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Original Article

A nerve growth factor-blocking antibody ameliorates ovalbumin-induced chronic allergic asthma by suppressing TGF-β/Smad signaling

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Abstract: Excessive airway remodelling that occurs as a consequence of repetitive injury-repair cycles plays an important role in the pathogenesis of chronic asthma. Our study aimed to determine whether anti-nerve growth factor (NGF) therapy plays a role in preventing airway remodelling via the transforming growth factor (TGF)-β/Smad signaling pathway in a murine model of chronic asthma. TGF-β_1 mRNA levels of were detected by quantitative real-time PCR (QRT-PCR). Histological examination and Masson's trichrome staining were used to evaluate pathological lung changes. The expression of TGF-β_1, P-Smad$_3$, Smad$_7$, and the downstream mesenchymal markers Snail, Slug, and α-SMA were measured using immunohistochemistry staining and Western blotting. Functional blockade of NGF in the asthmatic mice dramatically prevented lung inflammation and airway remodelling. TGF-β_1 and P-Smad$_3$ expression were decreased in the anti-NGF group. In bronchial epithelial cells, the TGF-β/Smad-induced expression of the mesenchymal markers Snail, Slug, and α-SMA were inhibited by the anti-NGF antibody. NGF exerts profibrotic effects on airways, which might be mediated by the TGF-β/Smad-induced epithelial-mesenchymal transition.

Keywords: Nerve growth factor, epithelial-mesenchymal transition, TGF-β_1, allergic asthma, signalling

Introduction

Nerve growth factor (NGF) is involved in the pathogenesis of allergic airway inflammation *in vivo*, and induces the proliferation of airway smooth muscle cells [1]. Airway remodelling and chronic inflammation lead to irreversible airway obstruction and persistent airway hyper-responsiveness. These changes cause further chronic allergic airway inflammation, stimulation of airway hyper-responsiveness, and additional airway remodelling, thus forming a vicious circle [2]. Previous studies have shown that anti-NGF therapy can inhibit the synthesis and release of inflammatory mediators in airway inflammation models of asthmatic rats [3, 4]. However, the molecular mechanism underlying anti-NGF treatment in asthma remains largely unclear. Several studies have shown that transforming growth factor (TGF)-β/Smad signaling plays an important role in the airway remodeling that occurs with asthma [5, 6]. Recently, NGF has been shown to promote renal tubular epithelial mesenchymal transformation (EMT) via the TGF-β_1 signaling pathway [7]. However, it remains unknown that whether TGF-β/Smad signaling is involved in the effect of anti-NGF therapy for asthma.

This study aimed to investigate whether anti-NGF therapy for asthma prevents inflammation and airway remodeling by regulating TGF-β/Smad signaling in a murine asthma model.

Materials and methods

Animals and groups

Six-to-eight-week-old female BALB/c mice were obtained from the Laboratory Animal Centre of Guilin Medical University (Guilin, China). All animals had free access to water, and were maintained in a clean, quiet, and dimly lighted environment at room temperature. All animal exper-

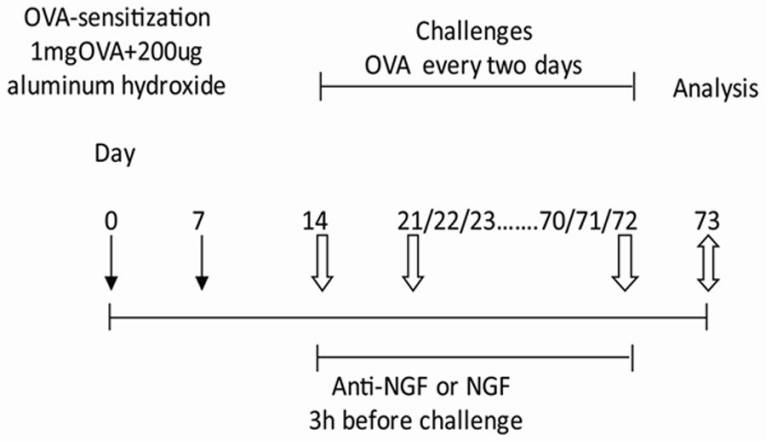

Figure 1. Effects of the anti-NGF antibody or NGF on experimentally induced chronic ovalbumin (OVA)-induced allergic asthma.

iments were performed according to the guidelines recommended by the Ministry of Science and Technology of the People's Republic of China [(2006)398].

