Original Article
A DNA vaccine encoding a chimeric allergen derived from major group 1 allergens of dust mite can be used for specific immunotherapy

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Abstract: Immunization with DNA-based constructs has been shown to be against the antigen and the response is skewed in such a way as to ameliorate the symptoms of allergic disease. This approach is particularly useful in the treatment of allergic inflammatory diseases, such as asthma. The major group 1 allergen from house dust mites is one of the triggers of allergic asthma. This study explores whether a chimeric gene R8, derived from the major group 1 allergen of house dust mite species (Dermatophagoides farinae and Dermatophagoides pteronyssinus), can be expressed in Human Embryonic Kidney 293 cells (HEK 293T) and whether such a construct can be used as a DNA vaccine in asthma therapy. The eukaryotic expression vector pcDNA3.1 was used to express the R8 molecule in HEK 293T cells and successful expression of R8 was confirmed using a fluorescence microscope and western blot analysis. The efficacy of R8 as DNA vaccine was also assessed in a mouse asthma model. The in vivo data showed that R8 rectified the TH1/TH2 imbalance typical of allergic inflammation and stimulated the proliferation of regulatory T (Treg) cells. Immunization with the R8 construct also decreased serum allergen-specific IgE production in this mouse asthma model. Our findings suggest that R8 may be a feasible potential DNA vaccine for specific immunotherapy (SIT) in the treatment of allergic asthma.

Keywords: Dermatophagoides allergen 1 group, chimeric gene, DNA vaccine, asthma

Introduction

Allergic asthma is one of the most common type 1 allergic inflammatory diseases and the prevalence of asthma continues to rise in most industrialized countries [1-3]. The pathogenesis of asthma remains unclear. Asthma is characterized by chronic airway inflammation, goblet cell hyperplasia, and variable airflow obstruction with airway hyperresponsiveness (AHR) [4]. Another well-recognized characteristic of asthma is an imbalance in the activation of T helper type 1 (TH1) and type 2 (TH2) [5], which is accompanied by the predominance of type 2 cytokines secreted by the increased number of TH2 cells. Moreover, regulatory T (Treg) cells, important in regulating the inflammatory response and maintaining the TH1 and TH2 balance, also play a central role in the pathogenesis of allergic asthma [6].

Specific immunotherapy (SIT) is a conventional treatment for allergic asthma [7, 8] and involves the repetitive application of an allergen by subcutaneous injection or sublingual application. This treatment approach is designed to modify the natural course of the asthmatic response and alleviate the severity of a patient’s asthma symptoms when they are re-exposed to the inhaled allergens [9].

Rodent models of allergic inflammation have shown that immunization with DNA encoding an allergen protein is a feasible form of SIT [9-12]. DNA vaccination has been shown to induce anti-allergic immune responses through the recruitment of allergen-specific TH1 cells and the establishment of a TH1 cytokine milieu, principally mediated by interferon γ (IFN-γ) production [13]. Therefore, the focus of many research groups has been to develop and opti-
mize DNA vaccines that are able to restore the balance to the TH1/TH2 allergic inflammatory response typical in allergic asthma.

Previously, the \textit{R8} gene that encodes the chimeric R8 allergenic protein was discovered from a screen of the major group 1 allergens of house dust mite (ProDer f1 and ProDer p1). The R8 protein is hypoallergenic and hyperimmunogenic [14] and exhibits properties similar to that of the parental allergen ProDer f1. Specifically, R8 has similar IgE immunoreactivity to ProDer f1 and restores the imbalance of TH1/TH2 cells during an allergic inflammatory response [15]. Although the \textit{R8} gene is an attractive therapeutic tool for immunotherapy, DNA vaccination using the chimeric R8 gene has not been previously explored. In the present study, the expression of R8 in HEK 293T cells was characterized and the use of the chimeric \textit{R8} gene as a DNA vaccine for the alleviation of allergic inflammation was explored using a mouse asthma model.

