Original Article
Specific immunotherapy with major group 5 allergen derived from *Dermatophagoides farinae* in murine model of asthma

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Abstract: Dust mite allergen is recognized as contributory cause for allergic asthma, and group 1 (*Der f* 1) and group 2 (*Der f* 2) are being extensively studied in the commonly defined allergens. However, few reports are available on the use of *Der f* 5 as allergen for specific immunotherapy (SIT). In our previous laboratory effort, we successfully developed *Der f* 5 allergen. In order to verify its immune efficacy, the present work was aimed at investigating the efficacy of SIT with the major group 5 allergen derived from *Dermatophagoides farina* (*Der f* 5). Forty specific pathogen-free BALB/c mice were randomized into group of either PBS, ovalbumin (OVA) treatment, asthma model (*Der f* 5 sensitization) or *Der f* 5 SIT (n=10 for each group). Our results demonstrated that compared to OVA and asthma models, the pulmonary inflammation was alleviated significantly for SIT group, and the number of total white blood cells, eosinophils, levels of IL-5 and IL-17 in BALF as well as supernatant of cultured splenocytes (SCSS) allergen-specific IgE and IgG_1 were greatly decreased in SIT group (all *P*<0.01), yet BALF IFN-γ levels and SCSS as well as serum IgG_{2a} were significantly increased (P<0.01). Therefore, *Der f* 5 vaccine developed from *Dermatophagoides farina* may effectively inhibit airway allergic inflammation, and the findings suggest that it can be a candidate vaccine for treatment of allergic diseases.

Keywords: *Dermatophagoides farina*, Der f 5, allergic asthma, specific immunotherapy

Introduction

Allergic asthma is defined as chronic inflammatory disorder of the airways, and characterized by airway inflammation, persistent airways hyperresponsiveness caused by dust mite allergen [1]. And *Dermatophagoides farina* (*D. farinae*) has been shown to be one of the most important allergen sources associated with asthma and other allergic conditions in humans [2, 3]. To date, more than 30 groups of dust mite allergens have been recognized globally [4], in which group 1 (*Der f* 1) and group 2 (*Der f* 2) allergens are being extensively studied [5-7]. However, *Der f* 5 is also thought to be one of the most important allergens capable of inducing allergic asthma, and bioinformatics analysis revealed that the matured *Der f* 5 allergen contains 132 amino acids with molecular mass of some 13-14 kD [8]. It was reported that the positive serum was detected in about 50%-70% of the allergic patients, which accounted for 25% in total specific IgE [9].

Contributory cause of asthma attack is primarily associated with imbalanced release of Th1/Th2 CD4+ T lymphocyte subsets that lead to initial T helper cells (Th0) in favor of Th2 differentiation, in which the regulatory T cells (Treg) and Th17 are also involved [10]. Allergen sensitization may facilitate Th2 to secrete cytokines of IL-4, IL-5 and IL-13 in increased manner, whereas secreted IL-2 and IFN-γ in Th1 may be demonstrated as immediate allergic inflammation in the target organs involved. IL-17 secreted by Th17 has been classified as a proinflammatory cytokine because of its capacity to induce the expression of many mediators of inflammation and tissue damage [11], a proof is that increased level of IL-17 was found in the serum and tissue samples from patients with asthma [12].

Allergen-specific immunotherapy (SIT) is the only available etiological therapy capable of affecting the natural course of allergy [13, 14]. In current clinical practice, this therapy mainly depends on administration of increasing doses of allergen extracts as the vaccine to patients, and has achieved positive clinical efficacy to a certain extent [15], yet use of the crude extract has been controversial because of its complex components and potential severe anaphylactic adverse reactions or sensitization towards new allergens present in the mixture. In order to eliminate the negative effects, different strategies have been created, including simply purified allergen or vaccination with its recombinants on *Der f 1* and *Der f 2* basis, and become intensive study subjects. Nevertheless, few reports are available on the use of *Der f 5* as allergen for SIT.

