



Therapeutic efficacy of a co-blockade of IL-13 and IL-25 on airway inflammation and remodeling in a mouse model of asthma

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ABSTRACT

Repeated airway inflammation and unremitting remodeling provoke irreversible pulmonary dysfunction and resistance to current drugs in patients with chronic bronchial asthma. Interleukin (IL)-13 and IL-25 play an important role in airway inflammation and remodeling in asthma. We aimed to investigate whether co-inhibiting IL-13 and IL-25 can effectively down-regulate allergen-induced airway inflammation and remodeling in mice. Mice with asthma induced by chronic exposure to ovalbumin (OVA) were given soluble IL-13 receptor $\alpha 2$ (sIL-13R) or soluble IL-25 receptor (sIL-25R) protein alone and in combination to neutralize the bioactivity of IL-13 and IL-25, and relevant airway inflammation and remodeling experiments were performed. We found that the co-blockade of IL-13 and IL-25 with sIL-13R and sIL-25R was more effective than either agent alone at decreasing inflammatory cell infiltration, airway hyperresponsiveness (AHR) and airway remodeling including mucus production, extracellular collagen deposition, smooth muscle cell hyperplasia and angiogenesis in mice exposed to OVA. These results suggest that the combined inhibition of IL-13 and IL-25 may provide a novel therapeutic strategy for asthma, especially for patients who are resistant to current treatments.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways in which the immune and structural cells (such as T cells, eosinophils, mast cells and epithelial cells) act together to cause airway inflammation, mucus overproduction and airway wall remodeling [1]. Asthma affects approximately 300 million people worldwide, causes 250,000 deaths annually and is responsible for billions of dollars in healthcare expenditures [2]. The vast majority of asthmatics are well controlled by established treatments, such as inhaled corticosteroids and $\beta 2$ -adrenergic agonists. Unfortunately, 5–10% of asthmatics are poorly controlled and often have an increased risk of hospitalization [3]. Frequently, these conditions can be associated with airway wall remodeling, including goblet cell metaplasia, subepithelial fibrosis, smooth muscle hyperplasia and neovascularization [4,5].

Traditionally, asthma has been viewed as the hallmark Th2 (adaptive immune response cell) disorder of the lungs and is characterized by increased levels of IL-4, IL-5, and IL-13. These cytokines are needed to induce class switching of B cells to produce allergen-specific IgE, recruit eosinophilic inflammation and promote airway wall remodeling [6]. Importantly, a number of investigators have reported that IL-13

plays a key role in the effects of airway remodeling induced in the airway epithelium [7], smooth muscle [8], fibroblasts [9], and endothelium [10]. Preliminary efficacy has been shown for several compounds (including anti-IL-13Fab' [11], an IL-13 vaccine [12], and sIL-13R $\alpha 2$ [13]) in basic studies aiming to modulate IL-13 responses, but anti-IL-13 (lebrikizumab [14]) therapies have not consistently demonstrated significant benefits in clinical trials. Additional mechanisms may therefore underlie the pathogenesis of asthma. Recent studies have reported that ILC2s (innate immune response cells) are critical for the initiation and development of asthma in mice. These cells, which were discovered in mice, reside in the mucosa and secrete the Th2-like cytokines IL-5, IL-4 and IL-13 in response to IL-25, IL-33 and TSLP released by a damaged epithelium [15]. Although ILC2 development and activation can be induced by IL-25, IL-33 and TSLP, IL-25 may be higher in patients with allergen-induced asthma. In addition, IL-33 may be higher in patients with a virus-associated disease, and TSLP may be higher in those with a toxin-associated disease (such as cigarette smoke) [16]. Furthermore, IL-25 also drives IL-33 and TSLP production in the lung [17]. Yao et al. showed that IL-25 alone is sufficient to induce airway inflammation, remodeling and hypersensitivity in a murine asthma model [18]. A few studies have examined the effect of neutralizing the activity of IL-25 by sIL-25R [19] or an anti-IL-25 mAb [20] in asthma, and the results of these studies have shown partial efficacy due to the multiple mechanisms involved in the pathogenesis of asthma. Consequently, the most effective approaches for asthma patients who are resistant to current

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treatments may be combination therapies that suppress multiple cytokines and a range of disconnected pathways that separately contribute to asthma pathogenesis [21].

The soluble isoforms of the IL-13 receptor [13] and IL-25 receptor [22] have been reported to down-regulate their respective signaling pathways as endogenous antagonist “decoy receptors” that have the benefits of being water-soluble, nonimmunogenic, and able to easily cross tissue barriers.

The objective of this study was to test the hypothesis that the co-inhibition of adaptive immune responses and innate immune responses by a combined blockade of IL-13 and IL-25 with soluble IL-13 receptor $\alpha 2$ (sIL-13R) protein and soluble IL-25 receptor (sIL-25R) protein can down-regulate allergen-induced airway inflammation and remodeling effectively in mice.

2. Materials and methods

2.1. Animals

Female BALB/c mice aged 6–8 weeks and weighing approximately 20 g were obtained from the Experimental Animal Centre of the Fourth Military University (Xi'an, China). The mice were randomly divided into the following 6 groups: a normal control group (PBS/PBS group), an asthma group (OVA/OVA group), a control IgG group (OVA/IgG group), two single blocking groups (OVA/sIL-13R group and OVA/sIL-25R group) and a combined blocking group (OVA/sIL-13R + sIL-25R). All animal protocols were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University.

