

Anti-IgE therapy inhibits chemotaxis, proliferation and transformation of circulating fibrocytes in patients with severe allergic asthma

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Abstract

Background and objective: Circulating fibrocytes act as precursors of myofibroblasts, contribute to airway remodelling in chronic asthma and migrate to injured tissues by expressing CXCR4 and CCR7. Anti-IgE therapy improves severe allergic asthma (SAA) control and airway remodelling in T2-high SAA. The effects of anti-IgE therapy on fibrocyte activities were investigated in this study.

Methods: The expression of CCR7, CXCR4, ST2 and α -SMA (α -smooth muscle actin) in both circulating and cultured fibrocytes from all patients with asthma was measured, and was repeated after omalizumab treatment in SAA. Fibrocytes recruitment, proliferation and transformation were also measured in response to anti-IgE therapy.

Results: Omalizumab effectively improved asthma control and pulmonary function in T2-high SAA, associated with a decline in serum levels of IL-33 and IL-13. Omalizumab down-regulates CXCR4 and CCR7 expression of fibrocytes, which could suppress fibrocyte recruitment into the lungs. Omalizumab also suppressed the increased number of fibrocytes and α -SMA⁺ fibrocytes within the cultured non-adherent non-T (NANT) cells after 3–7 days of culture. The decrease in serum levels of IL-33 by omalizumab contributed to the effectiveness in inhibiting fibrocyte recruitment, proliferation and myofibroblast transformation through IL-33/ST2 axis. The elevated IL-13 expression in SAA patients potentiated the effects of IL-33 by increasing ST2 expression.

Conclusion: Omalizumab reduced the number of circulating fibrocytes, cell and number of fibrocytes as well as α -SMA⁺ fibrocytes after 3–7 days of culture in SAA patients. IL-33 and IL-13 may be implicated in the effectiveness of omalizumab in inhibiting fibrocyte activation contributing partly to the clinical benefits in reducing lamina propria and basement membrane thickening.

KEYWORDS

airway remodelling, anti-IgE therapy, chemokine receptors, chemotaxis, fibrocyte activation, omalizumab, severe allergic asthma

INTRODUCTION

Circulating fibrocytes have been shown to act as precursors of bronchial myofibroblasts.¹ We have shown these cells differentiate into fibroblasts and myofibroblasts,² which emerge at the airway tissue in fibrotic change,³ and are associated with airflow obstruction.² Fibrocytes recruited

to tissue damage sites in response to specific chemokine/receptors axis.^{4,5} We have demonstrated CXCR4/CXCL12 axis mediates fibrocyte chemotaxis in asthmatics with acute exacerbation, whereas CCR7/CCL19 axis plays a more important role in chronic obstructive asthmatics.⁶ However, the underlying mechanisms in regulation of these chemokine receptors expression are not yet elucidated.

Omalizumab, a humanized recombinant monoclonal anti-IgE antibody reducing the crosslink between IgE and

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IgE receptor (FcεRI),^{7,8} has been widely used for the treatment of severe allergic asthma (SAA).^{9–12} Both animal^{13,14} and clinical studies^{15,16} have shown omalizumab treatment reduces collagen and fibronectin deposition, reticular basement membrane (RBM) thickness and bronchial wall thickness, especially in patients with eosinophilic airway inflammation.¹⁵ Omalizumab treatment has been shown to significantly decrease airway expression of IL-33, IL-25 and TSLP (Thymic stromal lymphopoietin),¹⁷ and also airway IL-13 expression in SAA.¹⁷ IL-13 is implicated in airway remodelling by directly promoting fibroblast proliferation.^{18–20} IL-33 is also an important contributor to airway remodelling in allergen-induced asthma by reducing IL-33-driven type 2 cytokines, including IL-13 release,²¹ but IL-33/ST2 axis may drive airway remodelling beyond the type 2 responses,²¹ by directly promoting collagen deposition from asthmatic fibroblasts.^{21,22} IL-33 also enhances IL-13, IL-5, α-SMA expression and pro-MMP-9 activity of fibrocytes,²³ and increases fibrocyte chemotaxis and migration in allergen exacerbated asthma.²³ Therefore, it is possible that the clinical benefits of omalizumab in airway remodelling in SAA may be partly attributed to the effects of IL-33 on circulating fibrocyte recruitment, proliferation and myofibroblast transformation.

