate the impact of IL-35 and IL-10 on lung fibrosis. As expected, rmIL-35, rm-IL-10, or rmIL-35 combined with rm-IL-10 intervention decreased BLM-induced collagen I and α-SMA mRNA expression (Fig. 1D-E). And surprising, neutralising antibodies to IL-35 or IL-10 had no effect on a range of measures.

Imbalance of Th2 and Th17 responsein IL-35 and IL-10 administrated mice

We evaluated whether IL-35 or IL-10 affects the differentiation of Th2, Th17 and nTreg cells in spleen of SSc-ILD mice, further depicting the inflammatory condition with Th2/nTreg and Th17/nTreg percentage. The number of nTreg cells was increased with rmIL-10 administration (Fig. 2D). Furthermore, rmIL-35 or rmIL-10 intervention significantly downregulated Th2/nTreg and Th17/nTreg percentage (Fig. 2(E-F)), having a notable impact on inflammation of SSc-ILD mice. The concentration of inflammatory factors in the BAL supernatant was further measured. Results suggested that BLM exposure upregulated IL-4 and IL-17A levels in the BAL supernatant of mice compared with the PBS control (Fig. 2G-H). Conversely, rmIL-35 or rmIL-35 combined with rm-IL-10 administrated mice showed downregulation of IL-4 levels in the BAL supernatant (Fig. 2G). Similarly, mice administrated with rmIL-35, rmIL-10 or rmIL-35 combined with rm-IL-10 showed reduced IL-17A levels (Fig. 2H).

Upregulated iTreg cells activities in the presence of IL-35 and IL-10

Next, we investigated the potential of IL-35 and IL-10 to stimulate the activity of iTreg cells (Fig. 3). Interesting, the number of iTr35 and Tr1 cells were significantly increased in the presence of rmIL-35, while they were not affected by the rmIL-10 only or combined action of rmIL-35 and rmIL-10. To our surprise, there was no significant difference in IL-35 and IL-10 expression in BAL supernatant of mice between BLM and PBS group. Results also demonstrated increased IL-35 levels in response to rmIL-35, but not rmIL-10 alone or rmIL-35 combined with rmIL-10. For up-regulation of IL-10 levels, administration with rmIL-35 or rmIL-10 alone was significantly more effective than when the cytokines were combined.

Correlation of the spleen iTr35 and Tr1 cells frequency with BAL supernatant cytokine levels, spleen inflammatory cell ratio and lung fibrosis markers expression

To visualise the potential role of Tr1 and iTr35 cells in inflammatory response and pulmonary fibrosis, we combined the data for correlation analysis (Fig. 4). As our expected, iTr35 cell frequency and IL-35 levels were significantly positively correlated (r=0.6298, *p*=0.0051), which indicates a positive feedback loop between each other. We found the proportion of iTr35 cells was negatively correlated with IL-17A and

