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Research Paper

Identification of a novel cofilin-related molecule (Der f 31) as an allergen from *Dermatophagoides farinae*

Jianli Lin^{a,c,1}, Nana Huang^{b,1}, Hui Wang^{b,1}, Qinghui Fu^b, Eryi Wang^b, Ping Li^b, Liteng Yang^c,
Xinping Luo^b, Xiaoyu Liu^{b,*}, Zhigang Liu^{a,b,*}

^a State Key Laboratory of Respiratory Disease, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, China

^b State Key Laboratory of Respiratory Disease for Allergy at Shengzhen University, School of Medicine, Shenzhen University, 3688 Nanhai Ave, Shenzhen 518060, Guangdong, China

^c The Affiliated Luohu Hospital of Shenzhen University, Shenzhen Luohu Hospital Group, Shenzhen 518000, China

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ABSTRACT

House dust mite (HDM) allergen is a major cause of allergic disease. In this study, two-dimensional immunoblot and Matrix-Assisted Laser Desorption Ionization tandem Time-of-flight mass spectrometry (MALDI-TOF-MS) were used to identify Der f 31. After Der f 31 was cloned, expressed and purified, skin prick test (SPT), Immune inhibitory assays, Western blot, ELISA and asthmatic mouse model were employed to examine the allergenicity of recombinant Der f 31. The gene of Der f 31 includes 447 bps, and encoded 148 amino acids. Positive responses of SPT to r-Der f 31 were 32.5% in 43 HDM-allergic patients. r-Der f 31 can induce allergic pulmonary inflammation in the mouse model. In conclusion, Der f 31 is a novel subtype of dust mite allergens.

1. Introduction

House dust mite (HDM) is considered to be the most important cause of allergic disease such as allergic rhinitis (Vrtala et al., 2014; An et al., 2013). Currently, specific immunotherapy (SIT) utilizing house dust mite crude extracts is considered to be the only therapy for the mite allergic disease (Raulf et al., 2015). However, the use of allergen extracts are often responsible for poor efficacy and sideeffects, which is one of the major bottlenecks of SIT (Valenta et al., 2011). Purified recombinant allergens have been recognized as to an alternative source of allergens, which may open possibilities for new treatment strategies.

Up to 30 sub-allergens have been recognized and characterized in *D. farinae* (Thomas et al., 2004; Su et al., 2013). Group 1 and group 2 allergens of mites are the major allergens that bind over 80% of the IgE antibody (Thomas et al., 2004; Veen et al., 2001; Trombone Aptobias et al., 2010; Meyer et al., 1994). However, about 20% of patients have no specific IgE to the two group allergens (Thomas et al., 2004). Therefore, to identify new allergens is benefit to diagnose and management of allergic diseases.

In this work, we identified a new subtype of allergens from *D. farinae*—Der f 31 by a procedure of proteomics combined with two-dimensional immunoblotting, and its allergenicity was evaluated in mice.

2. Materials and methods

2.1. *D. farinae* culture and extracts preparation

According to Ting-Fung Chan's method (Chan et al., 2015), dust mites were culture at 25 °C with 70% relative humidity. Mites were isolated from the medium by using a modified heat-escape method. Purity of the cultured dust mites was assessed by checking mite morphology. Dust mite bodies were washed with 1 × PBS and homogenized in liquid nitrogen, Weigh 2 g sample adding 1 ml lysate (9 M urea, 4% CHAPS, 60 mM DTT, 2% IPG buffer) and centrifuged at 15,000 rpm for 50 min under refrigeration. The supernatant was termed DME (dust mite extract) and lyophilized.

2.2. Allergenomics analysis of dust mite allergens

Inorder to achieve a more comprehensive characterization of the dust mite allergens, allergenomic was conducted in our study. The methods were the same with our previous research (Chan et al., 2015). Briefly, Whole dust mite proteins were separated by 2-dimensional (2-D) PAGE. Then, IgE-binding proteins were detected by immunoblotting with serum from patients allergic to HDM (house dust mite). After

* Corresponding authors at: State Key Laboratory of Respiratory Disease for Allergy at Shengzhen University, School of Medicine, Shenzhen University, 3688 Nanhai Ave, Shenzhen 518060, Guangdong, China

E-mail addresses: lx0901@163.com (X. Liu), lzg195910@126.com (Z. Liu).

¹ These authors equally contributed to this work.

