

Efficacy of local nasal immunotherapy for Dp2-induced airway inflammation in mice: Using Dp2 peptide and fungal immunomodulatory peptide

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Background: Local nasal immunotherapy (LNIT) is an effective immunotherapy. Peptides derived from the group 2 allergen of *Dermatophagoides pteronyssinus*, Dp2 28-40 and Dp2 28-40A, and fungal immunomodulatory peptide (FIP) have been shown to act as T_H1 potential and response-inducing adjuvant. LNIT by the use of Dp2 peptides in conjunction with FIP were investigated.

Objective: We sought to determine whether Dp2-induced airway inflammation in mice could be downregulated by Dp2 peptides or a mixture of Dp2 peptides with FIP.

Method: Mice were sensitized with rDp2 followed by LNIT with Dp2 peptides, FIP, or FIP and a mixture of Dp2 peptides. After intratracheal challenge with rDp2, the airway inflammation and hyperresponsiveness were determined by bronchoalveolar lavage fluid (BALF) analysis and methacholine challenge. **Results:** Both Dp2 peptides and FIP were able to inhibit rDp2-induced airway inflammation and airway hyperresponsiveness. An increase in IFN- γ and a decrease in IL-5 in BALF and sera were found after LNIT with Dp2 peptides, FIP, and mixtures of both. Serum levels of TGF- β were reduced after LNIT with FIP and Dp2 28-40. Penh values were significantly decreased after methacholine challenge in both the early and late phase.

Conclusions: LNIT with allergen-derived peptides and FIP can produce an anti-inflammatory effect on allergen-induced airway inflammation. LNIT with selected peptides and FIP might be a good alternative therapy for allergic airway disease. (J Allergy Clin Immunol 2003;112:301-10.)

Key words: Local nasal immunotherapy, group 2 allergen of *Dermatophagoides pteronyssinus*, Dp2 epitope peptides, fungal immunomodulatory peptide

Epidemiologic studies suggest that between 10% and 20% of the world's population exhibits some form of IgE-mediated hypersensitivity, which is manifested as asthma, atopic dermatitis, or allergic rhinitis.¹ House dust mite is the most important risk factor for asthma, and more than 10 mite allergens have been defined. The 14-kd group 2 allergens (Dp2 and Df2) are considered major allergens, because 80% to 90% of patients have

Abbreviations used

BALF:	Bronchoalveolar lavage fluid
DEX:	Dexamethasone
Dp2:	Group 2 allergen of house dust mite <i>Dermatophagoides pteronyssinus</i>
EU:	ELISA units
FIP:	Fungal immunomodulatory peptide
IT:	Intratracheal
LNIT:	Local nasal immunotherapy
NS:	Normal saline
Penh:	Enhanced pause

specific IgE antibodies to these allergens.^{2,3} Therapy for allergic disease includes allergen avoidance, pharmacotherapy, and allergen-specific immunotherapy.⁴

Allergen-specific immunotherapy is an established form of treatment for atopic allergic diseases.⁵ However, there are problems such as unpredictable anaphylactic reactions and the dose difficulties associated with the standardization of protein levels in complex allergic extracts.⁶⁻⁸ It has been previously shown that peripheral T-cell tolerance could be induced in naive and primed mice by the subcutaneous injection of peptides from the major cat allergen, Fel d1, without the requirement of an early IgE/mast cell-dependent response among patients who have cat-allergic asthma.^{9,10} It has been reported that the human peripheral T-cell response to Dp2 is primarily caused by 2 immunodominant regions, residues 28-40 and 101-109. The immunologic activity of an altered T-cell receptor ligand from the immunodominant T-cell epitope of Dp2, in which residues at positions 34 and 36 were substituted by alanine, Dp2 28-40A was demonstrated to induce elevated INF- γ synthesis at equimolar concentrations compared with native peptide (Dp2 28-40).¹¹⁻¹³ Recently, it has been demonstrated that intradermal administration of short allergen-derived T-cell peptide epitopes produces a reduction in the size of cutaneous late-phase reactions after whole allergen challenge.¹⁴

A fungal immunomodulatory peptide (FIP) isolated from the edible golden needle mushroom (*Flammulina velutipes*) has been reported to possess immunomodulatory activity. According to this study, this FIP not only suppresses a systemic anaphylactic reaction and the local swelling of mouse footpads, but it might also enhance the transcriptional expression of IL-2 and IFN- γ .¹⁵

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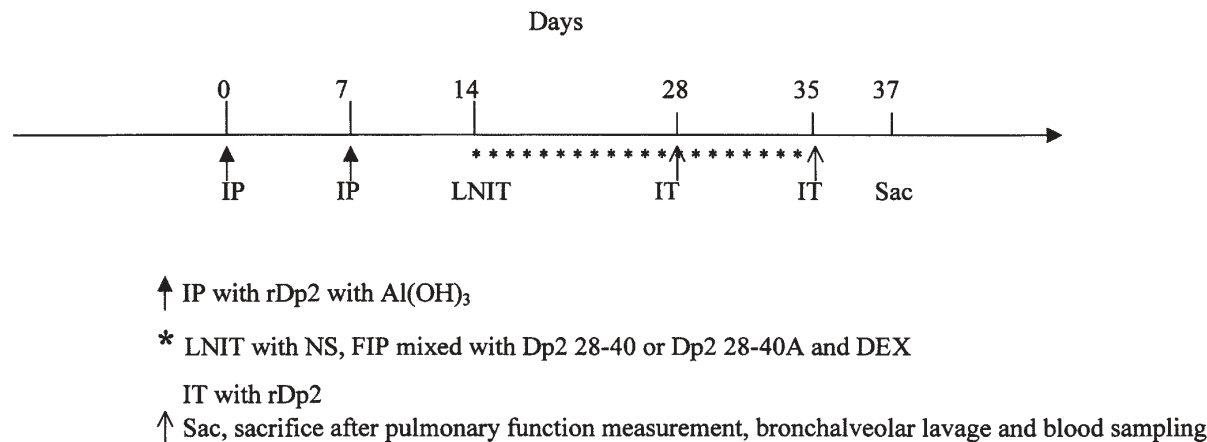


FIG 1. Experimental schedule for intraperitoneal sensitization with rDp2 and local nasal immunotherapy with Dp2-derived peptides after immunization and subsequent intratracheal challenge with rDp2. Normal saline and dexamethasone were used as controls.