\textbf{Materials and methods}

\textbf{Animals}

Female BALB/c mice (6-8 week-old) were purchased from the Center for Comparative Medicine, Yangzhou University (License No: SCXK 2007-0001). Mice were housed in the animal facility of Wannan Medical College and were provided with food and water \textit{ad libitum} under specific-pathogens free conditions. All procedures were approved by the Animal Research Ethics Board of Wannan Medical College.
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Transfections of recombinant DNA vectors in vitro

HEK 293T cells (Sangon Biotech) were cultured in RPMI-1640 media (Gibco®, Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) in a 5% CO₂ incubator at 37°C, overnight. Then, cells were transfected with the pcDNA3.1, pcDNA3.1-GFP, pcDNA3.1-GFP-ProDer f1 or pcDNA3.1-GFP-R8 plasmids. The transfection of the plasmids into the cells was performed using DNAfect Transfection Reagent (CWBiotech, Beijing, China) according to the manufacturer’s instructions. HEK 293T cells grew to 80%-90% confluence and were transfected with 10 μl (about 4 μg) of pcDNA3.1, pcDNA3.1-GFP, pcDNA3.1-GFP-ProDer f1 or pcDNA3.1-GFP-R8 plasmid, dissolved in 100 μl RPMI-1640 without FBS. After a 6 h transfection, the culture media was replenished with fresh RPMI-1640 containing 10% FBS. After 46 h in culture, the cells were observed under a fluorescent microscope (ix17, Olympus, Tokyo, Japan) using a 488 nm wavelength filter.

Western blot analysis

The HEK 293T cells were treated with trypsin (0.25%) and rinsed with 1x phosphate-buffered saline (PBS, 11.9 mM phosphate, pH 7.4, 137 mM NaCl, 2.7 mM KCl, pH 7.2). The cells were collected and lysed using lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1% SDS) containing 1 μl protein inhibitor and 1 μl phenylmethanesulfonyl fluoride (PMSF), at 4°C for 10 min. The lysates were centrifuged at 12000 ×g for 10 min at 4°C, and the supernatants were collected. The protein concentration of each sample was measured using the Quick Start™ Bradford protein assay (Bio-Rad, Berkeley, CA, USA), with bovine serum albumin (BSA) to generate a concentration curve for quantification. Equal volumes (about 80 μg of total soluble proteins) of each treatment were separated using 10% SDS-PAGE according to Laemmli’s method [16] in a Mini-PROTEAN 3 system (Bio-Rad), and then transferred onto an Immobilon-P membrane (EMD Millipore, Billerica, MA, USA). Membranes were incubated in blocking buffer (10% bovine serum, 3% BSA, in PBS, pH 7.2) for 2 h at 37°C. GFP-specific rabbit polyclonal antibody (1:500, Sangon Biotech) diluted in PBS was added to the blocking buffer and the membranes were incubated overnight at 4°C. The following day, the membranes were washed three times with blocking buffer for 10 min each and a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, ZSGB-BIO, Beijing, China) diluted in PBS was added as a secondary antibody, followed by three washes in blocking buffer (10 min each). Antibodies specific to β-actin (Sangon Biotech) served as an internal control. The target proteins were visualized using enhanced chemiluminescence (ECL) reagent (Sangon Biotech) according to the manufacturer’s instructions.

Immunization protocols

Sixty mice were randomly assigned to seven groups (n = 10 for each) as follows: (i) PBS group (PBS): mice treated, sensitized and challenged with PBS; (ii) asthma group (asthma): mice sensitized and challenged with the ProDer f1 allergen; (iii) pcDNA3.1 group (pcDNA3.1): mice treated with the pcDNA3.1 plasmid (100 μg/mouse), sensitized and challenged with the ProDer f1 allergen; (iv) pcDNA3.1-GFP group (GFP): mice treated with the pcDNA3.1-GFP plasmid (100 μg/mouse), sensitized and challenged with the ProDer f1 allergen; (v) pcDNA3.1-GFP-ProDer f1 group (ProDer f1): mice treated with the pcDNA3.1-GFP-ProDer f1 plasmid (100 μg/mouse), sensitized and challenged with the ProDer f1 allergen; (vi) pcDNA3.1-GFP-R8 group (R8): mice treated...
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with the pcDNA3.1-GFP-R8 plasmid (100 µg/mouse), sensitized and challenged with the ProDer f1 allergen.