Ovalbumin (OVA) sensitization and challenge

Mice were randomly assigned into the following four groups (eight animals/group): control, asthma, NGF, and anti-NGF. The sensitization and challenge protocols were performed according to the methods described by Li and Shang [8], with modifications described below. On days 0 and 7, all mice, except for mice in the control group, were sensitized with an intraperitoneal injection of 1 mg of OVA (Grade V; Sigma, St. Louis, MO, USA) and 200 μg of aluminum hydroxide (Aldrich, Milwaukee, WI, USA) in 0.5 ml sterile phosphate-buffered saline (PBS).

OVA-sensitized mice were exposed to 1% aerosolized OVA (1 g OVA in 100 ml sterile PBS in a nebulizer) for 30 min every 2 days from days 14 to 72. We used exogenous murine NGF (NGF-7S; Alomone Labs, Jerusalem, Israel), and blocked the activity of endogenous NGF using 100 μg/ml goat anti-NGF antibody (R&D Systems, Minneapolis, MN, USA). Mice in the NGF and anti-NGF groups received an intraperitoneal injection of NGF-7S (80 ng/kg) and anti-NGF antibody (4 ml/kg) diluted at a ratio of 1:1,000 in sterile PBS 3 h before the OVA aerosol challenge. Mice in the asthma group received an intraperitoneal injection of 4 ml/kg PBS 3 h before the OVA aerosol challenge. The intervention administration routes, timing, and doses of the two types of intervention agents were chosen based on a prior study [8]. Mice in the control group were subjected to the same protocol using sterile PBS (**Figure 1**).

Bronchial responsiveness

The responsiveness of the mice to methacholine (Mch; Sigma-Aldrich) was evaluated using whole-body plethysmography (EMKA, Paris, France). Increases in the average pulmonary resistance were measured and used as an index of airway obstruction. Airway reactivity was expressed as the fold-increase in the average pulmonary resistance to each Mch concentration compared with the average pulmonary resistance observed after the PBS challenge.

Total cell count and bronchoalveolar lavage fluid (BALF) collection

BALF was collected from the left lung by adjusting the trachea cannula and rinsing with 1 ml of saline three times. The concentrations of IL-4 and IL-13 in the cell-free fluid were determined using ELISA (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Detection of TGF-β_1 in BALF

The concentration of TGF-β_1 in the BALF was measured using ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For the TGF-β_1 assay, the activated samples were transferred into 96-well plates coated with TGF-β_1 soluble receptor Type II. After incubation for 15 min, TGF-β_1 was detected by a horseradish peroxidase-based colorimetric assay.

Histological examination of lung tissues

Sections of lung tissues were stained with hematoxylin and eosin (H&E). In addition, Masson's trichrome staining was used to measure collagen deposition, and the thickness of smooth muscle. Immunohistochemistry was performed to measure the levels of specific proteins. Collagen area measurements were

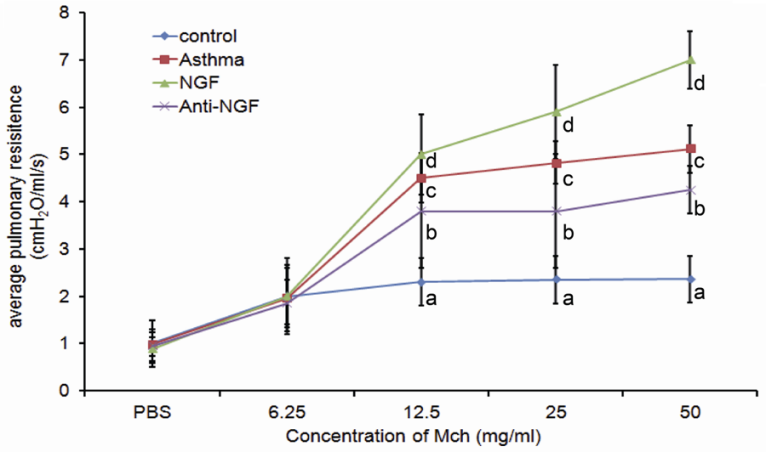

Figure 2. Comparison of the average pulmonary resistance following the administration of methacholine (Mch) in mice from each group before and after the challenge. Groups with different letters are significantly different ($P < 0.05$) in the same concentration.

performed as previously described [9]. A semi-quantitative immunohistochemical method was used to detect the expression level of TGF-β_1 protein. Lung tissue sections were incubated with 0.01 mol/l citric acid buffer (pH 6.0) for 15 min in a microwave for antigen retrieval, followed by incubation with H_2O_2 (3 g/l) for 30 min. The slides were then incubated with horse serum (1:10 dilution) for 30 min, followed by incubation with anti-TGF-β_1 antibody (1:400 dilution). Slides were then incubated with horseradish peroxidase-conjugated goat anti-rat secondary antibody, followed by addition of the diaminobenzidine chromogen substrate. The intensity of TGF-β_1 expression was detected by a microscope using Image-pro plus 6.0 software.

