Original Article
Characterization of Der f 29, a new allergen from dermatophagoides farinae

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Abstract: More than 30 allergens have been identified from Dermatophagoides farina (*D. farina*), which is one of the main species of house dust mites. The mite allergens are an important factor contributing to allergic disease in the world. As the detection and identification of new allergens is critical for the diagnosis or treatment of allergic diseases, we sought to characterize the profilin of *D. farina* (Der f 29) in this study. The results showed that 21% of allergic patients displayed positive results in skin prick test with recombinant Der f 29 (rDer f 29) as the specific allergen; specific IgE reactivity to rDer f 29 was shown by Western Blot and ELISA. In addition, rDer f 29 induced bone marrow-derived dendritic cells (DC) to produce T cells immunoglobulin domain and mucin domain protein 4 (TIM4). Moreover, an allergic asthma mouse model was established by challenging with rDer f 29. Airway hyperresponsiveness, serum specific IgE, IgG1, eosinophil infiltration in the allergic mice bronchoalveolar lavage fluid, the cytokines interleukin-4 (IL-4) and interferon-γ (INF-γ) from spleen cells were markedly increased; the histology showed severe inflammation in the lung. In conclusion, Der f 29 is identified as a new type of the house dust mite allergen.

Keywords: Profilin, der f 29, expression, purification, dendritic cell

Introduction

House dust mites (HDM) are responsible for the sensitization of more than 50% of allergic patients, such as asthma, allergic rhinitis and dermatitis [1, 2]. At present, the specific immunotherapy with house dust mite extracts as vaccine is the only specific therapeutic remedy for mite-related allergic diseases. In fact, the crude extracts of house dust mites not only contain the mite allergens, but also include other inflammatory molecules, such as kallikreins, ceramides and the ever popular endotoxins, which may cause additional allergic response or other side effects [3]. The group 1 and 2 of mite allergens have been proven to be the major allergens in *Dermtophagoides farina (D. farinae)* that have contributed to designing new strategies of immunotherapy for mite-allergic patients. However, about 20% of patients lack of IgE antibody to the group 1 and 2 allergens. There are a number of other house dust mite allergens that show IgE binding activity but present at low and variable concentrations in mite extracts. Thus, it is beneficial to characterize those mite allergens in order to design more effective therapeutic remedies for the treatment of mite-related allergic diseases [4].

To date, some recombinant dust mite allergens have been expressed and purified, including sixteen groups of allergens (Der f 1-3, 5-8, 10, 11, 13 to 18, and 22, 24), while Der f 29 was still under identified [5]. Der f 29s are highly conserved proteins in all eukaryotic organisms such as pollens and a wide variety of vegetable foods. They also have high degree of structural homology between different species with themolecular weight of about 14 kDa [6].

In this study, we cloned, expressed and purified the rDer f 29 with the genetic information from HDM, and the allergenicity of rDer f 29 was evaluated by an asthmatic mouse model.

Materials and methods

Patients and animals

A written, informed consent was obtained from each human participant for the use of blood samples and the skin test. In total of 14 allergic asthma patients and 2 healthy subjects were recruited from our allergy clinic. The study was approved by the ethics committee of the Institutional Review Board of the School of Medicine, Shenzhen University (Shenzhen, Guangdong, China).

Female BALB/c mice (6-8 week old, 18-20 g body weight) were purchased from Guangdong Laboratory Animal Center (Guangzhou, Guangdong, China). The mice were housed in a pathogen-free environment and under conditions of constant temperature (22-24°C) and humidity (60%), exposed to a 12 h light/dark cycle, and provided tap water to drink. The experimental design was approved by the Institutional Ethics Committee of Shenzhen University (Shenzhen, Guangdong, China).

Der f 29 gene synthesis and plasmid construction

The gene sequence of Der f 29 (AIO08866.1) was obtained from the whole genome of *D. farinae.* To generate a His-tagged Der f 29 expression plasmid, the PCR product of Der f 29 cDNA was obtained from pMD18-T-Der f 29 cloned by Shenzhen Huada Gene Research Institute, and cloned into the EcoRI and XhoI sites of pET-28a (+), and the resulted sequence was verified by DNA sequencing.

Expression and purification of rDer f 29

pET-28a (+)-Der f 29 were transformed into E. coli BL21. The transformed bacteria were grown on LB plates containing kanamycin (100 µg/ml) at 37°C overnight. The individual positive colonies were selected and inoculated into 5 mL LB containing kanamycin and cultured at 37°C. Then 1 mL bacteria were added to fresh LB medium and grew to OD_{600} nm of 0.6 to 0.8. The expression of rDer f 29 was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1 µM). After 4 h incubation, *E.coli* cells were harvested by centrifugation at 10,000 rpm for 10 min, and rinsed twice with PBS. After resuspension, cells were disrupted ultrasonically. After centrifugation, the supernatant was applied to a Ni-NTA-agarose column equilibrated in lysis buffer. After elution with lysis buffer containing 300 mM imidazole, the rDer f 29 proteins were collected and stored at 4°C.

