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Original Article

Generation of a chimeric dust mite hypoallergen using DNA shuffling for application in allergen-specific immunotherapy

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Abstract: Specific immunotherapy (SIT) is the only treatment that provides long lasting relief of allergy symptoms. Unfortunately, SIT-based traditional remedies carry the risk of producing local and/or systemic side effects. To improve the safety and efficacy of SIT, it has been proposed that SIT must utilize allergens that are hypoallergenic but hyperimmunogenic. Therefore, we used DNA shuffling to generate mutant genes encoding hypoallergens with potent immunogenicity and screened them for their capacity to modify the allergic response. We tentatively shuffled the major group 1 allergen genes from house dust mite, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, and discovered a novel chimeric gene, termed *C 1*. The gene was expressed in *Escherichia coli* (*E. coli*) and the chimeric protein C 1 was purified. An animal model of asthma demonstrated that C 1 not only decreased the production of serum IgE and IgG_1, and inhibited the production of IL-4 and IL-5 in the bronchoalveolar lavage fluid (BALF). C 1 also boosted the levels of IgG_{2a} and IFN-γ, which may demonstrate a rebalance of T_H1 and T_H2 allergic response. Additionally, flow cytometry showed that the immunogenicity of C 1 was higher than that of ProDer f 1, but was not significantly different from that of ProDer p 1. Our findings suggest that the C 1 is hypoallergenic and yet highly immunogenic, which makes it potentially safe and effective for use in SIT of allergic asthma.

Keywords: *Dermatophagoides* allergen 1 group, DNA shuffling, specific immunotherapy, allergenicity, immunogenicity

Introduction

Exposure to allergens from two species of house dust mites, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are associated with various allergic diseases such as asthma [1]. More than 80% of allergic patients sensitized to house dust mites have immunoglobulin E (IgE) antibodies against the group 1 allergens *D. farinae* and *D. pteronyssinus* [2, 3]. The group 1 allergens (Der f 1 and Der p 1), have cysteine protease activity [4], are involved in the pathogenesis of allergic inflamation [5, 6], and IgE specific to Der f 1 has been reported to cross-react with Der p 1 and *vice versa* [7, 8].

Since there has been a large increase in the prevalence of allergic disease in the past decades, there has been an increasing initiative to develop new and safe treatments for allergic inflammation. Some of these approaches have targeted IgE and the IgE-mediated allergic reaction [9-11]. However, specific immunotherapy (SIT) is the only current treatment that provides long-lasting relief of allergic symptoms. SIT is performed by injecting a patient with increasing doses of an often poorly-characterized allergen extract. Although the efficacy of allergen extract-based SIT has been well-documented, it bears the risk of sporadic IgE-mediated side effects, including local and systemic anaphylaxis, and induction of new sensitizations [12-14]. Therefore, allergens that have a reduced IgE-binding and increased T-cell epitopes have been proposed to improve the safety and efficacy of SIT [15, 16].

DNA shuffling along with large-scale screening provides an efficient method to select the can-

didates that have the desired properties [17-19]. This approach has been successfully applied in the discovery of hypoallergens that have potent immunogenicity for use in SIT. For example, DNA shuffling was used in multigene recombination of three group 2 allergen genes from the dust mites *Lepidoglyphus destructor* and *Glycyphagus domesticus* [15] and 14 allergen genes of the *Bet v 1* family [16]. However, there are few published studies that provide data concerning the homologue allergen genes from *D. farinae* and *D. pteronyssinus* recombined by DNA shuffling.

In this study, we applied DNA shuffling to two group 1 mite allergen genes: one group 1 allergen from *D. farinae* (ProDer f 1) and another group 1 allergen from *D. pteronyssinus* (ProDer p 1). The amino acid sequences of these two allergens have 82% similarity, and are well adapted templates for DNA shuffling for generating hypoallergens. On this basis, we successfully screened one chimeric gene, referred to as *C 1*, encoding a protein that had hypoallergenicity and high T-cell immunogenicity. The inhibitor effects of the C 1 protein on allergic inflammatory *in vivo* and *in vitro* were identical.

Materials and methods

Animals

Female BALB/c mice (6 weeks of age) were purchased from the Center for Comparative Medicine, Yangzhou University (License No.: SCXK 2007-0001) and provided with food and water *ad libitum* under specific-pathogen free conditions. All procedures were approved by the Research Ethics Board of Wannan Medical College.

DNA shuffling of allergen genes and screening

Two allergen genes, *ProDer f 1* (GenBank No. AB034946.1) and *ProDer p 1* (GenBank No. U11695.1) served as templates for DNA shuffling. They were amplified using PCR and specific primers as follows: 5'- TAT GGA TCC CGT CCA GCT TCA ATC AAA ACT -3' (*BamH* I) and 5'- GGC CTC GAG TCA CAT GAT TAC AAC ATA TGG -3' (*Xho* I) for *ProDer f 1*, 5'- GGC GGA TCC TCA TCG ATC AAA ACT TTT GAA -3' (*BamH* I) and 5'- GGC CTC GAG TCA GAG AAT GAC AAC ATA TGG ATA -3' (*Xho* I) for *ProDer p 1*. The chimeric library was constructed by DNA shuffling according to the protocols described by Stemmer [17]. The shuffled DNA library was inserted into a pUCm-T vector (Sangon Biotech, Shanghai, China), and sequenced on an ABI DNA sequencer (ABI, Foster City, CA, USA). Sequenced chimeric genes were aligned with parental genes to select for mutant clones. Amino acid sequences encoded by the selected clones were used to predict T- and B-cell epitopes with NetMHCII 2.2 software (http://www.cbs.dtu.dk/services/) in the HLA-DRB1*0301 and HLA-DRB1*0401 alleles which are associated with human asthma [20]. A shuffled gene, named *C 1*, was selected and its amino acid sequence was shown to have reserved T cell epitopes and decreased B cell epitopes.

