

# Assessment of tumor growth factor- $\beta_1$ neutralizing antibody in the treatment of allergic rhinitis and asthma

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**Abstract.** To identify a novel and effective therapy for allergic rhinitis and asthma (ARA), the present study focused on treatment with tumor growth factor (TGF)- $\beta_1$  neutralizing antibody. In the present study, four medications were administered to mice with ovalbumin-induced allergic inflammation. Allergic symptoms in the lungs and nasal mucosa were evaluated by detecting the secretion of cytokines from helper T cells (Th) in the peripheral blood, nasal lavage fluid and bronchoalveolar lavage fluid using ELISA. Defects in regulatory T (Treg) cells in peripheral blood mononuclear cells were also detected using flow cytometry. Furthermore, the expression of TGF- $\beta_1$  and activation of Smad2/3 pathways were assessed using immunohistochemical staining, reverse transcription-quantitative polymerase chain reaction, and western blotting. It was observed that TGF- $\beta_1$  neutralizing antibody inhibited symptoms of inflammation in the upper and lower airways. TGF- $\beta_1$  neutralizing antibody also restored the Th1/Th2 balance and ameliorated Treg cell defects induced by ARA. Furthermore, the therapeutic effects of TGF- $\beta_1$  neutralizing antibody were related to its inhibitory effects on TGF- $\beta_1$  expression and Smad2/3 signaling in nasal and lung tissues. Therefore, TGF- $\beta_1$  neutralizing antibody may be an effective medicine for the treatment of ARA.

## Introduction

Allergic rhinitis currently affects 20-40% of the global population and is increasing in prevalence (1). For patients with allergic rhinitis, quality of life is reduced by fatigue, cognitive impairment and many other symptoms associated with the

condition (2). Asthma is an airway disease associated with airway hyperresponsiveness, which causes wheezing, breathlessness and chest tightening (3). The incidence of asthma is markedly higher in developed countries, and >300 million individuals are estimated to have asthma worldwide (4,5). Both allergic rhinitis and asthma are chronic, inflammatory and reversible allergic airway diseases, which share similar pathophysiology features, including eosinophil infiltration, goblet cell hyperplasia and sub-epithelial fibrosis (6). Moreover, the probability of coexistence between allergic rhinitis and asthma in patients is high, and ~78% of patients with asthma also exhibit nasal inflammation (7,8). Allergic rhinitis and asthma (ARA) are substantial medical and financial burdens to healthcare systems worldwide. The pathogenesis of ARA is not well understood, and current research is focusing on more effective treatment methods.

Transforming growth factor- $\beta$  (TGF- $\beta$ ), so named for its ability to induce fibroblast proliferation, is involved in cell differentiation, developmental biology and immunological processes (9). The TGF- $\beta$  superfamily consists of >30 distinct molecules, including TGF- $\beta$  isoforms, bone morphogenetic proteins, activins, growth factors, inhibins and anti-müllerian hormone (10). Activated TGF- $\beta$  complexes may bind to corresponding TGF- $\beta$  receptors, which stimulates the receptors to induce signaling cascades that regulate the expression of different target genes involved in cell differentiation, growth and immune responses (11,12). TGF- $\beta_1$  is an isoform of TGF- $\beta$ , and is synthesized as a 390-amino-acid protein precursor that is proteolytically processed to produce a mature peptide containing 112 amino acids. TGF- $\beta_1$  is considered to serve a key role in regulating the immune system. In particular, it has been documented that TGF- $\beta_1$  exerts suppressive effects on the activities of many types of immune cells (13-16). For instance, TGF- $\beta_1$  may inhibit the proliferation of macrophages and monocytes and suppress their production of reaction oxygen and nitrogen intermediates (14). Furthermore, TGF- $\beta_1$  has been demonstrated to affect the differentiation of B cells and regulate the secretion of antibodies (15). In the regulation of helper T cells (Th), previous studies observed that TGF- $\beta_1$  suppressed the action of Th1 cells and inhibited the release of interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (16,17).

In recent studies into the pathogenesis of Th1/Th2 immune response imbalances, it was indicated that allergic rhinitis and

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asthma may be caused by a hypernomic immune reaction of Th2 cells mediated by various inflammatory cytokines (6,18). Therefore, restoration of the Th1/Th2 immune balance may be a potential therapeutic strategy. In the present study, TGF- $\beta_1$  neutralizing antibody was used to treat a mouse model of ARA, and was compared to treatments with dexamethasone (DXM), montelukast (MK) and budesonide (BUD). The effects of TGF- $\beta_1$  neutralizing antibody on inflammatory symptoms, secretion of interleukin (IL)-4 and IFN- $\gamma$ , regulation of regulatory T (Treg) cells and activation of Smad2/3 signaling pathways were also evaluated.

## Materials and methods

**Animals and ARA modeling.** The animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). A total of 60 female Balb/c mice (4-6 weeks old, weighing 18-20 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Mice were maintained under a constant ambient temperature (22-24°C) and relative humidity (55%) with a normal 12 h light/dark cycle and free access to food and water. A mouse model of ARA was established as described previously with minor modification (19). Briefly, as a sensitization treatment, mice were injected with 200  $\mu$ l phosphate-buffered saline (PBS) containing 40  $\mu$ g ovalbumin (OVA; 0.2 mg/ml; S7951; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 2 mg aluminum hydroxide every other day for 13 days (on days 1, 3, 5, 7, 9, 11 and 13). A rest phase was applied between days 14 and 20. From days 21 to 27, the mice were challenged daily with 5% OVA through an ultrasonic nebulizer (WH-2000; Yuehua Medical Instrument Factory Co., Ltd., Guangdong, China) for 30 min, followed by intranasal instillation of 20  $\mu$ l OVA (40 mg/ml). Mice in the normal control group were treated with PBS alone instead of OVA. Mice were divided into medication groups and were treated according to the experimental design below. All mice were sacrificed by cervical dislocation 24 h after the final treatments.

