

Absence of immunoglobulin E synthesis and airway eosinophilia by vaccination with plasmid DNA encoding ProDer p 1

A. Jacquet, M. Magi, M. Haumont, M. Jurado, L. Garcia and A. Bollen

Service de Génétique Appliquée, Université Libre de Bruxelles, Institut de Biologie et de Médecine Moléculaires, Gosselies, Belgium

Summary

Background Various studies have shown that immunization with naked DNA encoding allergens induces T helper 1(Th1)-biased non-allergic responses.

Objective To evaluate the polarization of the immune responses induced by vaccinations with plasmid DNA encoding the major mite allergen precursor ProDer p 1.

Methods A DNA vaccine was constructed on the basis of a synthetic cDNA encoding ProDer p 1 with optimized codon usage. The immunogenicity of ProDer p 1 DNA in CBA/J mice was compared with that of purified natural Der p 1 or recombinant ProDer p 1 adjuvanted with alum. Vaccinated mice were subsequently exposed to aerosolized house dust mite extracts to provoke airway inflammation. The presence of inflammatory cells was examined in bronchoalveolar lavage (BAL) fluids and allergen-specific T cell reactivity was measured.

Results Naive mice immunized with ProDer p 1 DNA developed Th1 immune responses characterized by high titres of specific IgG2a antibodies, low titres of specific IgG1 and, remarkably, the absence of anti-ProDer p 1 IgE. No specific responses were observed in animals vaccinated with the blank DNA vector. By contrast, natural Der p 1 or recombinant ProDer p 1 adsorbed to alum induced pronounced Th2 allergic responses with strong specific IgG1 and IgE titres. Spleen cells from DNA ProDer p 1-vaccinated mice secreted high levels of IFN- γ and low production of IL-5. Conversely, both adjuvanted allergens stimulated typical Th2-type cytokine profile characterized by high and low levels of IL-5 and IFN- γ , respectively. Whereas BAL eosinophilia was clearly observed in Der p 1-immunized animals, ProDer p 1 DNA as well as ProDer p 1 vaccinations prevented airway eosinophil infiltrations.

Conclusions These results suggest that vaccination with DNA encoding ProDer p 1 effectively fails to induce the allergen-induced IgE synthesis and airway cell infiltration. Plasmid DNA encoding ProDer p 1 may provide a novel approach for the treatment of house dust mite allergy.

Keywords allergy, DNA vaccination, eosinophilia, IgE, ProDer p 1

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Introduction

The major allergen Der p 1, from the house dust mite *Dermaphagoides pteronyssinus*, is a major cause of allergic disorders such as bronchial asthma, atopic dermatitis and allergic rhinitis [1]. The prevalence of anti-Der p 1 IgE antibodies in dust mite-allergic patients is higher than 80% [2, 3]. In animal models, Der p 1 was shown to induce specific IgE, pulmonary eosinophilic inflammation and airway hyper-responsiveness [4–7]. The molecular basis explaining the Der p 1 allergenicity is not fully understood but recent evidence suggests a correlation with its proteolytic activity [8]. Indeed, immunization of mice with proteolytically active Der p 1 results in a significant enhancement in total IgE and Der p 1-specific IgE synthesis compared with

animals immunized with Der p 1 that was irreversibly blocked with the cysteine protease inhibitor E-64 [9]. This up-regulation of IgE synthesis might result from the cleavage of human CD23 on B cells by Der p 1 [10]. The CD23 cleavage leads to disruption of the negative feedback signal delivered by IgE binding to CD23. In addition, Der p 1 can selectively cleave human and murine CD25 on T cells, reducing T cell proliferation and IFN- γ secretion [11, 12]. Moreover, a recent study demonstrated that cocultures of T cells, previously incubated with proteolytically active Der p 1, with autologous B cells enhanced specific IgE responses [13]. Altogether, these data clearly showed that Der p 1 biases the immune system in favour of the activation of CD4⁺-Th2 cells. Allergen-specific immunotherapy, involving sublingual or subcutaneous administration of increasing doses of allergen extracts, has been mainly used in the treatment of rhinitis and insect venom anaphylaxis [14]. The mechanism of specific immunotherapy is not fully understood but recent evidence suggested a shift of the Th2-type allergic responses towards a non-pathological Th1-type profile. However, desensitization with total allergen extracts can cause

Correspondence: Alain Jacquet, Department of Applied Genetics, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, rue des Professeurs Jeener et Brachet, 12 B-6041 Gosselies, Belgium. E-mail: alain.jacquet@ulb.ac.be

severe anaphylactic reactions during the treatment. The allergen extracts are often heterogeneous from batches to batches and consequently hard to standardize. Moreover, these extracts contain non-allergenic and toxic protein inducers of undesirable side-effects. Consequently, new immunotherapeutics, with reduced side-effects, higher efficacy and devoted to the reprogramming of immune responses towards a Th1 pathway, represent the most promising approaches for the treatment of allergic diseases.