It has long been recognized that local nasal immunotherapy (LNIT) is capable of controlling the symptoms of seasonal allergic rhinitis in a dose-dependent manner.^{16,17} At higher dosages, LNIT can sometimes cause local adverse reactions.¹⁸⁻²¹ On the other hand, the studies that used a low-dose administration schedule did not show clinical effectiveness, although tolerance was found to be good.^{17,22,23}

We hypothesized that through a mechanism of co-immunization with a mixture of Dp2 peptide and FIP, we would be able to bias an immune response toward a T_H1 response. To test this hypothesis, allergen was delivered by LNIT and evaluated for its effect in pre-existing T_H2 response mice.

METHODS

Animals

Male Balb/c mice between 6 and 8 weeks of age were obtained from the National Laboratory Breeding Research Center in Taiwan and were raised in a specific pathogen-free environment.

Reagents

Recombinant *Dermatophagoides pteronyssinus* group 2 (rDp2) was prepared as previously described.²⁴ Endotoxin-free Dp2 28-40 and Dp2 28-40A were obtained from Dr Jonathan Lamb (Respiratory Medicine Unit, Edinburgh University, Medical School, Edinburgh, UK).¹³ FIP was obtained from Dr Ko Jiunn-Liang (Institute of Toxicology, Chung Shan Medical University, Taichung, ROC),¹⁵ with endotoxin less than 30 endotoxin units (EU)/mL (1 EU = 0.1 ng, Limulus Amebocyte Lysate, Cape Cod, Mass).

Induction of allergic airway inflammation

Mice were immunized by intraperitoneal injection of 1 μ g/0.1 mL rDp2 emulsified in 4.0 mg/0.062 mL aluminum hydroxide (Al(OH)₃) (Whitehall Lab Ltd, Punchbowl, Australia) on day 0 and day 7. After immunization, LNIT was carried out daily from day 14 to day 35 with Dp2 28-40 or Dp2 28-40A at a concentration of 2 μ g/20 μ L. FIP alone or FIP mixed with either Dp2 28-40 or Dp2 28-40A (FIP, 50 μ g/mouse/day; Dp2 peptide, 2 μ g/mouse/day). Normal saline (NS) was used as a negative control. The other control

group of mice was fed with dexamethasone (DEX) 1 μ g/100 μ L/mouse. On day 28, the mice were lightly anesthetized with an IP injection of 60 mg/kg sodium pentobarbital (Sigma Chemical Co, St Louis, Mo) and intratracheally (IT) inoculated with 1 μ g/50 μ L of rDp2 on day 28 and on day 35. Two days after the second IT inoculation, the mice were killed after pulmonary function measurement (Fig 1).

Bronchoalveolar lavage fluid collection and preparation

After pulmonary function measurement, bronchoalveolar lavage was performed with the following procedure. BAL fluids (BALF) were collected using 1 mL of sterile endotoxin-free saline wash of the lung through the trachea of each mouse. BAL cells were washed once with PBS after centrifugation at 200g at 4°C. After washing, the cells were resuspended in RPMI-1640 (Sigma). Total leukocytes in the BALF were determined with a hemocytometer. The BALF was aspirated and stored at -70°C until assay. After total leukocyte counting, a cytospin preparation of 100 μ L of BALF (2×10^5 cells/mL) was carried out by cytocentrifuging onto glass slides at 350 rpm for 5 minutes using the Cytospin 3 cytocentrifuge (Shandon Scientific Limited, Astmoor, Runcorn, UK), followed by staining with Liu stain (Tonyar Diagnostic Inc, Taipei & Hien, Taiwan). Differential counts were performed on a total of 200 cells.

Determination of Dp2-specific immunoglobulins and cytokines

Blood was obtained from the retro-orbital venous plexus at the beginning and end of the experiments. The serum IgG1 and IgG2a titers of anti-Dp2 antibodies were determined by using an ELISA, during which plates (Nunc Lab, Copenhagen, Denmark) were coated with 100 μ L rDp2 overnight at a concentration of 0.5 μ g/mL in 4°C in a refrigerator. Detection antibodies (horseradish peroxidase-conjugated goat anti-mouse IgG2a Ab used at 1:800 and IgG1 antibody used 1:2000; Southern Biotech Assoc, Inc, Birmingham, Ala) were added separately for each assay, and the bound enzyme substrate was detected with TMB (Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md). The reaction was stopped with 50 μ L 4N H₂SO₄ after 15 minutes, and the optical density was measured at 450 nm in a multiscan spectrophotometer (model A-5682; SLT Lab Instruments, Salzburg, Austria). Results were expressed as EU. To ensure reproducibility, a known serum was run with each test as a

TABLE I. Leukocyte subpopulation in the bronchoalveolar lavage fluid derived from all groups of mice

	Total cell	Macrophage	Lymphocyte	Neutrophil	Eosinophil
Naive	14.3 ± 4.3*	13.3 ± 4.3*	1.0 ± 0.0*	0*	0*
NS	160.0 ± 23.5	56.4 ± 12.7	41.4 ± 6.5	36.8 ± 2.8	25.2 ± 4.5
Dp2 28-40	53.3 ± 4.1*	31.0 ± 2.5*	11.8 ± 0.8*	7.2 ± 1.5*	3.5 ± 0.5*
Dp2 28-40A	65.0 ± 3.2*	38.4 ± 7.2*	14.6 ± 3.3*	8.2 ± 1.1*	3.0 ± 1.0*
FIP	65.0 ± 10.5*	37.0 ± 7.4*	13.0 ± 1.8*	9.0 ± 2.4*	6.3 ± 1.3*
FIP + Dp2 28-40	61.0 ± 1.5*	27.7 ± 2.1*	20.6 ± 0.6*	9.3 ± 2.0*	4.0 ± 0.6*
FIP + Dp2 28-40A	61.0 ± 1.5*	23.3 ± 1.8*	16.7 ± 0.9*	13.7 ± 0.9*	7.3 ± 0.3*
DEX	46.4 ± 2.2*	29.2 ± 4.4*	9.4 ± 1.5*	6.6 ± 1.0*	3.2 ± 0.4*

10⁴ cells/mL, data represented as mean ± SEM of 6 animals.

NS, Normal saline; FIP, fungal immunomodulatory peptide; DEX, dexamethasone.

