

Original Article

Characterization of arginine kinase, a novel allergen of *dermatophagoides farinae* (Der f 20)

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Abstract: Objective: To characterize a novel allergen, the *Dermatophagoides farinae*-derived arginine kinase (Der f 20). Methods: The protein of Der f 20 was synthesized by genetic engineering approaches. The allergenicity of Der f 20 was tested by enzyme-linked immunosorbent assay and an airway allergy mouse model. Results: The Der f 20 gene was cloned and presented in the Gene Bank with an accession number of AAP57094. The Der f 20 is an arginine kinase (AK), which showed a close relationship with *D. pteronyssinus* AK and *Aleuroglyphus ovatus* AK. Western-blot and ELISA studies showed the IgE binding capacity of Der f 20 was 66.7% in the sera from 6 dust mite allergic patients. Immune inhibition assay results showed the IgE cross-reactivity between Der f 20 and DME (Dust mite extract). Positive responses to Der f 20 were 41.2% as shown by skin prick tests in 17 DME-allergic patients. In vitro experimental results showed that Der f 20 induced Th2 cell differentiation and the expression of T cell Ig mucin domain molecule-4 (TIM4) in DCs. Conclusions: The Der f 20 protein is a novel subtype of the dust mite allergen.

Keywords: Arginine kinase, *dermatophagoides farinae*, Der f 20, prokaryotic expression, allergy

Introduction

Allergic asthma and Allergic rhinitis are global health problems affecting all age groups. The incidence of asthma and allergic rhinitis is increasing worldwide in the recent decades. It is estimated that approximately 300-500 million people around the world suffer from asthma and/or allergic rhinitis [1, 2]. Compared with the outdoor allergens, sensitization to the indoor inhaled allergens, such as (dust mite allergens) is more important in the development of asthma (http://www.ginasthma.org/local/uploads/files/GINA_Report__2014_Aug12). Dust mites have been described as the most common allergen in allergic asthma and allergic rhinitis (AR) [3-7].

Arginine kinase (AK) is one of the HDM allergens we identified recently. AK is a phosphate kinase of invertebrates which plays a crucial role in energy metabolism [8, 9]. Although allergic diseases caused by AK have been reported [10-13], there are no reports about the charac-

terization of Der f AK. Therefore, it is necessary to characterize the Der f AK in the initiation of allergic responses. In this study, high purity Der f AK was cloned and its immunological characterization was analyzed. According to the nomenclature series of Der f allergens, the Der f AK was nominated as Der f 20.

Material and methods

Material

The sera of patients with allergic disorders were obtained from The First Affiliated Hospital of Guangzhou Medical University. Serum from non-allergic individuals were used as normal controls. *Escherichia coli* Top10 and *Escherichia coli* BL21 were provided by Institute of Allergy and Immunology, Shenzhen University. Female BALB/c mice (6 to 8 weeks old, weighed 18-20 g) were purchased from Guangdong Medical Laboratory Animal Center. All of the mice were maintained in a pathogen-free environment with free-access to rodent laboratory food and

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water. Eco R I and Bam H I (TaKaRa China), Horseradish Peroxidase (Southern Biotech USA), anti-TIM 4 antibody (abcam UK).

Ethic statement

The using human tissue in the present study was approved by the Human Ethic Committee at Guangzhou Medical University. A written informed consent was obtained from each human subject. The animal study was approved by the Animal Ethic Committee at Shenzhen University.

Der f 20 cloning

Der f 20 gene sequences were referred to our published data [18, 19]. The primers of Der f 20 with double enzyme loci included cgaattcatg-gttgatcaagctgtcatc (Eco RI) and gctcgagcatag-atTTTTcaattttaaTcaattc (Xho I). The cDNA was synthesized with a reverse transcription kit. PCR amplification reaction was performed to synthesize the Derf 20 gene segment. The cDNA segment was cloned into the pMD-18 T vector, and then transformed into E. coli top 10. Positive clones were picked out by Luria-Bertani (LB) plates containing ampicillin, and confirmed by double enzyme analysis with Eco R I and Xho I and Agarose gel electrophoresis for the product identification. The positive clones were sent to the Shanghai Sangon Company for DNA sequencing and the strains were stored at -80°C.