Vaccination with naked DNA encoding antigen induces cellular and humoral immunity characterized by the activation of specific Th1 cells and CD8⁺ cytotoxic lymphocytes (CTL) secreting predominantly IFN- γ [15]. In the context of allergy, administration of DNA encoding allergen can selectively prevent activation of Th2 cells and IgE generation by B cells and consequently abolishes the release of histamine in bronchoalveolar fluids and airway hyper-responsiveness in challenged animals [16–26]. Moreover, DNA vaccination with plasmid encoding allergens shifted typical Th2 allergic responses to Th1 when administrated in allergic animals [26, 27].

In the present study, the immunogenicity of naked DNA encoding ProDer p 1, the enzymatically inactive precursor form of Der p 1, was evaluated in mice. As we recently designed a synthetic gene encoding ProDer p 1 on the basis of the codon usage frequently found in highly human expressed genes, this coding cassette was cloned into a pJW4304 vector suitable for DNA vaccination [28]. The specific immune responses were compared with those obtained after immunizations with purified natural Der p 1 or recombinant ProDer p 1 (rec-ProDer p 1), both allergens being adjuvanted with aluminium hydroxide.

Materials and methods

Protein antigens

Natural Der p 1 from whole *Dermatophagoides pteronyssinus* culture and recProDer p 1 from CHO spent culture medium were purified to homogeneity as previously described [28].

Animals

Female CBA/J mice (6 weeks old) were obtained from Iffa-Credo Laboratories (Brussels, Belgium).

Construction of ProDer p 1 recombinant plasmid

ProDer p 1 expression plasmid pNIV4846 was digested with *Hind*III-*Bam*HI to isolate the 1070 bp ProDer p 1 coding cassette (full-length 1–302aa ProDer p 1 synthetic cDNA with optimized mammalian codon usage downstream to the VZV gE signal peptide) [28]. This DNA fragment was cloned into the pJW4304 vector (a kind gift from Mullins, University of Washington) restricted with *Hind*III-*Bgl*II. The resulting plasmid was named pNIV4868. Plasmids pNIV4868 and control pJW4304 were amplified in transformed *E. coli* DH5 α and purified by two consecutive caesium chloride gradients.

Endotoxin levels were determined using the limulus assay (Limulus amoebocyte lysate QCL 1000, BioWhittaker, Verviers, Belgium). DNA used for nucleic acid vaccination contained less than 0.2 EU/10 μ g DNA.

Transient transfections

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Merelbeke, Belgium) supplemented with 5% fetal calf serum, 10 mM Hepes (pH 7.3), 2 mM glutamine, 100 U/mL penicillin and 10 μ g/mL streptomycin. Confluent monolayers of COS-7 (containing approximately 7×10^5 cells/well) growing in six-well dishes were transfected with 1 μ g of the respective DNA construct using the Eugene 6 transfection reagent (Roche, Brussels, Belgium). Spent culture medium was harvested after 48 h and incubated overnight with 50 μ L of Concanavalin A- Sepharose (Amersham Biosciences, Roosendaal, the Netherlands) conditioned in PBS pH 7.2 to concentrate the recombinant secreted allergen. To detect ProDer p 1 expression, the resins were collected by centrifugation and applied onto SDS-PAGE. ProDer p 1 was immunodetected in Western blot using a mouse polyclonal antibody directed to recProDer p 1 (a kind gift from J. Pestel, Institut Pasteur de Lille, France) or with an ELISA kit using Der p 1 specific monoclonal antibodies 5H8 and 4C1 (Indoor Biotechnologies, Cardiff, UK). The Der p 1 standard (UVA 93/03) used in the ELISA assay was at a concentration of 2.5 μ g/mL.

Immunizations

Groups of 10 CBA/J mice were immunized intraperitoneally 4-weekly with 5 μ g of natural Der p 1 or recProDer p 1 adjuvanted with alum. The allergens were injected in the presence of alum as adjuvant. In one group, pNIV4868 plasmid was administrated intramuscularly (50 μ L of 150 mM NaCl solution containing 50 μ g ProDer p 1 into each tibialis muscle). As controls, mice were immunized with alum or pJW4304 vector alone. Mice were bled from the retro-orbital venous plexus on days 7, 14, 21, 28 and 39 and sera were collected.