METHODS

Study population

Fourteen non-smoking stable asthmatics under low-dose inhaled beclomethasone (0–1000 µg/day or equivalent, inhaled corticosteroid) with well control of their asthma symptoms (Asthma Control Test [ACT] scores >23) and normal pulmonary function (mild asthma) were recruited. Thirteen non-smoking patients with T2-high SAA with poor asthma control (ACT < 19) were recruited and treated with anti-IgE antibody therapy (omalizumab) monthly for >3 months. The clinical characteristics of those mild and SAA are listed in Table 1. All of asthmatic subjects had been followed up at our clinics for >3 years and stable for at least 3 months. Fibreoptic bronchoscopy was performed in 11 SAA patients and bronchial biopsies were obtained.

Measurement of IL-33, IL-13 in serum from asthma patients

The serum levels of IL-33, and IL-13 were measured by quantitative sandwich-type enzyme-linked immunoassay (ELISA) kits (R&D, MN, USA). The procedure was performed according to manufacturer's illustration.

Circulating progenitor fibrocyte preparation

Preparation of fibrocytes had been described previously.²⁴ In brief, peripheral blood mononuclear cells were separated using

SUMMARY AT A GLANCE

Omalizumab suppressed fibrocyte chemotaxis, proliferation and myofibroblast transformation in severe allergic asthma patients. IL-33 and IL-13 may be implicated in the effectiveness of omalizumab in inhibiting fibrocyte activation contributing partly to the clinical benefits in reducing lamina propria and basement membrane thickening.

Ficoll–Hypaque density gradient. After adherence, the non-adherent mononuclear cells were depleted of T cells by sheep red blood cells and anti-CD3 magnetic beads with the remaining T lymphocytes <1% (non-adherent non-T, NANT cells).

Fibrocyte expression of CXCR4, CCR7, and ST2

Fibrocytes in NANT cells were verified as described previously.²⁴ The NANT cells at 1×10^5 cells/ml were immersed with specific antibodies and quantitated for the expression of Col I, CD45, CXCR4, CCR7, ST2 and α-SMA by flow-cytometry (Becton Dickinson).

Fibrocyte treatment and culture

NANT cells (3×10^5) from mild or SAA patients were treated with IL-33, IL-13 or both in the presence or absence of anti-ST2 antibodies. After culture, cells were gently scraped off the dishes, viability measured and the number of

TABLE 1 Clinical characteristics

	Mild asthma (N = 14)	Severe allergic asthma (N = 13)
Age, years	56.8 ± 6.4	66.2 ± 5.1
Gender, M/F	6/8	3/10
FVC, L	3.1 ± 0.3	1.6 ± 0.2**
FVC, % predicted	92.5 ± 2.4	51.9 ± 3.4**
FEV ₁ , L	2.8 ± 0.2	1.2 ± 0.1**
FEV ₁ , % predicted	87.6 ± 2.6	41.4 ± 2.8**
FEV ₁ /FVC, %	81.6 ± 1.3	61.7 ± 3.6**
ACT scores	23.4 ± 0.6	11.3 ± 0.8**
FeNO, ppb	18.6 ± 3.5	42.3 ± 8.7*
IgE, IU/ml	378.9 ± 108.3	598.7 ± 135.6*
Absolute eosinophil counts	189.5 ± 52.6	549.1 ± 46.5*
Serum IL-33 (pg/ml)	2.3 ± 1.3	37.8 ± 1.1**
Serum IL-13 (pg/ml)	163.3 ± 6.2	218.8 ± 3.0**

Abbreviations: ACT, Asthma Control Test; F, female; FeNO, fraction of exhaled nitric oxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; M, male.

***p* < 0.001, **p* < 0.05 compared to asthmatics with mild asthma.

fibrocytes (CD45⁺/ColI⁺ cells) and the proportion of CXCR4, CCR7 and α -SMA expression in fibrocytes were determined after correction with viability.

