

Induction of specific Th1 responses and suppression of IgE antibody formation by vaccination with plasmid DNA encoding Der f 11

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Abstract

DNA vaccines encoding low-molecular-weight allergens have been used to prevent IgE responses. A high-molecular-weight mite allergen Der f 11 that was hardly to be purified for immunotherapy was used to develop a DNA vaccine here. Vaccination of mice with plasmid DNA encoding Df11 (pDf11) induced Th1 responses characterized by IgG2a responses and spleen cell secretion of IFN- γ . In contrast, sensitization with recombinant Der f 11 (rDf11) and alum induced Th2 responses characterized by IgE responses and spleen cell secretion of IL-4 and IL-5. Vaccination with pDf11 prevented the induction of IgE responses. Moreover, it could inhibit on-going IgE responses. The debate whether CD4⁺ or CD8⁺ T cells were the regulatory cells to inhibit IgE responses by DNA vaccination was also examined. First, sensitization of pDf11-vaccinated mice after depletion of CD8⁺ T cells still showed suppression of IgE responses. Secondly, adoptive transfer of either CD4- or CD8-depleted spleen cells from pDf11-vaccinated mice suppressed IgE responses. In conclusion, this is the first report to confirm the therapeutic effect of a DNA vaccine encoding a strong allergen on specific IgE responses. Both CD4⁺ and CD8⁺ T cells are crucial for the immunomodulation of IgE responses by pDf11. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: DNA vaccine; Th1 responses; Allergen; IgE; Th2 responses

1. Introduction

Aeroallergens derived from house dust mites *Dermatophagoides pteronyssinus* (Dp) and *Dermatophagoides farinae* (Df) have been recognized as common allergens of asthma, perennial rhinitis and atopic dermatitis [1]. Mite allergy affects about 10–20% of world population [1]. Most mite allergens have low-molecular-weight ranging from 14 to 60 kDa [2]. A unique high-molecular-weight mite allergen has been recently cloned by us and named as Der f 11 [3–5]. It is mite paramyosin, a muscle-associated protein [4,6]. Whereas, parasite paramyosin is a strong immunogen and has been regarded as a promising vaccine for parasite diseases [7], mite paramyosin Der f 11 is a major allergen in allergic patients and a strong immunogen in normal humans [6,8]. The recombinant Der f 11 (rDf11) was used to set up a mouse allergic model in this report.

Allergic diseases are characterized by sensitization of allergen-specific Th2 cells and Th2 cytokine-dependent IgE

production [9,10]. Since the regulation of IgE generation is controlled by the reciprocal activities of Th1 and Th2 cells and their cytokines, particularly IFN- γ and IL-4 [10,11], the aim of immunotherapy has been currently focused on modulation of Th2 responses to Th1 responses [12,13]. Recent studies in rodents have demonstrated that DNA vaccination is a simple approach to activate Th1 responses and to counteract Th2 responses [14–22]. Unlike protein vaccines, DNA vaccines induce long-lasting intracellular expression of recombinant allergens that can be presented onto MHC class I and class II molecules [23]. This leads to cellular and humoral immunity characterized by the generation of specific Th1 cells secreting predominantly IFN- γ [15–20]. IFN- γ can selectively inhibit activation of Th2 cells and IgE generation by B cells [24]. DNA vaccines have been thus postulated to be a promising candidate for allergic diseases [12,13].

Whereas, DNA vaccines have been used to prevent subsequent sensitization of IgE reactions [14–21], the therapeutic effect has not been studied using plasmid DNA encoding a well-established allergen. It was confirmed here that an on-going IgE antibody response to rDf11 could be inhibited by plasmid DNA encoding Df11 (pDf11). Moreover,

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the regulatory T cells underlying the suppression of IgE by DNA vaccination remain controversial. Whereas, two reports found that only CD8⁺ T cells were the immunoregulatory T cells in DNA vaccination [14,15], another report found that both CD4⁺ and CD8⁺ T cells played some roles in DNA vaccination [17]. To determine the potential therapeutic T cell population, we have further sought evidence here that both CD4⁺ and CD8⁺ T cells play crucial roles in pDf11-vaccinated mice.

2. Materials and methods

2.1. Animals

Female BALB/c (H-2d) mice purchased from the National Animal Center of Taiwan (Taipei) were first used at 6–8 weeks of age. They were maintained on a standard diet (Lab diet; PMI Feeds, St. Louis, MO, USA) in the Animal House of Veterans General Hospital-Taipei (Taiwan). All the animal experiments were performed at least twice.