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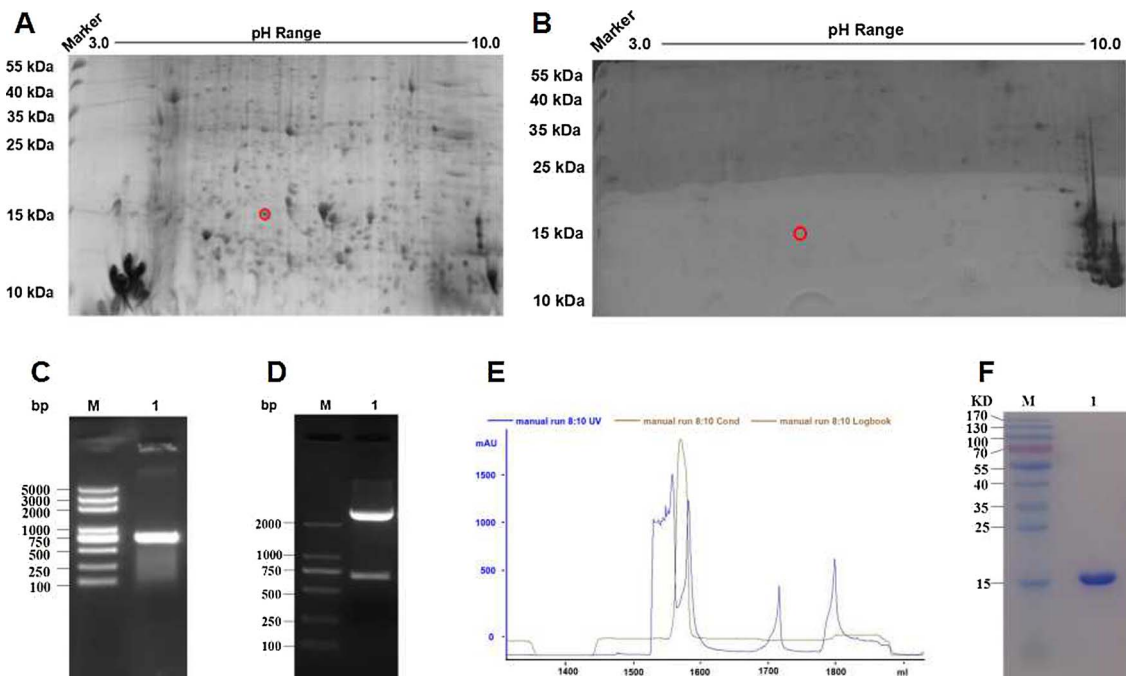


Fig. 1. Proteomics discovery, cloning and expression of cofilin.

(A) Two-dimensional PAGE demonstrating (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) IgE-immunopositive spots (red circles).
 (B) Corresponding IgE-immunoblot image.
 (C) Cloning and PCR of Der f 31 cDNA (M: DNA Marker; 1: Der f 31 gene).
 (D) Restriction enzymatic digestion results of recombinant plasmid (M: DNA Marker; 1: Der f 31 DNA).
 (E) Recombinant Der f 31 was purified by affinity chromatography.
 (F) SDS-PAGE analysis of the final purified r-Der f 31. Lane M: Protein Marker. Lane 1: purified r-Der f 31.

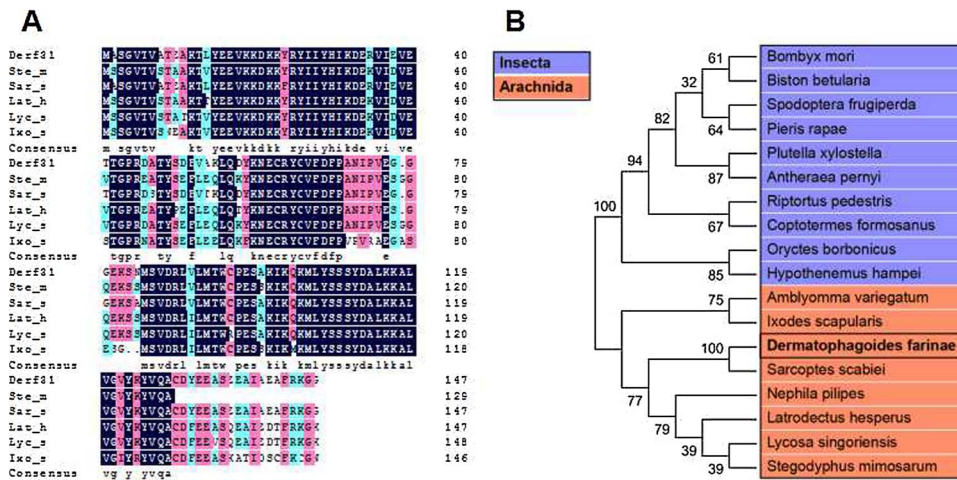


Fig. 2. The sequence alignment and evolutionary tree analysis of Der f 31.

