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Original Article

Artemisia sieversiana pollen allergy and immunotherapy in mice

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Abstract: This study developed a murine model of asthma using *Artemisia sieversiana* pollen extract (ASE) and subcutaneous immunotherapy (SCIT) without an adjuvant. BALB/c mice were sensitized subcutaneously with 25 μg of ASE and challenged with 0.1% ASE aerosol. To investigate the efficacy of SCIT, mice were subcutaneously injected with 0.3 mg ASE without adjuvant once a week for 8 weeks, followed by challenge for 3 additional days. Airway hyperresponsiveness (AHR) to methacholine, pulmonary inflammatory cell infiltration, cytokine levels of bronchoalveolar lavage fluid, histopathology of the lung, and serum allergen-specific serum IgE and IgG2a levels were assessed following the final challenge. Mice sensitized with ASE developed AHR and had significantly higher interleukin (IL)-4, IL-5, and IL-13 levels as well as lower IL-12 level than those of control mice. Moreover, mice sensitized with ASE showed increased plasma levels of allergen-specific IgE, and histologic analyses showed peribranchial infiltration of inflammatory cells and mucosal hyperplasia. After SCIT, allergic symptoms and immunological parameters were effectively improved, and the plasma level of allergen-specific IgG2a was significantly increased cmpared to that in the vehicle group. These findings described successful development of an *A. sieversiana* pollen-induced asthma model in BALB/c mice, with in vivo findings revealing that SCIT without adjuvant significantly improved the symptoms and pathophysiology of asthmatic mice.

Keywords: *Artemisia sieversiana* pollen, murine model of asthma, subcutaneous immunotherapy, adjuvant

Introduction

Asthma, an airway disease induced by multiple factors, including genetic background, allergens, and air irritants [1], places a huge social and economic burden on society. Pollen is an important outdoor allergen that causes asthma [2]. Jia et al. [3] conducted a study in 1120 patients from northern China with summer and autumn hay fever and found that 53.8% of the patients had seasonal allergic asthma, among which 30.4% needed emergency treatment. *Artemisia* (*Artemisia L., sensu stricto*) is a widespread weed in the temperate and subtropical regions of the northern hemisphere; however, its geographical distribution has been expanding due to global climate changes [4]. In Europe, *Artemisia* pollen accounts for 10-14% of pollen allergy cases [5], with 4.5% of people affected in Germany [6]. Surveys of pollen allergies in many regions of China [7] show that *Artemisia* pollen is the most abundant and longest-lasting airborne pollen. Jia et al. [8] reported that *Artemisia* pollen allergy accounts for 86.7% of cases with autumnal pollinosis in Beijing, China, and a recent study reported that *Artemisia* pollen is the main vector for airborne endotoxin and that *Artemisia* pollen-carrying endotoxin can cause strong allergic sensitization [9].

Almost 50% of patients with autumnal pollen allergic rhinitis develop seasonal allergic asthma within 9 years [10]. Specific immunotherapy, currently the only treatment that affects the natural course of allergic diseases [11], can improve symptoms and prevent the progression of allergic rhinitis to asthma.

In China, the genus *Artemisia* includes 187 species [7], and *Artemisia sieversiana* is one of the most widely distributed species [12]. Since the 1950s, Peking Union Medical College Hospital

has used the crude extract of *A. sieversiana* pollen for subcutaneous immunotherapy (SCIT) [13] and promoted its use throughout the country. Hundreds of thousands of people have been subsequently treated, with the percentage of those effectively treated at ~77.9% [14]. However, the mechanism associated with SCIT using *A. sieversiana* pollen extract (ASE) remains unclear, limiting its application.

Given the limitations and safety considerations associated with human trials, mouse models are commonly used as tools to study pollen allergies and specific immunotherapy. Currently, the mouse models used to study *Artemisia* pollen allergy include an airway inflammation model constructed by Chen et al. [15] using *Artemisia vulgaris* pollen exposed to diesel exhaust and the sublingual immunotherapy model established by Ma et al. [16] using *A. vulgaris* pollen. However, to the best of our knowledge, there is neither a SCIT model constructed using *Artemisia* pollen nor a murine asthma model constructed with *A. sieversiana* pollen. Although allergens of different species of *Artemisia* pollen share similarities, they also have their own specific protein components. For example, the major allergens of *A. sieversiana*, *A. vulgaris*, and *Artemisia annua* pollen are a 16-kDa fragment [17], 22-kDa fragment [18], and 43-kDa fragment, respectively [19]. Moreover, a previous study reported different reaction intensities to a skin prick test using pollen extract from different *Artemisia* sp. [20]. Therefore, there might also be differences in mouse asthma models constructed using pollen from different species of *Artemisia*.