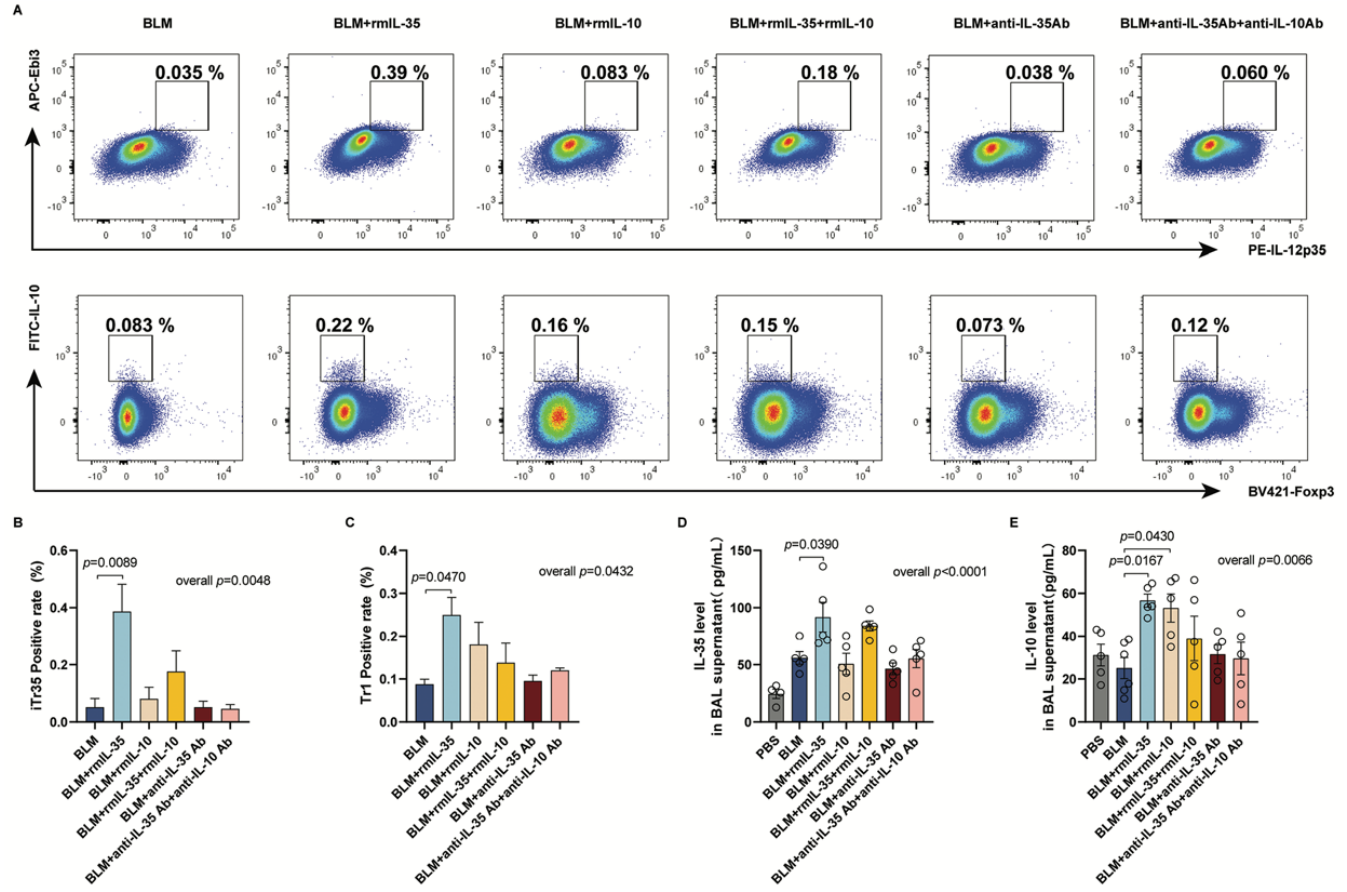

Fig. 3. Effects of IL-35 and IL-10 on regulatory T cell activity after BLM induction.
A: The proportions of iTr35 (CD4+Foxp3-Ebi3+IL-12p35+) and Tr1 (CD4+Foxp3-IL-10+) cells in the mouse spleen were quantified by flow cytometry. **B-C**: iTr35 and Tr1 ratio between groups. **D-E.** BAL supernatant fluid levels of IL-35 and IL-10 as determined by ELISA.
Data represent the mean ± SEM.
iTr35: inducible IL-35 producing regulatory T cell; Tr1: type 1 regulatory T cell.

IL-4 levels (r= -0.5212, p=0.0266; r= -0.4750, p=0.0464, respectively) and positively correlated with IL-10 levels (r=0.6017, p=0.0083). Similarly, iTr35 cells proportion was conversely correlated with Th17 and Th2 cell ratio (r= -0.6501, p=0.0035, r= -0.6326, p=0.0048, respectively) and positively correlated with Tr1 cell ratio (r=0.5671, p=0.0141). Moreover, the lung collagen I and α-SMA mRNA expression was inversely correlated with iTr35 cell frequency (r= -0.5191, p=0.0273, r= -0.6512, p=0.0034, respectively). The percentage of Tr1 cells was positively correlated with IL-35 levels (r=0.4290, p=0.0381) and negatively correlated with Th17 and Th2 cell ratio (r= -0.5425, p=0.0200, r= -0.5835, p=0.0110, respectively). Unexpectedly, there was no clear correlation between nTreg cells percentage and either iTr35 or Tr1 cells proportion. Besides, there was no significant correlation between the Tr1 cell frequency and IL-4, IL-17A and IL-10 levels, as well as the lung collagen I and α-SMA mRNA expression.