(A) Sequence alignment of Der f 31 from different sources. Ste m (*Stegodyphus mimosarum*), Sar s (*Sarcoptes scabiei*), Lat h (*Latrodectus hesperus*), Lyc s (*Lycosa singoriensis*), Ixo s (*Ixodes scapularis*).
 (B) Evolutionary tree of *D. farinae* Der f 31 with 17 other arthropod species.

tryptic digestion, the peptides of IgE-binding proteins were analyzed by Matrix-Assisted Laser Desorption Ionization tandem Time-of-flight mass spectrometry (MALDI-TOF-MS). Database searches were used to identify allergens in dust mite.

2.3. Cloning, expression and purification r-Der f 31

The methods were the same with our previous research (Lin et al., 2015a, 2015b). Briefly, Total RNA was isolated from the homogenate with Trizol (Invitrogen) following the manufacturer's instructions and reverse transcribed (TaKaRa). The cDNA fragment of Der f 31 was amplified with Taq DNA polymerase (TaKaRa). The full-length Der f 31 cDNA obtained was subcloned into expression plasmid vector pET-28a and expressed in *E. coli* BL21 cells. The individual positive colonies

were selected by LB medium with Kanamycin (50 µg/ml) at 37 °C, and then cultured in LB medium with Kanamycin at 37 °C. The LB medium were added isopropyl-β-D –thiogalactopyranoside (IPTG, 1 µM) to induce the expression of Der f 31 for 4 h. Finally the recombinant Der f 31 was purified by affinity chromatography.

2.4. Skin prick tests and serum samples

SPTs with purified and endotoxin removed r-Der f 31 was dissolved in Ph7.4 phosphate buffer (50 mM PB, 100 mM NaCl). Glycerin was added to 50% final concentration. The Sample concentration was 0.01 mg/ml controlled with both positive histamine phosphate (0.1%) and negative (saline). The “average diameter method” was used to characterize the wheal size 20 min after SPT. The judgments of the

Table 1
The results of SPT.

Subject	Gender/Age	Diagnosis	Net wheal size(mm), Level			
			DME	Histamine	PS	r-Der f 31
1	Male/11	BA, AR	9, + + +	6	0	1.5, +
2	Female/50	AR	2.25, +	5	0	0, -
3	Female/25	AR	6.75, + + +	3.75	0	0
4	Female/40	BA, AR	2, -	5.5	2	1.5, +
5	Male/16	AR	2, +	6.5	0	0, -
6	Male/43	BA, AR	6, + +	6	0	0, -
7	Male/26	AR	5, + +	6	0	0, -
8	Female/32	AR	1.75, +	6	0	0, -
9	Male/12	AR	4.5, + +	4.75	0	2.0, +
10	Male/46	BA, AR	4.5, + +	8.5	0	0, -
11	Female/17	AR	4.5, + + +	4.5	0	0
12	Male/45	BA	3, +	7	0	2.5, +
13	Male/19	BA	0.5, +	1	0	0
14	Male/38	BA, AR	6, + +	7.5	0	2.75, +
15	Male/8	BA, AR, FA	10, + + +	7	0	0
16	Female/19	BA, AR, AD, DA	1, +	6.5	0	0
17	Female/47	BA, AR, FA, DA	4.5/8, + +	8	0	0
18	Female/37	AR	2.5, + +	5	0	0
19	Female/33	AR	1.5, +	5.75	0	1.5, +
20	Male/24	BA, AR	2, +	5	0	0
21	Male/33	BA	10, + + +	6.5	0	2, +
22	Male/36	AR	7.5, + + +	5	0	0
23	Female/43	BA	3.5, + +	5	0	0
24	Female/51	BA, AR	2.5, + +	4.5	0	0
25	Female/18	BA, AR, FA, DA	3.5, + + +	2	0	0
26	Female/42	BA, AR, FA, DA	3.5, + +	5	0	1.5, +
27	Female/47	BA, AR, DA	3, + +	6	0	0
28	Male/48	BA, AR	5.5, + + +	4.5	0	2, +
29	Male/15	BA, AR	6, + +	7.75	0	1.5, +
30	Female/39	AR	12.5, + + +	8.5	0	0
31	Male/63	BA, DA	8.5, + + +	8	0	0
32	Male/15	BA, AR, FA	6.5, + + +	6	0	1.5, +
33	Male/12	BA, AR	11, + + +	6	0	0
34	Female/18	BA, AR	5.5, + + +	4.5	0	2.25, + +
35	Female/25	AR	6.5, + + +	6.5	0	0
36	Female/40	BA, AR	6.5, + + +	5	0	0
37	Male/75	BA, AR, FA	2.5, +	6.5	0	0
38	Male/11	BA, AR, FA	6.5, + + +	5.5	0	0
39	Female/26	BA	1, +	5.5	0	0
40	Male/15	BA, AR, AD	7, + +	8	0	0
41	Female/14	BA, AR	6.5, + + +	6.5	0	1.5, +
42	Male/8	BA, FA	1, +	4.5	0	0
43	Female/43	BA	2, +	4.5	0	2.5, + +

