



Optimization of a Der p 2-based prophylactic DNA vaccine against house dust mite allergy

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ARTICLE INFO

Article history:

Received 26 November 2012

Received in revised form 16 January 2013

Accepted 29 January 2013

Available online 8 February 2013

Keywords:

HDM allergy

Der p 2

DNA vaccine

Electroporation

Mice model

ABSTRACT

DNA vaccines encoding allergens are promising immunotherapeutics to prevent or to treat allergy through induction of allergen-specific Th1 responses. Despite anti-allergy effects observed in small rodents, DNA-based vaccines are weak immunogens in primates and humans and particularly when administered by conventional injection. The goal of the present study was to improve the immunogenicity of a prophylactic vaccine encoding the major house dust mite allergen Der p 2. In this context, we evaluated the influence of different DNA backbones including notably intron and CpG enriched sequence, the DNA dose, the *in vivo* delivery by electroporation as well as the heterologous prime boost regimen on the vaccine efficiency.

We found that a minimal allergen expression level threshold must be reached to induce the production of specific antibodies but beyond this limit, the intensity of the immune response was independent on the DNA dose and allergen expression. The *in vivo* DNA delivery by electroporation drastically enhanced the production of specific antibodies but not the IFN γ secretion. Vaccination of naïve mice with DNA encoding Der p 2 delivered by electroporation even at very low dose (2 μ g) prevented the development of house dust mite allergy through Th1-skewed immune response characterized by the drastic reduction of allergen-specific IgE, IL-5 and lung inflammation together with the induction of strong specific IgG2a titers and IFN γ secretion. CpG cassette in the DNA backbone does not play a critical role in the efficient prophylaxis. Finally, comparable protective immune responses were observed when using heterologous DNA prime/protein boost or homologous DNA prime/boost. Taken together, these data suggest that the potent Th1 response induced by DNA-based vaccine encoding allergens through electroporation provides the rationale for the evaluation of DNA encoding Der p 2 into HDM allergy clinical trials.

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1. Introduction

Perennial exposures to house dust mite (HDM) allergens induce inflammatory diseases in atopic patients such allergic asthma, perennial rhinitis as well as atopic dermatitis (AD) [1]. We can now consider that more than 20% of the population from industrialized countries develop sensitization to HDM allergens [1]. This strong Th2 response is characterized by the production of allergen-specific IgE, chronic airway inflammation through eosinophil recruitment, mucus production, and variable airflow obstruction.

Allergen-specific immunotherapy (SIT) is, to date, the unique treatment to cure the HDM allergic patients that induces active immunity to allergens. Following repeated exposures to HDM extracts, SIT was shown to revert the allergen-specific Th2 response by the induction of immunological tolerance and Th1 responses [2].

Although the beneficial effect of subcutaneous (SCIT) or sublingual (SLIT) immunotherapy was evidenced in the context of HDM allergy, such immune-modifying therapy presents some disadvantages as the long duration of the treatment (50–80 injections within 2–5 years), the risk of anaphylactic side-effects by repeated administrations of unmodified HDM allergens displaying IgE reactivity and the development of new allergic responses against allergens for which patients were not sensitized prior to these treatments [3]. The standardization from batch to batch together with the stability of the HDM extracts represent also important

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issues as the SIT efficacy is highly dependent on the quality of HDM vaccines [4]. But more importantly, the full characterization of HDM extracts remains a critical issue as HDM produce not only the yet identified allergens but thousands of proteins and macromolecules that might prime the allergic response through innate immunity activation. As example, it is now well recognized that the presence of contaminating LPS, chitin, β -glucan in HDM extracts play important roles for the allergenic sensitizations [5].

Through the cloning of mite allergens, new recombinant HDM vaccines can be now commonly produced with high purity and without irrelevant or unwanted components for the design of patient-tailored immunotherapies according to the patient's sensitization profile. In this context, the group 2 HDM allergen (Der p, f 2) is an important target for the development of recombinant vaccines against HDM allergy. Indeed, group 2 mite proteins are considered as major allergens according to the high prevalence of their IgE reactivity in HDM allergic patients world-wide [6,7]. Moreover, these LPS-binding proteins, structurally related to the TLR4 co-receptor MD-2, are strong activators of innate immunity through the production of proinflammatory cytokines (IL-8, IL-6, GM-CSF) but it was also demonstrated that Der p 2 associated with LPS triggered allergic sensitization in mice, even in MD2-deficient animals [8,9].

Immunizations with DNA encoding allergens preferentially induce Th1 responses while concomitantly inhibiting Th2 responses (notably through the activation of the TLR9 signaling pathway [10]), characterized in rodents by the production of IgG2a antibodies, induction of CD8+ and CD4+ type Th1 cells together with IFN γ secretion and inhibition of IgE [11–14]. Taken together, DNA vaccines based on group 2 HDM allergens could represent efficient approaches for the prevention and treatment of HDM allergy. However, due to their relatively modest immunogenicity, the development of such DNA-based vaccine candidates includes strategies to enhance the immune response through at least optimization of the plasmid backbone, the antigen expression and secretion, the use of new delivery methods as well as the evaluation of heterologous prime/boost vaccinations [15].