**P* < .05 compared with normal saline treatment.

standard. IFN- γ and IL-5 were measured using commercially available ELISA kits (R&D Minneapolis, Minn) containing mouse mAbs recognizing different epitopes of the cytokine molecules. The lowest range detectable was 10 pg/mL. Because of its low concentration in the BALF, IL-4 was not measured in this part of the study. TGF- β was measured using an ELISA method as described previously. Microtiter plates (Costar Lab, Cambridge, Mass) were coated with 50 μ L chicken IgY anti TGF- β (R&D) and detected with anti-TGF- β (50 μ L, 1:500, mouse IgG1 Moab, Genzyme, Cambridge, Mass). The lowest range detectable was 31.2 pg/mL. Results were expressed as pg/mL in sera.

Pulmonary function determination

Airway hyperresponsiveness was measured by means of methacholine-induced airflow obstruction. Each mouse was placed inside a barometric plethysmograph (Buxco Electronics, Troy, NY). Aerosol was generated by placing 5 mL of saline or methacholine (Sigma, 1.56-25.00 mg/mL) solution in the cup of an ultrasonic nebulizer (DeVilbiss, Somerset, Pa).²⁵ The percentage changes of Penh obtained from each dose of methacholine were accumulated by the area under the curve, and the values were calculated as means ± SEM. Differences in parameters among groups were analyzed by analysis of variance.

Cell culture, immunofluorescence staining, and flow cytometry analysis

Flow cytometric determination of the cytokine in activated murine T helper cells was assayed according to the method of Assenmacher et al.²⁶ Leukocytes from peripheral blood were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL), ionomycin (2 μ mol/L), and GolgiStop (Cytofix/Cytoperm Plus PharMingen, San Diego, Calif) for 5 hours and then washed twice with PBS. The cells were stained with CD4-PerCP, CD8-FITC, or IgG1-FITC (PharMingen) at room temperature for 30 minutes and then washed. Cells were fixed with cytofix/cytoperm at room temperature for 30 minutes and stained with anti-cytokine antibody and IgG2a-PE (PharMingen) at room temperature for 30 minutes and then washed. Both IgG1-FITC and IgG2a-PE were purchased from the same manufacturer as the anticytokine antibodies used as a negative control. Cells were resuspended in 0.5 mL PBS. Mean fluorescence was measured by Becton Dickinson flow cytometry (Becton Dickinson, San Jose, Calif). A total of 2000 cells was analyzed for each sample.

Pathology of lung and trachea tissue

The lung and trachea tissue from the experimental and control groups of mice were removed and rinsed with PBS. Tissue sections were fixed with 10% formalin and stained with hematoxylin-eosin, and the slides with these sections were mounted with a coverslip.

Statistical analysis

Results were expressed as an arithmetic mean ± SEM. Differences between values before and after saline or methacholine exposure were analyzed by the paired *t* test. Differences between the saline control and the naive groups were analyzed by the unpaired *t* test. Differences were considered significant if *P* was < .05.

RESULTS

Effects of Dp2 peptide and FIP on airway inflammation

The airway inflammation was compared in all experimental groups by analyzing the leukocyte subpopulation in the BALF. Free radicals in the BALF were decreased after Dp2 28-40 and FIP LNIT (data not shown). The results showed that Dp2 28-40 had the best anti-inflammatory effect on airway inflammation (53.3 ± 4.1 × 10⁴ cells/mL), although FIP was also able to diminish the total leukocyte counts in the BALF (65.0 ± 10.5 × 10⁴ cells/mL). Not only did the total leukocyte count decrease, but the same was also the case for the level of eosinophils, neutrophils, and lymphocytes subsequent to LNIT compared with the NS groups (Table I). Dp2 28-40A also had an anti-inflammatory effect, but it was weaker than Dp2 28-40. Similar findings were detected for the groups made up of a mixture of FIP with Dp2 28-40 and a mixture of FIP with Dp2 28-40A. The DEX group was decreased in total cell counts and all leukocytes of the subpopulation compared with the NS group.

Effects of Dp2 peptide and FIP on cytokine-producing cells in peripheral blood

The effects of Dp2 28-40 and FIP on cytokine-producing cells in peripheral blood were compared after LNIT. Both cytokine-producing CD4 and CD8 were assessed after phorbol 12-myristate 13-acetate and ionomycin stimulation. The results revealed that Dp2 28-40 can upregulate IFN- γ -producing CD4 and CD8 cells and downregulate IL-5-producing CD4 cells. FIP can upregulate IFN- γ -producing and downregulate IL-5-producing CD4 and CD8 cells. When the IL-5/IFN- γ ratio was analyzed, it was noted that the ratio was decreased in both CD4 and CD8 cells subsequent to Dp2 28-40 LNIT. However, it was decreased only in CD4 cells subsequent to FIP LNIT. When Dp2-immunized mice were treated

