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Original Article

Der f 21, a novel allergen from dermatophagoides farina

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Abstract: The Dermatophagoides farina (D. farina) allergens are an important factor contributing to allergic disease. To identify new allergens is important for diagnosis and treatment of allergic diseases. In this study, we sought to characterize the biological activity of Der f 21 of D. farina. The recombinant Der f 21 protein was characterized by western-blot, ELISA and Skin prick test using clinic patient's serum.An allergic asthma mouse model was established with the rDer f 21 as a specific antigen. The results showed that the sera from 28.9% in 38 dust mite allergic children displayed positive results in response to rDer f 21, and 42% in 98 dust mite allergic patients displayed positive response in skin prick test. In addition, Immune inhibition assays showed there was IgE cross-reactivity between rDer f 21 and rDer f 5. Moreover, an allergic asthma mouse model was established. Airway hyperresponsiveness, serum specific IgE, IgG1, eosinophil infiltration in the allergic mice, interleukin-4(IL-4) and interferon-γ (INF-γ) from spleen cells were markedly increased in the allergic mice. The results demonstrate that Der f 21 is a novel allergen.

Keywords: Allergen, Der f 21, biological activity, dust mite, airway

Introduction

Dust mites, especially Dermatophagoides farina and Dermatophagoides pteronyssinus are the main sources of allergens in airway allergy. About 20% of population in the world and more than half patients with allergic diseases are allergic to dust mites [1, 2]. Dust mites also have been described as the most common allergen in allergic asthma and allergic rhinitis [3, 4]. Published data indicate that about 59.0% and 57.6% patients sensitive to Dermatophagoides farina (Der f) and Dermatophagoides pteronyssinus (Der p) respectively. To date, at least 27 subtypes of dust mite allergens were listed in the International Union of Immunological Societies (IUIS) nomenclature data set (http://www.allergen.org), groups 1 and 2 are considered the major allergens of dust mites, which have been intensively studied [5-8]. In spite of the exact mechanism of antigen specific immunotherapy (SIT) is still unclear, utilizing crude dust mite extracts as vaccines is proposed as the only specific remedy for treatment of dust mite allergic diseases. Because of containing a large number of non-allergen substances in the dust mite crude extracts, side-effects occur often in the clinical practice of SIT. Therefore, recombinant allergens have been proposed to be a substitute for crude dust mite extracts in SIT.

Although the cloning, expression, and purification of rDer f 21 has been reported, the biological activity and the mechanism of Der f 21 have not been investigated [9]. It is necessary to characterize the biological activity and the mechanism of rDer f 21.

Materials and methods

Materials

Al(OH)3 and methacholine were purchased from Sigma-Aldrich. Biotin-labeled goat anti-mouse IgE and horseradish peroxidase (HPR)-labeled goat anti-mouse IgG1 were from eBioscience. Mouse interleukin (IL)-4, and interfer-

on gamma (IFN-γ) enzyme-linked immunosorbent assay (ELISA) kits were obtained from Bio-Legend.

Patients and animals

A written, informed consent was obtained from each human participant for the use of blood samples and skin prink test. The serum of 38 allergic children and 3 healthy subjects were recruited from Shenzhen Children's Hospital. 98 allergic patients were recruited form The First Affiliated Hospital of Guangzhou Medical University. The study was approved by the ethic Committee of the Institutional Review Board of the School of Medicine, Shenzhen University.

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 Ethic Committee at Shenzhen University.

Western blot and ELISA for analysis of IgE reactivity

Proteins were fractioned by 12% SDS-PAGE, followed by electro-transfer onto a PVDF membrane. Membranes were blocked with 5% (v/v) BSA-TBST for 2 h at room temperature (RT). The blots were incubated with the serum from dust mites allergic patients (diluted in 1:5 v/v in blocking buffer) overnight at 4°C. After washing, the membrane was incubated with a peroxidase-labeled streptavidin, and then the blots were visualized by diaminobenzidine (DAB) staining.

The 96 well plates were coated with 100 μl of rDer f 21 at a concentration of 1 μg/mL in carbonate buffer and stored at 4°C overnight with 3% BSA. The positive serum of 38 patients with dust mite allergy was used as primary antibodies (1:50 v/v). The plates were incubated at 37°C for 2 h. Then the plates were added with 100 ul of biotin labeled anti-human IgE (1:2000 v/v) and incubated at 37°C for 2 h. Next, 100 μL of peroxidase-labeled streptavidin (1:5000 v/v) were added after the plates were washed 5 times, and incubated at 37°C for 1 h. The plates were subjected to color development at 37°C for 10 minutes, and the reactions were terminated by adding 2 mol/L H_2SO_4. The absorbance at 450 nm were then measured on a microplate reader.

Skin prick test (SPT)

SPT of Der f 21 was done in patients allergic to dust mites by their physicians. The skin response was observed at 20 minutes after SPT; the results were defined as positive (+) when the prick spot became a wheal and fleck surrounding the wheal. 4+: the response was stronger than the histamine control; 3+: the response was almost the same as histamine control; 2+: the response was weaker than histamine, but stronger than negative control; 1+: the response was significantly weaker than histamine, but slightly stronger than the negative control; negative (-): No response. The positive responses were further confirmed by measuring D. farinae-specific IgE antibodies with the CAP System.

Der f 21, Der f 5 and crude extract inhibition assays

Serum allergic to both Der f 21 and Der f 5 were used for inhibition experiment to detect the cross reaction among rDer f 21, rDer f 5 and crude dust mite extracts. The pooled sera were premixed with different concentrations of rDer f 21, rDer f 5 or crude dust mite extracts. Serum was diluted at a ratio of 1:50, and incubated at 4°C overnight. The inhibition rate was calculated according to the absorbance value in accordance with the ELISA testprocedures.