Allergen challenge

Ten days after the last injection, mice were placed in a Plexiglas chamber (13 $\times$ 19 $\times$ 37.5 cm) and exposed to aerosolized crude *D. pteronyssinus* extracts during 30 min and for 7 consecutive days. Each aerosol generated by an ultrasonic nebulizer (Syst'AM) used 15 mL of crude mite extract at a concentration of 300 μ g/mL. The output of the nebulizer was 0.5 mL/min and the mean particle size of the aerosol was between 1 and 5 μ m.

Measurement of ProDer p 1-specific IgG, IgG1 and IgG2a

The presence of anti-ProDer p 1 IgG, IgG1 and IgG2a antibodies in the individual sera was detected by ELISA. Immunoplates were coated with recProDer p 1 (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 μ L of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 h at 37 °C. Plates were washed five times with TBS-Tween buffer and antigen-bound antibodies were detected with the second antibody (goat anti-mouse IgG, Promega, Leiden, the Netherlands) coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer). The enzymatic activity was measured using the p-nitrophenylphosphate substrate (Sigma, Bornem, Belgium) dissolved in diethanolamine buffer (pH 9.8). OD_{415nm} was measured in a Biorad Novapath ELISA reader.

Mouse antibody subclass was determined using immunoplates coated as described above and IgG1- or IgG2a-specific

biotin-labelled monoclonal antibodies (rat anti-mouse, dilution 1/7000 in TBS-Tween buffer and 1% BSA, Biosource) as second antibodies. Phosphatase alkaline-conjugated streptavidin (1/1000 dilution, Amersham) was added to each well. Assay of the enzymatic activity proceeded as described above.

In all cases, ELISA titres were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD₄₁₅ value.

Measurement of ProDer p 1-specific IgE

Immunoplates were coated with rat anti-mouse IgE (10 ng/well), for 16 h at 4 °C. Plates were washed five times with TBS-Tween and saturated for 1 h at 37 °C with 150 µL of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 h at 37 °C. RecProDer p 1 was then added at 500 ng/mL in saturation buffer. Bound recProDer p 1 was detected by addition of biotinylated anti-Der p 1 monoclonal antibody 4C1 (Indoor Biotechnologies). Plates were washed five times with TBS-Tween buffer and antibodies-bound antigen was detected with addition of streptavidin coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer). The enzymatic activity was measured using the p-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{415nm} was measured in a Biorad Novapath ELISA reader.

Proliferation assays

To measure Der p 1-specific T cell proliferative response, immunized mice were killed and lymphocytes were isolated from spleens. Cells (4×10^5 /well in triplicate), cultured in RPMI 1640 with 10% FCS containing 15 mM HEPES and 30 µM β-mercaptoethanol, were stimulated with serial dilutions of recProDer p 1 in 96-well plates (40, 4, 0.4 and 0.04 µg/mL). As control, cells were incubated with only RPMI medium. After 72 h, cells were pulsed with 1 µCi/well [³H] thymidine (Amersham) for 16 h. Cells were harvested and ³H-thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as the means of quadruplicate wells and were expressed as stimulation index (SI). A stimulation index of > 2 was considered positive.

Cytokines assay

To assess the cytokine production of proliferative T cells, splenocytes were stimulated with 20 µg/mL recProDer p 1 and culture supernatants were collected after 72 h. The levels of IFN-γ and IL-5 in the supernatants were measured in ELISA assays. Plates were coated with 1 µg/mL of anti-mouse IL-5 monoclonal (Pharmingen, Erembodegem, Belgium) or anti-mouse IFN-γ (Biosource, Nivelles, Belgium) polyclonal antibodies. Plates were washed five times with TBS-Tween and saturated for 1 h at 37 °C with 150 µL of TBS-Tween-BSA. Serial dilutions of splenocyte culture supernatants were added and incubated for 90 min at 37 °C. Biotinylated anti-mouse IL-5 (Pharmingen, 1 µg/mL) or anti-mouse IFN-γ (Biosource, 0.2 µg/mL) antibodies were applied to the plates for 1 h at 37 °C. The antigen-antibody complexes were detected by incubation with streptavidin coupled to horseradish peroxidase (dilution 1/10 000, Amersham). The enzymatic activity was measured using tetramethylbenzidine (TMB) as substrate (Sigma). The absorbance at 460 nm was measured in a Biorad Novapath ELISA reader.

Cytokine concentrations were determined by interpolation from a standard curve performed with purified mouse IL-5 or IFN-γ.

Bronchoalveolar lavage

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 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. Cytopspin preparations from 50 µL-aliquots were stained with May-Grünwald Giemsa stain for differential cell counts.

Statistical analysis

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

Results

Transient recProDer p 1 expression

To examine whether pNIV4868 was able to express recProDer p 1 in eukaryotic cells, COS-7 cells were transiently transfected with this DNA construct or with the parental plasmid (pJW4304), serving as a negative control. RecProDer p 1 expression was analysed by immunoblotting analysis of spent culture medium (Fig. 1a).

