

Induction of Specific Th1 Responses and Suppression of IgE Antibody Formation by Vaccination with Plasmid DNA Encoding Cyn d 1

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Key Words

DNA vaccine · IgE · Pollen allergen · Th1 responses · Th2 responses

Abstract

Background: DNA vaccines encoding allergens have been developed to prevent or to treat specific IgE responses. **Objective:** To evaluate the potential preventive and therapeutic effect of DNA vaccines encoding Cyn d 1 alone or combined with different adjuvants on specific allergies. **Methods:** Recombinant plasmid Cyn d 1 (pCyn d 1) was constructed by insertion of Cyn d 1 cDNA into the vector pcDNA3. BALB/c mice were injected with pCyn d 1 alone or plus adjuvants such as bupivacaine, bestatin, liposome, or CpG. Control mice were treated with pcDNA3 or PBS. They were boosted 3 weeks later and then sensitized twice with recombinant Cyn d 1 and alum. Their serum antibody responses and cytokine profiles of spleen cells were studied. Adoptive transfer of spleen cells of pCyn d 1-vaccinated mice was also performed. **Results:** Vaccination of mice with pCyn d 1 induced Th1 responses characterized by IgG2a responses and spleen cell secretion of interferon- γ . Vaccination with pCyn d 1 not only prevented the induction of specific IgE responses but also suppressed ongoing IgE responses. The mice receiving

untreated, CD4+ or CD8+-depleted spleen cells from pCyn d 1-vaccinated mice all had suppression of IgE responses. **Conclusion:** This study confirms the prophylactic and therapeutic effects of DNA vaccines encoding Bermuda grass pollen allergen Cyn d 1 on specific IgE responses. Both CD4+ and CD8+ T cells are crucial for the immunomodulatory effect of pCyn d 1 on specific IgE responses.

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Introduction

Airway allergy affects about 10–20% of the world's population [1–4]. Aeroallergens have been recognized as common allergens of asthma, perennial rhinitis, and atopic dermatitis [1–4]. Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an aeroallergen of worldwide importance. Cyn d 1 is a 32- to 34-kDa glycoprotein that gives a 76–100% positive reaction rate with IgE in sera from patients with BGP allergy [5]. The sequences of some cDNA clones of Cyn d 1 were first identified by Smith et al. [6]. However, the cDNAs were truncated in the coding regions [7]. Several full-length cDNA clones encoding Cyn d 1 were thereafter characterized by us [8, 9]. We have further confirmed that Cyn d 1 consists of 11 iso-

forms with sequence polymorphism and differences in isoelectric point or molecular weight [10, 11].

Its major allergen *Cyn d* I is a 32–34-kDa protein. Most patients allergic to BGP have IgE antibodies specific for *Cyn d* 1 [5–8]. Smith et al. [6] partially characterized cDNA clones encoding *Cyn d* 1, but the N-terminal amino acid sequence of *Cyn d* 1 was cloned by us [7]. Recombinant *Cyn d* 1 (r*Cyn d* 1) was used to set up a mouse allergic model in this study.

Allergy is characterized by sensitization of allergen-specific Th2 cells and IgE production [12, 13]. The aim of immunotherapy is focused on modulation of Th2 responses to Th1 responses [14–16]. Recent studies have shown that DNA vaccination is a simple approach to activate Th1 responses [17–24]. Unlike protein vaccines, DNA vaccines induce long-lasting intracellular expression of recombinant allergens and generate specific Th1 cells secreting predominantly interferon- γ (IFN- γ) [18–23]. IFN- γ can selectively inhibit activation of Th2 cells and IgE production by B cells [25]. DNA vaccines have been postulated as a promising candidate to prevent allergic diseases [18–22]. However, the therapeutic effect of DNA vaccines has not been extensively studied.

CpG oligonucleotides have been proved to drive Th1 responses for DNA vaccines [26]. Bestatin and bupivacaine have also proved their adjuvant effect in DNA vaccines [27, 28]. Liposome had been used as an adjuvant and activated Th1 immune responses for protein and DNA vaccines [29]. Alum is a well-known Th2 adjuvant [30]. We used different adjuvants in the present study to regulate the immune reaction in DNA vaccination.

It was confirmed here that ongoing IgE antibody responses to *Cyn d* 1 could be inhibited by recombinant plasmid *Cyn d* 1 (p*Cyn d* 1). Moreover, the role of T cells underlying the suppression of IgE by DNA vaccination remains controversial. Whereas Hsu et al. [17] found that only CD8⁺ T cells were the immunoregulatory T cells in DNA vaccination, the other reports found that both CD4⁺ and CD8⁺ T cells played some roles in DNA vaccination [20]. In the present study, we have also sought evidence that both CD4⁺ and CD8⁺ T cells play crucial roles in p*Cyn d* 1-vaccinated mice.

Materials and Methods

Animals

Female BALB/c (6–8 weeks) mice and Sprague-Dawley rats (300–450 g) were purchased from the National Animal Center of Taiwan. They were maintained on a standard diet (Lab diet; PMI Feeds, St. Louis, Mo., USA) in the Animal House of Taipei Veter-

ans General Hospital (Taipei, Taiwan). All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital and were performed at least twice. Whereas groups of 6 mice were used for most studies, groups of 4 mice were used for spleen cell production of cytokines.