In this study, the asthma model and vaccination protocols were the same as described by Maecker et al. [17], with some modifications (Figure 2). On day 0, the mice were injected with 100 µg of plasmid DNA (dissolved in 100 µl of PBS) intramuscularly (i.m.) in the tibialis anterior muscles. On day 8, the mice were once again injected i.m. with the same amount of plasmid DNA. The mice in the PBS and asthma groups were injected with PBS or alum only, respectively. On days 15, 22, and 29, the asthma, pcDNA3.1, GFP, ProDer f1, and R8 groups were injected intraperitoneally (i.p.) with 10 µg of the ProDer f1 allergen. The allergen mix was dissolved in 200 µl of PBS containing 2% (W/V) Al(OH)₃ suspension. The PBS group was injected with only PBS. On day 43, the PBS group was challenged with nebulized inhalation of PBS and the remaining groups were challenged with a nebulized inhalation of the ProDer f1 allergen (10 µg/ml). All groups were challenged for 30 min with PBS or the ProDer f1 allergen for next 7 successive days (days 43-49).

Histopathologic evaluation of the pulmonary tissues

Twenty-four hours after the last aerosol challenge, the lungs were harvested, fixed in 10% formalin overnight, and embedded in paraffin. The pulmonary sections (5 µm) were placed onto poly-L-lysine-coated slides and stained with hematoxylin and eosin (HE). The histological changes in the tissues were microscopically assessed according to the scoring protocol described by Underwood [18] which is based on the extent of eosinophil infiltration, epithelial damage, and edema in the lung.

Detection of IFN-γ, IL-5, IL-10, and TGF-β cytokines in the BALF

Twenty four hours after the final inhalation challenge, the mice were anesthetized with an i.p. injection of 100 µl of 0.5% pentobarbital sodium. The trachea of each mouse was cannulated and bronchoalveolar lavage fluid (BALF) was harvested and handled as previously described [15]. Enzyme-linked immunosorbent assay (ELISA) was performed on each sample to quantify the levels of IFN-γ, IL-5, IL-10, and TGF-β in the BALF according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA).

Determination of allergen-specific IgE, IgG₁ and IgG₂a antibodies in the sera

Blood sample from each mouse was collected and centrifuged at 4000 xg for 30 min. The sera was separated and stored at -80°C for further analysis. ELISA plate wells were coated overnight with ProDer f1 or R8 (500 ng/well) in coating buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6) at 4°C. The plates were then washed 5 times with 0.1% Tween-20 in PBS (PBST), and blocked for 1 h at 37°C with 150 µl of PBST supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich® Co. LLC., St. Louis, MO, USA). Sera samples were diluted in PBST to appropriate concentrations and then incubated for 1 h at 37°C. After washing 5 times with PBST, the amount of specific IgE, IgG₁ and IgG₂a was detected after incubation with HRP-goat anti-mouse IgE, IgG₁ and IgG₂a (1:1000 in PBST; Sigma-Aldrich® Co. LLC.) at 37°C for 2 h. The plates were then washed 5 times with PBST, and 3', 3', 5', 5'-Tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich® Co. LLC.) was added, allowing it to react 20 min at 37°C. Stop buffer of 50 µl for each well was immediately used to terminate the reaction. A450 nm was measured in a microplate reader (BioTek, Winooski, VT, USA).

Statistical analysis

The statistical data for each group was expressed as the mean ± SD and analyzed using SPSS for Windows, version 16.0 (SPSS, Chicago, IL, USA), to determine the one-factor analysis of variance. The group comparisons were performed using the least significant difference (LSD-t or Thammhane’s T²) method. A P-value of less than 0.05 was accepted as significant.