As shown in Fig. 1, pNIV4868 DNA-transfected cells express a secreted form of recProDer p 1 with an apparent molecular mass of 36–40 kDa, whereas the recombinant allergen was not detected in culture medium of control pJW4304-transfected COS-7 cells. Moreover, recProDer p 1 was shown to adopt a correct folding as the recombinant allergen was recognized by conformational anti-Der p 1 monoclonal antibodies (Fig. 1b).

Genetic vaccination prevents the development of ProDer p 1-specific IgE

The humoral immune response induced after vaccination with naked DNA ProDer p 1 was examined in naïve CBA/J mice. The specific antibody titres were compared with that induced with natural Der p 1 or recProDer p 1 adjuvanted with alum (Table 1). As expected, no ProDer p 1-specific responses were observed in mice injected with alum or pJW4304 plasmid alone. DNA ProDer p 1 induced significantly weaker global humoral responses than the corresponding purified allergens (*P* < 0.05). However, mice receiving DNA ProDer p 1 developed a production of anti-ProDer p 1 IgG2a together with a complete abolition of specific IgE antibodies and weak IgG1 antibody responses. In contrast, protein immunizations with Der p 1 or recProDer p 1 induced typical allergic Th2-biased immune responses characterized by high titres of anti-ProDer p 1 IgG1, large amounts of specific IgE antibodies as well as a weak ProDer p 1-specific IgG2a response (*P* < 0.001). Comparison of the IgG1 : IgG2a ratio between groups clearly confirmed that immune responses induced by DNA ProDer p 1 are preferentially Th1-orientated.

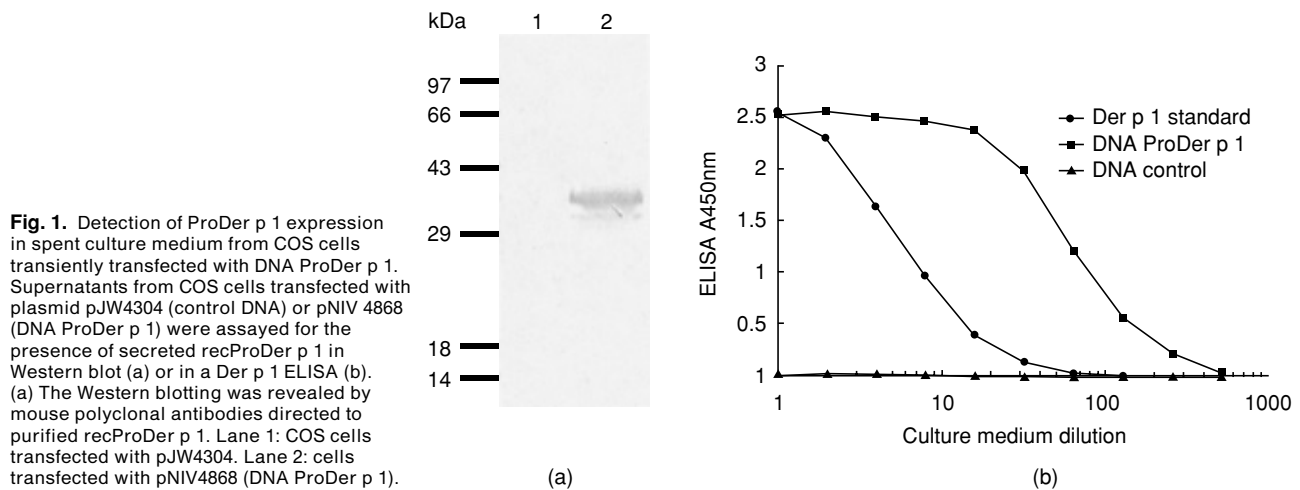


Table 1. Anti-recProDer p 1 antibodies from mice immunized with different antigens. Groups of 10 CBA/J mice were immunized intraperitoneally weekly for four times with 5 µg of natural Der p 1 or recProDer p 1 adjuvanted with alum or with pNIV4868 plasmid administered intramuscularly (50 µg DNA ProDer p 1 into each tibialis muscle). As controls, mice were immunized with alum or pJW4304 vector alone. Mice were bled from the retro-orbital venous plexus on days 7, 14, 21, 28 and 41 and sera were collected. Specific anti-ProDer p 1 IgG, IgG1 and IgG2a titres were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD_{415nm} value. Results are expressed as the mean of individual titre ± SD from 10 mice. For IgE titres, results are expressed as OD_{415nm} values for a 1/10 dilution of sera. Titres were also measured after bronchoprovocations with *D. pteronyssinus* extracts (HDM) (day 41). **P* < 0.05 compared with Der p 1- or recProDer p 1-vaccinated mice. ***P* < 0.001 compared with Der p 1- or recProDer p 1-vaccinated mice

