

Dust mite allergen, glutathione S-transferase, induces T cell immunoglobulin mucin domain-4 in dendritic cells to facilitate initiation of airway allergy

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Clinical & Experimental Allergy

Summary

Background Allergens from dust mites play a critical role in the pathogenesis of airway allergy. The mechanism by which dust mite allergens induce allergic diseases is not fully understood yet.

Objective This study tests a hypothesis that the eighth subtypes of *Dermatophagoides farina* allergen (Derf8) play an important role in the induction of airway allergy.

Methods The protein of Derf8 was synthesized via molecular cloning approach. Dendritic cells (DC) were stimulated with Derf8 in the culture, and then, the expression of T cell immunoglobulin mucin domain 4 (TIM4) in dendritic cells (DC) was analysed. The role of Derf8 in the induction of airway allergy was evaluated with a mouse model.

Results Exposure to Derf8 markedly induced the TIM4 expression in DCs by modulating the chromatin at the TIM4 promoter locus. Derf8 played a critical role in the expansion of the T helper 2 response in the mouse airway via inducing DCs to produce TIM4. Administration with Derf8-depleted dust mite extracts (DME) inhibited the allergic inflammation and induced regulatory T cells in mice with airway allergy.

Conclusion Derf8 plays an important role in the initiation of dust mite allergy. Vaccination with Derf8-deficient DME is more efficient to inhibit the dust mite allergic inflammation than using wild DME.

Keywords allergen, allergy, animal model, asthma, specific immunotherapy

Submitted 20 December 2015; **revised** 3 July 2016; **accepted** 17 July 2016

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Cite this as: L.-H. Mo, L.-T. Yang, L. Zeng, L.-Z. Xu, H.-P. Zhang, L.-J. Li, J.-Q. Liu, X.-J. Xiao, P.-Y. Zheng, Z.-G. Liu and P.-C. Yang, *Clinical & Experimental Allergy*, 2017 (47) 264–270.

Introduction

The prevalence of allergic diseases arises rapidly in the recent decades. The underlying mechanism has not been fully elucidated yet [1]. The major pathologic features of allergy include T helper (Th)2 polarization in the local tissue and over production of the antigen-specific IgE. The IgE binds to the high-affinity IgE receptors on mast cells to make mast cells sensitized [2]. Re-exposure to specific allergens induces sensitized mast cells to release allergy-related mediators to the local tissue to initiate allergic attacks [3]. Although the pathologic process of allergy is somewhat clear, the mechanisms

about how antigen-specific Th2 cells get polarization and how they sustain at a dominant status in the body are still unknown; the therapeutic efficacy on allergic diseases is unsatisfactory currently.

Dust mites are the most common allergen source of asthma, atopic dermatitis and perennial rhinitis [4]. Dust mites contain a vast 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: *Dermatophagoides farina-1* (Der f 1) and *Dermatophagoides pteronyssinus* (Der p 1) are important allergens in airway allergy [5, 6]. The group 8 allergen of *D. farina* (Derf8) was cloned and characterized by us; its DNA sequence is listed in NCBI database (www.ncbi.nlm.nih.gov/nucleotide/KM)

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010011.1). We found that the Derf8 gene sequence was similar to glutathione S-transferase; the latter has a close relation to allergic asthma [7]. Published data show that 40.9% of mite allergic patients had Derf8-specific IgE in the sera [8, 9]. It suggests that Derf8 may play an important role in the induction of allergy.

Dendritic cells (DCs) are antigen-presenting cells and also produce several co-stimulatory molecules; one of them is the T cell immunoglobulin mucin domain-4 (TIM). TIM4 plays a critical role in the Th2 polarization and initiates allergic disorders [10, 11]. Yet, the mechanism of the over expression of TIM4 by DCs in allergic diseases is unclear. The transcriptional coactivators, p300 and STAT6 (signal transducers and activators of transcription-6), are associated with the pathogenesis of allergic diseases [12]; whether these molecules are associated with TIM4 expression remains to be investigated. Thus, we hypothesize that Derf8 can induce TIM4 expression in DCs, which plays an important role in the initiation of dust mite allergy. In this study, we found that Derf8 induced TIM4 expression in DCs, which played an important role in the dust mite-related airway allergy. The Derf8-deficient dust mite extract (DME) vaccination resulted in much better therapeutic efficacy on airway allergy than the wild DME vaccination.

Materials and methods

A murine model of asthma

As shown by Fig. S1 in Supporting Information, mice were subcutaneously injected with Derf8 (100 µg/mouse) or DME (100 µg/mouse) or Derf8-deficient DME in 0.1 mL saline on days 1, 3 and 7, respectively. Saline was used as a negative control. From day 15 to day 21, the mice were challenged via nostril with Derf8 (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 analyse the allergic airway inflammation or treated with specific Immunotherapy (SIT).

Lung histology

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

Bronchoalveolar lavage cytokine analysis

Bronchoalveolar lavage (BAL) and BAL fluid (BALF) cell counts were performed with experimental mice following the published procedures [13]. Th2 cytokines in BALF were measured by ELISA with commercial reagent kits following the manufacturer's instructions.

Respiratory mechanics of mice

To evaluate the effect of lung function in the mice, we assessed respiratory mechanics using the flexiVent system following the published procedures [14]. Briefly, mice were anaesthetized by intraperitoneal injection with xylaxine and sodium pentobarbital. Measurements were performed using the flexiVent small animal ventilator (SCIREQ, Montreal, QC, Canada) by treating mice with increasing concentrations of aerosolized methacholine.