**Experimental design.** Mice were divided into the following six groups (n=10), according to a previously described method (20): i) Normal group (treated with PBS alone); ii) ARA group (ARA without any treatment); iii) Anti-TGF- $\beta_1$  group (ARA + TGF- $\beta_1$  neutralizing antibody), iv) DXM group (ARA + DXM); v) MK group (ARA + MK); and vi) BUD group (ARA + BUD). Mice in the anti-TGF- $\beta_1$  and DXM groups were treated with a daily intraperitoneal injection of TGF- $\beta_1$  neutralizing antibody (10 mg/kg; ab25121; Abcam, Cambridge, UK) and DXM (0.5 mg/kg; D1756; Sigma-Aldrich; Merck KGaA), respectively, at 30 min prior to OVA challenge for 7 days starting on day 21. The same treatment protocol was performed on mice in the MK and BUD groups, but with intragastric administration of MK (5 mg/kg; Lunan Beite Pharmaceutical Co. Ltd., Jinan, China) and nebulization of BUD (1 ml/2 ml; AstraZeneca, Cambridge, UK), respectively.

**Histological assay.** On day 28, 5 mice in each group were sacrificed, and lung and nasal mucosa tissues were collected. Sections of nasal mucosa were isolated at a distance of 5 mm

posterior to the nasal vestibule. All tissues were fixed with 10% formalin at room temperature overnight and embedded in paraffin. Lung and nasal mucosa sections were prepared at a thickness of 3  $\mu$ m. Lung sections were stained with hematoxylin and eosin to assess the infiltration of eosinophils. Periodic acid-Schiff (PAS) staining and Masson's trichrome (MT) staining were performed on nasal mucosa sections to visualize goblet cell hyperplasia and collagen deposition, respectively, in the nasal mucosa. The numbers of eosinophils were evaluated under a light microscope at x200 magnification. PAS-stained goblet cells and MT-stained areas were analyzed under a microscope at x400 magnification using ImageJ software.

**Immunohistochemistry (IHC).** To assess the levels of TGF- $\beta_1$  protein in nasal and lung tissues, IHC was performed as described previously (21). Briefly, nasal mucosa and lung sections were incubated with mouse monoclonal anti-TGF- $\beta_1$  antibody (1:20; rabbit monoclonal antibody; ab170874; Abcam) at 4°C overnight, followed by incubation with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:200; A0208; Beyotime Institute of Biotechnology, Haimen, China) at 37°C for 2 h. Sections were then developed with diaminobenzidine (DAB) in accordance with instructions of a commercial DAB Horseradish Peroxidase Color Development kit (P0202; Beyotime Institute of Biotechnology). Positive stained cells were counted under a light microscope at x200 magnification.

**Enzyme-linked immunosorbent assay (ELISA).** At 24 h after the final OVA challenge, the peripheral blood, nasal lavage fluid (NALF) and bronchoalveolar lavage fluid (BALF) were harvested from the remaining five mice of each group. After ligating the upper level of the trachea, the nasal cavities were gently rinsed with 1 ml cold PBS through a 22-gauge catheter inserted into the nasopharynx. The pulmonary alveoli were rinsed with cold PBS via a 20-gauge needle inserted through the main bronchus. NALF and BALF were collected from the nose and trachea, respectively. Peripheral blood, NALF and BALF were then centrifuged at 400 x g at 4°C for 10 min, and the supernatant was applied to ELISA plates coated with antibodies against IL-4 or IFN- $\gamma$ . The concentrations of IL-4 and IFN- $\gamma$  were measured according to the instructions of IL-4 (cat. no. BMS613TWO) and IFN- $\gamma$  (cat. no. BMS606TWO) ELISA kits (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Flow cytometry analysis.** To identify forkhead box P3 (Foxp3)<sup>+</sup> Treg cells and TGF- $\beta_1$ <sup>+</sup> T cells, flow cytometry analysis was performed using a flow cytometer (Accuri C6, BD Biosciences, Franklin Lakes, NJ, USA), according to a previously described method (22). Peripheral blood mononuclear cells (PBMCs; 10<sup>5</sup> cells per sample) were obtained from the isolated peripheral blood samples using lymphocyte separation medium (Cedarlane Laboratories, Burlington, Canada) in accordance with the manufacturer's instructions. PBMCs were blocked with 5% BSA-PBS solution (Beyotime Institute of Biotechnology) for 45 min at 4°C and then surface-stained with anti-cluster of differentiation (CD)4-fluorescein isothiocyanate (FITC; 1:20; 11-0043-82, eBioscience; Thermo Fisher Scientific, Inc.), anti-CD25-allophycocyanin (APC,