2.2. Construction of recombinant plasmid pDf11

Recombinant plasmid pDf11 was constructed by insertion of Der f 11 cDNA, a 2078 bp DNA fragment digested with enzymes *Bam*HI and *Apa*I from the plasmid pBlue-script/Df11 [4], into the *Bam*HI and *Apa*I sites in the vector pcDNA3 (Invitrogen, Groningen, The Netherlands). Plasmids pDf11 and pcDNA3 were grown, respectively, in transformed *Escherichia coli* DH5 α in Luria–Bertani broth supplemented with ampicillin (100 μ g/ml). Large-scale purification of both plasmids was conducted using a Qiagen maxiprep kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. The quality and quantity of the DNA were analyzed by absorption at 260 and 280 nm, and by agarose gel electrophoresis. The DNA was stored at -20°C and re-suspended immediately before use with sterile PBS (1 mg/ml).

2.3. Preparation of rDf11

The rDf11 was expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli* and purified as previously described [4,6]. In addition, rDf11 was purified through GST-specific cleavage by adding thrombin to GST-containing rDf11 when it was bound to glutathione Sepharose 4B.

2.4. Preparation of spleen cells

Mouse spleen cells were collected and washed by RPMI-1640 with 10% FCS, 2 mM L-glutamine, 100 u/ml of penicillin and 100 μ g/ml of streptomycin. RBCs were depleted using EDTA–NH₄Cl lysing buffer. The suspension was washed three times with medium at 4°C and spleen

cells were i.p. transferred into naïve recipients (5×10^7 cells per mouse).

2.5. Depletion of CD4⁺ or CD8⁺ T cells in vivo

Rat hybridoma cells GK1.5 and 3.155 (ATCC, Rockville, MD) were i.p. transplanted in pristane-primed scid mice (2×10^6 cells per mouse). Ascites containing IgG2b and IgM monoclonal antibodies specific for mouse CD4 and CD8 were collected by tapping and pooled, respectively (1.9 and 6.2 mg/ml). Mice were i.p. injected daily with 0.6 or 0.3 ml of ascites, respectively, for 3 days. One day after the third injection, depletion of CD4⁺ or CD8⁺ T cells was confirmed by FACS analysis ($<1\%$, respectively) using 1:20 diluted FITC-conjugated rat IgG specific for mouse CD4 or CD8, respectively (Caltag Lab., CA, USA).

2.6. Systemic sensitization

Mice were sensitized i.p. with 10 μ g rDf11 adsorbed onto 4 mg alum and suspended in 0.5 ml of PBS. They were bled weekly after sensitization. The separated serum was frozen at -20°C until required.

2.7. Assessment of specific IgG2a and IgG1 antibody responses

Antibody responses were assessed using an ELISA as previously described [25]. Briefly, 96-well plates (Nunc, Denmark) were coated with 4 μ g/ml rDf11 in 0.05 M carbonate buffer, pH 9.6 (100 μ l per well) overnight at 4°C . Free sites were blocked with 3% skimmed milk in PBS–Tween 20 for 1 h. Serum samples (1/50–1/1000) and standards (pooled sera collected after monthly sensitization for 4 months) were added in duplicate and the plates incubated for 5 h at RT. After washing, 100 μ l of horseradish peroxidase-conjugated goat anti-mouse IgG2a or IgG1 (1/4000; SBA, Birmingham, AL, USA) were added and the plates incubated overnight at 4°C . After washing, orthophenylenediamine (OPD; 0.5 mg/ml; Sigma) in citrate–carbonate buffer containing 0.015% H₂O₂ were added and the plates incubated in the dark. Color development was stopped by 4N H₂SO₄. Absorbance at 492 nm was read using a reader (SPECTRA-max 250; Molecular Devices, Sunnyvale, CA, USA) and unknowns were interpolated.

2.8. Passive cutaneous anaphylaxis

Serum IgE antibody responses to rDf11 were assessed as described [25] by passive cutaneous anaphylaxis (PCA) tests in Sprague–Dawley rats (300–450 g) obtained from the Animal Center, National Yang-Ming University (Taipei, Taiwan). Aliquots of 100 μ l of two-fold dilutions of pooled mouse serum samples (1/2.5–1/1280) were i.d. injected into the rats. They were challenged 48 h later by an i.v. injection of 0.5 mg rDf11 and 5 mg Evans Blue in 1 ml

PBS. The reaction was read 30 min later. The PCA titer was the highest dilution giving a positive reaction of at least 5 mm in diameter and was expressed as mean $\pm$ 1 S.E.M.