IL-35 decreased the activation of STAT3 expression in lung tissues

To investigate the mechanisms of IL35 and IL-10 in modulating lung fibrosis, we detected the changes in STAT3 phosphorylation, which has been supported to participate in the pathogenicity of ILDs (25). We observed increased p-STAT3 expression in lung tissues of BLM-induced mice compared to controls, while rmIL-35 and rmIL-35 combined with rm-IL-10 administrated mice showed significantly reduced levels of p-STAT3. Nevertheless, rmIL-10 administration alone could not block this pathway. Similarly, there were no effect by IL-35 or IL-10 neutralising antibody. The specific results are presented in Figure 5.

Discussion

The pathogenesis of SSc is a progressive process of amplification that starts with microvascular damage and progresses to inflammation and immunological response, and finally characterised by skin and visceral fibrosis (26). IL-10, an anti-inflammatory mediator, has been a potential target for anti-fibrosis therapy given the link between inflammation and fibrogenesis (27). IL-35, an immunosuppressive factor, has been found to be increased in SSc patients (17). There are, however, few researches elaborate the roles of IL-10 and IL-35 in pulmonary inflammation and fibrosis of SSc. Our findings displayed that the Ashcroft ratings, hydroxyproline content and collagen I and α-SMA mRNA expression in the lung tissue were increased in BLM-induced SSc mouse model, suggesting that pulmonary fibrosis was aggravated. After administrated with rmIL-35,