Skin prick test (SPT)

The sera were collected from 3 allergic rhinitis and asthma patients who had a positive skin reaction to rDer f 29. The skin response was observed at 15 min after the SPT; the results were defined as positive when the wheal was larger than the negative control. The positive responses were further confirmed by measuring *D. farinae*-specific IgE antibodies with the CAP System.

Western blot and ELISA for analysis of IgE reactivity

Proteins were fractioned by 12% SDS-PAGE, followed by electroblotting onto a PVDF membrane. Membranes were blocked with 3% (v/v) BSA-TBST for 2 h at room temperature (RT). Blots were incubated with the allergic serum (diluted in 1:5 V/V in blocking buffer) overnight at 4°C. After washing, the membrane was incubated with a HRP-conjugated anti-human IgE monoclonal antibody, and then proteins were visualized using diaminobenzidine (DAB) staining.

Mouse model of allergic asthma

Eighteen mice were divided into three groups: ① HDM group, ② rDer f 29 group, ③ Control group. In HDM group and rDer f 29 group, mice were subcutaneously injected with 100 µg extracts from D. farinae or rDer f 29 with 4 mg aluminum hydroxide as adjuvants on day 0, 3 and 7, respectively. PBS was used in control group. In the following week, HDM and rDer f 29 group mice were challenged with 50 µg crude extracts of *D. farinae* or rDer f 29 protein/ mouse via intranastril drops, respectively. The control mice were treated with the saline instead of allergens in the same procedures. Following challenge, airway hyperresponsiveness to methacholine was measured using the unrestained whole-body plethysmography (WBP). 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.

Analysis of specific IgE and IgG1 in the serum

IgE reactivity in the allergic serum was determined by ELISA. In brief, 100 ng purified rDer f 29 in 100 µL carbonate-biocarborate buffer

was incubated in a 96-well microtitre plate overnight at 4°C. The plate was blocked with PBS containing 3% BSA for 2 h at RT, followed by incubating with the allergic serum (diluted in 1:10 (V/V) with block buffer) overnight at 4°C. After washing three times with PBST, the plate was incubated with (HRP)-conjugated goat anti-human IgE monoclonal antibody for 1.5 h at 37°C. Then, the color was developed by adding 100 µl chromogenic substrate to each well and stopped by the addition of 50 µl 2 M sulphuric acid. The absorbance at 450 nm was measured by a microplate reader.

Bronchoalveolar lavage cytokine analysis

Bronchoalveolar lavage (BAL) and different cell counts were performed as previously described in detail [7]. IL-4 and IFN-γ levels in bronchoalveolar lavage fluid were measured by Multiplex cytokine assays with commercial reagent kits following the manufacturer's instructions.

Proliferative and cytokine responses of splenocytes

Spleen cells were prepared and incubated ($1×10^6$ cells/well) in the presence of HDM (0.5 mg/ml), rDer f 29 (0.5 mg/ml) or culture medium alone in a 96-well plate for 72 h at 37°C. MTS was added to the culture. Four hours later, the culture medium samples were collected and analyzed by a microplate reader at 490 nm. To analyze cytokine responses, splenocytes ($1×10^6$/well) were cultured in 24-well plate and stimulated with 0.2 mg/mL HDM, rDer f 29 or medium alone for 72 h. IL-4 and IFN-γ levels in the supernatants were measured by multiplex cytokine assay kits following the manufactuer's instructions.

Histology

The lung tissue was excised after sacrifice and fixed with 4% paraformaldehyde for 24 h. After dehydration, the lung tissue was embedded in paraffin. Tissues were cut into 5 µm thick sections, and then mounted onto glass slides, and were stained with Hematoxylin and Eosin (HE), and observed under a light microscope.

Preparation of bone marrow derived dendritic cells

The female mice with 6 weeks were sacrificed by cervical dislocation. The femur bone was removed and disinfected in 75% ethanol for 1 minute, washed twice with DPBS. Both ends of the femur were cut with scissors; the bone marrow was flushed out with Ca^{2+} and Mg^{2+} free DPBS using a 25-gauge needle. After repeated pipetting to break cell aggregates, cells were filtered through a 70 µm nylon mesh to remove debris, then spin down and gently resuspended in 2 ml ACK for 2 min before adding 10 ml DMEM. Cells were washed again with DMEM and resuspended in culture medium and counted. The cells were grown in macrophage differentiation medium GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) for 3 days. The induced DCs were assessed by flow cytometry; the $CD11c^+$ MHC II^+ cells were regarded as DCs.

Real time RT-PCR

Total RNA was extracted from cultured cells using Trizol, and the DNA was removed by the recombinant DNase I. cDNA was synthesized with 1 µg of total RNA using a reverse transcriptase kit (iScript™ cDNA synthesis kit) in accordance with the manufacturers' protocol. All PCR was carried out using the SsoFastTM EvaGreen Supermix. The PCR reaction included 39 cycles of amplification (15 seconds of denaturation at 95°C, 15 seconds annealing at 60°C, and 20 seconds of elongation at 72°C) with primers designed against the following mouse sequences: TIM4 (forward: 5'-AGTCAG-ATCTGGGTTTTTGGGCCAGCCGGTG-3', reverse: 5'-AGTCCTGCAGTCAGAGAGTGAAGATCCCG-3'); ACTB (forward: 5'-CATCCGTAAAGACCTCTATGC-CAAC-3', reverse: 5'-ATGGAGCCACCGATCCA-CA-3').