2.2. Protocol for asthma model and intervention

Chronic asthma was induced in mice according to the methods described previously [23] with minor modifications. Briefly, the mice were sensitized by intraperitoneal injections of 20 μ g of OVA (Sigma, Saint Louis, MO, USA) and 500 μ g of Al(OH)₃ suspended in 0.2 ml of PBS or 0.2 ml of PBS alone on days 0 and 7. On day 21, the mice were placed in a closed plastic box (26 $\times$ 19 $\times$ 17 cm) and were airway challenged with 5% aerosolized OVA or PBS alone for 30 min once a week for 5 weeks by an ultrasonic air nebulizer (402AI, Yuyue, Danyang, China). One hour before each OVA challenge, the mice in the OVA/sIL-13R group received intraperitoneal doses of 150 μ g of sIL-13R $\alpha 2$ (Sino Biological Inc., Beijing, China) and 150 μ g of control IgG (Sino Biological Inc.); the mice in the OVA/sIL-25R group received a 150- μ g dose of sIL-25R (Sino Biological Inc.) and 150 μ g of control IgG; the mice in the OVA/sIL-13R + sIL-25R group received a 150- μ g dose of sIL-13R $\alpha 2$ and 150 μ g of sIL-25R; and the OVA/IgG group received a 300- μ g dose of control IgG. The dose of soluble neutralizing receptors used is within the dose range described previously [19,24,25] with minor modifications, and the endotoxin in these agents was <1.0 EU per μ g of the protein as determined by the Limulus Amebocyte Lysate (LAL) method. Mice in the single treatment groups were also injected with control IgG to ensure that the total dose delivered was equivalent to eliminate the dose effect.

2.3. Measurement of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) to methacholine (Mch; Sigma, St Louis, MI, USA) was measured 24 h after the last OVA challenge with an AniRes 2005 animal lung function analysis system (Best Lab International Inc., Beijing, China) as previously described [26] with minor modifications. Briefly, after anesthetization, the mice were tracheostomized and connected to a rodent ventilation machine set at 120 breaths/min. The ratio of inspiratory to expiratory beats was 2:1. The Mch was infused via the caudal vein at concentrations of 10 to 270 μ g/kg. Data were obtained, and the lung resistance (RL) at

270 μ g/kg Mch was statistically analyzed and used to express changes in AHR.

2.4. Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected according to the methods previously described [27] with minor modifications. Briefly, the mice were euthanized after anesthetization. In total, 0.5 ml of ice-cold saline was injected slowly into the trachea three times, and approximately 1.2 ml of BALF was obtained. The BALF was then centrifuged at 1500 rpm for 10 min at 4 $^{\circ}$ C, and the pellets were resuspended for cellular analysis. The supernatants were stored at -80° C for cytokine analysis. The total number of cells in the BALF was counted using a hemocytometer. A differential count was performed using cyto-smear preparations. The cells were fixed and stained with Wright-Giemsa stain. Differential counts of 200 cells were carried out using standard morphological criteria to identify eosinophils.

2.5. Lung histology

Left lung lobes were inflated and fixed with 4% paraformaldehyde for 24 h. Paraffin-embedded sections (4 μ m) were stained with hematoxylin/eosin (H&E) to evaluate the general morphology as well as with periodic acid-Schiff (PAS) to identify goblet cells and evaluate PAS scoring [24] for goblet cell hyperplasia. Additionally, Sirius red was used to assess the extracellular collagen deposition. Five round cross-sections of bronchi (shortest diameter of lumen/longest diameter >0.6) per section were randomly selected in a blinded manner and then observed. Pictures were then taken, and the goblet cell area and the perimeter of the bronchial basement membrane (Pbm) of the mice were assessed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) to determine the thickness of goblet cells [28]. A quantitative analysis of the peribronchiolar collagen density [29] was conducted by measuring Sirius red-stained collagen in lung sections with Image-Pro Plus 6.0 software. All scoring and measurements were performed blindly by the same observer.

2.6. Lung immunohistochemistry

Immunohistochemistry (IHC) was used to detect airway remodeling biomarkers. Paraffin sections were stained with rabbit anti-mouse antibodies against alpha smooth muscle actin (α -SMA) (Abcam, Cambridge, UK), proliferating cell nuclear antigen (PCNA) (Abcam), and von Willebrand factor (vWF) (Affinity, Cincinnati, OH, USA) using an avidin/biotin stain. The thickness [29] of the airway smooth muscle layer around medium-sized conducting airways was measured from α -SMA-stained sections. Ten measurements were collected per airway, and a minimum of 10 airways were measured per section. The percentage [29] of PCNA-positive airway mesenchymal cells was calculated as an index of smooth muscle hyperplasia. Cells positive for PCNA stained dark brown and were counted per bronchiole in four bronchioles per mouse. The total number of vWF-positive vessels of entire lung sections was calculated to evaluate angiogenesis in the lungs.

2.7. Lung tissue homogenates and collagen assay

Right lung tissue was homogenized as previously described [18]. Briefly, right lung sections were homogenized in 2 ml of PBS containing 1% Triton X-100 and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The supernatant was collected after centrifugation to measure the total collagen and cytokines. The amount of lung collagen was measured by a biochemical assay according to the manufacturer's instructions (Sircol collagen assay; Biocolor, Belfast, UK) and normalized to lung weight.

2.8. Western blot analysis

Equal amounts of homogenate supernatants (20 μ g) were separated on 8% polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in 5% skim milk for 1 h and then incubated with specific rabbit anti-mouse primary antibodies, including TGF- β 1 (Santa Cruz Biotechnology, CA, USA), Smad2 (Abcam), phospho-Smad2 (Abcam), and β -actin (Abcam), overnight at 4 °C. The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Boshide Inc., Shanghai, China) at 37 °C for 1 h. The immunoreactions were visualized using an electrochemiluminescence (ECL) Western blotting kit (Amersham Pharmacia Biotech, Freiburg, Germany). Densitometry analysis was performed using Quantity One 4.5.2 image processing software (BioRad, Hercules, CA, USA).

2.9. Cytokine analysis

The levels of cytokines IL-4, IL-5, IL-13, IL-33, vascular endothelial growth factor (VEGF), and CXCL2 in BALF or lung homogenates were determined using a Magnetic Luminex Screening Assay (Luminex 200™ System, Life Technologies), which allows the simultaneous measurement of a panel of multiple analytes in a small-volume sample. The assay for the quantitative detection of the aforementioned cytokines was performed using a mouse premixed multi-analyte kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The concentrations of IL-25 and thymic stromal lymphopoietin (TSLP) were measured with ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. All the presented data have been normalized for tissue weight.