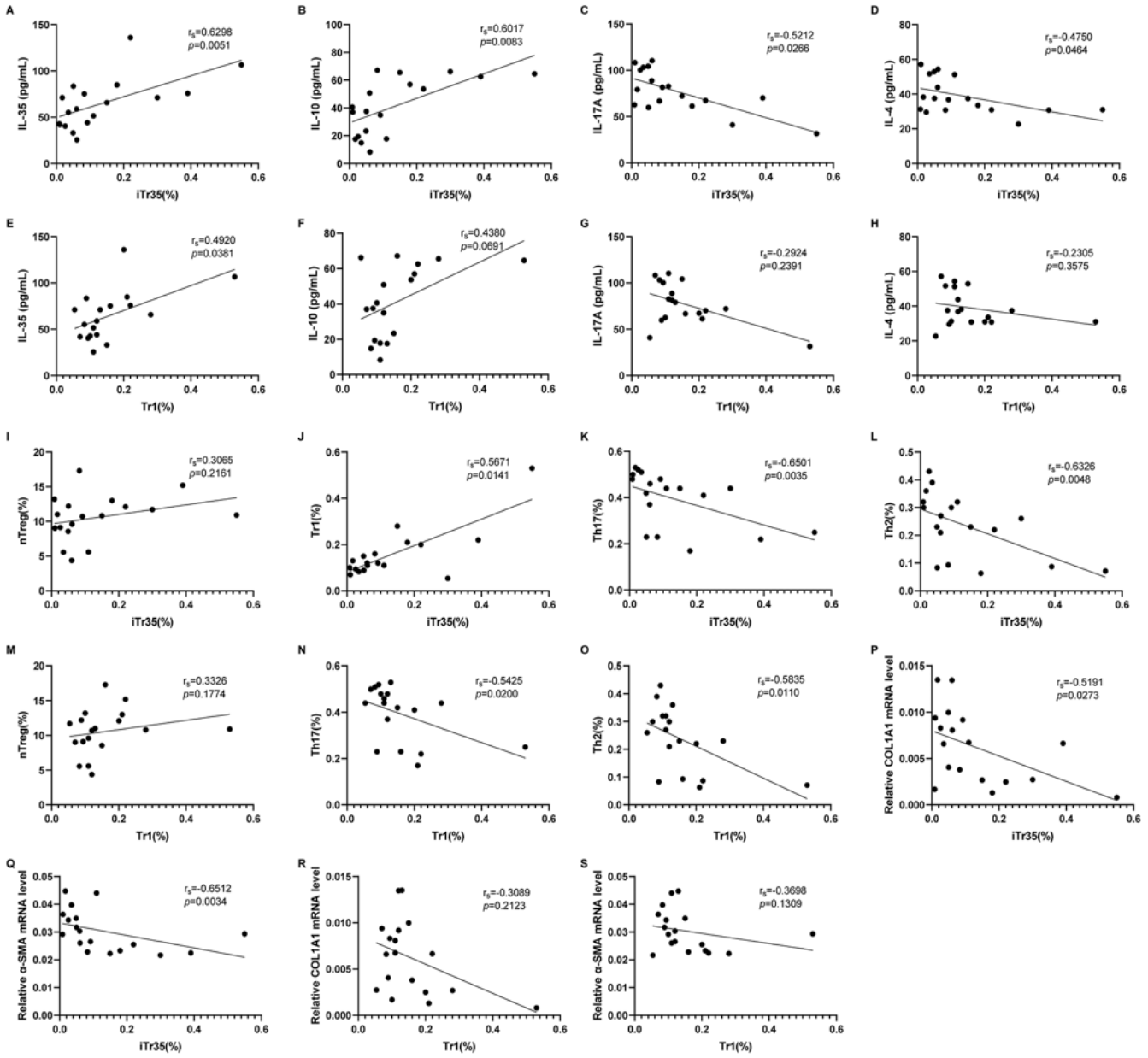

Fig. 4. Correlation analysis of BAL supernatant cytokine levels, spleen inflammatory cell ratio, lung fibrosis markers expression and the spleen iTreg cell ratio. **A-D.** Spearman correlation between expression of iTr35 cells in the spleen and IL-35, IL-10, IL-17A and IL-4 levels in BAL supernatant. **E-H**. Spearman correlation between expression of Tr1 cells in the spleen and IL-35, IL-10, IL-17A and IL-4 levels in BAL supernatant. **I-O**. Spearman correlation between the proportions of different inflammatory cells in the spleen. **P-S.** Spearman correlation between expression of collagen I and α-SMA in the lung and the proportions of iTr35 and Tr1 cells in the spleen.
iTreg: inducible Treg cell; iTr35: inducible IL-35 producing regulatory T cell; Tr1: Type 1 regulatory T cell; Th17: T helper cell 17; Th2: T helper cell 2; nTreg: naturally occurring regulatory T cell.

rm-IL-10 or rmIL-35 combined with rm-IL-10, these BLM-exposed mice' expanded pulmonary fibrosis was abrogated to a great extent and the collagen I and α-SMA mRNA expression in the lung tissue of mice was lessened. However, IL-35 and IL-10 do not show obvious collaboration.

Recent studies have shown similar results. Ebi3 (one subunit of IL-35) knockdown aggravated fibrosis in a mouse model of BLM-induced skin and lung fibrosis (19, 28). Kudo *et al*. also observed that Ebi3 downregulated the collagen I expression in normal and SSc fibroblasts (28). Administration of IL-10 partially reversed pulmonary fibrosis in mice treated with BLM (13). IL-10 has also been confirmed to degrade the abnormal deposition of ECM with downregulated collagen expression and upregulated expression of matrix metalloproteinases (MMPs) in dermic fibroblasts (29). Our results suggest that IL-35 and IL-10 can improve pulmonary fibrosis in SSc mouse models, but there is no synergistic effect. Here, we speculate that upregulated IL-35 expression in the patients of SSc is possibly compensatory but ineffective.