AD (Atopic dermatitis); AR (Allergic rhinitis); BA (Bronchial asthma); FA (Food allergy); DA (Drug allergy).

result were the same with our previous research (Lin et al., 2016).

2.5. Specific IgE reactivity to Der f 31

The proteins were subjected to SDS-PAGE (sodium dodecyl sulfate polyacryl-amide gel electrophoresis) and electro-transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) for immunoblotting analysis. The membrane was blocked for 2 h with 3% BSA (Bovine Serum Albumin) in 37 °C, incubated with the sera from patients sensitized to dust mite, diluted in 5% BSA-PBST (PBST: PBS containing 0.05% Tween 20) for an hour under constant agitation on a rotary shaker in room temperature. After washing three times with TBS-T (Tris-buffered saline containing triton X-100), the membrane was incubated with the secondary antibodies(mouse anti human IgE) for an hour. In the end, after washing with TBS-T for three times, the membrane was developed with DAB kit (Invitrogen, USA). Stop developing reaction with ddH₂O.

2.6. Enzyme-linked immunosorbent assay (ELISA) and ELISA inhibition

Antibodies specific for purified allergens were tested by ELISA, the methods were the same with our previous research. Briefly, the ELISA microtiter plates were coated with purified allergen at 4 °C with at 100 ng/well in Carbonate buffered solution (CBS, 15 mM Na₂CO₃ and 35 mM NaHCO₃, pH9.5) over night. Then blocked with 200 µl 3% bovine serum albumin (BSA) in PBS at 37 °C for 2 h. The serum (1:5 diluted with 3% BSA-PBS) or BSA (using as a negative control) were then added to each well (100 µl/well) and incubated for one hour at 37 °C. After IgE binding, the plates were incubated with peroxidase-labeled goat anti-human IgE (1:2000) for an hour at 37 °C. Each incubating step was followed by 3 washes with PBST. TMB (tetramethylbenzidine, 100uL/well) was used as the substrate and the reaction was stopped by 50 µl 2 M H₂SO₄. The plates were read by an ELx808 absorbance microplate reader (BioTek, Shanghai, China) at 450 nm. For the inhibition assay, serum allergic to r-Der f 31 were used for inhibition experiment to detect the cross reaction among r-Der f 31 and DME. Patients sera (1:5) were pre-incubated at 4 °C with purified r-Der f 31 or DME (final concentration: 0.0001, 0.001, 0.01, 0.1, 1 g/ml) overnight, and then were added to the microtiter platespre-coated with r-Der f 31 or DME (0.1 µg/well). Data from 3 measurements were accumulated. The inhibition rates were calculated according to the following formula: inhibition (%) = (OD₀-OD_{inhibitor})/(OD₀-OD_{BSA}), where OD₀ is the optical density of antigen without any inhibitor, OD_{inhibitor} is the optical density after adding an inhibitor and OD_{BSA} is the optical density with only BSA in the plate.