Specific immunotherapy (SIT)

As shown in Fig. S2, from day 28, the asthma mice were treated with DME vaccine, or Derf8-deficient 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 5–7), 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 intranasally challenged with 50 µg DME in 50 µL saline.

Isolation of mononuclear cells from lung tissue

The lung tissue was cut into small pieces (2 × 2 × 2 mm) and 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) and harvested by centrifugation. The mononuclear cells were further isolated by percoll density centrifugation and cultured in RPMI1640 medium (supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 2 mM glutamine). The cell viability was greater than 96% as assessed with trypan blue exclusion assay.

Statistical analysis

Data were presented as mean ± SD. Significance was determined by a one-way ANOVA followed by Bonferroni's multiple comparison test. $P < 0.05$ was set as a significant criterion.

Reagents, mice, Western blotting, qRT-PCR, flow cytometry, purification of immune cells, T cell proliferation assay, ELISA, generation of bone marrow-derived DC, preparation of mite proteins, luciferase assay, ChIP, bio-syntheticization of mite allergen components and depletion of Derf8 in DME are presented in Supporting Information.

Results

Exposure to Derf8 induces TIM4 expression in DCs

To test the effects of Derf8 on inducing TIM4 expression in DCs, we exposed BMDCs to DME in the culture.

The cells were analysed 48 h later. The results showed that DCs expressed high levels of TIM4. As there are a number of different components of DME allergens have been identified, to recognize which DME components induce TIM4 expression in BMDCs is of significance. Thus, we exposed BMDCs to 34 DME components individually in the culture for 48 h. The cells were analysed 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 BMDCs, in which the TIM4 mRNA was uniquely higher in the BMDCs exposed to Derf8 (Fig. 1a).

To corroborate the results, we exposed BMDCs to Derf8 in the culture at gradient concentrations for 48 h. It resulted in the increases in TIM4 expression in BMDCs in a Derf8 dose-dependent manner (Fig. 1b and c). The results suggest that Derf8 plays an important role in the TIM4 expression in BMDCs. On the other hand, we depleted Derf8 from DME (Fig. S3). BMDCs were stimulated by Derf8-deficient DME or Derf8-containing DME for 48 h. The results showed the wild-type DME induced TIM4 expression in BMDCs, and the Derf8-deficient DME did not (Fig. 1b and c). As exposure to Der f 1, 2 and Der p 1, 2 also increased the expression of TIM4 mRNA in BMDCs, we assessed the TIM4

protein levels in BMDCs after exposure to these four subtypes of mite allergen. The results showed that the TIM4 protein was not apparently increased in DCs (Fig. 1d). Taken together, the results indicate that Derf8 is the major component in mites to increase the expression of TIM4 in BMDCs.

Derf8 induces the TIM4 gene transcription

Next we transfected BMDCs with a TIM4 promoter luciferase reporter. The BMDCs were exposed to Derf8 (or BSA) in the culture for 3 h and then subjected to the analysis of luciferase activity at 24 h. The results showed that the exposure to Derf8, but not to BSA, markedly increased the luciferase activity (Fig. 2). The results indicate that Derf8 increases the TIM4 promoter activity.

Derf8 induces chromatin remodelling at the TIM4 gene locus

To elucidate the mechanism by which Derf8 alters the transcription of TIM4, we assessed the changes of the molecular components at the TIM4 promoter locus in BMDCs after exposure to Derf8 in the culture for

