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Dust Mite Allergen Der F 8 Promotes Th2 Polarization via Modulating Chromatin Structure at the *TIM4* Gene Locus in Dendritic Cells

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Abstract

Dust mites are an important source of allergens. The mechanism by which dust mite allergens induce allergic diseases is to be further investigated. This study aims to elucidate the role of one of the components of dust mite allergens, the Dermatophagoides farinae-8 (Der f 8), in the induction of airway allergy. In this study, the expression of T cell immunoglobulin and mucin domain 4 (*TIM4*) in dendritic cells (DC) s was analyzed after stimulating with Der f 8 in the culture. The role of Der f 8 in the induction of T helper (Th) 2 inflammation was assessed with an airway allergy mouse model. We observed that, in 34 subtypes of dust mite allergens, Der f 8 uniquely and markedly induced high levels of the TIM4 expression in DCs by modulating the chromatin at the TIM4 promoter locus. Der f 8 played a critical role in the expansion of the Th2 response in the mouse airway via inducing DCs to produce *TIM4*. Administration with Der f 8-null dust mite extracts (DME) efficiently inhibited the allergic inflammation and induced regulatory T cells in the airway of mice. We conclude that Der f 8 plays an important role in the pathogenesis of dust mite allergy. Vaccination with the Der f 8-null DME is more efficient to inhibit the dust mite allergic inflammation in the airway than the wild DME.

Key words:

Experimental allergy; Allergy mechanism; Specific immunotherapy

Introduction

Allergic diseases have become a worldwide health problem [1,2]. The T helper (Th)2 polarization in the local tissue is one of the major pathologic features of allergy; over production of the antigen specific IgE is another. The IgE binds to the high affinity IgE receptors on mast cells to make mast cells sensitized [3]. Upon re-exposure to specific allergens, the sensitized mast cells release allergy-related mediators to initiate allergic attacks [4]. Although the pathologic process of allergy is somewhat clear, yet, the mechanisms about how antigen-specific Th2 cells get polarization and how they sustain a dominant status in allergic inflammation are still unknown; the therapeutic efficacy on allergic diseases is unsatisfactory currently.

Allergens derived from dust mites are the most common allergens of asthma, atopic dermatitis and perennial rhinitis [5]. Mite allergy affects about 50% of the population with allergic diseases in the world [6]. The dust mite contains a large number of proteins; some of them are allergens. There are more than 30 subtypes of mite allergens have been recognized; some of them have been well characterized; such as Dermatophagoides farina-1 (Der f 1) and Dermatophagoides pteronyssinus (Der p) 1 are important allergens in airway allergy [7,8]. The group 8 allergen (Der f 8) of D. farina was cloned and characterized by us; the DNA sequence is listed in the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore/KM010011.1), from which we found that the gene sequence of Der f 8 was quite similar to the gene sequence of glutathione S-transferase; the latter has a close relation with the pathogenesis of allergic asthma [9]. Previous studies have shown that 40.9 % of mite allergic patients with asthma exhibited IgE reactivity to Der f 8 [10,11]. It reveals that Der f 8 is a significant allergen of mite allergy, but the role of Der f 8 leading to allergic disorders remains to be further investigated.

Besides being the major antigen presenting cells, dendritic cells (DCs) also produce a number of co-stimulatory molecules; one of them is the T cell immunoglobulin mucin domain (TIM)4. We and others previously reported that *TIM4* played a critical role in the expansion of Th2 response and allergic disorders [12-14]. Clinical data show that Der f 8-specific IgE can be detected in the patients with mite allergy [10]. Yet, the mechanism of the over expression of TIM4 by DCs in allergic diseases is unclear. It is reported that the transcriptional coactivators p300 and STAT6 (Signal transducers and activators of transcription-6) are associated with the pathogenesis of allergic diseases [15,16]; whether these molecules are associated with *TIM4* expression has not been investigated. Based on the above information, we hypothesize that Der f 8 modulates the expression of *TIM4* in DCs, which plays an important role in the pathogenesis of dust mite allergy. In this study, we observed that Der f 8 induced the *TIM4* expression in DCs and played an important role in the dust mite allergen-induced airway Th2 polarization and allergic inflammation. The Der f 8-null dust mite extracts (DME) vaccine showed much better therapeutic efficacy on allergic airway inflammation than the Der f 8-containing DME vaccine in the specific immunotherapy (SIT).