This study constructed the first murine asthma model using *A. sieversiana* pollen and then conducted SCIT for 8 weeks in asthmatic mice to simulate SCIT in clinical patients with *Artemisia* pollen allergy. Our findings may contribute to further understanding of the mechanism of sensitization and SCIT with ASE and provide a theoretical basis for clinical SCIT with ASE and a foundation for improving desensitization treatment methods.

Materials and methods

Mice

Female BALB/c mice (6-8 weeks old; 18-20 g) were purchased from the Academy of Military Medical Sciences of China (Beijing, China) and raised in a specific pathogen-free laboratory at the Animal Test Center of Peking Union Medical College Hospital with free access to water and standard laboratory chow. All animal feeding and operating procedures were approved by the Animal Experimentation Ethics Committee of Peking Union Medical College Hospital.

Allergen

A. sieversiana pollen was purchased from the Beijing Key Laboratory of Precision Medicine for Diagnosis and Treatment on Allergic Diseases (Beijing, China), and the endotoxin content was determined. ASE was prepared using a previously described extraction method [16]. Briefly, the *A. sieversiana* pollen was defatted with acetone at 4°C for 8 h, stirred with 0.125 M ammonium bicarbonate (1:20, w/v) for 24 h to extract protein, filtered, dialyzed against distilled water for 24 h, aliquoted, and lyophilized. The total protein concentration was determined using the Bradford method (ASE protein concentration: 0.93 μg/μL). Freeze-dried ASE was dissolved in phosphate-buffered saline (PBS) before use.

Experimental design

Establishment of a murine asthma model for A. sieversiana pollen allergy: Twenty mice were randomly divided into an asthma group (asthma) and a control group (control) (n=10 mice/group). The modeling process is shown in **Figure 1A**. Briefly, mice in the asthma group were sensitized via subcutaneous injection on the back of the neck with 25 μg of ASE adsorbed onto an alum adjuvant (Imject Alum; Pierce Biotechnology, Rockford, IL, USA) in a volume of 200 μL on days 1, 8, and 15, respectively. On days 18-24, the mice in the asthma group were challenged with aerosolized 0.1% ASE for 30 min daily for 7 consecutive days. The control group was sensitized and challenged with an equal volume of PBS. Airway hyperresponsiveness (AHR) was measured 24 h after the final challenge in all mice. On day 26, mice were anesthetized with 2% sodium pentobarbital, serum was collected and used to measure *A. sieversiana*-specific IgE antibodies, bronchoalveolar lavage fluid (BALF) was obtained from the right lung for inflammatory cell enumeration and cytokine quantitation, and the left lung was

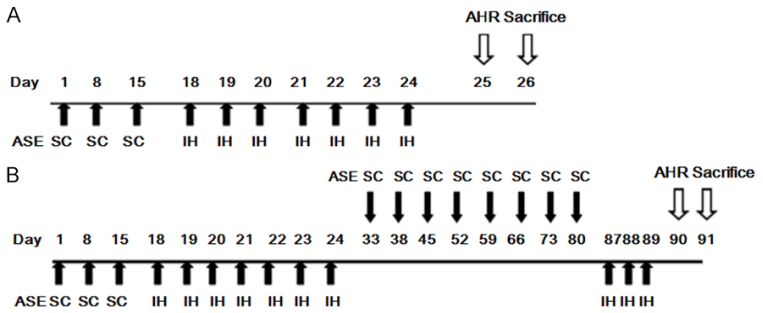

Figure 1. Overview of the experimental design. A. Establishment of the murine asthma model. Mice were sensitized subcutaneously (SC) with 25 μg *Artemisia sieversiana* pollen extract (ASE) or phosphate-buffered saline (PBS) on days 1, 8, and 15. On days 18-24, the mice were challenged with 0.1% ASE aerosol or PBS aerosol inhalation (IH). After 1 day, airway hyperresponsiveness (AHR) to methacholine (Mch) was assessed. After 24 h, mice were euthanized for further experiments. B. Subcutaneous immunotherapy (SCIT) for a murine asthma model. Mice were sensitized SC on days 1, 8, and 15 with 25 μg of ASE or PBS. Three days after the final sensitization, all animals were placed in a plastic box and challenged with aerosolized 0.1% ASE or an equal volume of saline (control group) IH for 30 min daily for 7 consecutive days. On days 31-80, mice in the treatment group underwent SCIT and received eight SC injections of 300 μg of ASE once weekly. The control and vehicle group animals received PBS. Mice were re-challenged with 0.1% ASE aerosol on days 87-89 and on day 90, and AHR to Mch was assessed. One day later, the mice were euthanized for further analysis.

used for pathologic analysis of paraffin sections.