Antigen	Bleeding	IgG	IgG1	IgG2a	IgE (OD dil 10x)	IgG1/ IgG2a
Der p 1	Day 14	201 ± 50	1135 ± 498	< 10	0.85 ± 0.33	> 113.5
	Day 21	3264 ± 1698	18002 ± 8664	< 10	0.34 ± 0.15	> 1800.2
	Day 28	8271 ± 3840	43306 ± 22881	< 10	0.59 ± 0.25	> 4330.6
	Day 39	7833 ± 3985	59010 ± 32490	< 10	0.66 ± 0.27	> 5901.0
recProDer p 1	Day 14	929 ± 381	7422 ± 2624	120 ± 54	0.80 ± 0.21	61.8
	Day 21	5061 ± 1946	27244 ± 12509	301 ± 68	0.37 ± 0.09	90.5
	Day 28	15110 ± 10099	68960 ± 18966	684 ± 141	1.19 ± 0.33	100.8
	Day 39	12225 ± 5323	63198 ± 16722	778 ± 125	0.46 ± 0.05	81.2
DNA ProDer p 1	Day 14	372 ± 214	855 ± 704	248 ± 158	0.01 ± 0.01	3.4
	Day 21	725 ± 759	728 ± 550	985 ± 1403	0.00 ± 0.00	0.7
	Day 28	782 ± 1172*	918 ± 653**	1407 ± 1820**	0.02 ± 0.01**	0.7
	Day 39	1123 ± 979*	1383 ± 1087**	1184 ± 1356**	0.03 ± 0.02**	1.2
Alum	Day 14	< 50	< 50	< 10	0.00 ± 0.00	–
	Day 21	< 50	< 50	< 10	0.00 ± 0.00	–
	Day 28	< 50	< 50	< 10	0.01 ± 0.01	–
	Day 39	< 50	< 50	< 10	0.01 ± 0.01	–
DNA control	Day 14	< 50	< 50	< 10	0.00 ± 0.00	–
	Day 21	< 50	< 50	< 10	0.00 ± 0.00	–
	Day 28	< 50	< 50	< 10	0.01 ± 0.01	–
	Day 39	< 50	< 50	< 10	0.02 ± 0.01	–

Immunization with DNA ProDer p 1 prevents airway eosinophilia after challenge

To determine whether DNA ProDer p 1-vaccinated mice developed allergen-induced airway inflammation after a challenge, all groups of mice were exposed to aerosolized house dust mite extracts for seven consecutive days. Cellular composition in bronchoalveolar lavage (BAL) fluids were analysed after cell staining (Table 2). Priming with Der p 1 emulsified with alum followed by repeated bronchoprovocations resulted in an

important cellular infiltrate in the pulmonary cavities. This cell accumulation was not observed in all the other vaccinated groups (*P* < 0.05). The great majority of cellular infiltrate in BAL fluids from mice sensitized with natural Der p 1 was eosinophils. Strikingly, eosinophils accumulated weakly in BAL fluids of mice vaccinated with DNA ProDer p 1 or recProDer p 1 adjuvanted with alum, the relative cellular composition of these BAL fluids was statistically similar to those of the control groups (mice vaccinated with alum or DNA control, *P* > 0.05).

DNA ProDer p 1 immunization up-regulates IFN- γ production by splenocytes

Twenty-four hours after the last nebulization, spleen cells from immunized mice were examined for T-cell proliferative responses after *in vitro* stimulation with recProDer p 1. Splenocytes from DNA ProDer p 1-immunized animals proliferated in a dose-dependent manner when incubated with increasing amounts of recProDer p 1 (Fig. 2). The deduced stimulation indexes (SI) were similar to those of Der p 1 or recProDer p 1-vaccinated animals ($P > 0.05$). To determine the nature of the cytokine secretion profiles, supernatants from cultured stimulated lymphocytes were harvested and assayed for IFN- γ and IL-5. Typical Th2 cytokine profile characterized with high production of IL-5 and low secretion of IFN- γ was elicited by Der p 1 or recProDer p 1 vaccinations ($P > 0.05$) (Fig. 3). In contrast, spleen cells from animals immunized with DNA ProDer p 1 secreted, respectively, large and very low amounts of IFN- γ and IL-5 upon restimulation with recProDer p 1. Mice vaccinated with pJW4304 or alum produced neither IFN- γ or IL-5 in the same experimental conditions.