Statistical analysis

All data are expressed as mean ± SD; analysed with SPSS 18.0 statistical software. One-way ANOVA was used for comparison of multiple groups and t-test was used for the mean differences between two groups, and the significance level was set at $P < 0.05$.

Results

Cloning, expression and purification of rDer f 29

We firstly carried out a homologous sequence alignment analysis between *D. farinae* profilin and other species. As shown in **Figure 1A**, the full length of *D. farinae* profilin cDNA, named

Figure 1. Purified rDer f 29. A. Profilin, named Der f 29, was compared with other species, amino acids; the identities to Der f 29 were indicated with stars and colon. B. The expression and purification of rDer f 29 were analyzed by SDS-PAGE. Lane 1, Pre-dyed protein maker; Lane 2, E.coli BL21 transformed with pET-Der f 29 without IPTG induction; Lane 3, E.coli BL21 transformed with pET-Der f 29 with IPTG induction; Lane 4, rDer f 29 after elution with imidazole.

Der f 29 (AIO08866.1), is 393 bp, encoding a 130 amino acid polypeptide. Der f 29 showed maximum sequence identity compared with profilins from other mites, such as *Suidasia medanensis* (83% identities, AAX34044.1), *Blomia tropicalis* (82% identities, AAQ24553.1). Significant identity was observed when compared with Caryota mitis (36% Identities, ABM53030.1), Mangifera indica (36% Identities, ABB76134.1).

In order to obtain Der f 29 protein for the subsequent experiments, we cloned Der f 29 cDNA into pET-28a (+), expressed and purified Der f 29 as described in Materials and Methods section. The result showed that His-tag Der f 29 was successfully expressed by induction with IPTG and purified using His-tag lane, and were detected by SDS-PAGE (**Figure 1B**).

The allergic activity of rDer f 29

We next investigated the allergic activity of rDer f 29. The results (**Figure 2A**) showed that three allergic patients had positive reaction to rDer f 29, accounting for 21.4%. To determine the allergenicity of rDer f 29 protein, immunoblot-

ting was performed using the serum from the three rDer f 29-allergic patients. The result demonstrated that the serum from allergic patients, but not that from healthy subjects, reacted to the rDer f 29 protein. The IgE-binding bands with molecular weight around 14.3 kDa (rDer f 29) were positive for patients, but completely negative in healthy volunteers (**Figure 2B**). The specific immunoreactivity of IgE against purified rDer f 29 was further confirmed by ELISA. Compared with the serum from healthy subjects, the IgE reactivity of rDer f 29 from allergic patients increased more than 3 folds (**Figure 2C**). These results suggested that rDer f 29 might be another allergen from dust mite allergen family that leading to type I hypersensitivity.

Establishment of mouse model with allergic asthma using rDer f 29

A rDer f 29-induced mouse model with allergic asthma was developed (**Figure 3A**). Airway hyperresponsiveness was assessed by the unrestained whole-body plethysmography (WBP). As shown by **Figure 3B**, the airway hyper-responsiveness of the mice in rDer f 29 group

A

Patients with serial number	Sex	Diagnosis	Profilin,net wheal size(mm)
1	Male	Bronchial asthma, allergic rhinitis	0
2	Female	Allergic rhinitis	0
3	Female	Allergic rhinitis	0
4	Male	Allergic rhinitis	1.5+
5	Male	Bronchial asthma, allergic rhinitis	0
6	Male	Allergic rhinitis	0
7	Female	Allergic rhinitis	0
8	Male	Allergic rhinitis	0
9	Male	Bronchial asthma, allergic rhinitis	0
10	Female	Allergic rhinitis	0
11	Male	Bronchial asthma	3.25+
12	Male	Bronchial asthma	0
13	Male	Bronchial asthma, allergic rhinitis	0
14	Female	Bronchial asthma, allergic rhinitis, urticaria	2.25+