Materials and Methods

Research ethic statement

The using mice in the present study was approved by the Research Ethic Committee at Shenzhen University (#SZU2015R032). The experiments were performed in accordance with the approved guidelines.

Luciferase reporter assay

The luciferase reporter of TIM4 promoter was constructed with the sequence betwee -500 and -1047 (or a non-specific sequence, using as a negative control) by the Sangon Biotech Limited Company (Shanghai, China) and transfected DCs following the manufacturer's instruction. In the reporter-gene analysis, the proteins were quantitatived with the Bradford reagent (Bio-Rad), and the relevant light intensity was measured with a luminometer with a Luciferase kit (Biomart, China). All assays were done in triplicate and protein expression was evaluated by immunoblot analysis.

Chromatin IP (ChIP)

The DCs were fixed with 1% formalin for 15 min and sonicated. The supernatant was collected by centrifugation and incubated with antibodies of interest and protein G overnight at 4°C. The beads were collected by centrifugation and washed for 3 times with PBS. The immune complexes on the beads were eluted by SDS buffer and collected by centrifugation. The DNA was purified from the immune complexes and subjected to qPCR with the TIM4 promoter primers, aggaagagagggagagagca and agaatcgcttgaacccagga. The results were calculated and presented as relevant changes against the DNA input.

A murine model of asthma

Mice were subcutaneously injected on day 1, 3, and 7 with Der f 8 (100 μg/mouse), or DME (100 μg/mouse), or Der f 8-null DME, in 0.1 ml saline respectively. Saline was used as a negative control. From day-15 to day-21, the mice were challenged via nostril with Der f 8 (50 μg/mouse) or DME (50 μg/mouse) in 50 μl PBS once a day for a week. The mice were sacrificed on day 22 to analyze the allergic airway inflammation, or treated with specific Immunotherapy (SIT).

Airway hypersensitivity response (AHR) measurement

Airway hyperresponsiveness to methacholine was measured with the unrestrained whole-body plethysmography. The mice were subjected to progressively increasing doses of methacholine (0, 6.25, 12.5, 25, 50, 100 mg/mL), and the Penh value was recorded.

Lung histology

Lung tissues were fixed in 4% phosphate-buffered formalin and embedded in paraffin wax. Sections (3 μm) were cut and stained with hematoxylin and eosin, and observed under a light microscope.

Specific immunotherapy (SIT)

From day 28, the asthma mice were treated with DME vaccine, or Der f 8-null vaccine, or saline (using as a control) for two weeks with the doses of 5 μg (days 1 and 2), 10 μg (days 3 and 4), 20 μg (days 5177), 30 μg (days 8 and 9), and 50 μg (days 10–14), in 0.1 ml saline by intraperitoneal injection. Two days before the sacrifice, AHR of the mice was assessed. One day before the sacrifice, the mice were intranostrilly challenged with 50 μg DME in 50 μl saline.

Isolation of mononuclear cells from the lung tissue

The lung tissue was cut into small pieces (2 × 2 × 2 mm), incubated with collagen IV (0.5 mg/ml) at 37°C with mild agitation for 2 h. The cells were filtered with a cell strainer (70 μm), harvested by centrifugation, and cultured in RPMI1640 medium.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from each mouse at the sacrifice of decapitation. The sera were isolated to store at -80 °C until use. The serum levels of Th2 cytokines (IL-4, IL-5, and IL-13) were determined by ELISA with commercial reagent kits following the manufacturer's instruction. The levels of specific IgE were determined by an in-house ELISA. Briefly, the microtiter plates were coated with purified allergens at 100 ng/well in 100 μl in carbonate buffered solution (pH 9.5) overnight at 4°C. Then blocked with 100 μl 3% bovine serum albumin (BSA) in PBS for 120 min at 37°C. The serum (diluted to 5 times) or BSA (using as a negative control) was then added to each well (100 μL/well) and incubated for 60 min at room temperature. The plates were incubated with peroxidase-labeled goat anti-mouse IgE (1:2000) for 60 min at 37°C. Each incubating step was followed by 3 washes with phosphate-buffered saline+Tween 20 (PBST). The color was developed by adding tetramethylbenzidine (TMB; 100 μL/well), stayed for 20 min at room temperature and stopped by addition of 2M H2SO4 (50 μL/well). The plates were read with an EL×808 absorbance microplate reader (BioTek, Shanghai, China) at 450 nm.