In our previous laboratory work, we successfully developed *Der f 5* allergen. In order to verify its immune efficacy, the current study was undertaken to apply the allergen to murine models induced by *Der f 5* with tentative SIT through examination of pathological changes in the pulmonary tissue biopsies, cytokine level variation in the bronchoalveolar lavage fluid (BALF) and the supernatant of cultured splenocytes (SCSS) as well as serum levels for specific IgG_1, IgG_{2a} and IgE, with an attempt to supply evidence for SIT in the allergic asthma on *Der f 5* basis.

Materials and methods

Animals

Forty specific pathogen-free (SPF) female BALB/c mice (aged 6-8 week, weighing 20 ± 2 g) were purchased from the Animal Center for Comparative Medicine, Yangzhou University, China (License No. SCXK/Su 2007-0001). Animal experiments were approved by the Animal Research Ethics Board of Wannan Medical College, and performed in compliance with the laboratory guidelines.

Reagents

Recombinant *Der f 5* allergen undergone prokaryotic expression was the preservation in our laboratory. Ovalbumin (Grade V) was purchased from Sigma (U.S.A), and IFN-γ, IL-5, IL-17, IgE, IgG_1 and IgG_{2a} as well as ELISA kit from R&D (U.S.A). Liu's haematocyte stain was a product of Basco Diagnostics Inc. (Zhuhai, China), and RPMI-1640 cell culture medium, fetal bovine serum, penicillin, streptomycin, EDTA-NH4Cl red blood cell lysate and trypan blue were obtained from Sangon Biotech (Shanghai, China). The remaining analytical reagents were domestic products.

Apparatus

Apparatus included CO_2 incubation box (SAN-YO, Japan), super-clean bench, Biotek enzyme marks (model ELx 800, U.S.A), Multiview Microscope (Olympus), Yuyue Atomizer, High-speed refrigerated Benchtop Centrifuge (Beckman).

Establishment of asthmatic murine models

Forty female BALB/c mice were equally randomized into four groups, namely PBS, ovalbumin (OVA), asthma model (*Der f 5* sensitization) and SIT with *Der f 5* allergen. Murine models were established in compliance with the protocol specified in our laboratory manual [16] as follows: Asthma models and mice in SIT group were sensitized with intraperitoneal injection of 100 μL/per caput [10 μg *Der f 5* and 2% (w/v) $Al(OH)_3$ were dissolved in PBS; pH 7.2)] solution at day 0, 7, and 14, respectively. By day 21, mice in the two groups were challenged with aerosol inhalation of *Der f 5* in dose of 10 μg/mL [2% (w/v) $Al(OH)_3$ dissolved in PBS; pH 7.2] for 30 min/per time, once a day for consecutive 7 days, with records on the asthma attacks being maintained. From day 25 to 27, mice in SIT group were additionally subjected to intraperitoneal injection of Der f 5 solution [10 μg Der f 5 and 2% (w/v) $Al(OH)_3$ were dissolved in PBS; pH 7.2)] in dose of 100 μL/per caput, 30 min prior to aerosol inhalation for SIT. Mice in PBS group and OVA group were exclusively given PBS and OVA via intraperitoneal injection and aerosol inhalation in accordance with the previous dosage and procedures. 24 h after the final intervention, mice in the four groups were sacrificed and undergone the following tests.

Measurement of the serum specific IgE, IgG_1 and IgG_{2a}

The blood samples were taken from individual mouse via removal of its eyeballs, stood for 30 min and centrifuged at 4°C by 3000 ×g for 5 min. The supernatant was obtained and stored at -80°C for spare use.
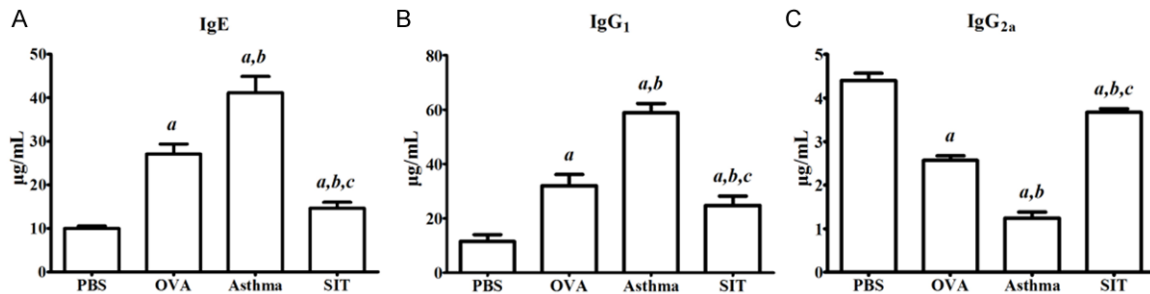
Figure 1. Variation of allergen-specific IgE, IgG$_1$ and IgG$_{2a}$. A: Compared with PBS group ($P<0.01$). B: Compared with OVA group ($P<0.01$). C: Compared with asthma models ($P<0.01$).