2.10. Statistical analysis

Statistical analyses were performed using SPSS 13.0 (IBM) and Prism 5.0 (GraphPad). Multiple comparisons were compared using the non-parametric Kruskal-Wallis test. If differences were observed as significant, Nemenyi's non-parametric multiple comparison was applied to compare differences between two groups. The data are presented as the mean $\pm$ SEM. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Co-blockade of IL-13 and IL-25 attenuates airway inflammation and AhR in OVA-induced asthma

Characteristic features of asthma, including marked elevation of airway infiltration of inflammatory cells, were observed in the OVA/OVA group. Treatment with control IgG did not have any effects on these changes (Fig. 1A–C). Compared to the OVA/IgG group, mice treated with sIL-13R alone and sIL-25R alone exhibited a significant decrease in the number of recruited cells in BALF, and the combination treatment caused a further decrease (Fig. 1A). In particular, eosinophilia, a typical feature of asthma, was significantly reduced by the combination treatment compared to the groups treated with sIL-13R and sIL-25R alone (Fig. 1B). As illustrated by the H&E-stained lung sections, the combined treatment also resulted in a remarkable improvement in the peribronchial accumulation of inflammatory cells compared to the two single treatment groups (Fig. 1C). To determine whether co-blocking IL-13 and IL-25 can interfere with AhR, lung resistance (RI) was tested in mice challenged with Mch (Fig. 1D). A greater improvement in AhR at 270 μ g/kg Mch was observed in the combined treatment group than in the two single treatment groups compared with the OVA/IgG group (Fig. 1E).

3.2. Co-blockade of IL-13 and IL-25 alleviates mucus hypersecretion in airways

Lung sections were stained with PAS to identify mucus-containing goblet cells, which represent the differentiation of the epithelium to a mucus-secreting phenotype. Compared with the PBS/PBS group, there was a remarkable increase in goblet cell hyperplasia and mucus hypersecretion in the lung tissue from the OVA/OVA- and OVA/IgG-treated mice (Fig. 2A and B). The change in the epithelium to a mucus-secreting phenotype was associated with an increase in the thickness of the epithelium [29], as demonstrated by the goblet cell area/Pbm value (Fig. 2C). Compared with the OVA/IgG group, treatment with sIL-13R alone and sIL-25R alone led to a significant decrease in PAS scoring and goblet cell area/Pbm values; moreover, the combination treatment caused further statistical decreases (Fig. 2B and C).

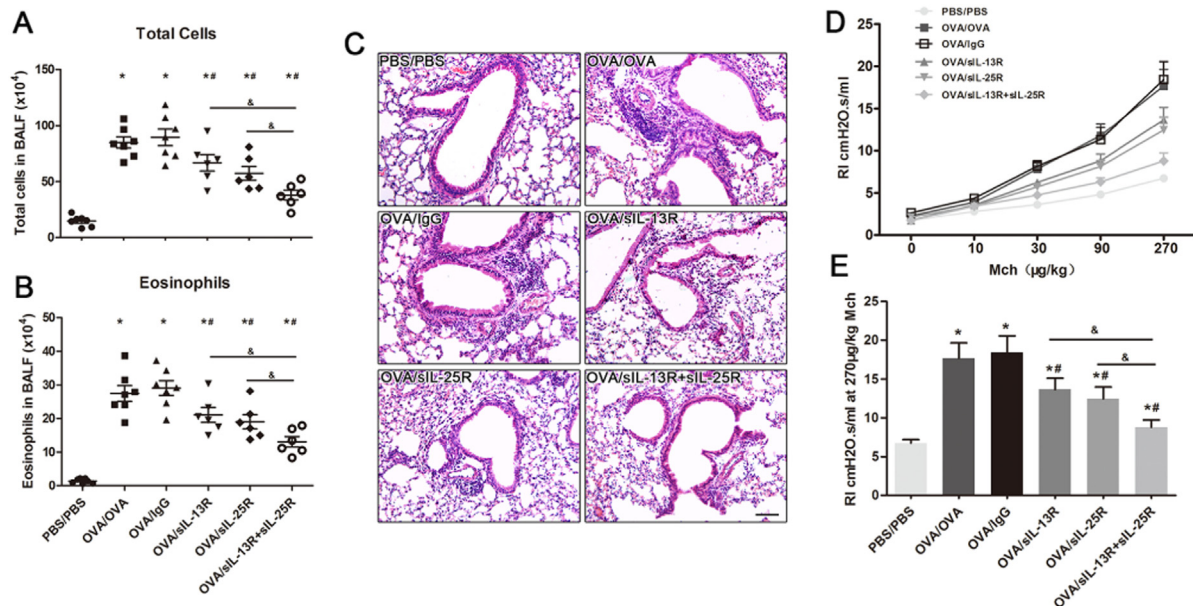


Fig. 1. Blockade of IL-13 and/or IL-25 attenuates airway cell infiltration, tissue pathology and AhR in mice. (A) Total cells in BALF. (B) Eosinophils in BALF. (C) Representative photographs of H&E-stained lung sections from each group. Original magnification $\times 20$. Scale bar = 50 μ m. (D) Airway resistance (RI) to methacholine (Mch) in mice. (E) RI at 270 μ g/kg Mch. The data are presented as the mean $\pm$ SEM ($n = 6-7$ per group). * $P < 0.05$ compared to the PBS/PBS group, # $P < 0.05$ compared to the OVA/IgG group, and & $P < 0.05$ compared to the single blocking group (OVA/sIL-13R or OVA/sIL-25R).

