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Original Article

Design of a *ProDer f* 1 vaccine delivered by the MHC class II pathway of antigen presentation and analysis of the effectiveness for specific immunotherapy

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Abstract: *Dermatophagoides farinae* (*Der f* 1) is one of leading cause for allergic asthma, and allergen-specific immunotherapy (SIT) is currently recognized as the only etiological therapy to ameliorate asthmatic symptom. The current study was designed on the major histocompatibility complex (MHC) class II pathway, invariant chain (Ii)-segment hybrids as vaccine basis to explore the efficacy of *Der f* 1 hybrid vaccine by virtue of Ii as carrier in enhancing the protective immune response to asthma. Initially, we engineered a fused molecule, DCP-IhC-*ProDer f* 1, to deliver *ProDer f* 1 antigen via specific dendritic cell-targeting peptides to dendritic cells (DCs). Then the DCP-IhC-*ProDer f* 1 was immunized to the asthmatic models of murine induced by *ProDer f* 1 allergen. The findings showed that the cytokine repertoire in the murine model was shifted after SIT, including stronger secretion of IFN-γ and IL-10, and a decreased production of IL-4 and IL-17. ELISA determination revealed that the hybrid displayed weak IgE and IgG_1 reactivities, and IgG_{2a} levels were elevated. Furthermore, DCP-IhC-*ProDer f* 1 treatment inhibited inflammatory cell infiltration in the lung tissues. Our results suggest that the DCP-Ihc-*ProDer f* 1 may be used as a candidate SIT against asthma.

Keywords: *Dermatophagoides farinae*, major histocompatibility complex, specific immunotherapy, dendritic cell, invariant chain

Introduction

Allergic asthma is the most common type I hypersensitivity, characterized by chronic airway inflammation, mucus production, and airway hyperresponsiveness [1, 2]. The group 1 allergens of House dust mites (HDMs) are the prevalent causes of asthma and responsible for IgE-mediated sensitization, which is a potent risk factor for developing allergic diseases, including asthma [1, 3, 4]. Patients allergic to HDMs (> 80%) may have IgE antibodies against the group 1 mite allergen *Der f 1* derived from the *Dermatophagoides farinae* (*D. farinae*), one of the HDM species [5, 6].

Although the origin of asthma remains unclear, it is well acknowledged that asthma attack is associated with imbalanced Th1/Th2 paradigm [7], marked by the predominance of type 2 cytokines secreted by Th2 cells. However, some studies demonstrated that certain $CD4^+$ T cell subsets also play pivotal roles in pathogenesis of asthma, including regulatory T (Treg) cells and Th17 cells [8-12]. For example, Treg cells are critical players in controlling the development of asthma and allergy via several mechanisms [10, 13], and IL-17 secreted by Th17 was also proved to be an indirect role in airway inflammation through stimulating fibroblasts to produce inflammatory mediators [14].

Allergen specific immunotherapy (SIT) is the sole allergen-specific, causative treatment of allergy [1, 15], and the therapy involves repetitive allergen use either by subcutaneous injection or sublingual administration. This regimen is able to modify the natural course of asthma and alleviate the symptoms of asthmatic patients, yet commonly accompanied by severe side-effects such as anaphylactic shock [16].

Table 1. Primers used in this project

Primers	Sequences	Restrict site
DCP-F	5'-**GATCT**TTTTATCCGAGTTATCATAGTACTCCTCAGCGGCCT**GGATCC*ACTAGT***-3'	*Bgl* II, *Bam*H I, *Spe* I
DCP-R	5'-**TCGAG*ACTAGT*GGATCC**AGGCCGCTGAGGAGTACTATGATAACTCGGATAAAA-3'	*Xho* I, *Spe* I, *B*amHI
IhC-F	5'-GA**AGATCT**ATG GATGACCAGCGCGACC-3'	*Bgl* II
IhC-R	5'-GG***ACTAGT*GGATCC** CCCCTGGGGCAGGGCTCC-3'	*Spe* I, *Bam*H I
ProDer f 1-F	5'-TAT**GGATCC**CGTCCAGCTTCAATCAAAACT-3'	*Bam*H I
ProDer f 1-R	5'-GGC**CTCGAG**TCACATGATTACAACATATGG-3'	*Xho* I

AGCTT in bold in the DCP-F and DCP-R primers indicates the restriction site for *Bgl* II and *Hind* III, which are isocaudomers; ACTAGT in bold italics in the DCP-F, DCP-R, IhC-R primers denotes the restriction site for *Spe* I. *BamH* I and *Xho* I restriction sites are marked in bold.