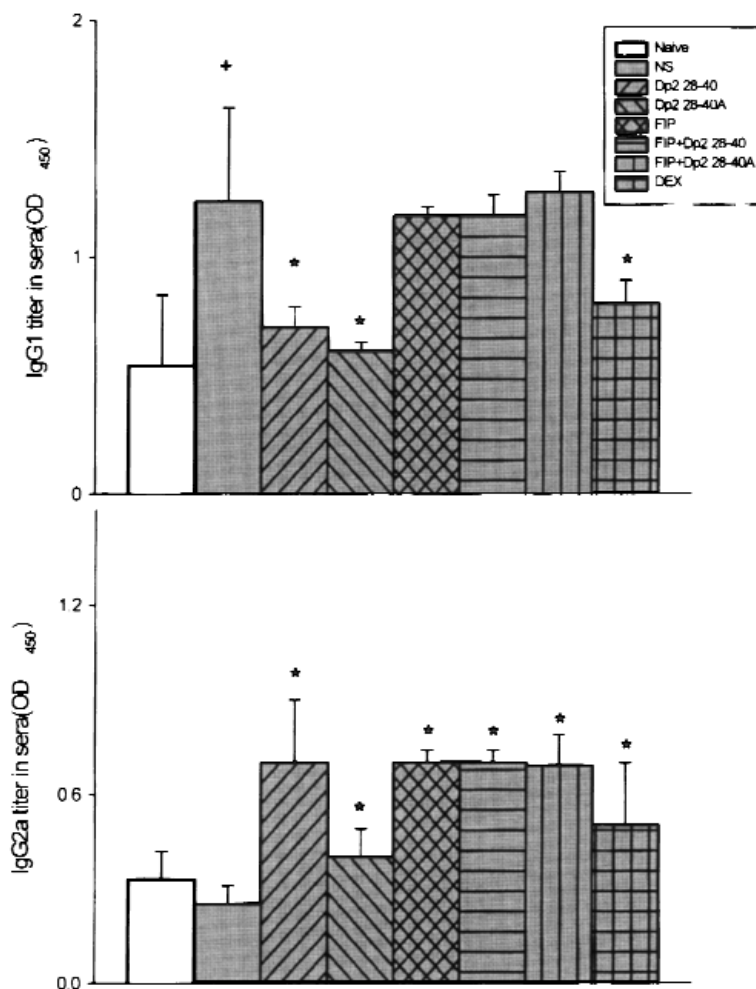


FIG 2. Effects of fungal immunomodulatory peptide, Dp2 28-40, and Dp2 28-40A on Dp2-specific IgG1 and IgG2a production in the sera. In IgG1, the serum was diluted 1:100 and IgG2a was diluted 1:5 at 4°C overnight. Detection antibodies were added separately for each assay, and the bound enzyme substrate was detected with TMB. The reaction was stopped with 50 μ L 4N H₂SO₄ and measured at 450 nm. * P < .05 compared with normal saline. + P < .05 compared with naive.

with a mixture of FIP with either Dp2 28-40 or Dp2 28-40A, the percentage of IL-5-producing CD4 and CD8 cells was noted to have decreased, but IFN- γ -producing cell levels increased. The IL-5/IFN- γ ratio was also noted to have decreased for both CD4 and CD8 cells (Table II).

Effects of Dp2 peptide and FIP on Dp2-specific immunoglobulins in the sera

In the NS-treated mice, IgG1 was significantly higher in the sera compared with naive controls (Fig 2). However, there were no significant changes in IgG2a level. In LNIT with Dp2 28-40 and Dp2 28-40A, the IgG1 levels were significantly reduced, and the IgG2a increased in both groups compared with that of the NS group. IgG2a levels were significantly increased for both FIP mixed with Dp2 28-40 and FIP mixed with Dp2 28-40A. The decreased IgG1 and increased IgG2a were also found in the DEX group.

Effects of Dp2 peptide and FIP on cytokine levels in sera and BALF

In the NS-treated mice, significantly increased IL-5 and decreased IFN- γ were observed in both sera and BALF compared with the naive controls. When mice were treated with LNIT with Dp2 28-40 and FIP, significantly increased IFN- γ and decreased IL-5 were observed in both sera and BALF compared with the NS-treated mice. When the effects of Dp2 28-40 and Dp2 28-40A were compared, the reduction in IL-5 and increase in IFN- γ were similar in the 2 groups, both in the sera (Fig 3) and in the BALF (Fig 4). FIP was also able to elicit a decrease in IL-5 and an increase in IFN- γ in both sera and BALF. When Dp2 28-40 or Dp2 28-40A was combined with FIP, the modulatory effect was altered. However, the reduction in IL-5 was decreased when mice were treated with LNIT with Dp2 28-40 mixed with FIP. The reduction

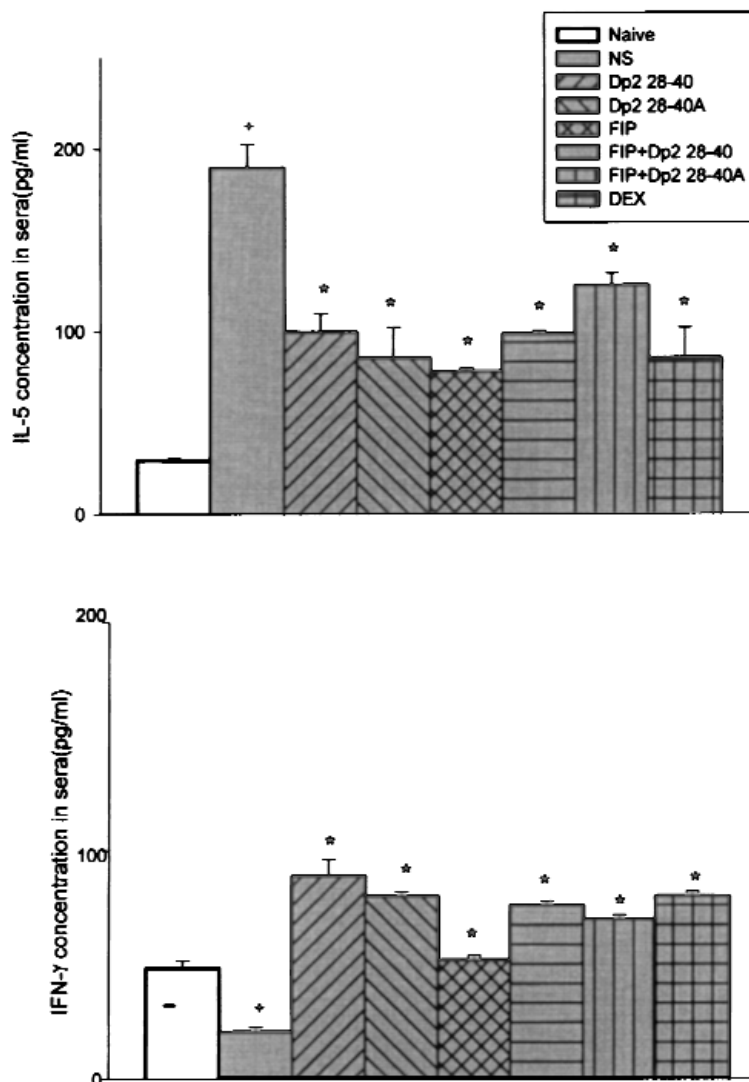


FIG 3. Effects of fungal immunomodulatory peptide, Dp2 28-40, and Dp2 28-40A on the serial concentration of cytokines in sera. IFN- γ and IL-5 were measured using commercially available ELISA kits. The lowest range detectable was 10 pg/mL. For the protocols see "Methods." * $P < .05$ compared with normal saline. + $P < .05$ compared with naive.

of IL-5 and increase of IFN- γ were also found not only in serum but also in BALF in the DEX group.