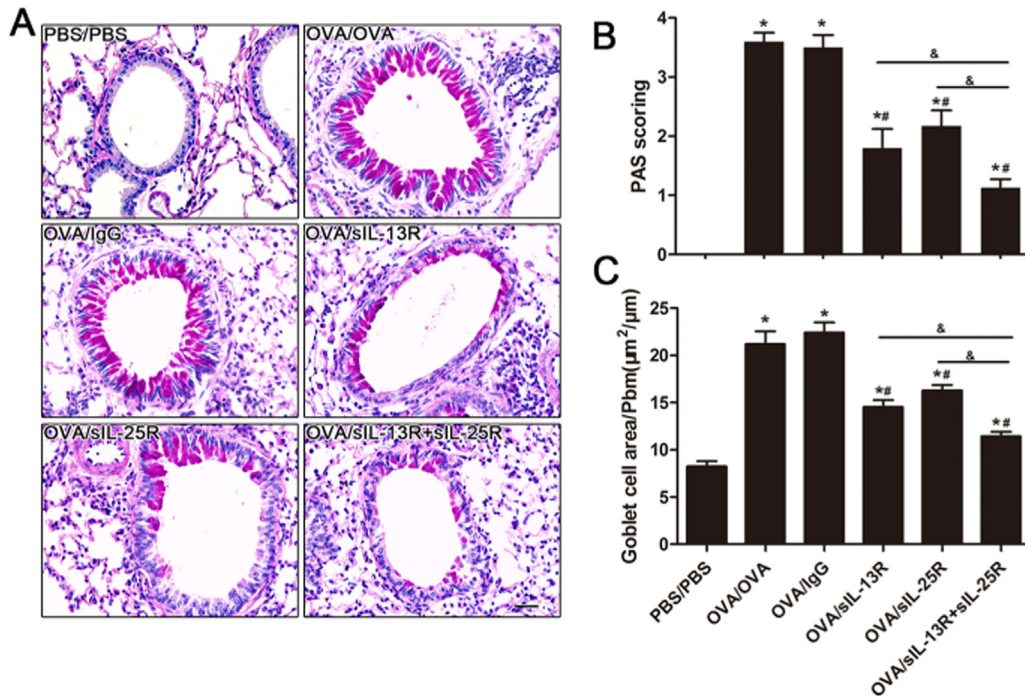


Fig. 2. Blockade of IL-13 and/or IL-25 alleviates mucus production in airways. (A) Representative photographs of periodic acid-Schiff (PAS)-stained lung sections from each group. PAS staining is depicted by pink/purple-colored mucin-containing cells in the epithelium. Original magnification $\times 40$. Scale bar = 20 μm . (B) Scoring of PAS-stained sections. (C) Quantitative analysis of goblet cell area/Pbm in lung sections. The data are presented as the mean $\pm$ SEM ($n = 6-7$ per group). * $P < 0.05$ compared to the PBS/PBS group, ** $P < 0.05$ compared to the OVA/IgG group, and $\Delta P < 0.05$ compared to the single blocking group (OVA/sIL-13R or OVA/sIL-25R). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Co-blockade of IL-13 and IL-25 reduces subepithelial fibrosis in airways

To examine the subepithelial fibrosis induced by OVA exposure, we analyzed the perivascular and peribronchial collagen deposition using Sirius red staining. Collagen deposition was markedly increased in the OVA/OVA- and OVA/IgG-treated mice compared with the PBS/PBS group (Fig. 3A). The collagen density and the total amount of collagen were significantly decreased in both the single treatment and combined treatment groups compared with the OVA/IgG-treated mice. In the

combined treatment group, the collagen density was further decreased only compared to the OVA/sIL-25R group (Fig. 3B), while the total amount of collagen was further decreased compared to both of the single treatment groups (Fig. 3C). To confirm whether blockade of IL-13 and IL-25 could inhibit the collagen synthesis promoted by activation of the TGF- $\beta 1$ signaling pathway, we examined the expression of TGF- $\beta 1$ and its downstream molecules (Smad2 and p-Smad2) in lung tissues. The expression of TGF- $\beta 1$ and p-Smad2 were significantly decreased in the OVA/sIL-13R and OVA/sIL-25R groups and particularly