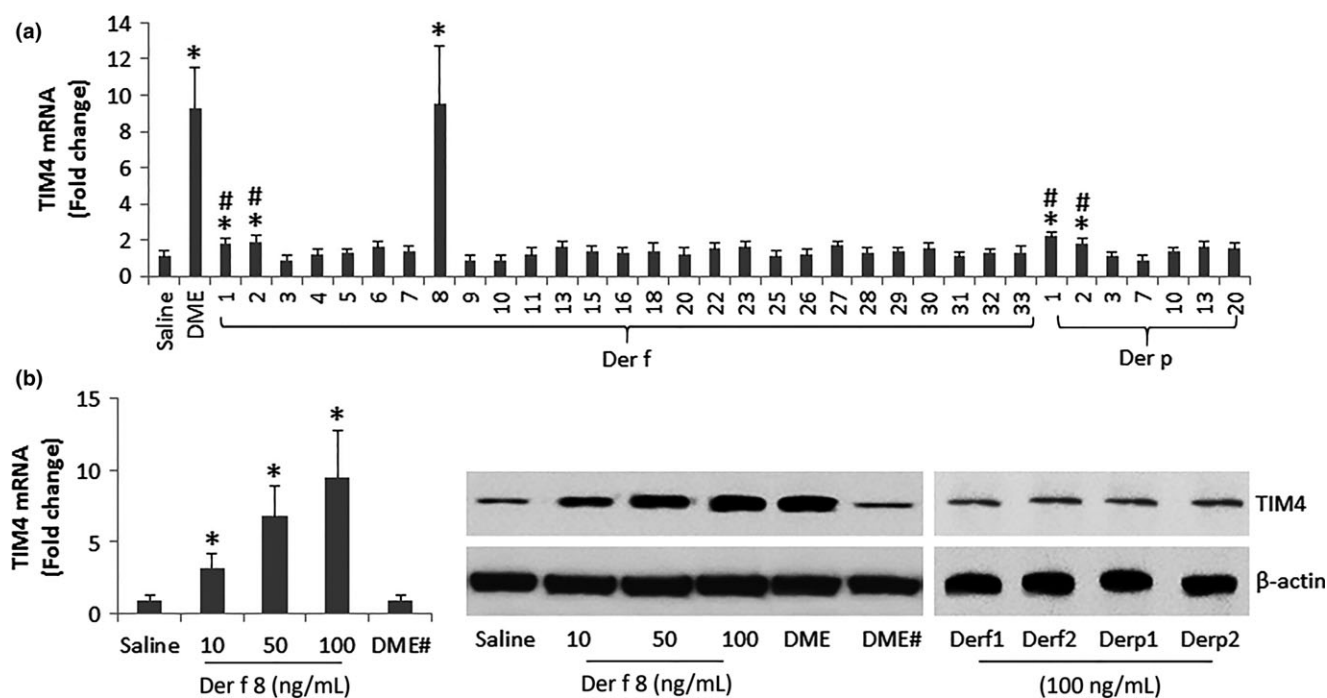


Fig. 1. Der f 8 increases TIM4 expression in DCs. CD11c⁺ MHC II⁺ BMDCs were prepared from the bone marrow. The BMDCs were cultured in the presence of dust mite extract (DME), or the subtypes of Der f allergens or Der p allergens (denoted on the X axis of panel a; 100 ng/mL) for 48 h. The BMDCs were collected and analysed. (a) The bars indicate the mRNA levels of TIM4 (by qRT-PCR). (b–d) BMDCs 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 three independent experiments. The full-length gel graphs are presented in Supporting Information.

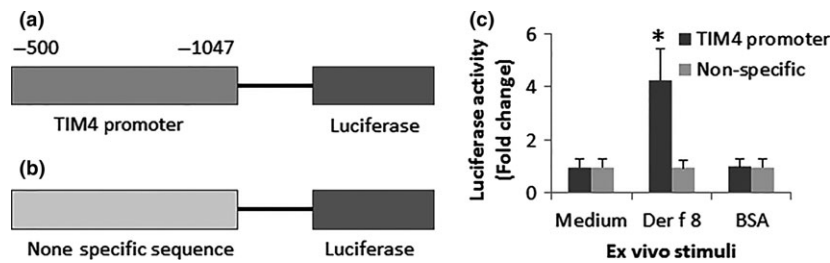


Fig. 2. Der f 8 activates TIM4 promoter. A reporter gene with TIM4 promoter sequence (a) or a non-specific sequence (b) was constructed. (c) BMDCs were transfected with the reporter gene. The cells were exposed to Der f 8 (100 ng/mL) in the culture medium for 3 h and analysed 24 h later. The bars indicate the luciferase activity (mean $\pm$ SD; * P < 0.01, compared with the medium group). The data are representatives of three independent experiments.

60 min. The results showed that the levels of pp300, pSTAT6, acetylated H2A, H2B, H3 and H4 were significantly increased at the TIM4 promoter locus (Fig. 3a). The TIM4 mRNA and proteins in BMDCs were also increased after exposure to Derf8 for 48 h (Fig. 3b and c).

Derf8 facilitates induction of airway allergy

To test the role of Derf8 in the induction of airway allergy, we sensitized mice with Derf8 or DME or Derf8-deficient DME. As shown in Fig. 4, the Derf8-specific CD4⁺ T cell proliferation, serum levels of Th2 cytokines, serum Derf8-specific IgE, lung tissue Th2 cytokines, BALF Th2 cytokines, BALF inflammatory cells (eosinophils, neutrophils and macrophages) and Th2-cell frequency in lung tissue were significantly increased in mice immunized with Derf8 than control mice, which were higher in mice sensitized to DME than those sensitized to Derf8. The allergic airway inflammation could be blocked by anti-TIM4 antibody. The data demonstrate that Derf8 can induce airway allergy in the lung.

Derf8-deficient DME vaccination Inhibits airway allergic inflammation

The above data suggest that Derf8 is a critical molecule in the induction of skewed dust mite-specific Th2 polarization. We hypothesized that the removal of Derf8 from the DME vaccine might greatly improve the efficacy of the DME-specific immunotherapy (SIT). To test this, 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 Derf8-deficient DME vaccine. As shown in Fig. 5, the immunization with DME induced allergic inflammation in the lung, 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, none of them reached the significant levels, while those mice treated with Derf8-deficient DME SIT showed a significant attenuation in the lung allergic inflammation. Furthermore, treatment with the Derf8-deficient DME SIT, but not the DME SIT, significantly up-regulated the frequency of antigen-