Results

Expression of R8 in vitro in HEK 293T cell line

To determine whether the R8 protein could be successfully expressed in the HEK 293T cell line, the sequences encoding for GFP and R8 were inserted into the pcDNA3.1 vector and the resulting plasmid (pcDNA3.1-GFP-R8) was used to transfect HEK 293T cells. Forty-six
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hours later, the cells were collected and further analyzed under a fluorescent microscope. The pcDNA3.1-GFP, pcDNA3.1-GFP-ProDer f1 and pcDNA3.1-GFP-R8 plasmids were successfully expressed in the HEK 293T cells (Figure 3).

To evaluate the expression of recombination proteins in the transfected cells, we examined the expression of R8 protein by western blot analysis. Distinctive bands were observed in the lysates from the cells transfected with the pcDNA3.1-GFP-ProDer f1 and pcDNA3.1-GFP-R8 plasmids (lanes 3 and 4 in Figure 4) and these bands corresponded to the predicted molecular weight of GFP-ProDer f1 and GFP-R8. These bands were not detected in the cell lysates from the control cells that had been transfected with the pcDNA3.1 or pcDNA3.1-GFP plasmids (lanes 1 and 2 in Figure 4) and only GFP was found in the cells that had been transfected with the pcDNA3.1-GFP plasmid (lane 2 in Figure 4).

**Pathological changes in the pulmonary tissue**

Histological examination of the lung tissues from mice in the asthma group (Figure 5B) showed intensive peribronchial inflammatory infiltration and submucosal airway wall thickening. The bronchial epithelial cells were overtly hypertrophied. Epithelial shedding and goblet cells hyperplasia were also noted. The same conditions were also present in the pcDNA3.1 and GFP groups (Figure 5C, 5D). Conversely, the negative controls exhibited minimal peribronchial cellular infiltration and airway wall thickening (Figure 5A). ProDer f1- and R8-immunized mice that had received DNA vac-

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**Figure 3.** Detection of R8 gene expression in HEK 293T cells by fluorescent microscopy, 46 hours after treatment with the DNA-liposome complex (×200).

**Figure 4.** Western blot analysis of total proteins from pcDNA3.1-GFP-R8 transfected cells. In each lane, 80 µg of extracted protein was loaded. Lane 1: negative control (pcDNA3.1 vector transfected cells); lanes 2-4: total protein in cells transfected with plasmid pcDNA3.1-GFP, pcDNA3.1-GFP-ProDer f1, and pcDNA3.1-GFP-R8.
A DNA vaccine encoding chimeric hypoallergen also showed a general alleviation of these inflammatory markers (Figure 5E, 5F), compared with the asthma group. Moreover, fewer pathological changes were observed in the R8 group compared to the ProDer f1 groups (Figure 5F).

Evaluation of the prophylactic potential of R8 in immunized mouse models

An imbalance in the activation of TH1/TH2 cells is considered an important pathogenic mechanism of asthma in most animal asthma models. To determine whether pcDNA3.1-GFP-R8 immunization could alter the TH1/TH2 cytokine profile characteristic of allergic inflammation in our mouse model, ELISA was performed to measure the amount of IFN-γ and IL-5 in the BALF. In the R8 group, IFN-γ production (409.54 ± 26.33 pg/ml) was significantly increased compared with the PBS group (146.77 ± 43.68 pg/ml, P < 0.001), the asthma group (55.31 ± 19.76 pg/ml, P < 0.001), the pcDNA3.1 group (58.96 ± 14.55 pg/ml, P < 0.001), and the GFP group (74.27 ± 12.5 pg/ml, P < 0.001), as shown in Figure 6A. Mice immunized with R8 also produced a higher amount of IFN-γ than mice in the ProDer f1 group (294.13 ± 71.76 pg/ml, P < 0.05). Conversely, animals vaccinated with R8 produced a lower amount of IL-5 (118.65 ± 24.23 pg/ml) than the mice in the asthma group (319.10 ± 57.36 pg/ml, P < 0.001), the pcDNA3.1 group (305.11 ± 48.87 pg/ml, P < 0.001), the GFP group (345.11 ± 59.53 pg/ml, P < 0.001), and the ProDer f1 group (209.58 ± 21.67 pg/ml, P < 0.001), as shown in Figure 6B. The amount of IL-5 in the ProDer f1, and R8 groups were still higher than in the PBS group (48.11 ± 15.76 pg/ml, P < 0.001).