Der f 21 monoclonal antibody preparation and its antigen localization

rDer f 21 was used as allergen to immune BALB/c mice. After fusion spleen cells of immunized mice with mouse myeloma NS-1 cell, the specific hybridoma was obtained through rapid screen by the combination of semisolid culture medium and limited dilution. The ascites were induced with hybridoma cell lines, and purified by affinity chromatography. Ig subtype of the monoclonal antibody was identified by Ig class and a subclass kit. Indirect ELISA was used for the identification of the characteristics and multi-disciplines of the monoclonal antibody.

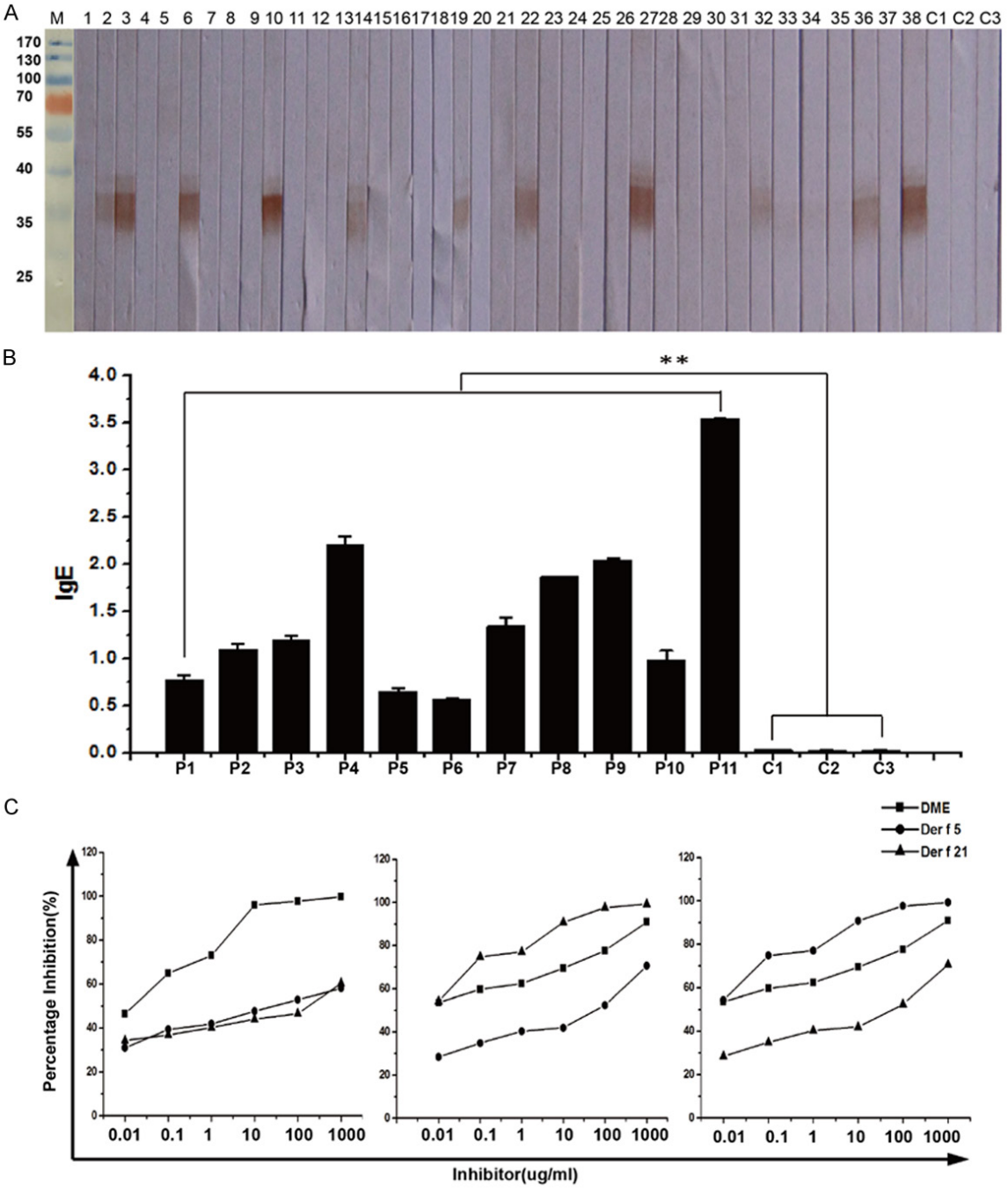

Figure 1. The allergic activity of rDer f 21. A. Immunoblotting analysis of specific IgE reactivity to allergen rDer f 21 in the serum from dust mite allergic children. B. The specific IgE reactivity to allergen rDer f 21 by ELISA. P1-P11, the serum from rDer f 21 positive allergic children; C1-C3, the serum from healthy children. C. IgE binding inhibition to extracts, rDer f 21, and rDer f 5. rDer f 21 (▲) , rDer f 5 (●) and extracts (■).