Major histocompatibility complex (MHC) class II molecules are recognized work efficiencies in antigen presentation for initiating the specific immune response [17]. In this process, the invariant chain (Ii), a type II integral membrane protein, acts as a chaperone for preventing self-peptide binding to MHC II in the endoplasmic reticulum (ER) [18, 19]. With the occurrence of proteolyzed degradation of Ii in lysosomes/endosomes, the class II-associated invariant chain peptide (CLIP) is replaced by an antigen peptide [20].

Previous studies reported that the linkage of Ii-Key to antigen or related epitope was capable of enhancing MHC class epitope charging and Th cell activation [18, 21-24]. The Ii-Key hybrids are composed of the Ii moiety linked to the N-terminus of an MHC class II epitope via a simple polymethylene as a bridge [23]. The novel technique for the induction of T helper (Th) cell activity has drawn growing attention [18, 25, 26]. Crameri *et al*. [18] described that Ii-allergen hybrids targeting to the MHC class II pathway enhanced $CD4^+$ T cell proliferation, increased IFN-γ and IL-10 secretion, but decreased IL-4 and IL-5 production.

Apart from the Ii-Key, some of the Ii segments also have a potential immune function, such as the first 110 amino-acids of Ii (IhC) [18] and the DN (Asn-Asp) segment [17]. Hypothetically, IhC would promote epitopes in *ProDer f* 1 association with MHC charging and enhance specific immune responses. Therefore, we tentatively constructed such hybrid to analyze its ability to treat the murine asthmatic model after SIT. The hybrid consists of dendritic cell peptide (DCP, FYPSYHSTPQRP) for binding fused allergen to the surface of DC [27, 28], IhC for enhancing epitope charging from fusion protein with MHC class II, and *ProDer f* 1 allergen.

Materials and methods

Cloning and construction of the hybrids

We cloned *DCP* cDNA fragment through anneal using complementary synthetic oligonucleotides (**Table 1**). The cDNA fragment was used to encode a hypothetical peptide sequence FYPSYHSTPQRP (GenBank No. AJ544526.1). The *DCP* fragment was inserted into the *Bam*H I-*Xho* I-digested pET28a to create recombinant vector pET28a-*DCP*. We also cloned the $IhC_{1\text{-}110}$ coding fragment (GenBank No. K01144.1) by PCR using specific primers (**Table 1**). The PCR product was digested with *Bgl* II/*Spe* I and cloned into *BamH* I/*Spe* I digested *pET28a-DCP* to generate the recombinant *pET28a-DCP-IhC* vector. The full-length *ProDer f* 1 coding sequence was amplified by PCR using the specific primers (**Table 1**). Subsequently, the amplicon was digested with *BamH* I/*Xho* I and inserted into the *pET28a-DCP*, *pET28a-DCP-IhC* vectors to create recombinant plasmids *pET-28a-DCP-ProDer f 1*, *pET28a-DCP-IhC-ProDer f 1*, respectively (**Figure 1**). Nucleotide sequences were identified by sequencing.

Expression and purification of the recombinant antigens

The recombinant plasmids, *pET28a-ProDer f 1, pET28a-DCP-ProDer f 1* and *pET28a-DCP-IhC-ProDer f 1* were transfected into *E. coli* expression strain BL21 (DE3). Antigen expression was induced by 1 mmol/L isopropyl-β-thiogalactopyranoside (IPTG). The total protein was purified under denaturing conditions with Ni^{2+}-chelate affinity chromatography with Ni^{2+}-NTA His·Bind® Resins (Invitrogen, Carlsbad, CA, USA). Eluted protein was dialyzed into 1000 volumes of 1× PBS (pH7.2) at 4°C. The proteins were assessed by SDS-PAGE using 12.5% poly-

