IFN-γ and IL-12 plasmid DNAs as vaccine adjuvant in a murine model of grass allergy

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Background: Plasmids encoding cytokines such as IFN-γ and IL-12 are potential genetic adjuvants that might increase the effectiveness of allergen vaccines.

Objective: The role of plasmids expressing the cytokines IFN-γ (pIFN-γ) and/or IL-12 (pIL-12) as adjuvants in modulating allergic immune responses, inflammation, and asthma was investigated in a murine model of Kentucky blue grass (KBG) allergy.

Methods: Groups of naive B6D2F1 mice were vaccinated subcutaneously with KBG allergens and administered intramuscularly with pIFN-γ, pIL-12, pIFN-γ plus pIL-12, or a vector control. Mice were then sensitized with KBG allergens in alum (intraperitoneally) and later challenged intranasally. Mice were examined for modulation of specific immunity, prevention of the development of airway hyperresponsiveness, and inflammation.

Results: Mice vaccinated with cytokine plasmid adjuvants had relatively lower levels of total serum IgE and higher levels of grass allergen–specific IgG2a in comparison with control mice. The lowest IgE and highest IgG2a levels were found in mice vaccinated with the combination of pIFN-γ and pIL-12 as an adjuvant. The vaccination of mice with both pIFN-γ and pIL-12 as an adjuvant induced the highest level of T_H1 cytokines, IFN-γ, and IL-2 in comparison with mice given either of the plasmids alone. The most profound decrease in airway hyperresponsiveness and pulmonary inflammation was observed in mice receiving both pIFN-γ and pIL-12 as an adjuvant.

Conclusion: These results demonstrate that pIFN-γ and pIL-12 together provide an effective adjuvant to parenteral grass allergen vaccines and show that this adjuvant can significantly enhance the effectiveness of allergen immunotherapy in human beings. (J Allergy Clin Immunol 2001;108:402-8.)

Key words: *Allergen, adjuvant, cytokines, plasmids, T_H1, T_H2, inflammation, airway hyperresponsiveness*

The effectiveness of allergen immunotherapy (AIT) is often compromised by the use of high doses of allergens for treatment, which can lead to severe systemic reactions and the inconvenience and discomfort of frequent dosing.[1] It has been suggested that the utilization of an adjuvant in conjunction with an allergen vaccine might enhance both the safety and the effectiveness of AIT.[2] Two approaches are under investigation. The first is coimmunization with a mixture of antigen(s) and bacterial DNA or immunostimulatory oligodeoxynucleotides (ODNs), which contain CpG motifs[3] and elicit a protective T_H1 response.[4] The potential of CpG-containing ODNs as adjuvants in AIT is under intense investigation; however, it has been reported that the immunostimulatory activity of these ODNs might be blocked by certain non-CpG motifs.[5,6] In addition, these ODNs might not suppress established allergic responses.[7] In a second approach, the potential of plasmid DNA (pDNA)–encoding cytokine(s) as genetic adjuvants has been examined, with varying levels of success, for modulating the immune response stimulated by administered antigen vaccines.[8]

Cytokines IFN-γ and IL-12 are known to mediate T-cell differentiation toward a T_H1-like phenotype.[9] Despite numerous studies in which pure or recombinant IFN-γ and IL-12 were used, their in vivo use has been limited by the short half-life of these molecules and the associated severe adverse effects.[10] Mucosal IFN-γ gene transfer has earlier been shown to inhibit both antigen- and T_H2 cell–induced pulmonary eosinophilia and airway hyperreactivity.[11] Vaccinia virus–mediated IL-12 gene transfer to the mouse airway abrogated airway eosinophilia and IgE synthesis.[12] However, the direct effects of these cytokine plasmids as genetic adjuvants in the allergen vaccines used for AIT have not been addressed.