Figure 2. Specific IgE reactivity to rDer f 29. A. The information of 14 allergic patients and the skin test results of rDer f 29. B. Immunobloting analysis of specific IgE reactivity to allergen rDer f 29 in the serum from rDer f 29 positive individuals. Lanes 1-3 indicated as P, serum from allergic subjects. Lane 4 and 5 indicated as C, serum from healthy subjects as negative controls. C. The specific IgE reactivity to allergen rDer f 29 by ELISA. P1-P3, the serum from rDer f 29 positive patients; C1 and C2, the serum from healthy subjects.
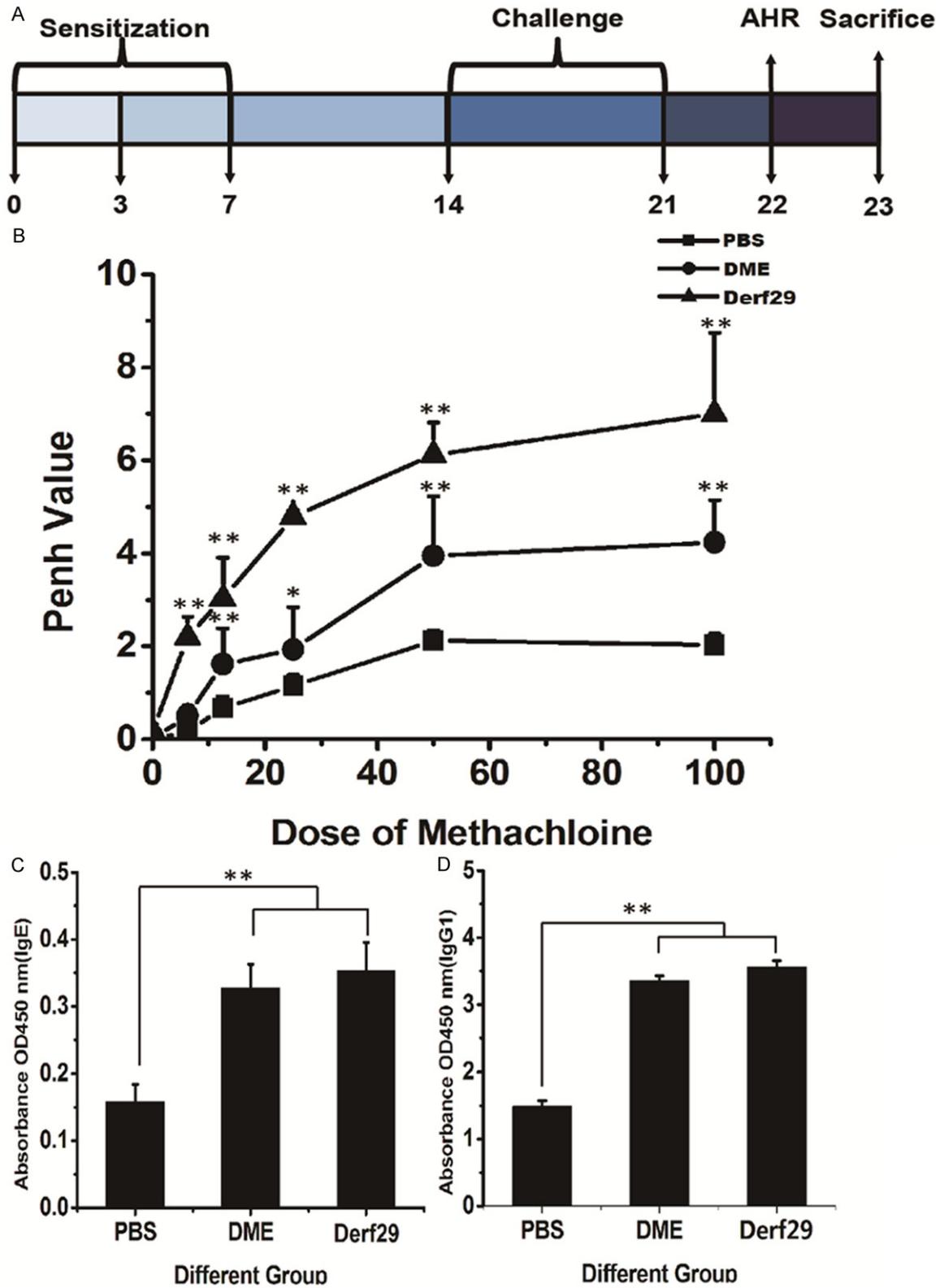
Figure 3. Establishment of mouse model of allergic asthma by induction with rDer f 29. A. Protocol of mouse model of allergic asthma. B. Airway hyper-responsiveness