Immunohistochemistry

The tissue sections were used for the immunehistochemical analysis after dewax to hydration. The sections were incubated with 1% hydrogen peroxide to inactivate endogenous-peroxidases. After washing, the sections were incubated with normal sheep serum at RT for 20 minutes. The sections were added anti-Der f 21 mAb and incubated overnight at 4°C. The sections were incubated with biotinylated second antibodies for 1 h at room temperature;

Table 1. The serum of 38 allergic children

Patient Number	Sex/Age	Total IGE (KU/L)	Der f Specific IgE	Der p Specific IgE
1	Male/5.6	554.35	97.8	>100
2	Male/7.8	1119.26	>100	>100
3	Male/16	-	>100	>100
4	Male/10.5	1881.23	>100	>100
5	Female/7.8	-	>100	>100
6	Female/11.5	1764.36	>100	>100
7	Male/5.9	598.94	-	>100
8	Female/6.7	-	>100	>100
9	Female/7	1736.6	>100	>100
10	Male/9H	2562.03	>100	>100
11	Male/10.4	1037.3	>100	>100
12	Male/12.1	-	>100	>97.4
13	Male/7.7	-	86.9	>100
14	Female/11	735.9	-	99.2
15	Female/4.6	1159.08	>100	>100
16	Male/4.8	-	>100	>100
17	Male/6	25.5	>100	>100
18	Male/10	446.62	56.2	57.8
19	Male/13.7	-	80.8	59.9
20	Female/14	127.11	19.6	45.7
21	Male/7.7	-	>100	>100
22	Male/9.10	950.16	>100	>100
23	Male/6.9	-	-	>100
24	Male/6.7	1316.39	-	>100
25	Female/5.2	-	68.4	>100
26	Male/6.1	1501.28	-	>100
27	Male/7.6	-	>100	>100
28	Male/8	-	>100	>100
29	Male/5.1	-	>100	>100
30	Male/11	389.92	57.3	>100
31	Male/11.1	-	>100	>100
32	Male/5.11	707.82	-	>100
33	Male/5.10	697.21	>100	>100
34	Female/6.9	2133.8	>100	>100
35	Female/4	2326.31	>100	>100
36	Male/4.1	656.97	-	93.6
37	Female/9.9	196.9	-	35.9
38	Male/9	1819.93	>100	>100

and then incubated with streptavidin-peroxidase for 40 minutes after washing. Development was carried out by diaminobenzidine chromogen. Then the section was strained with hematoxylin. The sections were observed under an optical microscope. All of the incubation was operated in humidified box.

A mouse model of allergic asthma

Twenty-four mice were randomly divided into three groups: ① DME group, ② rDer f 21 group, ③ Control group. In DME group and rDer f 21 group, mice were subcutaneously injected with 50 μg crude dust mite extracts or 50 mg rDer f 21 with 2 mg aluminum hydroxide as adjuvants on day 0, 3 and 7, respectively. PBS was used in control group. In the following week, DME and rDer f 21 group mice were challenged daily with 50 μg crude dust mite extracts or rDer f 21 protein/mouse via intranastril drops, respectively, from day 9 to day 13. The control mice were treated with the saline instead of allergens in the same procedures. On day 13, following allergen challenge, airway hyperresponsiveness to methacholine was measured using the unrestrained 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

The levels of IgE in the serum were determined by ELISA. In brief, 100 ng purified rDer f 21 in 100 μL carbonate-biocarbonate buffer was incubated in a 96 wells 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 mol/L H_2SO_4. 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

Table 2. The serum of 98 allergic patient for skin prick test

Patient Number	Sex/Age	Diagnosis	Skin prick test	
			Extract	rDer f 21
1	Male/9	AR	2+	4+
2	Male/7	AR	3+	2+
3	Male/6	AR	3+	-
4	Male/20	AR/BA	3+	1+
5	Male/8	AR	1+	-
6	Female/40	AR	2+	-
7	Female/16	AR/BA	4+	2+
8	Female/28	AR	3+	-
9	Male/36	AR	2+	-
10	Female/17	AR	4+	-
11	Female/17	AR/BA	3+	3+
12	Female/13	AR/BA	3+	2+
13	Female/37	BA	1+	-
14	Female/34	AR/BA	2+	-
15	Female/19	AR	4+	-
16	Male/45	AR	3+	-
17	Male/23	AR	3+	3+
18	Male/43	AR	2+	-
19	Female/29	AR	3+	-
20	Male/45	AR/BA	3+	-
21	Male/23	AR	3+	4+
22	Female/30	AR/BA	3+	-
23	Male/4.5	AR	3+	-
24	Male/6	AR	1+	-
25	Female/33	AR/BA	3+	-
26	Male/18	AR/BA	3+	-
27	Male/10	AR	2+	3+
28	Male/8	AR/BA	3+	4+
29	Female/30	AR/BA	2+	-
30	Female/33	AR	3+	-
31	Male/37	AR	3+	-
32	Male/29	AR	1+	-
33	Male/9	BA	2+	-
34	Male/20	AR/BA	2+	2+
35	Male/32	AR/BA	2+	3+
36	Female/31	BA	1+	3+
37	Female/8	AR/BA	3+	1+
38	Male/13	AR/BA	4+	1+
39	Female/15	AR/BA	3+	-
40	Female/43	AR	3+	-
41	Female/7	Other disease	4+	-
42	Female/28	AR/BA	2+	2+
43	Female/5	AR	3+	-
44	Male/8	AR	2+	3+
45	Female/34	AR/BA	1+	-
46	Female/31	AR/BA	2+	-
47	Female/47	AR/BA	2+	-
48	Male/5	AR	2+	-
49	Female/47	AR	3+	-
50	Male/7	AR/BA	3+	-
51	Male/13	AR/BA	1+	-
52	Female/34	AR/BA	1+	-
53	Female/37	AR	2+	-
54	Female/34	AR/BA	2+	-
55	Female/14	AR/BA	4+	4+
56	Female/5.5	AR/BA	2+	-
57	Male/8	AR/BA	3+	1+
58	Female/19	AR/BA	1+	2+
59	Female/47	AR/BA	2+	-
60	Female/37	AR	2+	-
61	Female/37	AR	1+	-
62	Male/24	AR/BA	2+	-
63	Male/33	BA	3+	3+
64	Male/36	AR	3+	1+
65	Female/43	BA	2+	1+
66	Female/51	AR/BA	2+	-
67	Female/18	AR/BA	3+	2+
68	Female/42	AR/BA	2+	-
69	Female/47	AR/BA	2+	2+
70	Male/48	AR/BA	3+	1+
71	Male/15	AR/BA	2+	2+
72	Female/39	AR	3+	1+
73	Male/63	BA	3+	-
74	Male/15	AR/BA	3+	-
75	Male/12	AR/BA	3+	1+
76	Female/18	AR/BA	3+	-
77	Female/25	AR	3+	1+
78	Female/40	AR/BA	3+	1+
79	Male/75	AR/BA	1+	-
80	Male/11	AR/BA	3+	3+
81	Female/43	BA	1+	-
82	Male/15	AR/BA	2+	3+
83	Female/14	AR/BA	3+	1+
84	Male/8	AR	1+	1+
85	Female/43	BA	1+	-
86	Male/47	AR/BA	2+	-
87	Male/45	AR	1+	2+
88	Female/52	AR	1+	-
89	Male/20	BA	1+	-
90	Female/20	BA	2+	-
91	Female/13	AR	4+	2+
92	Female/66	AR/BA	3+	2+
93	Female/64	BA	1+	1+