2.7. Induction of allergic airways disease

Female BALB/c mice (6–8-week old) were obtained from Guangdong Experimental Animal Center (Guangzhou, China). The experimental procedures were approved by the Ethics Committee at Shenzhen University (Shenzhen, China). Mice were exposed to 100 g of DME or r-Der f 31 with 4 mg aluminum hydroxide as adjuvants, or PBS on days 0, 3, and 7 for sensitization. Mice were then challenged via nostril with 50 g of DME or r-Der f 31 once a day for a week. On day 22, airway hyperresponsiveness (AHR) in response to increasing concentration (0, 6.25, 12.5, 25, 50, 100 mg/ml) of methacholine. Bronchoalveolar lavage (BAL) and cell counts were performed as published procedures (Lee et al., 2013). Cytokine levels in BALF (bronchoalveolar lavage fluid) were measured by ELISA with commercial reagent kits (Ebioscience) following the manufacturer's instructions. The lung tissue were fixed with 4% paraformaldehyde. After dehydration, lung tissue was embedded in paraffin. The tissues were cut into 4 µm thick sections and stained with Hematoxylin and Eosin (HE), and observed under a light microscope.

2.8. Data statistics

All data were expressed as means ± SD and processed with Graphpad software, and the T-test was used for the mean differences between two groups. *, P < 0.05. **, P < 0.01.

3. Results

3.1. Potential allergen Der f 31 is identified by allergenomics

Two-dimensional electrophoresis was used to characterize the proteins in the dust mite extracts. After 2-DE gels were blotted and incubated with the pooled sera, we found a new allergen from *D. farinae* (Fig. 1A, B). Subsequently, its gene sequence was identified by MALDI-TOF mass spectrometry (MS) analysis. The new allergen belonged to Cofilin family, encoding a 148 amino acid polypeptide, and named Der

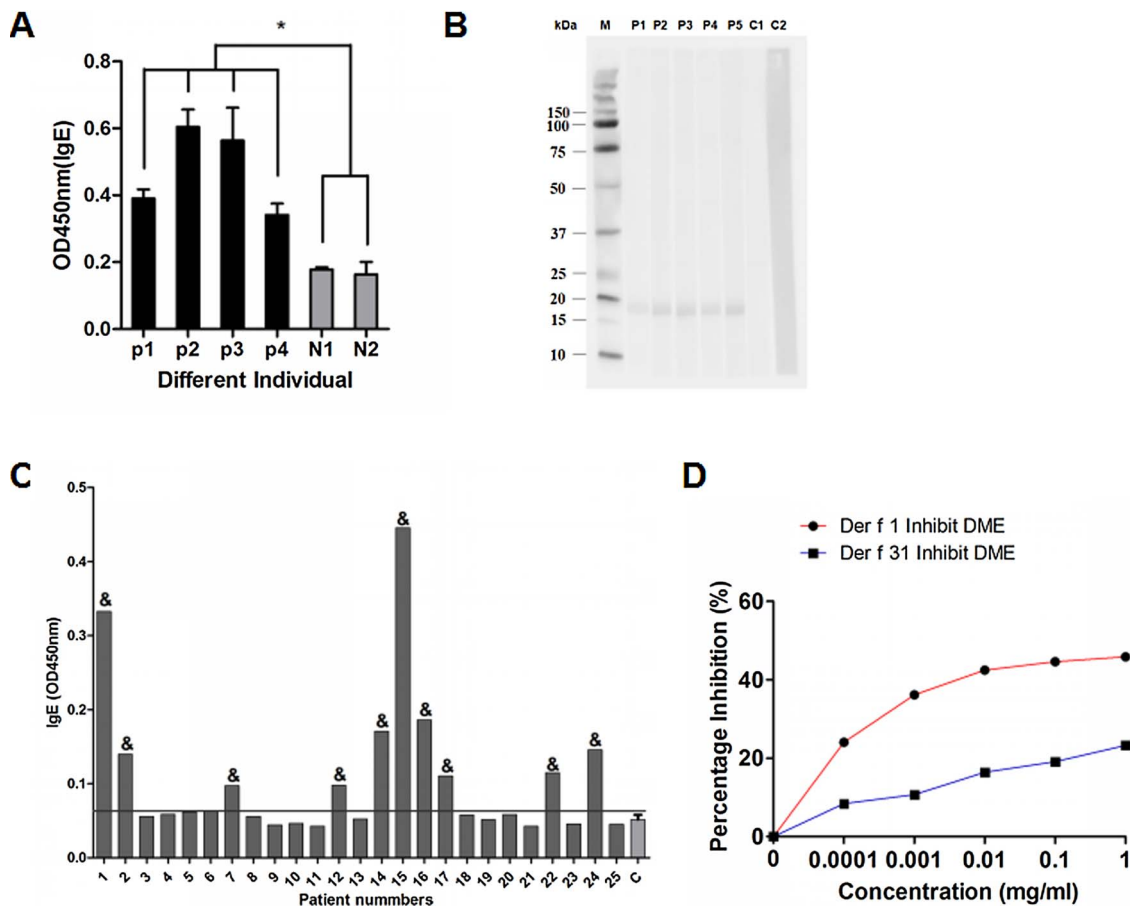


Fig. 3. Characterization of *Dermatophagoides farinae* Der f 31.