Quantitative real-time PCR (QRT-PCR)

Total RNA was extracted using a PicoPure RNA isolation kit (RR037A, Takara, Tokyo, Japan) according to the manufacturer's instructions, and was reverse transcribed into complementary DNAs (Robo Cycler, Stratagene, USA) using random hexamers and AMV reverse transcriptase (Promega, USA). The complementary DNAs were amplified by QRT-PCR (Rotor-Gene 3000, Corbett Research, Australia) using the SYBR Green PCR Master Mix Reagent (Invitrogen, Carlsbad, CA, USA). The primers were as follows, and based on prior literature [13]. TGF-β_1 (5'-ACCTGCAAGACCATCGACAT-3', 5'-GGTTTTCTCATAGATGGCGT-3', 279 bp); α-SMA (sense sequence 5'-GTC CAC CGC AAA TGC TTC TAA-3', anti-sense sequence 5'-AAA ACA CAT TAA CGA GTC AG-3'); Snail (sense sequence 5'-TCTAGGCCCTGGCTGCTACAA-3', anti-sense sequence 5'-GCCTGGCACTGGTACTTCTTGAC-3', 152 bp); Slug (sense sequence 5'-ATGCATATTCGGACCCACACATTA-3', anti-sense sequence 5'-AGAATTTGACCTGTCTGCAAATGCT-3', 158 bp); β-actin (5'-CAGAAGGACTCCTACGTG-3', 5'-GCTCGTCAGGATCTTCATG-3', 440 bp). Scion Image software (version 1.6; National Institutes of Health, Bethesda, MD, USA) was used to detect the intensity of each band. The target gene's relative mRNA expression was normalized to that of β-actin of the control group. The relative gene expression levels in each sample were normalized to β-actin, and analysed by Scion Image software (version 1.6; National Institutes of Health).

Western blot analysis

Lung homogenate was prepared in sodium dodecyl sulfate (SDS) sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis and transfer, the membranes were incubated with anti-P-Smad$_3$ (1:1,000; Cell Signaling Technology, Danvers, MA, USA) or anti-Smad$_7$ (1:200, Santa Cruz Biotechnology, CA, USA), anti-Snail (1:500; BIOSS; Beijing, China; cat. no. bs-56-18R), anti-α-SMA (1:400; Santa Cruz Biotechnology; cat. no. Sc-10688), anti-TGF-β_1 (1:200; Santa Cruz Biotechnology; cat. no. sc-145), anti-Slug (1:500, BIOSS; cat. no. bs-0716R), or β-actin (1:1,000; Santa Cruz Biotechnology; cat. no. sc-47276) antibodies. A goat anti-rat antibody (1:1000, Santa Cruz Biotechnology) was used as the secondary antibody. Membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody. Bands were detected using the Western blotting luminol reagent (ELIPIS Biotech., Inc., Daejeon, Korea).

Cell culture and anti-NGF treatment

The anti-NGF antibody was prepared as described above, and diluted in cell culture medium to the working concentrations. Recombinant human TGF-β_1 (00-21-10; PeproTech, Rocky

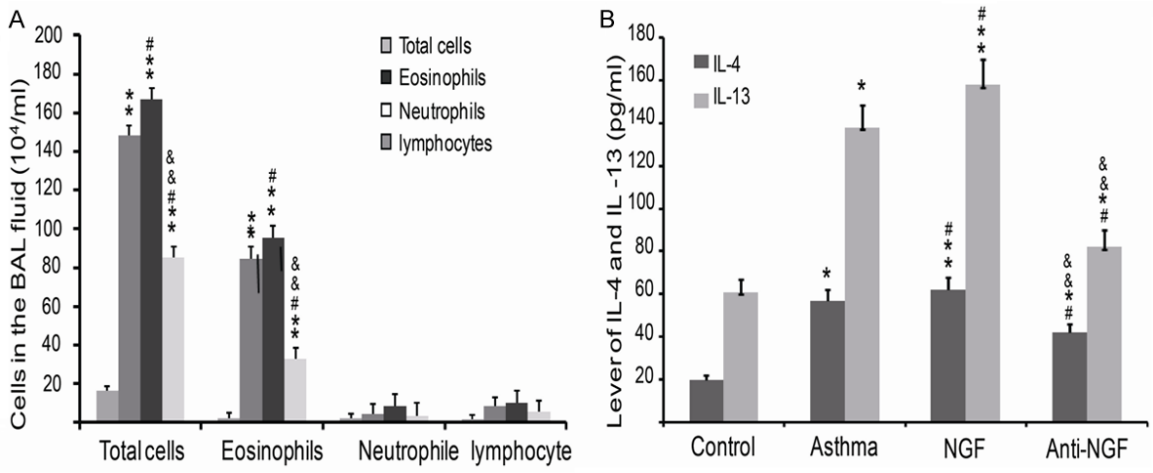

Figure 3. A. Effects of the anti-NGF treatment on the total and differential cell counts in the BALF. B. Effects of anti-NGF treatment on the levels of IL-4 and IL-13. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs*. control group; $^{\#}P < 0.05$, *vs*. Asthma group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, *vs*. NGF group.