94	Female/41	AR/BA	2+	-
95	Female/44	AR/BA	3+	2+
96	Female/43	BA	3+	3+
97	Male/47	AR/BA	2+	-
98	Male/45	AR	4+	4+

AR: allergicrhinitis. BA: bronchial asthma. Positive: ≥1+, 1+: the response was significantly weaker than histamine, 2+: the response was weaker than histamine, 3+: the response was almost the same as histamine control, 4+: the response was stronger than histamine control; Negative: -.

[10]. 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 DME (0.5 mg/ml), rDer f 21 (0.5 mg/ml) or culture medium alone in a 96 wells 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 cells /well) were cultured in 24 wells plate and stimulated with 0.2 mg/mL DME, rDer f 21 or medium alone for 72 h. IL-4 and IFN-γ levels in the supernatants were measured by multiplex cytokine assay kits following the manufacturer's instructions.

Histology

The lung tissue was excised after sacrifice and fixed with 4% parafor maldehyde 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.

Data statistics

All data are expressed as mean ± standard error; analysed with SPSS 18.0 statistical software. One-way ANOVA was used for comparison of multiple groups and Student's t test was used for the mean differences between two groups, and a *P*-value less than 0.05 was considered significant.

Results

The allergic activity of rDer f 21

In order to characterize the biological activity of Der f 21, we firstly cloned, expressed and purified the rDer f 21, the results was shown in the Supplementary Figure 1. We next investigated the allergic activity of rDer f 21 in dust mite allergic children. The results (**Table 1** and **Figure 1A**) showed that 11 allergic children had positive reaction to rDer f 21, accounting for 28.9%. To determine the allergenicity of rDer f 21 protein, immunoblotting was performed using the serum from the 11 rDer f 21 positive allergic children. The result showed that the serum from allergic children, but not that from healthy subjects, reacted to the rDer f 21 protein. The IgE binding bands with molecular weight around 35 kDa (rDer f 21) were positive in the sera from allergic patients, but negative in healthy volunteers (**Figure 1B**). Immunoblotting inhibition experiments showed that the concentration of rDer f 21, rDer f 5 and crude dust mite extracts reached 1000 mg/ml used as inhibitor could suppress their own response. Crude dust mite extracts were able to inhibit 86% and 90.9% of rDer f 21 and rDer f 5. On the contrary, rDer f 21 and rDer f 5 were able to inhibit 60% and 58% of crude dust mite extract respectively. rDer f 21 were able to inhibit 70% of rDer f 5, while rDer f 5 were able to inhibit 91% of rDer f 21, which indicate a cross reaction between rDer f 21 and rDer f 5 (**Figure 1C**).

Then we observed the allergic activity of rDer f 21 in allergic patients by SPT. The results (**Table 2**) showed that 42 allergic patients had positive reaction to rDer f 21, accounting for 42.9%, including 14 allergic patients were 1+, 13 allergic patients were 2+, 10 allergic patients were 3+, 5 allergic patients were 4+ (**Figure 2A**). The clinical data showed that the 42 Der f 21 allergic patients, including 13 patients had allergic rhinitis, 3 patients had bronchial asthma, 26 patients had both asthma and rhinitis (**Figure 2B**). These results suggest that rDer f 21 is an important allergen.

Antigen localization of Der f 21 in mites

The allergen Der f 21 localization in the D. farina was tested by immune-histochemical analysis. The result demonstrated that Der f 21 had strong positive in foregut (stomach) and midgut and hind-