Expression and purification of the proteins

The chimeric gene (*C 1*) was inserted into vector pET-28a(+) (Merck KGaA, Darmstadt, Germany) and transformed into the *E. coli* line BL21 (DE3) (Merck KGaA, Darmstadt, Germany). C 1 expression was induced with 1 mM isopropyl 1-thio-b-D-galactopyranoside (IPTG) (Sigma-Aldrich® Co. LLC. St Louis, MO, USA) at 37°C for 5 h. The C 1 protein in cell pellets was purified with a Ni^{+}-NTA affinity column chromatography kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Expression and purification of rProDer f 1 and rProDer p 1 was also carried out as described above. The endotoxin levels in the protein preparations were analyzed using a HEK-Blue™ LPS Detection Kit (Invivogen, San Diego, CA, USA).

Western blotting

Equimolar amounts (2.0 mmol/L) of the 3 recombinant proteins, rProDer f 1, rProDer p 1, and C 1, were analyzed on a 12.5% SDS-PAGE gel according to Laemmli's method [21] in a Mini-PROTEAN 3 system (Bio-Rad, Berkeley, CA, USA) and transferred onto an Immobilon-P membrane (EMD Millipore, Billerica, MA, USA). The membranes were incubated in blocking buffer (5% dried milk, 0.5% Tween-20 in PBS, pH 7.2) for at least 30 min. Afterward, the membranes were incubated for 2 h in blocking buffer containing Der f 1-specific rabbit polyclonal antiserum (obtained after immunization of rabbits with the purified native Der f 1 protein) diluted 1:10000 with PBS (pH 7.2). A horseradish peroxidase-conjugated goat anti-rabbit IgG

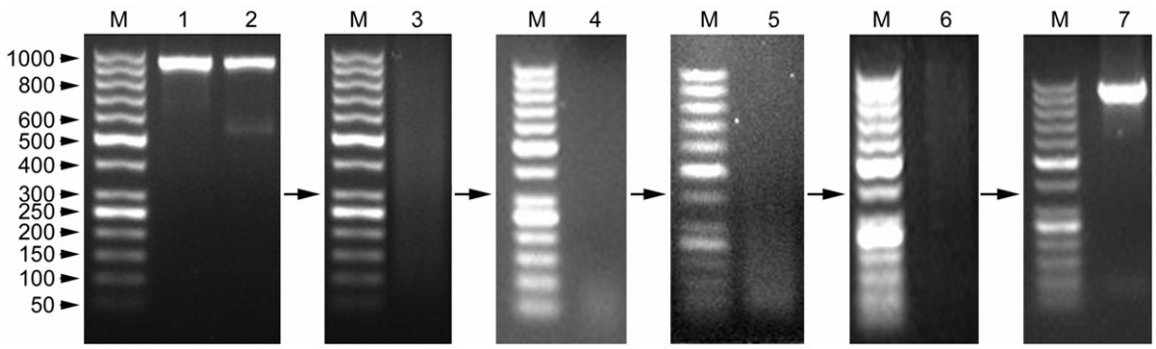

Figure 1. Procedures of DNA shuffling. M: DNA Marker; 1-2: PCR products of *ProDer f 1* and *ProDer p 1*, respectively; 3: Products from enzyme digestion of *ProDer f 1* and *ProDer p 1* with equivalent mixture; 4-6: Amplification of the small segments (50-150 bp) in consecutive three rounds of primerless PCR; 7: Chimeric gene pools after amplification by PCR with *ProDer f 1* primers.

(Sigma-Aldrich® Co. LLC., St Louis, MO, USA) diluted 1:10000 with PBS (pH 7.2) was used as a secondary antibody, followed by three washes with blocking buffer (20 min each). Transferred proteins were visualized using a DAB Horseradish Peroxidase Color Development Kit (Sangon Biotech, Shanghai, China) in PBS (pH 7.2) according to the manufacturer's instructions.

Determination of the IgE-binding activity

IgE-binding was measured using an Enzyme Linked Immunosorbent Assay (ELISA). Twenty-five serum samples were obtained from *D. farinea*-allergic patients at the Yijishan Hospital of Wannan Medical College. The Medical Ethics Committee of Wannan Medical College approved collection of these samples. Immunoplates were coated overnight with rProDer f 1, rProDer p 1 or C 1 (500 ng/well) at 4°C. Plates were washed with 100 µl per well of Tris-buffered saline (TBS)-Tween buffer (TBST, 50 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 0.1% Tween-20) five times and blocked for 1 h at 37°C with 150 µl of TBST supplemented with 1% BSA (Sigma-Aldrich® Co. LLC.). Sera from 25 patients allergic to *D. farinea* (diluted 1:8 in TBST buffer; all of the individual sera had a RAST value > 100 kU/L) were then incubated for 1 h at 37°C. Plates were washed 5 times with TBST buffer; the allergen-IgE complexes were detected by incubation with HRP-sheep anti human IgE (Sigma-Aldrich® Co. LLC.; diluted 1:1000 in TBST buffer) at 37°C for 2 h. The plate was then washed 5 times with TBST, and 3,3', 5,5'-Tetramethylbenzidine (TMB) substrate solution was added and the plate for incubated for 20 min at 37°C. Fifty microliter of stop buffer was added to each well to terminate the reaction and the absorbance of the plate was measured at 450 nm on a microplate reader (BioTek, Winooski, VT, USA). The standard curve control was performed according to the manufacturer's instructions provided by ELISA kit (R&D System, Minneapolis, MN, USA).