Discussion

New immunotherapeutic approaches against house dust mite allergy could be based on the immunomodulation of the Th2-biased allergic responses to non-allergic Th1-orientated responses by genetic vaccination with plasmid DNA encoding mite allergens [14, 29, 30]. Indeed, DNA immunization has been reported to induce preferentially strong Th1-biased immune responses. In this context, we constructed an expression vector suitable for nucleic acid vaccination and carrying the coding cassette of ProDer p 1, a precursor form the major house dust mite allergen Der p 1. ProDer p 1 could be less allergenic than Der p 1 for two reasons. First, Der p 1 maturation was not observed during allergen expression by DNA ProDer p 1-transfected CHO or COS cells [28]. Consequently, the absence of enzymatic activity in secreted ProDer p 1 could likely alter the Th2-bias of the immune responses provoked partly by the enzymatic activity of mature Der p 1. Secondly, we recently demonstrated that, although the overall IgE-binding epitopes of Der p 1 are conserved in ProDer p 1, the

Table 2. Cellular composition in bronchoalveolar lavage fluids of immunized and non-immunized animals challenged with aerosolized house dust mite extracts. Ten days after the last injection (day 31), sensitized mice were exposed to aerosolized crude *D. pteronyssinus* extracts during 30 min and for 7 consecutive days. 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. The lavage fluid was centrifuged and the cell pellet was resuspended in Hank's balanced salt solution (HBSS). Cytospin preparations from 50 μ L-aliquots were stained with May-Grünwald Giemsa stain for differential cell counts. Values are expressed as the means $\pm$ SD from 10 mice. Total cell counts were performed in a Thoma chamber. * $P < 0.05$ compared with other groups of vaccinated mice. ** $P > 0.05$ compared with recProDer p 1-, alum- or DNA control-vaccinated mice

Antigen	Cell numbers $\times 10^5/\text{mL}$	Cell percentage			
		Macrophages	Lymphocytes	Neutrophils	Eosinophils
Der p 1	91.2 $\pm$ 71.3*	9.9 $\pm$ 5.4	27.6 $\pm$ 22.0*	3.6 $\pm$ 3.6	58.8 $\pm$ 22.9*
recProDer p 1	5.2 $\pm$ 2.8	13.1 $\pm$ 7.2	73.4 $\pm$ 9.6	6.5 $\pm$ 6.7	6.5 $\pm$ 4.8
DNA ProDer p 1	10.6 $\pm$ 6.1**	11.1 $\pm$ 3.6**	81.7 $\pm$ 3.6**	3.6 $\pm$ 3.4**	3.5 $\pm$ 2.9**
Alum	7.7 $\pm$ 4.4	12.8 $\pm$ 4.8	78.3 $\pm$ 6.7	7.8 $\pm$ 4.9	1 $\pm$ 1.6
DNA control	12.0 $\pm$ 6.3	8.4 $\pm$ 4.1	88.4 $\pm$ 5.1	2.6 $\pm$ 4.2	0.6 $\pm$ 0.9

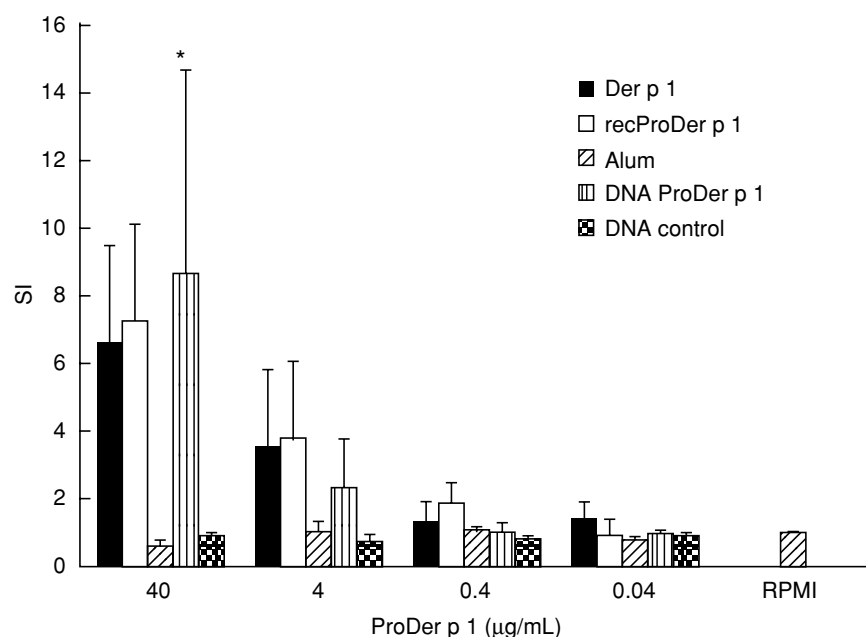


Fig. 2. Lymphoproliferative response from splenocytes after *in vitro* stimulations. Splenocytes from mice vaccinated with adjuvanted allergens or with DNA were stimulated with serial dilutions of recProDer p 1 as relevant antigen. The Concanavalin A (ConA) positive sample showed a stimulation index of 20 to 30. Samples were assayed in triplicate. Results are expressed as mean stimulation index $\pm$ SD from 10 mice. * $P > 0.05$ compared with Der p 1- or recProDer p 1-vaccinated mice.