The goal of the present study was to design an efficient and optimized prophylactic Der p 2-based DNA vaccine against HDM allergy in mice. For that, we assessed the importance of key parameters which could greatly contribute to the efficacy of Der p 2-encoded DNA vaccinations: the optimization of allergen expression, the DNA delivery by *in vivo* electroporation, the DNA dose as well as the heterologous prime/boost strategy.

2. Materials and methods

2.1. Plasmid constructions and preparation

The full length Der p 2.0101 (leader+mature sequence) cDNA was amplified from the recombinant *P. pastoris* expression vector pPIC9K-mature Der p 2 by PCR using the following primers: Leader Der p 2 forward 5'-ATCGGCTAGCCTCAGGCCACC ATGTACAAAATTTTGTGCTTTTCATTGTTGGTCGCAGCCGTTGCTCGT – GATCAAGTCGATGTCAAAGAT-3' (*NheI* restriction site double underlined, *XhoI* restriction site underlined, Kozak sequence in bold and Der p 2 leader sequence in bold and underlined) and reverse 5'-CTATCGCGGCCGGAATTCTTAATCGCGGATTTTAGCATGAGTAGC-3' (*NotI* restriction site double underlined, *EcoRI* restriction site underlined). The amplified PCR product was digested respectively with *NheI*–*NotI*, *NheI*–*EcoRI* or *XhoI*–*EcoRI* for the cloning into the mammalian expression vectors pCMV/Kan/(NCI, NIH, USA) [16], pcDNA3.1+ (Invitrogen, Carlsbad, USA) and pHIS-MCS (Pfizer,

Vaccine Research, Canada) [17] respectively. The pHIS vector contained a kanamycin-selection, a CMV promoter, the bovine growth hormone polyA signal, a synthetic intron placed immediately prior to multiple cloning site and 64 primate-optimized CpG immunostimulatory sequences. The presence and identity of the full length Der p 2 cDNA was verified by DNA sequencing. All plasmid DNA constructs were produced and purified under endotoxin free condition using GigaPrep Kits (QIAGEN, Germany).

2.2. In vitro transient transfection assays

HEK 293 cells line were cultured onto 6 well plates (and with coverslip for immunofluorescence assay) in Dulbecco's modified Eagle's medium (DMEM) GlutaMAXTM (Invitrogen, Carlsbad, USA) supplied with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, USA) at a density of 1×10^6 cells/well. At a 90% confluency, the purified plasmid DNA (4 μ g of each DNA) were diluted in 250 μ L of serum free DMEM medium, gently mixed and incubated with 250 μ L of a diluted solution of LipofectamineTM 2000 (Invitrogen, Carlsbad, USA), 10 μ L of LipofectamineTM 2000 diluted in 240 μ L of Opti-MEM I (Invitrogen, Carlsbad, USA). The lipoplexes were then added into each well containing cells and 2 mL medium, then plates were gently mixed and incubated at 37 °C in a CO₂ incubator for 48 h.

2.3. Detection of Der p 2 expression by immunoblotting

The Der p 2 secretion was assayed using supernatants from transfected cells. The culture media from transfected cells were loaded onto a 12% SDS PAGE polyacrylamide gel. The different protein bands were then transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, USA). The membrane was saturated with 5% skim milk (BD, USA) and incubated with anti-Der p 2 mouse polyclonal antibodies at 1:2000 dilution for 1 h. The membrane was washed with phosphate buffer saline–Tween (PBST) (PBS, 0.5% Tween 20) and then incubated for 1 h with horseradish peroxidase (HRP) conjugated anti-mouse IgG (dilution 1:5000) (KPL, USA). The immunoreactive bands were detected using the chemiluminescent HRP substrate (Millipore, USA) and following X-ray film exposition.

2.4. Detection of intracellular Der p 2 expression by immunofluorescence

HEK 293-transfected cells on coverslip were fixed by acetone at –20 °C for 10 min. Fixed cells were incubated with anti-Der p 2 mouse polyclonal antibodies at 1:5000 dilution at 37 °C for 1 h and washed with PBS. Cells were subsequently stained for 1 h with rabbit-anti mouse IgG-FITC (dilution 1:20) (Dako, Denmark) and washed with PBS. Cells were stained with Evans blue for 10 min and then washed with water. Cells were observed under fluorescence microscope (Olympus, Japan).

2.5. Immunizations

The present study was approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC). Plasmid DNA vaccinations were performed by *in vivo* electroporation using the TriGridTM Delivery System (rodent model, Ichor Medical Systems, www.ichorms.com). The i.m. DNA injection into one tibialis anterior muscle was directly followed by the stimulation of the site of injection with an electrical pulse that had an amplitude of 250 V per cm of electrode spacing for 40 ms over a 400 ms interval. To evaluate the immunogenicity of the different plasmids encoding Der p 2, groups of 6 Balb/C mice were immunized 3 times at

2-week intervals with 20 µg of the DNA vaccines. Two weeks after the last vaccination, animals were sacrificed to collect serum as well as the spleen. In the vaccination prophylactic effect experiment, animals were immunized three times by 2 or 20 µg pHIS-Der p 2 or pCMV/Kan-Der p 2. In another set of experiments, such homologous prime/boost regimen was also compared with two *in vivo* electroporations only followed or not by one injection of 5 µg unadjuvanted recombinant Der p 2. As a control, group of mice was immunized with saline alone. Two weeks after the last vaccination, animals were first bled from the retro-orbital venous plexus to collect serum and then sensitized at weekly intervals for 3 weeks with 5 µg recombinant Der p 2 formulated with alum (ratio allergen/adjuvant 1/100, intraperitoneal injection). To induce airway inflammation, mice were challenged 10 days after the last sensitization by intranasal administrations of crude *D. pteronyssinus* extract (Siriraj dust mite center, Mahidol University, Thailand) for 4 consecutive days. The amount of Der p 2 was estimated to 5 µg/mL by western blotting. Three days post-last challenge, the mice were sacrificed to collect serum, bronchoalveolar lavage fluid (BALF) as well as the spleen.