To evaluate whether R8 could stimulate Treg cell proliferation, the amount of IL-10 and TGF-β in the BALF was also measured by ELISA. The data showed that IL-10 production (709.47 ± 120.08 pg/ml) in the R8 group was significantly higher compared with the PBS group (77.18 ± 25.10 pg/ml, P < 0.001), the asthma group (413.42 ± 93.10 pg/ml, P < 0.001), the pcDNA3.1 group (301.18 ± 46.75 pg/ml, P < 0.001), and the GFP group (380.18 ± 59.28 pg/ml, P < 0.001). However, there was no statistically significant difference between the ProDer f1 group (744.03 ± 66.14 pg/ml), and the R8 group (P > 0.05) in terms of IL-10 in the BALF, as shown in Figure 6C. A similar trend was observed with the amount of TGF-β in the BALF of the immunized mice (Figure 6D).

In addition, there was no statistically significant difference in IFN-γ, IL-5, IL-10, and TGF-β in the BALF of mice in the PBS, pcDNA3.1, and GFP treatment groups.

We examined the levels of specific IgE, IgG₁, and IgG₂a to determine possible mechanisms
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Figure 6. Detecting cytokines in the BALF of DNA vaccinated mice. Cytokines in the BALF from all animals were detected by quantitative ELISA. Data are shown as the mean ± SD of ten mice from each group. Note: a, compared with the PBS group, $P < 0.001$; b, compared with the asthma group, the pcDNA3.1 group, and the GFP group, $P < 0.001$; c, compared with the PBS group, $P < 0.05$; d, compared with the asthma group, the pcDNA3.1 group, and the GFP group, $P < 0.05$; e, compared with the ProDer f1 group, $P < 0.05$; f, compared with the ProDer f1 group, $P < 0.001$.

Figure 7. The levels of specific IgE, IgG1, and IgG2a in the sera of mice immunized with an R8-containing plasmid. Note: a, compared with PBS group, $P < 0.001$; b, compared with asthma group, pcDNA3.1 group, and GFP group, $P < 0.001$; c, compared with asthma group, pcDNA3.1 group, and GFP group, $P < 0.05$; d, compared with asthma group, $P = 0.003$. 

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behind the effects of DNA-based vaccines. The resulting serum IgE, IgG1, and IgG2a antibody levels suggested that the IgE level in the R8 group (48.80 ± 5.94 ng/ml) was significantly lower than that of the PBS group (5.71 ± 2.29 ng/ml, P < 0.001), the asthma group (78.02 ± 8.63 ng/ml, P < 0.001), the pcDNA3.1 group (80.21 ± 9.00 ng/ml, P < 0.001), and the GFP group (82.70 ± 10.40 ng/ml, P < 0.001; Figure 7A). The IgG1 levels were also significantly different between the R8 group (46.67 ± 7.89 ng/ml) and the PBS group (7.44 ± 4.36 ng/ml, P < 0.001), the asthma group (85.11 ± 11.48 ng/ml, P < 0.001), the pcDNA3.1 group (93.44 ± 11.37 ng/ml, P < 0.001), and the GFP group (84.54 ± 10.37 ng/ml, P < 0.001; Figure 7B). Finally, the level of IgG2a in the R8 group (70.21 ± 16.22 ng/ml) was significantly higher compared to the PBS group (15.68 ± 7.81 ng/ml, P < 0.001), the asthma group (35.31 ± 7.31 ng/ml, P = 0.003), the pcDNA3.1 group (37.48 ± 9.92 ng/ml, P < 0.001) and the GFP group (34.28 ± 8.98 ng/ml, P < 0.001; Figure 7C). However, there was no statistically significant difference in the amount of specific IgE, IgG1, and IgG2a in the serum of mice in the ProDer f1 and the R8 groups (P > 0.05; Figure 7A-C).