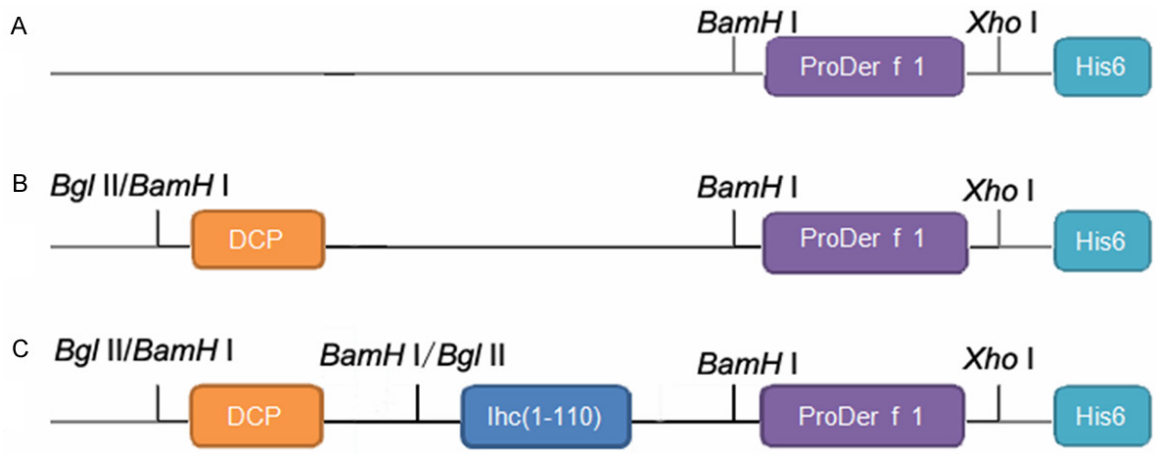

Figure 1. Schematic diagram of hybrids for expressing *ProDer f* 1, DCP-*ProDer f* 1 and DCP-IhC-*ProDer f* 1. $[His]_6$-tag is used to purify hybrids.

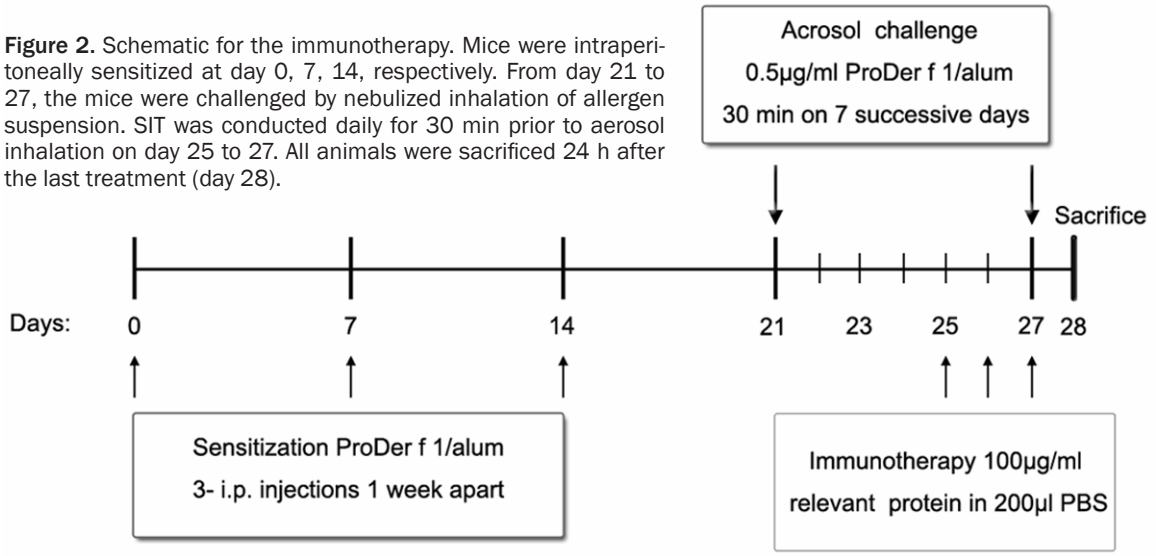

Figure 2. Schematic for the immunotherapy. Mice were intraperitoneally sensitized at day 0, 7, 14, respectively. From day 21 to 27, the mice were challenged by nebulized inhalation of allergen suspension. SIT was conducted daily for 30 min prior to aerosol inhalation on day 25 to 27. All animals were sacrificed 24 h after the last treatment (day 28).

acrylamide gel and Coomassie-blue staining. Protein concentration was measured by the Bradford protein assay according to the manufacturer's instructions (Sangon Biotech, Shanghai, China).

Western blot

In order to detect the hybrids prepared previously, 5 μg of total soluble proteins were boiled for 10 min in SDS buffer containing 5% β-mercaptoethanol (β-ME) and separated by 12.5% SDS-PAGE, and then transferred onto a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA), and the membranes were subjected to immunoblotting with anti-rabbit His_6-tag polyclonal antibodies (Sangon Biotech, Shanghai, China).

Animals and immunization

BALB/c female mice, aged 8 to 10 weeks, were obtained from the Animal Center for Comparative Medicine, Yangzhou University (License No. SCXK 2007-0001) and bred under conventional pathogen-free conditions. Food and water were supplied *ad libitum*. The protocol was approved by the Animal Research Ethics Board of Wannan Medical College.