was measured using the unrestrained whole-body plethysmography. The Penh values were recorded when the mice were stimulated with the increasing concentration of methacholine after the challenge. C, D. The level of IgE and IgG1 were detected by ELISA. The serum was collected on day 23, and the levels of specific serum IgG1 and IgE were measured.
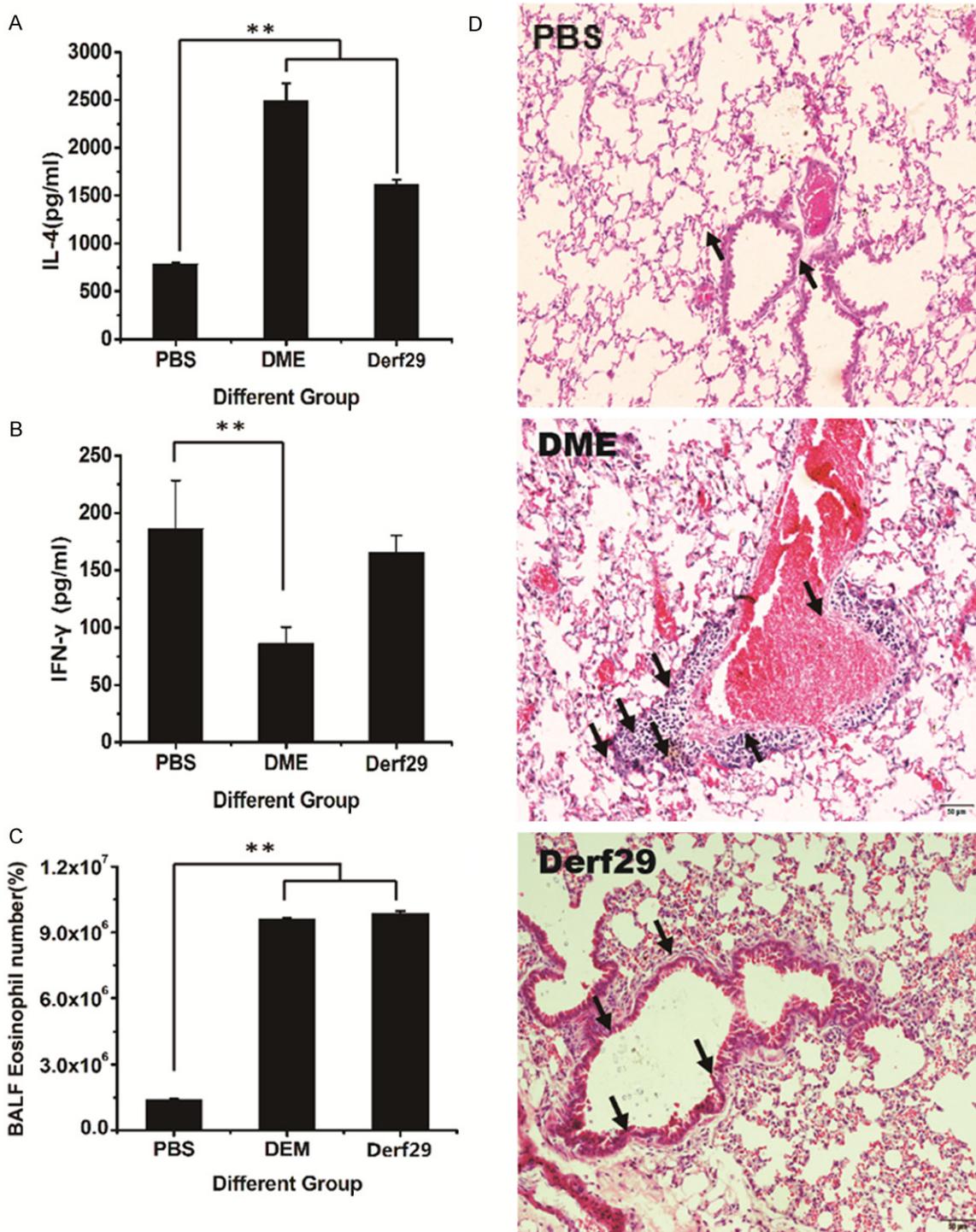
Figure 4. Effect of rDer f 29 on lung tissue inflammation. A, B. The level of IL-4 and INF-γ in BLAF were detected by ELISA. Samples of BLAF were collected on day 23, and levels of IL-4 and INF-γ were measured by ELISA. C. The number of eosinophil cell was counted in the BLAF. D. Light microscopy revealed the inflammatory cells (arrows) of the lung tissue.