Fibrocyte immunofluorescence labelling

Purified fibrocytes of SAA patients were incubated with specific antibodies for α -SMA-conjugated PE (Phycoerythrin) and ST2-conjugated FITC (Fluorescein isothiocyanate). The fluorescence-labelled slides were examined by confocal laser scanning microscopy.²⁵

Study of fibrocyte migration

The fibrocyte migration was determined as described previously.⁶ In brief, fibrocytes were purified from NANT cells by positive selection with CD34 microbeads (MACS, Miltenyi Biotech), and were labelled with carboxyfluorescein succinimidyl ester (CFSE) for migration assay. The purified fibrocytes from SAA asthmatics were cultured in non-coating round-bottom falcon tubes to prevent adherence and differentiation, and stimulated with IL-33 or vehicle for 3 h or 7 days. After being cultured, purified fibrocytes were incubated with anti-CXCR4 or anti-CCR7 antibodies for 30 min. The 1×10^4 pretreated CFSE-labelled fibrocytes were seeded onto the top channel of chip and CXCL12 or CCL19 was injected in the bottom channel. Two hours later, the cells migrated to the bottom channel were counted, collected and immunofluorescence labelled for the expression of collagen I (FITC) and CD45 (PE). Fibrocytes occupied more than 90% of migrated cells.

Statistical analysis

Nonparametric statistical analyses were employed, and the probability of differences between groups was initially assessed by Kruskal–Wallis analysis, or Mann–Whitney *U*-test (two-tailed) to assess the significance of differences between groups. Wilcoxon matched-pairs signed rank test was used for comparison within groups. Data are represented as means $\pm$ SD. The null hypothesis was rejected at $p < 0.05$.

RESULTS

Clinical characteristics of study subjects

SAA patients had increased serum IgE levels (598.7 ± 135.6 IU/ml), high blood eosinophil counts (502.9 ± 74.5 /ml), high fraction of exhaled nitric oxide (42.3 ± 8.7 ppb) and persistent asthma symptoms (ACT scores 11.3 ± 0.8), impaired pulmonary function (forced vital capacity $46.5 \pm 9.4\%$ predicted and forced expiratory

volume in 1 s $37.4 \pm 6.8\%$ predicted) and higher serum levels of IL-33 and IL-13 compared with mild asthmatics (Table 1). SAA patients had improved asthma control and pulmonary function after 3 months of omalizumab treatment, and decreased thickness of RBM and lamina propria, and eosinophil infiltration (Figure 1A) as well as serum levels of IL-33 and IL-13 (Figure S1 in the Supporting Information). The total number of circulating fibrocytes, CXCR4 and CCR7 expression in circulating fibrocytes were also decreased after omalizumab treatment in SAA patients (Figure 1B–D). After 3–7 days of culture, a decrease in the number of fibrocytes (3 days of culture) and the number of α -SMA⁺ fibrocytes (7 days of culture) within the cultured NANT cells were found in SAA patients after omalizumab treatment (Figure 1E,F).

Effects of IL-33 on circulating fibrocytes

Higher number of circulating fibrocytes in SAA patients expressed IL-33 receptor, ST2 (Figure 2A). The ST2 expression increased with time (Figure 2B), and was further up-regulated by IL-33 (10 ng/ml) in the fibrocytes within cultured NANT cells of SAA (Figure 2C). The ST2 expression was most prominent in α -SMA⁺ fibrocytes (Figure 2D). Stimulation of NANT cells from SAA patients with IL-33 (10 ng/ml) concentration-dependently increased CXCR4 expression on fibrocytes at 3–6 h of culture (Figure 3A). IL-33 also concentration-dependently induced CCR7 expression at 3–7 days of culture (Figure 3B). Anti-ST2 antibodies completely inhibited the effects of IL-33 (10 ng/ml) on CXCR4 or CCR7 expression of cultured fibrocytes (after 3–6 h and 7 days, respectively) (Figure 3C,D).

Functional relevance of IL-33 induced CXCL4 and CCR7 expression on fibrocytes

After 3-h stimulation with IL-33 (10 ng/ml), purified fibrocytes from SAA patients increased their migratory activities in response to CXCL12, but not CCL19 (Figure 3E). The enhanced migratory activity was suppressed by anti-CXCR4, but not anti-CCR7 antibodies (Figure 3E). Seven days after stimulation with IL-33, purified fibrocytes from SAA patients increased their migratory activities in response to CCL19, but not CXCL12 (Figure 3F). Anti-CCR7, but not anti-CXCR4, antibodies completely inhibited the IL-33 enhanced migratory activities (Figure 3F).