2.6. Measurement of Der p 2-specific IgE, IgG1 and IgG2a

The presence of anti-Der p 2 IgE, IgG1 and IgG2a antibodies in the individual sera was detected by ELISA. Immunoplates were coated with recDer p 2 (500 ng/well), for 16 h at 4 °C. Plates were washed five times with TBS–Tween (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 80) and saturated for 1 h at 37 °C with 150 µL of the same buffer supplemented with 1% BSA. For the detection of specific IgE, individual sera were applied at 1/8 dilution whereas serial dilutions of sera (40–125,000) were performed for the anti-Der p 2 IgG1 and IgG2a assays. In both cases, sera were diluted in saturation buffer and were incubated for 1 h at 37 °C. Plates were washed five times with TBS–Tween buffer and antigen-bound antibodies were detected following incubation with rat biotinylated anti-mouse IgE, IgG1 or IgG2a (BD, dilution 1/1000 for each antibody) and HRP-conjugated streptavidin (BD, dilution 1/1000). The enzymatic activity was measured using the TMB substrate (BD, USA). OD_{450 nm} was measured in a Thermo Scientific ELISA reader. The ELISA specific IgG1 or IgG2a titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD_{450 nm} value.

2.7. Cytokines assay

To assess the cytokine production of proliferative T cells, splenocytes (4×10^5 /well in triplicate) were stimulated with 20 µg/mL recDer p 2 in RPMI 1680 medium and culture supernatants were collected after 72 h. The levels of IFNγ and IL-5 in the supernatants were measured in ELISA assays using the corresponding BD OptiEIA kits (BD, USA) and following the manufacturer's instructions.

2.8. Bronchoalveolar lavage and analysis

One day after the final aerosol exposure, mice were bled and killed. The lungs were immediately washed via the trachea cannula with 1 mL PBS, which was instilled and gently recovered by aspiration three times. The lavage fluid was centrifuged at $400 \times g$ for 10 min at 4 °C. The cell pellet was resuspended in 300 µL Hank's balanced salt solution (HBSS) and cells were counted in a Thoma haemocytometer. Cytospin preparations from 50 µL-aliquots were stained with May–Grünwald–Giemsa stain for differential cell counts.

Table 1

Specific antibody titers and IFNγ production following intramuscular or electroporation delivery of pHIS-Der p 2.

	Intramuscular	Electroporation	Control
Specific IgG1	<30	1216 ± 988*	56,785 ± 14,566
Specific IgG2a	<30	2028 ± 1560*	<50
Specific IgE	0.01 ± 0.01	0.03 ± 0.02	0.83 ± 0.43
IFNγ (pg/mL)	650 ± 243	929 ± 127	<50

Control: specific immune response from recDer p 2/alum immunized mice.

* $P < 0.05$ compared with intramuscular.

2.9. Statistical analysis

Student's *t*-test was used to compare data between pairs of groups. Differences were considered significant for *P*-values < 0.05.

3. Results

3.1. Constructions of DNA vaccines and Der p 2 expression

Because it is generally believed that optimization of antigen expression is one of the most promising approaches proposed to enhance the immunogenicity of DNA vaccine candidates, the full length (including the leader sequence) Der p 2 cDNA was cloned into three different mammalian expression vectors: pCDNA3.1+, pHIS and pCMV/Kan. Whether all the three vectors contain the same CMV early promoter, pHIS not only contained a synthetic intron placed immediately prior to Der p 2 cDNA but also a primate-optimized CpG 2006 motif, an immunostimulatory sequence able to activate the innate immunity through TLR9 which is active in mice [18]. In order to evaluate expression and secretion of Der p 2 by the constructed DNA vaccine vectors, HEK cells were transfected with DNA-lipofectamine 2000 lipoplexes and antigen expression was analyzed by western blotting and immunofluorescence (Fig. 1). Surprisingly, following transfection with pcDNA-Der p 2, an extremely low amount of cells could express intracellularly the allergen. Moreover, the Der p 2 level of secretion was under the limit of detection using this recombinant DNA construct. Whether Der p 2 expression could be detected in transfected cells as well as in the culture supernatant with both pHIS- and pCMV/Kan-Der p 2 DNA vaccines, much higher Der p 2 production was however observed using the pHIS-based construction. Similar results were obtained when the transfection agent Extreme (Roche, Germany) was used to transfect the cells (data not shown).