Counting of white blood cells in BALF and eosinophils

In order to ensure recovery rate over 90%, endotracheal intubation was applied to individual mouse in each group for three repeated aspiration of the BALF by using pre-cooled PBS in dose of 0.3, 0.3 and 0.4 mL, respectively. The BALF was centrifuged at 2000 ×g for 5 min, and the supernatant was obtained and store at -80°C for following use. PBS was used to resuspend the cell sedimentation, and the cell concentration was adjusted to 1×10^6/mL. Then the cell suspension was taken and applied to a smear that was stained with Liu's solution and microscopically counted.

Culturing the splenocytes

After the blood was obtained, mice were sacrificed by cervical displacement. The spleen was taken and cut into pieces under sterile condition, and made to pass the 300-mesh stainless steel cell sieve for preparation of the single cell suspension. The erythrocyte was lysed with EDTA-NH$_4$Cl, stood for 5 min and centrifuged at 1000 ×g for 5 min. RPMI-1640 (10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin) was used to precipitate the cell pellets that was subjected to resuspension to harvest the spleen cells. Then the cell concentration was adjusted to 1×10^6/mL. Trypan blue staining was performed, and live cell count >98% was considered eligible for subsequent experiment. The adjusted spleen cell suspension was applied to the 48-well plate by 1 mL for each well. Corresponding 10 μg antigen was added, and incubated in CO$_2$ incubator (37°C, 5% CO$_2$) for 72 h. Then the supernatant was taken and stored at -80°C for spare use.

Detection of the allergen specific cytokines and serum allergen specific antibody

ELISA was performed to determine the content of IL-5, IL-17 and IFN-γ in BALF and SCSS and serum level of antigen specific IgE and IgG$_1$ and IgG$_{2a}$. The procedures were performed according to the user's instructions.

Preparation of the pulmonary tissues

Lung tissues were taken from each group of mice, fixed in 10% formaldehyde solution, embedded in paraffin, and sectioned in conventional technique. The tissue sections were stained with HE, and observed under light microscope to examine the EOS infiltration, incidence of edema and bronchial epithelial damage.

Statistical analysis

Data in each group were expressed with mean ($\bar{x} \pm S$). Software SPSS16.0 was used to analyze the single factor, and two samples were compared using the LSD-t method and the Tamhane s T$_2$. $P<0.05$ was considered statistically significant.

Results

*Antibody concentration of the serum antigen specific IgE, IgG$_1$ and IgG$_{2a}$*