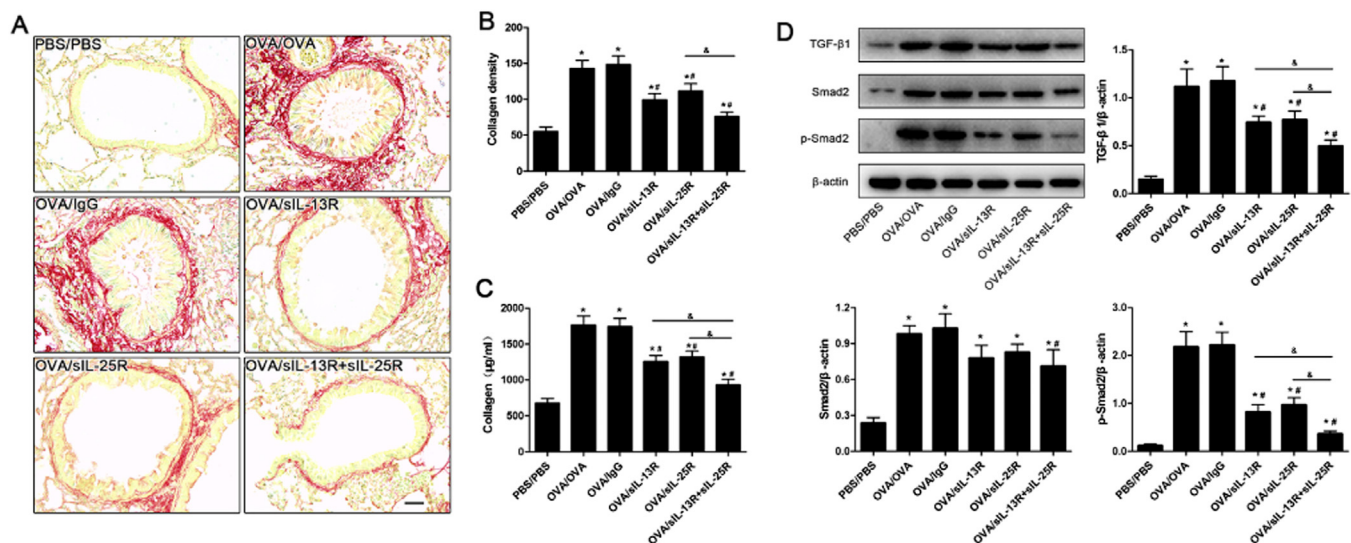


Fig. 3. Blockade of IL-13 and/or IL-25 decreases subepithelial fibrosis in airways. (A) Representative photographs of Sirius-red-stained perivascular and peribronchial collagen from each group. Original magnification $\times 40$. Scale bar = 20 μm . (B) Quantitative analysis of subepithelial peribronchiolar collagen density. (C) Total lung collagen was quantified by a biochemical Sircol assay. (D) Protein levels of TGF- $\beta 1$, Smad2 and phosphorylated Smad2 (p-Smad2) were determined in the lung by Western blotting and normalized to β -actin. The data are presented as the mean $\pm$ SEM ($n = 6-7$ per group). * $P < 0.05$ compared to the PBS/PBS group; ** $P < 0.05$ compared to the OVA/IgG group, and $\Delta P < 0.05$ compared to the single blocking group (OVA/sIL-13R or OVA/sIL-25R).

in the OVA/sIL-13R + sIL-25R group (Fig. 3D). These results revealed that sIL-13R, sIL-25R, and especially sIL-13R + sIL-25R could reduce subepithelial fibrosis by suppressing the TGF- β 1 signaling pathway.

3.4. Co-blockade of IL-13 and IL-25 ameliorates airway smooth muscle cell hypertrophy and hyperplasia

Increased airway smooth muscle mass, due to both smooth muscle hypertrophy and hyperplasia, is a very significant component of airway remodeling [30]. To assess these changes, lung sections were immunostained with antibodies against α -SMA and PCNA (Fig. 4A and C). The smooth muscle thickness surrounding the bronchioles was increased in OVA/OVA- and OVA/IgG-treated mice but decreased significantly in mice treated with sIL-13R, sIL-25R, and particularly sIL-13R + sIL-25R (Fig. 4B). The percentage of PCNA-positive cells, an index of smooth muscle hyperplasia, was also significantly increased in the OVA/OVA and OVA/IgG groups but decreased in the sIL-13R, sIL-25R and combined treatment groups. Moreover, a further decrease was observed in the co-treatment group compared to the mice treated with sIL-13R or sIL-25R alone (Fig. 4D).

3.5. Co-blockade of IL-13 and IL-25 decreases angiogenesis in the lung

The number of vWF-positive vessels was calculated to measure the angiogenesis in the lung (Fig. 5A). There was a significant increase in the number of vWF-positive blood vessels following OVA exposure. Compared with the OVA/IgG group, the number of vWF-positive vessels was significantly decreased in the mice treated with sIL-25R alone and especially in the mice that received the combined treatment. However, there was no significant difference between the sIL-13R and OVA/IgG groups in the number of vWF-positive vessels (Fig. 5B). We next investigated the levels of VEGF in BALF and lung homogenates. A significantly elevated VEGF level was observed in the OVA/IgG-treated mice, but the VEGF level decreased to different extents in the single and combined treatment groups (Fig. 5C and D). Thus, co-blockade of IL-13 and IL-25 could effectively decrease angiogenesis in the lung.

3.6. Co-blockade of IL-13 and IL-25 reduces the production of inflammatory mediators

Airway inflammation in asthma is identified partly by a local release of cytokines by immune and structural cells. Consequently, we