Development of mouse models with asthma and ASIT

Seventy-five BALB/c mice were randomly assigned to the following 5 groups with 15 mice in each group: PBS, asthma, rProDer f 1, rProDer p 1 and C 1. On days 0, 7 and 14, the mice in the PBS group received an intraperitoneal injection of 100 µl PBS, and the mice in the asthma group received an intraperitoneal injection of 10 µg mixed allergens (equal measures of rProDer f 1 and rProDer p 1). The mice in the rProDer f 1, rProDer p 1, and C 1 groups were intraperitoneally injected with 10 µg of rProDer f 1, rProDer p 1, and C 1, respectively. These injected protein were dissolved in 100 µl PBS containing 2% (W/V) $Al(OH)_3$ suspension. On day 21, the mice were caged in a home-made airway challenge apparatus, and challenged by nebulized inhalation of allergen suspension for 30 min. The mice were subsequently challenged this way for the next consecutive days. The mice in the PBS group were challenged with PBS, and the mice in the asthma group were challenged with 0.5 µg/ml of rProDer f 1. The mice in the rProDer f 1, rProDer p 1, and C 1 groups were challenged with 0.5 µg/ml of rProDer f 1, rProDer p 1, and C 1, respectively. Subsequently, the mice in the PBS group

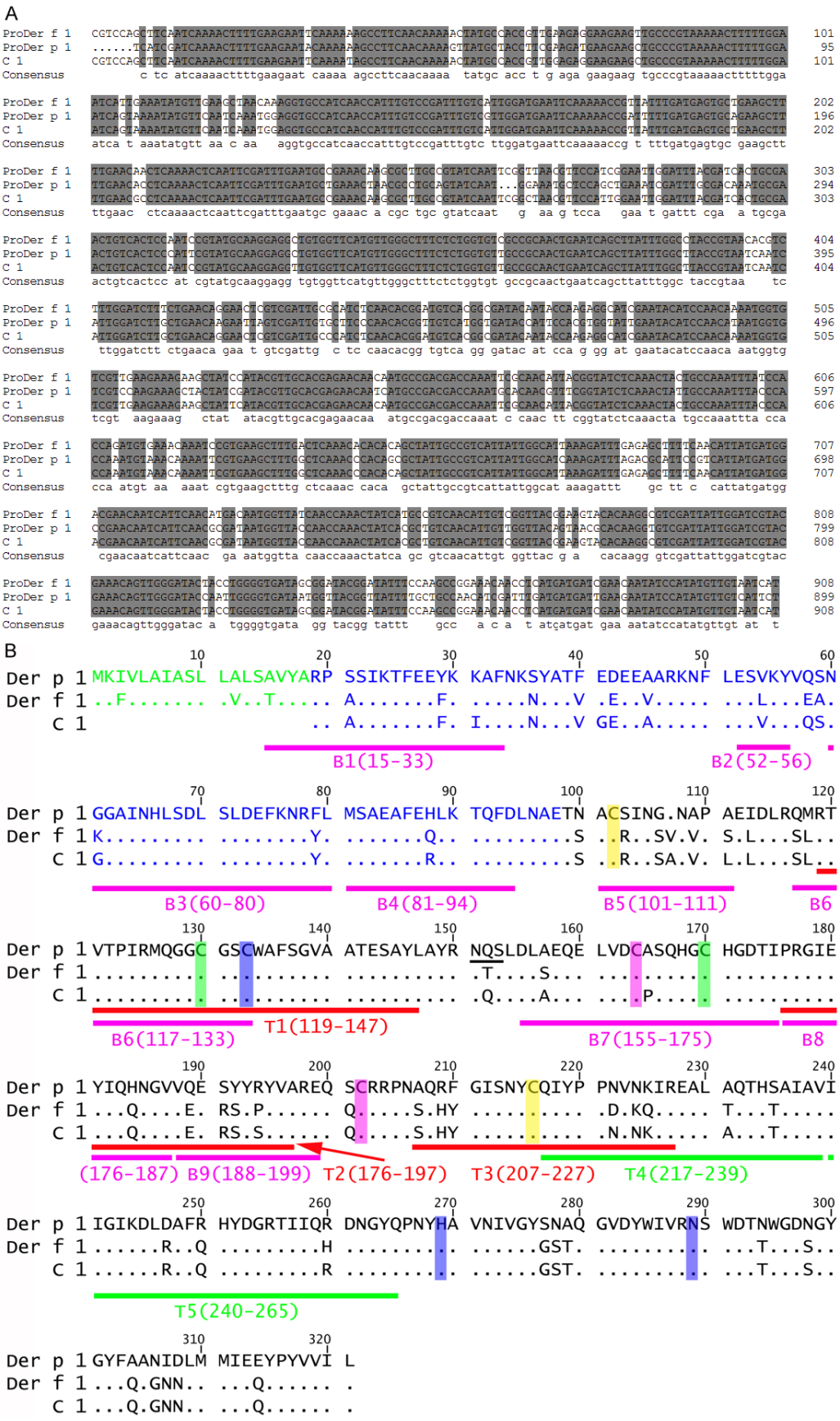
A
ProDer f 1
ProDer p 1
C 1
Consensus
B
Der p 1
Der f 1
C 1
B1(15-33)
B2(52-56)
B3(60-80)
B4(81-94)
B5(101-111)
B6
T1(119-147)
B6(117-133)
B7(155-175)
B8
(176-187)
B9(188-199)
T2(176-197)
T3(207-227)
T4(217-239)
T5(240-265)

Figure 2. Biochemical and immunological characterization of C 1. A. Alignment of the two parental genes (*ProDer f 1* and *ProDer p 1*) with the chimeric gene *C 1*. B. Amino acid sequence comparison among Der f 1 and Der p 1, and C 1. The sequences above match those used to locate the B-cell epitopes (underlined in pink) and the T-cell epitopes (underlined in red or green). Green amino acids indicate a signal peptide sequence; blue amino acids indicate a propeptide sequence; black amino acids indicate a mature sequence; a blue background represents active sites, while green, yellow, or pink backgrounds represent disulfide bridges. Potential N-glycosylation sites are underlined in black.