2.9. Cytokine assays

Spleen cells collected as before were pooled from three to five mice and plated in 24-well tissue culture plates (4×10^6 cells per ml). Cells were cultured with rDf11 (20 μ g/ml) or medium only. Supernatants were harvested at 24 and 72 h, and cytokines IL-4, IL-5, and IFN- γ were measured using sandwich ELISA kits (Endogen, Woburn, MA, USA).

2.10. Statistical analysis

Specific IgG1 and IgG2a antibody and cytokine titers were expressed as means $\pm$ 1 S.D. and all group comparisons for specific IgG2a and IgG1 responses were made using two-tailed Wilcoxon tests.

3. Results

3.1. Vaccination with pDf11 induced IgG2a but not IgE responses

The effect of DNA vaccination with pDf11 was first studied. Groups of six BALB/c mice were i.m. injected with 100 μ g pDf11, 10 μ g rDf11 alone or precipitated with 4 mg alum. They were boosted 3 weeks later. Their serum specific IgE, IgG2a and IgG1 antibody responses were tested 3 weeks after the last injection (Fig. 1). Compared with the controls receiving rDf11 only, the mice receiving pDf11 had increased IgG2a ($P < 0.005$) but similarly weak IgG1 antibody responses. Both had none of IgE antibody responses. In contrast, those receiving rDf11 in alum had very strong IgG1 ($P < 0.005$) and IgE antibody responses, but weak IgG2a antibody responses. In addition, those receiving pcDNA3 had no responses (data not shown).

3.2. Vaccination with pDf11 enhances spleen cell secretion of IFN- γ

The cytokine profiles after vaccination with pDf11 was further studied. As before, groups of eight mice were i.m. injected triweekly with 100 μ g pDf11 or pcDNA3, or PBS only twice. Three weeks after the last treatment, half of each group were sensitized with 10 μ g rDf11 precipitated with 4 mg alum. Their spleen cells were collected 12 days after sensitization, and their spleen cell cytokine profiles when cultured with 100 μ g rDf11 were shown in Fig. 2. Compared with the controls receiving pcDNA3 or PBS, those receiving 100 μ g pDf11 showed higher production of IFN- γ , but little of IL-4 or IL-5. After sensitization, those receiving 100 μ g

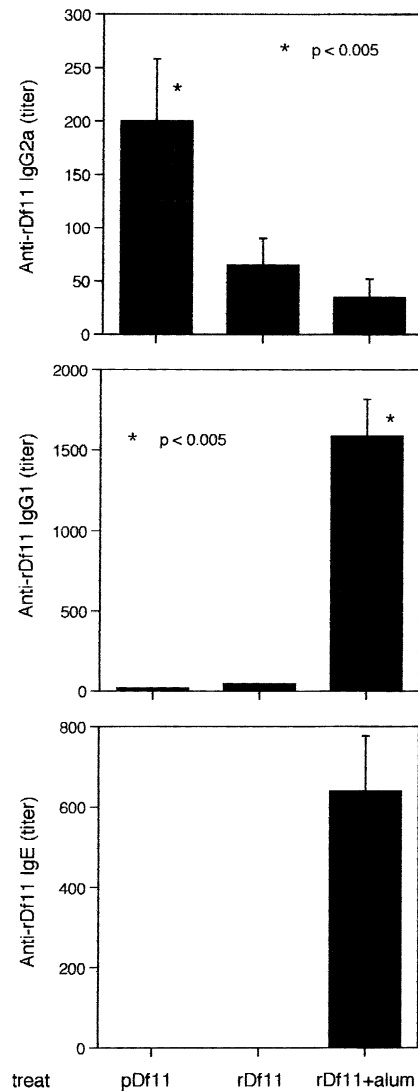


Fig. 1. Specific antibody responses after vaccination with pDf11 and rDf11. Groups of six BALB/c mice were i.m. injected triweekly with pDf11, rDf11 or rDf11 plus alum twice. Their specific antibody responses were measured 3 weeks after the second injection.

pDf11 showed stronger production of IFN- γ , but weak production of IL-4 and IL-5. In contrast, both sensitized controls had enhanced production of IL-4 and IL-5, but very weak secretion of IFN- γ .