When TGF- β concentrations in sera were measured, significantly increased TGF- β was observed in the NS-treated mice (NS, 342.3 ± 21.5 pg/mL; naive, 128.3 ± 21.5 pg/mL; $P < .05$). However, when mice were treated with LNIT, significantly decreased TGF- β was observed in the sera for all the therapy groups compared with the NS-treated mice (FIP, 242.4 ± 57.2 pg/mL; FIP mixed with Dp2 28-40, 135.4 ± 50.0 pg/mL; FIP mixed with Dp2 28-40A, 213.9 ± 14.4 pg/mL; DEX, 57.0 ± 32.3 pg/mL; $P < .05$ compared with NS). When the effects of Dp2 28-40 and Dp2 28-40A were compared, there were more TGF- β reductions in the mice treated with FIP mixed with Dp2 28-40.

Effects of Dp2 peptide and FIP on the pulmonary function

The airway hypersensitivity to methacholine was measured both 30 minutes and 24 hours after rDp2 challenge. In the NS-treated mice, the Penh values were significantly increased after methacholine challenge at both 30 minutes and 24 hours after rDp2 IT challenge compared with those of naive mice. For all of the experiment groups, except FIP, when mice were treated with LNIT, the Penh values were significantly decreased after methacholine challenge in both early phases and late phases compared with the NS-treated mice. When mice were treated with LNIT with FIP, the Penh significantly decreased after methacholine challenge 30 minutes after rDp2 IT chal-

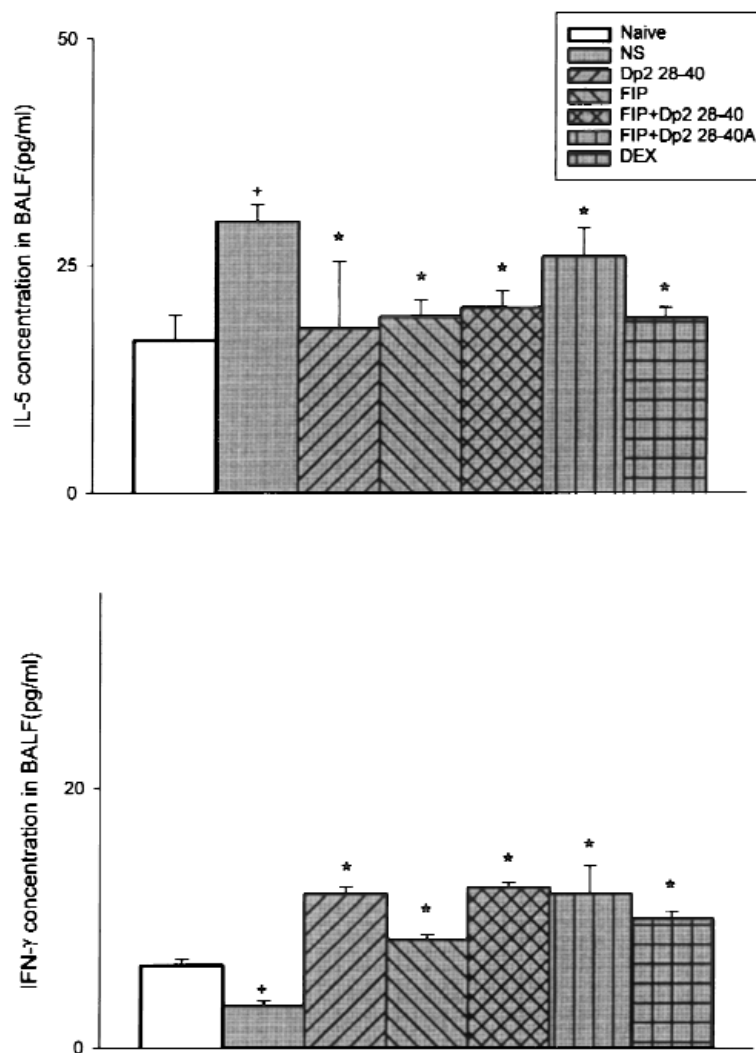


FIG 4. Effects of fungal immunomodulatory peptide, Dp2 28-40, and Dp2 28-40A on the serial concentration of cytokines in bronchoalveolar lavage fluid. * $P < .05$ compared with normal saline. + $P < .05$ compared with naive.

TABLE II. Effect of Dp2 28-40, fungal immunomodulatory peptide, and fungal immunomodulatory peptide mixture with Dp2 28-40A on cytokine-producing cells in peripheral blood*

	Naive	Normal saline	Dp2 28-40	Fungal immuno- modulatory peptide	Fungal immunomodulatory peptide+ Dp2 28-40A	Dexamethasone
CD4	47.1 ± 3.7	52.9 ± 2.6	56.0 ± 0.2	32.4 ± 3.3	48.9 ± 10.9	48.4 ± 2.8
IL-5/CD4	16.2 ± 0.8	31.1 ± 4.8	17.7 ± 9.7	12.2 ± 0.4	11.7 ± 4.1	12.5 ± 3.3
IFN-γ/CD4	20.0 ± 2.1	12.9 ± 3.7	27.8 ± 13.7	14.6 ± 0.9	21.8 ± 0.9	12.8 ± 4.9
IL-5/ IFN-γ CD4	0.8	2.4	0.6	0.8	0.5	1.0
CD8	7.0 ± 0.6	7.3 ± 2.4	9.8 ± 0.5	7.1 ± 0.1	5.6 ± 2.6	7.5 ± 0.5
IL-5/CD8	10.0 ± 0.5	8.6 ± 3.1	9.5 ± 7.2	4.6 ± 3.9	3.3 ± 2.5	11.2 ± 3.0
IFN-γ/CD8	16.0 ± 0.3	6.1 ± 1.2	16.8 ± 11.2	2.8 ± 2.2	6.8 ± 6.2	11.0 ± 3.1
IL-5/ IFN-γ CD8	0.6	1.4	0.6	1.6	0.5	1.0

These data did not reach statistical levels.

*Data represented as percentage of positive stained cells in the PBMC of 6 mice; mean ± SEM.