was lower than that in HDM group,the specific antibody levels of IgE and IgG1 were detected in the serum (**Figure 3C**), which were signifi-

cantly higher than that in the normal control group. These results suggested that just like the crude extracts of dust mites, rDer f 29
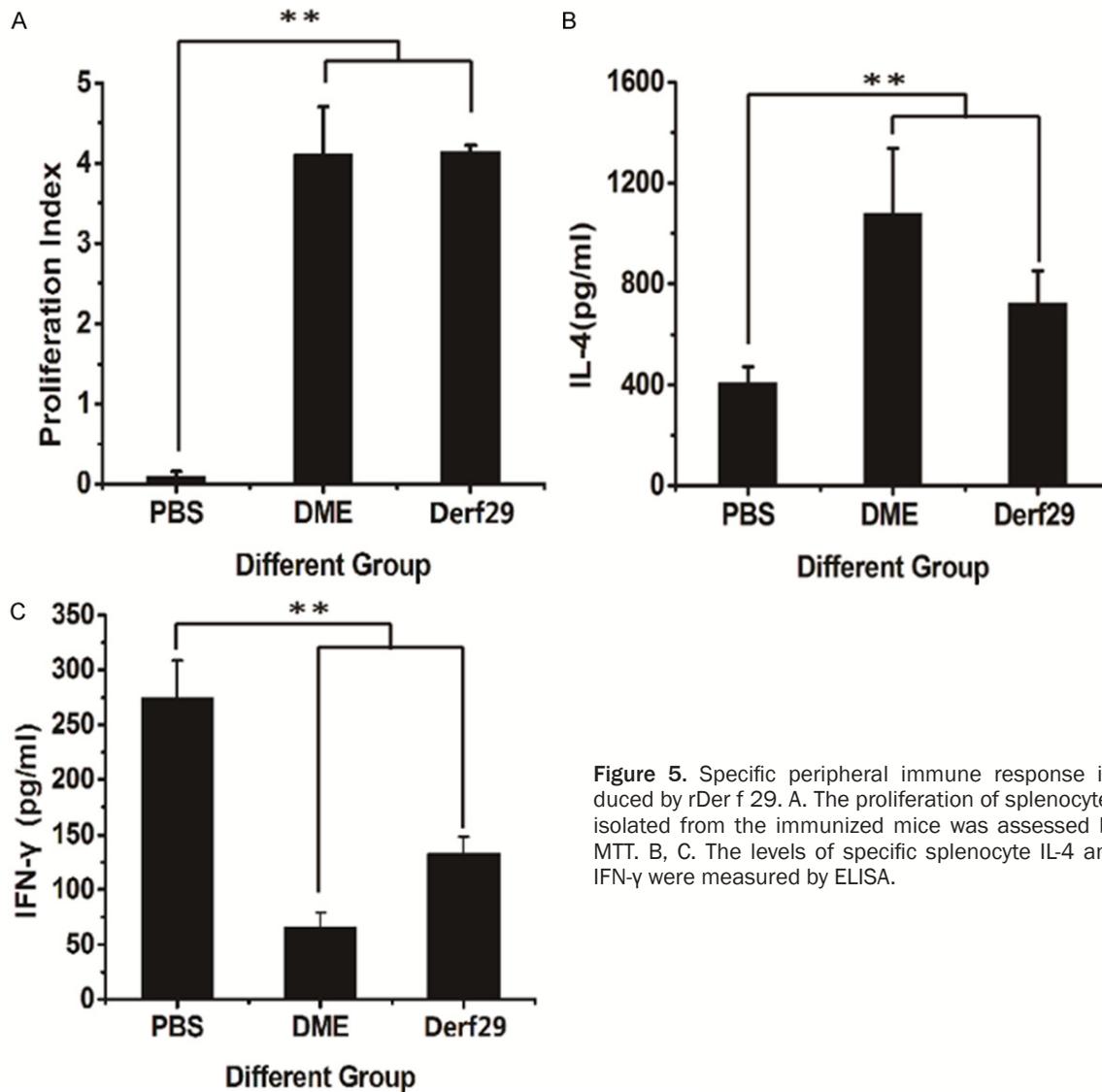
Figure 5. Specific peripheral immune response induced by rDer f 29. A. The proliferation of splenocytes isolated from the immunized mice was assessed by MTT. B, C. The levels of specific splenocyte IL-4 and IFN-γ were measured by ELISA.

could also stimulate the antigen specific IgE and IgG1 in mice.

rDer f 29 induced lung inflammation in mice

After sensitization, compared with control group, the levels of IL-4 in bronchoalveolar lavage (BAL) of HDM group and rDer f 29 group were increased significantly (**Figure 4A**). However, the level of IFN-γ in rDer f 29 group-showed no significant difference compared with the control group (**Figure 4B**). The total cells and eosinophils in BAL were counted with Wright Giemsa staining under optical microscope. The proportion of eosinophils in HDM group and rDer f 29 groups was higher than that in naive control group (**Figure 4C**). The

result was further confirmed by the histopathologic examination. In HDM group and rDer f 29 group, a large quantity of exudates was observed in the mice airway lumen; profound inflammatory cell infiltration was also observed in the lung tissue (**Figure 4D**). The results suggested that rDer f 29 can induce pulmonary inflammations.