3.2. Optimization of the route of immunization

Numerous studies highlighted the interest of *in vivo* electroporation to improve the immunogenicity of DNA-based vaccines [19]. According to our transient transfection assay results, the pHIS-Der p 2 recombinant plasmid was selected to evaluate the interest of the *in vivo* DNA delivery by electroporation for the optimization of the vaccine immunogenicity. Immune responses to the pHIS-Der p 2 DNA vaccine delivered by electroporation or conventional intramuscular injection were then assessed in Balb/C mice following three DNA administrations of 20 µg of the plasmid. The specific humoral as well cellular responses were analyzed through anti-Der p 2 IgG1, IgG2a and IgE detection as well as IFNγ and IL-5 from Der p 2-specific proliferative assays (Table 1). Specific antibodies were undetectable in animals immunized with conventional i.m. injection. In contrast, electroporation drastically increased the production of specific IgG1 and IgG2a but did not induce the development of anti-Der p 2 IgE. To validate the allergen-specific IgE assay, we used as positive control a serum from mice immunized with alum-adjuvanted rec Der p 2 which always gave strong signal (Table 1). An elevated IFNγ production together with the absence

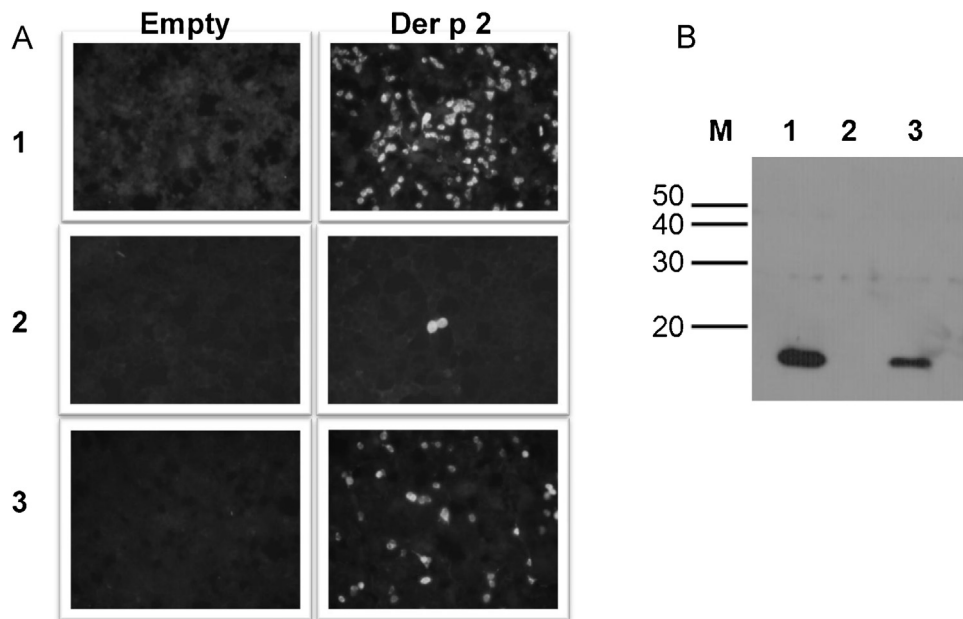


Fig. 1. Der p 2 expression from transiently transfected HEK cells. (A) HEK cells were transiently transfected with empty vector (Empty) or vector encoding Der p 2 (Der p 2). Intracellular Der p 2 expression was monitored by immunofluorescence with mouse anti-Der p 2 polyclonal antibodies and with anti-mouse IgG-FITC. Lane 1: pHIS, lane 2: pcDNA3.1+, lane 3: pCMV/Kan. (B) Der p 2 secretion was detected in the supernatant from transfected cells using mouse anti-Der p 2 polyclonal antibodies and anti-mouse IgG-HRP in chemiluminescent western blot. Lane 1: pHIS-Der p 2, lane 2: pcDNA3.1+-Der p 2, lane 3: pCMV/Kan-Der p 2.

of IL-5 in the T-cell proliferative assays was observed whatever the type of DNA delivery used. Even though there was a tendency to have higher IFN γ secretion following electroporation, this finding was not statistically significant ($P > 0.05$). The absence of specific IgE together with low specific IgG1/IgG2a ratio and high IFN γ production confirmed that DNA immunizations through electroporation induced a strong anti-Der p 2 Th1-biased response. Taken together, our data suggested that *in vivo* DNA delivery by electroporation had a positive impact of the production of Der p 2-specific IgG but not on the Th1-type cytokines.

3.3. Influence of antigen expression on the immunogenicity of Der p 2 encoding DNA vaccines

Transient transfection assay clearly evidenced large difference in Der p 2 expression between three different vector backbones, even though the same Der p 2 cDNA as well as the pCMV promoter were present in the plasmid constructs. To demonstrate whether the intensity of the specific anti-Der p 2 immune response following DNA vaccinations was correlated with the allergen expression, antibody titers as well as IFN γ production were assayed in mice receiving pHIS-, pCMV/Kan- or pcDNA-Der p 2 through electroporation.