Statistical analysis

Data were presented as mean ± SD. Differences between two groups were analyzed Student t-test or by ANOVA if more than two groups. The Newman– Keuls was used for post hoc analysis. A $p<0.05$ were set as statistically significant. Reagents, mice, the procedures of DC preparation and treatment, Western blotting, qRT-PCR, flow cytometry, purification of immune cells, T cell proliferation assay, preparation of mite proteins, bio-synthetization of mite allergen components and depletion of Der f 8 in DME are presented in supplemental materials.

Results

Der f 8 enforces the expression of TIM4 in DCs

To test if Der f 8 had any effect on regulating TIM4 expression, we exposed DCs to DME in the culture. The cells were analyzed 48 h later. The results showed that DCs expressed high levels of TIM4. Since there are a number of different components of DME allergens have been identified, to recognize which DME components induces TIM4 expression in DCs is of significance. Thus, we exposed DCs to 34 DME components in the culture for 48 h. The cells were analyzed by RT-qPCR. The results showed that the exposure to Der f 1, 2, 8 and Der p 1, 2 markedly enhanced the expression of TIM4 in DCs, in which the TIM4 mRNA was uniquely higher in the DCs exposed to Der f 8 (Figure 1A).

To enforce the results, we exposed DCs to Der f 8 in the culture at gradient doses for 48 h. It resulted in the increases in TIM4 expression in DCs in a Der f 8 dose-dependent manners (Figure 1B-C). The

results suggest that Der f 8 plays a critical role in the expression of TIM4 in DCs. To strengthen the results, we removed Der f 8 from DME. DCs were stimulated by the Der f 8-null DME or Der f 8-containing DME for 48 h. The results showed that although the wild type DME induced TIM4 expression in DCs, the Der f 8-null DME did not (Figure 1B-C). Since exposure to Der f 1, 2 and Der p 1, 2 also increased the expression of TIM4 mRNA in DCs somehow; we assessed the TIM4 protein levels in DCs after exposure to these 4 subtypes of mite allergen. The results showed that the TIM4 protein was not apparently increased in DCs (Figure 1D). Taken together, the results indicate that Der f 8 can increase the expression of TIM4 in DCs.

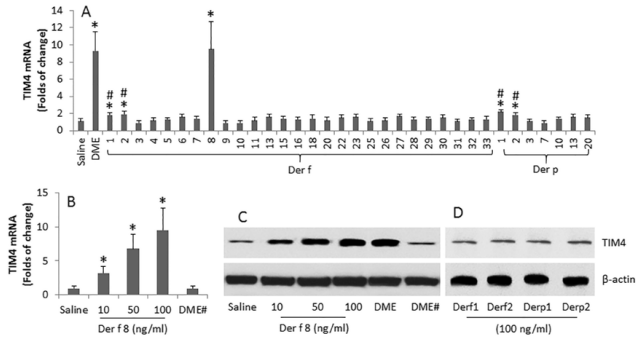

Figure 1: Der f 8 increases TIM4 expression in DCs. CD11c+ MHC II+ DCs were prepared from the bone marrow. The DCs were cultured in the presence of dust mite extract (DME), or the subtypes of Der f allergens or Der p allergens (denoted on the X axis of panel A; 100 ng/ml) for 48 h. The DCs were collected and analyzed. A, the bars indicate the mRNA levels of TIM4 (by qRT-PCR). B-D, DCs were stimulated in the culture as denoted in the Figure for 48 h. B, the bars indicate the TIM4 mRNA levels. C-D, the Western blots indicate the protein levels of TIM4 after stimulated with the indicated allergens (denoted below the blots; 100 ng/ml) for 48 h. The data of bars are presented as mean ± SD. *, p<0.01, compared with the saline group. #, p<0.01, compared with the Der f 8 group. The data are representatives of 3 independent experiments. The full length gel graphs are presented in supplemental materials.