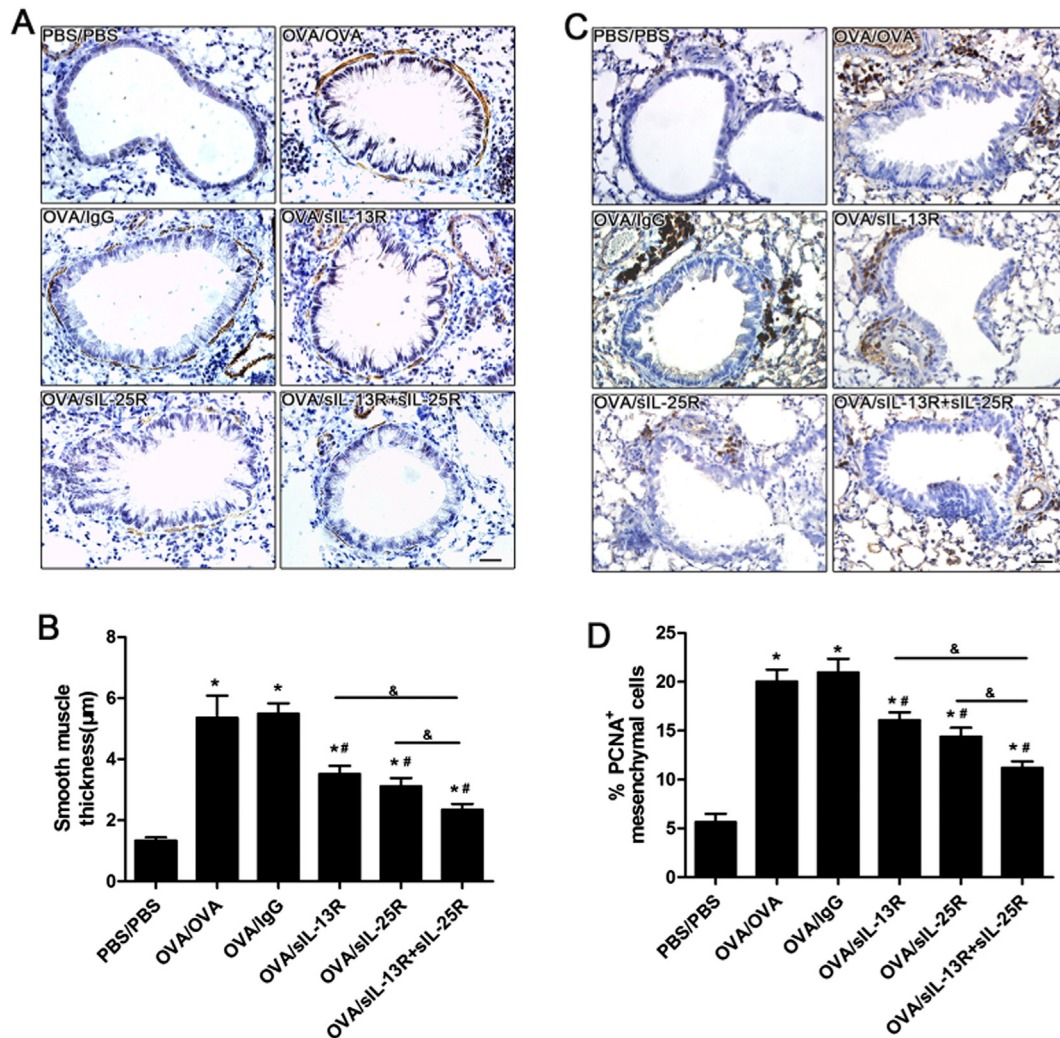


Fig. 4. Blockade of IL-13 and/or IL-25 ameliorates airway smooth muscle cell hypertrophy and hyperplasia. (A) Representative photographs of α -smooth muscle actin (α -SMA) immunoreactivity in lung sections from each group. Smooth muscle cells and myofibroblasts were identified by immunohistochemical staining of α -SMA (brown stain). (B) Quantitative analysis of the thickness of the α -SMA-stained peribronchial smooth muscle layer. (C) Representative photographs of proliferating cell nuclear antigen (PCNA)-positive (brown/dark stained) proliferating cells in lung sections from each group. (D) Percentage of PCNA-positive peribronchial mesenchymal cells. Original magnification $\times 40$. Scale bar = 20 μ m. The data are presented as the mean $\pm$ SEM ($n = 6-7$ per group). * $P < 0.05$ compared to the PBS/PBS group, # $P < 0.05$ compared to the OVA/IgG group, and & $P < 0.05$ compared to the single blocking group (OVA/sIL-13R or OVA/sIL-25R). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

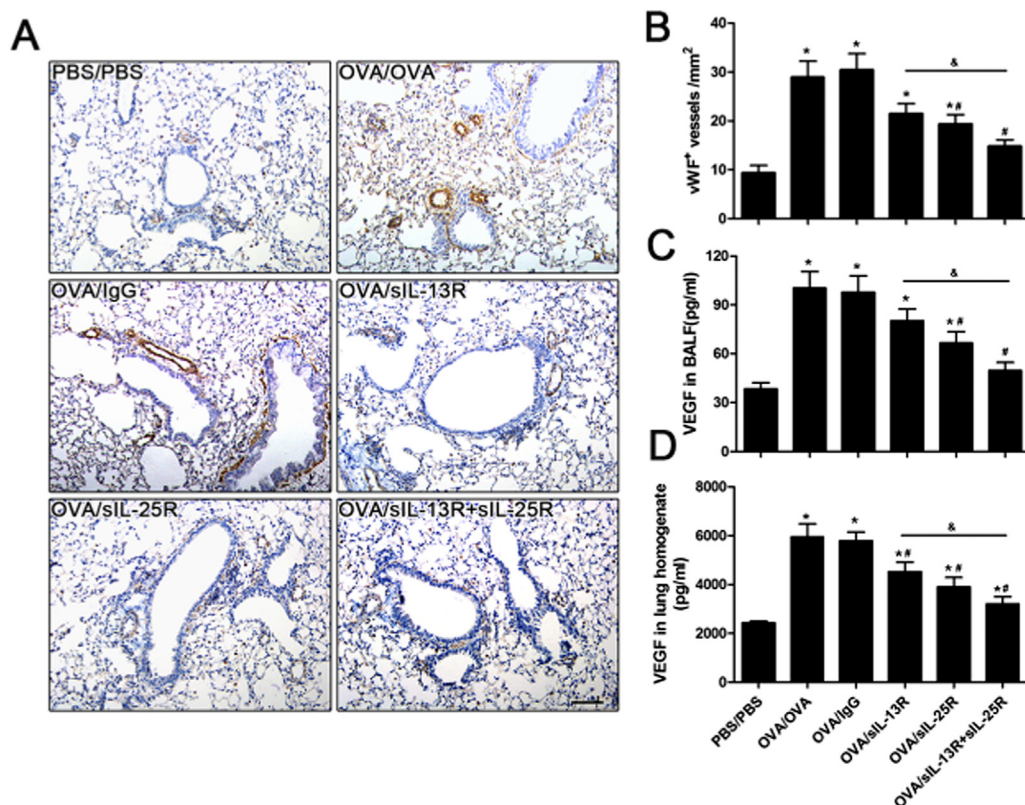


Fig. 5. Blockade of IL-13 and/or IL-25 decreases angiogenesis in the lung. (A) Representative photographs of von Willebrand factor (vWF)-positive (brown stained) peribronchial vessels. Original magnification $\times 20$. Scale bar = 50 μ m. (B) vWF-positive vessels per square millimeter. (C) Vascular endothelial growth factor (VEGF) levels in BALF. (D) Levels of VEGF in lung homogenates. The data are presented as the mean $\pm$ SEM ($n = 6-7$ per group). * $P < 0.05$ compared to the PBS/PBS group, # $P < 0.05$ compared to the OVA/IgG group, and ** $P < 0.05$ compared to the single blocking group (OVA/sIL-13R or OVA/sIL-25R). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

measured the concentrations of the Th2 cytokines IL-4, IL-5 and IL-13 and the epithelial cytokines IL-25, IL-33, and TSLP in BALF and lung tissue homogenates. As illustrated in Fig. 6, OVA challenge induced an increased production of Th2 cytokines and epithelial cytokines. IL-4 was reduced significantly in sIL-25R- and sIL-13R + sIL-25R-treated mice. IL-5 and IL-13 were also reduced significantly by sIL-13R and/or sIL-25R. More importantly, further reductions in the cytokine levels in both BALF and lung homogenates were often observed in the combined treatment group compared to the single treatment groups (Fig. 6A–D). The concentrations of IL-25, IL-33 and TSLP in the lung were significantly reduced by sIL-25R but not by sIL-13R. Co-blocking IL-13 and IL-25 could significantly reduce the levels of IL-25, IL-33 and TSLP not only in BALF but also in lung tissue homogenates (Fig. 6G–L). These results suggested that co-blocking IL-13 and IL-25 was more effective in abrogating the inflammatory mediators induced by allergens than blocking IL-13 or IL-25 alone.