As mentioned before, ILD might be the final result of the excessive acti-

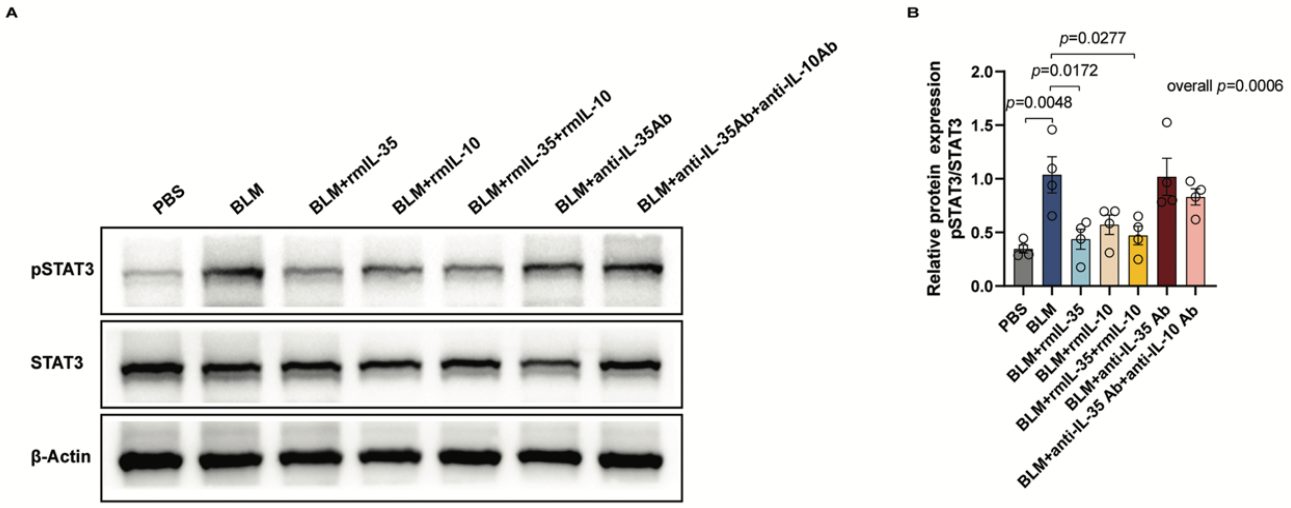

Fig. 5. The impact of IL-35 and IL-10 on STAT3 signalling in the lung after BLM induction.
A-B: Western blotting analysis of the lung levels of p-STAT3/STAT3 in the mouse model. Data represent the mean ± SEM.

vation of immune system in SSc (30). As found previously, Th2 and Th17 responses were hyperactivated in SSc and contribute to fibrosis (31). Our results also demonstrated that exposure to BLM significantly induced IL-17A and IL-4 production. Accumulating evidences have shown that IL-35 and IL-10 can induce the production of Treg cells and inhibit the activity of $CD4^+$ effector T cells including Th1, Th2 and Th17 cells (16, 32). Our findings are consistent with the literature described above. The results confirmed that IL-35 could promote Treg cells generation and limit Th2 and Th17 response, as indicated by the increased IL-35, IL-10, iTr35 and Tr1 cells and decreased IL-17A and IL-4 levels, as well as the downregulated Th2/nTreg and Th17/nTreg percentage. IL-10 exhibited a comparable effect as shown by the increased IL-10 and nTreg cells expression and the downregulated IL-17A levels, Th17/nTreg and Th17/nTreg percentage. Though these two anti-inflammatory cytokines largely overlapped in regulating immune response of $CD4^+$ T cells, IL-35 seemed to play a broader and stronger role. Studies have suggested that IL-10 and IL-35 acted in cooperation to inhibit T cells activation and maintain immune tolerance (21, 22). Our previous study also discovered that IL-35 and IL-10 cooperatively induced the differentiation of iTr35 and Tr1 cells *in vitro* (33). Here we showed that both IL-35 and IL-10 can modulate immune response in SSc mouse models, but there is no cooperative impact. We found an upregulation in the levels of IL-10 and Tr1 cells following rmIL-35 administration, suggesting that IL-35 may intensify its immunosuppression by increasing IL-10 levels.

Both Tr1 and iTr35 cells have potent roles in suppressing autoimmune response and chronic inflammation (34, 35). We have previously noted an increased iTr35 cells and decreased Tr1 cells expression in SSc patients' peripheral blood, and iTr35 cells could prevent T cell multiplication and fibroblast-induced α-SMA expression *in vitro* (33). Comparing Tr1 with iTr35 cells, we observed that elevated iTr35 cells expression was more strongly related with T cell immune response and reduced collagen and α-SMA production, which suggests that lung fibrosis was likely regulated by iTr35 cells via inflammation response. iTr35 cells may mediate the T cells-fibroblasts communication. Furthermore, there was an obvious positive relationship between the expression of iTr35 cells and IL-35, implicating iTr35 cells work in an IL-35-dependent manner. Studies by Wang *et al*. also verified the favourable interaction between IL-35 and iTr35 cells (36). SSc-ILD is a chronic autoimmune disease that can seriously impair life quality of patients and even lead to death (3). Combined therapy is generally adopted with immunosuppressants, biological and antifibrotic therapies, but it has limited effect. iTr35 cells are found to be very stable in the circulation of human body (35), which makes it to be a promising therapeutic target.

Previous studies have shown that the JAK/STAT pathway is involved in the pathogenesis of SSc-ILD (37). Between different STAT subtypes, it seems that STAT3 is predominant in the progression of ILDs (25), which prompt us to concentrate on the impact of IL-35 and IL-10 on STAT3 signalling. Here, we also found that BLM exposure can increase p-STAT3 expression. Indeed, STAT3 can mediate the biological effects of many cytokines, including IL-35 and IL-10. Some studies have revealed that IL-35 can attenuate the phosphorylation of STAT3 (38), while other studies have demonstrated a promoting effect of IL-35 on STAT3 (39). Whereas IL-10 exerts biological effects via upregulated expression of p-STAT3 levels (40). In our study, IL-35 markedly suppressed STAT3 phosphorylation in the lung tissue of the mice induced by BLM, suggesting that IL-35 ameliorates pulmonary fibrosis via the STAT3/p-STAT3 pathway. However, we failed to discover perceptible differences in lung p-STAT3 levels with or without IL-10 treatment, which suggested that the STAT3/p-STAT3 pathway was not necessary for the protective effects of IL-10 on pulmonary fibrosis.