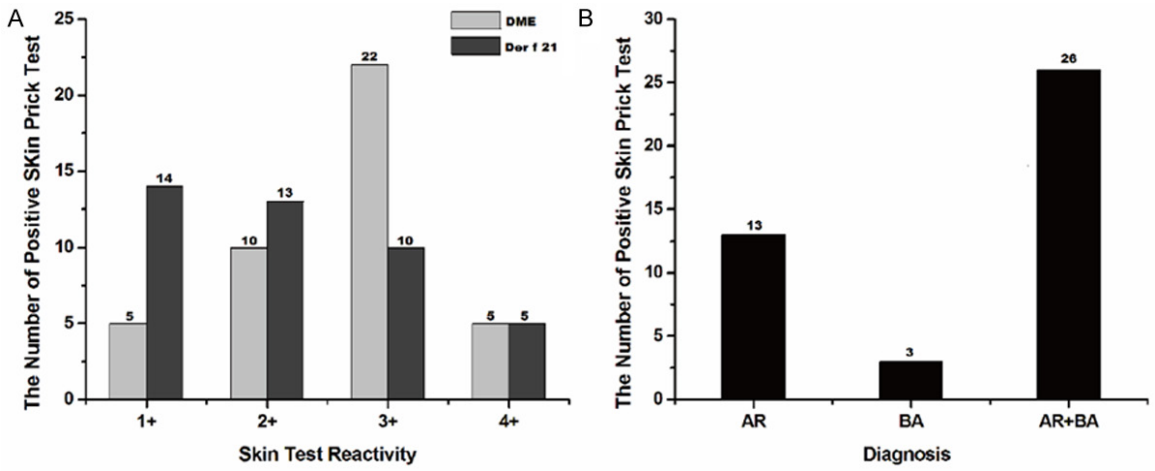

Figure 2. The allergic activity of rDer f 21 in allergic patients. A. Skin reactivity and severity of DME and rDer f 21 on mite allergic patients. The number of patients with severity of skin reactivity. B. Skin reactivity and severity of rDer f 21 on mite allergic patients. AR: Allergic rhinitis, BA: Bronchial asthma.

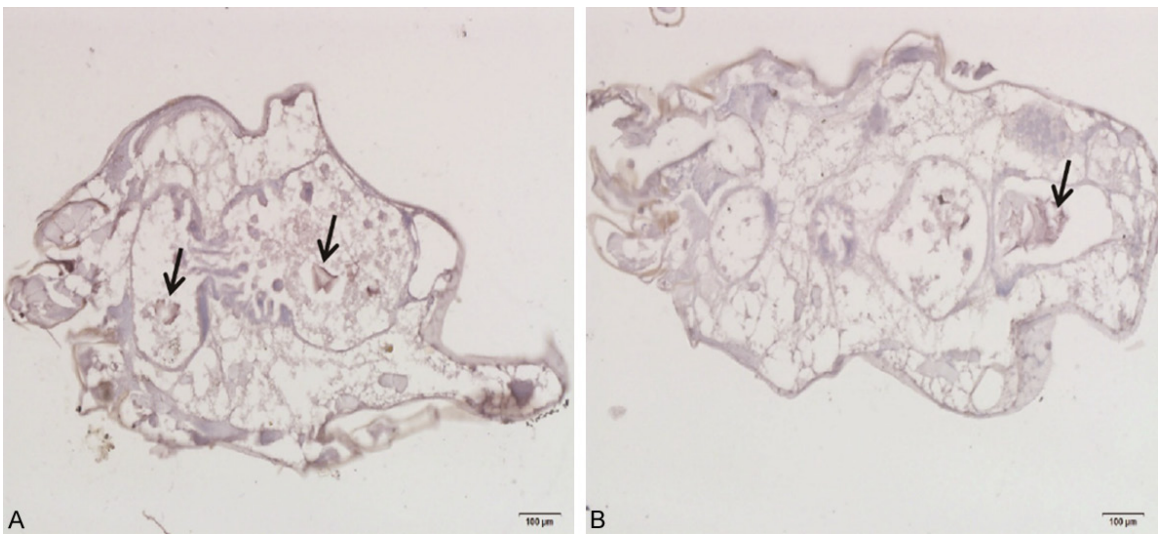

Figure 3. Immunostaining of Der f 21 in paraffin-embedded sections of D. farina. A, B. Probe with anti-Der f 21 monoclonal antibody in longitudinal and sagittal mite sections.

gut, while negative in body wall, reproductive system and body cavity of dust mites (**Figure 3**).

Establishment of a mouse model of allergic asthma using rDer f 21 as an allergen

A rDer f 21-induced mouse model of allergic asthma was developed (**Figure 4A**). Airway hyperresponsiveness was assessed by the unrestrained whole-body plethysmography (WBP). As shown by **Figure 4B**, the airway hyperresponsiveness of the mice in rDer f 21 group was lower than that in DME group, the specific antibody levels of IgE and IgG1 were detected in the serum (**Figure 4C**), which were significantly higher than that in the control group. The results suggest that similar to the crude dust mite extracts, rDer f 21 can induce antigen specific IgE and IgG1 in mice.

rDer f 21 induces lung inflammation in mice

After sensitization, the total cells and eosinophils in BAL were counted with wright giemsa staining under a light microscope. The frequency of eosinophils in the DME group and the rDer f 21 group was higher than that in the control group (**Figure 5A**). The result was further con-