Construction of Recombinant Plasmid *Cyn d* 1

Recombinant plasmid *Cyn d* 1 (p*Cyn d* 1) was constructed by insertion of *Cyn d* 1 cDNA, a 764-bp DNA open reading frame derived from digestion of the plasmid pBluescript/*Cyn d* 1 with enzymes *Eco*R1 and *Xba*I, into the *Eco*R1 and *Xba*I sites in the vector pcDNA3 (Invitrogen, Groningen, The Netherlands). Both p*Cyn d* 1 and pcDNA3 were grown respectively in transformed *Escherichia coli* DH5 α in Luria-Bertani broth supplemented with ampicillin (100 μ g/ml). Purification of plasmids was conducted using a Qiagen maxiprep kit (Qiagen, Inc., Chatsworth, Calif., USA) according to the manufacturer's instructions. Their quality and quantity were analyzed by absorption at 260 and 280 nm and by electrophoresis. They were stored at –20°C and resuspended immediately before use with sterile PBS (2 mg/ml).

Preparation of Recombinant *Cyn d* 1

Recombinant *Cyn d* 1 (r*Cyn d* 1) was expressed as a glutathione-S-transferase (GST) fusion protein in *E. coli* and purified as previously described [6]. In addition, r*Cyn d* 1 was purified through GST-specific cleavage by adding thrombin to GST-containing r*Cyn d* 1 when it was bound to glutathione Sepharose 4B.

Adjuvants

Bestatin (100 μ g per mouse; Calbiochem, San Diego, Calif., USA), liposome (50 μ l per mouse), CpG (50 μ g per mouse; CpG ODN 1826: 5'-TCCATGACGTTCTGACGTT-3'; Sigma), or alum (Amphojel; 50 μ g per mouse; Whitehall Laboratory, Punchbowl, N.S.W., Australia) were injected together with 100 μ g p*Cyn d* 1 or 10 μ g r*Cyn d* 1 as adjuvants. On the other hand, 50 μ l of 0.5% bupivacaine (Sigma) was i.m. injected at the same site 3 days before p*Cyn d* 1 or r*Cyn d* 1 inoculation.

p*Cyn d* 1 Vaccination

Groups of 6 mice were i.m. injected with 100 μ g p*Cyn d* 1 alone or plus bupivacaine, bestatin, liposome, or CpG. Control mice were injected with 100 μ g pcDNA3 or 100 μ l PBS only. They were boosted again triweekly. Whereas the mice in the prevention protocol received p*Cyn d* 1 vaccination twice triweekly (weeks –6 and –3) before systemic sensitization (weeks 0 and 2), those in the treatment protocol had p*Cyn d* 1 injection three times triweekly (week 3, 6, and 9) after systemic sensitization.

Systemic Sensitization

Mice were i.p. sensitized twice biweekly (weeks 0 and 2) with 10 μ g r*Cyn d* 1 adsorbed onto 4 mg alum and suspended in 0.5 ml PBS twice. They were bled biweekly or triweekly after sensitization. The separated serum was frozen at –20°C until required. The PBS-treated group was used as a sensitization model.

Preparation of Spleen Cells

Mouse spleen cells were collected and washed by RPMI-1640 with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin. Red blood cells 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/mouse).

Depletion of CD4+ or CD8+ T Cells

CD4+ or CD8+ T cells were depleted as previously described [20]. Briefly, rat hybridoma cells GK1.5 and 3.155 (ATCC, Rockville, Md., USA) were i.p. transplanted in pristane-primed scid mice (2×10^6 cells/mouse). Ascites containing IgG2b or IgM monoclonal antibodies specific for mouse CD4 or CD8 were collected by tapping and pooled, respectively (1.9 and 6.2 mg/ml). Mice were i.p. injected daily with 0.6 ml of ascites respectively for 3 days. Depletion of CD4+ or CD8+ T cells was confirmed by FACS analysis (<1%, respectively) using 1:20 diluted fluorescein isothiocyanate (FITC)-conjugated rat IgG specific for mouse CD4 or CD8, respectively (Caltag Laboratories, Burlingame, Calif., USA).

Specific Antibody Assessment

Specific IgG antibodies were assessed using ELISA as described [31]. Briefly, plates were coated with 4 µg/ml rCyn d 1 in 0.05 M carbonate buffer, pH 9.6 (100 µl/well), overnight at 4°C. Free sites were blocked with 3% skimmed milk in PBS-Tween 20 for 1 h. Serum samples (1/50–1,000) and standards (pooled sera collected after monthly sensitization with 10 µg rCyn d 1 adsorbed onto 2 mg alum for 4 months) were added in duplicate and the plates incubated for 5 h at RT. After washing, horseradish peroxidase-conjugated goat anti-mouse IgG2a or IgG1 (1/4,000; SBA, Birmingham, Ala., USA) were added and the plates incubated overnight at 4°C. After washing, orthophenylenediamine (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 4 N H₂SO₄. Absorbance at 492 nm was read using a reader (SPECTRAMax 250; Molecular Devices, Sunnyvale, Calif., USA) and unknowns were interpolated.