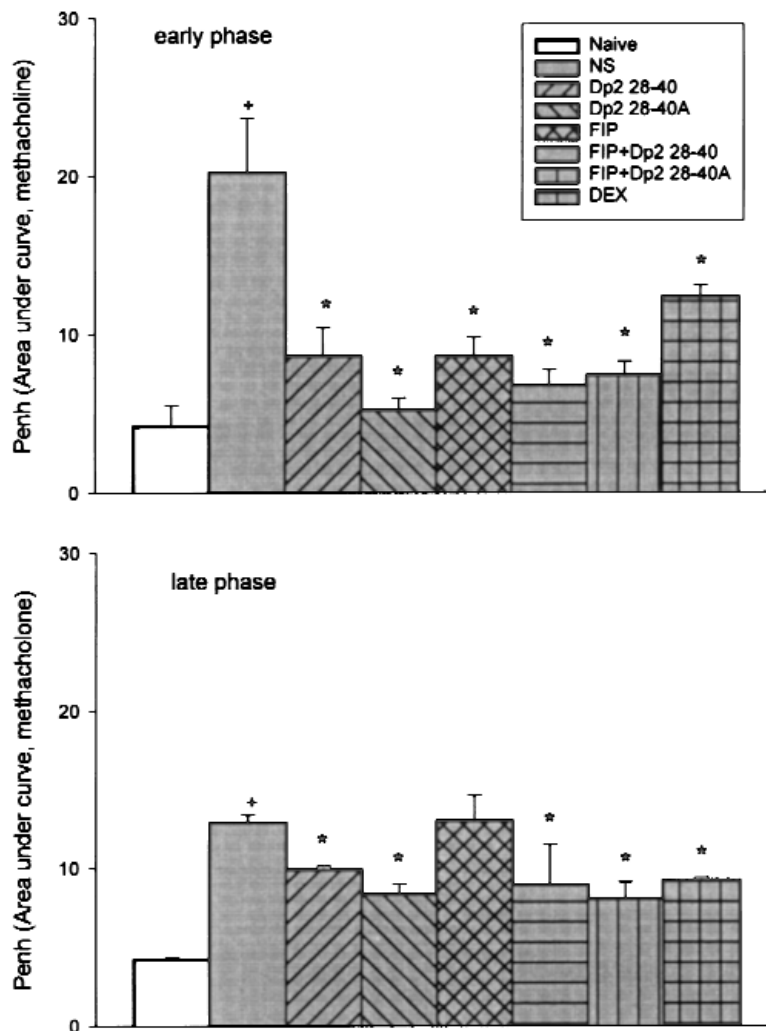


FIG 5. The effects of fungal immunomodulatory peptide, Dp2 28-40, and Dp2 28-40A on pulmonary function. The airway hypersensitivity to methacholine was measured both early and late after rDp2 IT challenge. The Penh values were accumulated after each dose of methacholine challenge and were expressed as the area under the curve. * $P < .05$ compared with normal saline. + $P < .05$ compared with naive.

lenge. There were no differences in Penh observed 24 hours after rDp2 IT challenge (Fig 5).

The pathology of different groups of mice after LNIT

The inflammatory cell infiltration and epithelium damage in the airway were compared among all experiment groups after LNIT. The results showed that there was a reduction of airway inflammatory cell infiltration and a decrease of epithelium damage in all experimental groups compared with that of NS-treated mice (Fig 6).

DISCUSSION

In this study we demonstrated that a dominance of the T_H2 response by rDp2-immunized mice can be modulated by LNIT with the Dp2 peptide epitope and FIP. LNIT using not only FIP but also the dominant peptide Dp2 28-

40 or the altered peptide Dp2 28-40A were noted to be able to suppress ongoing airway inflammation induced by rDp2. This LNIT-induced suppression of airway inflammation was associated with an increase in IFN- γ and a decrease in IL-5 and TGF- β production. The reduction of TGF- β indicated that suppression regulatory T cells might be involved.

It has been previously reported that co-immunization with a mixture of antigens and the immunostimulatory DNA sequences ISS elicits a T_H1 response,^{8,27,28} and this has also been shown to be effective in animal models considered to be relevant to human allergic disease states.²⁹⁻³³ Amb A1, a major ragweed allergen that had been conjugated to ISS, has been recently reported to reflect an enhanced T_H1 -biased immunogenicity and a reduced antigenicity.³⁴ On the basis of the aforementioned system, the delivery of an allergen peptide with a T_H1 response-inducing adjuvant might amplify the ther-

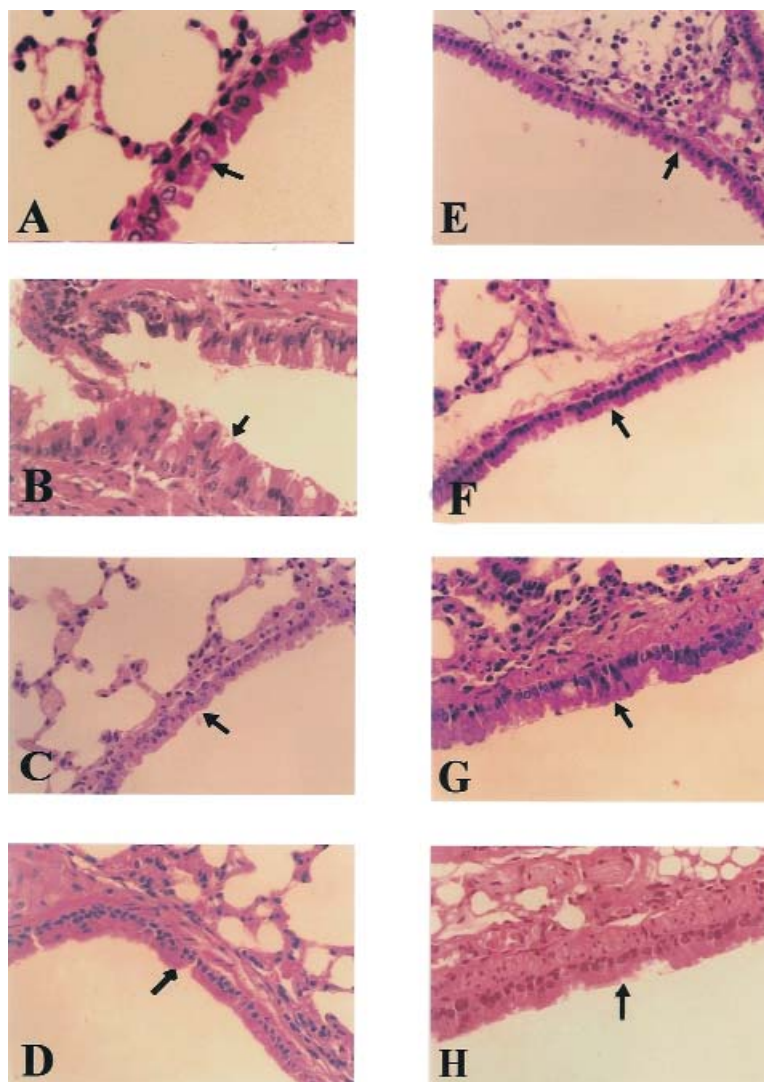


FIG 6. The pathology of different groups of mice after local nasal immunotherapy. The lung tissues were obtained 2 days after intrathecal rDp2. **A**, Naive mice. **B**, Mice treated with normal saline. **C**, Mice treated with dexamethasone. **D**, Mice treated with fungal immunomodulatory peptide (FIP). **E**, Mice treated with Dp2 28-40. **F**, Mice treated with Dp2 28-40A. **G**, Mice treated with FIP mixed with Dp2 28-40. **H**, Mice treated with FIP mixed with Dp2 28-40A (hematoxylin-eosin stain, original magnification $\times 400$).