Whereas administration of pcDNA-Der p 2 did not induce an anti-Der p 2 IgG2a response, strong allergen specific IgG2a antibody titers were elicited with pHIS- or pCMV/Kan-Der p 2 (Table 2). Although this pHIS-Der p 2-induced IgG response was still slightly higher than that measured with pCMV/Kan-Der p 2 electroporation, the difference was not statistically significant

($P > 0.05$). As expected, very few allergen-specific IgE and IL-5 were elicited whatever the plasmid used. Mice vaccinated with any of the three recombinant plasmids secreted IFN γ on restimulation. Whether pCMV/Kan- or pHIS-Der p 2 promoted comparable IFN γ response ($P > 0.05$), the levels of IFN γ was significantly lower following pcDNA-Der p 2 immunizations ($P < 0.05$). Our results suggested that the *in vivo* DNA delivery by electroporation can elicit potent allergen-specific immune responses even with a DNA backbone not optimized for antigen expression.

3.4. Influence of DNA dose on the prophylactic effect of Der p 2 encoding DNA vaccines

To determine whether DNA-Der p 2 can prevent the development of HDM allergy, a first experiment of prophylactic DNA vaccinations was scheduled using a Der p 2 sensitization mouse model (Fig. 2). According to the absence of specific antibody response following pcDNA-Der p 2 electroporations, groups of 6 naïve mice were immunized by three electroporations with pHIS-Der p 2 or pCMV/Kan-Der p 2 used at 2 or 20 μ g/delivery to assess the contribution of DNA dose in the efficacy of DNA vaccines against HDM allergy. As controls, unvaccinated mice were administrated with saline alone. The pretreated animals were all subsequently sensitized with three i.p. injections of Der p 2/alum followed by four consecutive intranasal challenges using HDM extracts to provoke airway inflammation. Whether the delivery of pHIS-Der p 2 at low dose (2 μ g) induced a strong specific IgG2a response, no production of anti-Der p 2 IgG2a could be measured using the same amount of pCMV/Kan-Der p 2 ($P < 0.05$). By contrast, both DNA constructs used at 20 μ g/injection stimulated a similar specific IgG2a response, which were higher than those measured from immunizations using 2 μ g DNA ($P < 0.05$). Whatever the DNA dose or the vector used, no specific IgE could be detected. The subsequent Der p 2 sensitizations and intranasal challenges were shown to increase the specific IgG2a level ($P < 0.05$ compared with post-vaccination titers) to reach comparable antibody titer in any DNA-vaccinated groups ($P > 0.05$ compared between groups post-challenge). Strikingly, the specific IgE levels were kept very

Table 2
Specific antibody titers and IFN γ production induced by pcDNA-Der p 2, pHIS-Der p 2 or pCMV/Kan-Der p 2 delivered *in vivo* by electroporation.

	Specific IgG2a	Specific IgE	IFN γ (pg/mL)
pcDNA-Der p 2	<30	0.01 $\pm$ 0.01	459 $\pm$ 204
pHIS-Der p 2	2439 $\pm$ 1523*	0.02 $\pm$ 0.02	1014 $\pm$ 391*
pCMV-Der p 2	1105 $\pm$ 458*	0.03 $\pm$ 0.01	738 $\pm$ 226*

* $P < 0.05$ compared with pcDNA-Der p 2.