3.3. Immunoprophylaxis of specific IgE responses by pDf11 vaccination

Whether DNA vaccination with pDf11 could prevent the induction of specific IgE antibody responses or not was first studied. Groups of six mice were i.m. injected triweekly with 100 μ g pDf11 or pcDNA3, 10 μ g rDf11, or PBS only twice. Three weeks after the second treatment, they were sensitized with 10 μ g rDf11 in alum. Their specific IgE, IgG1 and IgG2a antibody responses were sequentially assessed (Fig. 3). Three weeks after the second vaccination, those

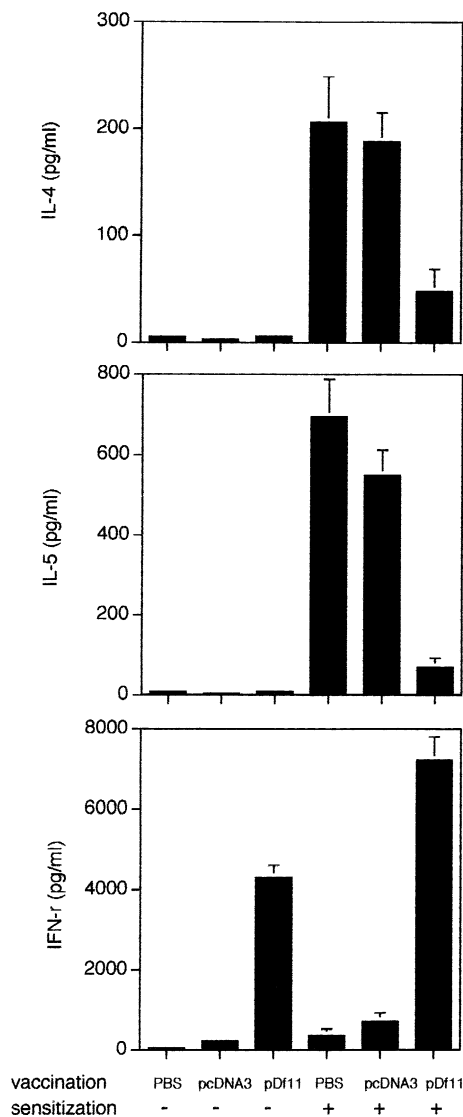


Fig. 2. Cytokine profiles after vaccination with pDf11 and rDf11. Groups of eight BALB/c mice were i.m. injected triweekly with pDf11, pcDNA3 or PBS twice. Three weeks after the last injection, half of each group ($n = 4$) were i.p. injected with PBS or rDf11 in alum, respectively. Their spleen cells were collected 12 days later and cultured with rDf11 in vitro for 1 or 3 days. Their cytokines IFN- γ , IL-4 and IL-5 in the supernatant were measured in duplicate by ELISA kits.

receiving pDf11 showed strong IgG2a, weak IgG1 and no IgE antibody responses to rDf11, whereas those receiving rDf11 showed mild IgG1 and IgG2a but no IgE antibody responses. After sensitization, those receiving pDf11 had moderate increases of IgG2a and IgG1 antibody responses, but conversely suppressed IgE antibody responses. In contrast, those receiving rDf11 showed strong increases of IgE, IgG1 and IgG2a antibody responses. Whereas the controls receiving pcDNA3 or PBS had rapidly increased but still much lower IgG1 and IgG2a antibody responses than those previously treated with pDf11 or rDf11, their specific IgE antibody responses were increased as high as those previously