4. Discussion

In this study, we investigated the effect of a co-blockade of IL-13 and IL-25 on airway inflammation, AhR and remodeling in a murine model of asthma. Our observations suggest that combination therapy with sIL-13R and sIL-25R is a very promising immunotherapeutic strategy for improving the symptoms associated with exposure to allergens in mice.

IL-13 is a well-established driver of airway responses in mouse models of allergic asthma. Studies of IL-13 deficiency or the blockade of IL-13 with specific antagonists have confirmed that IL-13 plays a pivotal role in airway inflammation, AhR and remodeling within the adaptive immune system [25,31,32]. IL-25 has been reported to be secreted by epithelial cells and is thought to be a more crucial mediator of the

epithelium in the development of airway inflammation, AhR and remodeling independently or by promoting a Th2 response than other cytokines [18,33].

Our findings are similar to those in previous studies of Kasaian et al. [13], who observed that inflammation was significantly reduced by IL-13 blockade with sIL-13R, and of Tamachi et al. [19], who reported the amelioration of eosinophilic inflammation by sIL-25R in a dose-dependent manner. Moreover, in our study, the combined therapy reduced eosinophilia more substantially than either agent alone. The most likely reason for this result is that the recruitment of eosinophils via the secretion of eotaxin induced by IL-13 [32] and via the IL-5-dependent pathway [34] was ablated by the combined treatment with sIL-13R and sIL-25R (Fig. 6B–C and E–F). Meanwhile, eosinophils have been reported to be substantially involved in allergen-driven airway remodeling (in both collagen and smooth muscle changes) [35]. Therefore, the ablation of eosinophilia by co-blockade of IL-13 and IL-25 might contribute indirectly to the attenuation of airway remodeling. In addition, AhR has been reported to be prevented by sIL-13R [13] or an anti-IL-25 mAb [20] alone and is a crucial symptom in clinical asthma patients that is closely correlated with airway remodeling [36]. Interestingly, in our study, co-blockade of IL-13 and IL-25 was more effective in preventing OVA-induced AhR than blocking IL-13 or IL-25 alone.

Mucus hyperproduction and goblet cell hyperplasia are the main pathological markers of airway remodeling in asthma and contribute to mortality in severe asthma because of airflow obstruction [37]. The induction of goblet cell metaplasia and mucus hyperproduction by IL-13 [24] or IL-25 [20] has been confirmed by previous studies. The current findings are consistent with and extend previous observations that neutralization of IL-13 [31] and IL-25 [17] alone both successfully attenuate mucus production and goblet cell hyperplasia. Moreover, the combination therapy exhibited additional effectiveness in our study.