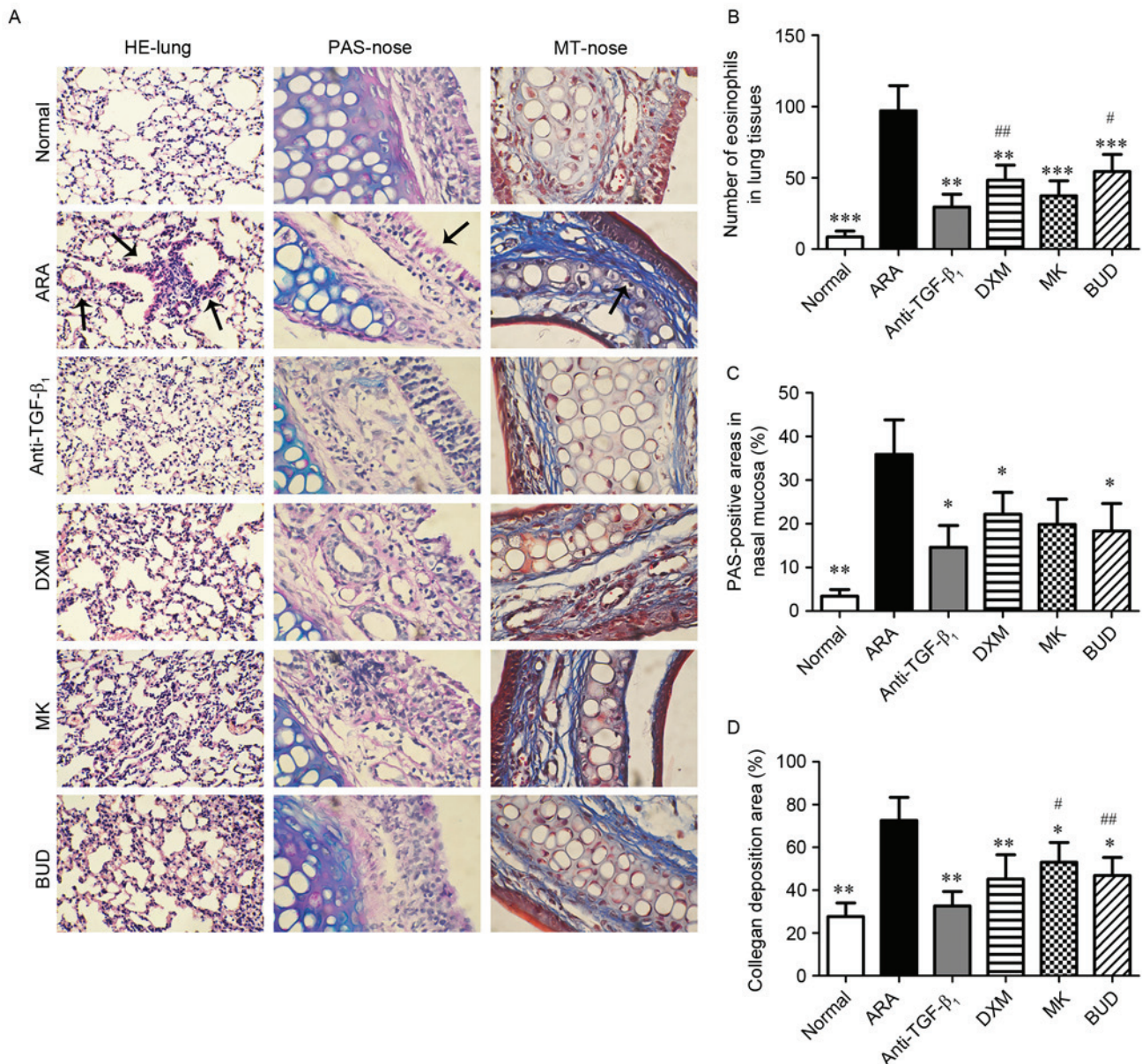


Figure 1. Effect of drug treatments on the symptoms of ovalbumin-induced ARA in the lung and nasal mucosa. (A) Representative photos of H&E-stained lung tissue sections (magnification, x200), and PAS- and MT-stained nasal mucosa sections (magnification, x400) from the six experimental groups. Stained eosinophils (H&E), goblet cells (PAS) and collagen (MT) are indicated by the arrows. (B) Analysis of eosinophilia infiltration in each group. (C) Analysis of PAS-positive areas in nasal mucosa. (D) Analysis of positive-stained collagen areas in each group. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. ARA; # $P < 0.05$  and ## $P < 0.01$  vs. anti-TGF- $\beta_1$  group. ARA, allergic rhinitis and asthma; anti-TGF- $\beta_1$ , tumor growth factor- $\beta_1$  neutralizing antibody; DXM, dexamethasone; MK, montelukast; BUD, budesonide; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff; MT, Masson's trichrome.

1:20; 17-0251-82; eBioscience; Thermo Fisher Scientific, Inc.) and anti-CD3-FITC (1:20; ab34275; Abcam) at 4°C for 1 h. After washing with ice-cold PBS twice, cells were fixed with 10% formalin at 4°C for 30 min and then permeabilized with 0.5% Triton X-100 in PBS at 4°C for 30 min. The cells were then stained intracellularly with anti-Foxp3-phycoerythrin (PE; 1:20; 12-5773-82, eBioscience; Thermo Fisher Scientific, Inc.) and anti-TGF- $\beta_1$  (1:20; MAB240-100; R&D Systems, Inc., Minneapolis, MN, USA) at 4°C for 1 h. Cells were incubated with Alexa Flour 555 conjugated donkey anti-mouse secondary antibody (1:400; A0460; Beyotime Institute of Biotechnology) to detect intracellular staining. All the antibodies were diluted in PBS. Data were analyzed using FlowJo software Version 7.6 (Tree Star, Inc., Ashland,

OR). A minimum of 10,000 events was collected in each analysis.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the isolated nasal mucosa tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription was carried out using a cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) with incubation for 60 min at 42°C. qPCR was conducted with a SYBR Green PCR Master Mix (Fermentas; Thermo Fisher Scientific, Inc.) in an ABI-7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). All procedures were conducted in accordance with the manufacturer's instructions. GAPDH was used as an

internal control. The primer sequences used were as follows: For TGF- $\beta_1$  (accession number, NM\_001312868.1), forward, 5'-CGAGAGGCAGAGATTTATCAG-3' and reverse, 5'-ATG TGAAGATGGGCAAGAC-3'; and for GAPDH (accession number, NM\_008084.2), forward, 5'-ATCACTGCCACC CAGAAG-3' and reverse, 5'-TCCACGACGGACACATTG-3'. Thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec. for 40 cycles. Data were analyzed with the ABI Prism 7500 SDS software (Applied Biosystems; Thermo Fisher Scientific, Inc.). The experiments were repeated three times in triplicate. TGF- $\beta_1$  mRNA expression was calculated relative to the expression of GAPDH.