In a previous study, a combination of allergen and IFN-γ effectively redirected allergen-specific T-cell cytokine production toward elevated IFN-γ production in human PBMC cultures.[13] In a murine model of Kentucky blue grass (KBG) allergy, parenteral administration of 1 mg of recombinant allergen induced effective immune deviation.[14] On the basis of these observations and the results of cytokine gene transfer studies,[11,12] it was reasoned that a provision for the elevated in vivo expression of IFN-γ and/or IL-12 as an adjuvant at the onset of AIT might enhance the effectiveness of allergen vaccines. The results reported herein demonstrate that a substantially

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Abbreviations used
AIT: Allergen immunotherapy
BGH: Bovine growth hormone
CMV: Cytomegalovirus
KBG: Kentucky blue grass
ODN: Oligodeoxynucleotide

reduced dose of allergens given with an adjuvant comprising both pIFN-γ and pIL-12 induces the most effective immune deviation and protection in a murine model of KBG allergy.

MATERIALS AND METHODS

Animals

Female B6D2F1 mice, 6 to 8 weeks old, from Jackson Laboratory (Bar Harbor, Me) were maintained in pathogen-free conditions at the animal center at the James A. Haley Veterans Hospital. All procedures were reviewed and approved by the committee on animal research at the James A. Haley VA Medical Center and the University of South Florida College of Medicine.

Vaccination protocol

Four groups of naive mice (n = 12) were intramuscularly vaccinated 3 times at intervals of 2 days, each in its right quadriceps muscle, with 100 μg of pIFN-γ, 100 μg of pIL-12, or a mixture of pIL-12 and pIFN-γ (50 μg of each), along with subcutaneous injection of 50 μg per mouse of crude KBG extract given at the back of the animal. Control mice each received 100 μg of pcDNA3.1 plasmid and 50 μg of KBG allergen extract. Seven days after the last DNA and KBG vaccinations, control and experimental groups of mice were immunized intraperitoneally with 10 μg of KBG allergen and 1 mg of alum.

Construction of the pIL-12 and pIFN-γ plasmids

To clone murine IL-12, the IL-12 p40 subunit was amplified from a mouse cDNA library as an *Nhe*I-*Xho*I cassette through use of the following set of primers: forward primer 5′-dCCA GGC AGC TAG CAG CAA AGC AA-3′ and reverse primer 5′-dTCC CTC GAG GCA TCC TAG GAT CGG AC-3′. The amplified product was ligated to the mammalian expression vector pcDNA 3.1 (Invitrogen, San Diego, Calif) at the corresponding sites. The resulting plasmid, pcP40, was used as the template to amplify the p40 subunit and bovine growth hormone (BGH) poly A sequences derived from the pcDNA 3.1 vector as an *Hind*III–*Kpn*I cassette and ligated to pcDNA 3.1 at the corresponding sites to generate the plasmid pc40. The following primers were used: 5′-dACC CAA GCT TGC TAG CAG CAA A-3′ and 5′-dGAA GCC ATA GAG GGT ACC GCA TC-3′. Through use of forward primer 5′-dTGC GGA TCC AGC ATG TGT CAA T-3′ and reverse primer 5′-dGCA GAG GGC CTC GAG CTT TCA G-3′, the p35 subunit of murine IL-12 was amplified as a *Bam*HI-*Xho*I fragment and cloned into pcDNA3.1 to generate the pcP35 vector. With pcP35 used as a template, the cytomegalovirus (CMV) promoter and p35 subunit was amplified as an *Eco*RI-*Eco*RV cassette and ligated to the corresponding site in the vector pc40. The resulting plasmid, pIL-12, had each of the 2 subunits of IL-12 under a separate CMV promoter and a BGH gene poly A sequence. Murine IFN-γ was cloned in pcDNA3.1; the construction of this plasmid has been described elsewhere.[15] Large-scale plasmid preparation was performed through use of a Qiagen kit (Qiagen, Valencia, Calif).