(A) Evaluation of sIgE reactivity to r-Der f 31. P1-P4, the serum from Der f 31 SPT positive patients; N1 and N2, the serum from healthy subjects.

(B) The Western blots indicate that the r-Der f 31 binds to IgE in the serum from allergic subjects, Lanes P1-P5: serum from Der f 31 SPT positive patients. Lane C1 and C2: serum from healthy subjects.

(C) ELISA was used to determine the IgE reactivity of Der f 31 to sera collected from HDM sensitive allergic patients ($n = 25$, total IgE > 100 IU/ml). mite-insensitive healthy donors ($n = 5$) were also detected as a negative control. Ten of them (40%) were considered Der f 31-positive.

(D) r-Der f 31 inhibited the mite-allergic patients' serum IgE binding to DME.

f 31 (KM009993). The amplification PCR products showed a bright band at around 450 bp by agarose gel electrophoresis (Fig. 1C) and double enzymatic digestion (Fig. 1D). To obtain r-Der f 31 protein for the subsequent experiments, Der f 31 was cloned and expressed (Fig. 1E). The results showed that His-tag Der f 31 were successfully expressed by induction with IPTG (Fig. 1F). Compared with other species, we found the amino acid sequence homology of Der f 31 were 87%, 97%, 84%, 84% and 74% with *Stegodyphus mimosarum* coflin (NCBI accession number KFM71703.1), *Sarcoptes scabiei* coflin (NCBI accession number KPM08623.1), *Latrodectus hesperus* coflin (NCBI accession number ADV40203.1), *Lycosa singoriensis* coflin (NCBI accession number ABX75507.1) and *Ixodes scapularis* coflin (NCBI accession number XP_002435134.1), respectively (Fig. 2A). The MEGA5.1 was used to construct the molecular evolutionary tree (Fig. 2B).

3.2. Allergenicity assessment of Der f 31

We next determined the allergenicity of r-Der f 31. The results (Table 1) showed that fourteen of 43 (32.5%) dust mite allergic patients had positive reactions to r-Der f 31. The results of Elisa and immunoblotting indicated that serum IgE from Der f 31 SPT positive patients interacted with Der f 31 (Fig. 3A and B). Sera samples from 25 HDM allergic patients and from five HDM insensitive healthy donors were used to determine IgE reactivity of r-Der f 31, those OD values of the allergic sera were higher than the cut off ELISA value (mean ELISA value of healthy subjects $\pm 3SD$) were set as the IgE positive. As

shown in Fig. 3C, ten of them (40%) were considered Der f 31-positive. Elisa inhibition assays indicated there were IgE cross-reactivity between r-Der f 31 and DME (Fig. 3D). The results suggested that Der f 31 was an subtype of allergen in *D. farinae*.

3.3. Der f 31 induces allergic asthma in the mouse model

To test the role of Der f 31 in the induction of airway allergy, a Der f 31-induced asthmatic mouse model was developed in the present study (Fig. 4A). AHR (airway hyperresponsiveness) was tested 24 h after the last challenge. Mice sensitized with Der f 31 and HDM showed much higher Penh than the PBS group (Fig. 4B). The number of eosinophils in the BALF from mice exposed to Der f 31 were significantly increased compared with PBS groups (Fig. 4C). The histology of lung tissue from Der f 31 and HDM groups showed peribronchial inflammatory cell infiltration (Fig. 4D, E and F). The data suggested that Der f 31 can induce allergic pulmonary inflammation in the mouse model.

3.4. Der f 31 facilitates Th2 immune responses

As shown by Fig. 5, serum Der f 31 specific IgE and IgG1, the splenocytes culture supernatant and BALF Th2 cytokines IL-4 were significantly increased in mice immunized with Der f 31 than control mice, while the secretion of Th1 cytokine IFN- γ was suppressed in Der f 31 groups.