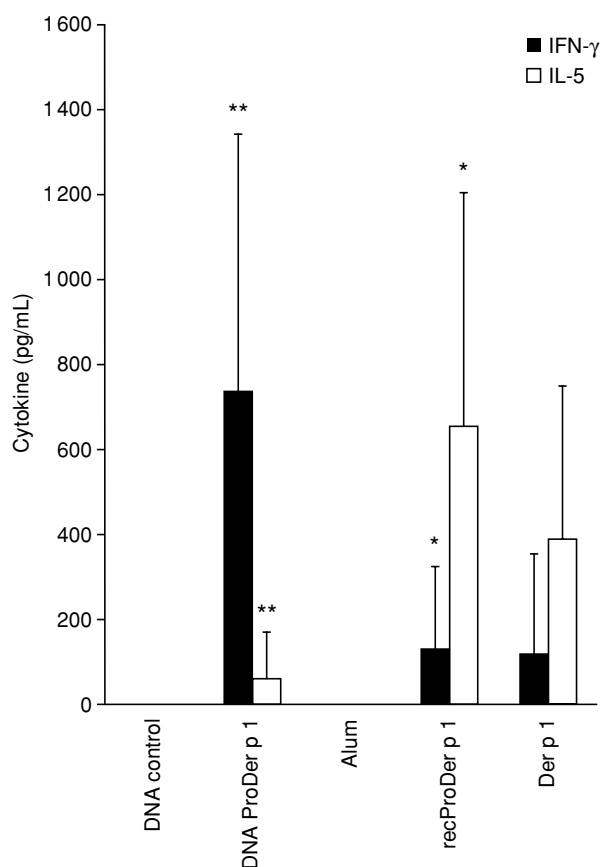


Fig. 3. IL-5 and IFN- γ concentrations in supernatants from recProDer p 1-restimulated splenocytes. Splenocytes were stimulated with 20 μ g/mL recProDer p 1, and culture supernatants were collected after 72 h. The production of IL-5 and IFN- γ in supernatants was measured by ELISA. Results are expressed as the means $\pm$ SD from 10 mice. * P > 0.05 compared with Der p 1-vaccinated mice. ** P < 0.05 compared with Der p 1- or recProDer p 1-vaccinated mice.

histamine releasing capacity of the Der p 1 precursor was drastically reduced [28]. The IgE-facilitated antigen presentation of ProDer p 1 in dendritic cells and B cells leading to Th2-type cytokines could thus be impaired.

Our results clearly demonstrated that DNA vaccination with plasmid encoding ProDer p 1 prevents not only the development of allergen-specific IgE but also the induction of airway inflammation after challenge with aerosolized house dust mite extracts. These results are consistent with those previously reported for several DNA vaccines encoding allergens, notably the mite allergens Der p 5, Der f 11 or murine T cell epitopes on Der p 1 and 2 [23, 25, 26]. DNA ProDer p 1 induced predominantly Th1-biased specific responses characterized by strong IgG2a, low IgG1 and, strikingly, no IgE antibodies. Conversely, purified Der p 1 or recProDer p 1 adjuvanted with alum elicited typical allergic responses as strong IgG1 and IgE but no IgG2a anti-ProDer p 1 antibodies were produced, suggesting that the adjuvanted Der p 1 precursor is allergenic. The development of Th1-biased specific responses after DNA ProDer p 1 vaccination was confirmed by the cytokine pattern of T cell proliferation. Indeed, high-level secretion of IFN- γ together with low IL-5 production was detected in DNA ProDer p 1-vaccinated mice. An opposite cytokine profile was observed with adjuvanted Der p 1 or recProDer p 1 immunizations.

Whereas Der p 1-sensitized mice developed airway eosinophilia subsequent to exposure to aerosolized mite extracts, animals vaccinated with DNA ProDer p 1 showed very low level of eosinophilic infiltrates in BAL. The absence of eosinophils in BAL from DNA ProDer p 1-immunized mice was not correlated with the absence of allergen-specific IgE as no airway inflammation was observed in adjuvanted recProDer p 1-vaccinated animals, despite the fact that high titres of specific IgE were detected in this group. The absence of correlation between specific IgE titres and eosinophilia was previously reported in several studies developing murine models of allergy [5, 31].