Passive Cutaneous Anaphylaxis

Specific IgE antibodies were assessed in triplicate by passive cutaneous anaphylaxis tests in Sprague-Dawley rats as described [14]. Aliquots of 100 µl of two-fold dilutions of pooled mouse serum samples (1/2.5–1,280) were i.d. injected into the rats. They were challenged 48 h later by an intravenous injection of 1 mg rCyn d 1 and 5 mg Evans Blue in 1 ml of PBS. The reaction was read 30 min later. The passive cutaneous anaphylaxis titer was the highest dilution giving a positive reaction of at least 5 mm in diameter and was expressed as means $\pm$ 1 SEM.

Cytokine Assays

Spleen cells were pooled from four mice and plated in 24-well tissue culture plates (4×10^6 cells/ml). Cells were cultured with rCyn d 1 (20 µg/ml) or medium only. Supernatants were harvested at 24 and 72 h and cytokines interleukin-4 (IL-4) and IFN- γ were measured using sandwich ELISA kits (Endogen, Woburn, Mass., USA).

Statistical Analysis

Specific antibody and cytokine titers were expressed as means $\pm$ 1 SD, and all group comparisons for specific antibody responses between DNA-vaccinated groups and the pcDNA-treated controls were made using Mann-Whitney U tests.

Results

Specific Antibody Responses after pCyn d 1 Vaccination

The effect of DNA vaccination with pCyn d 1 was first studied. Groups of 6 mice were vaccinated twice respectively with pCyn d 1 alone or plus bupivacaine, bestatin, liposome, or CpG. Control mice were injected with pcDNA3 or PBS only. Their serum-specific antibody responses were tested every 3 weeks after the first vaccination. Whereas the controls had neither IgG2a nor IgG1 responses, those receiving pCyn d 1 only had significant IgG2a and IgG1 responses. The mice receiving pCyn d 1 plus adjuvants bupivacaine, bestatin, CpG, or liposome had greatly enhanced IgG2a and IgG1 antibody responses compared to the mice vaccinated with pcDNA3, PBS, or pCyn d 1 only ($p < 0.005$; fig. 1a, b, respectively). Bestatin had the most powerful adjuvant effect on IgG2a responses. Specific IgE responses were not detected after DNA vaccination (data not shown).

Spleen Cell Cytokine Production after pCyn d 1 Vaccination

The cytokine profiles after vaccination with pCyn d 1 were further studied. Groups of 4 mice were vaccinated as described above. Five weeks after the last treatment, their spleens were collected and the cytokine profiles of pooled spleen cells are shown in table 1. Compared with the controls, those receiving pCyn d 1 alone or plus adjuvants showed a higher production of IFN- γ but little of IL-4. Vaccination with pCyn d 1 together with CpG induced the highest level of IFN- γ .

Prophylactic Effects of pCyn d 1 Vaccination on Allergy

Whether DNA vaccination could prevent the induction of specific IgE responses was first studied. Groups of 6 mice were vaccinated as a preventive protocol described in figure 1. Three weeks after the second vaccination, they were sensitized with rCyn d 1 in alum twice biweekly. Their specific antibodies were sequentially assessed (fig. 2). After sensitization, those receiving pCyn d 1 alone or plus adjuvants had increases in IgG2a and IgG1 responses compared to the controls ($p < 0.05$, respectively; fig. 2a, b) but conversely suppressed IgE responses (fig. 2c). Vaccination with pCyn d 1 plus bestatin had the best increase in specific IgG2a and IgG1 responses (fig. 2a, b). Vaccination with pCyn d 1 and adjuvant CpG or bestatin profoundly suppressed IgE responses (fig. 2c). The controls had much lower IgG1 and IgG2a responses, but