Hill, NJ, USA) was prepared as a stock solution at the concentration of 0.1 mg/ml in 10 ml citric acid. After reaching 70-80% confluence, cells were switched to serum-free DMEM/F-12 for 24 h, and then incubated with 5 μM TGF-β_1 in the presence or absence of anti-NGF antibody for 48 h. The mRNA and protein levels of EMT markers, i.e., Slug, α-SMA, and Snail, were measured. Each group was detected to facilitate the analysis of the expression of the EMT markers and the Smad proteins in bronchial epithelial (BEAS)-2B cells that were treated with 5 ng/ml TGF-β_1 for 24 h. The cell culture methods and anti-NGF treatments were performed as previously described [9].

Statistical analysis

All data were expressed as means ± standard error of the mean (SEM) for each group. Comparisons between multiple groups were made by ANOVA and the nonparametric Kruskal-Wallis test, followed by post hoc testing with Dunn's multiple comparisons of means. Data analyses were performed with GraphPad Prism version 6 software (GraphPad Software, San Diego, CA, USA). A *P* value of < 0.05 was considered to indicate statistical significance.

Results

Inhibitory effects of anti-NGF therapy on average pulmonary resistance

Compared with the control group, the asthma group exhibited an increase in average pulmonary resistance after the Mch challenge. In addition, exogenous NGF treatment further elevated the Mch-induced pulmonary resistance. However, when mice were treated with the anti-NGF antibody, the Mch-induced pulmonary resistance was significantly attenuated ($P < 0.05$) (**Figure 2**).

Anti-NGF therapy decreased airway inflammation

To investigate the effect of anti-NGF on airway inflammatory cell infiltration in the asthma mice, we measured inflammation-related cells and cytokines in the BALF. As shown in **Figure 3A**, the NGF group had more total cells, and eosinophils in the BALF than the control group. By contrast, anti-NGF treatment significantly attenuated the increase in the number of total cells, and eosinophils in the BALF. Compared with the asthma group, the total cell count in the BALF and the levels of eosinophils, IL-4, and IL-13, were significantly lower in the anti-NGF group ($P < 0.05$) (**Figure 3B**).

Anti-NGF therapy prevented inflammatory cell infiltration and airway remodelling

To investigate the effect of anti-NGF on the development of airway remodelling, we assessed the thickness of airway smooth muscle, the deposition of collagen, and peribronchial cell infiltration. Lung sections were stained with H&E (**Figure 4Aa-d**) and Masson's trichrome stain (**Figure 4Ae-h**) to evaluate pathological changes. Lower numbers of epithelial lesions, lower levels of mucosal edema and inflamma-