SCIT for induction of ASE allergy in a murine asthma model: Thirty mice were randomly divided into control, vehicle, and treatment groups (n=10 mice/group). The experimental procedure was as follows (**Figure 1B**). Mice in the vehicle and treatment groups were sensitized and stimulated with ASE as described. SCIT was started on day 31 and comprised eight treatments once weekly. The mice in the treatment group received a subcutaneous injection of 300 μg ASE (2 μg/μL; 150 μL) into the neck, and mice in the vehicle group were injected subcutaneously with 150 μL PBS. Mice in the vehicle and treatment groups were re-challenged with 0.1% ASE aerosol on days 87, 88, and 89. Simultaneously, control mice received PBS rather than ASE at each step. On day 90, AHR was assessed in all mice, and 1 day later, all mice were sacrificed. Sera were used to detect specific IgE and IgG2a antibodies against *A. sieversiana*, and other samples used for analysis were harvested as described.

AHR measurement

AHR was measured in mice 24 h after the final ASE challenge with a FinePointe system for non-invasive airway measurement (Buxco, Wilmington, NC, USA) according to the manufacturer's instructions. Mice were acclimated for 5 min and then exposed to aerosolized PBS to establish a baseline value, followed by increasing concentrations of aerosolized methacholine (Mch; 3.125, 6.25, 12.5, and 25 mg/mL in PBS for 2 min; Sigma-Aldrich, Steinheim, Germany). The specific airway resistance (sRAW), which is directly related to AHR, was measured for 3 min after each Mch aerosol application using a non-invasive airway device.

Preparation of BALF and cell enumeration

After the mice were anesthetized, the trachea was surgically exposed and cannulated, and the right lung was lavaged three times with a single volume of warm PBS. BALF was centrifuged at 400 *g* for 10 min at 4°C, and the supernatant was stored at -80°C until cytokine measurement. Cell pellets from BALF were resuspended in PBS, and leukocytes were classified and enumerated using an automatic blood analyzer (ADVIA 2120; Siemens, Munich, Germany). Results were expressed as the number of each cell type per 1 mL of BALF.

A. sieversiana pollen-specific serum IgE (ASE-sIgE) measurement

ASE-sIgE levels were measured via enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 μL/well ASE (50 μg/mL), and reference wells were coated with purified anti-mouse IgE capture antibody (BD Biosciences Pharmingen, San Jose, CA, USA). One the next day, the plates were blocked and washed, and then 100 μL of each serum sample (diluted 1:5 in blocking buffer) was applied to the sample wells. A series of seven two-fold dilutions of purified mouse IgE (BD Biosciences Pharmingen) was used in conjunction with reference wells as standards. After overnight incubation, the plates were sub-

sequently treated with 100 μL of biotinylated monoclonal anti-mouse IgE antibody (1 μg/mL; BD Biosciences Pharmingen) and horseradish peroxidase (HRP)-streptavidin (1:1000; BD Biosciences Pharmingen) for 1 h each at 37°C. Finally, 100 μL/well of TM Blue (CW Biotech, Beijing, China) was applied as a substrate, and the reactions were developed at room temperature for 20 min. The plates were read at 450 nm using an ELISA plate reader (ELX800; BioTek, Winooski, VT, USA), and a standard curve of murine IgE was used as a reference.

ASE-sIgG2a measurements

ASE-sIgG2a levels were measured via ELISA. Briefly, plates were coated with 100 μL/well ASE (5 μg/mL) overnight at 4°C, followed by washing three times, addition of 200 μL blocking buffer (5% skim milk in PBS), and incubation for 2 h at room temperature (22-25°C). After another three washes, serum samples were diluted (1:2000) with blocking buffer, and 100 μL/well was added to the plate for 1 h incubation at 37°C. The serum samples were washed again, and secondary antibodies (1:3000) were added for 1 h incubation at 37°C. HRP was then added, and the plates were read at 450 nm using an ELISA plate reader.

Measurement of cytokine levels in BALF

The concentrations of interleukin (IL)-4, IL-5, IL-13, interferon (IFN)-γ, and IL-12 in BALF were measured via sandwich ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Values were interpolated from standard curves using recombinant cytokines and expressed as pg/mL.