Der f 8 activates the TIM4 promoter

The data of Figure 1 implicate that Der f 8 modulates the expression of TIM4. To test the inference, we transfected DCs with a luciferase reporter of TIM4 promoter. The cells were exposed to Der f 8 (or BSA, using as a negative control) in the culture for 3 h and then subjected to the analysis of the luciferase activity at 24 h. The results showed that the exposure to Der f 8, but not to BSA, markedly increased the luciferase activity (Figure 2). The results indicate that Der f 8 can modulate the TIM4 promoter activity.

Der f 8 modulates *TIM4* gene chromatin in DCs

We next assessed the phosphorylation status of p300 and STAT6 in DCs after exposure to Der f 8. The results showed that the exposure to Der f 8 increased the phosphorylation of p300 and STAT6 in DCs (Figure 3A-B). Further analysis by ChIP showed that the levels of acetylated H2A, H2B, H3 and H4 at the TIM4 promoter were increased (Figure 3C-H). Finaly we observed that the exposure to Der f 8 increased the expression of TIM4 in DCs, which was abolished by the presence of either p300 inhibitor or STAT6 inhibitor (Figure 3I-J). The results indicate that exposure to Der f 8 triggers the expression of TIM4 in DCs.

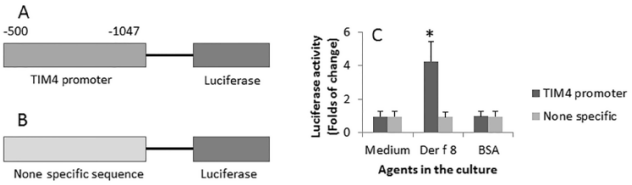

Figure 2: Der f 8 activates *TIM4* promoter. A reporter gene with TIM4 promoter sequence (A) or a non-specific sequence (B) was constructed. C, BMDCs were transfected with the reporter gene. The cells were exposed to Der f 8 (100 ng/ml) in the culture medium for 3 h and analyzed 24 h later. The bars indicate the luciferase activity (mean ± SD; *, p<0.01, compared with the medium group). The data are representatives of 3 independent experiments.

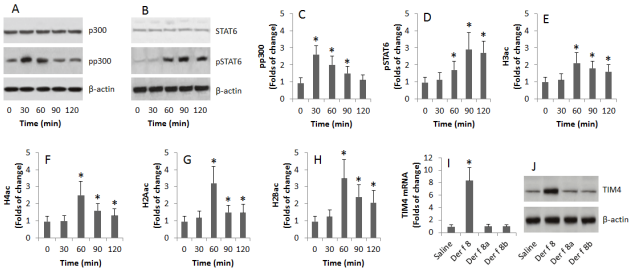

Figure 3: Der f 8 modulates chromatin of TIM4 gene in DCs; DCs were treated with Der f 8 (100 ng/ml) in the culture for 2 h. The nuclear extracts of DCs were analyzed. A-B, the Western blots indicate the levels of p300/pp300 and STAT6/pSTAT6. C-H, the nuclear extracts were analyzed by ChIP. The bars indicate the binding to the TIM4 promoter by the molecules denoted on the Y axis. I-J, DCs were treated with Der f 8 in the culture for 48 h. I, the bars indicate the mRNA levels of TIM4. J, the Western blots indicate the protein levels of TIM4. Der f 8a: In the presence of both Der f 8 and p300 inhibitor (garcinol: 3 μM). Der f 8b: In the presence of Der f 8 and STAT6 inibitor (A77-1726: 10 nM)The data of bars are presented as mean ± SD. *, p<0.05, compared with the time point of "0" (C-H) or the saline group (I). The data are representatives of 3 independent experiments. The full length gel graphs are presented in supplemental materials.

Der f 8 promotes antigen specific Th2 response

We also assessed the role of Der f 8 in the induction of the antigen specific Th2 reponses. We sensitized mice with Der f 8, or DME, or Der f 8-null DME. As shown by Figure 4, the Der f 8 specific CD4+ T cell proliferation, serum levels of Th2 cytokines and Der f 8 specific IgE were higher in mice immunized with Der f 8 than control mice, while much higher serum levels of IL-4, DME specific IgE and DME specific CD4+ T cell proliferation were detected in mice immunized with DME, but not in those immunized with the Der f 8-null DME. The

results suggest that Der f 8 plays an important role in the development of the DME specific Th2 responses. To elucidate if TIM4 is the molecule by which Der f 8 facilitates the DME specific Th2 responses, a group of mice was treated with anti-TIM4 antibody in the course of immunization with DME. Indeed, the Th2 responses were abolished. We also stimulated the sensitized CD4+ T cells with an irrelevant antigen, OVA, in the culture; it did not induce the T cell proliferation. The results demonstrate that that Der f 8 induces TIM4 production by DCs plays a critical role in the development of DME allergy.