The resulted Der f 20 sequence was converted to an amino acid sequence.

Compared with the amino acids sequence of twelve different sub-cloned AK in GenBank, molecular evolutionary tree was constructed by software MEGA5.1.

After sequencing alignment, the vector Der f 20-PMD18-T was subject to double enzyme digestion. The vector was connected with pET-32a(+) vector with double enzymatic digestion by EcoRI and XhoI after agarose electrophoresis separation and recycling to complete the construction of recombinant expression vector of Der f 20- pET-32a(+). The E. coli Top10 clone bacteria was then transformed, positive clones were selected by ampicillin and identified it by double enzyme analysis.

The expression and purification of recombinant Der f 20

Based on our previous studies [14, 15], the full-length gene of Der f 20-pET-32-a-(+) plasmid were transformed into Ecoli BL21 expression bacterium by the calcium chloride approach. Positive clones were selected by ampicillin and cultured overnight. IPTG was added to a final concentration of 1 mM. The bacteria were harvested after 20 h culture. Both precipitation and supernatant samples were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. FPLC was used for Ni²⁺ affinity chromatography purification.

Western blot assay of Der f 20

The sera from dust mites allergic patients were used as the primary antibodies. The biotinylated goat anti-human IgE as the second antibody. The membrane was incubated with the primary antibodies, the second antibody and HRP-labeled streptavidin, and then visualized by developing with diaminobenzidine substrate solution. The immunogenicity of Der f 20 protein was determined according to the coloration.

ELISA

The 96 well plates were coated with 100 µl of Der f 20 at a concentration of 1 µg/ml in carbonate buffer and incubated at 4°C overnight. The positive serum of 6 patients with dust mite allergy was used as primary antibodies (1:50 dilution). The plates were incubated at 37°C for 2 h. Then the plates were added with 100 µl of biotinylated anti-human IgE (1:2000 dilution) and incubated at 37°C for 2 h. Next, 100 µL of HRP labeled with streptavidin (1:5000 dilution) were added washing 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 H₂SO₄. The absorbances at 450 nm were then measured with a microplate reader.

Der f 20 and crude extract inhibition assays

The Der f 20 specific human serum was used for the inhibitory experiment to detect the cross reaction for Der f 20 and DME. The sera were diluted (1:50), pooled at different concentra-