Lung histopathology

Mouse lungs were fixed with 10% formaldehyde, and paraffin sections were prepared by the histology core at Peking Union Medical College Hospital. Lung tissues were stained with hematoxylin and eosin or Alcian Blue-periodic acid-Schiff for histologic analysis. Peribronchial and perivascular eosinophil infiltration and goblet cell hyperplasia in airway epithelium were evaluated semiquantitatively under a light microscope (20× magnification) in a blinded manner. Inflammatory and mucus scores were graded (0= no inflammation to 4), as described previously [21, 22].

Statistical analysis

Statistical analyses were performed using SPSS (v.16.0; SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of the mean (SEM). Analysis of variance and Student's t-test were used to assess differences between groups. $P<0.05$ was considered significant. All experiments were performed independently at least twice, and the results were similar.

Results

Murine model of asthma induced by A. sieversiana pollen

Compared with the control group, mice in the asthma group showed obvious AHR to Mch, characterized by significantly greater sRAW values at 6.25 mg/mL, 12.5 mg/mL, and 25 mg/mL Mch inhalation (**Figure 2A**; $P<0.01$). Additionally, the numbers of white blood cells [WBCs; $(1908\pm335)\times10^3$/mL], eosinophils [Eos; $(746\pm241)\times10^3$/mL], and neutrophils [Neu; $(566\pm209)\times10^3$/mL] in the BALF from ASE-sensitized mice were all significantly higher than those in the control group [$(133\pm12)\times10^3$/mL, $(1.35\pm1.58)\times10^3$/mL, and $(4.05\pm0.16)\times10^3$/mL, respectively] (**Figure 2B**; $P<0.01$).

ASE-sIgE level of mice in the asthma group was 337±55 ng/mL, which was significantly higher than that in the control group (15±6 ng/mL) (**Figure 2C**; $P<0.01$). Additionally, mice in the asthma group produced significantly higher levels of T helper cell (Th)-2 cytokines (IL-4, IL-5, and IL-13) in BALF ($P<0.05$) and lower levels of IL-12, a Th-1 cytokine ($P<0.05$) than those of mice in the control group (**Figure 2D**). Notably, the level of IFN-γ (another Th-1 cytokine) in BALF did not change significantly between the two groups.

Lung histopathology in the asthma group showed inflammatory cell infiltration, airway-wall edema and thickening, and airway epithelial cell shedding around the small bronchus, vascular submucosa, and surrounding lung tissues. Specifically, the airway wall was widely stained blue-purple, with diffuse blue-purple staining observed in the airway, indicating the presence of abundant goblet-cell metaplasia and mucus in the airway; however, this was not