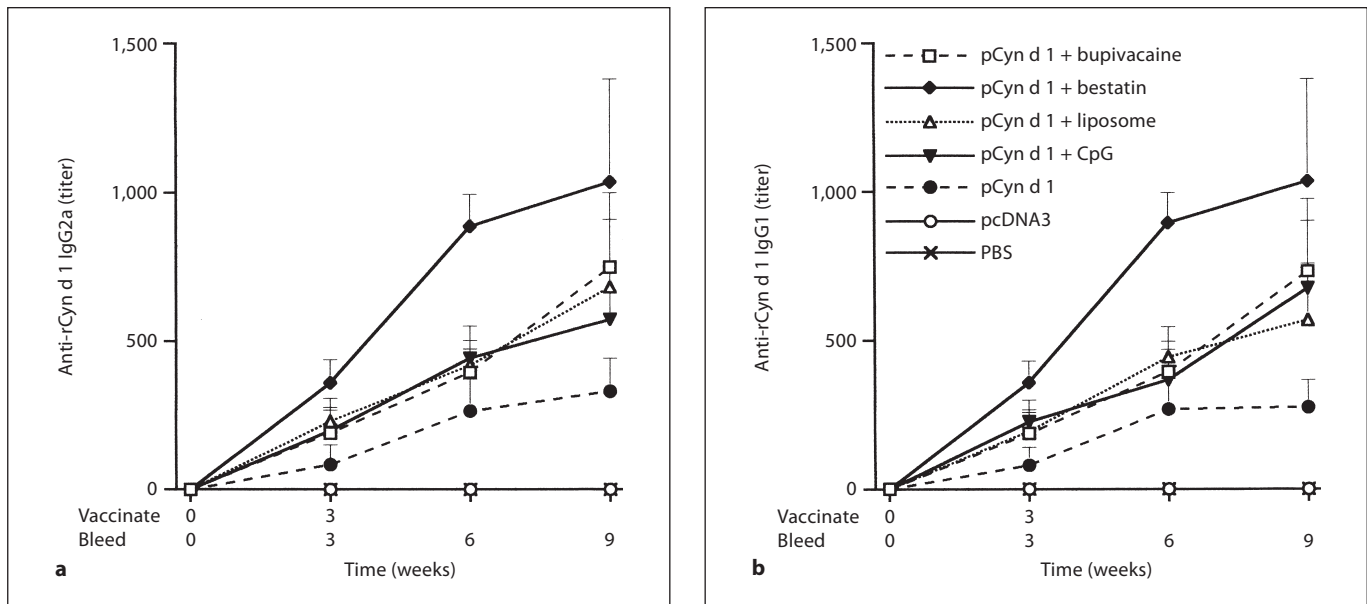


Fig. 1. Specific antibody responses after vaccination with pCyn d 1. Groups of 6 BALB/c mice were i.m. injected with pCyn d 1 alone or plus bupivacaine, bestatin, liposome, or CpG on weeks 0 and 3. Control mice were similarly treated with pcDNA3 or PBS only. Their serum-specific IgG2a (**a**) and IgG1 (**b**) antibody responses were tested every 3 weeks after the first vaccination.

Table 1. Cytokine secretion after DNA vaccination in naïve mice

Vaccine	Sensitization	IFN- γ pg/ml	IL-4 ng/ml
Bupivacaine + pCyn d 1	rCyn d 1	1,346.5 $\pm$ 417.4	4.9 $\pm$ 1.8
Bestatin + pCyn d 1	rCyn d 1	1,400.5 $\pm$ 42.5	11.0 $\pm$ 1.9
CpG + pCyn d 1	rCyn d 1	1,602.8 $\pm$ 464.1	19.6 $\pm$ 4.3
Liposome + pCyn d 1	rCyn d 1	965.2 $\pm$ 230.6	20.5 $\pm$ 0.1
pCyn d 1	rCyn d 1	715.4 $\pm$ 191.2	23.8 $\pm$ 2.9
pcDNA 3	rCyn d 1	9.5 $\pm$ 0.2	51.1 $\pm$ 15.8
PBS	rCyn d 1	6.8 $\pm$ 1.2	95.1 $\pm$ 21.7

much higher IgE responses, than those vaccinated with pCyn d 1 alone or plus adjuvants (fig. 2a–c, respectively).

Therapeutic Effects of pCyn d 1 Vaccination on Allergy

Whether DNA vaccination could suppress ongoing specific IgE antibody responses or not was further studied. Mice were sensitized twice with 10 μ g rCyn d 1 in alum biweekly. One week after the last sensitization, they were vaccinated three times with 100 μ g pCyn d 1 alone or plus bupivacaine, bestatin, liposome, or CpG triweekly. Control

mice were treated with pcDNA3 or PBS only. Their specific antibodies were assessed before vaccination and tri-weekly after vaccination (fig. 3). Compared with the controls, those receiving pCyn d 1 alone or plus any adjuvant had suppression of specific ongoing IgE responses (fig. 3c) and, in contrast, profound enhancement of specific IgG2a and IgG1 responses ($p < 0.05$, respectively; fig. 3a, b). Those receiving pCyn d 1 plus bupivacaine induced the highest production of specific IgG2a antibodies (fig. 3a). There was little difference between the two control groups.

Spleen Cell Cytokine Production after pCyn d 1 Immunotherapy

The cytokine profiles after vaccination with pCyn d 1 were further studied. Groups of 6 mice were sensitized and treated as described in figure 3. All of the spleens were collected 7 weeks after the last treatment, and their spleen cell cytokine profiles were analyzed when cultured with rCyn d 1. The mice receiving pCyn d 1 alone or plus any adjuvant showed stronger production of IFN- γ than the controls (table 2) but weak production of IL-4. Those receiving pCyn d 1 with liposome showed the highest production of IFN- γ but the lowest secretion of IL-4. In contrast, the control mice had enhanced production of IL-4 but very weak secretion of IFN- γ .