Immunohistochemistry

Groups of naive mice were vaccinated intramuscularly 3 times with 100 μg of pIFN-γ, pIL-12, or pcDNA3.1 at intervals of 2 days each. Seven days after the last vaccination, the mice were killed and their right quadriceps muscles were removed and subjected to paraffin embedding, as described previously.[15] Immunostaining for IFN-γ and IL-12 was performed through use of polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif) against murine IFN-γ and the murine IL-12 p40 subunit, as described previously.[16]

Splenocyte culture and assay for cytokines

Seven days after the KBG immunization, one half of the animals in each group were killed and their spleens removed aseptically. Single-cell suspensions were cultured with 1 mg/mL KBG allergen for 48 hours. Supernatants were collected from the cultures, and the production of IFN-γ, IL-2, and IL-4 was determined by ELISA (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

Antibody assays

Mice were bled 21 days after KBG immunization, and their sera were collected. Total IgE and antigen-specific IgG1 and IgG2a were estimated by ELISA through use of purified antimouse mABs (Pharmingen, San Diego, Calif). Microtiter plates (Costar, Cambridge, Mass) were coated overnight with 0.1 μg per well of purified KBG allergen in a bicarbonate buffer (0.05 mol/L, pH 9.6) or antimouse IgE mAbs. Wells were washed with washing buffer (0.5% Tween-20 in PBS, pH 7.4) and blocked with 200 μL per well of PBS (pH 7.4) containing 1% BSA for 1 hour at 37°C. After 3 washes, serum samples were added at 100 μL per well and incubated for 2 hours at 37°C. After washing, biotinylated antimouse IgG1, IgG2a, and IgE antibodies were added to each well and incubated at 37°C for 1 hour. Streptavidin-peroxidase conjugate (1:10,000 dilution, Sigma Chemicals, St Louis, Mo) was added after washing, and the wells were further incubated at 37°C for 1 hour. Finally, the plates were washed and color was developed by the addition of substrate tetramethyl benzidine (Pharmingen) at room temperature for 30 minutes. The reaction was stopped and the absorbance read at 450 nm through use of an automated ELISA reader.

Pulmonary function

To assess pulmonary function, naive mice (n = 4) were vaccinated 3 times as described in connection with the vaccination protocol. Seven days after the last cytokine pDNA and KBG vaccinations, animals were sensitized intraperitoneally with 10 μg of KBG allergen and 1 mg of alum. Ten days later, these mice were challenged intranasally with 50 μg of KBG allergen once daily for 3 consecutive days. Airway responsiveness was measured with a whole-body plethysmograph (Buxco Electronics, Troy, NY) in conscious, unrestrained mice at 24 hours after allergen challenge; response to methacholine was expressed as enhanced pause, as previously described.[16]

Histologic analysis

Mice were killed with an overdose (0.6 g/kg) of pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill) 24 hours after the final allergen challenge, and lung sections were subjected to paraffin embedding. Lung inflammation was assessed after the sections were stained with hematoxylin and eosin; this was followed by scoring for severity of inflammation on a scale of 0 to 3 (0 being the minimum degree of inflammation and 3 the maximum), as described previously.[15] Pathologic scores were expressed as means ± SDs. The slides were coded and scored in a blinded fashion twice each by 3 different individuals. Intraobserver variation was <5%.