**Western blot analysis.** The nasal mucosa tissue samples from 5 mice in each group were homogenized, and the tissue homogenate was disrupted in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) at 4°C for 30 min. Following centrifugation at 13,000 x g for 30 min at 4°C, the supernatant was collected and protein concentration was determined by using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Following normalization for protein concentration, samples (30  $\mu$ g protein per lane) were separated by 10% SDS-PAGE. The nitrocellulose membranes (Merck KGaA) of electrotransferred samples were block with 5% skim milk at room temperature for 30 min and probed with primary antibodies, then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. Immunolabeling was detected with an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control. The antibodies used were as follows: Anti-Smad2/3 (1:1,000; cat. no. 8685), anti-phospho-Smad2/3 (1:1,000; cat. no. 8828; both from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-TGF- $\beta_1$  (ab170874; 1:100; Abcam), anti-GAPDH (1:1,500; cat. no. 5174; Cell Signaling Technology, Inc.) and HRP-labeled secondary antibodies (1:1,000; A0208; Beyotime Institute of Biotechnology). Experiments were repeated three times. The protein expression was quantified using ImageJ software (version 1.6; National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Five independent experiments were performed for each assay, and the results were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean  $\pm$  standard deviation and analyzed with a one-way analysis of variance followed by a Sidak's t-test for multiple comparisons.  $P < 0.05$  was considered to indicate a statically significant difference.

## Results

**TGF- $\beta_1$  neutralizing antibody attenuates upper and lower airway inflammation in ARA.** The effect of TGF- $\beta_1$  neutralizing antibody on inflammatory symptoms in the lungs and nasal mucosa were investigated by histological analysis (Fig. 1A). Following sensitization and challenges with OVA, mice with ARA exhibited a significant infiltration of eosinophils in the bronchi ( $P < 0.001$  vs. normal group), and significant goblet cell hyperplasia and collagen deposition in the nasal mucosa

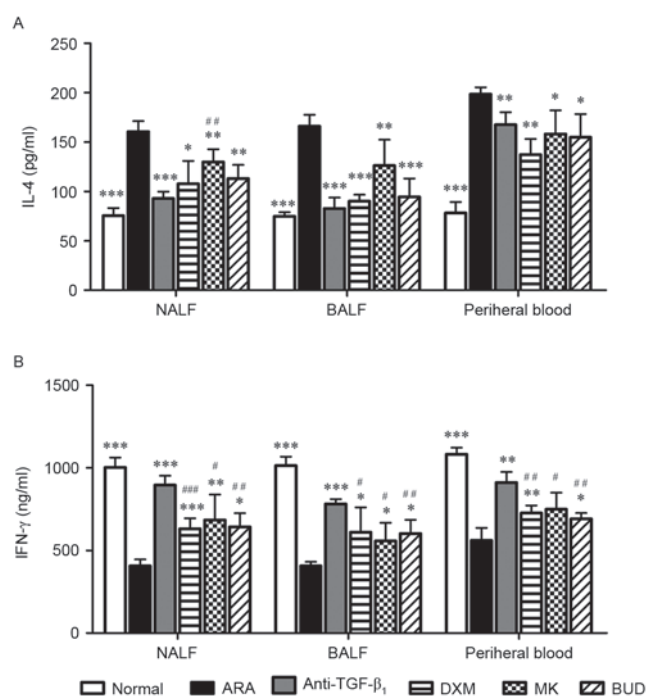


Figure 2. Effect of drug treatments on cytokine secretion by Th1 and Th2 cells in NALF, BALF and peripheral blood. Assessment of (A) IL-4 and (B) IFN- $\gamma$  levels in NALF, BALF and peripheral blood in ARA mice following treatments with anti-TGF- $\beta_1$ , DXM, MK and BUD. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. ARA; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. anti-TGF- $\beta_1$  group. Th, helper T cells; NALF, nasal lavage fluid; BALF, bronchoalveolar lavage fluid; IL, interleukin; IFN, interferon; ARA, allergic rhinitis and asthma; anti-TGF- $\beta_1$ , tumor growth factor- $\beta_1$  neutralizing antibody; DXM, dexamethasone; MK, montelukast; BUD, budesonide.

( $P < 0.01$  vs. normal group; Fig. 1B-D). Groups administered with different drug treatments (TGF- $\beta_1$  neutralizing antibody, DXM, MK and BUD) prior to OVA challenges exhibited significant decreases in eosinophil infiltration in the lower airways ( $P < 0.01$ ), and in goblet cell hyperplasia and collagen deposition in the upper airways ( $P < 0.05$ ). Moreover, treatment with TGF- $\beta_1$  neutralizing antibody exerted a significantly greater inhibitory effect on eosinophil infiltration relative to the DXM and BUD groups ( $P < 0.05$ ), and on collagen deposition relative to the MK and BUD groups ( $P < 0.05$ ; Fig. 1B-D). These results indicated that TGF- $\beta_1$  neutralizing antibody reduced inflammation of the upper and lower airways in ARA mice.