As compared with PBS group, expression level of antigen specific IgE and IgG$_1$ was significantly increased in groups of OVA, asthma models and SIT ($P<0.01$), yet IgE and IgG$_1$ contents in SIT group were significantly decreased, whereas IgG$_{2a}$ was increased compared to the asthma models ($P<0.01$, **Figure 1**). Although the
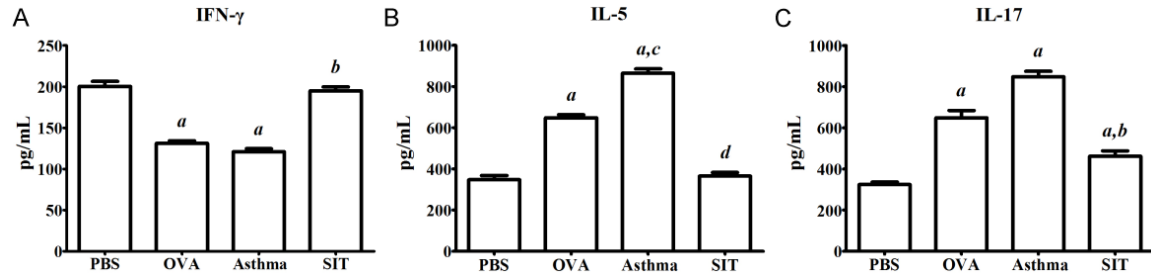
Figure 2. Changes of IFN-γ, IL-5 and IL-17 levels in BALF. A: Compared with PBS group (*P*<0.01). B: Compared with OVA group (*P*<0.01). C: Compared with asthma models (*P*<0.01).
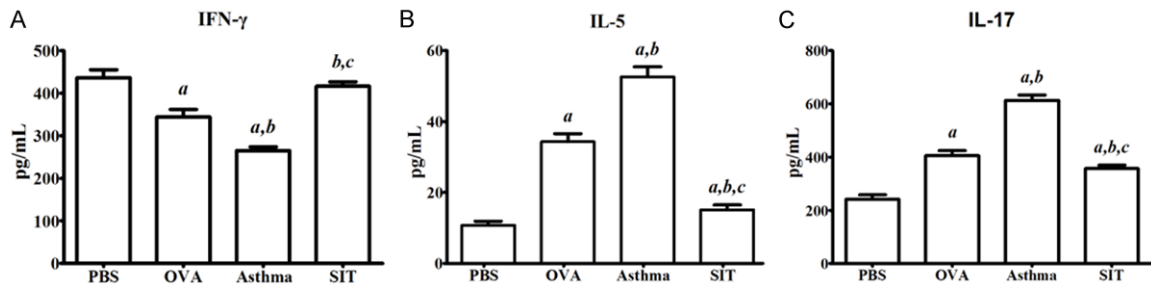
Figure 3. Changes of IFN-γ, IL-5 and IL-17 levels in supernatant of cultured splenocyte. A: Compared with PBS group (*P*<0.01). B: Compared with OVA group (*P*<0.01). C: Compared with asthma models (*P*<0.01).
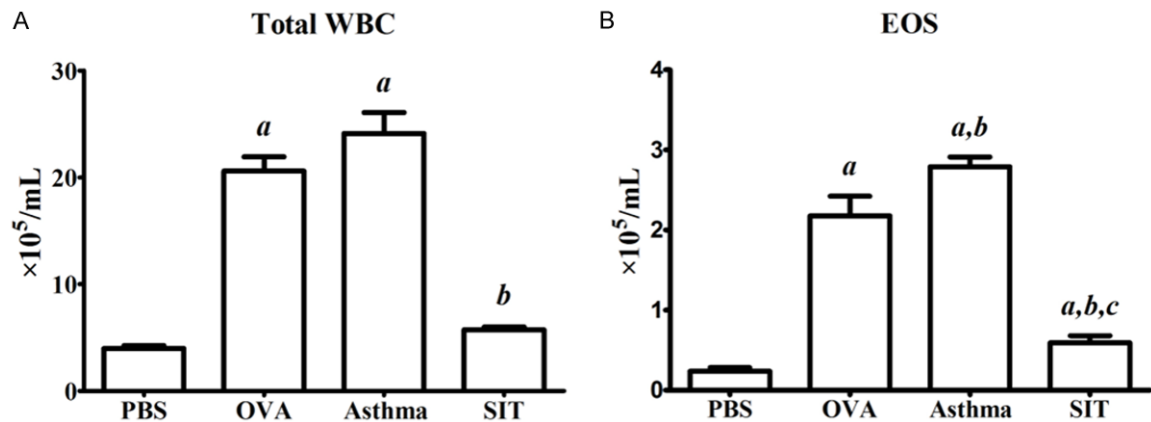
Figure 4. Count of total white blood cells (WBC) and eosinophils (EOS) in BALF. A: Compared with PBS group, the total WBC were lower than OVA group and asthma models (P<0.01). B: Compared with PBS group, the total number of EOS were lower than OVA group and asthma models (P<0.01).

IgG$_{2a}$ antibody level in groups of OVA, asthma models and SIT was significantly higher than that of PBS group (*P*<0.01), IgG$_{2a}$ level was strikingly higher in the OVA group and asthma models by comparison with SIT group intervened with *Der f* 5 (*P*<0.01).