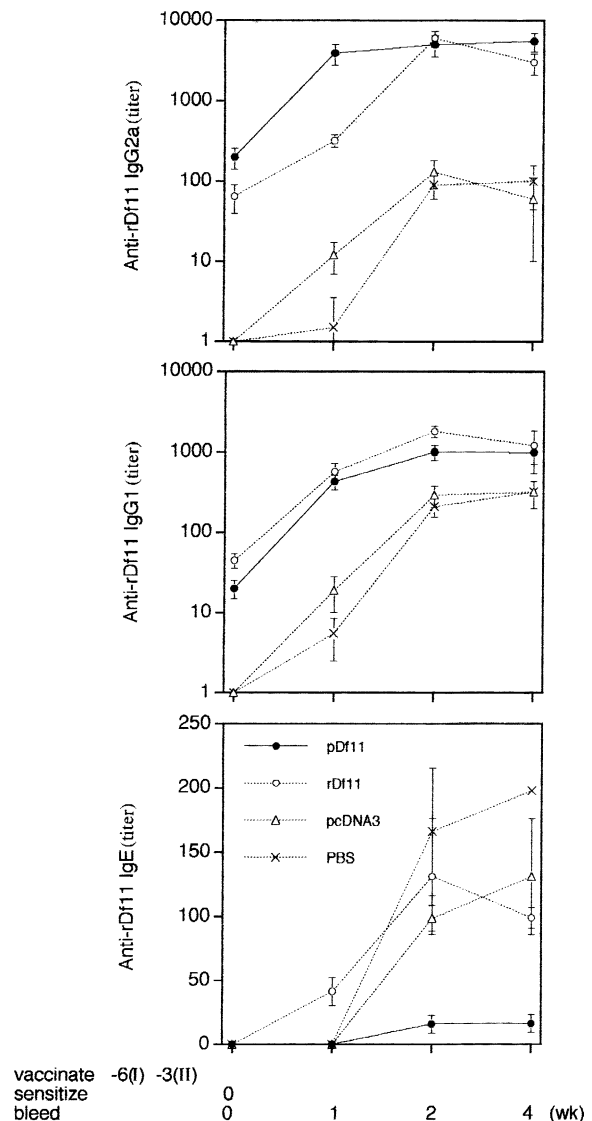


Fig. 3. DNA vaccination with pDf11 can prevent the induction of specific Th2 responses. Groups of six mice were i.m. injected triweekly with pDf11, pcDNA3, rDf11 or PBS only twice. Three weeks after the second injection, they were sensitized with rDf11 in alum. Their specific antibody responses were weekly examined.

treated with rDf11 and both were much higher than those previously treated with pDf11.

3.4. Immunotherapy of on-going IgE responses by pDf11 vaccination

Whether DNA vaccination with pDf11 could suppress on-going IgE antibody responses to rDf11 or not was further studied. Groups of six mice were sensitized twice with 10 μ g rDf11 in alum triweekly. One week after the second sensitization, they were i.m. injected biweekly with 100 μ g pDf11 or pcDNA3, or PBS alone twice. Their serum specific IgE, IgG1 and IgG2a antibody responses were tested before each vaccination and monthly after vaccination (Fig. 4).

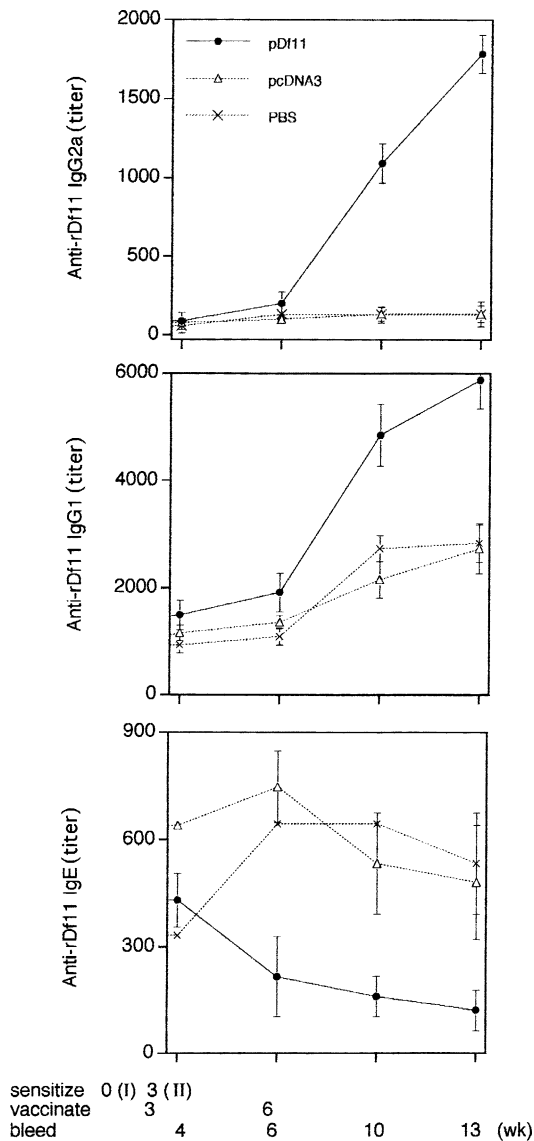


Fig. 4. DNA vaccination with pDf11 can inhibit on-going Th2 responses. Groups of six mice were sensitized twice with rDf11 in alum triweekly. One week after the second sensitization, they were i.m. injected biweekly with pDf11, pcDNA3 or PBS twice. Their specific antibody responses were sequentially examined before and after vaccination.