**TGF- $\beta_1$  neutralizing antibody alters levels of IL-4 and IFN- $\gamma$  in the NALF, BALF and peripheral blood.** The levels of cytokines secreted by Th cells in the NALF, BALF and peripheral blood. As depicted in Fig. 2A, mice with ARA exhibited significantly increased levels of IL-4 in the NALF, BALF and peripheral blood ( $P < 0.001$  vs. normal group), while IL-4 levels were significantly decreased in ARA mice post-medication ( $P < 0.05$ ). The degree of IL-4 inhibition did not differ significantly among the TGF- $\beta_1$  neutralizing antibody, DXM and BUD groups. TGF- $\beta_1$  neutralizing antibody exerted a greater inhibitory effect on IL-4 levels in NALF ( $P < 0.001$ ), but not in BALF or peripheral blood. By contrast, significantly reduced levels of IFN- $\gamma$  were identified in



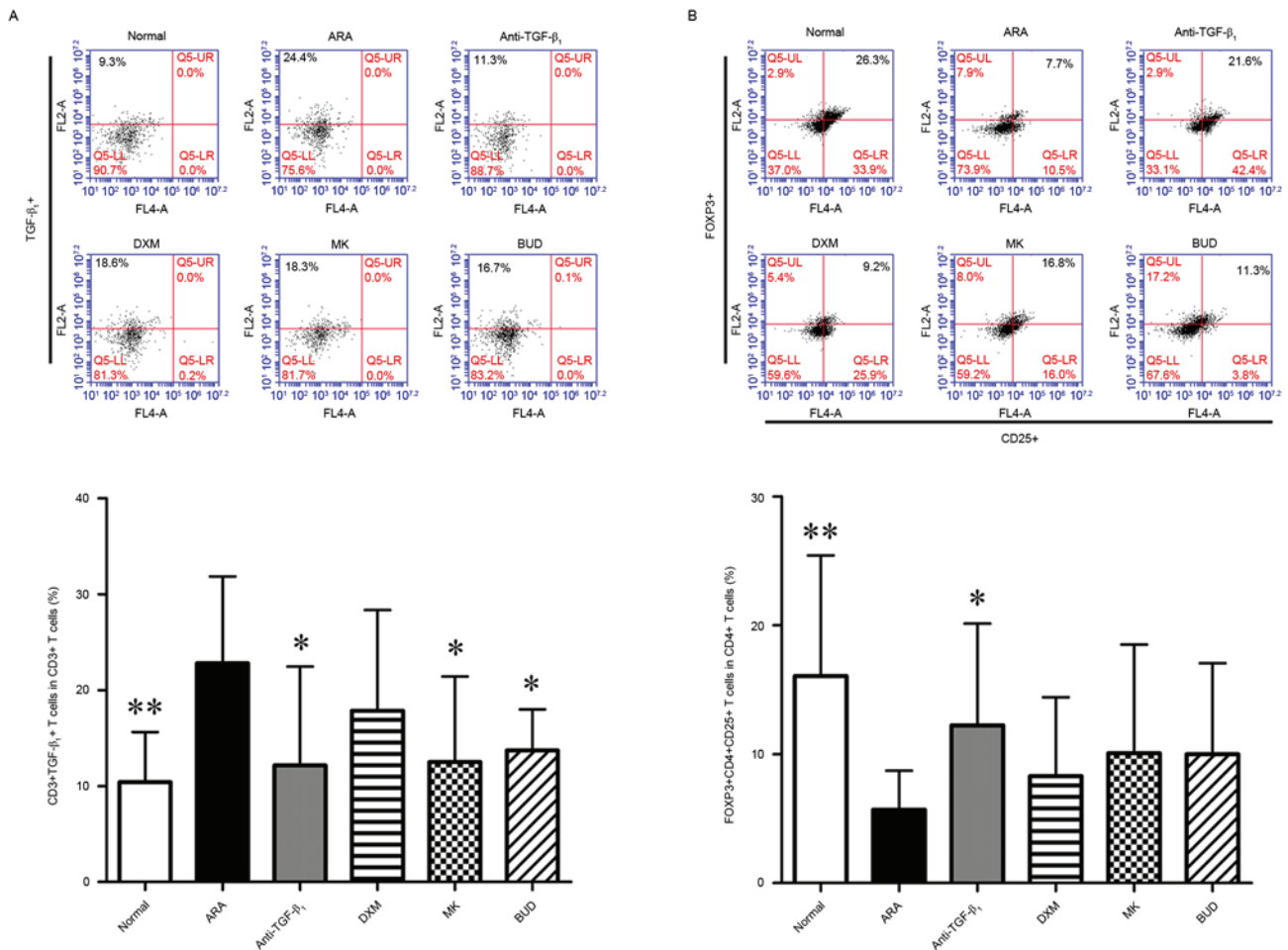


Figure 3. Effect of drug treatments on the regulation of T cells in peripheral blood mononuclear cells. (A) Representative flow cytometry histograms and analysis of TGF-β<sub>1</sub><sup>+</sup> cells in the CD3<sup>+</sup> subpopulation of each group. (B) Representative flow cytometry histograms and analysis of CD25<sup>+</sup> Foxp3<sup>+</sup> cells in the CD4<sup>+</sup> subpopulation of each group. Data are presented as the mean ± standard deviation. \*P<0.05 and \*\*P<0.01 vs. ARA. TGF-β<sub>1</sub>, tumor growth factor-β<sub>1</sub>; CD, cluster of differentiation; Foxp3, Forkhead Box P3; ARA, allergic rhinitis and asthma; anti-TGF-β<sub>1</sub>, TGF-β<sub>1</sub> neutralizing antibody; DXM, dexamethasone; MK, montelukast; BUD, budesonide.

the NALF, BALF and peripheral blood of the ARA group (P<0.001 vs. normal group), and the medications significantly increased IFN-γ in ARA mice (P<0.05). Moreover, TGF-β<sub>1</sub> neutralizing antibody exerted a significantly greater stimulatory effect on IFN-γ levels when compared with the other drug treatments in all three samples (P<0.05). As IL-4 is characteristic of Th2-mediated allergic inflammation and IFN-γ is a primary secretory product of Th1 (23), these results indicated that TGF-β<sub>1</sub> neutralizing antibody suppressed the action of Th2 cells while elevating the action of Th1 cells in the nasal mucosa, bronchia and peripheral blood.