IL-33 increased fibrocyte and α -SMA⁺ fibrocyte cell numbers

SAA patients had increased cell number of fibrocytes and α -SMA⁺ fibrocytes within the cultured NANT cells, compared with mild asthmatics, at 3 or 7 days of culture (Figure S3 in the Supporting Information). IL-33 (10 ng/ml)

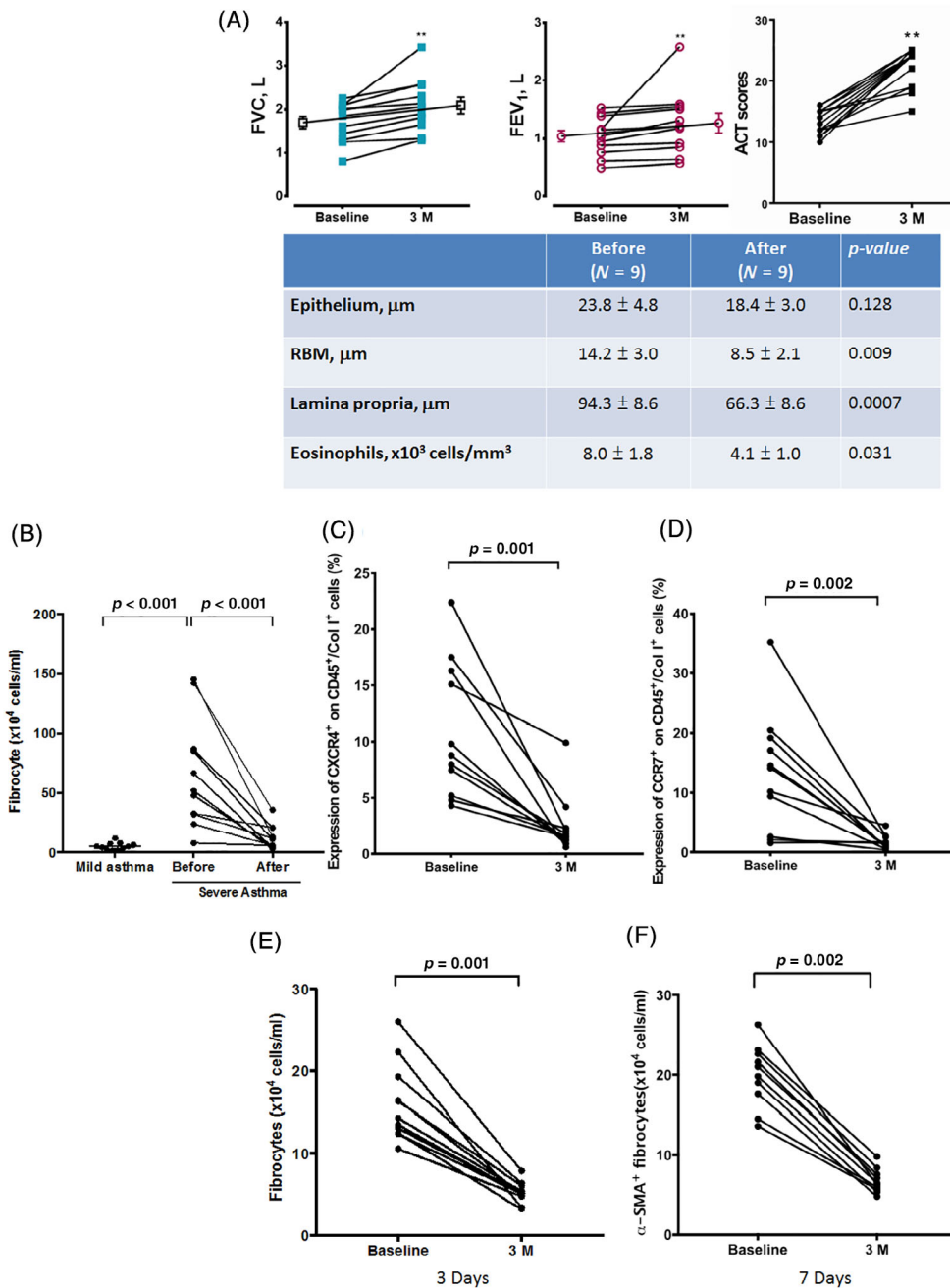


FIGURE 1 (A) Treatment with omalizumab significantly improved pulmonary function and Asthma Control Test scores ($N = 13$), subepithelial fibrosis and eosinophil infiltration determined by measuring the thickness of reticular basement membrane and lamina propria areas, and eosinophil counts in immunohistochemical staining of bronchial biopsied tissues from nine patients with severe allergic asthma (SAA) before and 3 months (3 M) after omalizumab treatment. (B) The number of circulating fibrocytes ($\text{CD45}^+/\text{Col I}^+$ cells) of patients with mild asthma ($N = 14$) and severe asthma ($N = 13$) before and 3 M after omalizumab treatment. The horizontal line is presented as median, and each dot represents an individual patient. $**p < 0.01$ compared with before treatment. p -Values are also indicated in the figures. The number and proportion of circulating fibrocytes ($\text{CD45}^+/\text{Col I}^+$ cells) expressing (C) CXCR4 and (D) CCR7 in patients with mild asthma (mild asthma, $N = 14$) and SAA (severe asthma, $N = 13$) before (baseline) and 3 M after omalizumab treatment. The number of fibrocytes (E) and $\alpha\text{-SMA}^+$ fibrocytes (F) within the cultured NANT cells of SAA (severe asthma, $N = 13$) after 3 and 7 days of culture, respectively, before (baseline) and 3 M after omalizumab treatment. Each dot represents an individual patient, and horizontal lines indicate median values. p -Values are indicated in the figures. The Wilcoxon matched-pairs signed rank test was used for comparison within groups

significantly increased cell number of fibrocytes and $\alpha\text{-SMA}^+$ fibrocytes within the cultured NANT cells of SAA patients (Figure S3 in the Supporting Information), but not in mild asthmatics (Figure S2 in the Supporting Information). Anti-ST2 antibodies reduced both baseline and IL-33-induced increase in cell number of fibrocyte and $\alpha\text{-SMA}^+$ fibrocytes in SAA patients (Figure S3 in the Supporting Information).

Synergy between IL-13 and IL-33

Stimulation of NANT cells from mild asthmatics with IL-13 (10 ng/ml) did not increase the proportion of cultured

fibrocytes expressing CXCR4 (Figure S4 in the Supporting Information), but induced CCR7 expression after 72 h of culture (Figure S4 in the Supporting Information). IL-13 (10 ng/ml) did not increase cell number of fibrocytes (Figure S4 in the Supporting Information), but increased the proportion of $\alpha\text{-SMA}^+$ fibrocytes within the cultured NANT cells after 7 days of culture (Figure S4 in the Supporting Information). IL-13 (10 ng/ml) increased the proportion of fibrocytes expressing ST2 after 6 h of culture (Figure 4A). Neither IL-13 (1 ng/ml) nor IL-33 (0.1 ng/ml) increased CXCR4 and CXCR7 expression, proliferation or transformation in fibrocytes of mild asthmatics. However, there was a synergistic effect between IL-13 (1 ng/ml) and