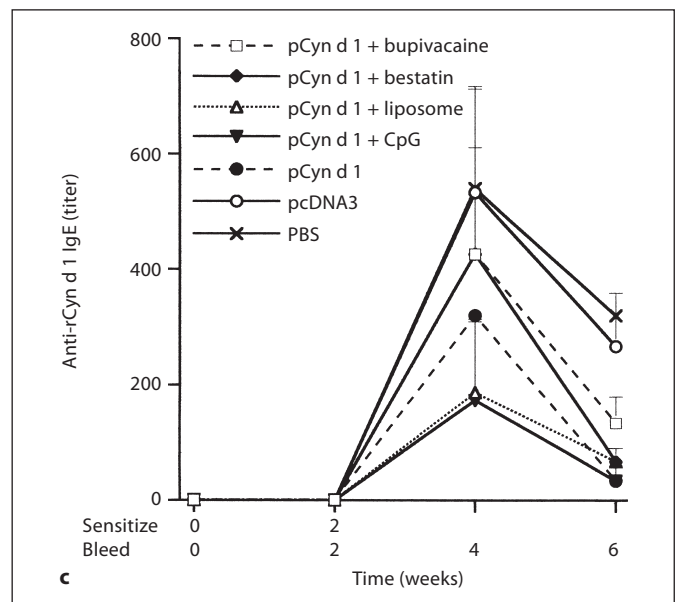
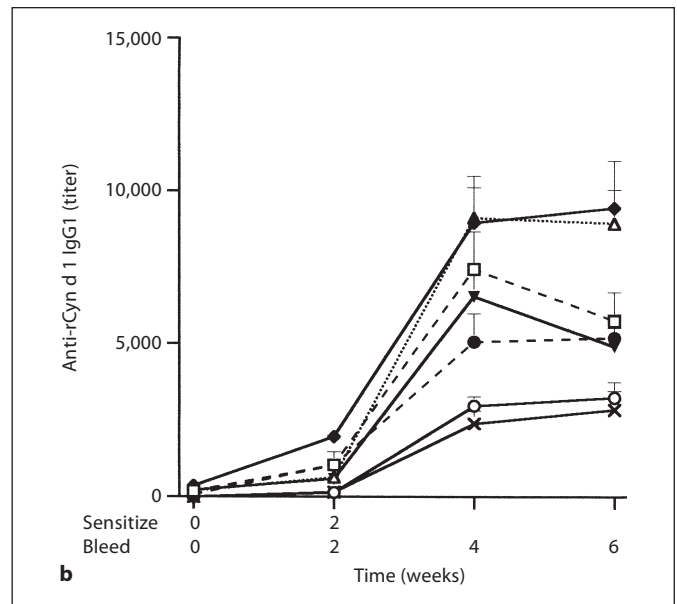
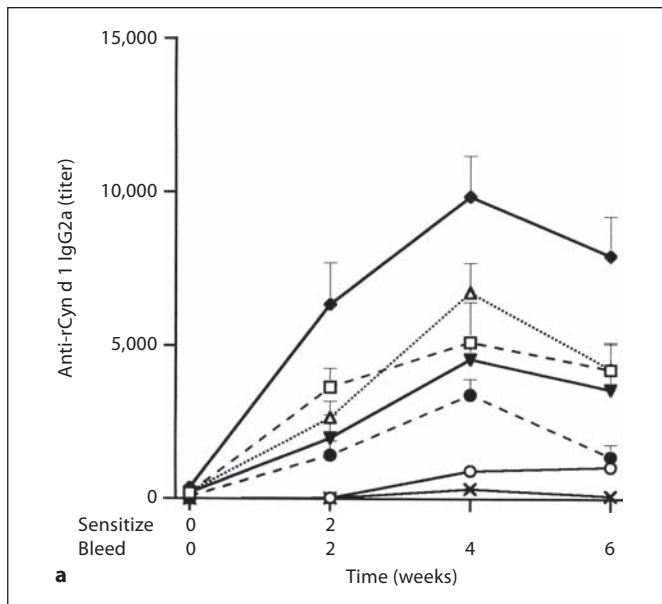


Fig. 2. DNA vaccination effectively prevents the induction of specific Th2 responses. Groups of 6 BALB/c mice were vaccinated as described in figure 1. Three weeks after the second vaccination, they were sensitized with rCyn d 1 in alum twice every 2 weeks (weeks 0 and 2). Their specific IgG2a (a), IgG1 (b), and IgE (c) antibody responses were sequentially assessed biweekly.

Immunomodulatory T Cells during pCyn d 1 Vaccination

Whether CD4+ or CD8+ T cells suppress specific IgE responses after DNA vaccination was also studied. Groups of 6 donor mice were vaccinated twice with pCyn d 1 triweekly. Four weeks after the last vaccination, two groups of donor mice were injected daily with the ascites containing monoclonal antibodies against CD4 or CD8 T cells respectively for 3 days. Their spleen cells were transferred respectively into groups of 6 naïve recipients 1 day after the last injection. The recipients were imme-

diately sensitized and boosted 2 weeks later. Their antibody responses are shown in figure 4. When compared with the controls, those receiving vaccinated spleen cells or vaccinated spleen cells with CD4 or CD8 depletion all had suppressed IgE but enhanced IgG2a responses ($p < 0.05$; fig. 4c, a, respectively). Among recipients, the group receiving vaccinated spleen cells with CD8 depletion had greatly enhanced IgG2a responses, but the group receiving vaccinated spleen cells with CD4 depletion had decreased IgG1 responses (fig. 4b).