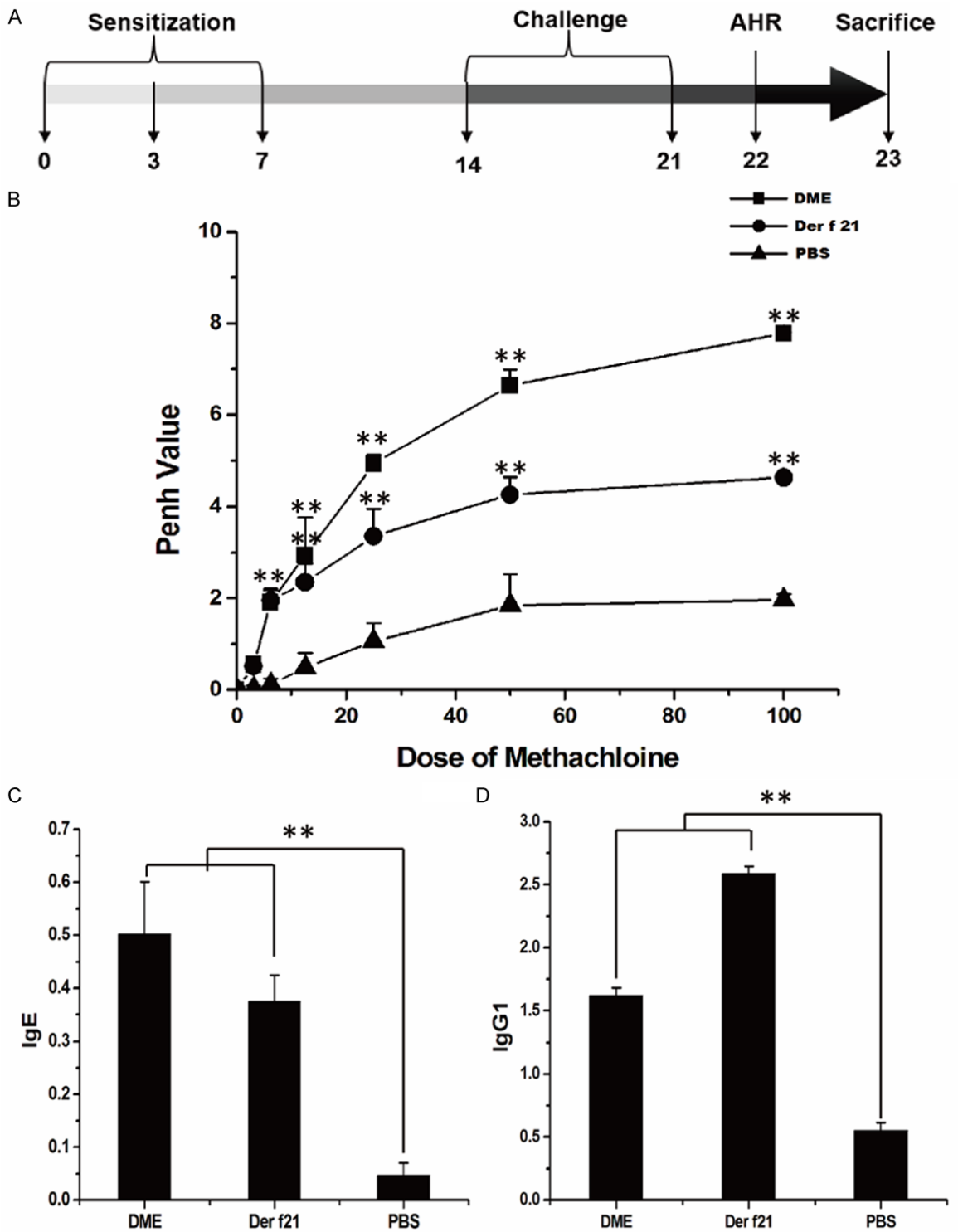

Figure 4. Establishment of a mouse model of allergic asthma by rDer f 21. A. Protocol of mouse model of allergic asthma. B. Airway hyper-responsiveness was measured with the unrestrained whole-body plethysmography. The Penh values were recorded when the mice were stimulated with increasing concentration of methacholine after the challenge. C, D. The level of IgE and IgG1 were detected by ELISA.

firmed by the histopathologic examination. In DME group and rDer f 21 group, a large quantity of exudates was observed in the the mouse airway lumen; profound inflammatory cell infil-