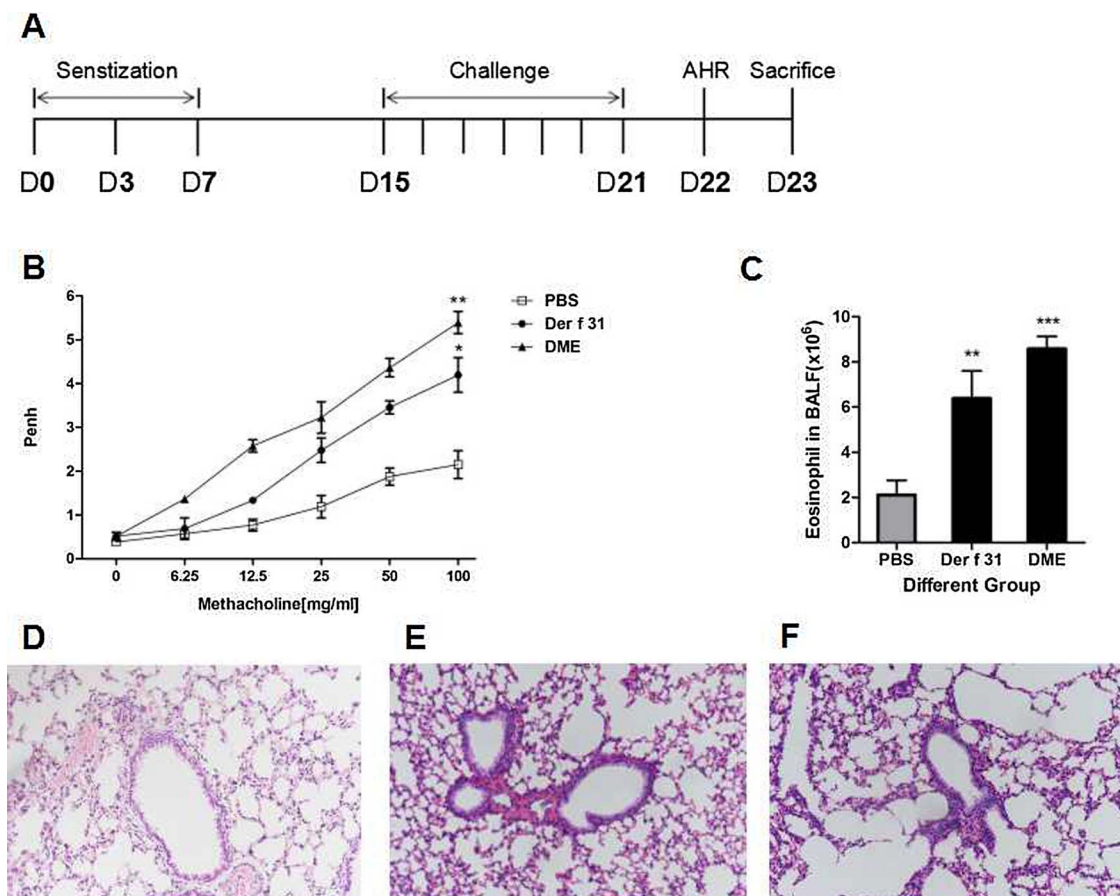


Fig. 4. Allergic airway inflammation induced by Der f 31.

(A) The protocol for mouse model of r-Der f 31-induced allergic asthma.

(B) Airway hyper-responsiveness were 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) The number of eosinophil cell was counted in the BLAF.

(D, E and F) The pathologic changes of pulmonary tissue. F: The PBS group. G: The Der f 31 group. H: The DME group.

4. Discussion

D. farinae is one of the most important perennial allergen sources worldwide (Chang et al., 2013). *D. farinae* produce a number of allergens, and its some allergens have been identified. However, HDM allergic patients have variable profiles of IgE reactivity, so distinct allergic responses were indicated to be in response to different allergens. Characterization of new allergens are important in the diagnosis and immunotherapy of mite-related diseases (Lin et al., 2015a, 2015b).

Using a procedure of allergenomics combined with high-resolution two-dimensional IgE immunodetection from *D. farinae* extracts, a new allergen-Der f 31 was identified. Through cloning, expression, and purification, we acquire a recombinant Der f 31.