Fifty mice were randomly divided into four groups (n = 10 for each), i.e. PBS group, asthma group, DCP-*ProDer f* 1 group, DCP-Ihc-*ProDer f* 1 group, and *ProDer f* 1 group. Mice were sensitized with intraperitoneal injection of 10 μg relevant allergen in 100 μl PBS (pH7.2) containing 2% (W/V) $Al(OH)_3$ at day 0, 7 and 14, respec-

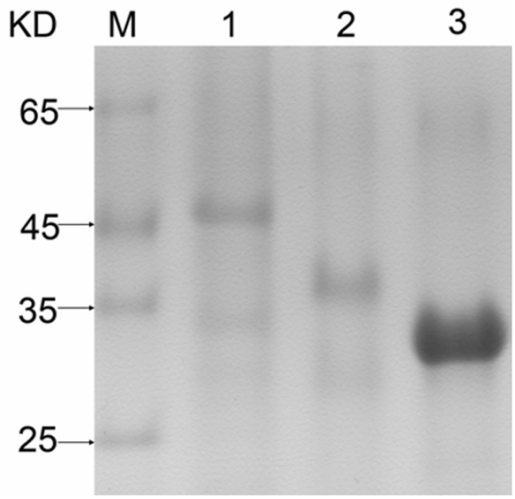

Figure 3. SDS-PAGE analysis of purified recombinant proteins expressed in *E. Coli* BL21. M: Protein marker (kD); Lane 1: DCP-Ihc-*ProDer f* 1; Lane 2: DCP-*ProDer f* 1; Lane 3: *ProDer f* 1.

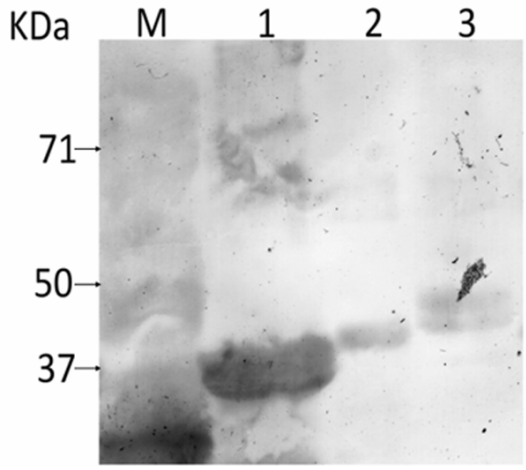

Figure 4. Western blot analysis of the purified recombinant allergens. M: Protein marker (kD); Lane 1: *ProDer f* 1; Lane 2: DCP-*ProDer f* 1; Lane 3: DCP-Ihc-*ProDer f* 1.

tively. The PBS groups were exclusively treated with PBS. The mice in asthma, DCP-*ProDer f* 1, DCP-Ihc-*ProDer f* 1 and *ProDer f* 1 groups were exposed daily for 30 min to nebulized *ProDer f* 1 (0.5 μg/ml) from day 21 to 27. The PBS groups were challenged by nebulized inhalation of PBS instead. After model development, the mice in DCP-*ProDer f* 1, DCP-Ihc-*ProDer f* 1 and *ProDer f* 1 groups underwent ASIT via intraperitoneal injection of DCP-*ProDer f* 1, DCP-Ihc-*ProDer f* 1 and *ProDer f 1* (100 μg/ml), respectively in dose of 200 μl. SIT was performed daily for 30 min prior to aerosol inhalation on day 25 to 27. PBS group were treated both intraperitoneal injection and nebulized inhalation of PBS. Asthma group were devoid of management. 24 h after the final inhalation challenge, the serum and the bronchoalveolar lavage fluid (BALF) were collected according to previous description [29], and were stored at -80°C for further analysis (**Figure 2**).

Measurement of antibodies in sera and cytokines in BALF

ELISA was performed to measure the serum levels of IgE, IgG_1 and IgG_{2a} as well as levels of IFN-γ, IL-4, IL-10, and IL-17 in BALF, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

Histopathologic analysis of the pulmonary tissues

The mouse lung tissues were fixed in 4% neutral formalin and embedded in paraffin, and the paraffin sections were cut into 5 μm thickness using a microtome. The pulmonary sections were put onto poly-L-lysine-coated slides and stained with hematoxylin and eosin (HE). The inflammatory changes were microscopically assessed based on the extent of eosinophils infiltration, epithelia damage, and edema in the lung.

Statistical analysis

The statistical data for each group were expressed in mean ± SD and analyzed using the GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were analyzed using the one-way analysis of variance and Dunnett comparison. Differences were considered to be statistically significant when $P < 0.05$.