However, the absence of eosinophils in BAL fluids from recProDer p 1-sensitized mice was, however, surprising, given the induction of high titres of specific IgE, IgG1 and IL-5 in this group of mice. The recruitment of eosinophils in the airways is mediated by IL-5, and the central role of Th2 lymphocytes as a major source of IL-5 has been well established [32]. On the other hand, the role of mast cells and IgE in the airway inflammation remains controversial, although that mast cell activation is known to regulate T cell functions [33].

Recent studies showed that mice, deficient in B cells, IgE, CD23 or mast cells, systemically sensitized and challenged, displayed similar accumulation of eosinophils in the airways to wild-type animals [34–41]. On the other hand, administration of anti-IgE monoclonal antibody in house dust mite antigen-sensitized mice before antigen challenge inhibited the infiltration of eosinophils in the bronchoalveolar lavages [42]. These data suggest the existence of IgE-dependent and IgE-independent mechanisms in the recruitment of eosinophils in the airways. A recent murine model of asthma using Fc ϵ RI α -chain-deficient (Fc ϵ RI α ^{-/-}) mice clearly demonstrated that IgE-dependent mast cell activation is important in airway inflammation [43]. The diminished mast cell activation could thus result in down-regulation of T cell activation in the airways and consequently reduced Th2 cytokine production leading to weaker eosinophil recruitment.

We showed that, although the IgE antibodies directed to Der p 1 recognized similarly recProDer p 1 and the natural allergen, the reactivity of the anti-ProDer p 1 IgE towards Der p 1 is weak (about 50% of the reactivity towards ProDer p 1, data not shown). Consequently, the mast cell activation mediated by anti-ProDer p 1 IgE could be reduced after aerosol exposures, explaining, at least partly, the failure of recProDer p 1-vaccinated mice to develop airway eosinophilia.

It is generally accepted that, following intramuscular DNA injections, antigen-presenting cells (APC) uptake the DNA vaccine and small amounts of the corresponding antigen are synthesized. Consequently, the intensity of the immune response is related to the optimization of the antigen expression. In this context, the ProDer p 1 DNA expression vector described in this study offers two advantages. First, the Der p 1 prosequence likely influences drastically the allergen expression and processing as we previously failed to express a mature form of Der p 1 in mammalian cells (unpublished results) [28]. Secondly, the ProDer p 1 cDNA was synthesized on the basis of the codon usage frequently found in highly human expressed genes as we previously demonstrated that codon usage optimization up-regulated ProDer p 1 expression. Moreover, a recent study showed that DNA vaccination with an optimized synthetic gene

increased immune responses compared with that elicited with the respective wild-type gene [44].

However, and in spite of the plasmid characteristics, the global humoral response induced by DNA ProDer p 1 vaccinations was shown to be statistically weaker than that obtained with the corresponding purified allergen adjuvanted with alum. The DNA ProDer p 1 formulation could be optimized by complex formation with cationic lipids to enhance specific immune responses as previously reported with DNA vaccines complexed to vaxfectin or mannan-coated diC14-amidine [45, 46]. A DNA vaccine encoding murine T cell epitopes on Der p 1 has already been described as an efficient prophylactic vaccine against dust mite allergy [23]. However, although DNA encoding single or multiple T cell epitopes of allergens could prevent B-cell activation and subsequently the production of specific IgE and IgE-mediated anaphylaxis, the production of allergen-specific IgG induced by these Der p 1 epitopes could be weaker than that induced with DNA encoding whole allergen. The intensity of the production of specific blocking antibodies, particularly of the IgG4 class, was shown to be important to impair IgE-facilitated antigen presentation [47, 48].

As DNA vaccines lead to allergen presentation through MHC class I and II molecules, both CD4⁺ Th cells and CD8⁺ cytotoxic T cells are activated and can secrete IFN- γ , which inhibits the development of CD4⁺ Th2 cells and the production of specific IgE [25–27]. Depletion experiments using anti-CD4 or anti-CD8 monoclonal antibodies are currently underway to determine the nature of regulatory T cells after ProDer p 1 DNA vaccination and involved in the suppression of IgE induction.

In conclusion, DNA ProDer p 1 vaccination could represent a new approach for the treatment of house dust mite allergy. To clearly demonstrate that DNA ProDer p 1 vaccine constitutes an efficient prophylactic vaccine, subsequent immune responses after Der p 1 sensitization and airway challenge in previously DNA ProDer p 1 vaccinated mice are currently under study. The therapeutic effect of DNA ProDer p 1 vaccination is also, at the moment, evaluated as an alternative to conventional immunotherapy. This vaccination could shift the Th2 immune responses induced by Der p 1 sensitization and airway exposure towards Th1.

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