Specific peripheral immune response induced by rDer f 29

To characterize the specific cellular immune response, splenocytes were isolated and stimulated with rDer f 29 in the culture. An increase of splenocyte proliferation was detected in the cells isolated from the rDer f 29-treated mice
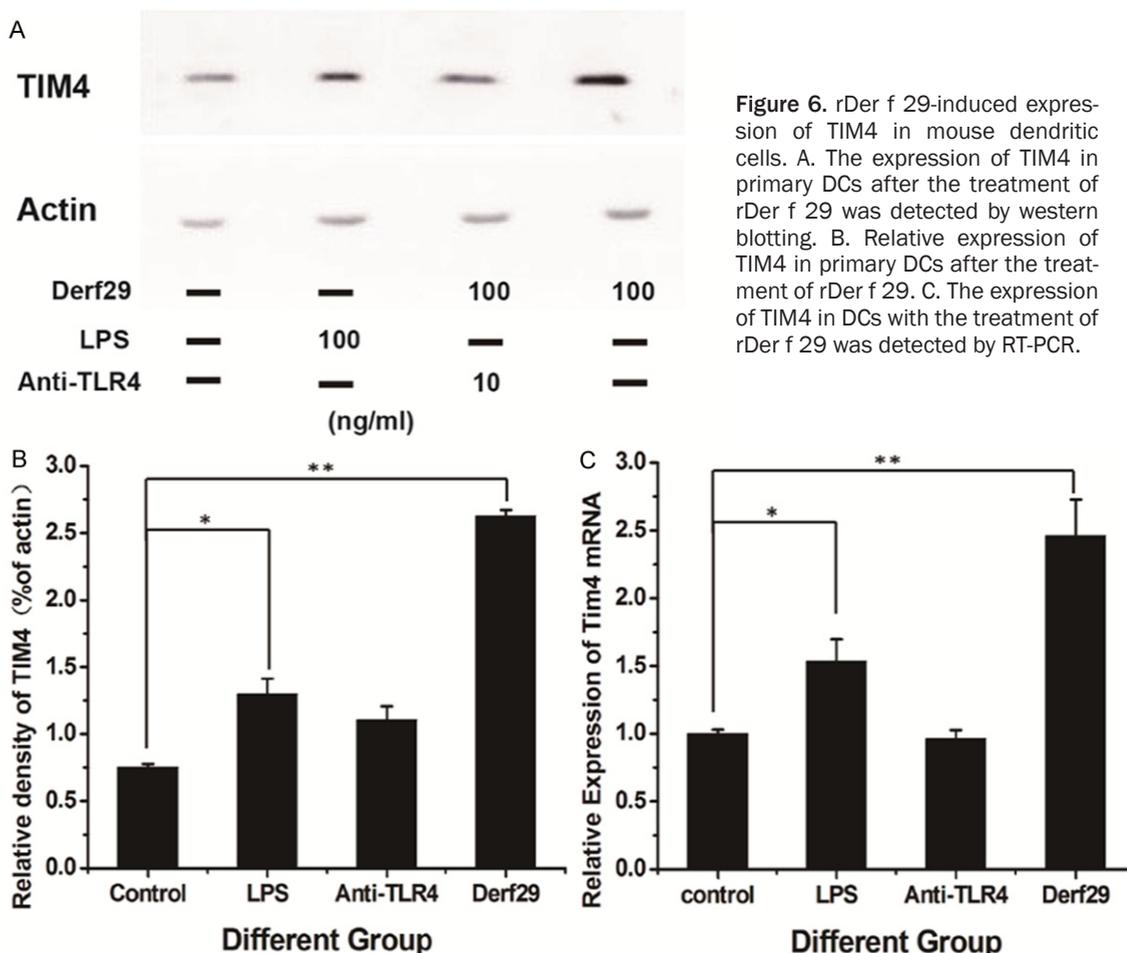
Figure 6. rDer f 29-induced expression of TIM4 in mouse dendritic cells. A. The expression of TIM4 in primary DCs after the treatment of rDer f 29 was detected by western blotting. B. Relative expression of TIM4 in primary DCs after the treatment of rDer f 29. C. The expression of TIM4 in DCs with the treatment of rDer f 29 was detected by RT-PCR.

after exposure to rDer f 29 in the culture (**Figure 5A**). It also induced a significant increase in IL-4 and IFN-γ in the culture medium (**Figure 5B** and **5C**).

rDer f 29 induced expression of TIM4 in mouse dendritic cells

Many studies have shown that the costimulatory molecule TIM4 play a critical role in inducing Th2 polarization in allergic disease. In this study, primary dendritic cells (DCs) were obtained from bone marrow mononuclear cells in mice treated with 20 ng/ml GM-CSF and 10 ng/ml IL-4. As shown in Supplementary Figure 1, the purity of primary DCs was over 80%. The result showed that the stimulation of rDer f 29 could enhance the expression of TIM4 in DCs compared with stimulated with LPS, while the costimulation of rDer f 29 and TLR4 blocking antibody suppressed its' expression (**Figure 6A** and **6B**). This result was also confirmed by RT-PCR (**Figure 6C**). The finding suggested that

rDer f 29 might modulate the expression of TIM4 in DCs.

Discussion

D. farinae is a globally important source of inhalant and contact allergen. There are more than 30 allergens have been identified from dust mite [2]. The relationship between house dust and HDM in bronchial asthmatics has been demonstrated in many studies [8]. Although specific immunotherapy with HDM extracts is somewhat effective at present, allergen extracts have not been fully standardized and severe side-effects occur sometimes in the course of treatment with the allergen crude extracts [9]. The investigation of molecular characterization of various proteins from HDM has provided new insight and strategies for the development of vaccines that could be used in the specific immunotherapy. Profilin, named Der f 29 is a pan-allergen from different sources including pollen and food but has not been

reported as an allergen in HDM [10, 11]. In this study, through cloning, expression, and purification of full length recombinant Der f 29 (rDer f 29), we characterized Der f 29; the results showed that Der f 29 was also an allergen in *D. farinae*.