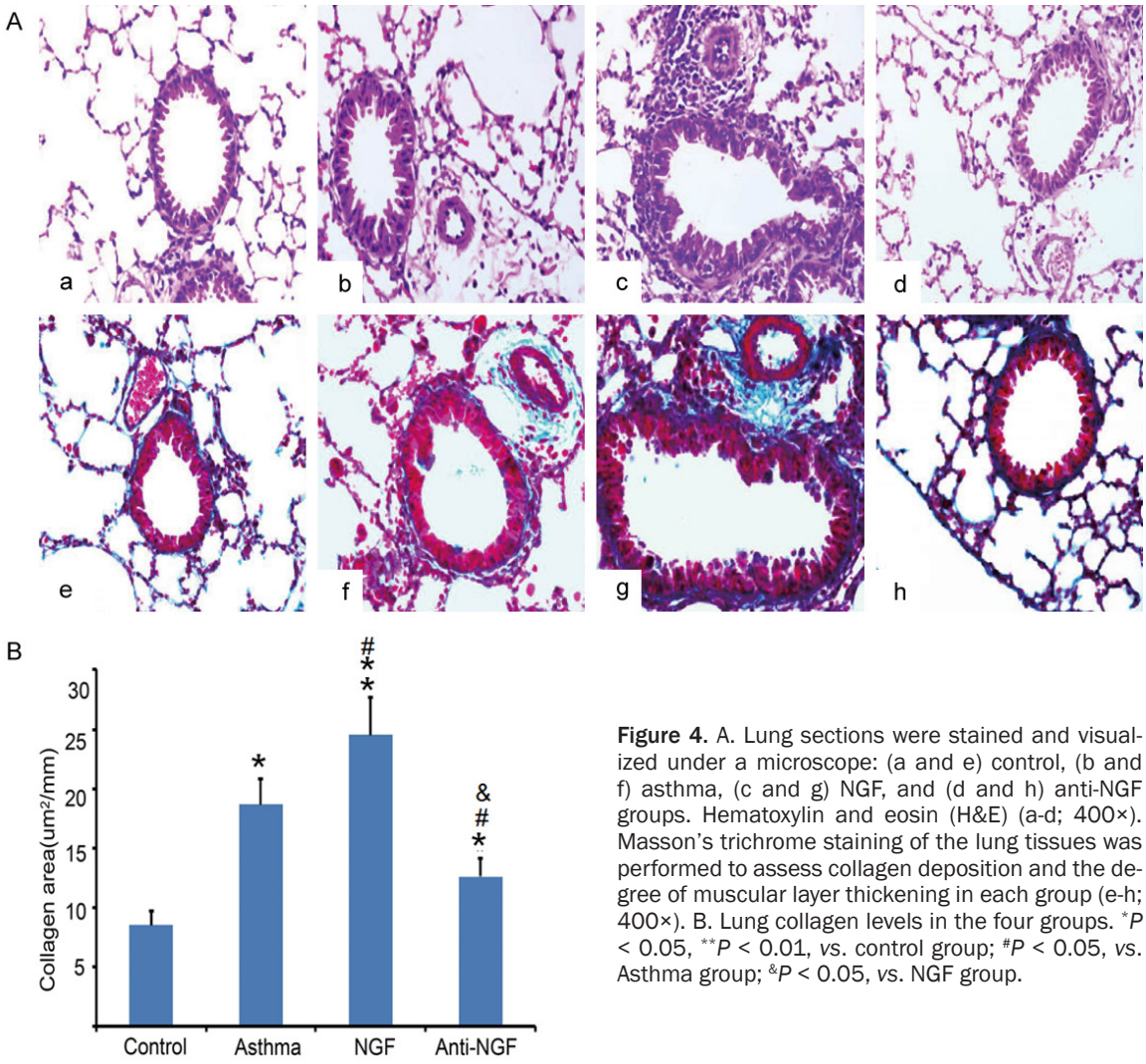

Figure 4. A. Lung sections were stained and visualized under a microscope: (a and e) control, (b and f) asthma, (c and g) NGF, and (d and h) anti-NGF groups. Hematoxylin and eosin (H&E) (a-d; 400×). Masson's trichrome staining of the lung tissues was performed to assess collagen deposition and the degree of muscular layer thickening in each group (e-h; 400×). B. Lung collagen levels in the four groups. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs*. control group; $^{\#}P < 0.05$, *vs*. Asthma group; $^{\&}P < 0.05$, *vs*. NGF group.

tion were seen in the anti-NGF group (**Figure 4Ad**), whereas the asthma group displayed moderate-to-severe inflammation, epithelial lesions, and mucosal edema (**Figure 4Ab**). Specifically, only the NGF group developed very severe inflammation, including large lymphoid aggregates and interstitial infiltration (**Figure 4Ac**). The collagen area was quantitatively estimated (**Figure 4B**). Compared with the control group, the asthma group and NGF group exhibited increases in the trichrome-stained peribronchial area and deposition of collagen in the lung (**Figure 4Ae-g**). Compared with the asthma group, the anti-NGF group showed reduced peribronchial collagen staining (**Figure 4Ah**). The levels of lung collagen were obviously decreased in the anti-NGF group (14.8±1.2 μg lung tissue) as compared with the asthma group (25.5±1.3 μg lung tissue, $P < 0.05$, **Figure 4B**).