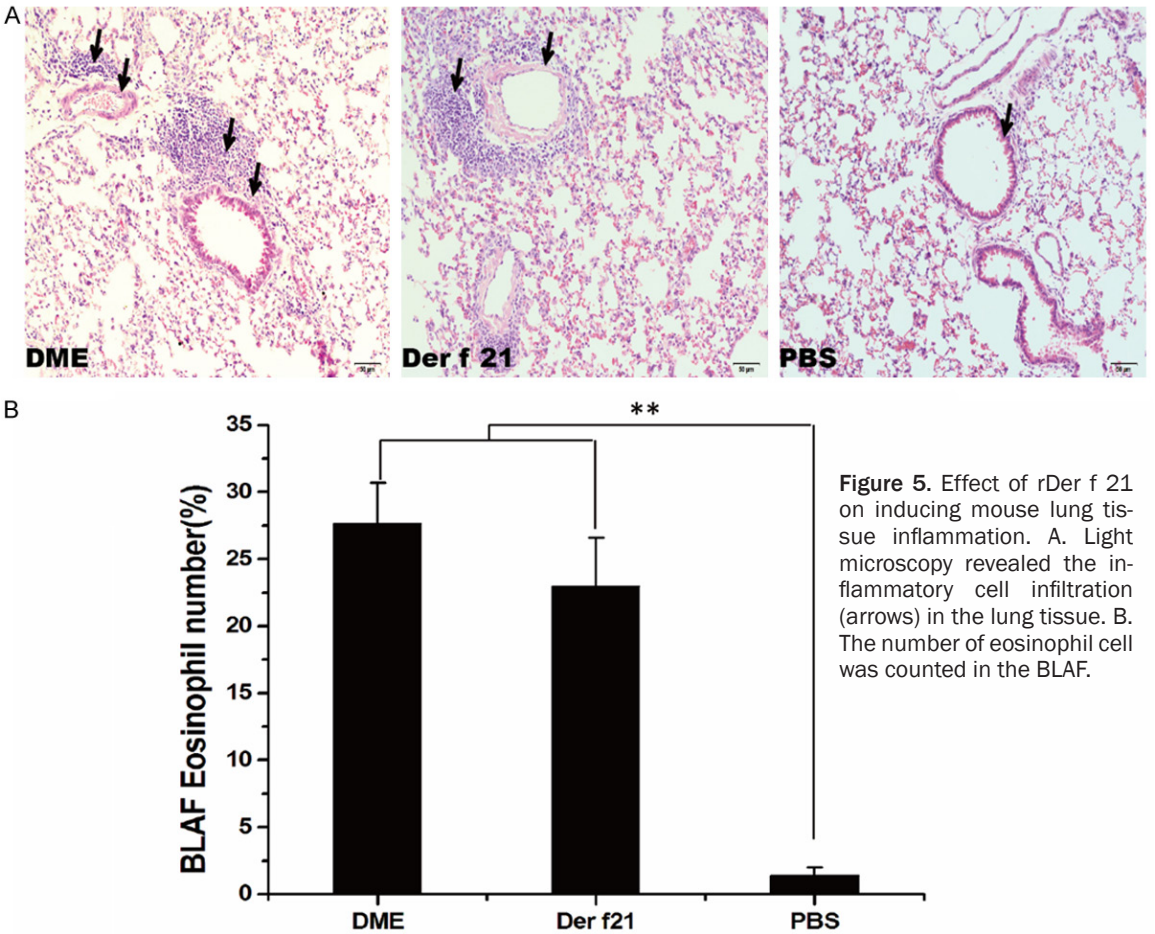

Figure 5. Effect of rDer f 21 on inducing mouse lung tissue inflammation. A. Light microscopy revealed the inflammatory cell infiltration (arrows) in the lung tissue. B. The number of eosinophil cell was counted in the BLAF.

tration was also observed in the lung tissue (**Figure 5B**). The results suggested that rDer f 21 can induce pulmonary inflammations.

Specific peripheral immune response induced by rDer f 21

To characterize the specific cellular immune response, splenocytes were isolated and stimulated with rDer f 21 in the culture. An increase of splenocyte proliferation was detected in the cells isolated from the rDer f 21 treated mice after exposure to rDer f 21 in the culture (**Figure 6A**). It also induced a significant increase in IL-4 and IFN-γ in the culture medium (**Figure 6B** and **6C**).

Discussion

Cumulative reports indicate that dust mite allergen proteins can induce the type I allergy response. Studies have shown that Der p 2 and Der p 7 can activate B lymphocytes to induce immune response through activating TLR4 (or TLR2, 3, 4). There are more than 30 subtype allergens have been identified from dust mite. Although specific immunotherapy with crude dust mite 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 mite crude extracts [11].

With the development of molecular methods, recombinant allergens have been used in the diagnosis of allergic diseases [12]. Molecular characterization of various recombinant allergens from dust mite has provided a new insight and new strategies for the diagnostics of allergic diseases. In this study, through cloning, expression, and purification of full length recombinant Der f 21. The results showed that rDer f 21 could be another allergen from dust mite allergen family in the induction of type I hypersensitivity. The inference is supported by the present data. Using rDer f 21, we success-