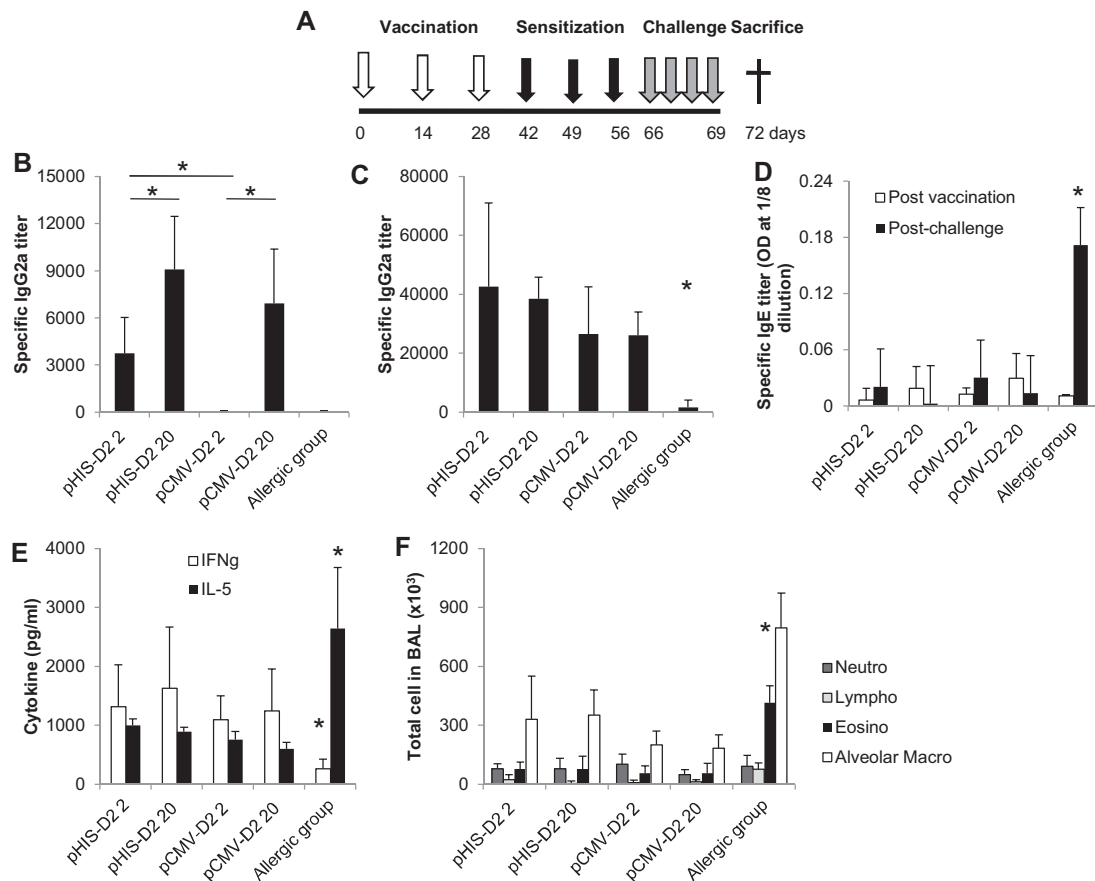


Fig. 2. Prevention of experimental HDM allergy by pHIS-Der p 2 or pCMV/Kan-Der p 2 prophylactic vaccines. (A) Immunization protocol for the evaluation of prophylactic DNA-based vaccinations, (B) specific IgG2a titers post-vaccination, (C) specific IgG2a titers post-challenge, (D) specific IgE titers post-vaccination and post-challenge, (E) restimulated splenocyte IL-5 and IFN γ production, (F) airway cellular infiltrates. All the values were plotted as mean $\pm$ SEM. One representative experiment out of 2 is shown. * $P < 0.05$ (comparisons between treatments, B), * $P < 0.05$ (compared with vaccinated mice, C–F).

low in vaccinated animals following these treatments. As expected, the unvaccinated control animals clearly developed respectively poor and strong anti-Der p 2 specific IgG2a and IgE responses.

We next analyzed IL-5 and IFN γ secretions by Der p 2-restimulated spleen cells. Der p 2-sensitized and HDM-challenged control mice developed high IL-5 and low IFN γ production respectively. High level of IFN γ secretion was similarly measured in Der p 2-restimulated spleen cells from the four vaccinated animal groups ($P > 0.05$). Interestingly also, any of the DNA vaccination schedules impaired equivalently the IL-5 production. Finally, BAL fluids analysis demonstrated that non-vaccinated but sensitized and challenged animals developed an important airway eosinophilia. In contrast, DNA vaccinations with low or high doses of pHIS-Der p 2 or pCMV/Kan-Der p 2 significantly as well as similarly decreased the eosinophil influx in the airways ($P < 0.05$). Taken together, our results suggested that Der p 2-encoded DNA delivered by electroporation maintained its capacity to prevent the HDM allergic response even at a very low dose.

3.5. Comparison of vaccine efficacy elicited with heterologous DNA–protein prime–boost versus homologous DNA–DNA

The heterologous prime–boost vaccinations, an immunization protocol using different types of vaccines for the delivery of the same antigen in the priming and the boost, can elicit higher immune responses than the homologous prime–boost using same type of vaccines [20]. Consequently, we compared in another set of experiments three different DNA immunization regimens using pHIS-Der

p 2 as DNA vaccine candidate: two or three electroporations (homologous prime/boost) and two electroporations combined with one intramuscular immunization with unadjuvanted recDer p 2 (heterologous prime/boost) (Fig. 3). The vaccinated animals as well as the unvaccinated control group were subsequently sensitized and challenged as described above. Whereas comparable specific IgG2a antibody titers were elicited following three DNA electroporations or two DNA electroporations + one protein immunization, two DNA electroporations triggered lower production of anti-Der p 2 IgG2a ($P < 0.05$). Whatever the immunization regimen, very low levels of specific IgE were detected. Following sensitization and challenge steps, the specific IgG2a titers were increased in all the vaccinated animals but no significant difference was observed between groups ($P > 0.05$). As expected, the allergic control group developed the highest anti-Der p 2 IgE response. Although a significant reduction in the production of specific IgE was detected following the three different immunization regimens ($P < 0.05$), the lowest specific IgE amount was measured in animals vaccinated by three DNA electroporations. The heterologous/prime/boost regimen induced an increase in the allergen-specific IgE production compared with the homologous prime/boost and this increment was more pronounced following prophylactic vaccinations by two DNA electroporations. Compared with control allergic group, all the three prime/boost vaccination regimens similarly triggered strong IFN γ secretion and prevented the IL-5 production as well as eosinophil infiltration in the airways.