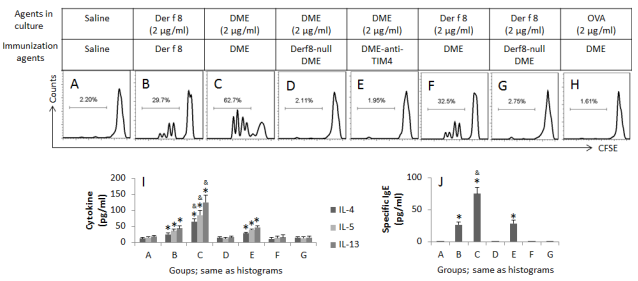

Figure 4: Der f 8 facilitates antigen specific Th2 polarization; BALB/c mice (6 mice per group) were sensitized to Der f 8 or DME. A group of mice was injected with anti-TIM4 Ab at 0.2 mg/mouse on day 3 and day 7 respectively. A-H, lung CD4+ T cells were isolated, stained with CFSE and treated as denoted above each subpanel in the presence of DCs (T cell: DC=5:1). The gated histograms indicate the frequency of proliferated CD4+ T cells. I-J, the bars indicate the serum levels of Th2 cytokines (I) and Der f 8-specific IgE or DME-specific IgE (J). The data of bars are presented as mean ± SD. *, p<0.01, compared with group A. &, p<0.01, compared with group B. Samples from each mouse were processed separately.

Inhibition of airway allergic inflammation with Der f 8-null DME vaccine

The above data suggest that Der f 8 is a critical molecule in the induction of skewed dust mite-specific Th2 polarization. We hypothesized that the removal of Der f 8 from the DME vaccine might greatly improve the efficacy of the DME-specific immunotherapy (SIT). To test the hypothesis, we developed an airway allergy mouse model with DME as the specific antigen. The mice were treated with a two-week SIT with DME vaccine or Der f 8-null DME vaccine. As shown by Figure 5, the immunization with DME induced lung inflammation, airway hypersensitivity to methacholine challenge, high levels of serum IL-4 and specific IgE, and specific CD4+ T cell proliferation. After DME SIT, although the parameters of lung allergic inflammation were somewhat down regulated, yet none of them reached the significant levels; while those mice treated with the Der f 8-null DME SIT showed a significant attenuation in the lung allergic inflammation. Furthermore, treatment with the Der f 8-null DME SIT, but not the DME SIT, significantly up regulated the frequency of antigen specific CD4+ CD25+ Foxp3+ regulatory T cells (Treg) in the lung.

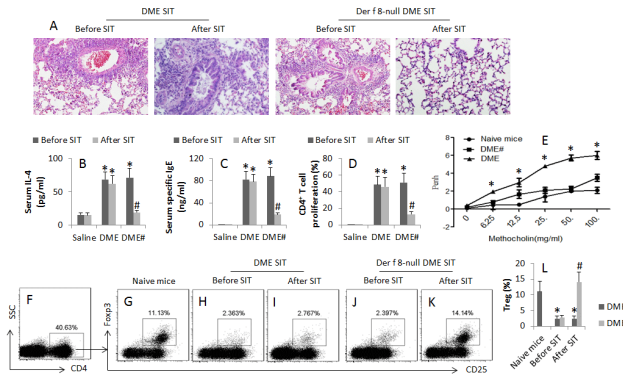

Figure 5: Der f 8-null DME SIT attenuates mite allergic lung inflammation; Mice (6 mice per group) were sensitized to DME and treated with DME SIT or Der f 8-null DME SIT; A, the histology images (x200) show lung inflammation. B-C, the bars indicate the serum levels of IL-4 (B) and specific IgE; D, the bars indicate the CD4+ T cell proliferation after stimulation with DME in the culture; E, the results of airway hypersensitivity; F, mononuclear cells were isolated from the lung. The gated cells are CD4+ T cells. G-K, the gated cells are CD25+ Foxp3+ Tregs in the gated CD4+ T cells of panel F. L, the bars indicate the summarized data of G-K. *, p<0.01, compared with the saline group (B-D), or with the naive mice (E and L); #, p<0.01, compared with the DME group (B-D) or with naive mice (L); The data of bars are presented as mean ± SD. Samples from each mouse were processed separately.