In this study, there was no amplification effect when IL-35 and IL-10 were used together, implicating that they did

not depend on each other for their anti-inflammatory and anti-fibrotic effects. Unexpectedly, there were no significant differences on a range of measures after IL-35 or IL-10 neutralising antibody intervention. One possible explanation for this is that we used neutralising antibody only against p35, one subunit of IL-35, which could not completely neutralise IL-35. In addition, IL-10 neutralising antibody could not work well as a result of the very short half-life (13) and low expression of IL-10 *in vivo*.

In summary, we demonstrate that IL-35 and IL-10 attenuated pulmonary inflammation and fibrosis by inducing Tregs (including nTreg, iTr35 and Tr1 cells) and repressing IL-17A and IL-4 levels in BAL supernatant in a mouse model of SSc. Moreover, IL-35 might play a role in pulmonary inflammation and fibrosis through STAT3 signalling pathway. Furthermore, our results demonstrate that iTr35 cells may be a potential immunomodulatory molecule with anti-fibrotic effects, which offers valuable theoretical basis for the treatment of SSc-ILD. But our research results also indicate that blocking IL-35 and IL-10 does not regulate inflammation and fibrosis in the lungs of SSc mice. In addition to the previously mentioned reasons, it is speculated that the mechanism of lung inflammation and fibrosis in SSc mice is relatively complex, and not only one cell or factor acts, but multiple cells and factors participate in the action. Simply blocking one or two cytokines does not have a significant effect.

Acknowledgments

The authors thank all the participants of this study for their generous assistance.