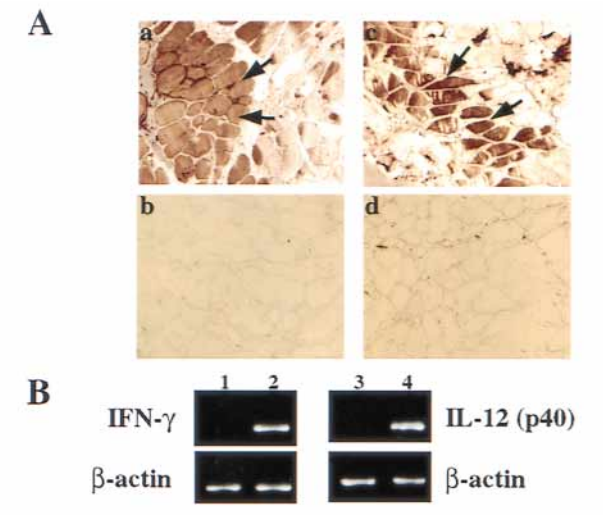


FIG 1. Expression of murine IFN-γ and IL-12 p40 subunit in the mouse muscle. Mice were vaccinated as described in the Methods section. Seven days after the last DNA injection, expression for IFN-γ and IL-12 p40 subunit was checked by immunohistochemistry (*A*) and RT-PCR (*B*). **A,** Mice vaccinated with pIFN-γ (*a*) and pIL-12 (*c*) showed expression of IFN-γ and IL-12 p40 subunits, respectively, as indicated by positive staining (*arrows*). Mice vaccinated with control empty vector did not show expression for IFN-γ (*b*) and IL-12 p40 (*d*). **B,** Total RNA was isolated from the muscle, and RT-PCR was performed for IFN-γ and IL-12 p40 subunit. Mice vaccinated with pIFN-γ and pIL-12 showed IFN-γ (*panel 1, lane 2*) and IL-12 p40–specific mRNA amplification (*panel 1, lane 4*). No amplification was observed in the mice vaccinated with control empty vector (*panel 1, lanes 1 and 3*). β-actin was used as internal control (*lower panel*).

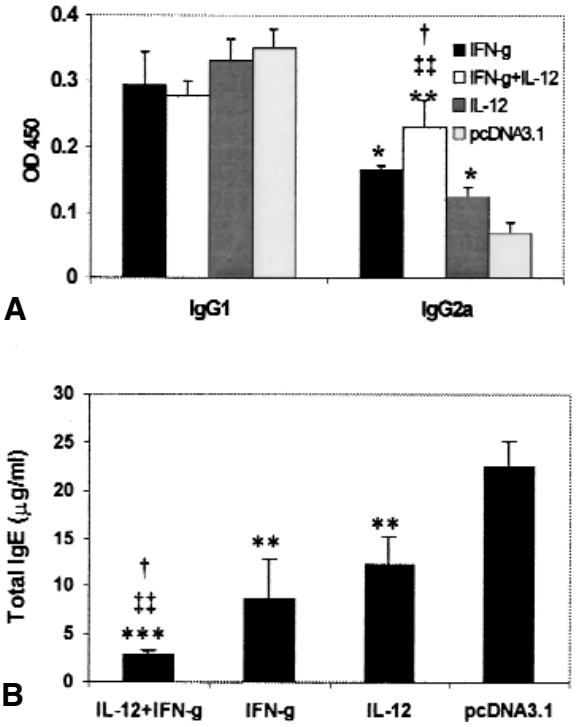


FIG 2. Analysis of the total IgE and KBG-specific IgG subtypes. Four groups of mice (n = 6) were vaccinated as described in the Methods section. On day 21 after immunization with alum and KBG allergen, their serum was analyzed for total IgE **(B)** and KBG-specific IgG2a and IgG1 **(A)** antibodies by ELISA. *Bars* represent the means ± SDs. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ in comparison with pcDNA3.1 control. $^{\dagger}P < .05$; $^{\dagger\dagger}P < .01$; $^{\dagger\dagger\dagger}P < .001$ in comparison with pIFN-γ group. $^{\ddagger}P < .05$; $^{\ddagger\ddagger}P < .01$; $^{\ddagger\ddagger\ddagger}P < .001$ in comparison with pIL-12 group.

Statistical analysis

Pairs of groups were compared through use of Student *t* tests. Differences between groups were considered significant at $P < .05$. Values for all measurements are expressed as means ± SDs.