apeutic effect of allergen immunotherapy. We prepared the mixture of FIP with or without Dp2 peptides to compare their effects by way of LNIT on mice primed with rDp2 in aluminium hydroxide to induce a pre-existing T_H2 response. Similar results were found in our study: LNIT using FIP mixed with the dominant peptide of Dp2 28-40 was able to enhance T_H1 -biased immunogenicity and reduce T_H2 -biased allergenicity. These results indicated that FIP is a T_H1 response-inducing adjuvant.

The relatively high level of IFN- γ and the corresponding relatively low level of IL-5 in both BALF and sera suggest that LNIT could modulate allergen-specific T cells, activating T_H1 and suppressing T_H2 ; this process is possibly similar to subcutaneous immunotherapy. The previous clinical observations of immunotherapy-treated patients during the ragweed pollen season have revealed that

immunotherapy can decrease IL-5 and IL-5-secreting T_H2 cell levels and increase IFN- γ -secreting T_H1 cells for an allergen-stimulated T-cell culture.³⁵⁻³⁷ Although LNIT with FIP also indicated a shift in the T_H1 and T_H2 response for test mice on the basis of the results of cytokine, it was weaker than that induced as a result of subjecting test mice to a mixture of FIP and Dp2 epitope peptides that resulted in a decrease of the IL-5/IFN- γ ratio for both CD4 and CD8 cells. The value of the ratio of IL-5/IFN- γ for CD8 cells was observed to be greater for the test group FIP mixed with Dp2 28-40 than that for FIP mixed with Dp2 28-40A. An alteration of allergic epitopes, Dp2 28-40A, has previously been reported to be able to modulate the T-cell response.¹³ Our results have revealed that there were fewer IL-5-secreting CD4 and CD8 cells for those mice treated with a mixture of FIP with Dp2 28-40A than was

the case for test mice exposed to a combination of FIP and Dp2 28-40; these results also suggest that Dp2 28-40A is more effective at downregulating T_H2 cells. Whether a mixture of FIP with Dp2 28-40A is more efficient than a mixture of FIP and Dp2 28-40 for rDp2-sensitized mice, however, remains to be investigated.

LNIT with FIP caused an increase in production of IgG2a and a decrease in production of IgE (data not shown). However, the modulatory effect on IgG1 production did not occur. Similar findings were found when LNIT with FIP was mixed with Dp2 allergic epitope peptides. Antigen-specific variation in the effect of LNIT was noted by Wiedermann et al,³⁸ who showed that a regimen that inhibited responses to Bet v1 did not affect responses to ovalbumin. This variation might occur because the outcome of LNIT is balanced between activation and inhibition events. Our results revealed that LNIT with FIP was effective for the prevention of an early phase reaction. Similar results have been reported that FIP can abolish immediate anaphylaxis reaction.¹⁵ LNIT with FIP was not effective for the prevention of late-phase airway hyperreactivity despite the fact that the inflammatory cells and IL-5 in the BALF were decreased. These results suggested that late-phase airway hyperreactivity is only partially correlated with airway inflammatory cell infiltration. Although it is now widely accepted that airway inflammation is the basis of airway hyperreactivity, there are at least 3 independent studies of airway hyperreactivity in mice.³⁹ The first pathway is dependent on IL-4 and mast cells, the second is dependent on IL-5 and eosinophils, and the third is mediated by IL-13. Whether IL-4 and IL-13 can be downregulated by FIP LNIT remains to be clarified.

Recently, endotoxins have been reported to possibly induce airway hypersensitivity.⁴⁰⁻⁴³ In Kerstin et al, endotoxins (12.5 µg LPS/25 g mouse/100 µL) can prevent murine IgE production and T_H2 immune responses. In our materials, which have been tested for the endotoxin contaminations, there were less than 30 EU/mL (3 ng/mL) in FIP, and they were undetectable in Dp2 peptides. The effects of FIP and Dp2 peptides were unlikely caused by endotoxin. The slight inflammation in these groups of mice treated with DEX might be because the concentration we used was not enough to suppress the inflammation.

In summary, these studies have demonstrated that FIP in combination with Dp2 28-40 or 28-40A makes a good alternative candidate for LNIT.