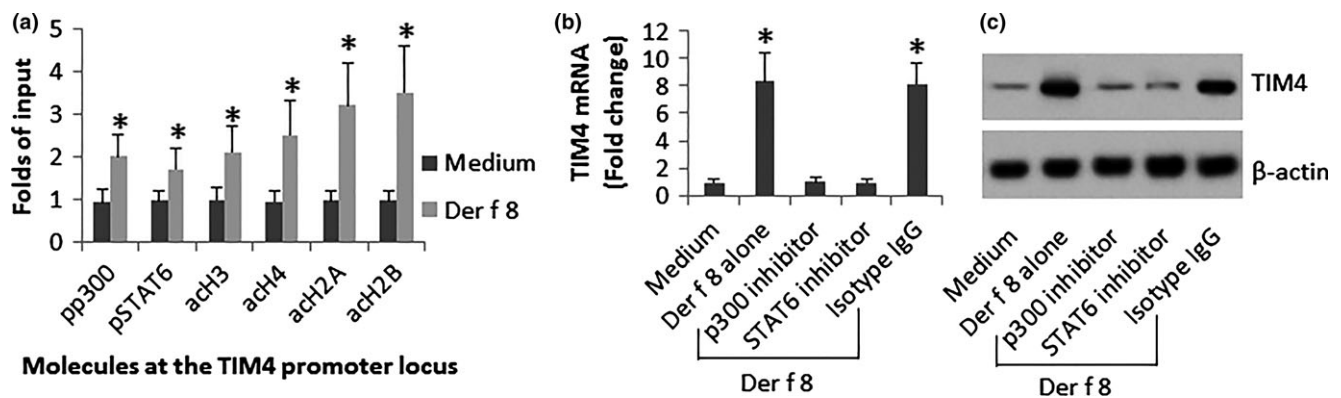


Fig. 3. Der f 8 modulates chromatin of TIM4 gene in BMDCs. (a) BMDCs were treated with Der f 8 (100 ng/mL) in the culture for 1 h. The nuclear extracts of BMDCs were analysed by ChIP assay. The bars indicate the molecules (denoted on the X axis of panel a) at the TIM4 promoter locus. (b, c) BMDCs were treated with Der f 8 (100 ng/mL) in the culture for 48 h. The cytosolic extracts were prepared and analysed by RT-qPCR and Western blotting. (b) The bars indicate the mRNA levels of TIM4 in BMDCs. (c) The immune blots indicate the TIM4 protein in BMDCs. Data of bars are presented as mean $\pm$ SD. * P < 0.05, compared with the 'medium' samples. The data are representatives of three independent experiments. p300 inhibitor: garcinol (3 μ M). STAT6 inhibitor: A77-1726 (10 nM).

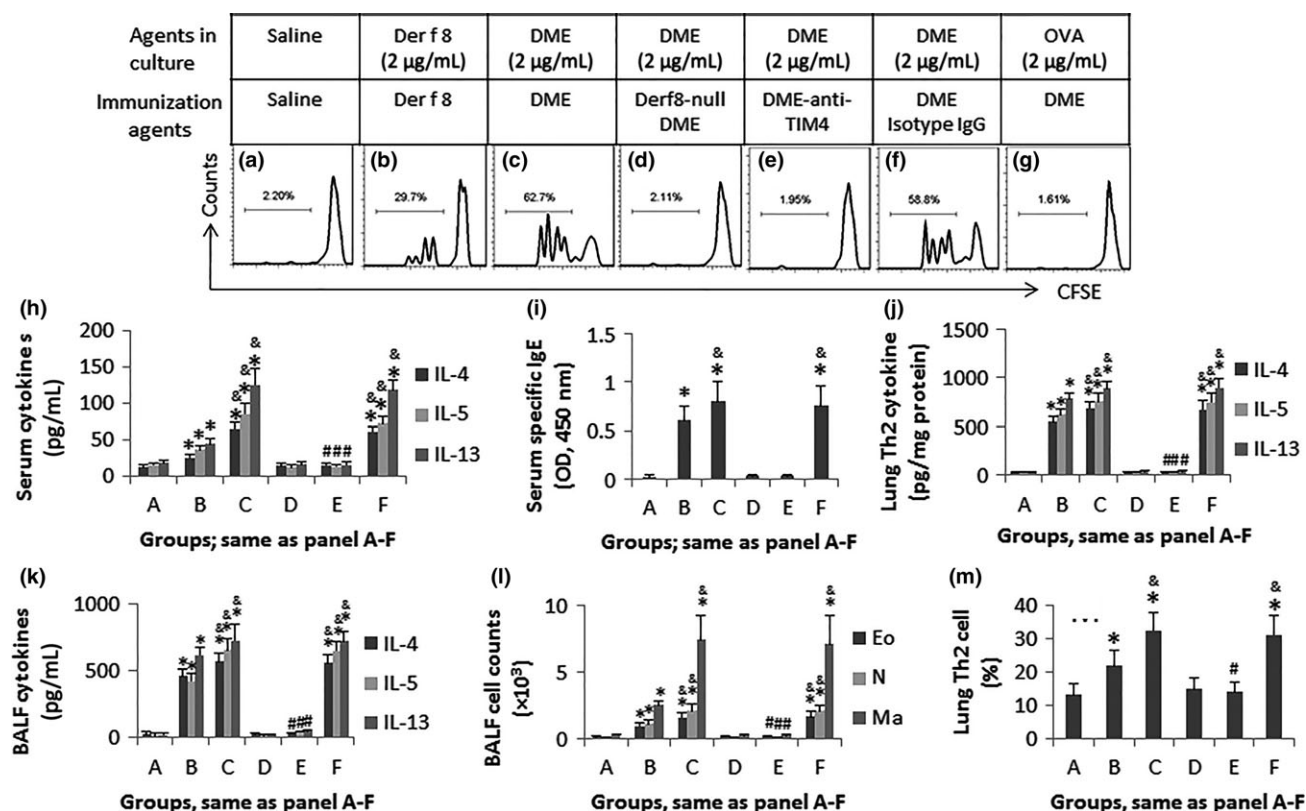


Fig. 4. Der f 8 facilitates antigen-specific Th2 polarization. (a–g) The histograms indicate the frequency of proliferated lung CD4⁺ T cells after treatment (denoted in the table above each panel) in the culture. (h–i) The bars indicate the serum levels of Th2 cytokines (h) and specific IgE (i). (j–l) The bars indicate the Th2 cytokine levels in lung tissue (j) and BALF (k) and inflammatory cell counts/mL in BALF (k). (m) The bars indicate the frequency of lung CD4⁺ IL-4⁺ T cells. Data of bars are presented as mean ± SD. **P* < 0.01, compared with group A. [†]*P* < 0.01, compared with group B. #*P* < 0.01, compared with group C. Samples from each mouse were processed separately. The data are representatives of six independent experiments.

specific CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Treg) in the lung.