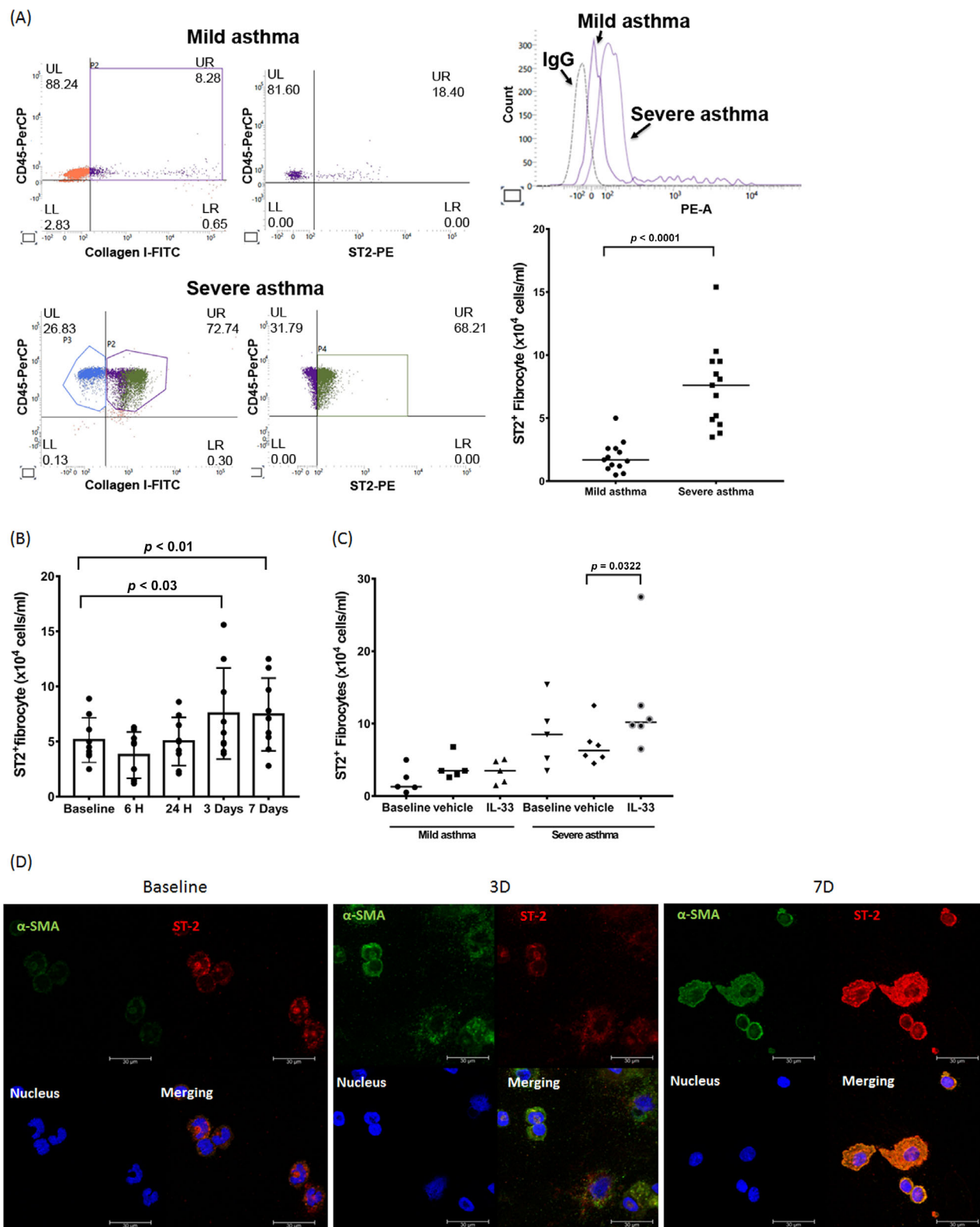


FIGURE 2 (A) Represented dot plots showing the ST2 percentage of CD45⁺/Coll⁺ fibrocytes isolated from mild and severe asthma, and histogram plot indicated increase of ST2 fluorescence intensity in severe asthma. The number of circulating fibrocytes expressing ST2 in patients with mild asthma (mild asthma, $N = 14$) and severe allergic asthma (SAA) (severe asthma, $N = 13$). (B) Fibrocytes of SAA ($N = 9$) within the cultured NANT cells for 6 h, 24 h, 3 days and 7 days increased ST2 expression with time. (C) Fibrocytes of mild asthmatics ($N = 5$) or SAA (severe asthma, $N = 6$) within NANT cells cultured with IL-33 (10 ng/ml) or its vehicle for 3 days. (D) Representative immunofluorescence staining for α -SMA (green), ST2 (red) and DAPI (4',6-diamidino-2-phenylindole) (blue) in fibrocytes of SAA. Data are means $\pm$ SD, and each dot represents an individual. The horizontal lines indicate median values. The Mann-Whitney U -test (A) or Kruskal-Wallis analysis (B,C) was used to assess the significance of differences between groups. p -Values are indicated in the figures