IL-5, IL-17 and IFN-γ level in BALF and SCSS

IFN-γ levels in OVA group and asthma models were significantly lower compared to PBS group

(*P*<0.01), whereas IFN-γ level in SIT group was markedly higher than that in OVA group and asthma models (*P*<0.01, **Figure 2A**). Although IL-5 levels in OVA group and asthma models overtopped PBS group, IL-5 level remained significantly lower in SIT group than the asthma models (*P*<0.01, **Figure 2B**). IL-17 level was found with similar trend to IL-5 (*P*<0.01, **Figure 2C**). The cytokine levels of IL-5, IL-17 and IFN-γ in the supernatant of splenocytes remained comparable to those detected in BALF (**Figure**
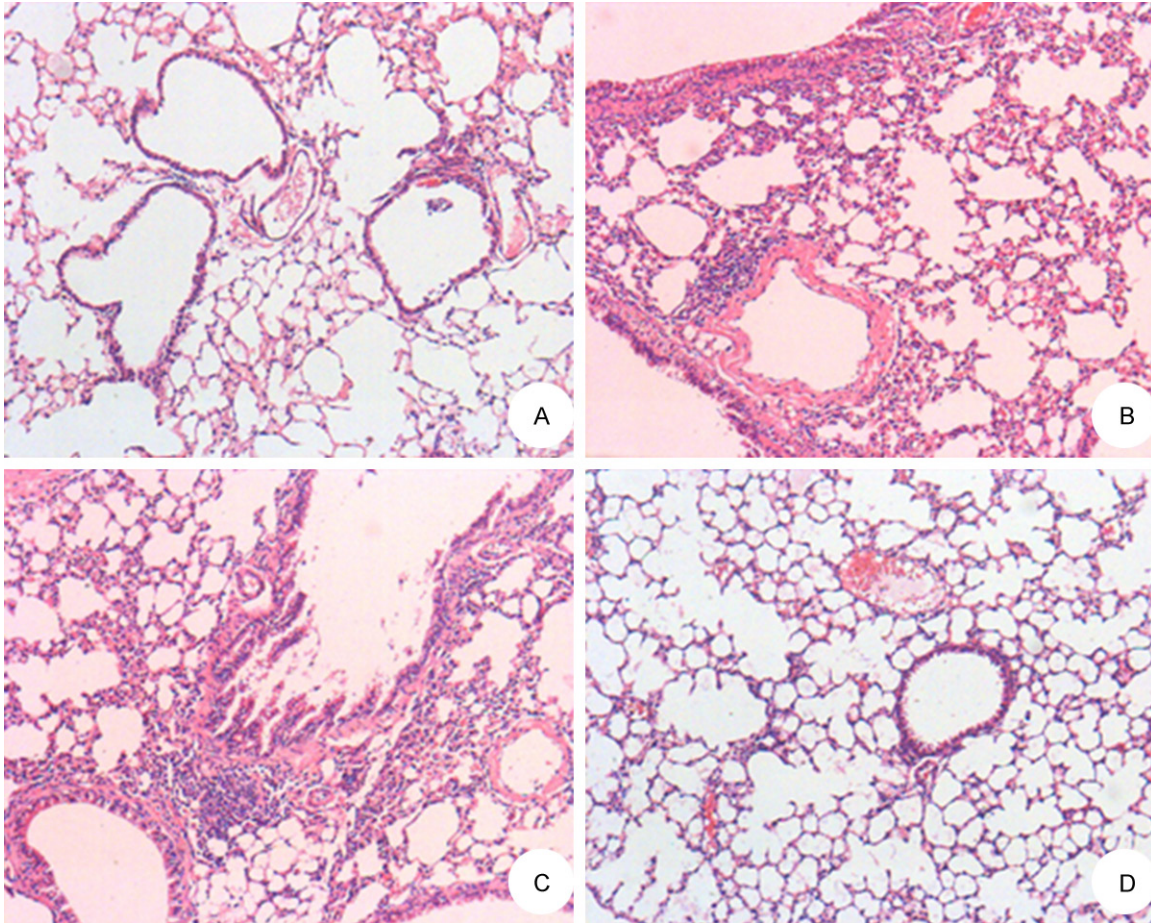
Figure 5. Lung tissue sections for mice in the four groups (HE, × 100). A: PBS group. B: OVA group. C: Asthma group. D: SIT group.