Compared with the controls receiving pcDNA3 or PBS, those receiving pDf11 showed suppression of the specific on-going IgE antibody response and, in contrast, profound enhancement of specific IgG2a and IgG1 antibody responses. On the other hand, there was little difference between two control groups.

3.5. $CD4^+$ T cells can suppress IgE responses during pDf11 vaccination

Whether $CD4^+$ T cells might have a role to suppress specific IgE antibody responses during pDf11 vaccination was indirectly studied. Groups of 12 mice were i.m. injected

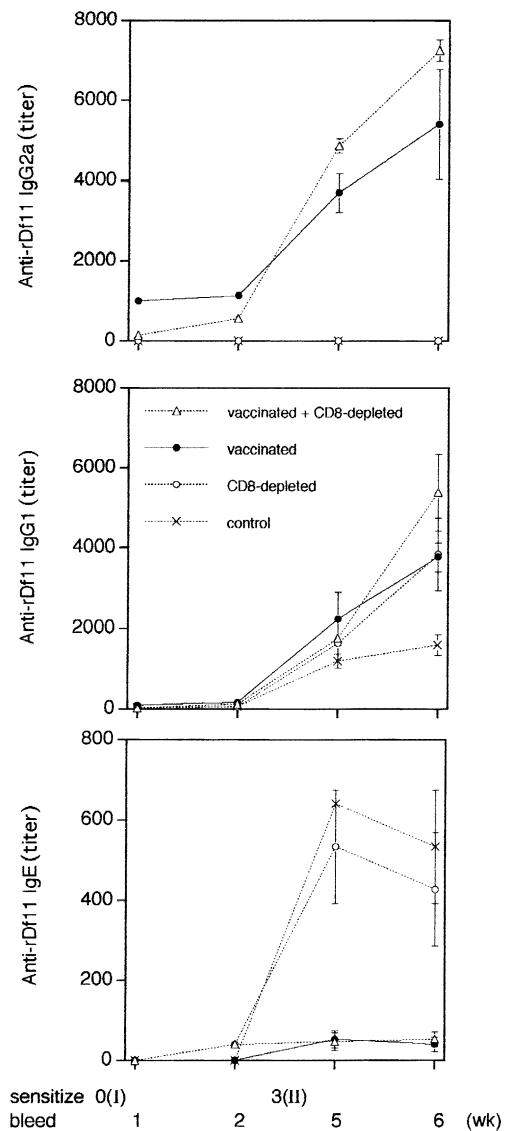


Fig. 5. Suppression of specific IgE responses after depletion of $CD8^+$ T cells in pDf11-vaccinated mice. Groups of 12 mice were i.m. injected monthly with pDf11 or pcDNA3, respectively, twice. They were s.c. sensitized with rDf11 in alum 1 month after the last injection. Meanwhile, half of them were i.p. injected with ascites containing monoclonal antibodies against CD8 for 3 days. They were boosted again 3 weeks later and their antibody responses were sequentially assessed.

monthly with pDf11 or pcDNA3 twice. One month later, they were s.c. sensitized with $10 \mu\text{g}$ rDf11 in alum. At the same time, half of them were i.p. injected with ascites containing monoclonal antibodies against CD8 for 3 days. They were sensitized again 3 weeks later and their antibody responses were examined (Fig. 5). Compared with pcDNA3-treated controls either with or without CD8-depletion, both pDf11-vaccinated groups with or without CD8-depletion showed highly suppressed IgE, greatly enhanced IgG2a and moderately increased IgG1 antibody responses to rDf11.

3.6. Both CD4⁺ and CD8⁺ T cells play some roles to suppress specific IgE antibody responses during pDf11 vaccination

Whether CD8⁺ T cells could suppress specific IgE responses during pDf11 vaccination was also studied. Donor mice were vaccinated as above. One month after the last vaccination, two groups were i.p. injected with ascites containing monoclonal antibodies against CD4 or CD8, respectively, for 3 days and their spleen cells were transferred, respectively, into naïve recipients. They were immediately

s.c. sensitized with 10 µg rDf11 in alum and boosted 3 weeks later. Their antibody responses were shown in Fig. 6. When compared with the controls receiving normal spleen cells, those receiving pDf11-vaccinated spleen cells or pDf11-vaccinated spleen cells with CD4- or CD8-depletion all had suppressed IgE but enhanced IgG2a responses. Among recipients, the group receiving spleen cells with CD8-depletion had increased IgG1 responses but the group receiving spleen cells with CD4-depletion had decreased IgG1 responses.