RESULTS

Plasmid constructs and expression of IL-12 and IFN-γ

The 2 subunits of murine IL-12, p35 and p40, were cloned into the same pcDNA3.1 vector. The cloning strategy was such that each subunit was under the transcriptional control of an individual CMV immediate-early promoter and also had its own BGH poly A sequences derived from the vector pcDNA3.1. Mice given pIFN-γ or pIL-12 exhibited expression of the IFN-γ or IL-12 p40 subunit, respectively, in their muscle, as observed by immunohistochemical staining of the muscle tissues (Fig 1, *A*, *a* and *c*). No immunostaining was observed in a control group of mice that received the empty vector pcDNA3.1 (Fig 1, *A, b* and *d*). The results of RT-PCR analyses of muscle mRNAs revealed that mice given either pIFN-γ or pIL-12 (Fig 1, *B*, *lane 2* and *lane 4*), but not the control group of mice (Fig 1, *B*, *lane 1* and *lane 3*), expressed IFN-γ and IL-12 p40 subunit-specific mRNAs, respectively.

Cytokine genetic adjuvants inhibit production of IgE antibodies and enhanced production of IgG2a antibodies

Four groups of mice (n = 6) were vaccinated 3 times, as described in connection with the vaccination protocol. Sera were collected on day 21 after KBG immunization for an antibody assay. As shown in Fig 2, mice given cytokine plasmid(s) as an adjuvant exhibited significantly lower total IgE levels than control mice ($P < .01$). The group of mice given both pIL-12 and pIFN-γ constructs revealed a significantly lower amount of total IgE than mice given pIL-12 ($P < .01$) or pIFN-γ ($P < .05$) alone (Fig 2, *B*). There was also an increase in antigen-specific IgG2a levels ($P < .01$) in mice given pIFN-γ plus pIL-12 as an adjuvant in comparison with the control mice and the mice receiving either plasmid alone (Fig 2, *A*). However, no significant difference was observed for antigen-specific IgG1 antibody levels among the cytokine pDNA–vaccinated and control groups of mice (Fig 2, *A*). These results indicate that administration of cytokine pDNA adjuvants along with allergen vaccines resulted in a shift in the antibody production from the IgE type to the IgG2a type.

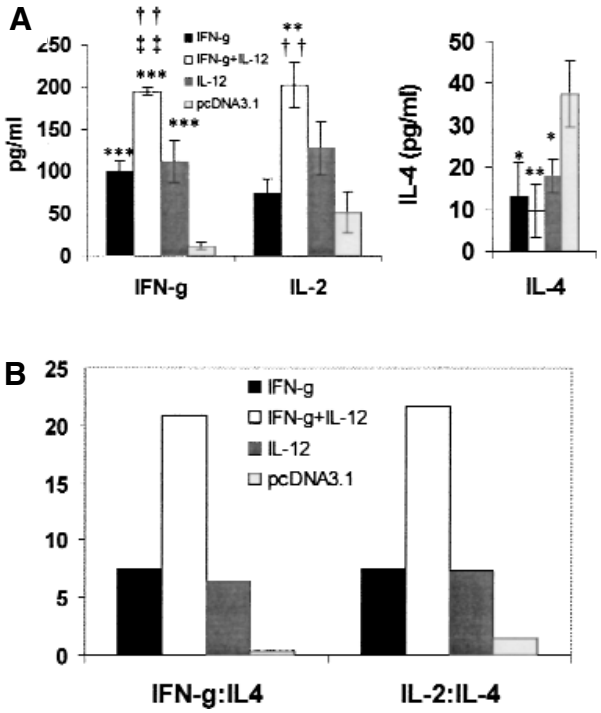


FIG 3. A, Analysis of the cytokine production. Mice (n = 6) were vaccinated as described in the Methods section. On day 7 after KBG and alum immunization, their spleens were cultured in vitro for 48 hours in the presence of KBG allergen and cytokines were measured by ELISA. *Bars* represent the means ± SDs. **P* < .05; ***P* < .01; ****P* < .001 in comparison with pcDNA3.1 control. †*P* < .05; ††*P* < .01; †††*P* < .001 in comparison with pIFN-γ *(IFN-g)* group. ‡*P* < .05; ‡‡*P* < .01; ‡‡‡*P* < .001 in comparison with pIL-12 *(IL-12)* group. **B,** Analysis of the dominant cytokine pattern after cytokine DNA vaccination. Dominant cytokine pattern was determined from the IFN-γ/IL-4 and IL-2/IL-4 ratios. *Bars* represent means.