Th2 polarization is typical change in allergic asthma [12]. However, the mechanism of allergy by which stimulates naïve CD4 T cells shift to Th2 cells and leads to the imbalance between Th1 cells and Th2 cells remains unclear. Recent studies showed that the expression level of TIM4 on DCs was closely related to the allergic pathogenesis [13]. The interaction of TIM1-TIM4 can produce Th2 polarization [14]. Liu T et al have proved that SEB-induced TIM4 enhancement in human DCs [15]. Food antigen peanut also increased the expression of TIM4 in DCs, which played a key role in the initiation of Th2 polarization and allergy in the intestine [16]. Blag7, as a panallergen like profilin from cockroach, promoted the expression of TIM4 in DCs and led to the Th2 polarization [17]. Consistent with the previous studies, the results in this study showed Der f 29 enhanced the expression TIM4 expression in DC cells. Taken together, Der f 29 as a minor allergen from *D. farinae* may induce Th2 polarization through the enhancement of TIM4 expression on DCs.

The current work has characterized a novel type of allergen, Der f 29, in *D. farinae*, which may be used for HDM allergy diagnosis and therapy, especially for patients without response to HDM major allergens and provide more insights to understand the diversity, characterization and allergy mechanism of *D. farinae* allergens.

Disclosure of conflict of interest

None.

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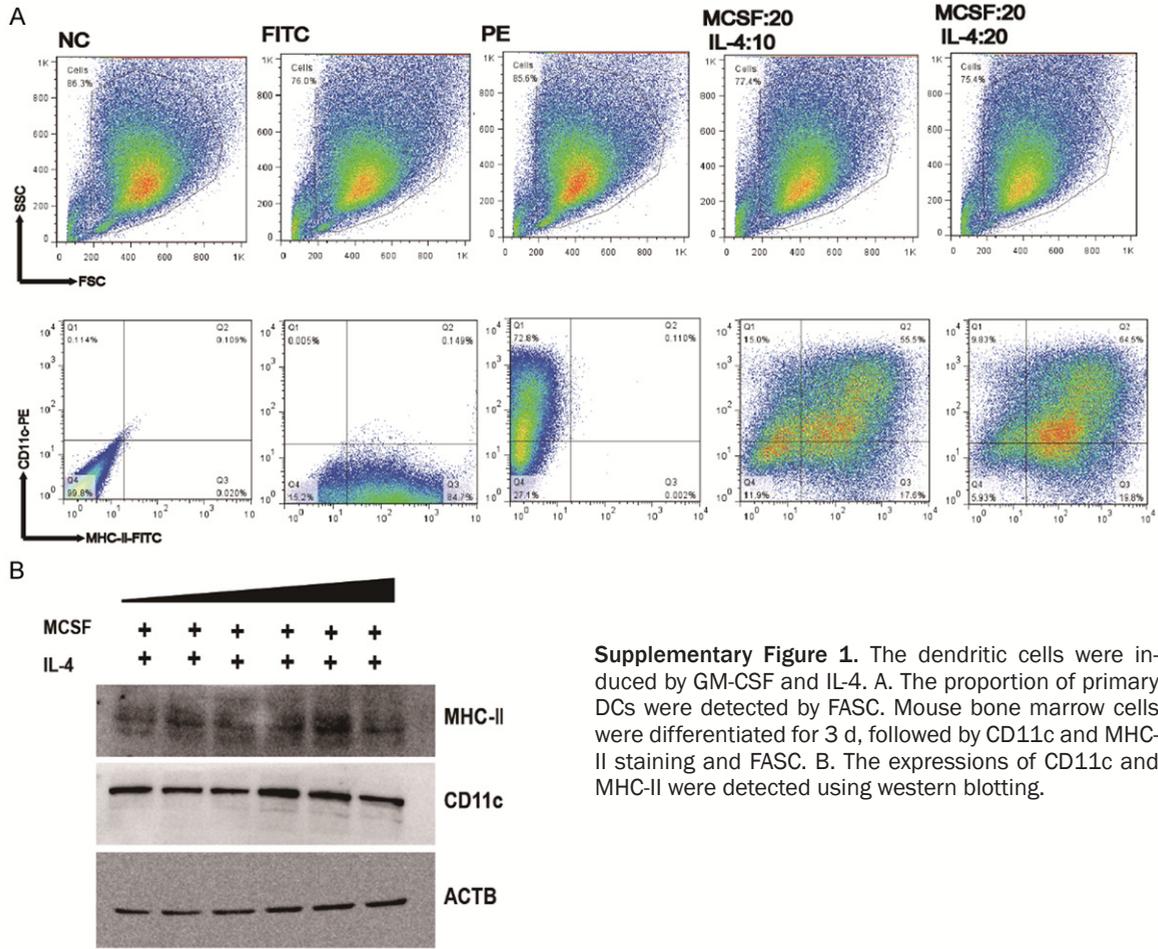
Supplementary Figure 1. The dendritic cells were induced by GM-CSF and IL-4. A. The proportion of primary DCs were detected by FASC. Mouse bone marrow cells were differentiated for 3 d, followed by CD11c and MHC-II staining and FASC. B. The expressions of CD11c and MHC-II were detected using western blotting.