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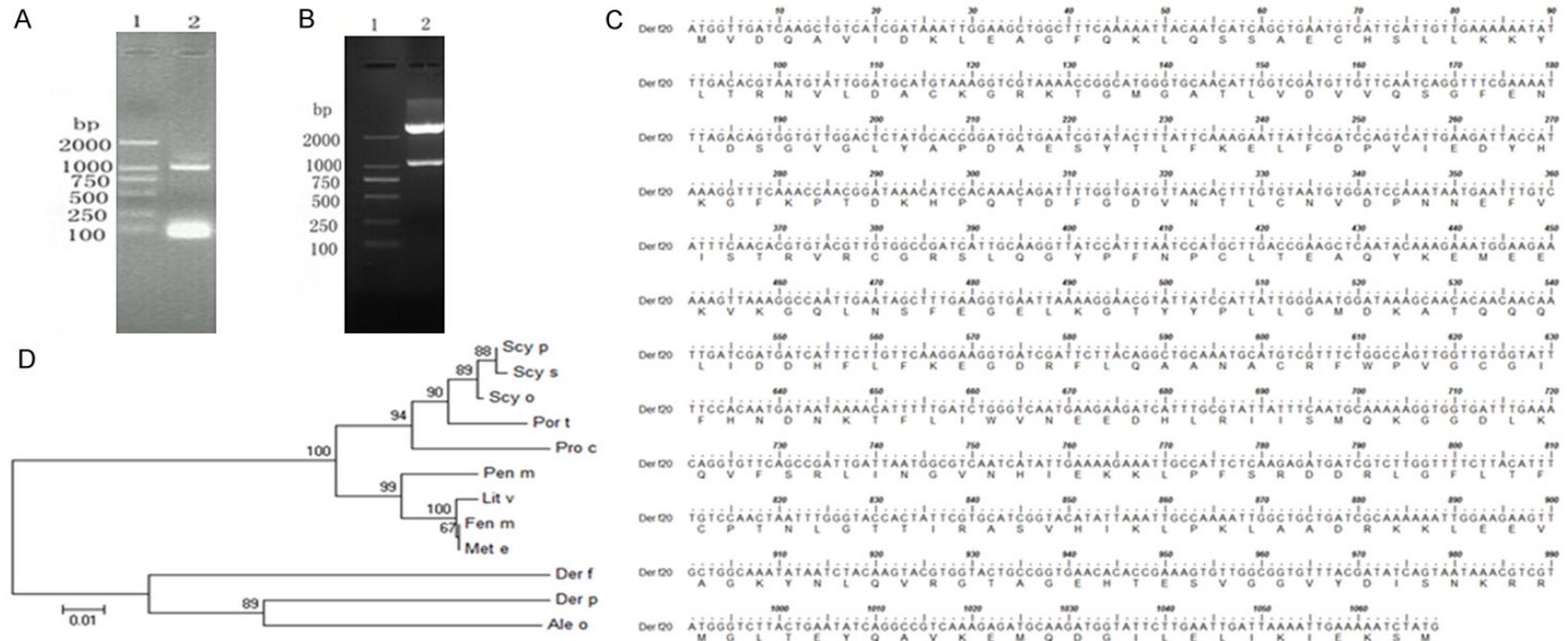


Figure 1. Cloning and sequence alignment of the Der f 20 gene. A. Cloning and PCR of Der f 20 Cdna (1; DNA Marker 2; Der f 20 gene); B. Restriction enzymatic digestion results of recombinant Hyastatin-pET-32a(+) plasmid (1: DNA Marker; 2: Der f 20 DNA); C. The amino acid sequences of Der f 20; D. The phylogenetic tree analysis; Der f (*Dermatophagoides farinae*), Der p (*Dermatophagoides pteronyssinus*), Ale o (*Aleuroglyphus ovatus*), Por t (*Portunus trituberculatus*), Pro c (*Procambarus clarkii*), Scy o (*Scylla olivacea*), Scy s (*Scylla serrata*), Pen m (*Penaeus monodon*), Scy p (*Scylla paramamosain*), Lit v (*Litopenaeus vannamei*), Met n (*Metapeneus mercurialis*), Fen m (*Fenneropenaeus merguensis*).