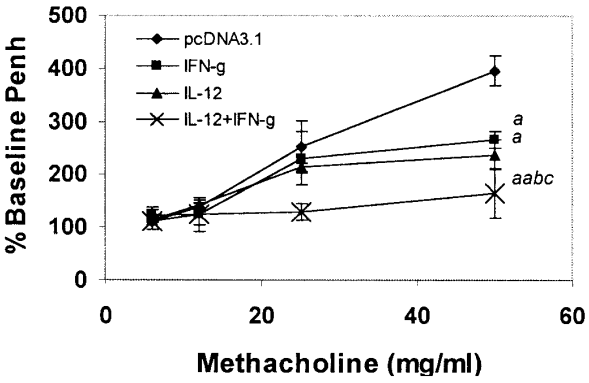


FIG 4. Measurement of the airway hyperresponsiveness in KBG-sensitized and -challenged mice after cytokine DNA vaccination. Naive mice (n = 4) were vaccinated as described in the Methods section and sensitized with the allergen 7 days later. Ten days after the sensitization, animals were challenged intranasally 3 times with 50 μg of KBG allergen. Airway reactivity to inhaled methacholine (6 to 50 mg/mL) was measured 24 hours later. Results are expressed as means ± SDs of enhanced pause values. *a, P* < .05; *aa, P* < .01 in comparison with pcDNA3.1 group. *b and c, P* < .05 in comparison with pIFN-γ *(IFN-g)* and pIL-12 *(IL-12)* groups, respectively.

Cytokine genetic adjuvants alter the cytokine profiles of splenocytes

Four groups of mice (n = 6) were vaccinated 3 times as described in connection with the vaccination protocol. Spleens were removed on day 7, after KBG immunization, and cultured in vitro for assessment of T_H1-like cytokines IL-2 and IFN-γ or T_H2-like cytokine IL-4. Mice given cytokine pDNA adjuvants produced more T_H1-like cytokines than did the controls (Fig 3). Mice given combined pIFN-γ and pIL-12 produced higher amounts of IFN-γ (194.64 pg/mL) than mice given pIFN-γ (100.14 pg/mL) or pIL-12 (111.87 pg/mL) alone. Control mice produced only 11.71 pg/mL IFN-γ (Fig 3, *A*). Mice vaccinated with pDNA cytokine(s) adjuvant produced more IL-2 than did control mice. IL-2 levels after vaccinations were as follows: pIFN-γ, 73.68 pg/mL; pIL-12, 128.43 pg/mL; pIFN-γ plus pIL-12, 202.57 pg/mL; and pcDNA3.1 (control), 51.88 pg/mL (Fig 3, *A*). In contrast, control mice, which were vaccinated only with the empty vector plasmid, produced more IL-4 (37.55 pg/mL) than the groups injected with the pDNA cytokine adjuvant, which produced 9.71 pg/mL (pIFN-γ plus pIL-12), 13.05 pg/mL (pIFN-γ) and 17.99 pg/mL (pIL-12) of IL-4 (Fig 3, *A*). No significant difference was observed in IL-4 production among the pIFN-γ, pIL-12, and pIFN-γ plus pIL-12 treatment groups.

To examine the dominant pattern of cytokine responses, IFN-γ:IL-4 and IL-2:IL-4 ratios were compared among different groups of mice (Fig 3, *B*). The IFN-γ:IL-4 ratios in the mice vaccinated with pIFN-γ, pIL-12, and pIFN-γ plus pIL-12 as adjuvants were 7.48, 6.33, and 20.86, respectively, whereas the empty pcDNA3.1-vaccinated control exhibited a ratio of only 0.31. The ratios of IL-2:IL-4 in pIFN-γ, IL-12, pIFN-γ plus IL-12, and pcDNA3.1 controls were 7.50, 7.32, 21.71, and 1.4, respectively. These results indicate that the net cytokine balance shifted in favor of the T_H1-like response in cytokine plasmid–vaccinated mice; however, this shift was greater in the group vaccinated with the adjuvant that was a combination of pIFN-γ and pIL-12 plasmid DNAs.