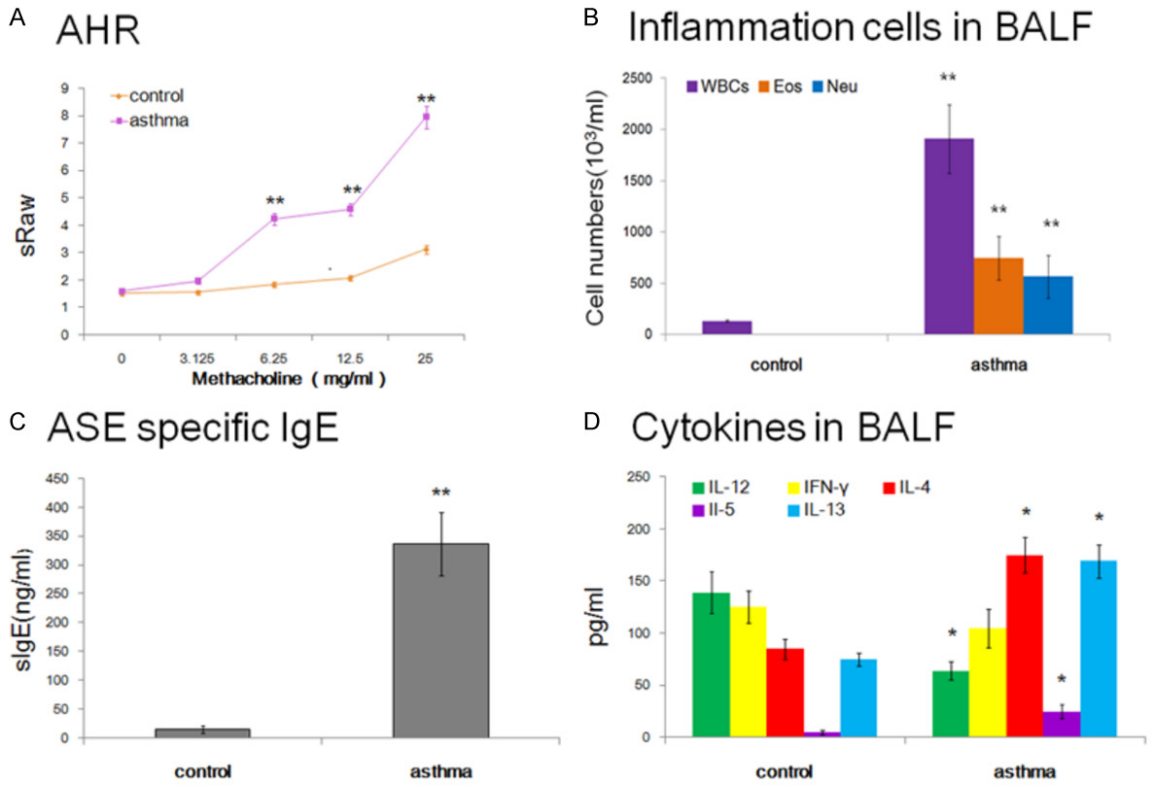

Figure 2. Allergen sensitization. A. Airway hyperresponsiveness (AHR) to methacholine (Mch) on day 25. B. Cells in bronchoalveolar lavage fluid (BALF). C. ELISA measurement of *Artemisia sieversiana* pollen extract (ASE)-specific IgE levels. D. Cytokines in BALF. Data represent the mean ± SD (n=10 mice/group). *P<0.05 and **P<0.01, asthma vs. control group.

present in the control group. Moreover, semi-quantitative scoring showed that inflammatory cell infiltration and airway mucus secretion in mice of the asthma group were significantly higher than those in mice of the control group (**Figure 3**; P<0.01).

Effects of SCIT

Compared with the vehicle group, SCIT with ASE treatment significantly reduced sRAW values at 6.25 mg/mL, 12.5 mg/mL, and 25 mg/mL Mch inhalation (**Figure 4A**; P<0.01), indicating that SCIT effectively improved lung function in asthmatic mice. Additionally, the numbers of WBCs (1508±708)×10^3/mL and Eos (423±144)×10^3/mL in BALF from treated mice were significantly lower than those of mice in the vehicle group [(3223±619)×10^3/mL and (1970±977)×10^3/mL, respectively] (**Figure 4B**; P<0.01). Interestingly, the number of Neu in BALF from treated mice [(968±317)×10^3/mL] did not differ significantly from that in the vehicle group [(1108±698)×10^3/mL] (**Figure 4B**).

After SCIT, the levels of IL-4, IL-5, and IL-13 in BALF from treated mice were significantly lower than those of mice in the vehicle group (P<0.01, P<0.05, and P<0.01, respectively; **Figure 4C**), whereas IL-12 level increased significantly (**Figure 4C**; P<0.01). Furthermore, IFN-γ level did not change significantly between the treatment and vehicle groups (**Figure 4C**), and SCIT significantly inhibited ASE-sIgE production and increased ASE-sIgG2a production (**Figure 4D**; P<0.01).

Lung histopathology (**Figure 5**) identified airway edema, airway mucus secretion, and less inflammatory cell infiltration around the trachea in treated mice compared with those in the vehicle group; semi-quantitative scoring results supported these observations (**Figure 5**; P<0.01).