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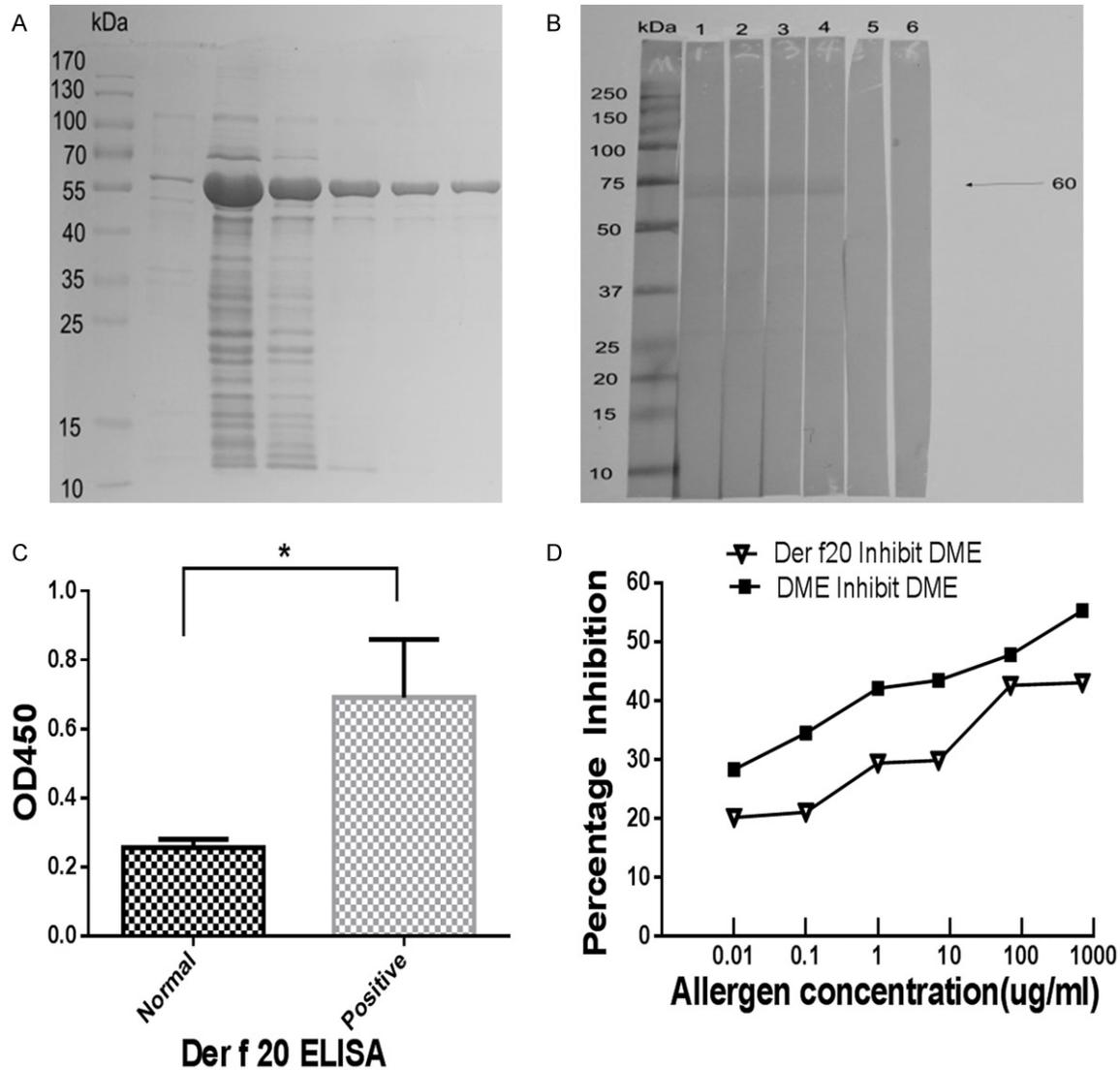


Figure 2. Immunological characterization of *Dermatophagoides farinae* AK. A. The protein band indicates the purified *Dermatophagoides farinae* AK (Der f 20); B. The Western blots indicate that the rAK binds to IgE in the sera from patients allergic to *Dermatophagoides farinae*; C. Allergenicity of *Dermatophagoides farinae* AK detected by ELISA, *means $P < 0.05$ in comparison with Normal control; D. Mutual inhibition between dust mite crude extract (DME) and Der f 20.

tions of Der f 20 or DME, and incubated at 4°C overnight. The inhibitory rate was calculated according to the absorbance value.

Skin prick test

The rDer f 20 protein was dissolved in phosphate buffered saline (50 mM PB, 100 mM NaCl; pH 7.4). Glycerin was added to the solution at 50% asthefinal concentration. The Der f 20 concentration was 0.01 mg/ml, controlled by histamine phosphate (0.1%; positive) and saline (negative). The results were checked 20 minutes after SPT. The judgement of the result:

if the prick spot became a wheal and fleck surrounding the wheal, it was positive (+). 4+: the response was stronger than 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.