Cytokine genetic adjuvants prevent the development of airway hyperresponsiveness in allergen-sensitized and -challenged mice

Mice (n = 4) were vaccinated, sensitized, and challenged as described in connection with pulmonary function. The airways of control mice that received the empty vector as vaccine adjuvant were significantly more reactive to 50 mg/mL of methacholine than those of the mice that received pIFN-γ and/or pIL-12 (Fig 4). Mice vaccinated with pIFN-γ plus pIL-12 exhibited the least reactivity to the inhaled methacholine challenge in comparison with the pcDNA3.1 (*P* < .01), pIFN-γ, and pIL-12 groups (*P* < .05). These results suggest that the vaccination of mice with combined pIFN-γ and pIL-12 as an adjuvant significantly reduces airway reactivity.

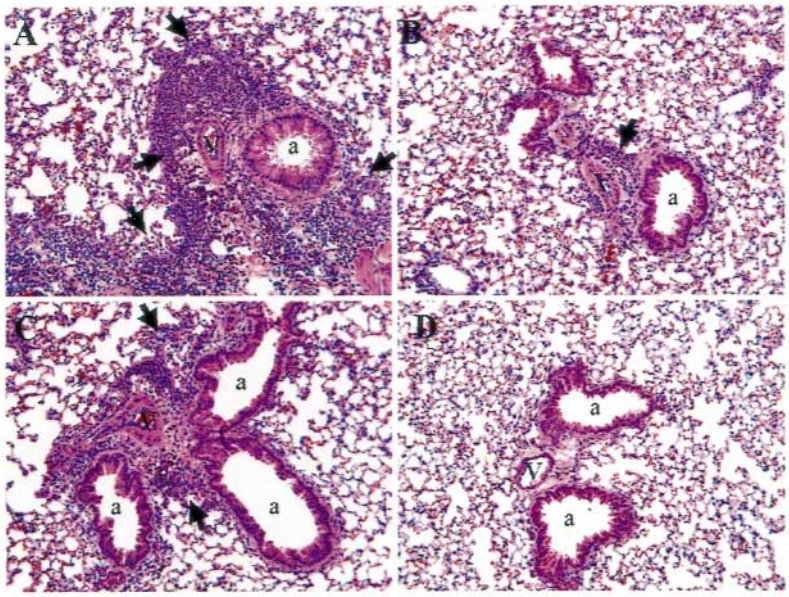


FIG 5. Assessment of the lung inflammation in KBG-sensitized and -challenged mice after cytokine DNA vaccination. Lung tissue was removed from the different groups of mice (n = 4) 24 hours after the last intranasal allergen challenge and was stained with hematoxylin and eosin. A representative photomicrograph from each group is shown. **A,** pcDNA3.1. **B,** pIFN-γ. **C,** pIL-12. **D,** pIFN-γ + pIL-12. *Arrows* indicate cellular infiltration. *a,* Airway; *v,* vessel.

TABLE I. Quantification of pulmonary inflammation in mice after allergen vaccination with various cytokine adjuvants

Pathology	pcDNA3.1	Adjuvants pIFN-γ	pIL-12	pIFN-γ + pIL-12
Epithelial damage	2.8 ± 0.12	2.23 ± 0.23*	2.3 ± 0.16*	1.36 ± 0.48*†‡
Interstitial-alveolar infiltrate	2.73 ± 0.20	2.4 ± 0.25*	2.56 ± 0.42	1.76 ± 0.36*†‡
Peribronchovascular infiltrate	2.83 ± 0.26	2.16 ± 0.23*	2.16 ± 0.32*	1.26 ± 0.20*†‡

Each value represents the mean ± SD of 5 fields from 6 individual lung sections from each mouse in a group (n = 4). Values were considered significant when the *P* value was less than .05 (*†‡). Statistical differences are indicated as follows: *in comparison with pcDNA3.1 control; †in comparison with pIFN-γ control; ‡in comparison with pIL-12 control.