The effects of anti-NGF therapy on TGF-β_1 and Smad expression levels in BALF and lung tissue

To assess the effect of anti-NGF on TGF-β_1 signalling, the expression levels of TGF-β_1 in the BALF and lung tissue samples were evaluated. As illustrated in **Figure 5D**, TGF-β_1 immunohistochemical staining showed that TGF-β_1 was colored canary yellow to brown, and mainly distributed in the cytoplasm of smooth muscle cells, in airway epithelial cells, the submucosa, inflammatory cells, and the vascular smooth muscle layer. The asthma group exhibited high expression of TGF-β_1 in the epithelial cells of the airways compared to the control group. The anti-NGF group showed lower expression of TGF-β_1 than the asthma group. TGF-β_1 expression was significantly reduced in the BALF by anti-NGF treatment (**Figure 5A**). In the lung

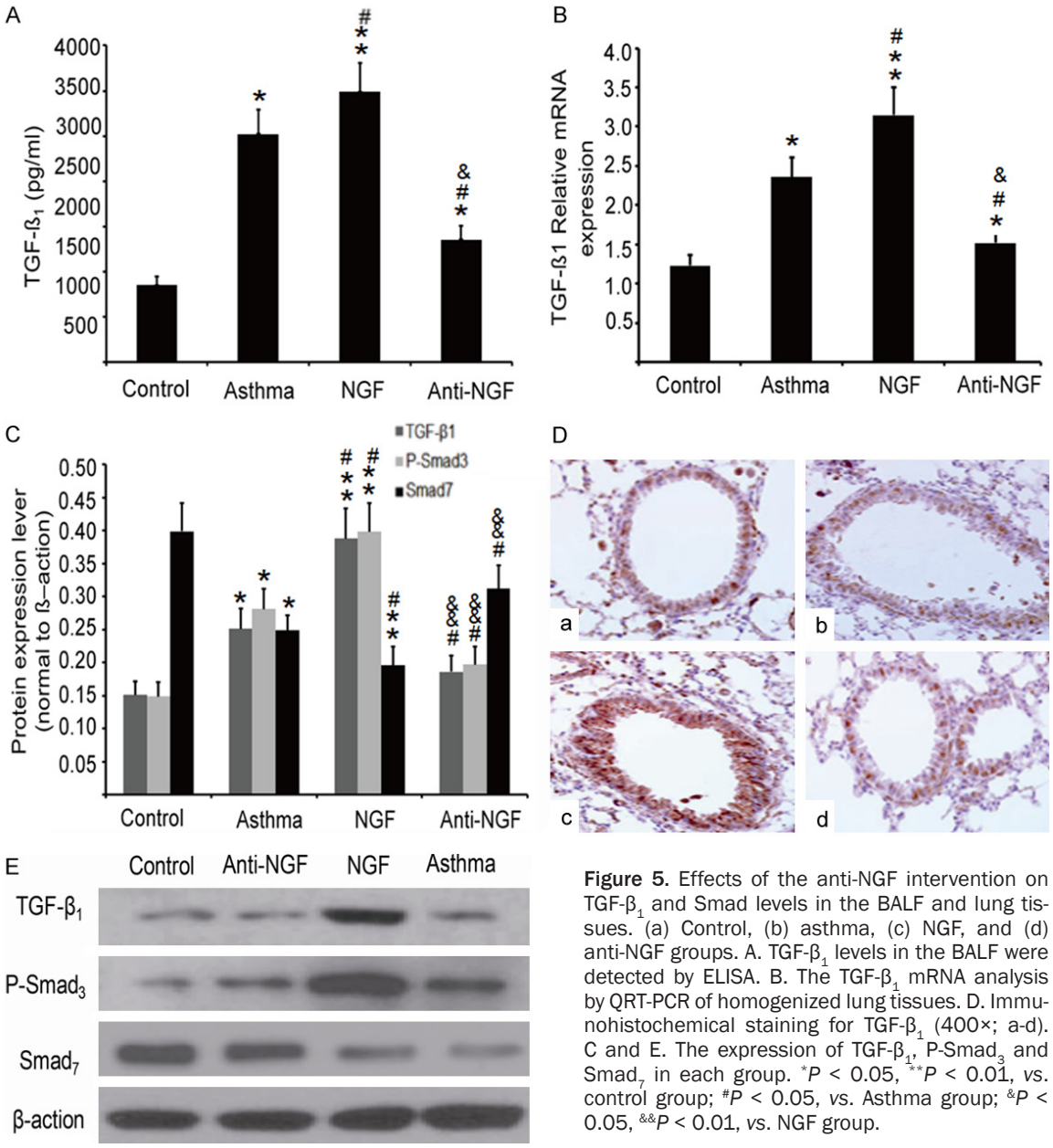

Figure 5. Effects of the anti-NGF intervention on TGF-β_1 and Smad levels in the BALF and lung tissues. (a) Control, (b) asthma, (c) NGF, and (d) anti-NGF groups. A. TGF-β_1 levels in the BALF were detected by ELISA. B. The TGF-β_1 mRNA analysis by QRT-PCR of homogenized lung tissues. D. Immunohistochemical staining for TGF-β_1 (400×; a-d). C and E. The expression of TGF-β_1, P-Smad$_3$ and Smad$_7$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs*. control group; $^{\#}P < 0.05$, *vs*. Asthma group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, *vs*. NGF group.

homogenized tissue, the asthma group exhibited high expression of TGF-β_1 and P-Smad$_3$, but low expression of Smad$_7$. TGF-β_1 protein and mRNA expression were suppressed by anti-NGF treatment, and the protein expression TGF-β_1 and P-Smad$_3$ were also suppressed by anti-NGF treatment (**Figure 5B**, **5C**, **5E**).