Discussion

In previous work, we observed that DCs produced TIM4 upon exposure to staphylococcal enterotoxin B (SEB) [10] that played a critical role in the induction of intestinal allergy [15]. Rodriguez-Manzanet et al. [16] reported that DCs produced TIM4 after activation to induce T cell expansion and survival. Li et al. [17] reported recently that hepatic ischaemia reperfusion injury could increase TIM4 expression by DCs. 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; this was much higher in DCs after exposing to Derf8.

The present results showed that exposure to Derf8 increased the phosphorylation of p300, followed by the acetylation of histone at the TIM4 promoter locus, and then, the STAT6 phosphorylation was increased in DCs. The results suggest that Derf8 can induce the chromatin

remoulding at TIM4 promoter locus to induce the TIM4 gene transcription and expression in DCs. Both p300 and STAT6 play an important role in the Derf8-induced TIM4 expression because the presence of either a p300 inhibitor or a STAT6 inhibitor abolished the TIM4 expression.

Derf8 is similar to the glutathione S-transferase (GST) in structure. GST can 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 on the substrate [18]. This action of Derf8 is of significance because it initiated TIM4 gene transcription in DCs, induced Th2 polarization and facilitated the DME-specific allergic inflammation in the lung, as shown in the present study. Supportive data have also published that GST is associated with the pathogenesis of asthma [7].

In line with the previous studies [13, 19], we developed 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

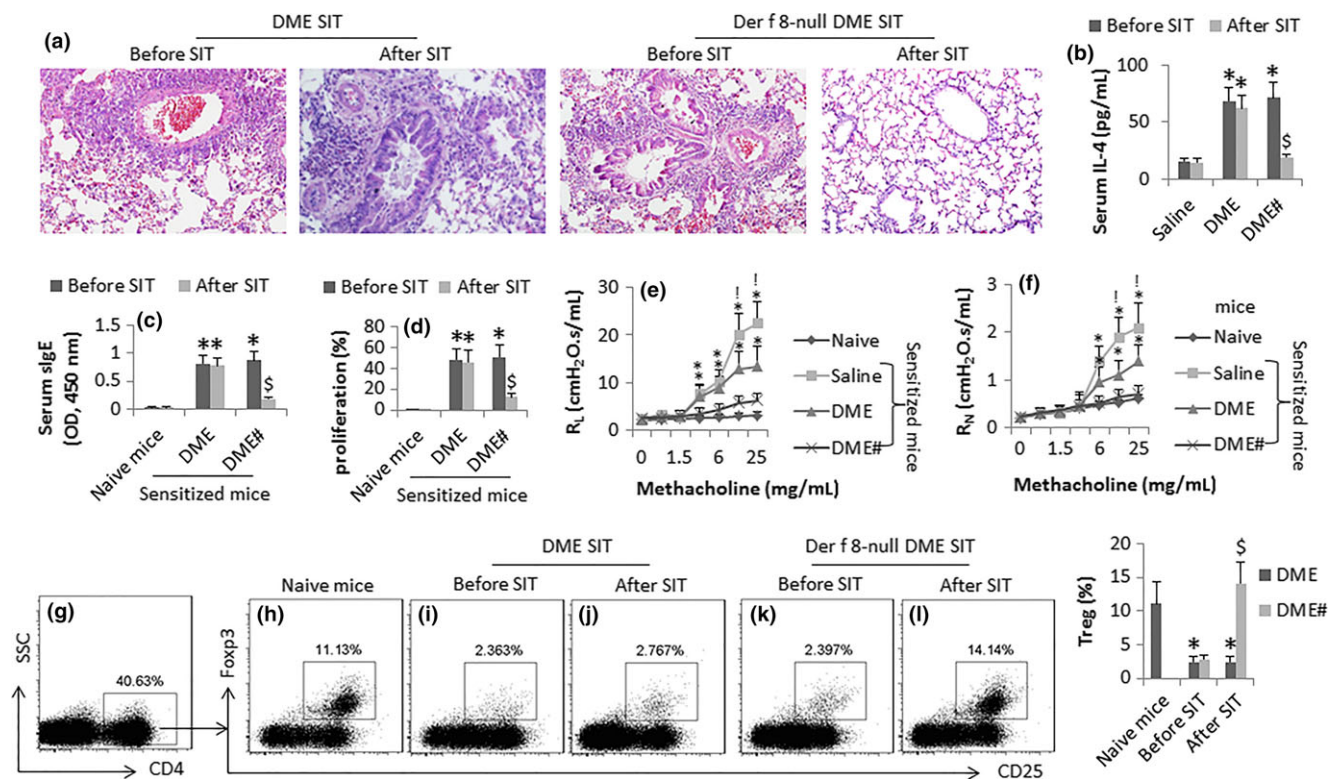


Fig. 5. Der f 8-null DME SIT attenuates mite allergic lung inflammation. Mice (six mice per group) were sensitized to DME and treated with DME SIT or Der f 8-null DME (DME#) SIT. (a) the histology images (×200) show lung histology. (b, c) the bars indicate the serum levels of IL-4 (b) and specific IgE (c). (d) The bars indicate the CD4⁺ T cell proliferation after stimulation with DME in the culture. (e, f) The curves indicate the total lung resistance (e) and central airway resistance (f). (g) mononuclear cells were isolated from the lung. The gated cells are CD4⁺ T cells. (h-l) the gated cells are CD25⁺ Foxp3⁺ Tregs in the gated CD4⁺ T cells of panel g. (m) the bars indicate the summarized data of (h-l). * $P < 0.01$, compared with the saline group (b-d), or with the naive mice (k). $^{\S}P < 0.01$, compared with the DME group (b-d) or with the dose 0 (e, f) or with naive mice (m). $^{\dagger}P < 0.01$, compared with the DME group (e, f). The data of bars and curves are presented as mean $\pm$ SD. Samples from each mouse were processed separately. The data are representatives of six independent experiments.