Table 1. Comparison of T-cell epitope counts in proteins deduced from C 1, ProDer f 1, and ProDer p 1

	HLA-DRB10301		HLA-DRB10401		Total
	Strong bond*	Weak bond*	Strong bond*	Weak bond*	
ProDer f 1	6	28	15	85	134
ProDer p 1	3	37	10	105	155
C 1	10	24	2	111	147

*According to the annotation given on http://www.cbs.dtu.dk/services/.

Table 2. Comparison of B cell epitope counts in proteins deduced from C 1, ProDer f 1, and ProDer p 1

	ProDer f 1	ProDer p 1	C 1
No. of B cell epitope	103	99	82

received both an intraperitoneal injection and aerosol inhalation of PBS. The asthma group was not subjected to a treatment. The mice in the rProDer f 1, rProDer p 1 and C 1 groups underwent SIT by intraperitoneal injection of 200 μl of rProDer f 1, rProDer p 1, and C 1 (100 μg/ml), respectively. SIT was performed each day for 30 min prior to the inhalation treatment on days 25-27.

Detection of cytokines in BALF and antibodies in sera

Twenty-four hours after the final aerosol challenge, the mice were anesthetized with an intraperitoneal injection of 100 μl of 0.5% pentobarbital sodium. The trachea of each mouse was cannulated, a syringe with 19-gauge needle was used to inject 0.3 ml of sterilized PBS into the airway through the trachea and the resulting bronchoalveolar lavage fluid (BALF) was withdraw. This was repeated 2 more times, resulting in a total volume of 0.9 ml BALF per mouse. Subsequently, the BALF was centrifuged at 3000 ×*g* for 5 min at 4°C and the supernatant was collected and stored at -80°C. Blood samples were collected via the orbital cavity, centrifuged at 4000 ×*g* for 5 min at 4°C and stored at -80°C. The amounts of IFN-γ, IL-4, IL-5, IL-10 and TGF-β in the BALF, and the amounts of serum antibodies of IgE, IgG_1 and IgG_{2a} in the serum were measured using ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

In vitro flow cytometry (FCM) determination

Twenty-four hours after the final challenge of each group of mice, their spleen was aseptically removed and processed into a splenocyte suspension using 300-steel mesh filtration. Contaminating red blood cells were lysed with an erythrocyte lysate for 5 min and the suspension was centrifuged at 1000 ×*g* for 5 min at room temperature. The precipitate was re-suspended in RPMI-1640 medium containing 10% FBS (fetal bovine serum) and the cell pellet was washed twice with RPMI-1640. The cells were resuspended in RPMI-1640 medium at a concentration 1 × 10^6/ml, and 200 μl of splenocyte suspension and 50 μl C 1 (rProDer f 1/rProDer p 1/PBS) was added to each well of a 96-well culture plate. The splenocytes isolated from the mice in the asthma group were left untreated. The cultures were maintained at 37°C and 5% CO_2 for 60 h. After the incubation, 5 μl of mouse phycoerythrin (PE)-labeled anti-CD4 and 5 μl of phycoerythrin cyanin 5.1 (PC5)-labeled anti-CD3 antibody (BD Biosciences, San Jose, CA, USA) were added to each well and incubated for 30 min in an iced bath in the dark. The samples were centrifuged at 1200 ×*g* for 10 min at 4°C. After removal of the supernatant, the sample was washed twice with 500 μl of PBS, and resuspended in 200 μl of PBS. The cell viability and purity was analyzed by flow cytometry (Coulter® Epics XL-MCL™ Flow Cytometer, Beckman Coulter, Inc., Brea, CA, USA) and the data were analyzed using Cellquest software.

Statistical analysis

Statistical analysis was done using SPSS for Windows, version 16.0 (SPSS, Chicago, IL,

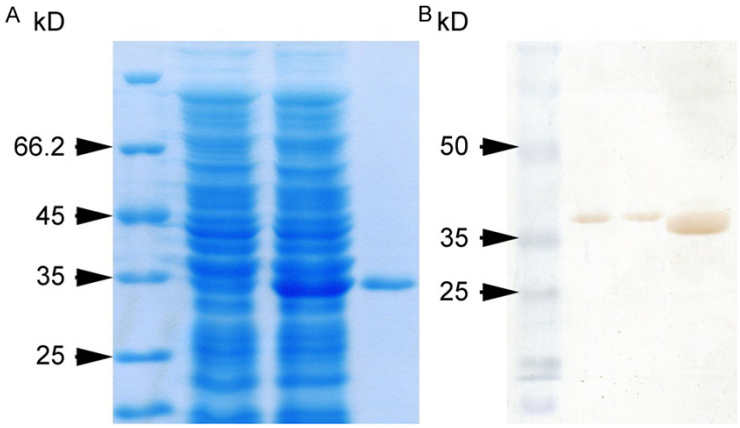

Figure 3. Purification and analysis of C 1. A. Expression and purification of C 1. Products of empty vector pET28a(+) (lane 1), products of pET28a(+)-*C 1* induction (lane 2) and purified C 1 (lane 3); B. Western blot analysis of rProDer f 1 (lane 1), rProDer p 1 (lane 2) and C 1 (lane 3).