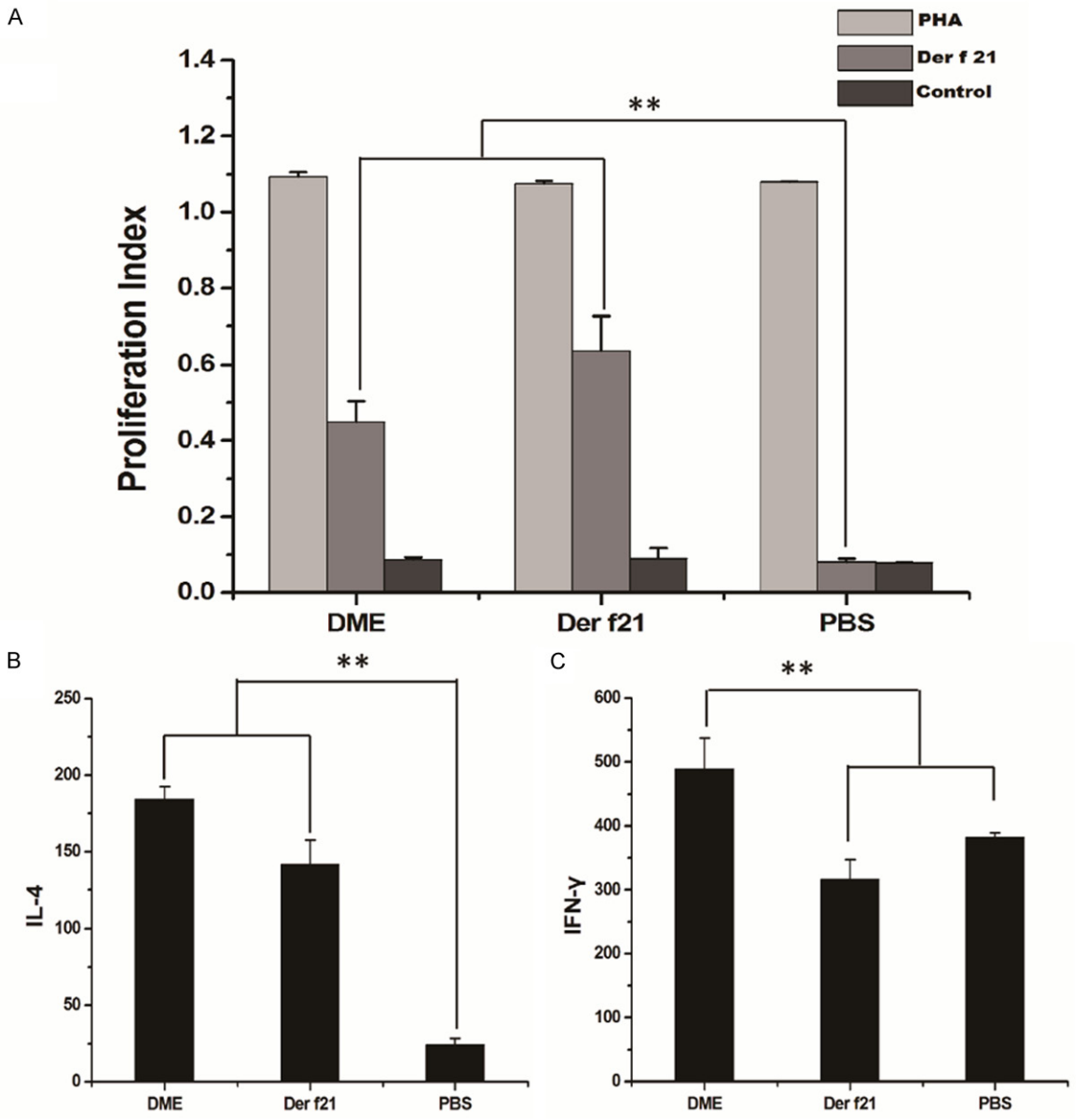

Figure 6. Specific peripheral immune response induced by rDer f 21. A. The proliferation of splenocytes isolated from the immunized mice was assessed by MTT. B, C. The levels of specific splenocyte IL-4 and IFN-γ were measured by ELISA.

fully developed a mouse model of airway allergy. The results showed that Der f 21 is a new allergen in D. farinae.

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Disclosure of conflict of interest

None to declare.

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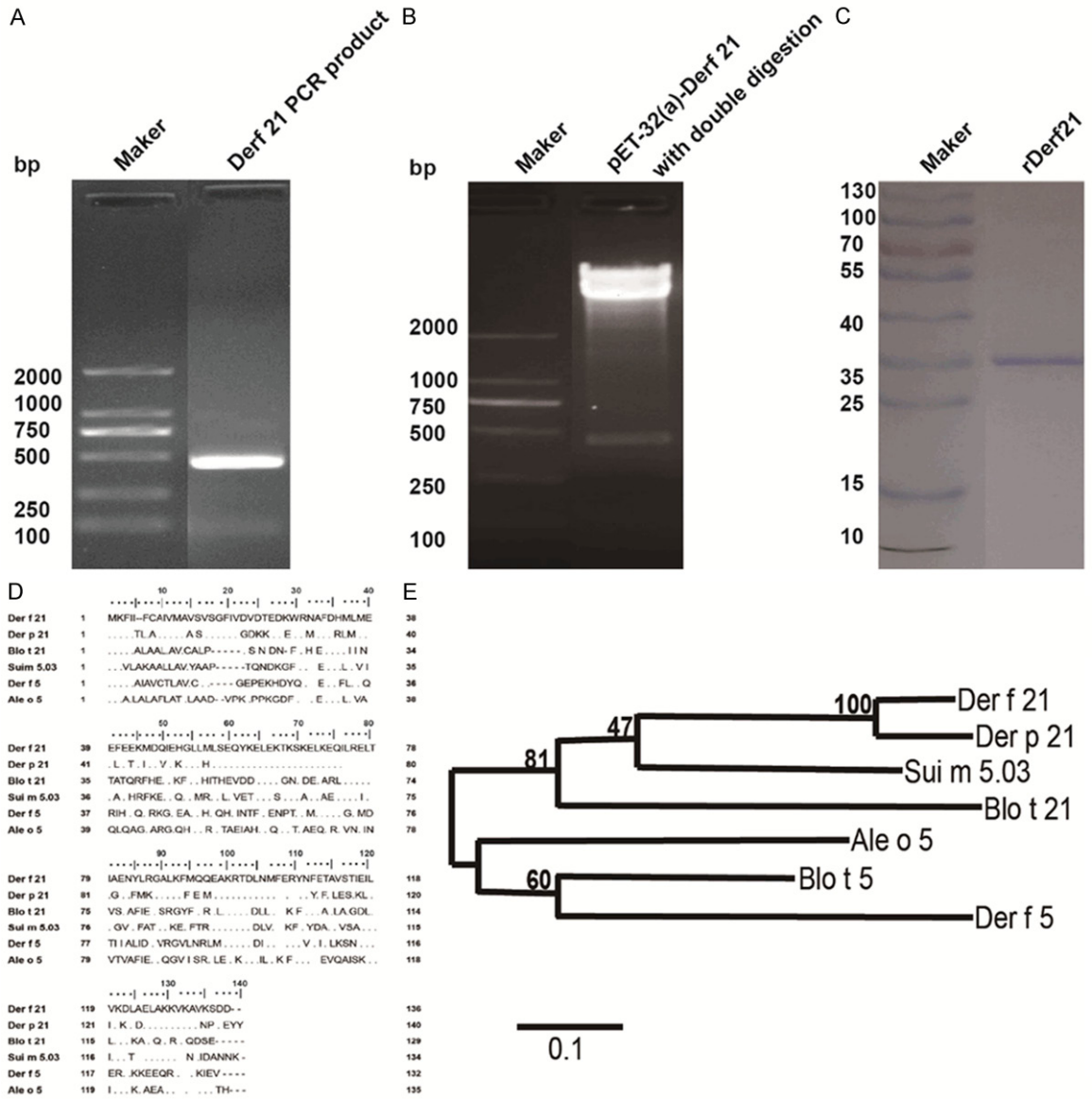

Supplmentary Figure 1. Cloned and purified rDer f 21. A. The PCR product of Der f 21. B. The identified of pET-32a(+)-Der f 21 by restriction enzymes digestion. C. SDS-PAGE analysis of rDer f 21 after affinity chromatography. D. Alignment of Der f 21 from different sources. E. The Phylogenic Tree of Der f 21 from Mite.