**TGF-β<sub>1</sub> neutralizing antibody modulates Treg cell expansion in the PBMCs of ARA mice.** As TGF-β<sub>1</sub> neutralizing antibody altered the levels of Th1- and Th2-related cytokines, the effect of TGF-β<sub>1</sub> neutralizing antibody on Treg cells in ARA were evaluated using flow cytometry. Firstly, the expression of TGF-β<sub>1</sub> in immunological cells was assessed. No significant difference in the frequency of TGF-β<sub>1</sub><sup>+</sup> PBMCs was observed among all six groups (data not shown). By contrast, the frequency of TGF-β<sub>1</sub><sup>+</sup> cells in the cluster of differentiation (CD)3<sup>+</sup> subpopulation, and CD3<sup>+</sup> is a marker of T cells (24) of the ARA group was significantly elevated compared with

the normal group (P<0.01; Fig. 3A). Furthermore, treatment with TGF-β<sub>1</sub> neutralizing antibody, MK and BUD significantly decreased the frequency of TGF-β<sub>1</sub><sup>+</sup> cells in the CD3<sup>+</sup> subpopulation when compared with the ARA group (P<0.05; Fig. 3A). Next, the modulatory effects of ARA and drug treatments on Treg cells, which were defined by the expression of CD4, CD25 and Foxp3 (25), were investigated. As depicted in Fig. 3B, the numbers of Treg cells were significantly decreased in the ARA group when compared with the normal group (P<0.01). In turn, treatment with TGF-β<sub>1</sub> neutralizing antibody significantly increased the frequency of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> cells in the CD4<sup>+</sup> subpopulation compared with the ARA group (P<0.05). The numbers of Treg cells did not differ significantly between the ARA group and remaining treatment groups. These data suggested that TGF-β<sub>1</sub> neutralizing antibody inhibited the expression of TGF-β<sub>1</sub> in T cells and prevented Treg cell deficiency induced by ARA.

**TGF-β<sub>1</sub> neutralizing antibody reduces the level of TGF-β<sub>1</sub> in nasal and lung tissues.** To further analyze the function of TGF-β<sub>1</sub> neutralizing antibody in ARA, levels of TGF-β<sub>1</sub> in nasal and lung tissues were assessed using IHC and RT-qPCR. As depicted in Fig. 4A-C, levels of TGF-β<sub>1</sub> were significantly