Effect of anti-NGF therapy on epithelial-mesenchymal transition signaling (EMT)

We hypothesized that the mechanism by which anti-NGF therapy decreases airway inflammation involved a decrease in the levels of TGF-β_1 and downstream signalling proteins. Therefore, we examined one of the TGF-β_1 signalling pathways that is associated with bronchial asthma pathophysiology, i.e., the TGF-β_1-induced EMT pathway in primary airway epithelial cells [12], to clarify the effects of anti-NGF treatment on the EMT. Moreover, we also performed an in vitro study using a bronchial epithelial line (BEAS-2B) and evaluated the levels of mesenchymal EMT markers using QRT-PCR and Western blotting. To confirm if TGF-β_1 induced EMT in primary airway epithelial cells, bronchial epithelial cells were treated with TGF-β_1 (5 μM) for 48 h with or without anti-NGF antibody (4

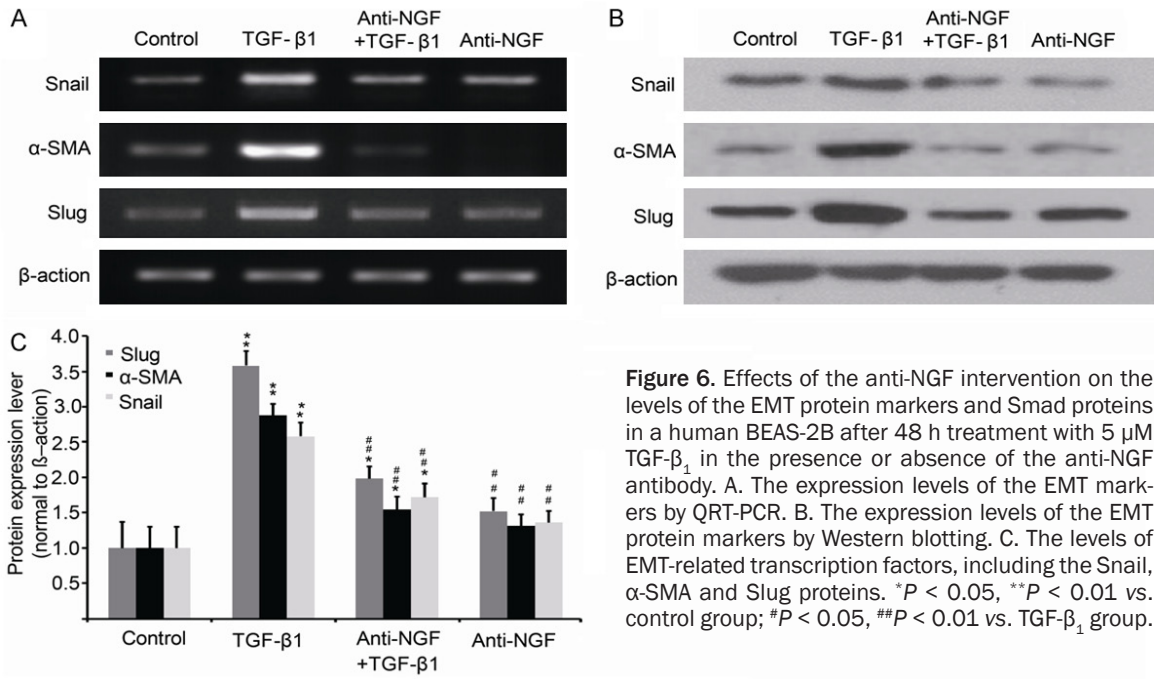

Figure 6. Effects of the anti-NGF intervention on the levels of the EMT protein markers and Smad proteins in a human BEAS-2B after 48 h treatment with 5 μM TGF-β_1 in the presence or absence of the anti-NGF antibody. A. The expression levels of the EMT markers by QRT-PCR. B. The expression levels of the EMT protein markers by Western blotting. C. The levels of EMT-related transcription factors, including the Snail, α-SMA and Slug proteins. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. TGF-β_1 group.