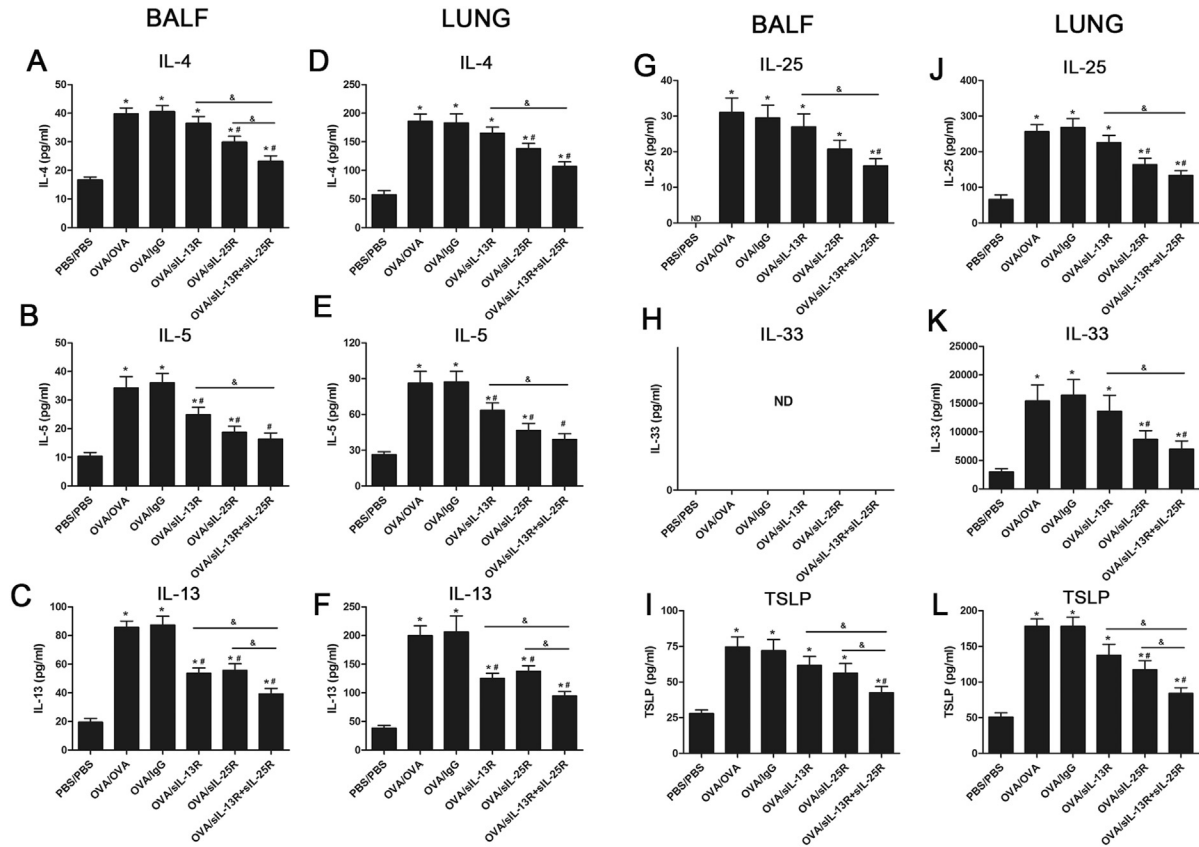


Fig. 6. Blockade of IL-13 and/or IL-25 reduces the production of inflammatory mediators. Levels of the allergen-induced Th2 cytokines IL-4 (A), IL-5 (B), and IL-13 (C) in BALF and IL-4 (D), IL-5 (E), and IL-13 (F) in lung homogenates. Levels of the allergen-induced epithelial cytokines IL-25 (G), IL-33 (H), and TSLP (I) in BALF and IL-25 (J), IL-33 (K), and TSLP (L) in lung homogenates. The data are presented as the mean $\pm$ SEM ($n = 6-7$ per group). * $P < 0.05$ compared to the PBS/PBS group, ** $P < 0.05$ compared to the OVA/IgG group, and # $P < 0.05$ compared to the single blocking group (OVA/siL-13R or OVA/siL-25R). ND, non-detectable.

Airway sub-epithelial collagen, another component driving airway remodeling, is rapidly secreted by fibroblasts in response to stimulation with IL-13 [9] or IL-25 [38]. One emerging theory is that airway remodeling involves reactivation of the epithelial-mesenchymal trophic unit (EMTU), which increases the production of extracellular matrix (ECM) proteins within the reticular basement membrane by activating myofibroblasts [39]. We show here that collagen density and the total collagen amount are significantly reduced when IL-13 or IL-25 is neutralized in mice exposed to chronic OVA stimulation. These changes may be the result of siL-13R and siL-25R interacting with additional cell types and mediators likely present in the EMTU. TGF- β is a potent pro-fibrotic mediator that regulates fibroblast/myofibroblast function and controls the production of collagen. In this study, we also show that the combination pharmacotherapy was more effective than either drug alone in down-regulating the expression of TGF- β 1 and p-Smad2. According to our results, the suppression of collagen synthesis mediated by the co-blockade of IL-13 and IL-25 likely occurs through inhibition of the TGF- β 1/Smad signaling pathway in the EMTU, because the EMTU is a rich source of preformed TGF- β 1 [40].

Hypertrophy and hyperplasia of smooth muscle cells within the airway wall are also responsible for the increased thickness of airway tissues and are thought to contribute to altered asthmatic airway function [17]. IL-13 [41] or IL-25 [18] airway challenges alone could increase airway smooth muscle thickness, which has been previously observed. In our experiments, mesenchymal cell hyperplasia and the subsequent increase in smooth muscle mass exhibited in mice exposed to chronic OVA challenge were effectively abrogated by treatment with either siL-13R or siL-25R alone. In addition, we observed that the combination treatment exerted greater inhibitory effects on airway smooth muscle thickness and mesenchymal cell hyperplasia than either treatment alone.

Increased bronchial vascularization is another recognized feature of airway remodeling, which is thought to contribute to airflow obstruction in patients with asthma [17]. In previous studies, it has been reported that IL-25 could enhance the proliferation of vascular endothelial cells and angiogenesis in vitro and in vivo by up-regulating the expression of VEGF and its receptors [42,43]. However, the role of IL-13 in the angiogenesis of asthmatic mice remains undetermined. Our data showed that mice exposed to OVA have elevated levels of VEGF and subsequent airway angiogenesis compared with PBS-treated mice. In addition, neutralizing IL-25 alone substantially abolished angiogenesis and the level of VEGF in vivo, consistent with earlier research conducted by Gregory et al. [17]. Moreover, we also observed that neutralizing IL-13 with siL-13R could effectively decrease the level of VEGF in lung homogenates. Most importantly, the co-blockade of IL-13 and IL-25 completely abolished angiogenesis at a basal level. These data suggest that the combination of the two agents has a therapeutic advantage in the treatment of neovascularization compared to either agent alone.

Complex interactions between cytokines from the immune and structural cells are thought to drive the formation and development of asthma [21]. The administration of siL-13R alone significantly decreased the production of the Th2 cytokines IL-5 and IL-13. The reduced levels of these cytokines may indirectly ameliorate eosinophilia and goblet cell hyperplasia. Similarly, IL-25 neutralization alone significantly abrogated the OVA-induced increases in the Th2 cytokines IL-4, IL-5 and IL-13, which further confirms the effect of IL-25 on enhancing Th2 responses reported in previous studies [22,44]. Moreover, a more effective abrogation of the Th2 cytokines was observed when the combined treatment was administered in our study. The epithelial cytokines IL-25, IL-33 and TSLP have been reported to collectively drive Th2 polarization through complementary and sometimes synergistic mechanisms and

are also associated with tissue remodeling [16,17]. In our study, sIL-13R alone did not have a significant effect but did result in a downward trend in the expression of these cytokines, perhaps due to the attenuation of the epithelial damage caused by IL-13. The effects of neutralizing IL-25 on epithelial cytokines are consistent with a previous study [17]. Additionally, we noticed that there were further decreases in the concentrations of epithelial cytokines in the combination treatment group. In summary, these data illustrate that a combination treatment with both sIL-13R and sIL-25R effectively reduces the production of inflammatory mediators from Th2 and epithelial cells.

In conclusion, the co-blockade of IL-13 and IL-25 could effectively attenuate airway inflammation, AhR and airway remodeling including mucus production, extracellular collagen deposition, peribronchial smooth muscle hyperplasia and neovascularization induced by allergens compared to the blockade of IL-13 or IL-25 alone. Therefore, our results suggest that the combined inhibition of IL-13 and IL-25 may provide a novel therapeutic strategy for asthma patients, especially those who are resistant to current treatments.

Conflicts of interest

The authors declare no conflicts of interest.

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