3A and **3C**). These results showed that *Der f 5* allergen was able to boost IFN-γ content in BALF from asthmatic mice, but inhibited the expression of IL-5 and IL-17.

Total white blood cell count and the EOS count in BALF

The results showed that the total leukocytes and EOS count in BALF of OVA group were significantly increased compared to PBS group ($P<0.01$), whereas the total number of white blood cells had no change in mice received SIT ($P>0.05$), and were lower than OVA group and asthma models ($P<0.01$, **Figure 4A**). Similar change trend was seen regarding EOS count (**Figure 4B**).

Pathological changes of the lung tissues

Mice in OVA group and asthma models demonstrated evident bronchitis and vasculitis asso-

ciated with EOS and other inflammatory cell infiltration as well as fractured or shed bronchial epithelial cells in certain locations (**Figure 5B** and **5C**). In contrast, SIT had effectively inhibited the infiltration of inflammatory cells and alleviated the inflammation (**Figure 5D**). The airway epithelial structure in SIT group remained similar to that of PBS group (**Figure 5A**).

Discussion

Dust mites are the major causative sources of allergic asthma, rhinitis and atopic dermatitis, and over 80% of asthma victims are allergic to house dust mites [17, 18]. Allergic asthma is a chronic entity characterized by pulmonary infiltration with eosinophils, hypersecretion of mucus and airway hyperresponsiveness (AHR), and its incidence rate tends to grow in annual fashion [19]. This condition is primarily associ-

ated with the immune response to Th2 cytokines [20], while Th1 cytokines are inhibited [21, 22]. The sustained airway inflammation is frequently the result of Th2 cells sensitized and activated by dust mite allergens [20]. IL-17 secreted by Th17 cells is also an important pro-inflammatory cytokines, tending to unusually increase during the acute stage of asthma attack and exacerbation [23].

The current specific immunotherapy is the only allergen-specific approach to the treatment of mite allergy. The principle for SIT relies on administration of increasing doses of allergen extracts to the patients in order to alleviate the symptoms caused by allergen exposure. The therapeutic mechanisms are primarily involved in correcting imbalanced Th1/Th2 cells, regulating serum IgE and inhibiting the effector cells responsible for allergic inflammation, including eosinophils and mast cells [24, 25]. Clinical efficacy by SIT was confirmed in treatment of allergic rhinitis and asthma, and importantly, SIT can not only reduce the allergic sensitization, but also the incidence of asthma after therapy [15, 26, 27].

Previous work primarily focused on the *Der f 1* and *Der f 2*. In current study, we attempted to verify the efficacies of SIT by using *Der f 5* in mouse models sensitized with *Der f 5* allergen. The results showed that *Der f 5* protein can credibly inhibit the number of white blood cells in BALF from the asthmatic mice, leading to reduced serum antibody-specific IgE, IgG_1 level, up-regulated IgG_{2a} content and corrected Th1/Th2 imbalance. A good case in point is that the IL-5 level was reduced in the BALF supernatant from the asthma models, whereas IFN-γ expression was up-regulated, and IL-17 level from Th17 cells was decreased. As compared with the simple asthmatic animal, lung tissue sections demonstrated no significant pathological changes in mice undergone SIT with *Der f 5*, and the bronchial and alveolar structure remained relatively intact, with no obvious inflammatory cell infiltration in around the small bronchi and vessels. Still, the bronchial wall mucosa appeared free of any fracture and epithelial shedding. These findings indicate that *Der f 5* allergen derived from *D. farinae* can effectively check the progress in mice after allergen-specific immunotherapy.

In conclusion, our work confirms that *Der f 5* allergen from *D. farinae* can achieve in SIT pur-pose, and efficaciously alleviate lung inflammation and asthmatic symptoms to a certain extent. The findings suggest that *Der f 5* gene can be used as a candidate vaccine for the treatment of allergic diseases, which will lay a foundation for further research of such vaccine for wider clinical use.

Disclosure of conflict of interest

None.

Abbreviations

Der f, *Dermatophagoides farinae*; SIT, specific immunotherapy; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; EOS, eosinophils; SCSS, supernatant of cultured splenocytes; SPF, specific pathogen-free.

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