Discussion

Asthma is one of the most serious health problems worldwide. It has long been recognized that there exists an imbalance in the number of activated TH1 and TH2 cells during the allergic inflammatory response that characterizes asthma [6]. In addition, Treg cells play an important role in the development of asthma [19], and patients with asthma have a significant decrease in the number of Treg cells [20].

SIT is the only etiologic treatment available to patients with allergies [21]. Successful SIT is accompanied by a reduction in allergen-specific IgE production, a shift in the balance of TH1/TH2, as well as induction of allergen-specific CD4+ CD25+ Treg cells [22]. Several recombinant allergens such as Der p1 or Der p2 from D. pteronyssinus, Bet v 1a from birch pollen, and Api m 1 and Api m 2 from bee venom have been introduced as possible immunotherapy allergens for use in pre-clinical studies [23-25].

However, traditional SIT has frequent side-effects, making it difficult to achieve the desired therapeutic effects [9]. Allergen DNA immunizations are considered to be one of the most promising immunotherapeutic strategies for the treatment of allergic diseases [26, 27], because they have fewer side-effects and are easier to use [28]. DNA vaccination has been shown to induce anti-allergic immune responses [29, 30] that are induced through the recruitment of allergen-specific TH1 cells and establishment of a TH1 cytokine milieu, most notably IFN-γ [13].

In this study, we began by establishing the expression of R8 recombinant protein in HEK 293T cells through plasmid transfection. This proof-of-concept experiment showed stable expression of R8 in HEK 293T cells was possible with this system. Next, we used introduced this transfection system into a mouse asthma model and measured several allergic inflammatory biomarkers in vivo. As expected, the amount of IFN-γ, a cytokine predominantly secreted by TH1, in the BALF of mice that had been treated with the R8 gene was higher compared to mice that had been treated with a plasmid that carried the gene that encoded the ProDer f1 allergen. The amount of IL-5, primarily produced by TH2 cells, found in the BALF of these mice was significantly lower, when compared to the BALF of mice that had been treated with plasmid carrying the gene encoding the parental allergen. The shift in the TH1/TH2 cytokine profile from predominantly TH2 towards TH1 implies that the chimeric DNA vaccine R8 can activate TH1 cell differentiation, while downregulating TH2 cell growth. This data agrees with previous studies. For example, Jarman et al. reported that a Der p1 DNA vaccine could reverse airway inflammation caused by the Der p1 allergen, as indicated by lower levels of IL-5 in BALF [31]. Pulsawat et al. also found that a Der p1 DNA vaccine candidate could induce an allergen-specific TH1 response [12].

Treg cells, mainly CD4+ CD25+ Treg, play an important regulatory role in the pathogenesis of allergic diseases and asthma [32-34] via the secretion of IL-10 and TGF-β [33, 35, 36]. Some studies have reported an association between the elevated production of IL-10 and TGF-β by Treg cells and the suppression of allergic responses after DNA immunization [32, 37, 38]. Our results support these observations because they show an increased production of IL-10 and TGF-β after treatment with the R8 DNA vaccine in our mouse model.
Additionally, the results of our study show that the level of IgG1 was significantly down-regulated in the R8 group but the level of IgG2a was significantly higher compared with the other experimental groups (P < 0.001). This evidence strongly suggests that R8 has the potential to modify the imbalance in the TH1 and TH2 immune response that is associated with asthma. Furthermore, the treatment of sensitized and challenged mice with the R8 DNA vaccine resulted in a predominately TH1-biased allergic response. The results agree with our previous study which demonstrated that using R8 allergen as a vaccine for SIT can rectify the imbalance in TH1/TH2 cells [15].

In conclusion, this study demonstrates that the chimeric gene R8 was more effective than its parental allergens in reversing an allergen-induced Th2-skewed immune response. Likewise, R8 DNA vaccine was also more effective in preventing allergic pulmonary inflammation through a decrease in specific IgE levels. Therefore, it can be considered an extremely promising candidate vaccine for clinical SIT trials in patients with house dust mite allergy in future.

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Disclosure of conflict of interest

None.

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