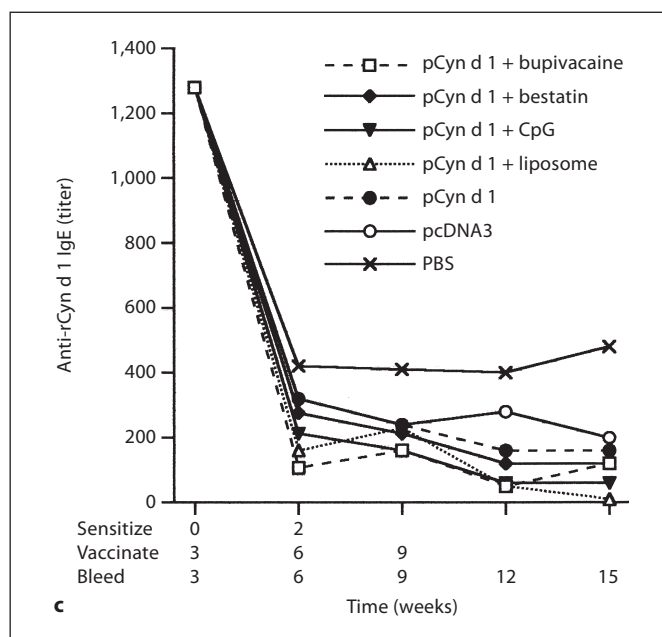
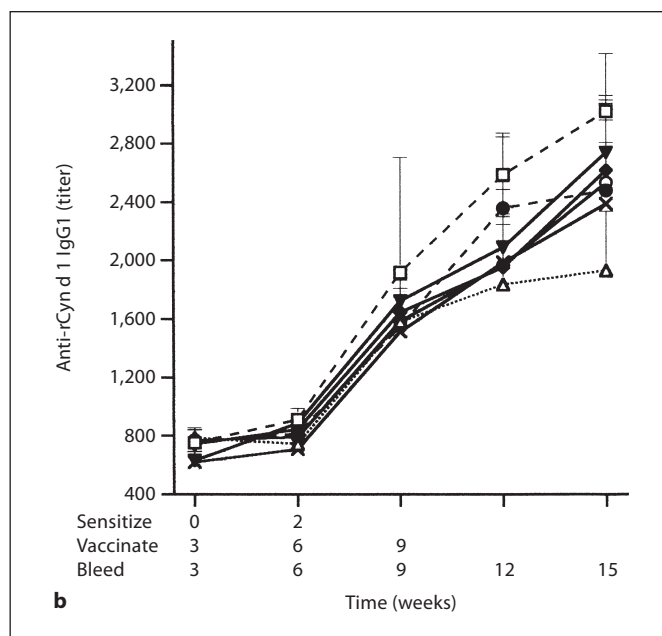
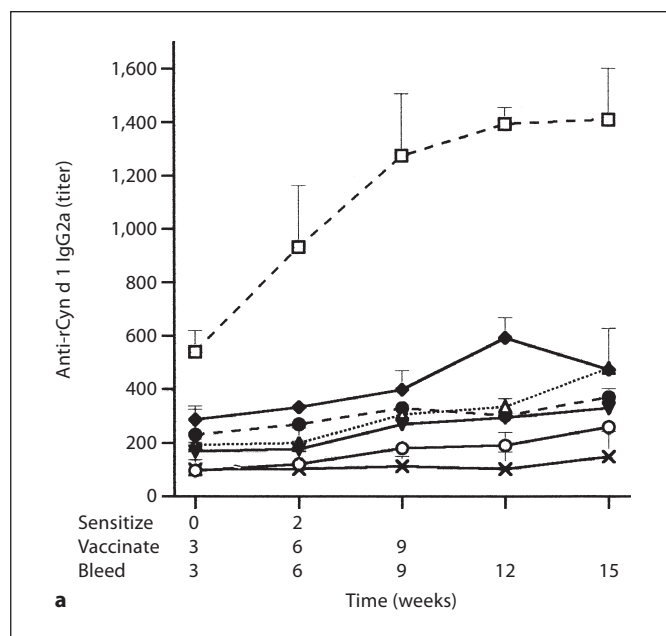


Fig. 3. DNA vaccination inhibits ongoing Th2 responses. Groups of 6 mice were sensitized twice with 10 μ g rCyn d 1 in alum bi-weekly (weeks 0 and 2). One week after the second sensitization, they were i.m. injected triweekly with pCyn d 1 alone or plus bupivacaine, bestatin, liposome, or CpG three times (weeks 3, 6, and 9). Control mice were similarly treated with pcDNA3 or PBS only. Their serum-specific IgG2a (a), IgG1 (b), and IgE (c) antibody responses were tested before vaccination and triweekly after vaccination.

Discussion

The present study has shown that vaccination of mice with plasmid DNA encoding Cyn d 1 can preferentially stimulate specific Th1 responses. Moreover, it prevents subsequent sensitization of specific Th2 responses. These results are consistent with other reports that DNA vaccination is a potential approach for the prophylaxis of al-

lergic diseases [17–24]. We have further shown that vaccination with pCyn d 1 alone or plus adjuvant CpG, liposome, bestatin, and bupivacaine can effectively enhance Th1 responses during an ongoing Th2 response. It down-regulates specific IgE production. We have also demonstrated that both CD4⁺ and CD8⁺ T cells play important roles for the immunomodulatory effect of DNA vaccination.