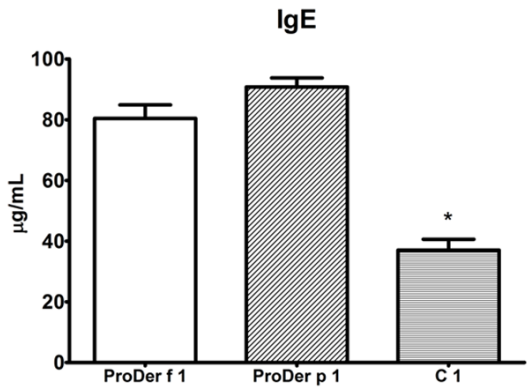

Figure 4. IgE reactivity to rProDer f 1, rProDer p 1, and C 1. **Compared with rProDer f 1 and rProDer p 1 groups, $P < 0.001$.

USA), and the statistical data for each group was expressed in $\bar{x} \pm s$ in terms of one-factor analysis of variance. The group comparisons were performed using least significant difference-*t* (LSD-*t*) and Thamhane's T_2 analysis. A *P*-value of less than 0.05 was accepted as significant.

Results

DNA shuffling

The chimeric gene pool, with *ProDer f 1* and *ProDer p 1* as templates, was successfully shuffled by PCR amplification using *ProDer f 1* primers (**Figure 1**). DNA sequencing revealed that about 10% of the clones in the library exhibited shuffled sequences and/or point mutations, based on a comparison with two parental genes. The frequency of clones without expression of full-length protein was more than 90% according to the amino acid sequences encoded by DNA assemblies. In these colonies, the *C 1* gene exhibited obvious mutation (**Figure 2A**). The amino acid sequence encoded by *C 1* was also characterized by a number of T cell epitopes which was similar to the two parental allergens (**Table 1**), but the number of B cell epitopes was reduced (**Table 2**). A comparison with the parental proteins showed that some of the characterizations of C 1 were changed, including the T cell epitopes [22] and B cell epitopes (**Figure 2B**) [23], but the basic structure of the protein (such as the disulfide backbones) were retained.

Expression and purification of C 1

C 1 was expressed as a C-terminus His6-tagged protein that accumulated in inclusion bodies in *E. coli* and was purified to homogeneity by nickel affinity chromatography under denaturing conditions. The purified C 1 was refolded using successive dialysis against decreasing concentrations of urea, and migrated as a single band at 35 kD on SDS-PAGE (**Figure 3A**). The band was also recognized by polyclonal antibodies produced in rabbits immunized with the Der f 1 allergen. This result confirmed that C 1 was homologous with two parental allergens, rProDer f 1 and rProDer p 1 (**Figure 3B**). The endotoxin content in the wild-type allergen and the shuffled allergen C 1 preparations ranged between 0.29 and 7.32 ng/mg protein (median 1.07 ng/mg). The amount of endotoxin in the preparations was not correlated with their proliferative capacity when they were analyzed in the lymphoproliferation assay.

C 1 displaying a reduced IgE-binding reactivity

The sera were tested by ELISA using plates coated with equivalent amounts of rProDer f 1, rProDer p 1 or C 1. Results (**Figure 4**) showed that the binding capacity of C 1 to IgE from the sera of *D. farinea*-allergic patients (37.03 ± 12.46 μg/ml; $P < 0.001$) was strikingly reduced as compared to the binding capacity of the

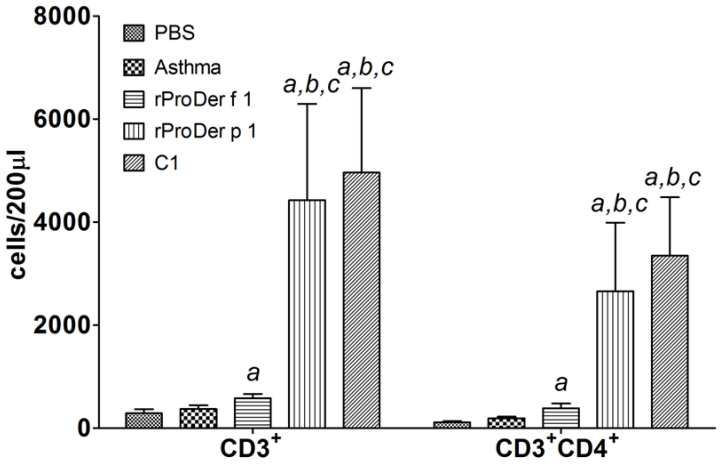

Figure 5. Number of $CD3^+CD4^+$ T cells in each experimental group as determined by FCM. Note: *a*, compared with PBS group, $P < 0.01$; *b*, compared with asthma group, $P < 0.01$; *c*, compared with rProDer f 1 group, $P < 0.01$.

parental allergens, rProDer f 1 (80.44 ± 15.50 μg/ml) and rProDer p 1 (90.79 ± 10.38 μg/ml).