Collectively, our results demonstrated that all regimens studied were capable of preventing the development of the HDM

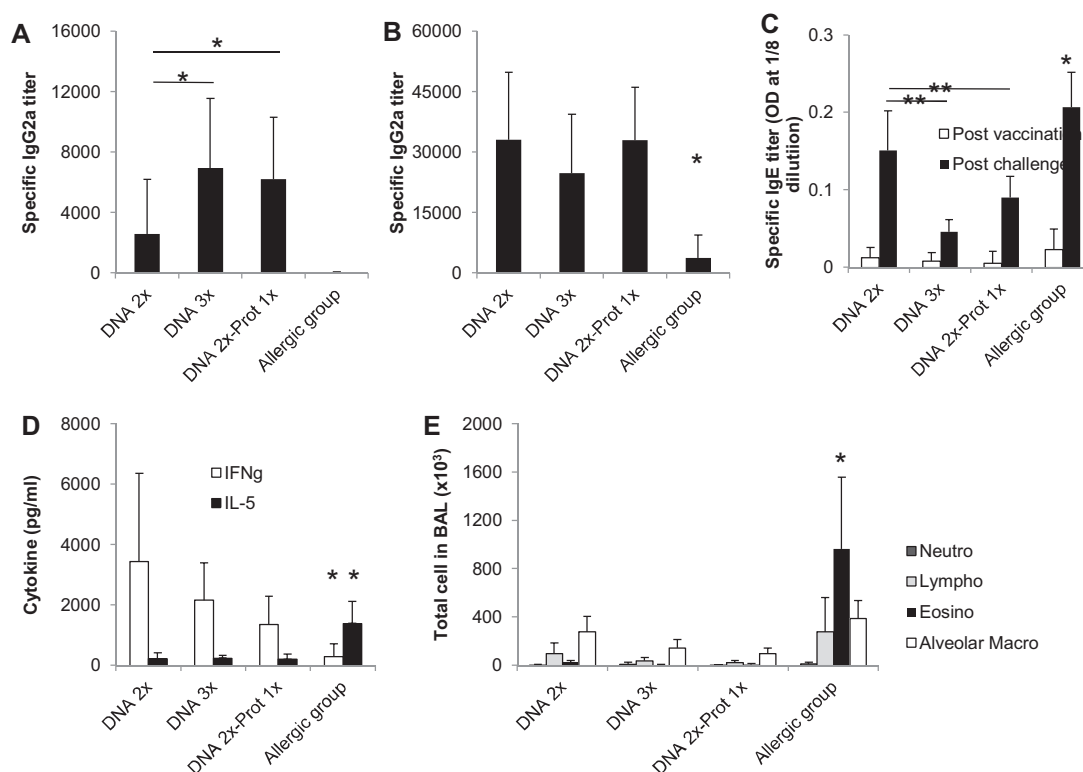


Fig. 3. Efficacy of prophylactic DNA vaccinations using different prime-boost regimens in the prevention of HDM allergy. (A) Specific IgG2a titers post-vaccination, (B) specific IgG2a titers post-challenge, (C) specific IgE titers post-vaccination and post-challenge, (D) restimulated splenocyte IL-5 and IFN γ production, (E) airway cellular infiltrates. All the values were plotted as mean $\pm$ SEM. One representative experiment out of 2 is shown. * $P < 0.05$ (comparisons between treatments, A), ** $P < 0.05$ (comparisons between treatments, C), * $P < 0.05$ (compared with vaccinated mice, B–E).

allergic response although we could consider that the homologous prime/boost immunization regimen based on three DNA electroporations generates the highest Th1 specific response.

4. Discussion

Vaccinations with allergen encoding DNA plasmids were shown to represent a promising approach to prevent allergy through notably the induction of strong IFN γ production which prevents Th2 bias. Whereas the efficacy of DNA vaccines against extracellular pathogens is primarily based on the stimulation of potent neutralizing antibody responses, the contribution of the allergen specific humoral response in protective anti-allergy DNA vaccinations is questionable. Indeed, allergen-specific Th1 memory generated by DNA encoding allergens could be sufficient to prevent allergy as judged by the efficacy of vaccinations with DNA plasmids encoding allergen not carrying leader sequence including vaccines expressing ubiquitin-allergen fusion molecules or replicase-based DNA which elicit very weak or no antibody response [21–23].

However, the generation of blocking IgG antibodies which impair allergen-IgE interactions following DNA vaccinations could be of importance and particularly for the immunotherapeutic treatment of allergy. Such blocking antibody responses, generated in mice by recombinant hypoallergenic protein vaccines [24,25], were shown to be essential for successful allergen-specific immunotherapy (SIT) [26]. The development of allergen-specific IgG response by DNA vaccinations would be greatly facilitated by the allergen secretion through the use of efficient leader sequence in plasmid constructs. Although intracellular allergen expression could reduce the risk of the priming of allergen-specific IgE response, as demonstrated in one study with a Der p 2 encoding DNA [27], numerous anti-allergy DNA vaccines based on DNA constructs able to secrete

allergens [28–30] triggered nevertheless protective Th1/Treg responses. The allergen secretion could result in a higher MHCII antigen presentation through cross-priming, a process initiated by the transfer of the expressed antigen from transduced myocytes or other bystander cells to DCs. This exogenous antigen presentation can also be accompanied with the delivery of endogenous antigens for MHCII presentation by autophagy in directly transfected DCs [31]. Noteworthy, the DNA vaccine immunogenicity could be improved by incorporation of an autophagy inducing system [32].

In the context of HDM allergy, previously published studies evaluated the immunogenicity as well as the protective efficiency of Der p 2 encoding DNA vaccine prototypes [21,27,33]. Whether some partial reduction of the Der p 2-specific allergic response was observed, the optimization of such DNA vaccine candidates and immunization regimens remained poorly investigated so far.