Discussion

A. sieversiana is among the most widespread *Artemisia* sp. in China and an important cause

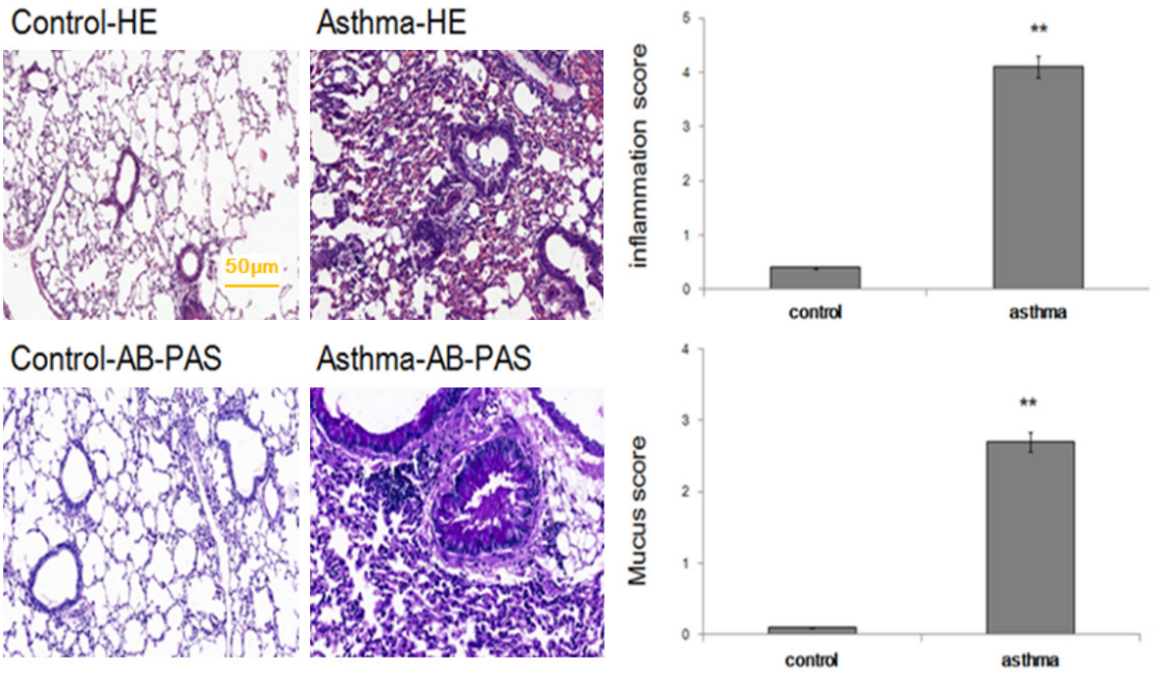

Figure 3. Lung tissue eosinophilia and mucus production (20×). Lung specimens were stained with hematoxylin and eosin (H&E) and Alcian Blue periodic acid-Schiff (AB-PAS). Data represent the mean ± SD (n=10 mice/group). **P<0.01, asthma vs. control group.

of allergic asthma in summer and autumn. In this study, we established the first model of asthma induced by *A. sieversiana* pollen through subcutaneous sensitization and nebulization challenge. This modeling method previously demonstrated an ability to produce higher allergen-specific IgE [23] and lower modeling animal mortality [24]. After ASE induction, mice in the asthma group showed obvious AHR, high levels of ASE-sIgE, high levels of WBCs and Eos in BALF, increased inflammatory cell infiltration, and airway goblet cell metaplasia. These findings suggested successful construction of the mouse model of asthma induced by *A. sieversiana* pollen, the pathological characteristics of which are basically analogous to those of human allergic asthma [25].

The immune-centered theory of asthma pathogenesis proposes that the process of asthma is accompanied by Th2 cell-prone T cell differentiation, leading to a decrease in Th1 cytokines and an increase in Th2 cytokines [26, 27]. Th2 cytokines can stimulate B-/T cell proliferation, increase IgE synthesis, induce Eos infiltration and AHR, and cause airway inflammatory cell infiltration and excessive mucus secretion [28, 29]. By contrast, Th1 cytokines play a protective role during asthma development [30, 31]. In the present study, we found that Th2 cytokine (IL-4, IL-5, and IL-13) levels were significantly higher and those of Th1 cytokines (IL-12) were significantly lower in BALF from treated mice than those in BALF from control mice. This suggests that Th1/Th2 imbalance is an important asthma-related mechanism [32]. Notably, IFN-γ levels in mice from the asthma group did not change significantly, which is inconsistent with results obtained from murine models of asthma established by Yali et al. [33] and Hua et al. [34] using sensitization via intraperitoneal injection, where IFN-γ levels in BALF from asthmatic mice were substantially lower than those in control mice. However, in murine models of asthma established by Xie & Yin [35] and Conejero et al. [36] using sensitization via subcutaneous injection, IFN-γ levels in BALF did not change significantly between treated and control mice. Therefore, the role of different sensitization pathways in these different outcomes remains to be elucidated.

Currently, commonly used SCIT preparations, such as Mites Allergen Product, contain alumi-