Results

Expression and purification of hybrids

The pET28a vector was modified by inserting the sequences encoding for *ProDer f* 1, DCP-*ProDer f* 1, and DCP-IhC-*ProDer f* 1. The harvested plasmids pET28a-*ProDer f 1*, pET28a-*DCP-ProDer f 1*, and pET28a-*DCP-IhC-ProDer f 1* were used to transfect *E. coli* BL21 (DE3).

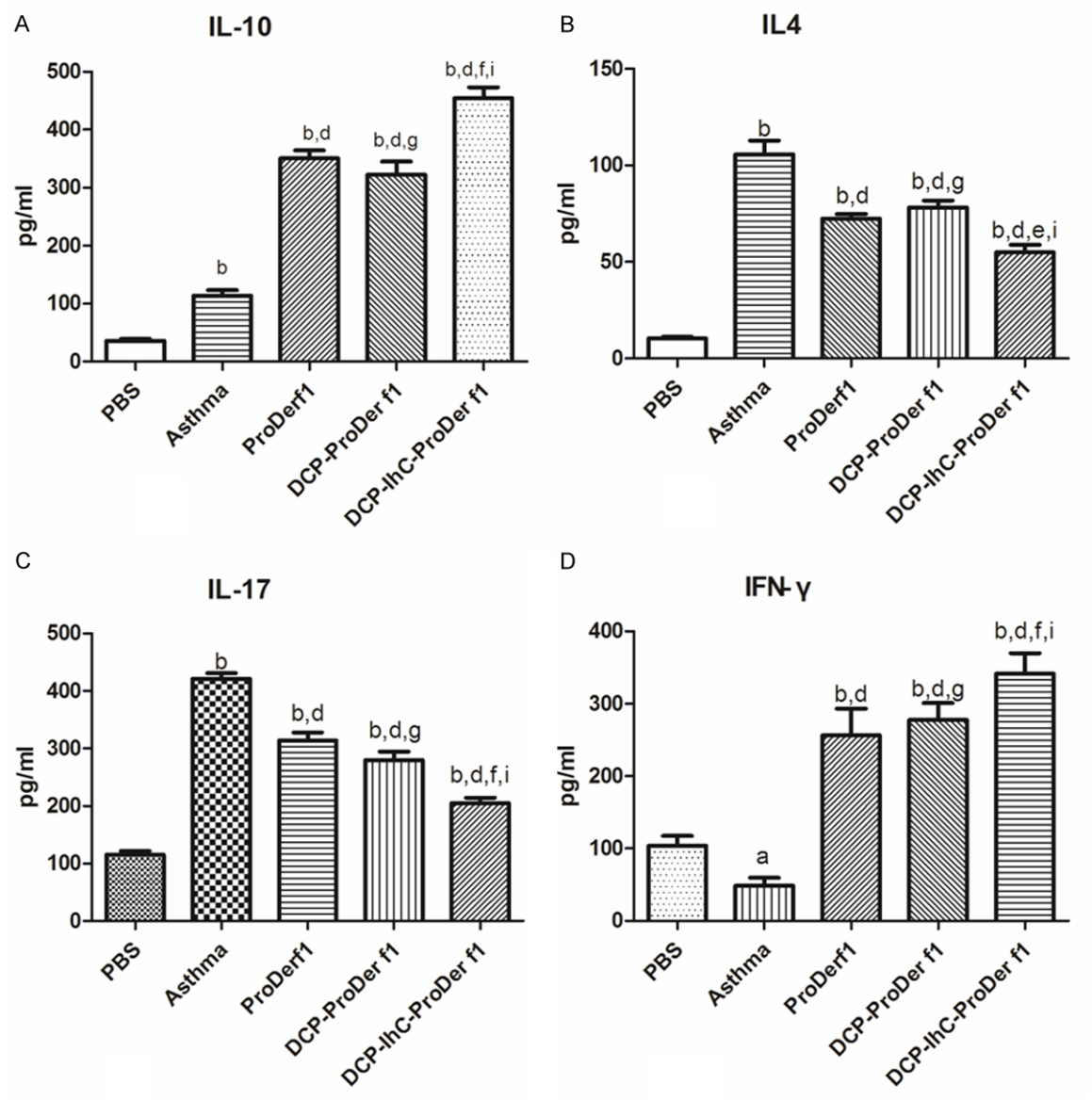

Figure 5. The levels of IL-4, IL-10, IL-17 and IFN-γ in BALF. Note: Compared with PBS group, [a]$P < 0.05$, [b]$P < 0.01$; Compared with asthma group, [c]$P < 0.05$, [d]$P < 0.01$; *Vs.* the *ProDer f* 1 group, [e]$P < 0.05$, [f]$P < 0.01$, [g]$P > 0.05$; *Vs.* the DCP -*ProDer f* 1 group, [h]$P < 0.05$, [i]$P < 0.01$.