In the present study, three expression plasmids (pcDNA 3.1, pCMV/Kan and pHIS) were used to clone the natural full-length Der p 2 cDNA, including the sequence encoding the Der p 2 leader peptide. Whereas all these plasmids drive antigen expression through the same CMV promoter, the pHIS plasmid was considered as an optimized vector for DNA vaccination through the presence of a synthetic intron placed immediately prior to Der p 2 coding sequence as well as a stretch of CpG motifs. Whereas very weak Der p 2 expression could be detected following transient transfection assays with pcDNA-Der p 2, the two other recombinant constructions could clearly induced the expression and secretion of the allergen. The higher allergen production observed with pHIS expression plasmid could be more likely explained by the enhancing effect of the intron present in the vector, as previously reported [34].

Our data highlighted that electroporation combined with intramuscular injection of pHIS-Der p 2 drastically enhanced the overall

level of anti-Der p 2 humoral response through, more likely, a prolonged allergen expression by transfected cells and/or a more efficient cross-priming of DCs. In contrast to previously published reports, the IFN γ production in mice immunized by electroporation or intramuscular injection was comparable [35–38]. This result suggested that the higher or more prolonged allergen expression triggered by electroporation modulate only the specific antibody response but not the Th1-bias.

The correlation between the intensity of the immune response and the allergen expression was next addressed through the comparison of the immunogenicity of the three DNA vaccine candidates expressing differently Der p 2. The absence of specific antibodies following electroporations with pcDNA-Der p 2 indicated that the threshold level of expressed Der p 2 to elicit the allergen-specific antibody response was not reached *in vivo* with this recombinant plasmid. Nevertheless, pcDNA-Der p 2 was able to induce a production of IFN γ from restimulated splenocytes but at a lower level than observed with pCMV/Kan-Der p 2 or pHIS-Der p 2. By contrast, pCMV/Kan-Der p 2 and pHIS-Der p 2 generated a potent Der p 2-specific Th1-biased response characterized by high specific IgG2a response and IFN γ production which prevented the development of specific IgE and IL-5 but also eosinophilia in a mouse model of HDM allergy (Fig. 2). Our results suggested that, once a critical threshold of antigen expression is reached, the expression level is not correlated to the vaccine efficiency beyond this limit. Surprisingly, our data evidenced that the stretch of CpG motifs in the pHIS backbone does not improve also the specific Th1-biased immune response. Although such CpG-containing sequences in the pHIS vector were optimized for primate vaccination, their immunostimulatory capacity is maintained in mice [39]. Our observations are in contrast with previous results showing the up-regulation of T-cell response through the use of CpG-enriched plasmids [40]. Interestingly, *in vivo* electroporation of DNA results in cytosolic localization of nucleic acids whereas the triggering of TLR9 pathways through CpG requires the accumulation of DNA into the endosomes. Consequently, it is likely that pHIS and pCMV/Kan DNA plasmids can be similarly sensed by TLR9-independent DNA-sensing PRRs as the cytosolic helicases [41].

We observed also that the DNA dose did not influence the global Th1-biased prevention of HDM allergy. Indeed we remarkably demonstrated that an allergen-encoding DNA vaccine delivered even at very low dose (2 μ g) by electroporation was able to prevent the development of HDM allergy following Der p 2 sensitization and intranasal challenge with HDM extracts. It must be pointed out that previous report demonstrated that similar *in vivo* protein expression level could be generated whatever the DNA amount electroporated [42].

Although it was previously reported that heterologous prime/boost vaccination regimens were more efficient than homologous prime/boost modality to generate antigen-specific responses [43], comparable specific Th1 immunity preventing HDM allergy was observed following homologous (DNA/DNA/DNA) or heterologous (DNA/DNA/protein) prime boost prophylactic vaccinations (Fig. 3). Even through two electroporations, the intensity of the Th1 bias generated by homologous prime boost regimen is sufficient to prevent efficiently the development of HDM allergy in our mice model.

In conclusion, through a robust Der p 2 sensitization mice model using the potent Th2 alum adjuvant, our data emphasized that DNA encoding Der p 2 delivered by electroporation even at very low dose as 2 μ g represents an efficient prophylactic vaccine against HDM allergy through induction of potent Th1-skewed immune response. The *in vivo* electroporations drastically improved the development of allergen-specific antibodies which could represent an advantage for the efficiency of the Der p 2-based DNA vaccine through induction of blocking IgG antibodies. Whether this humoral response

participates to the observed protective effect remains to be demonstrated through notably passive transfer antibodies experiments as well as *in vitro* competition assays.

These key results suggest that *in vivo* DNA delivery by electroporation, currently testing in clinical trials, could represent an efficient technology to reduce the dose and the frequency of allergen encoding DNA vaccines to achieve appropriate immune responses to prevent or to revert HDM allergic response in humans.

Acknowledgements

This work was funded by National Science and Technology Development Agency (NSTDA, BIOTEC Grant P-09-00324), the National Research university Project of CHE and the Ratchadaphiseksomphot Endowment Fund (HR1164A) and the CU-Cluster-Emerging H-8-68-53. Dr. Pinya Pulsawat was sponsored by a Post-doctoral fellowship from the Chulalongkorn University Graduate School. The authors thank respectively Pfizer and the National Cancer Institute (NIH, USA) for providing the pHIS and the pCMV/Kan DNA plasmid vectors used in this study

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