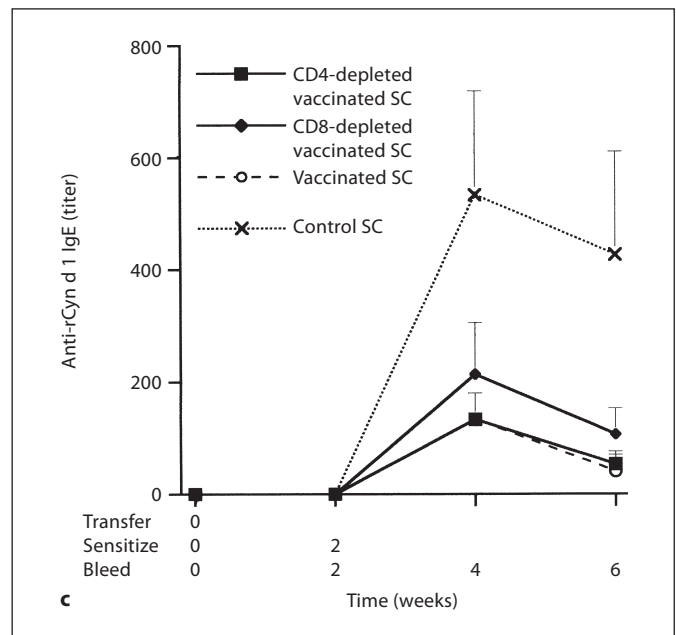
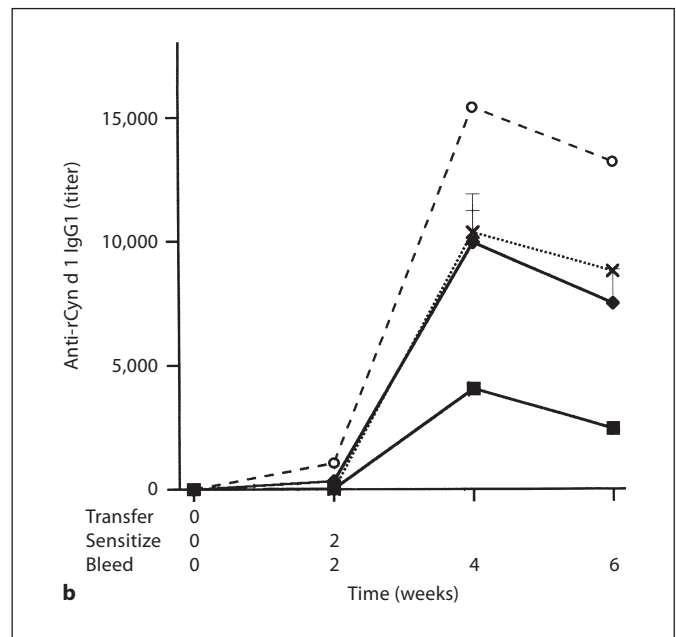
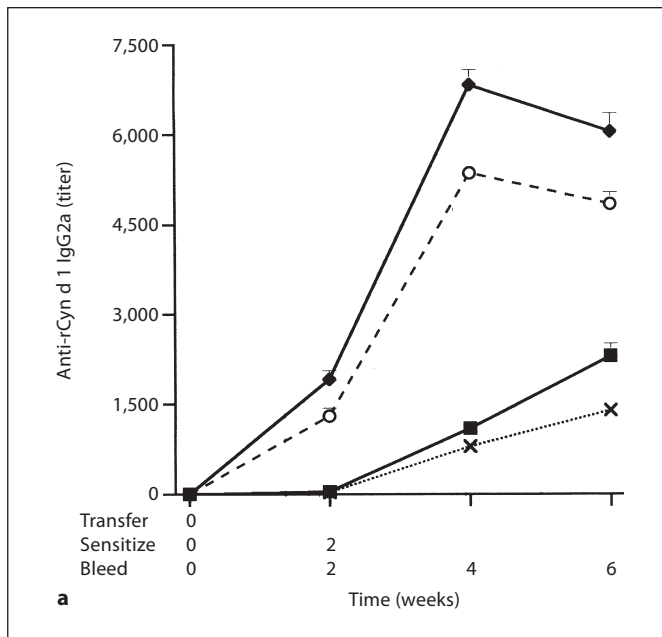


Fig. 4. Suppression of specific IgE responses after transfer of CD4- or CD8-depleted spleen cells from pCyn d 1-vaccinated donors to naïve recipients. Groups of 6 donor mice were vaccinated with pCyn d 1 alone as described in figure 1. One month after the last vaccination, two groups of donor mice were i.p. injected daily with ascites containing monoclonal antibodies against CD4 or CD8 T cells respectively for 3 days and their spleen cells were transferred respectively into groups of 6 naïve recipients 1 day after the last injection (week 0). The recipients were immediately subcutaneously sensitized with rCyn d 1 in alum and boosted 2 weeks later (weeks 0 and 2). Their serum-specific IgG2a (a), IgG1 (b), and IgE (c) antibody responses were shown every 2 weeks (weeks 0, 2, 4, and 6). SC = Spleen cells.