After 5 h induction with 1mmol/L IPTG, the amplicons were collected and subjected to further analysis. *ProDer f* 1, *DCP-ProDer f* 1, and DCP-IhC-*ProDer f* 1 were purified to homogeneity by nickel affinity chromatography under denaturing conditions. The purified hybrids were generated to migrate as a single band, respectively on SDS-PAGE (**Figure 3**). The bands were recognized with rabbit polyclonal antibodies raised to $[His]_6$ (**Figure 4**). Subsequently, the hybrids were refolded by seccessive dialysis and against decreasing concentrasions of urea.

Modulatory potency of DCP-Ihc-ProDer f 1 in sensitized murine model with ProDer f 1 allergen

To assess the potential utility of the hybrid DCP-Ihc-*ProDer f* 1 in modulating the immune response, ELISA was performed to measure the level variation in IL-4, IL-10, IL-17 and IFN-γ in BALFs, as well as in serum IgE, IgG_1 and IgG_{2a}.

Compared with the asthma group (105.7 ± 16.21 pg/ml), mice vaccinated with DCP-Ihc-

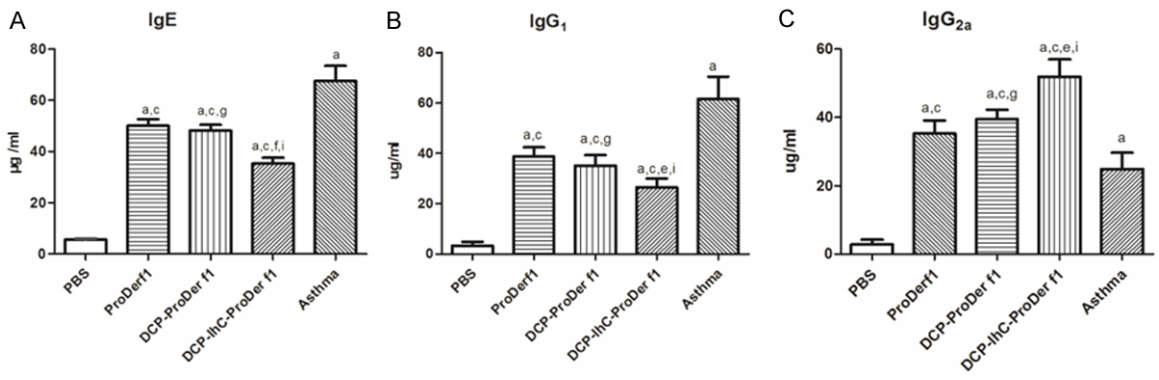

Figure 6. Level of specific IgE, IgG_1 and IgG_{2a} in sera of mice Note: *Vs.* PBS group, $^{a}P < 0.01$, $^{b}P < 0.05$; *Vs.* the asthma group, $^{c}P < 0.01$, $^{d}P < 0.05$; *Vs.* the *ProDerf* 1 group, $^{e}P < 0.01$, $^{f}P < 0.05$, $^{g}P > 0.05$; *Vs.* the DCP-*ProDer f* 1 group, $^{h}P < 0.01$, $^{i}P < 0.05$.