4. Discussion

The present study has shown that i.m. immunization of BALB/c mice with plasmid DNA pDf11 encoding Der f 11 can preferentially activate specific Th1 immune responses and thus prevents subsequent sensitization of Th2 cell-regulated specific IgE responses. This finding is consistent with recent reports that DNA vaccination is a potential approach for the prophylaxis of allergic diseases [14–22]. In addition, we have further revealed that pDf11 vaccination can extraordinarily induce a Th1 cell-predominated immune response during an on-going Th2 cell-associated allergic reaction and thus down-regulates specific IgE production.

Similar to other reports [14–21], twice or more injections of pDf11 DNA with 3 or 4 weeks apart had stronger immune responses and were used throughout our experiments. Vaccination with pDf11 alone induced exclusively specific Th1 responses characterized by serum strong IgG2a and mild IgG1 but no IgE antibody reactions and by spleen cell secretion of high levels of IFN-γ but not of IL-4 or IL-5. Although the sensitizing capacity of rDf11 is much weaker than that of native Der f 11 (data not shown), it was used as a control protein vaccine since native Der f 11 is very difficult to purify. Indeed, mice injected twice with rDf11 in PBS had weak IgG1 and IgG2a but no IgE antibody responses. Conversely, mice sensitized with rDf11 plus adjuvants induced specific Th2 responses characterized by strong IgE and IgG1 but weak IgG2a antibody reactions. More intriguingly, their IgG2a to IgG1 ratio was conversely inverted when compared with that of pDf11-vaccinated mice. Whereas a newly published report has shown that gene gun inoculation of plasmid DNA encoding pollen allergen Cry j 1 fails to induce specific Th1 responses [20], our data is consistent with those reports that i.m. or i.d. administration of DNA vaccines can predominantly induce specific Th1 responses [14–19].

When further sensitized with rDf11 plus adjuvants, pDf11-vaccinated mice had a strong secondary Th1 response characterized by a much higher IgG2a antibody reaction and by spleen cell secretion of doubly higher levels of IFN-γ and very low levels of IL-4 and IL-5. Moreover, their IgE antibody response was much lower than the primary IgE antibody response of control mice previously treated with PBS or pcDNA3. This finding confirms previous reports

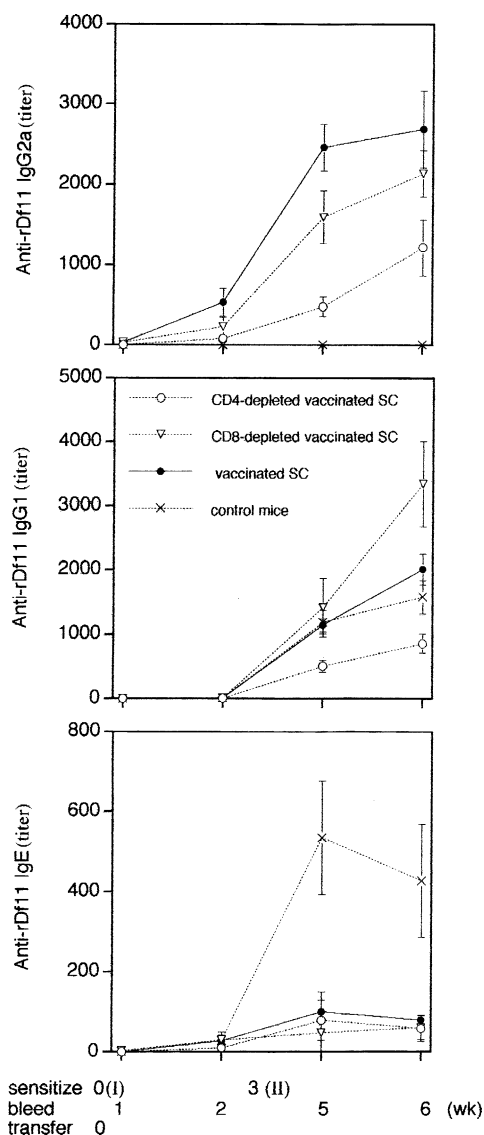


Fig. 6. Suppression of specific IgE responses after transfer of CD4- or CD8-depleted spleen cells from pDf11-vaccinated donors to naïve recipients. Three groups of six mice were i.m. injected monthly with pDf11 twice. Two month later, two groups were i.p. injected with ascites containing monoclonal antibodies against CD4 or CD8, respectively, for 3 days and their spleen cells were transferred, respectively, into naïve recipients. The recipients were s.c. sensitized twice and their antibody responses were assessed.