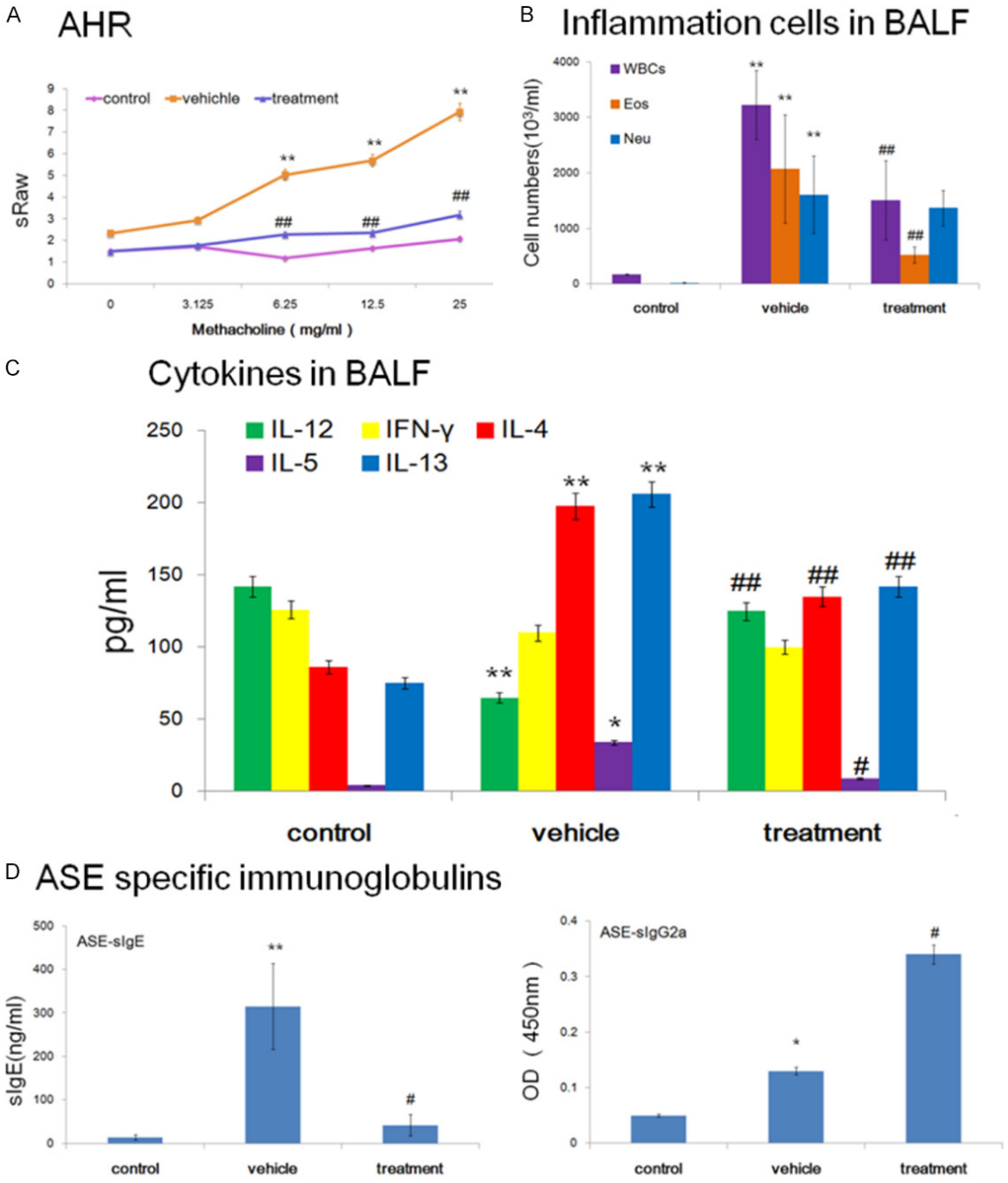

Figure 4. Subcutaneous immunotherapy provides therapeutic benefit. A. Airway hyperresponsiveness (AHR) to methacholine. B. Inflammatory cells in BALF. C. Cytokines in BALF. D. ELISA measurement of *Artemisia sieversiana* pollen extract (ASE)-specific immunoglobulin (sIgE and sIgG2a) levels. Data represent the mean ± SD (n=10 mice/group). *P<0.05 and **P<0.01, vehicle vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, treatment vs. vehicle group.

num hydroxide adjuvants, whereas the SCIT preparations of *A. sieversiana* pollen used in clinical practice in China do not contain adjuvants [37]. In the present study, we used ASE without adjuvant to perform SCIT (eight treatments once per week) and effectively alleviated AHR, pulmonary inflammation, and goblet cell metaplasia around the airway, as well as reduced the amount of WBCs and Eos in the BALF of asthmatic mice. These results suggest that SCIT can effectively reduce the allergic inflammatory response in the airway. Moreover,