Asthma mouse model

Eighteen BALB/c mice were divided into three groups randomly: Normal (PBS) group, asthma

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Table 1. The result of Skin prick tests (SPT)

Gender/Age	Diagnosis	Dust mites	Histamine	PS	Der f 20 (mean diameter (mm), Level)
Male/47	B A, A R	3.5, ++	5.5	0	0
Female/9	A R, D A	7, +++	5.5	0	0
Female/72	B A, D A	2, +	5	0	0
Male/45	A R	2, +	7.5	0	1.5, +
Female/28	B A	2, +	4.5	0	0
Female/52	A R, F A	1.5, +	5.5	0	0
Male/20	B A, F A	1.5, +	5	0	0
Female/20	B A	2, ++	4	0	2.5, ++
Female/13	A R	1.25, ++++	6	0	3, ++
Male/62	A R, Urticaria	2.25, +	4.75	0	0
Female/66	B A, D A, A R	9, +++	5.5	0	2.5, +
Female/64	B A	2.25, +	6	0	0
Male/17	B A, A R	14, +++	7.5	0	0
Female/84	B A, M A	2.5, ++	3.5	0	2.25, ++
Male/11	B A, A R	9, +++	5.5	0	2.5, +
Female/41	B A, A R	3.5, ++	5.5	0	0
Female/44	B A, A R	4.5, +++	4.5	0	3, ++

B A (Bronchial asthma); A R (Allergic rhinitis); D A (Drug allergy); M A (Mango allergy); F A (Food allergy); PS: Physiological saline; Positive: ≥ 1 ; Negative: 0.

(DME) group and rDerf 20 group. On day 1, day 3 and day 7, the mice were treated with subcutaneous injection of 50 μ g DME, or 100 μ g rDerf 20 mixing with 0.3 ml aluminum hydroxide, or PBS. From 14 days to 21 days, the mice of asthma group, rDerf 20 group and PBS group were challenged with 50 μ g DME, or rDerf 20 or PBS, respectively via intranasal instillation. In 24 hours after the final challenge, AHR was recorded by the double-chambered whole-body plethysmograph (Buxco, USA). The baseline response was recorded for 5 minutes in the event of stabilized respiration after 10 minutes of adaptation. Then the mice were subjected to inhaling methacholine (Mch) at increasing concentrations (0, 6.25, 12.5, 25, 50, and 100 mg/mL PBS). Tests at two different concentrations were separated temporarily to allow the respiratory intensity to drop back to baseline. The percentage curves for Penh values at different methacholine concentrations were plotted based on the value from PBS stimulation. The mice were sacrificed on day 23. The lungs were removed and processed for HE staining. The levels of IgE in the serum and the level of IL-4 in the cultured splenocyte supernatant were determined by ELISA. The number of eosinophil in bronchoalveolar lavage fluid (BALF) of each groups were counted under a light microscope.

Stimulation of DCs with Der f 20 in the culture

DCs were prepared from the mouse bone marrow following published procedures [16], and cultured in RPMI-1640 medium supplemented with 0.1 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 12% FBS (fetal bovine serum), IL-4 and GM-CSF (20 ng/ml). Three days later, the DCs were stimulated with graded concentrations of Der f 20 (0-2000 ng/ml) or LPS (20 ng/ml) for 48 h at 37°C, the cells were then collected. The cell extracts were analyzed by Western blotting and RT-qPCR; the culture medium was analyzed by ELISA to determine the level of TIM4.

Real-time RT-PCR (RT-qPCR)

Total RNA was extracted from the DCs with TRIzol reagent. The cDNA was synthesized with a reverse transcription kit. qPCR was performed in a real time PCR device using the SYBR Green Master Mix. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative gene expression.

Statistical analysis

All data are expressed as mean \pm SD. Statistical significance between different groups was determined using the SPSS 13.0 software One-way ANOVA or t-test. A $p < 0.05$ was set as a significant criterion.

Results

Cloning and sequence alignment of the dust mite Der f 20 gene

The amplification products showed a bright band around 1068 bp in agarose gel after electrophoresis (**Figure 1A**) and double enzymatic digestion (**Figure 1B**). According to the nucleic acid sequence of Der f 20, amino acid sequence was deduced by BioEdit (**Figure 1C**). The molecular evolutionary tree was constructed by software MEGA5.1. The results showed that Der f AK has a close relationship with Der p AK and Aleuroglyphus ovatus AK (**Figure 1D**).