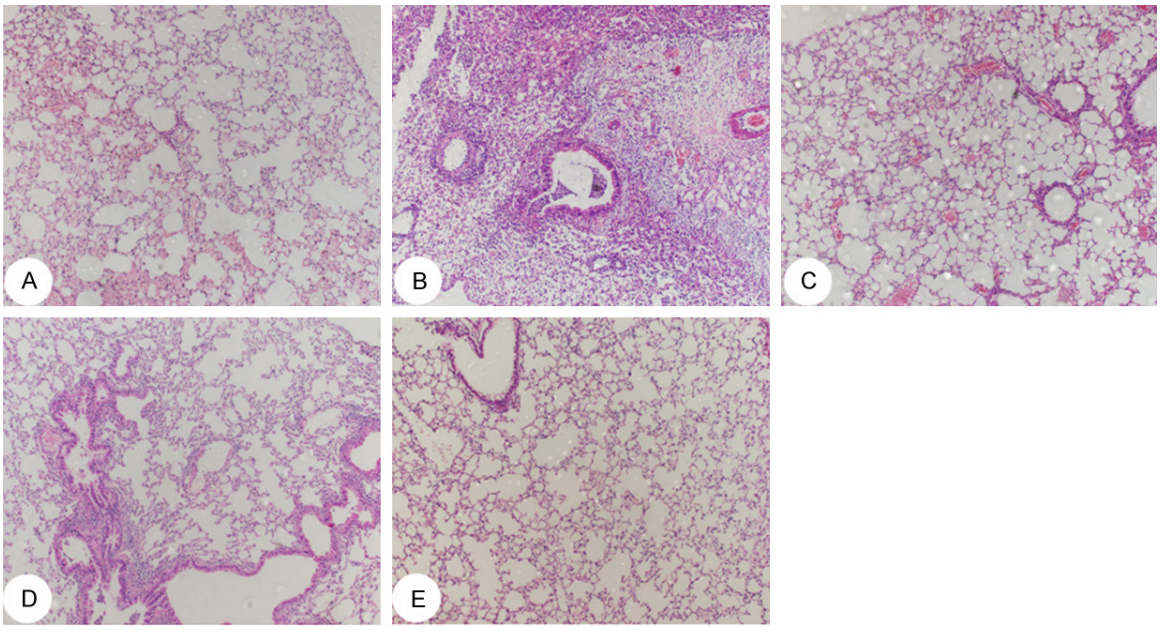

Figure 7. The effect of DCP-IhC-*ProDer f* 1 treatment on lung pathology (HE staining ×100). The mice were sensitized and treated as described in the Materials and Methods section. No evidence of histological injury was noted in naïve animals (A). Severe pulmonary inflammation in the areas adjacent to various sized airways in *ProDer f* 1-sensitized/challenged mouse (B), whereas the inflammation, goblet cell hyperplasia and mucus production were relatively minor in the lungs of mice treated with *ProDer f* 1, DCP-*ProDer f* 1 or DCP-IhC-*ProDer f* 1 (C-E). (A) PBS group; (B) Asthma group; (C) *ProDer f* 1 group; (D) DCP-*ProDer f* 1 group; (E) DCP-Ihc-*ProDer f* 1 group.

ProDer f 1 produced a lower level of IL-4 (55.08 ± 8.6 pg/ml) ($P < 0.01$), *ProDer f* 1 (72.53 ± 4.9 μg/ml) ($P < 0.05$), and DCP-*ProDer f* 1 (78.31 ± 7.84 pg/ml) ($P < 0.01$) (**Figure 5B**). Similar trends were seen regarding the levels of IL-17 ($P < 0.01$) (**Figure 5C**). Conversely, the mice immunized by DCP-Ihc-*ProDer f* 1 generated higher level of IFN-γ (341.6 ± 28.17 pg/ml) than asthma group (48.75 ± 10.82 pg/ml), *ProDer f* 1 group (256.2 ± 37.38 pg/ml) and DCP-*ProDer f* 1 (277.6 ± 23.38 pg/ml) group ($P < 0.01$) (**Figure 5D**). Similarly, such trends were also seen regarding the levels of IL-10 ($P < 0.01$, **Figure 5A**). However, there was no statistical difference between *ProDer f* 1 and DCP-*ProDer f* 1 groups by the levels of cytokines, including IL-4, IL-10, IL-17 and IFN-γ ($P > 0.05$).

The measured serum IgE, IgG_1 and IgG_{2a} antibody suggested that the level of IgE (35.26 ±

5.02 μg/ml) in DCP-Ihc-*ProDer f* 1 group was significantly lower than that of the asthma group (67.56 ± 13.09 μg/ml, $P < 0.01$), DCP-*ProDer f* 1 group (48.09 ± 5.29 μg/ml, $P < 0.05$) and *ProDer f* 1 group (50.02 ± 5.75 μg/ml, $P < 0.05$) (**Figure 6A**). Moreover, The IgG_1 level in the DCP-Ihc-*ProDer f* 1 group (26.48 ± 3.53 μg/ml) were also significantly decreased compared to asthma group (61.51 ± 8.9 μg/ml, $P < 0.01$), *ProDer f* 1 group (38.68 ± 3.64 μg/ml, $P < 0.01$) and DCP-*ProDer f* 1 (35.09 ± 4.12 μg/ml, $P < 0.05$) (**Figure 6B**). In contrast, significant higher IgG_{2a} level was found in the DCP-Ihc-*ProDer f* 1 group (51.9 ± 5.06 μg/ml) than asthma group (24.95 ± 4.71 μg/ml, $P < 0.01$), the *ProDer f* 1 group (35.29 ± 3.77 μg/ml, $P < 0.01$) and the DCP-*ProDer f* 1 group (39.54 ± 2.70 μg/ml, $P < 0.05$) (**Figure 6A**). However, there was no statistical difference between the *ProDer f* 1 and the DCP-*ProDer f* 1 groups ($P > 0.05$) concerning the levels of IgE, IgG_1, and IgG_{2a} (**Figure 6A-C**).