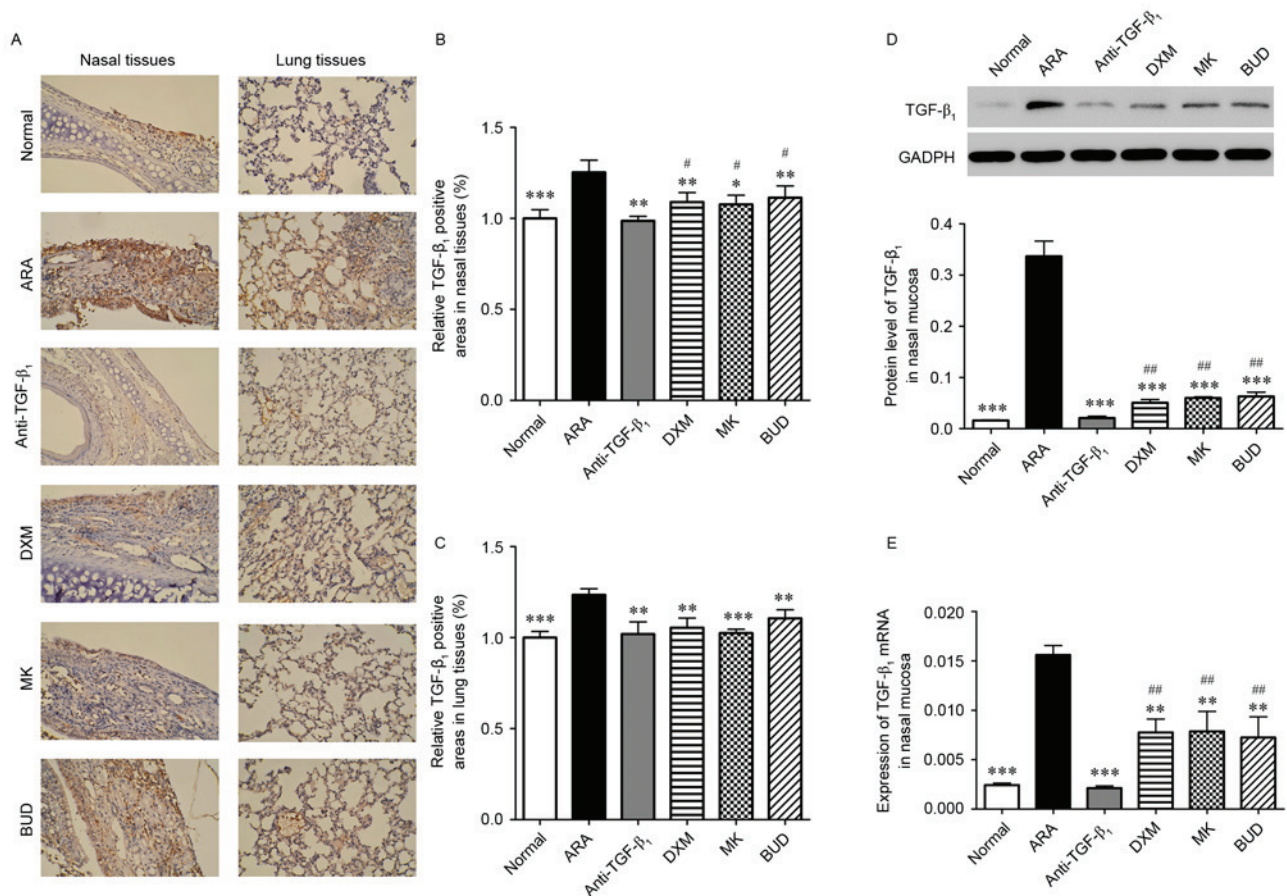


Figure 4. Expression of TGF-β<sub>1</sub> in the lungs and nasal mucosa following treatment with TGF-β<sub>1</sub> neutralizing antibody, DXM, MK and BUD. (A-C) TGF-β<sub>1</sub> expression in the lungs and nasal mucosa analyzed by immunohistochemistry (brown DAB staining). Magnification, x200. (D) Expression of TGF-β<sub>1</sub> protein in the nasal mucosa measured by western blot analysis. (E) Expression of TGF-β<sub>1</sub> mRNA in the nasal mucosa measured by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard deviation. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. ARA; #P<0.05 and ##P<0.01 vs. anti-TGF-β<sub>1</sub> group. TGF-β<sub>1</sub>, tumor growth factor-β<sub>1</sub>; ARA, allergic rhinitis and asthma; anti-TGF-β<sub>1</sub>, TGF-β<sub>1</sub> neutralizing antibody; DXM, dexamethasone; MK, montelukast; BUD, budesonide.

higher in the nasal and lung tissues of the ARA group compared with the normal group (P<0.001). In turn, the medications induced significant decreases in the TGF-β<sub>1</sub>-positive areas in the nasal mucosa and lung of ARA mice (P<0.05). Similar results of western blotting and RT-qPCR demonstrated that the protein and mRNA expressions of TGF-β<sub>1</sub> were significantly reduced in the nasal mucosa of ARA mice by the medications (P<0.01), particularly by TGF-β<sub>1</sub> neutralizing antibody treatment (P<0.001; Fig. 4D and E). These data suggested that TGF-β<sub>1</sub> levels were significantly higher in ARA mice, and TGF-β<sub>1</sub> neutralizing antibody inhibited the expression of TGF-β<sub>1</sub> in the nasal and lung tissues.

*TGF-β<sub>1</sub> neutralizing antibody inhibits the activation of the Smad2/3 signaling pathway.* Finally, the Smad2/3 signaling pathway was investigated using western blot analysis. The protein expression of Smad2/3 and the phosphorylation of Smad2/3 were significantly higher in the ARA group compared with the normal group (P<0.01). It was observed that the protein expression of Smad2/3 and the phosphorylation of Smad2/3 were significantly inhibited by the medications compared with the ARA group (P<0.05; Fig. 5). Furthermore, TGF-β<sub>1</sub> neutralizing antibody had a significantly greater inhibitory effect on the phosphorylation of Smad3 (P<0.05) compared with the

other drug treatments and a greater inhibitory effect on Smad2 expression than DXM and MK (P<0.05).

## Discussion

TGF-β<sub>1</sub> is as a multifunctional cytokine involved in many biological processes. TGF-β<sub>1</sub> is typically considered to be an anti-inflammatory cytokine that exerts inhibitory effects on T cells, macrophages and dendritic cells (13). It may also lead to the differentiation of Treg cells (16,26). However, facilitated expression of TGF-β<sub>1</sub> has also been detected in the airways of some chronic respiratory diseases, including allergic rhinitis and asthma (27,28). It has been suggested that TGF-β<sub>1</sub> may function in several pro-inflammatory processes, and may serve complex roles in the pathophysiology of ARA (13-16). The complex effects of TGF-β<sub>1</sub> in allergic diseases may provide novel clinical targets. In the present study, TGF-β<sub>1</sub> neutralizing antibody as was used to treat allergic inflammation in upper and lower airways of mice with ARA induced by OVA. Treatments with three other medicines, namely DXM, MK and BUD, served as positive control groups. Firstly, the effects of the medications on the pathological symptoms of ARA were investigated. It was observed that TGF-β<sub>1</sub> neutralizing antibody exerted significant therapeutic effects in ARA,