C 1 enabling T-cell stimulation

To further observe whether C 1 was sufficient to stimulate the differentiation of ProDer f 1/ ProDer p 1-specific T cells, we analyzed spleen cells taken from mice that had been immunized to rProDer f 1, rProDer p 1 or C 1 *in vitro*. The number of $CD3^+CD4^+$ T cells in each group was detected using flow cytometry. The results demonstrated that C 1 (3347 ± 1136 cells/200 μl) was able to re-stimulate the $CD3^+CD4^+$ T cells isolated from mice immunized with C 1, but there had little effect on $CD3^+CD4^+$ T cells isolated from mice in the PBS group (112 ± 20 cells/200 μl, $P < 0.01$), asthma group (188 ± 34 cells/200 μl, $P < 0.01$) and rProDer f 1 group (383 ± 93 cells/200 μl, $P < 0.01$). This result suggests that C 1 retained the relevant ProDer f 1-specific T cell epitopes (**Figure 5**). However, there was no statistically significant difference between the C 1 group and rProDer p 1 group (2658 ± 1130 cells/200 μl, $P = 0.775$).

Evaluation of the prophylactic potential of C 1 in mouse model

To verify the effects of C 1 on ASIT in an animal asthma model, mice were sensitized and challenged with these allergen proteins and their BALF was analyzed for the amount of IFN-γ, IL-4, and IL-5 and the amount of IgE, IgG_1 and IgG_{2a} in their serum was analyzed by ELISA. Similar to mice immunized with rProDer f 1 (322.98 ± 30.36 pg/ml) and rProDer p 1 (314.97 ± 33.89 pg/ml), mice immunized by C 1 produced a higher amount of IFN-γ (343.43 ± 38.79 pg/ml) than mice in the asthma group (208.44 ± 46.11 pg/ml, $P < 0.01$) (**Figure 6A**). Conversely, the results in **Figure 6B** and **6C** show a lower productions of IL-4 (37.01 ± 4.00 pg/ml), and IL-5 (118.65 ± 24.23 pg/ml) by mice immunized with C 1, compared to IL-4 and IL-5 levels for the asthma group (74.29 ± 9.70 pg/ml, $P < 0.01$; 379.10 ± 38.39 pg/ml, $P < 0.01$; respectively). However, there was no statistically significant difference in IL-4 and IL-5 production between rProDer f 1 (43.97 ± 10.13 pg/ml in IL-4; 241.58 ± 37.18 pg/ml in IL-5) and rProDer p 1 (55.06 ± 9.68 pg/ml in IL-4, $P > 0.05$; 304.70 ± 39.90 pg/ml in IL-5, $P > 0.05$) immunized mice. The results in **Figure 6D** also show that mice immunized with C 1 produced more IL-10 (341.10 ± 79.09 pg/ml) compared to mice in the PBS (68.84 ± 14.73 pg/ml, $P < 0.01$), asthma (103.14 ± 19.59 pg/ml, $P < 0.01$), rProDer f 1 (232.01 ± 51.22 pg/ml, $P < 0.01$), and rProDer p 1 groups (248.12 ± 80.13 pg/ml, $P < 0.05$). As shown in **Figure 6E**, a similar trend was observed in the level of TGF-β produced by mice in the C 1 group (76.89 ± 11.13 pg/ml, $P < 0.01$), compared with mice in the PBS (0.35 ± 0.46 pg/ml, $P < 0.01$), asthma (31.78 ± 8.82 pg/ml, $P < 0.01$), rProDer f 1 (57.17 ± 9.40 pg/ml, $P < 0.01$), and rProDer p 1 groups (59.37 ± 16.0 pg/ml) ($P < 0.05$).

An analysis of the serum IgE, IgG_1 and IgG_{2a} antibody levels in **Figure 7** suggests that the amount of IgE in the serum of mice in the C 1 group (26.84 ± 5.09 μg/ml) was significantly lower than in the mice in the asthma (53.55 ± 9.37 μg/ml, $P < 0.01$), rProDer f 1 (34.07 ± 4.72 μg/ml, $P < 0.01$) and rProDer p 1 groups (38.48 ± 4.40 μg/ml, $P < 0.01$). The serum IgG_1 levels were also significantly different between the mice in the C 1 group (28.84 ± 3.77 μg/ml) and the PBS (7.79 ± 3.40 μg/ml, $P < 0.01$), asthma (56.48 ± 8.27 μg/ml, $P < 0.01$), rProDer f 1 (35.47 ± 3.77 μg/ml, $P < 0.01$) and rProDer p1 groups (37.55 ± 4.52 μg/ml, $P < 0.01$).