that DNA vaccination can effectively prevent the subsequent sensitization of a Th2 response [14–19]. In contrast, the mice previously treated with rDf11, a protein vaccine, had a secondary Th2 reaction characterized by increased IgE and IgG1 responses 7 days after sensitization. Our data support the reports that DNA vaccines are more effective than protein vaccines to induce Th1 responses [14–19].

Many controlled clinical trials of desensitization have confirmed the efficacy of specific immunotherapy that involves stepwise subcutaneous injections of a specific protein allergen in allergic patients [19,20]. As allergic symptoms are common during immunotherapy, it is thus considered as a potentially dangerous treatment. In general, it takes a long time but is not always successful [19,20]. Allergic diseases are mainly derived from Th2 cytokine-stimulated IgE production [9–11]. Recent data have revealed that successful immunotherapy leads to an immunomodulation of specific Th2-orientated responses to Th1-biased responses [19,20]. As DNA vaccination can preferentially enhance specific Th1 reactions, it is regarded as a potential alternative for conventional immunotherapy [12,13].

Although six DNA vaccines have been characterized to suppress subsequent sensitization of allergic reactions [14–21], there is only one group to further examine the therapeutic effect of plasmid DNA encoding *E. coli* β -galactosidase, a non-allergen [17]. We have further shown here that an on-going IgE antibody response to rDf11 can be suppressed by a single i.m. inoculation of plasmid DNA pDf11. When compared with sensitized mice further treated with PBS or pcDNA3, much stronger secondary IgG1 and IgG2a antibody responses are found in sensitized experimental mice subsequently treated with pDf11.

The regulatory T cells underlying the modulation of IgE responses by DNA vaccination are so far undetermined. Two studies in Brown Norway rats and BALB/c mice have shown, respectively, that the suppression of IgE can only be adoptively transferred with CD8⁺ T cells from donors i.m. injected with the DNA vaccine encoding Der p 5 [14,15]. In contrast, the other report have shown that the suppression of IgE can be passively transferred into naïve recipients by both CD4⁺ and CD8⁺ T cells collected from BALB/c donors i.d. injected with plasmid DNA encoding β -galactosidase [17]. The present result is consistent with the latter and confirms that both CD4⁺ and CD8⁺ T cells are regulatory T cells after DNA vaccination. It is likely that both CD8⁺ T cells and CD4⁺ Th1 cells can secrete IFN- γ to inhibit the activation of Th2 cells and the production of IgE.

Der f 11 is a strong allergen that has positive serum IgE reactivity in up to 77.8% of asthmatic patients with positive mite skin tests [6,8]. Since preparation of allergen Der f 11 is so laborious and inconvenient, a DNA vaccine encoding Der f 11 would be particularly useful. There is so far no report of inflammatory or allergic side effects of DNA vaccines [26]. In contrast to protein vaccines which may contain contaminants and/or induce allergic or anaphylactic reactions, DNA vaccines can be well purified and induce continuous

expression of minute quantity of recombinant allergens but without adverse effects [26,27].

The present data support the existence of a dynamic and mutually inhibitory network between Th1 and Th2 cells [10,11]. The mechanisms underlying the preferential activation of Th1 responses by DNA vaccines are still unclear [12,13]. It is possible that somatic cells transfected by DNA vaccines will intracellularly express and then secrete recombinant proteins into the microenvironment of injection sites in which secretion of Th1 cytokines IL-12, IL-18, IFN- α , IFN- β and IFN- γ are greatly triggered by the CpG motifs contained in the plasmid vector [28,29]. This implication is supported by the recent report that unmethylated CpG motifs can be used as an adjuvant to preferentially activate a Th1 response [29,30]. Moreover, other adjuvants such as liposome, bestatin and bupivacaine may also have adjuvant effects on the suppression of specific IgE antibody responses by pDf11 vaccination and need further investigation.

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