The allergenicity of Der f 31 was evaluated by SPT. Fourteen of 43 (32.5%) dust mite allergic patients showed positive reaction to this new allergen, suggesting that Der f 31 is an allergen with moderate IgE-binding capacity against mites-related individuals. Immunoblotting and ELISA indicated that our rDer f 31 had IgE-binding capacity to serum IgE from allergic patients. Moreover, a well characterized asthmatic mouse model induced by r-Der f 31 was developed successfully. Exposure to Der f 31 can lead to a hypersensitivity reaction.

Th1/Th2 imbalance is the important reasons for allergic diseases (Jeon et al., 2015; Abrahamsson et al., 2011). Cytokines secreting by Th2 cells, such as IL-4, an important mediator of inflammatory cytokine derived from T helper cells which triggers IgE synthesis (Gour and Willskarp, 2015; Wynn, 2015). This study found that mice exposed to r-Der f 31 showed significantly higher levels of Th2 cytokine IL-4 and

specific IgE, which suggested that Der f 31 induce allergic airway inflammation via modulation of Th1/Th2 cytokines.

In summary, our current study demonstrates that Der f 31 is a new allergen from *D. farinae* and induces allergic airway inflammation by facilitating Th2 immune responses. This research will be useful for diagnosis and immunotherapy of HDM allergy.

Conflict of interest statements

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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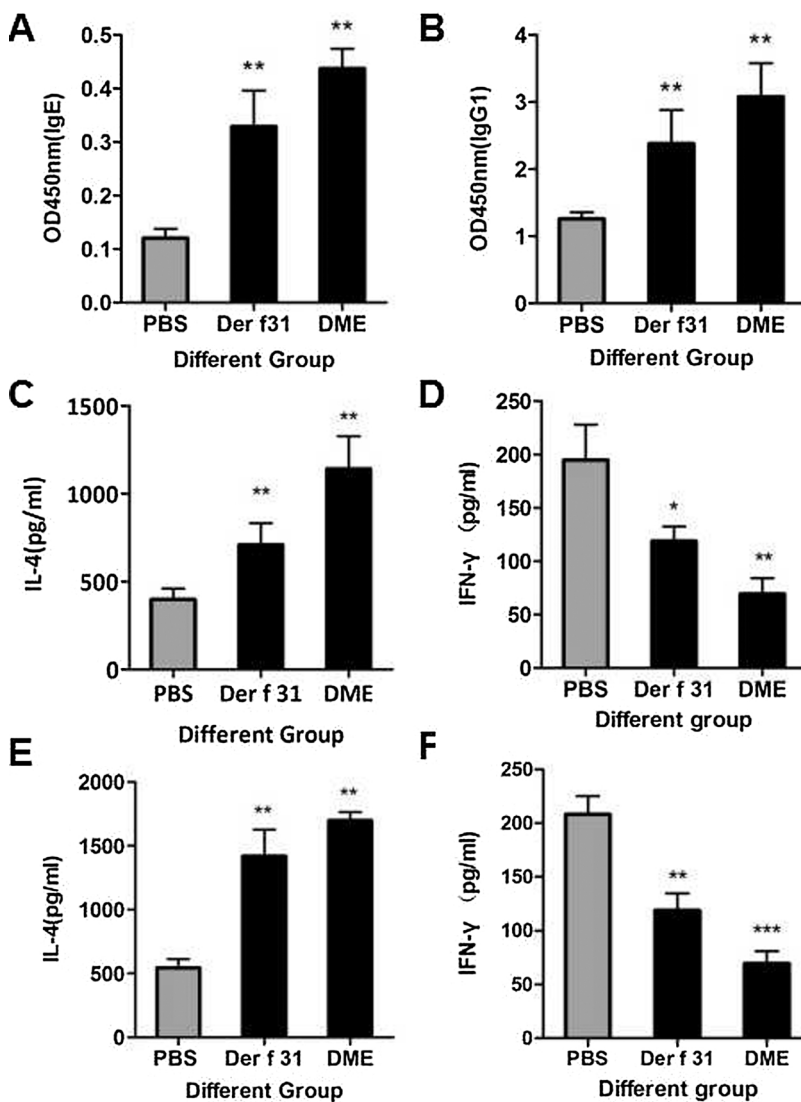


Fig. 5. The profiles of the allergen-specific cytokine response.

(A) and (B) Serum levels of allergen-specific IgE and IgG1 were measured by ELISA as optical density (OD).

(A) and (B) The level of IL-4 and INF-γ in BLAF were detected by EILSA.

(D, E) The levels of specific splenocyte IL-4 and IFN-γ were measured by EILSA.

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