specific Th2 polarization in the airway was significantly attenuated with the Derf8-deficient DME, pinpointing the importance of Derf8 in the allergy induction capacity of dust mite. Such a feature of Derf8 implicates that the removal of Derf8 from DME may be useful in the SIT. The results from vaccination with the Derf8-deficient 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 [20]. The present data also show that using the Derf8-deficient 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 Derf8-containing DME. This is in line with the previous studies. Li et al. [17] 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. Yeung et al. [21] indicate that the interruption of DC-mediated TIM4 signalling induces regulatory T cells and promotes skin allograft survival. 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, and one of the components of the dust mite allergens, the Derf8, 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 Derf8 from DME vaccine significantly improves the inhibitory efficacy on the dust mite-specific allergic airway inflammation. Thus, the Derf8-deficient DME may be a promising vaccine to obtain a better therapeutic effect on inhibition of the dust mite-specific allergic inflammation.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of SZU (No. 000004), the Innovation of Science and Technology Commission of Shenzhen Municipality (No. JCYJ20140418095735611),

the Natural Science Foundation of China (81373176, 31400856, 31570932, 81571790 and 81501573).

Conflict of interest

The authors declare no conflict of interest.

Author contribution

LHM, LTY, LZ, LZ, HPZ, LJJ, JQL and XJX performed the experiments, analysed data and reviewed the manuscript. PCY and ZGL organized the study and supervised the experiments. PCY designed the project and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Time course of sensitization.

Figure S2. Time course of SIT.

Figure S3. Assessment of immune response of purified Der f 8.

Table S1. Information of mite allergen components.

Supplemental materials

Dust mite allergen, glutathione S-transferase, induces T cell immunoglobulin mucin domain-4 in dendritic cells to facilitate initiation of airway allergy

Running title: mite allergens induce TIM4 in DCs

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Reagents

Garcinol, A77-1726, antibodies of TIM4, H2A, H2B, H3, H4, p300 and pp300 were purchased from Biomart (Shanghai, China). The reagents for quantitative real-time RT-PCR (qRT-PCR) and Western blotting were purchased from Thermo China (Shenzhen, China). The ELISA kits for IL-4, IL-5 and IL-13 were purchased from Ebioscience (Shenzhen, China). The IgE ELISA kit was purchased from Shanghai Yantuo Biotech (Shanghai, China). Fluorescent labeled antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). The ChIP kit and protein G were purchased from Sigma Aldrich (St. Louis., MO). The immune cell isolation kits were purchased from Miltenyi Biotech (San Diego, CA). The anti-TIM4 Ab was purchased from R&D Systems (Minneapolis, MN). The components of Der f and Der p proteins were synthesized by molecular cloning were produced in our laboratory following our established procedures (Jiang C, Fan X, Li M, Xing P, Liu X, Wu Y, Zhang M, Yang P, Liu Z. Characterization of Der f 29, a new allergen from dermatophagoides farinae. Am J Transl Res. 2015 Jul 15;7(7):1303-13). The monoclonal antibody of Der f 8 was produced by our laboratory.

Mice

Male BALB/c mice (6-8 week old) were purchased from the Guangdong Experimental Animal Center (Guangzhou, China). The mice were maintained in a pathogen-free environment with accessing food and water freely. The using mouse 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.

Western blotting

Extracted total proteins from the cells were fractioned by SDS–PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk solution for 30 min, the membrane was incubated with antibodies of TIM4 or β -actin (300 ng/ml) for 1 h at room temperature, and followed by incubation with the horseradish peroxidase labeled secondary antibodies (50 ng/ml) for 1 h at room temperature. The immune blots on the membrane were developed with the enhanced chemiluminescence (ECL) reagents. The results were photographed with an image device (UVI, Shenzhen, China).

qRT-PCR

The total RNA was extracted from DCs with the TRIzol reagents. cDNA was sensitized using a reverse translation kit. qPCR was performed on a MiniOpticon PCR device (Bio-Rad). The results were calculated with the $2^{-\Delta\Delta C_t}$ method and normalized to folds of change against the controls. The primers used were: TIM4 (agtcagatctgggtttttgggccagccggtg and agtcctgcagtcagagagtgaagatcccg); β -actin (catccgtaaagacctctatgccaac and atggagccaccgatccaca).

Flow cytometry

In the surface staining, cells were stained with fluorochrome-labeled antibodies of interest for 0.5 h. After washing with PBS, the cells were fixed and permeabilized with Fix/Perm buffer and permeabilization buffer 0.5 h at 4°C. The cells were then incubated with the fluorochrome-labeled antibodies for 1.5 h at 4°C. After washing with PBS thoroughly, the cells were analyzed

with a flow cytometer (FACSCanto I, BD Biosciences). The data were analyzed with the software Flowjo.

Purification of immune cells

Immune cells were purified with magnetic cell sorting (MACS) with commercial reagent kits following the manufacturer's instruction. The cell purity was checked by flow cytometry. If the purity was below 95%, the purification was repeated.