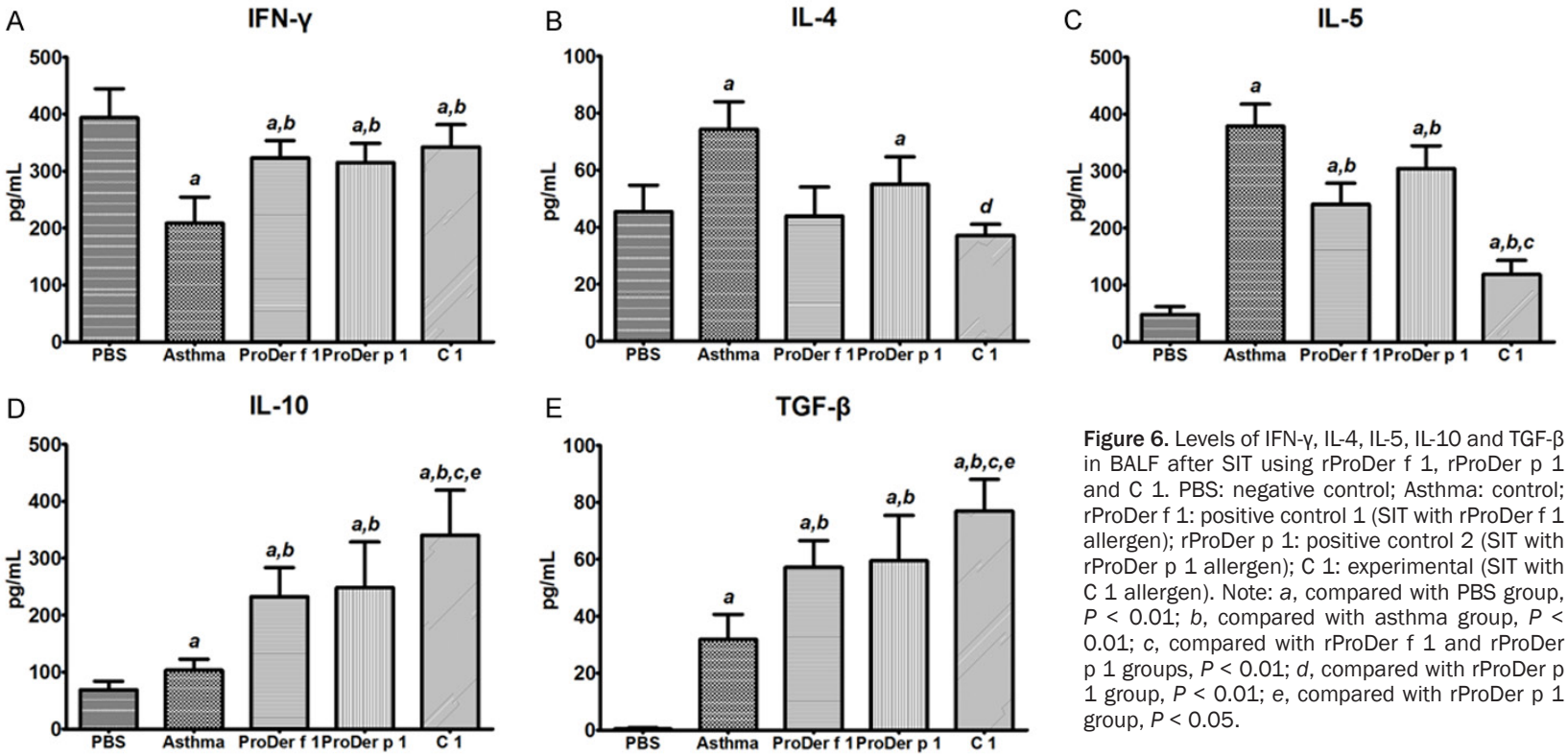

Figure 6. Levels of IFN-γ, IL-4, IL-5, IL-10 and TGF-β in BALF after SIT using rProDer f 1, rProDer p 1 and C 1. PBS: negative control; Asthma: control; rProDer f 1: positive control 1 (SIT with rProDer f 1 allergen); rProDer p 1: positive control 2 (SIT with rProDer p 1 allergen); C 1: experimental (SIT with C 1 allergen). Note: *a*, compared with PBS group, $P < 0.01$; *b*, compared with asthma group, $P < 0.01$; *c*, compared with rProDer f 1 and rProDer p 1 groups, $P < 0.01$; *d*, compared with rProDer p 1 group, $P < 0.01$; *e*, compared with rProDer p 1 group, $P < 0.05$.

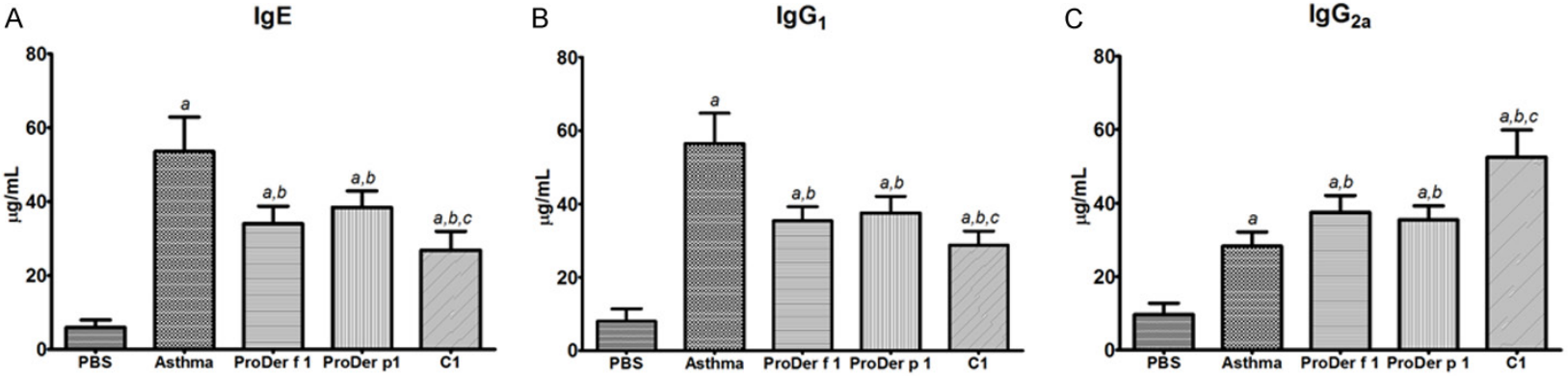

Figure 7. The levels of IgE, IgG_1 and IgG_{2a} antibody in sera after SIT using rProDer f 1, rProDer p 1 and C 1. PBS: negative control; Asthma: control; rProDer f 1: positive control 1 (SIT with rProDer f 1 allergen); rProDer p 1: positive control 2 (SIT with rProDer p 1 allergen); C 1: experimental (SIT with C 1 allergen). Note: *a*, compared with PBS group, $P < 0.01$; *b*, compared with asthma group, $P < 0.01$; *c*, compared with rProDer f 1 and rProDer p 1 groups, $P < 0.01$.