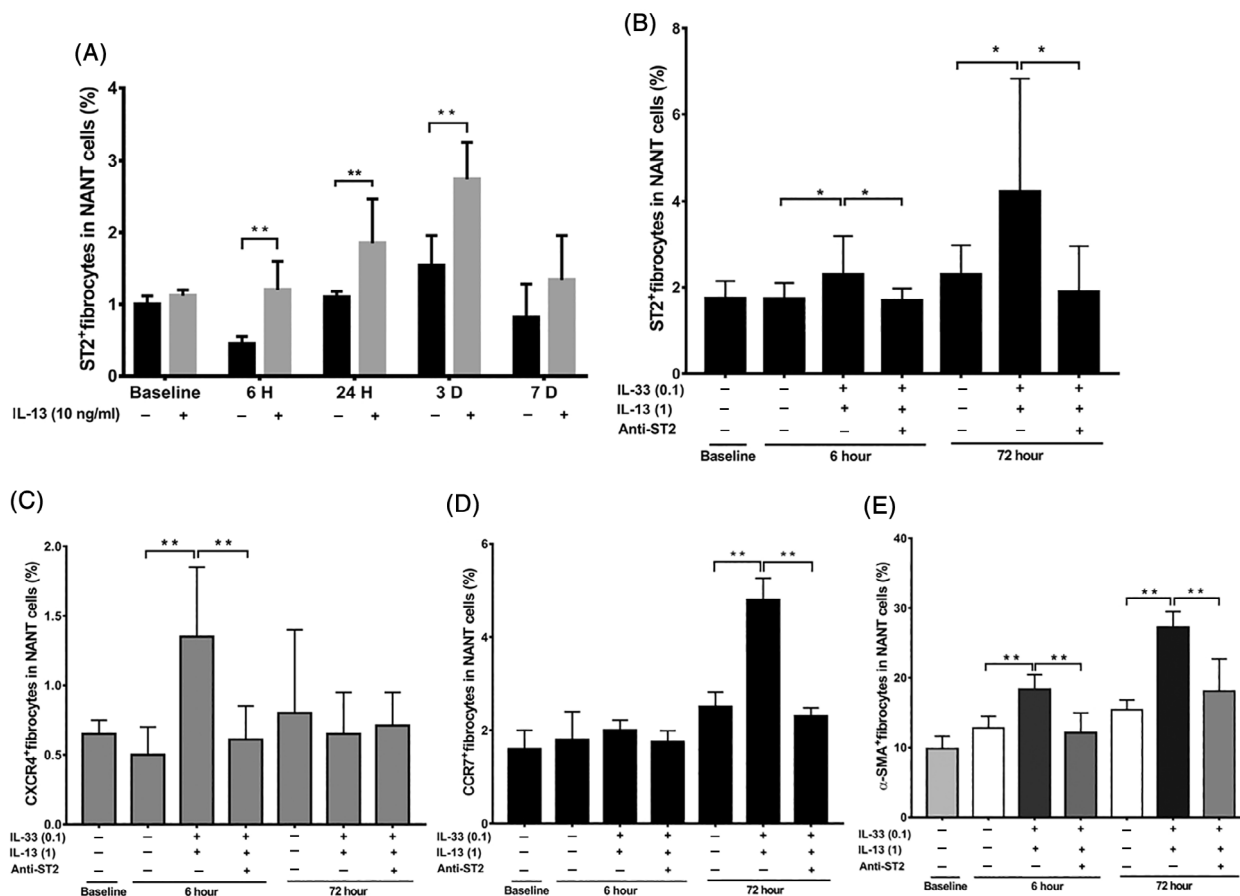


FIGURE 4 (A) The number of fibrocytes from mild asthmatics ($N = 6$) expressing ST2 within NANT cells cultured with IL-13 (10 ng/ml) or its vehicle for 6 h, 24 h, 3 and 7 days. (B) The number of fibrocytes from mild asthmatics ($N = 7$) expressing ST2 within NANT cells cultured with IL-13 (1 ng/ml) and IL-13 (0.1 ng/ml) or their vehicles for 6 and 72 h in the presence or absence of anti-ST2 antibodies (anti-ST2). The number of fibrocytes expressing CXCR4 (C), CCR7 (D) or α -SMA (E) within NANT cells cultured with IL-13 (1 ng/ml) and IL-13 (0.1 ng/ml) or their vehicles for 6 and 72 h in the presence or absence of anti-ST2 antibodies (anti-ST2). Data are means $\pm$ SD. Each dot represents an individual. The horizontal line indicates median. The Kruskal-Wallis analysis was used to assess the significance of differences between groups. * $p < 0.05$; ** $p < 0.01$ compared with the corresponding vehicle controls

were responsive to IL-33 to increase both CXCR4 and CCR7 expression, and enhanced their migratory activity in response to chemotactic factors, CXCL12 and CCL19, respectively. CXCR4 expression appeared as early as 3–6 h after IL-33 stimulation, and then declined thereafter. Previous studies have indicated that CXCR4 protein expression may be induced after 24 h of stimulation.^{28,29} The CXCR4 protein may be internalized and recycled back to plasma membrane through an actin-dependent manner.^{30,31} The rate of CXCR4 recycling is cell type specific, and the time for 80% recycling is up to 90 min in the immune cells.³¹ In our study, the duration of IL-33 stimulation was 3–6 h, not long enough for de novo synthesis. Therefore, the increased CXCR4 expression by IL-33 may be through the relocalization effect in fibrocytes. In contrast, CCR7 did not appear until 3 days after IL-33 stimulation, and reached the maximum at 7 days of stimulation. The expression of chemokine receptors appeared to be related to fibrocyte maturation, where CXCR4 expression regressed when fibrocytes differentiated into α -SMA⁺ fibrocytes, whereas CCR7 expression emerged. The stimulatory effects of IL-33 were

suppressed by anti-ST2 antibodies, mediated through IL-33/ST2 axis. The increased number of fibrocyte and α -SMA⁺ fibrocytes in SAA patients was partly inhibited by anti-ST2 antibodies, suggesting the contribution of IL-33 in SAA patients, and the up-regulated ST2 expression appeared a prerequisite for IL-33 to induce fibrocyte chemotaxis, proliferation and fibrotic phenotype transformation.