Discussion

We previously found that DCs produced TIM4 upon the exposure to Staphylococcal enterotoxin B (SEB) [12] that played a critical role in the induction of intestinal allergy [17]. Rodriguez-Manzanet et al also reported that after activation, DCs produced TIM4 that induced T cell expansion and survival [14]. Li et al reported recently that hepatic ischemia reperfusion injury could increase TIM4 expression by DCs [18]. The present data have expanded the knowledge in the studies of TIM4. After exposing to Der f 1, Der f 2, Der p 1 and 2, the transcripts of TIM4 in DCs were markedly increased,which was much higher in DCs after exposing to Der f 8.

From the time course we found that, upon exposure to Der f 8, the phosphorylation of p300 occurred, followed by the acetylation of histone at the TIM4 promoter locus, and then the STAT6 phosphorylation was increased. These data suggest that Der f 8 can modulate the chromatin of the TIM4 promoter, and induces the *TIM4* gene transcription and expression in DCs. Both p300 and STAT6 play an important role in the Der f 8-induced TIM4 expression because the presence of either a p300 inhibitor or a STAT6 inhibitor abolished the TIM4 expression.

Structurally, Der f 8 is similar to the glutathione S-transferase (GST). One of the GST functions is to bind both the substrate at the enzyme's hydrophobic H-site and glutathione at the adjacent, hydrophilic G-site, which together form the active site of the enzyme; and subsequently to activate the thiol group of glutathione, enabling the nucleophilic attack upon the substrate [19]. Our data show that Der f 8 has a similar feature of GST that activates p300 in DCs and

induced p300 phosphorylation. This action of Der f 8 is of significance because it initiated *TIM4* gene transcription in DCs, induced an Der f 8-specific Th2 polarization and facilitated the DME-specific allergic inflammation in the lung, as shown by the present study. Supportive data have also published that GST is associated with the pathogenesis of asthma [9].

In line with previous studies [20,21], we created a DME-specific airway allergy mouse model without an extra adjuvant. A significant finding in the present study is that the capacity of inducing the specific Th2 polarization in the airway was significantly attenuated with the Der f 8-null DME, pinpointing the importance of Der f 8 in the allergy-induction capacity of dust mite. Such a feature of Der f 8 implicates that the removal of Der f 8 from DME may be useful in the SIT using DME as a vaccine. The results from vaccination with the Der f 8-null DME on inhibiting DME-specific allergic airway inflammation support the inference.

To induce Tregs in the allergic subjects is one of the therapeutic purposes of SIT [22]. The present data also show that using the Der f 8-null DME vaccine recovered the induction of Tregs in the mice with dust mite allergy, which did not occur in those allergic mice treated with the Der f 8-containing DME. This is in line with previous studies. Li et al reported that in a DC: $CD4^+$ T cell co-culture system, blockade of TIM4 on DCs significantly inhibited Th2 cell differentiation and facilitated the induced $CD4^+$ $CD25^+$ $Foxp3^+$ Treg expansion [18]. Yeung et al indicate that interruption of DC-mediated TIM4 signaling induces regulatory T cells and promotes skin allograft survival [23]. To induce Tregs in an allergic environment is generally refractory. The present data provide a possible strategy to enhance the efficacy in the induction of Tregs by suppressing the production of TIM4.

In summary, the present data have revealed a previously unknown feature of dust mite allergens, one of the components of the dust mite allergens, the Der f 8, is capable of inducing TIM4 expression in DCs, which plays a critical role in the initiation of skewed Th2 polarization and allergic inflammation. Removal of Der f 8 from DME vaccine significantly improves the inhibitory efficacy on the dust mite-specific allergic airway inflammation. Thus, the Der f 8-null DME may be a promising vaccine to obtain a better therapeutic effect on inhibition of the dust mite-specific allergic inflammation.

Acknowledgement

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