ml/kg). As illustrated in **Figure 6A** and **6B**, the mRNA levels of the mesenchymal markers Slug, α-SMA, and Snail in the bronchial epithelial cells increased in response to the increased expression of TGF-β_1, but decreased in the presence of anti-NGF. The expression levels of the Slug, Snail, and α-SMA proteins were detected using QRT-PCR and Western blotting, and the results revealed similar changes in the levels of these mesenchymal markers (**Figure 6**). Compared with the control group, levels of the Slug, α-SMA, and Snail proteins were significantly increased in the TGF-β_1 group (all, $P < 0.05$). However, the enhancing effect of TGF-β_1 was significantly attenuated by anti-NGF treatment (TGF-β_1 group vs. anti-NGF$_1$+TGF-β_1 group, $P < 0.05$) (the expression of Slug, Snail, and α-SMA were higher in the TGF-β_1 group than the anti-NGF group). The inhibitory effects of anti-NGF were significant (TGF-β_1 group vs. anti-NGF$_1$+TGF-β_1 group, $P < 0.05$; TGF-β_1 group vs. anti-NGF group, $P < 0.05$; **Figure 6C**). There was no significant difference in these protein levels between the control group and the anti-NGF$_1$ group.

Discussion

NGF has been shown to play a crucial role in the pathogenesis of asthma, and the level of circulating NGF is related to the severity of asthma [10]. Reasonable regulation of NGF can effectively reduce airway remodeling and the inflammatory response, and delay or even reverse the pathogenesis of asthma [2]. However, the effect of anti-NGF therapy on asthma, and its underlying molecular mechanism is not fully understood. Previous studies have shown that anti-NGF therapy can reduce NGF levels, and produce beneficial effects in an allergic asthma model [9]. It has also been reported that anti-NGF therapy can reduce pulmonary inflammation and airway remodeling [9, 11], possibly related to inhibition of the MMP-9 pathway [12]. Our previous studies demonstrated that anti-NGF inhibits airway inflammation [4, 12]. In this study, we further demonstrated that the anti-NGF antibody inhibited OVA-induced chronic allergic asthma by suppressing the TGF-β/Smad signaling pathway. Moreover, we found the inhibitory effect of anti-NGF therapy on pulmonary resistance, inflammatory cell infiltration, airway hyper-responsiveness, and airway remodeling in asthmatic mice was at least partially via EMT and TGF-β_1/Smad signaling. To the best of our knowledge, this is the first study reporting that EMT and TGF-β/Smad signaling is involved in the molecular mechanism underlying the inhibitory effect of anti-NGF therapy on airway inflammation and hyper-responsiveness.

Recent research has also suggested that an excessive level of circulating NGF promotes re-

nal fibrosis, and causes damage to the renal tubules, and NGF promotes renal fibrosis by activating the TGF-β_1 signaling pathway [7]. $Smad_3$ is known to be a receptor-regulated Smad protein (R-Smad) that is phosphorylated in response to the activation of TGF-β_1 signaling in chronic asthma [13]. In this study, anti-NGF therapy significantly reduced TGF-β_1 and $Smad_3$, decreased collagen deposition, and reduced the infiltration of inflammatory cells in the airway. TGF-β_1 treatment up-regulated the mesenchymal markers Slug, Snail, and α-SMA in the lung and P-$Smad_3$ protein in human bronchial epithelial cells. The enhancing effect of TGF-β_1 can be significantly inhibited by anti-NGF antibody. It has been shown that anti-NGF antibody treatment induces nuclear translocation of P-$smad_3$ [7], an intracellular mediator of TGF-β_1 signaling [14]. NGF has been shown to bind to nerve endings in damaged airway endothelium, inhibiting neurotransmitter release [15].

TGF-β_1 regulates the mRNA and protein expression of EMT markers, including Snail, α-SMA, and Slug in cells [16]. It has been demonstrated that EMT play a role in the pathogenesis of airway fibrosis and epithelial cell remodeling [17]. An *in vitro* study by Doerner and Zuraw demonstrated that TGF-β_1 can induce EMT in respiratory epithelial cells [18]. In this study, we found that anti-NGF therapy can significantly reduce EMT. Therefore, the anti-NGF therapy may exert its function through inhibiting TGF-β_1 expression, the downstream Smad activation, and EMT in the lung tissue.

In summary, this study showed that intraperitoneal injection of anti-NGF antibody can inhibit airway remodeling. The mechanism of action of anti-NGF antibody may be associated with inhibition of TGF-β/Smad signalling and EMT. Our findings may help better understand the mechanism underlying anti-NGF antibody treatment for chronic asthma.

Acknowledgements

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Disclosure of conflict of interest

None.

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