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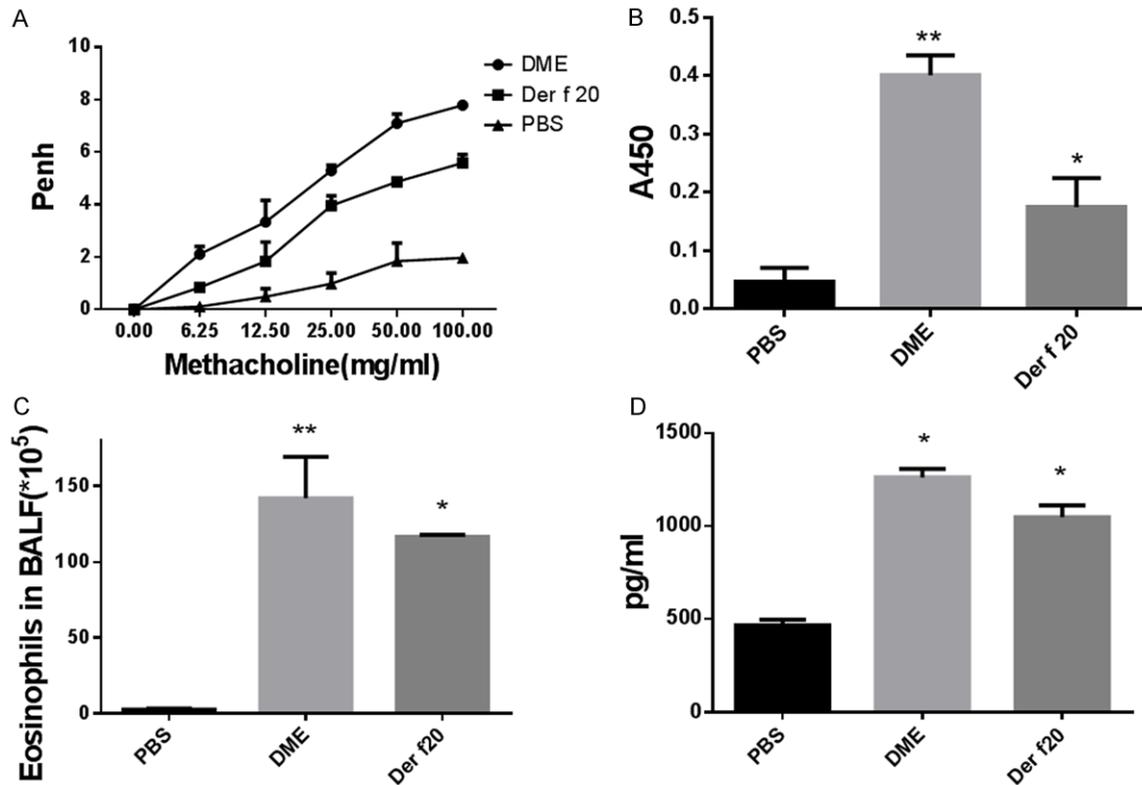


Figure 3. Evaluation of AHR. A. The evaluations of AHR were performed with Resistance and Compliance (Buxco, USA) in 24 h after the last challenge with Der f 20. A. The curves indicate the Penh (Enhanced pause) values. B. The bars indicate the levels of IgE in the serum (by ELISA). C. Eosinophil counts in BALF of each group. D. The bars indicate the levels of IL-4 in the cultured splenocyte supernatant (by ELISA). The results are representative of 3 independent experiments. * $P < 0.05$ in comparison with PBS group. ** $P < 0.01$ in comparison with PBS group.

Recombinant Der f 20 (AK) has Immunological activity

The rDerf 20 was purified by affinity chromatography (Figure 2A). The immune activity of the purified rDerf 20 was shown by Western blot (Figure 2B), ELISA (Figure 2C), and inhibition experiments (Figure 2D). At concentration of 70 $\mu\text{g}/\text{ml}$, the inhibition rate of Der f 20 against DME was about 50%.