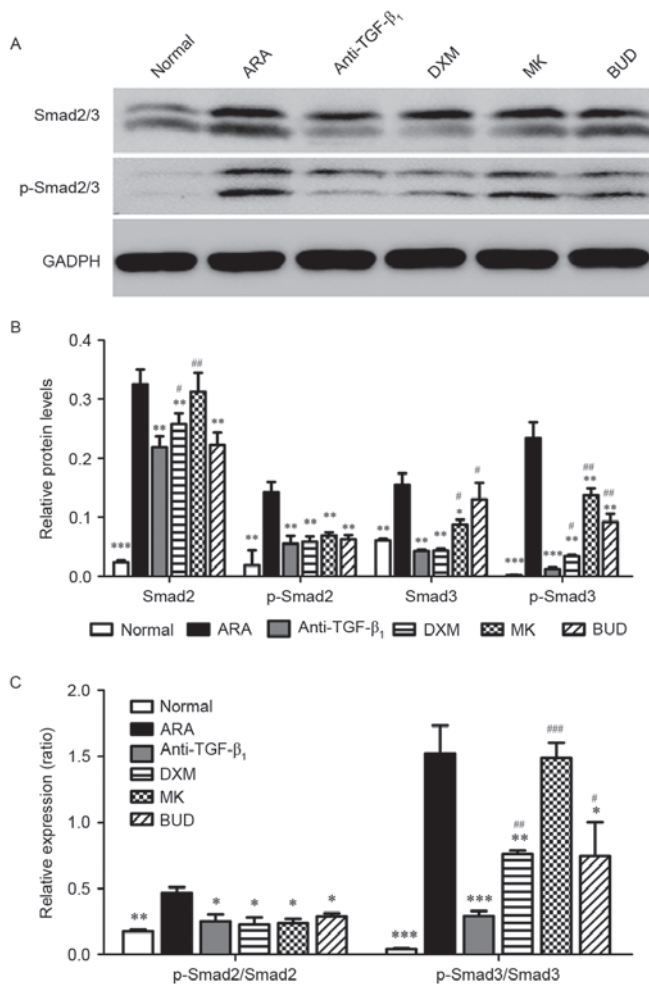


Figure 5. Effect of TGF- $\beta_1$  neutralizing antibody on Smad2/3 signaling pathways. (A and B) Western blot analysis of the relative protein levels in the Smad2/3 pathways. (C) Analysis of Smad2/3 pathway activation. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. ARA; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. anti-TGF- $\beta_1$  group. ARA, allergic rhinitis and asthma; anti-TGF- $\beta_1$ , tumor growth factor- $\beta_1$  neutralizing antibody; DXM, dexamethasone; MK, montelukast; BUD, budesonide; p-, phosphorylation.

as demonstrated by significant inhibition of eosinophil infiltration, goblet cell hyperplasia and collagen deposition in the upper and lower airways.

Cytokine secretion was subsequently assessed in the NALF, BALF and peripheral blood. It was observed that treatment with TGF- $\beta_1$  neutralizing antibody significantly decreased levels of IL-4. Moreover, relative to DXM, MK and BUD, TGF- $\beta_1$  neutralizing antibody reversed the ARA-induced decrease in IFN- $\gamma$  to a significantly greater extent. Results of flow cytometry also suggested that TGF- $\beta_1$  neutralizing antibody inhibited the expression of TGF- $\beta_1$  in T cells, and significantly restored the ratio of Treg cells in CD4<sup>+</sup> T cells. Imbalance in the Th1/Th2 immune response is considered to be involved in the pathogenesis of allergic airway diseases (29). Secretions of Th1 cells, containing IL-2, IFN- $\gamma$  and TNF, serve roles in cell-mediated immunity and delayed-type hypersensitivity occurrence (30). However, cytokines released by Th2 may also contribute to the initiation and maintenance of inflammation in allergic immune responses (31,32). For instance, IL-4 has been demonstrated to regulate the isotype switching of IgE (33).

Mutual antagonism between Th1 and Th2 cells means that restoration of the Th1/Th2 balance is necessary for effective ARA therapy. More recently, Treg cell immunodeficiency has been observed in allergic diseases (34). Treg cells may regulate the Th1/Th2 immune balance by inhibiting the function of Th2 cells (35,36). The data in the present study indicated that TGF- $\beta_1$  neutralizing antibody restored the Th1/Th2 immune balance and alleviated the deficiency in Treg cells induced by ARA.

To elucidate the functional mechanism of TGF- $\beta_1$  neutralizing antibody, the expression of TGF- $\beta_1$  was measured in lung and nasal tissues, and Smad2/3 signaling pathway activation was assessed in the nasal mucosa by western blot analysis. Despite the anti-inflammatory functions of TGF- $\beta_1$  (16), results have suggested that the expression of TGF- $\beta_1$  is increased in ARA. Previous studies have also demonstrated that TGF- $\beta_1$  was involved in the promotion of eosinophil infiltration and goblet cell hyperplasia (37,38). Moreover, TGF- $\beta_1$  may serve a role in the differentiation of effector Th17 cells, which are related to the pathogenesis of several inflammatory diseases (39). The Smad2/3 signaling pathways are associated with many biological processes. Rosendahl *et al* (40) documented that the TGF- $\beta$ /Smad2 pathway may be activated during allergic airway inflammation. Furthermore, the activation of Smad2/3 signaling pathways promote IL-6 release, as a critical process in the induction of subepithelial fibrosis and airway remodeling in asthma (41). Activated Smad2/3 pathways also serve roles in collagen deposition and airway remodeling (40,42). Moreover, Antoni *et al* (43) suggested that a lack of Smad3 may induce an upregulation in Foxp3 mRNA and increased numbers of Foxp3<sup>+</sup> cells during contact hypersensitivity responses. In the present study, treatment with TGF- $\beta_1$  neutralizing antibody significantly downregulated TGF- $\beta_1$  in the lung and nasal tissues, and all medications with the exception of MK inhibited the protein expression of Smad2/3 and phosphorylation of the Smad2/3 pathway. Notably, TGF- $\beta_1$  neutralizing antibody exerted a significantly greater inhibitory effect on the Smad3 signaling pathway when compared with the other three medicines. These results indicated that the therapeutic effects of TGF- $\beta_1$  in ARA were based on the downregulation of TGF- $\beta_1$  and inhibition of Smad2/3 signaling in the airways.

In conclusion, the present study used TGF- $\beta_1$  neutralizing antibody as a novel therapeutic approach for the treatment of ARA. It was demonstrated that TGF- $\beta_1$  neutralizing antibody inhibited allergic symptoms, restored the Th1/Th2 immune balance and ameliorated Treg cell deficiencies. The functional mechanism of TGF- $\beta_1$  neutralizing antibody may involve the repression of TGF- $\beta_1$  expression and Smad2/3 signaling in airway tissues. The results of the present study suggest that the clinical implications of TGF- $\beta_1$  neutralizing antibody in ARA therapy although the side effects have not been characterized.

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