References

1. DENTON CP, KHANNA D: Systemic sclerosis. *Lancet* 2017; 390(10103): 1685-99. https://doi.org/10.1016/s0140-6736(17)30933-9
2. LEPRI G, DI BATTISTA M, CODULLO V *et al*.: Systemic sclerosis: one year in review 2024. *Clin Exp Rheumatol* 2024; 42(8): 1517-28. https://doi.org/10.55563/clinexprheumatol/is29he
3. PERELAS A, SILVER RM, ARROSSI AV, HIGHLAND KB: Systemic sclerosis-associated interstitial lung disease. *Lancet Respir Med* 2020; 8(3): 304-20. https://doi.org/10.1016/s2213-2600(19)30480-1
4. TYNDALL AJ, BANNERT B, VONK M *et al*.: Causes and risk factors for death in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. *Ann Rheum Dis* 2010; 69(10): 1809-15. https://doi.org/10.1136/ard.2009.114264
5. SAHINOGLU M, SARGIN G, YAVASOGLU I, SENTURK T: The relationship between peripheral T follicular helper cells and disease severity in systemic sclerosis. *Clin Exp Med* 2024; 24(1): 19. https://doi.org/10.1007/s10238-023-01286-9
6. YANG C, LEI L, PAN J *et al*.: Altered CD4+ T cell and cytokine levels in peripheral blood and skin samples from systemic sclerosis patients and IL-35 in CD4+ T cell growth. *Rheumatology* 2022; 61(2): 794-805. https://doi.org/10.1093/rheumatology/keab359
7. HASHIMOTO S, GON Y, TAKESHITA I, MARUOKA S, HORIE T: IL-4 and IL-13 induce myofibroblastic phenotype of human lung fibroblasts through c-Jun NH2-terminal kinase–dependent pathway. *J Allergy Clin Immunol* 2001; 107(6): 1001-8. https://doi.org/10.1067/mai.2001.114702
8. MI S, LI Z, YANG H-Z *et al*.: Blocking IL-17A promotes the resolution of pulmonary inflammation and fibrosis via TGF-β1-dependent and -independent mechanisms. *J Immunol* 2011; 187(6): 3003-14. https://doi.org/10.4049/jimmunol.1004081
9. XING X, LI A, TAN H, ZHOU Y: IFN-γ+IL-17+Th17 cells regulate fibrosis through secreting IL-21 in systemic scleroderma. *J Cell Mol Med* 2020; 24(23): 13600-8. https://doi.org/10.1111/jcmm.15266
10. OUYANG W, RUTZ S, CRELLIN NK, VALDEZ PA, HYMOWITZ SG: Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Ann Rev Immunol* 2011; 29(1): 71-109. https://doi.org/10.1146/annurev-immunol-031210-101312
11. OUYANG W, O'GARRA A: IL-10 family cytokines IL-10 and IL-22: from basic science to clinical translation. *Immunity* 2019; 50(4): 871-91. https://doi.org/10.1016/j.immuni.2019.03.020
12. MARTINEZ JA, KING TE, BROWN K *et al*.: Increased expression of the interleukin-10 gene by alveolar macrophages in interstitial lung disease. *Am J Physiol* 1997; 273(3): L676-L83. https://doi.org/10.1152/ajplung.1997.273.3.L676
13. SHAMSKHOU EA, KRATOCHVIL MJ, ORCHOLSKI ME *et al*.: Hydrogel-based delivery of Il-10 improves treatment of bleomycin-induced lung fibrosis in mice. *Biomaterials* 2019; 203: 52-62. https://doi.org/10.1016/j.biomaterials.2019.02.017
14. YE H, PAN J, CAI X *et al*.: IL-10/IL-10 receptor 1 pathway promotes the viability and collagen synthesis of pulmonary fibroblasts originated from interstitial pneumonia tissues. *Exp Ther Med* 2022; 24(2): 518. https://doi.org/10.3892/etm.2022.11445
15. COLLISON LW, VIGNALI DAA: Interleukin-35: odd one out or part of the family? *Immunol Rev* 2008; 226(1): 248-62. https://doi.org/10.1111/j.1600-065x.2008.00704.x
16. LIU J, SU B, TAO P *et al*.: Interplay of IL-33 and IL-35 modulates Th2/Th17 responses in cigarette smoke exposure HDM-induced asthma. *Inflammation* 2023; 47(1): 173-90. https://doi.org/10.1007/s10753-023-01902-6
17. DANTAS AT, GONÇALVES SMC, PEREIRA MC *et al*.: Increased IL-35 serum levels in systemic sclerosis and association with pulmonary interstitial involvement. *Clin Rheumatol* 2015; 34(9): 1621-5. https://doi.org/10.1007/s10067-015-3006-y
18. TANG J, LEI L, PAN J, ZHAO C, WEN J: Higher levels of serum interleukin-35 are associated with the severity of pulmonary fibrosis and Th2 responses in patients with systemic sclerosis. *Rheumatol Int* 2018; 38(8): 1511-19. https://doi.org/10.1007/s00296-018-4071-8
19. CHEN D, ZHENG G, YANG Q, LUO L, SHEN J: IL-35 subunit EBI3 alleviates bleomycin-induced pulmonary fibrosis via suppressing DNA enrichment of STAT3. *Respir Res* 2021; 22(1): 280. https://doi.org/10.1186/s12931-021-01858-x
20. TOMCIK M, ZERR P, PALUMBO-ZERR K *et al*.: Interleukin-35 is upregulated in systemic sclerosis and its serum levels are associated with early disease. *Rheumatology* 2015; 54(12): 2273-82. https://doi.org/10.1093/rheumatology/kev260
21. KOGA MM, ENGEL A, PIGNI M *et al*.: IL10- and IL35-secreting MutuDC lines act in cooperation to inhibit memory T cell activation through LAG-3 expression. *Front Immunol* 2021; 12: 607315. https://doi.org/10.3389/fimmu.2021.607315
22. SAWANT DV, YANO H, CHIKINA M *et al*.: Adaptive plasticity of IL-10+ and IL-35+ Treg cells cooperatively promotes tumor T cell exhaustion. *Nat Immunol* 2019; 20(6): 724-35. https://doi.org/10.1038/s41590-019-0346-9
23. LU Y, ZHAO C, LEI L *et al*.: Effects of thalidomide on Th17, Treg cells and TGF-β1/Smad3 pathway in a mouse model of systemic sclerosis. *Int J Rheum Dis* 2019; 23(3): 406-19. https://doi.org/10.1111/1756-185x.13769
24. ASHCROFT T, SIMPSON JM, TIMBRELL V: Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 1988; 41(4): 467-70. https://doi.org/10.1136/jcp.41.4.467
25. MONTERO P, MILARA J, ROGER I, CORTIJO J: Role of JAK/STAT in interstitial lung diseases; molecular and cellular mechanisms. *Int J Mol Sci* 2021; 22(12): 6211. https://doi.org/10.3390/ijms22126211
26. CUTOLO M, SOLDANO S, SMITH V: Pathophysiology of systemic sclerosis: current understanding and new insights. *Expert Rev Clin Immunol* 2019; 15(7): 753-64. https://doi.org/10.1080/1744666x.2019.1614915
27. STEEN EH, WANG X, BALAJI S, BUTTE MJ, BOLLYKY PL, KESWANI SG: The role of the anti-inflammatory cytokine interleukin-10 in tissue fibrosis. *Adv Wound Care* 2020; 9(4): 184-98. https://doi.org/10.1089/wound.2019.1032
28. KUDO H, WANG Z, JINNIN M *et al*.: EBI3 Downregulation contributes to type I collagen overexpression in scleroderma skin. *J Immunol* 2015; 195(8): 3565-73. https://doi.org/10.4049/jimmunol.1402362
29. SHI J-H, GUAN H, SHI S *et al*.: Protection against TGF-β1-induced fibrosis effects of IL-10 on dermal fibroblasts and its potential therapeutics for the reduction of skin scarring.