Clinical skin prick tests (SPT)

The allergic activity of rDerf 20 was evaluated by SPT and it shows that 7 (41.2%) out of 27 allergic patients showed positive reaction to Der f 20 (Table 1).

Der f 20 induces airway allergic response in mice

Following published procedures, Der f 20 induced pulmonary allergic inflammation in the mouse model. Upon challenge with methacholine, the airway hyperresponsiveness (AHR) was induced. As shown in (Figure 3A): DME

group had the highest Penh (Enhanced pause) value, indicating the airway hyperresponsiveness is the strongest among the three groups. The Der f 20 group mice also showed the airway hypersensitivity, which was weaker than the DME group, while PBS group showed the lowest reaction. DME group and Der f 20 group showed high serum levels of the antigen-specific IgE (Figure 3B). Compared with PBS group, the frequency of BALF eosinophils of the DME group and the Der f 20 group was increased significantly (Figure 3C). The levels of IL-4 in the splenocyte culture supernatant were much higher in DME group and Der f 20 group than PBS group (Figure 3D).

Allergic inflammation of mouse lungs induced by rDerf 20

After sensitized and challenged with Der f 20, bronchial and lung tissue had profound inflammatory cell infiltration. A large number of inflammatory cells migrated to bronchial and alveolar spaces. The vascular wall showed apparent edema (Figure 4).

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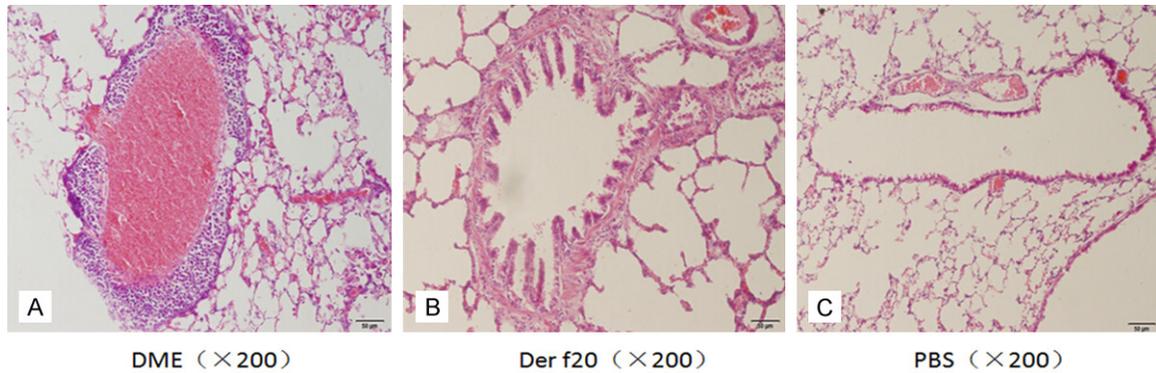


Figure 4. The pathologic changes of pulmonary tissue (hematoxylin and eosin staining, $\times 200$).