T cell proliferation assay

CD4⁺ T cells were purified from the isolated lung mononuclear cells by MACS, stained with CFSE, cultured with DCs (purified from the spleen) at $5 \times 10^5 : 1 \times 10^5$ cells/ml in the presence of DME (5 µg/ml) for 3 days. The cells were analyzed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from each mouse at the sacrifice by 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 (DME, or Derf8) at 100 ng/well in 100 µl in carbonate buffered solution (pH 9.5) overnight at 4 °C. The plates were 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 μ L/well), stayed for 20 min at room temperature and stopped by addition of 2M H₂SO₄ (50 μ L/well). The plates were read with an ELx808 absorbance microplate reader (BioTek, Shanghai, China) at 450 nm.

Bone marrow derived DC (BMDC) preparation and treatment

BMDCs were generated from the mouse bone marrow following our established procedures {Feng, 2008 #7}. CD11c⁺ MHC II⁺ BMDCs were isolated with a commercial kit following the manufacturer's instruction. The BMDCs were cultured in RPMI 1640 medium at 37 °C in 5% CO₂. Per 1 $\times$ 10⁶ BMDCs were exposed to 34 subtypes of dust mite allergen respectively at 100 ng/ml, or DME (100 ng/ml, using as a positive control), or saline (using as a negative control). After culturing for 48 h, the BMDCs were harvested by centrifugation at 300 $\times$ g for 10 min and subjected to analysis of Western blotting and real-time RT-PCR (qRT-PCR).

Luciferase reporter assay

The luciferase reporter of TIM4 promoter was constructed with the sequence between -500 and -1047 (or a non-specific sequence, using as a negative control) by the Sangon Biotech Limited Company (Shanghai, China) and transfected bone marrow-derived DCs (BMDC) following the manufacturer's instruction. In the reporter-gene analysis, the proteins were quantified 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 immunoprecipitation (ChIP)

BMDCs were fixed with 1% formalin for 15 min and sonicated. After preclearing, the supernatant was collected by centrifugation and incubated with antibodies of interest or isotype IgG (used as controls) and protein G agarose beads 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 agaatcgcttgaaccagga. The results were calculated and presented as relevant changes against the DNA input.

Preparation of mite proteins

D farinae mites were isolated from indoor dust samples from Shenzhen City in southern China and cultured as we reported previously (Chan TF, et al. The draft genome, transcriptome, and microbiome of *Dermatophagoides farinae* reveal a broad spectrum of dust mite allergens. J Allergy Clin Immunol. 2015; 135: 539-48). Mites in lysis buffer [In 100 ml: 10mM HEPES (PH7.9 238.4mg), 10mM KCL (74.5mg), 0.1mM EGTA (MW = 380.35; 3.8mg), 0.5mM DTT (7.7mg; added before use), 0.2 mM PMSF (3.4mg, added before use), 0.1mM EDTA (2.9mg; added before use)] were homogenized with a Kimble Dounce Homogenizer (Beijing, China) for 10 min by placing the sample tubes in ice water. The lysates were centrifuged for 10 min at 13,000 rpm

at 4 °C. Supernatant was collected and designated dust mite extracts (DME). Proteins in the supernatant were quantitated with Bio Rad protein assay.

Bio-synthetization of mite allergen components

The gene sequences of 33 mite components were cited from the Gene Bank of NCBI. The relative information of the mite component gene is presented in Table S1. The procedures of mite component cloning and protein purification were performed by our laboratory as reported previously (Jiang C, et al. Characterization of Der f 29, a new allergen from dermatophagoides farinae. Am J Transl Res. 2015; 7: 1303-13).

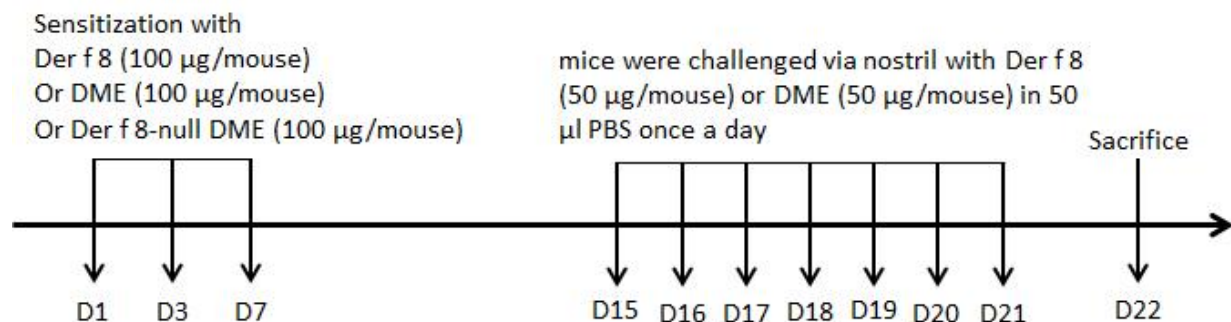


Figure S1. Time course of sensitization.

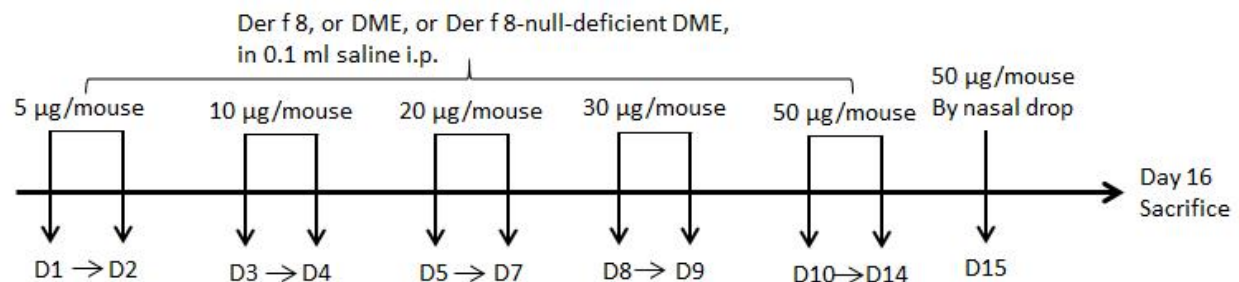


Figure S2. Time course of SIT.