The previous finding that triweekly DNA vaccination has better responses [17–24] was adopted in the present study. Vaccination with pCyn d 1 alone induced serum IgG2a and IgG1 but no IgE responses (fig. 1). Vaccination with pCyn d 1 plus adjuvant bupivacaine, bestatin, lipofectin, or CpG induced extraordinarily stronger IgG2a and IgG1 responses but no IgE production (fig. 1). Whereas an early report found that gene gun inoculation of plas-

mid DNA failed to stimulate Th1 responses [23], our results are consistent with the other reports that i.m. injection of DNA vaccines can effectively induce specific Th1 responses [17–22].

pCyn d 1 plus bestatin or bupivacaine had the best effect on induction of IgG2a antibody in preventive or therapeutic studies, respectively. In addition, they did not trigger strong IgE antibody responses. Both of them seem

to be good candidate adjuvants for DNA vaccines. In agreement with the report that DNA vaccines conjugated with CpG could enhance immune responses, the present study confirmed that CpG effectively reduce IgE antibody responses. Consistent with a previous report [29], liposomes showed their adjuvant effect on DNA vaccines and thus reduced IgE antibody production.

After sensitization, pCyn d 1-vaccinated mice had very strong secondary Th1 responses characterized by much higher IgG2a reactions and by spleen cell secretion of higher levels of IFN- γ but little IL-4. Moreover, their IgE response was much lower than the primary IgE response of the control mice previously treated with PBS or pcDNA3. This finding confirms our previous report that DNA vaccination can effectively prevent the subsequent sensitization of Th2 responses [17–22]. Clinical trials of desensitization have documented the efficacy of immunotherapy that involves stepwise subcutaneous injections of specific allergens in allergic patients [32]. As allergic symptoms are common during immunotherapy, it is considered a potentially dangerous treatment. In addition, it takes a long time but is not always successful [32]. Allergic diseases are mainly derived from Th2 cytokine-stimulated IgE production [9–12], but successful immunotherapy can modulate ongoing Th2-orientated responses to Th1-biased responses [33]. Since DNA vaccination can preferentially enhance specific Th1 reactions, it is a potential alternative to immunotherapy [17].

In addition to the study to examine the therapeutic effect of plasmid DNA encoding *E. coli* β -galactosidase, a nonallergen [20], we have previously shown that ongoing IgE responses to aeroallergen Der f 11 can be suppressed by DNA vaccination. We have further shown here that ongoing IgE responses to aeroallergen Cyn d 1 can be also suppressed by DNA vaccination. When compared with the controls, marked enhancement of specific IgG responses and suppression of specific IgE responses are found in previously sensitized mice subsequently vaccinated with pCyn d 1 alone or plus adjuvant. Whereas two studies disclosed that suppression of IgE can only be adoptively transferred by CD8 $^{+}$ T cells collected from DNA-vaccinated rats and mice [17, 18], we have repeatedly shown that suppression of IgE can be transferred either by CD4 $^{+}$ or by CD8 $^{+}$ T cells collected from donor mice vaccinated with plasmid DNA encoding Der f 11 or Cyn d 1 [20]. The present finding confirms that both CD4 $^{+}$ and CD8 $^{+}$ T cells are crucial T cells after DNA vaccination. It is likely that both CD8 $^{+}$ T cells and CD4 $^{+}$ Th1 cells can secrete IFN- γ to inhibit Th2 cell activation. It seems CD4 $^{+}$ T cells play a more dominant role in driving Th1 responses.

Table 2. Cytokine secretion after DNA vaccination in sensitized mice

Vaccine	Sensitization	IFN- γ pg/ml	IL-4 ng/ml
Bupivacaine + pCyn d 1	rCyn d 1	1,352.7 $\pm$ 206.4	15.8 $\pm$ 0.6
Bestatin + pCyn d 1	rCyn d 1	1,043.1 $\pm$ 239.3	24.9 $\pm$ 3.2
CpG + pCyn d 1	rCyn d 1	540.1 $\pm$ 10.8	31.6 $\pm$ 3.5
Liposome + pCyn d 1	rCyn d 1	1,440.9 $\pm$ 115.3	2.1 $\pm$ 0.3
pCyn d 1	rCyn d 1	634.7 $\pm$ 146	23.8 $\pm$ 6.9
pcDNA 3	rCyn d 1	9.5 $\pm$ 2.8	21.4 $\pm$ 0.9
PBS	rCyn d 1	69.4 $\pm$ 6.2	21.0 $\pm$ 3.5

The present study supports the existence of a dynamic and mutually inhibitory network between Th1 and Th2 cells [12–13]. The mechanisms underlying the preferential activation of Th1 responses by DNA vaccines are still unclear. It is possible that somatic cells transfected by DNA vaccines will generate recombinant proteins in the microenvironment of injection sites in which secretion of Th1 cytokines IL-12, IL-18, IFN- α , IFN- β , and IFN- γ is greatly triggered by the CpG motifs of the plasmid vector [34, 35]. This implication is supported by recent reports that unmethylated CpG motifs of viral, bacterial, and insect origins are strong adjuvants to activate Th1 responses [36]. On the other hand, we have also shown that other adjuvants such as liposome, bestatin, and bupivacaine have adjuvant activity for DNA vaccines. Cyn d 1 is a common aeroallergen with high positive rates of IgE reactivity in asthmatic patients [8]. Since preparation of allergen Cyn d 1 is a slow and laborious process, a DNA vaccine encoding Cyn d 1 would be particularly useful. In contrast to protein vaccines that may contain contaminants and/or induce allergic or anaphylactic reactions, DNA vaccines can induce continuous expression of a minute quantity of recombinant allergens but without adverse effects in rodent studies [37].

Acknowledgments

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