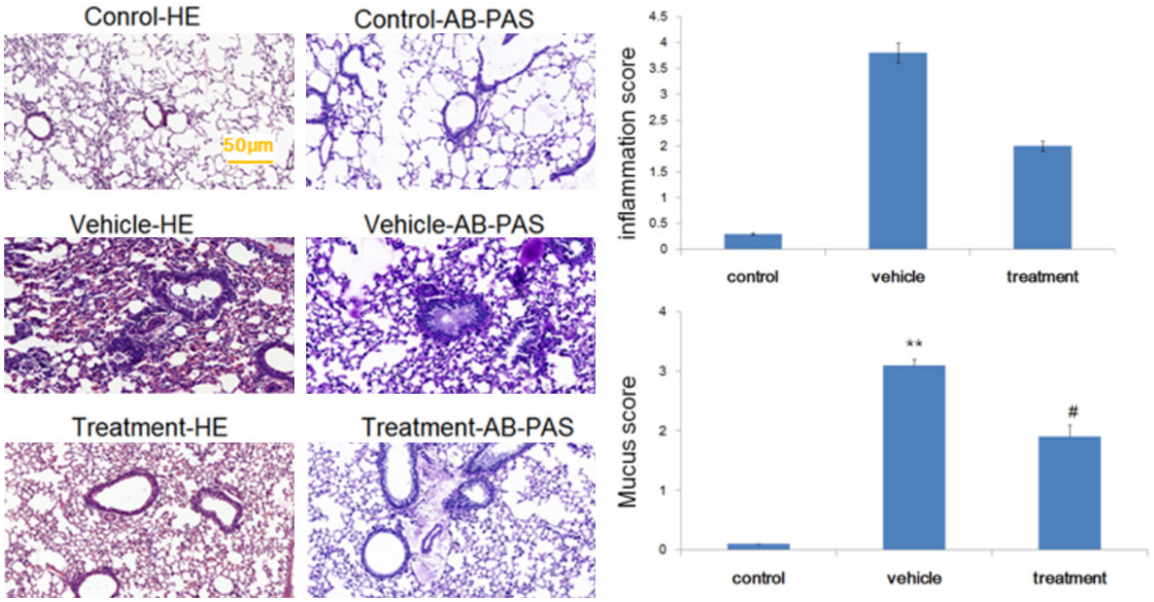

Figure 5. Lung tissue eosinophilia and mucus production (20×). Lung specimens were stained with hematoxylin and eosin (H&E) and Alcian Blue periodic acid-Schiff (AB-PAS). Data represent the mean ± SD (n=10 mice/group). $^{**}P<0.01$, vehicle vs. control group; $^{\#}P<0.05$, treatment vs. vehicle group.

the data confirmed the therapeutic effectiveness of desensitization preparations without adjuvant in an animal model.

Notably, SCIT did not significantly reduce the number of Neu in BALF from asthmatic mice. A previous study reported that some patients receiving SCIT showed unsatisfactory outcomes [14]. Additionally, studies have proposed the concept of Neu phenotypic asthma [38] and speculated that the hormone resistance of some patients with refractory asthma is related to Neu infiltration in the airway [39]. Therefore, we speculate that the reason for the unsatisfactory response to SCIT by some patients might be due to the insignificant improvement of airway Neu infiltration.

Regarding changes in sIgE levels after allergen-specific immunotherapy, existing research results are inconsistent [40-42]. In the present study, sIgE levels were significantly reduced after SCIT. Notably, previous studies [40-42] found that regardless of sIgE changes after immunotherapy, allergic symptoms were significantly alleviated. This suggests that sIgE level is not directly related to symptom improvement, implying that sIgE level cannot be used as a biomarker for evaluating immunotherapeutic efficacy. By contrast, in the present study, sIgG2a levels increased significantly after SCIT, which is consistent with the results of other studies [43-45]. Thus, sIgG2a, which plays a protective role [46], might represent a valuable predictor of immunotherapeutic efficacy.

After SCIT, we found that Th2 cytokine levels in mice were significantly reduced, whereas Th1 cytokine levels were significantly increased, which finding was in line with previous studies [16, 47]. This suggests that correcting an imbalanced Th1/Th2 ratio might represent a mechanism of immunotherapy. Among these cytokines, IL-12 can stimulate the production of sIgG2a [48], which might be related to the significant increase in sIgG2a levels observed after SCIT in this study.

Due to limitations in experimental condition, we did not comprehensively explore the mechanism of *Artemisia* pollen sensitization and desensitization treatment. We will use the model conditions and methods of this study to further explore the mechanism underlying both sensitization and desensitization to *Artemisia* pollen using a mouse model with targeted gene knockout.

In conclusion, we successfully established for the first time a murine asthma model induced by *A. sieversiana* pollen. Using this model, we

also constructed an *Artemisia* pollen SCIT model. Our findings may promote further understanding of the mechanisms of *A. sieversiana* pollen sensitization and SCIT and provide a reference for improving immunotherapy and developing new treatment strategies in the future.

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Disclosure of conflict of interest

None.

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