Pathological change in pulmonary tissues

Histological examination of the lung tissues obtained from the mice of asthma group showed intensive peribronchial inflammatory infiltration, submucosal airway wall thickening, overt hypertrophied bronchial epithelial cells, and notable epithelial shedding (**Figure 7B**). Yet, the negative controls exhibited minimal peribronchial cellular infiltration and airway wall thickening (**Figure 7A**). Alleviated inflammatory symptoms were found in mice immunized by *ProDer f* 1, DCP-*ProDer f* 1 and DCP-Ihc-*ProDer f* 1 (**Figure 7C-E**).

Discussions

Recurrent aeroallergen exposure may boost the production of allergen-specific IgE antibodies [30] and facilitate degranulation and release of inflammatory mediators as well as pro-inflammatory cytokines from mast cells and basophils [31].

Allergen SIT is the only disease-modifying treatment for allergy with long-lasting effects [32]. DCs are also crucial players in the immunoregulatory mechanisms underlying successful SIT [33]. In an attempt to enhance the allergen presentation of *ProDer f* 1 via MHC II pathway to make SIT more effective, we assessed the power of a hybrid DCP-IhC-*ProDer f* 1 allergen as vaccine. Ii plays multiple roles in the MHC-II processing pathway, and has been shown that truncated forms of Ii through physical interaction with MHC-II molecules that were fused to allergens can induce efficient antigen presentation [18, 34] and increase immune responses [17]. Previous studies have provided that fused DCP-antigen can be presented to T cells, and induce potent antigen-specific T cell activation [28], for which we verified hybrid DCP-IhC-*ProDer f* 1 as vaccine for SIT. The results demonstrated superior efficacy of rectifying the imbalance of Th1/Th2 by the hybrid to that with P*roDer f* 1 and DCP-*ProDer f* 1, and that the fused allergen induced a strong IFN-γ secretion of Th1-type cytokines and reduced IL-4 secretion of Th2- type cytokine, as well as inhibited IL-17 production of Th17-type cytokines.

IL-10 appears to be an important cytokine in successful SIT [35]. Previous study has shown that SIT for HDM is accompanied by increases in IL-10 production by peripheral $CD4^+CD25^+$ Treg cells, following stimulation with Der p 1 allergen [36]. Exclusive of shifting the balance of T-lymphocyte subsets from a Th2- towards a Th1-phenotype with major production of IFN-γ and decreased production of IL-4, increase of IL-10 produced by $CD4^+CD25^+$ Treg cells also coexists in SIT [37, 38]. Our findings revealed that the levels of IFN-γ and IL-10 were higher in DCP-Ihc-*ProDer f* 1 group than those in *ProDer f* 1 and DCP-*ProDer f* 1 groups. Nevertheless, the IL-4 level was significantly lower as compared with the previous two groups. This is associated with IFN-γ suppressing IL-4 synthesis required for IgE production and an increased IL-10 secretion inhibiting IgE production [39]. These results are consistent with the previous work by Martinez-Gomez *et al.* that Immunotherapy with modified Fel d 1 could enhance protection against anaphylaxis, as indicated by lower levels of IL-4 and increased IL-10 levels [40].

Furthermore, measurement of IgG_1 and IgG_{2a} revealed that IgG_1 level was strikingly downregulated in DCP-Ihc-*ProDer f* 1 group, whereas IgG_{2a} level was markedly raised compared to other experimental groups. These findings suggest that the hybrid DCP-Ihc-*ProDer f* 1 has the potential to modify the imbalance of Th1/Th2 cells characterized by suppression of IL-4, and an increase of IFN-γ and IL-10 in agreement

with the therapeutic principles for allergic disorders.

In conclusion, we found that the DCP-Ihc-*ProDer f* 1 is more effective than other allergens in reversing an allergen-induced Th2-skewed immune response, and DCP-Ihc-*ProDer f* 1 is a potent vaccine in preventing allergic pulmonary inflammation through lowering specific IgE levels. However, further studies are necessary to verify the safety and efficacy of the DCP-Ihc-*ProDer f* 1 as a useful vaccine for SIT.

Acknowledgements

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Disclosure of conflict of interest

None.

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