Arch Dermatol Res 2013; 305(4): 341-52. https://doi.org/10.1007/s00403-013-1314-0
30. TALOTTA R: The rationale for targeting the JAK/STAT pathway in scleroderma-associated interstitial lung disease. *Immunotherapy* 2021; 13(3): 241-56. https://doi.org/10.2217/imt-2020-0270
31. JIN W, ZHENG Y, ZHU P: T cell abnormalities in systemic sclerosis. *Autoimmun Rev* 2022; 21(11): 103185. https://doi.org/10.1016/j.autrev.2022.103185
32. COOMES SM, KANNAN Y, PELLY VS *et al*.: CD4+ Th2 cells are directly regulated by IL-10 during allergic airway inflammation. *Mucosal Immunol* 2017; 10(1): 150-61. https://doi.org/10.1038/mi.2016.47
33. YANG C, LU C, PAN J *et al*.: The role of iTr35 cells in the inflammatory response and fibrosis progression of systemic sclerosis. *Rheumatology* 2023; 62(10): 3439-47. https://doi.org/10.1093/rheumatology/kead053
34. POT C, APETOH L, KUCHROO VK: Type 1 regulatory T cells (Tr1) in autoimmunity. *Semin Immunol* 2011; 23(3): 202-8. https://doi.org/10.1016/j.smim.2011.07.005
35. YANG C, DONG L, ZHONG J: Immunomodulatory effects of iTr35 cell subpopulation and its research progress. *Clin Exp Med* 2024; 24(1): 41. https://doi.org/10.1007/s10238-024-01303-5
36. WANG W, WEI C, CHENG Z, YANG J: Aberrant Th2 immune responses are associated with a reduced frequency of IL-35-induced regulatory T cells after allergen exposure in patients with allergic asthma. *Allergy Asthma Immunol Res* 2020; 12(6): 1029-45. https://doi.org/10.4168/aair.2020.12.6.1029
37. WANG W, BHATTACHARYYA S, MARANGONI RG *et al*.: The JAK/STAT pathway is activated in systemic sclerosis and is effectively targeted by tofacitinib. *J Scleroderma Relat Disord* 2020; 5(1): 40-50. https://doi.org/10.1177/2397198319865367
38. KANAI K, PARK A-M, YOSHIDA H, TSUNODA I, YOSHIE O: IL-35 Suppresses lipopolysaccharide-induced airway eosinophilia in EBI3-deficient mice. *J Immunol* 2017; 198(1): 119-27. https://doi.org/10.4049/jimmunol.1600506
39. XIE M, ZHU Y, ZHOU Y *et al*.: Interleukin-35-producing B cells rescues inflammatory bowel disease in a mouse model via STAT3 phosphorylation and intestinal microbiota modification. *Cell Death Discov* 2023; 9(1): 67. https://doi.org/10.1038/s41420-023-01366-5
40. GUO D, KAN S, ZHANG L *et al*.: IL-10 enhances cell-to-cell communication in chondrocytes via STAT3 signaling pathway. *Cell Signal* 2023; 105. https://doi.org/10.1016/j.cellsig.2023.110605