The mechanistic causes for up-regulated expression of ST2 in SAA fibrocytes were not yet elucidated. In the present study, ST2 expression in fibrocytes increased with time of culture, paralleling with fibrocyte maturation. Thus, the increased number of circulating ST2⁺ fibrocytes in SAA patients may represent increased number of α -SMA⁺ fibrocytes in SAA, compared with mild asthmatics, as that has been reported in unstable asthmatics.³² IL-33 was shown to increase ST2 expression in SAA fibrocytes, suggesting IL-33 might self-perpetuate its response by up-regulating ST2 expression. Th2 cytokines, such as IL-4 and IL-13, have been shown to induce ST2 expression in several cell types, including airway epithelial cells,^{33,34} fibroblasts³⁵ and endothelial cells through MAPK

(mitogen-activated protein kinase) pathway.³³ Compatible with previous studies,^{36,37} IL-13 was shown in the present study to induce α -SMA expression and myofibroblastic differentiation, and increased CCR7 expression after 3–7 days of stimulation. Stimulation of fibrocytes with a combination of IL-13 and IL-33 at low concentrations, but not alone, increased ST2 expression after 6 h of culture, and persisted for more than 3 days. The up-regulated ST2 expression by IL-13 greatly potentiated IL-33 to increase both chemokine receptor expression and the capacity for α -SMA⁺ fibrocyte transformation.

One of the major limitations of our study was that it was not performed with purified fibrocytes. As fibrocytes are recognized as ColI⁺CD45⁺ cells, the intracellular staining for ColI causes purified fibrocytes not viable for functional study. The results of fibrocytes from NANT cell culture might be influenced by cells other than fibrocytes.

In conclusion, our present study demonstrated that omalizumab reduced the number of circulating fibrocytes, and inhibited the increase in cell number of total fibrocytes and α -SMA⁺ fibrocyte after 3–7 days of NANT cell culture in SAA with T2-high phenotype. The effectiveness of omalizumab in down-regulating chemokine receptor expression may be attributable to the effects of IL-33 on both CXCR4 and CCR7, and the effects of IL-13 on CCR7. Omalizumab inhibited the increase in cell number of total fibrocytes and α -SMA⁺ fibrocyte after 3–7 days of NANT cell culture possibly through an inhibition of the direct effect of IL-33 on fibrocytes. IL-13 may potentiate the effects of IL-33 by increasing ST2 expression. The inhibitory effects of omalizumab on circulating fibrocytes in patients with SAA may contribute in part to the clinical benefits in reducing lamina propria and RBM thickening.

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AUTHOR CONTRIBUTIONS

Chun-Hua Wang: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing-original draft; writing-review & editing. **Chih-Ming Weng:** Data curation; formal analysis; investigation; methodology; project administration; validation; visualization; writing-original draft; writing-review & editing. **Tzu-Ting Huang:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing-review & editing. **Meng-Jung Lee:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing-review & editing. **Chun-Yu Lo:** Formal analysis; resources; writing-review & editing. **Mei-Chuan Chen:** Formal analysis; resources; writing-review & editing. **Chung-Liang Chou:** Formal analysis; resources; writing-

review & editing. **Han-Pin Kuo:** Conceptualization; funding acquisition; project administration; resources; supervision; writing-original draft; writing-review & editing.

CONFLICT OF INTEREST

Han-Pin Kuo is a consultant for NovartisTaiwan; a speaker for GSK, Novartis, Elli Lilly, Pfizer, Roche, Boehringer Ingelheim, Astra-Zeneca, Sanofi-Aventis and MSD; and has received research grants from Elli Lilly, Sanofi-Aventis, Roche, Astra-Zeneca and GSK. The other authors declare that they have no conflicts of interest.

HUMAN ETHICS APPROVAL DECLARATION

This study was performed in accordance with the Declaration of Helsinki and approved by TMU-Joint Institutional Review Board (N201807020). All adult participants provided written informed consent to participate in this study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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