Finally, the serum IgG_{2a} levels in C 1 immunized mice (52.55 ± 7.37 μg/ml) was significantly higher compared to the mice in the PBS (9.76 ± 3.03 μg/ml, $P < 0.01$), asthma (28.37 ± 3.82 μg/ml, $P < 0.01$), rProDer f 1 (37.55 ± 4.52 μg/ml, $P < 0.01$) and rProDer p 1 groups (35.47 ± 3.77 μg/ml) ($P < 0.01$).

Discussion

House dust mites of the *Dermatophagoides* genus (*D. pteronyssinus* and *D. farinae*) are an important causative factor of various allergic diseases, such as bronchial asthma [24, 25]. Group 1 (Der p 1 and Der f 1) allergens are considered to be one of the major house dust mite allergens, based on the frequency of sensitization in allergic patients, their ability to induce high amounts of specific IgE, and their high content in mite extracts [26]. SIT for mite allergy is routinely carried out using mite extracts that are prepared from whole dust mite extracts. However, the use of such crude extracts has numerous disadvantages [27], including the presence of undefined nonallergenic materials, difficulties in extract standardization, the risk of inducing anaphylactic side-effects and induction of new IgE sensitizations [28-30]. However, the use of DNA technologies can address these problems and recombinant allergens with desirable properties have become available [31, 32]. Hypoallergens with poor IgE-binding capacity but sustained immunogenicity have been obtained by recombinant DNA technologies [33-35]. In this study, we attempted to experimentally recombine the group 1 allergen genes (*ProDer f 1* and *ProDer p 1*) *in vitro* using DNA shuffling and then we screened for proteins that had reduced IgE reactivity while retaining their T-cell epitopes. A sequence analysis showed some mutants in B cell epitopes, but the basic structure was unaffected. The screening result suggested that the chimeric gene *C 1* met the criteria for potential use for SIT.

To further verify the low allergenicity but preserved immunogenicity of C 1, we determined the binding capacity of C 1 to IgE in sera from mite-sensitized patients and showed that C 1 had a weakened binding to IgE, compared with the two parental allergens, and suggested that C 1 bears the property of hypoallergenicity. Group 1 mite allergens display strictly conformational IgE-binding epitopes [36, 37]. However, the process of shuffling carried frame-shifts and point-mutations that produced a C 1 variant which essentially reduced IgE binding compared with the parental proteins ProDer f 1 and ProDer p 1. One possible explanation for the decreased IgE binding capacity of C 1 is that the substituted amino acid residue(s) is/are part of a dominant B-cell epitope [15]. Another plausible explanation for the decreased IgE reactivity is that the single amino acid substituted in C 1 is important for the tertiary structure of the entire allergen and that the substitution disrupted one or more conformational epitopes [15]. To determine the immunogenicity of C 1, we examined the number of T_H cells isolated from the spleen of mice immunized with the different protein constructs, and found that the number of T_H cells in the mouse spleens was higher in the C 1 group compared with the asthma and rProDer f 1 groups, but was not significantly different from the rProDer p 1 group. These findings strongly suggest that C 1 possesses immunogenicity greater than or equal to that of the two parental proteins. Also, the T cell epitopes in C 1 represent the preservation or recruitment of T cell epitopes from the parental proteins.

Successful SIT leads to allergen non-responsiveness and symptom relief [12, 38], including major changes in the T-cell response to allergens, either through immune deviation of T_H1/T_H2 imbalance or by induction of allergen-specific antibodies to prevent immediate-type reactions. In previous studies, hypoallergenic derivatives from parental allergens have produced high levels of IgG recognizing wild-type allergens [15, 16, 28, 39]. To further understand the differentiation of subtypes of T_H cells, we measured the amount of IFN-γ, IL-4 and IL-5 produced by T_H1/T_H2 cells in the BALF of these mice. We also measured the amount of IL-10 and TGF-β associated with regulatory T cell differentiation. As expected, the levels of IFN-γ, a

cytokine predominantly secreted by T_H1, were not strikingly different between the two groups of mice immunized with the parental proteins, while IL-4 and IL-5 levels, primarily generated by T_H2 cell, were greatly reduced in the C 1 immunized mice compared with mice immunized with the two parental proteins. This implies that C 1 can activate T_H1 cell differentiation and inhibit T_H2 cell differentiation in the same way as the parental proteins, in agreement with a previous study [40]. An increase in IL-10 and TGF-β suggests that SIT with C 1 promotes Treg cell differentiation/proliferation. Furthermore, the results of our study show that IgG_1 levels were significantly down-regulated in C 1 group but IgG_{2a} levels were strikingly raised compared with the other experimental groups. This data strongly suggests that C 1 can modify the imbalance of T_H1 and T_H2 cells and rectify T_H1-biased allergic responses, a necessary process for any therapy that hopes to ameliorate the process of allergic inflammation.

In conclusion, this study recombined the group 1 allergen genes from dust mites using DNA shuffling and the chimeric gene *C 1* was successfully screened and expressed. A series of observations confirmed that the chimeric protein C 1 inhibited IgE production, possessed properties of hypoallergenicity, and had the ability to activate T cell differentiation, similar to its two parental proteins. Treatment with the C 1 construct resulted in T_H1 cell predominance, suggesting that C 1 may restore the balance between T_H1/T_H2 cells. Most importantly, our study offers further evidence that DNA shuffling is an effective method for *in vitro* recombination of homologous genes in a directed evolution experiment. Successfully screening high-grade chimeric C 1 favorable to SIT will form a basis for further investigation of eligible treatment options for allergic disorders involving anaphylactic dust mites.

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Disclosure of conflict of interest

None.

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