Depletion of Der f 8 in DME

The anti-Der f 8 mAb was generated by our laboratory. The mAb was biotinylated by using a biotinylation kit (Elaboscience, Wuhan, China) following the manufacturer's instructions. The biotinylated Der f 8 mAb was conjugated with streptavidin magnetic beads (Miltenyi Biotech) at a ratio of 1:4. The beads were collected by centrifugation and resuspended in PBS; the Der f 8 mAb contents were calculated based on the amount of mAb processed and the final volume. To deplete Der f 8 in DME, the Der f 8 mAb conjugates were added to DME at a final concentration of 1 μ g/ml and incubated for 1 h at room temperature. The samples were selected using an MS column and MACS separator (Miltenyi). The Der f 8-free samples were passed through the column. As analyzed by ELISA, Der f 8 was below the detectable levels in the purified samples. The DME with Der f 8 depleted was designated Der f 8-null DME.

To demonstrate the effect of the Der f 8 purification, ELISA was performed with the purified Der f 8 or Der f 8-null DME as the primary antigens to coat the microplates. After incubated at 4 °C overnight, Der f 8-specific monoclonal antibody was added to each well. The rest procedures of ELISA were the same as described in the main text. The results showed that specific immune response was detected in the wells coated with purified Der f 8, but not the Der f 8-null DME (Fig. S3).

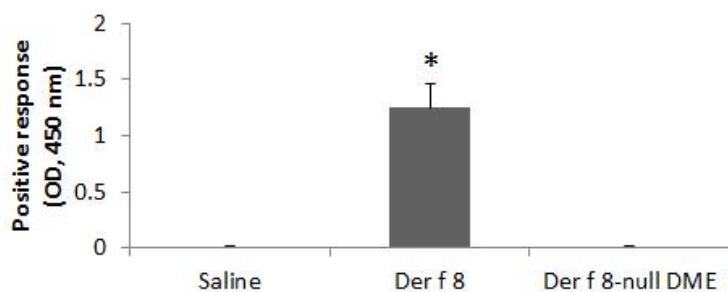


Figure S3. Assessment of immune response of purified Der f 8. The bars indicate the positive immune response of Der f 8. Data are presented as mean $\pm$ SD. *, $p < 0.01$, compared with the saline group. The data are summarized from 3 independent experiments.

Table S1. Information of mite allergen components

Allergen	Biochemical name	MW	accession number		vector
Der f 1	Cysteine protease	27	BAC53948	inclusion body	PET-24a
Der f 2	NPC2 family	15	BAA01240	inclusion body	PET-24a
Der f 3	Trypsin	29	BAA09920	inclusion body	PET-His
Der f4			AJF93907.1		
Der f5			ABO84972.1	inclusion body	PET-28a

Der f 6	Chymotrypsin	25	AAF28423	inclusion body	PET- 24a
Der f 7		30-31	AAB35977	inclusion body	PET- 32a
Der f 8	Glutathione S-transferase	32 kD	AGC56215	soluble protein	PET- 32a
Der f9			AAP35067.1		
Der f 10	Tropomyosin	37	BAA04557	soluble protein	PET- 32a
Der f 11	Paramyosin	98	AAK39511		
Der f 13	Fatty acid binding protein		AAP35078		
Der f 14	Apolipoporphin	177	BAA04558		
Der f 15	Chitinase	98/109	AAD52672	inclusion body	PET- 32a
Der f 16	Gelsolin/villin	53	AAM64112	inclusion body	PET- 24a
Der f 17	Calcium binding protein	53			
Der f 18	Chitin-binding protein	60	AAM19082	inclusion body	PET- 24a
Der f 19					
Der f 20	Arginine kinase	40 kD	AIO08850.1	inclusion body	PET-

					32a
Der f 21		14 kDa	AHC94806	inclusion body	PET-32a
Der f 22			ABG35122	soluble protein	PET-44a
Der f23			AIO08855.1	soluble protein	PET-44a
Der f 24	Ubiquinol-cytochrome c reductase binding protein homologue	13	AGI78542		
Der f 25	Triosephosphate isomerase	34 kDa	AGC56216	soluble protein	PET-28a
Der f 26	Myosin alkali light chain	18 kDa	AIO08852.1		
Der f 27	Serpin	48 kD	AIO08851.1		
Der f 28	Heat Shock Protein	70 kD	AGC56218.1	inclusion body	PET-32a
Der f 29	Peptidyl-prolyl cis-trans isomerase (cyclophilin)	16 kD	AAP35065	inclusion body	PET-His
Der f 30	Ferritin	16 kD	AGC56219.1	inclusion body	PET-His
Der f 31	Cofilin	15 kD	AIO08870.1	inclusion body	

Der f 32	Secreted inorganic pyrophosphatase	35 kD	AIO08870.1	inclusion body	
Der f 33	alpha-tubulin	52 kDa	AIO08861	soluble protein	
Der f34	profilin		AIO08866	soluble protein	PET- 28a