REFERENCES

- Platts-Mills TA, Vervloet D, Thomas WR, Aalberse RC, Chapman MD. Indoor allergens and asthma: report of the Third International Workshop. *J Allergy Clin Immunol* 1997;100:S2-24.
- Sporik R, Chapman M, Platts-Mills TA. House dust mite exposure as a cause of asthma. *Clin Exp Allergy* 1992;22:897-906.
- Heymann PW, Chapman MD, Aalberse RC, Fox JW, Platts-Mills TA. Antigenic and structural analysis of group II allergens (Der f II and Der p II) from house dust mites (*Dermatophagoides* spp). *J Allergy Clin Immunol* 1989;83:1055-67.
- Smith AM, Chapman MD. Allergen-specific immunotherapy: new strategies using recombinant allergens. In: Bousquet J, Yssel H, editors. *Immunotherapy in asthma*. New York: Marcel Dekker; 1999. p. 99-118.
- Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, et al. Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;341:468-75.
- Varney VA, Hamid QA, Gaga M, Ying S, Jacobson M, Frew AJ, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest* 1993;92:644-51.
- Secrist H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4⁺ T cells from allergic individuals. *J Exp Med* 1993;178:123-30.
- Jutel M, Pichler WJ, Skrbic D, Urwyler AA, Dahinder C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 increase of IFN-γ secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;154:4187-94.
- Briner TJ, Kuo MC, Keating KM, Rogers BL, Greenstein JL. Peripheral T-cell tolerance induced in naïve and primed mice by subcutaneous injection of peptides from the major cat allergen Fel d 1. *Proc Natl Acad Sci U S A* 1993;90:7608-12.
- Haselden BM, Kay AB, Larche M. IgE-independent MHC-restricted T cell peptide epitope-induced late asthmatic reactions. *J Exp Med* 1999;189:1885-94.
- Mueller GA, Benjamin DC, Rule GS. Tertiary structure of the major house dust mite allergen Der p 2: sequential and structural homologies. *Biochemistry* 1998;37:12707-14.
- Tsitoura DC, Verhoef CM, Gelder RE, O'Hehir RE, Lamb JR. Alter T cell ligands derived from a major house dust mite allergen enhance IFN-γ but not IL-4 production by human CD4⁺ T cells. *J Immunol* 1996;157:2160-5.
- Verhoef A, Lamb JR. Threshold signaling of human Th0 cells in activation and anergy: modulation of effector function by alter TCR ligand. *J Immunol* 2000;164:6034-40.
- Oldfield WLG, Kay AB, Larche M. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. *J Immunol* 2001;167:1734-39.
- Ko JL, Hsu CI, Lin RH, Kao CL, Lin JY. A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, *Flammulina velutipes* and its complete amino acid sequence. *Eur J Biochem* 1995;228:244-9.
- Fox PC, Siraganian RP. IgE antibody suppression following aerosol exposure to antigens. *Immunology* 1981;43:227-34.
- Georgitis JW, Clayton WF, Wypych JJ, Barde SH, Reisman RE. Further evaluation of local intranasal immunotherapy with aqueous and allergoid grass extracts. *J Allergy Clin Immunol* 1984;74:694-700.
- Nickelsen JA, Goldstein S, Mueller UR, Wypych J, Reisman RE, Arbesman CE. Local intranasal immunotherapy for ragweed allergic rhinitis. II. Immunologic response. *J Allergy Clin Immunol* 1981;68:41-5.
- Nickelsen JA, Georgitis JW, Mueller UR, Kane J, Wypych J, Goldstein S, et al. Local intranasal immunotherapy for ragweed allergic rhinitis. A second year for treatment. *Clin Allergy* 1983;13:509-19.
- Welsh PW, Butterfield JH, Yunginger JW. Allergen-controlled study of intranasal immunotherapy for ragweed hay fever. *J Allergy Clin Immunol* 1983;71:454-60.
- Bjorksten B. Local immunotherapy is not documented for clinical use. *Allergy* 1994;49:299-301.
- Georgitis JW, Nickelsen JA, Wypych JJ. Local nasal immunotherapy: efficacy of low dose aqueous extract. *J Allergy Clin Immunol* 1985;75:496-500.
- Bertoni M, Cosmi F, Bianchi I, Berardino L. Clinical efficacy and tolerability of a steady dosage schedule of local nasal immunotherapy. Results of preseasonal treatment in grass pollen rhinitis. *Ann Allergy Asthma Immunol* 1998;82:47-51.
- Tsai JJ, Shen HD, Chua KY. Purification of group 2 *Dermatophagoides pteronyssinus* allergen and prevalence of its specific IgE in asthmatics. *Int Arch Allergy Immunol* 2000;121:205-10.
- Hamelmann E, Schwarze J, Takada K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766-75.
- Assenmacher M, Schmitz J, Radbruch A. Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-γ and in interleukin-4 expressing cells. *Eur J Immunol* 1994;24:1097-101.

27. Secrist H, Chelen CJ, Wen J, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4⁺ T cells from allergic individuals. *J Exp Med* 1993;178:2123-30.
28. McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to TH-1 dominant pattern: comparison of rush and conventional immunotherapy. *Clin Exp Allergy* 1995;25:828-38.
29. Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998;102:157-64.
30. Roman M, Martin-Orozco E, Goodman JS, Nguyen MD, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as Th1 inducing adjuvants. *Nat Med* 1997;3:849-54.
31. Chu RS, Targoni OS, Krieg AM, Lehman PV, Harding CV. CpG oligodeoxynucleotides act as adjuvant that switch on T helper 1 (Th 1) immunity. *J Exp Med* 1997;186:1623-31.
32. Sun S, Kishimoto H, Spretn J. DNA as an adjuvant: capacity of insect DNA and synthetic oligonucleotides to augment T cell responses to specific antigen. *J Exp Med* 1998;187:1145-50.
33. Jahn-Schmid B, Wiedermann U, Bohle B, Repa A, Kraft D, Ebner C. Oligodeoxynucleotides containing CpG motifs modulate the allergic Th2 response to BALB/c mice to Bet v 1, the major birch pollen allergen. *J Allergy Clin Immunol* 1990;104:1015-23.
34. Santeliz JV, Nest GV, Traquina P, Larsen E, Wills-Karp M. A β -linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol* 2002;109:455-62.
35. Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999;162:6284-93.
36. Broide D, Schwarze J, Tighe H, Gifford T, Nguyen MD, Malek S, et al. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;161:7054-62.
37. Kline JN, Waldshmidt TJ, Businga TR, Lemish JE, Weinstock JV, Thorne PS, et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998;160:2555-9.
38. Wiedermann U, Jahn-Schmid B, Lindblad M, Rask C, Holmgren J, Kraft D, et al. Suppressive versus stimulatory effects of allergen/cholera toxinoid (CTB) conjugates depending on the nature of allergen in a murine model of type I allergy. *Int Immunol* 1999;11:1717-24.
39. Leong KP, DP Huston. Understanding the pathogenesis of allergic asthma using mouse models. *Ann Allergy Asthma Immunol* 2000;87:96-110.
40. Jarnicki AG, T Tsuji, Thomas WR. Hypersensitivity reactions after respiratory sensitization: Effect of intranasal peptides containing T cell epitopes. *J Allergy Clin Immunol* 2002;110:610-6.
41. Gerhold K, Katharina B, Anja B, Christine S, Philippe S, Tilmann K, et al. Endotoxins prevent murine IgE production, Th2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 2002;110:110-6.
42. Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, et al. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996;154:1641-6.
43. Reed CE, Milton DK. Endotoxin-stimulated innate immunity: a contributing factor for asthma. *J Allergy Clin Immunol* 2001;108:157-66.