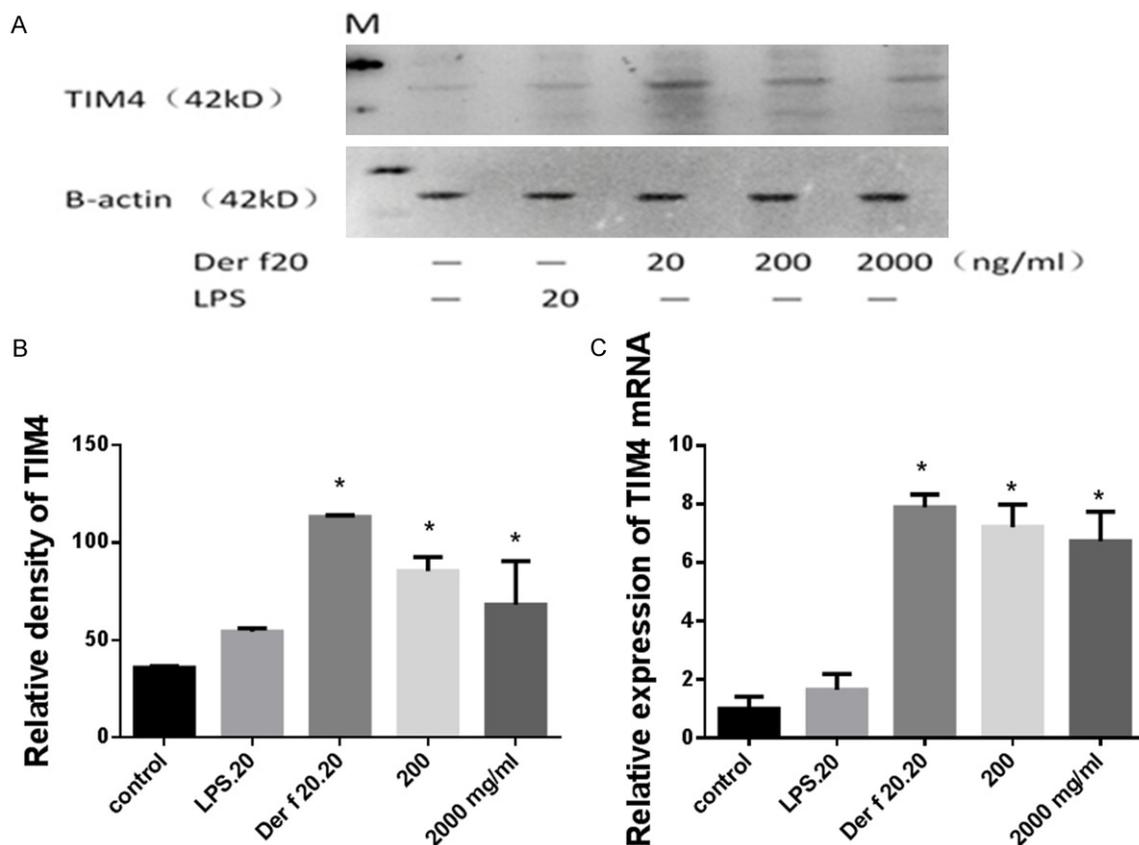


Figure 5. rDerf 20 up-regulates the expression of TIM4 in DCs. DCs were cultured in the presence of different concentrations of Der f 20 or LPS (20 ng/ml) for 48 h. A. The Western blots indicate the TIM4 protein in DCs. B. The bars indicate the integrated density of the blots in panel A. C. The bars indicate the mRNA levels of TIM4 in DCs (by Real-Time RT-PCR). The results are representative of 3 independent experiments. * $P < 0.05$ in comparison with PBS group, **means $P < 0.01$ in comparison with PBS group.

Der f 20 up-regulates the expression of TIM4 in DCs

After exposure to Der f 20 in the culture, higher levels of TIM4 were detected in DCs. Compared with control group, Der f 20 induced higher levels of TIM4 in the DC culture supernatant (Figure 5).

Discussion

The present results showed that Der f 20 had immunogenicity, and could induce airway hyperreactivity (AHR) in an animal model. Skin Prick Tests (SPT) with Der f 20 showed specific allergic activity. Furthermore, analysis of bioinformatics showed that Der f 20 had a close rela-

tionship with *D. pteronyssinus* AK and *Aleuroglyphus ovatus* AK.

Within our knowledge, there have been no reports about the relationship between sensitization mechanism of Der f 20 and TIM4. TIM4, a member of TIM (T cell immunoglobulin domain and mucin domain) family, is mainly expressed by dendritic cells and macrophages, which can promote Th0 cell to differentiate to Th2 cells leading to type I allergic disorders [17, 18], such as rhino conjunctivitis, atopic dermatitis, and bronchial asthma. In the present study, both Western blot and qRT-PCR analysis showed Der f 20 up-regulates the expression of TIM4 in DCs.

In conclusion, Derf 20 was purified and recombined, which induced the expression of TIM4 in DCs.

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Disclosure of conflict of interest

None.

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