Combination of IFN-γ and IL-12 plasmid DNAs prevents lung inflammation in the allergen-sensitized mice

Mice (n = 4) were vaccinated, sensitized, and challenged as described in connection with pulmonary function. Lung inflammation was examined 24 hours after the final allergen challenge. Representative pathologic features are shown in Fig 5. The group of mice receiving the combined constructs as an adjuvant (Fig 5, *D*) exhibited less epithelial damage and less infiltration of mononuclear cells and polymorphs in the interstitial and peribronchovascular region than was seen in the control group (Fig 5, *A*) and the other vaccinated groups (Fig 5, *B* and *C*). A semiquantitative analysis using a scoring system for inflammatory cells in the lung is shown in Table I. Groups of mice that received pIFN-γ, pIL-12, or a combination of the two exhibited reduced pulmonary inflammation in comparison with the empty vector (pcDNA3.1) control. The group of mice that received the combination of pIFN-γ and pIL-12 showed significantly less ($P < .05$) pulmonary inflammation than the pIFN-γ and pIL-12 groups. No statistically significant difference was found between the pIFN-γ and pIL-12 groups.

DISCUSSION

Previously, it was shown in a mouse model that effective parenteral vaccination of mice with grass allergens required subcutaneous injection of a high dose (250 μg to 1 mg per mouse) of allergens.[14] By corollary, an even higher dose of allergen mixture would be required to induce an effective immune deviation from an allergic response. The results of this study demonstrate that in a mouse, a substantially lower dosage (50 μg) of allergens, when administered along with a pDNA cytokine(s) adjuvant, can induce effective immune deviation and a protective airway response in comparison with allergens alone. Furthermore, a comparison of pIFN-γ and/or pIL-12 as adjuvants indicates that the combination of pIFN-γ and pIL-12 is a more effective adjuvant than either of these plasmids alone. Although we did not examine the effect of these adjuvants in mice with an established allergic response, a number of studies have reported the effective-

ness of IFN-γ and of IL-12 in modulating established allergic responses.[11,12,17,18] The results of this study, therefore, have significant implications for "time-honored" AIT, which might be rendered more safe and effective by including these cytokine plasmids as adjuvants.

A major finding of this study is that a vaccine adjuvant comprising both pIFN-γ and pIL-12 inhibited total IgE levels in mice with a concomitant increase of allergen-specific IgG2a antibody production. The level of IgE synthesis in mice vaccinated with a cocktail adjuvant was significantly ($P < .001$) more suppressed than that of the levels obtained by vaccination with pIFN-γ or pIL-12 alone as an adjuvant. Both systemic and local IFN-γ gene delivery was earlier shown to decrease serum IgE levels,[17] whereas the role of the IL-12 protein as an adjuvant on serum IgE levels has been controversial.[19-22] The precise mechanism underlying a higher reduction in IgE after the use of both pIFN-γ and pIL-12 is unclear. The results of this study indicate that an adjuvant comprising the IFN-γ and IL-12 plasmids enhances the production of IFN-γ. This finding is in agreement with a report that mice vaccinated with IL-12 exhibited enhanced local IFN-γ production.[23]

The deviation of the cytokine production profile from T_H2-like to T_H1-like is a significant marker of the effectiveness of a vaccine. Analysis of the cytokine pattern of mice after vaccination with allergens and different plasmid adjuvants revealed that the group of mice receiving pIFN-γ plus pIL-12 as the adjuvant had a more profound T_H1-like effect—ie, it produced higher levels of IFN-γ and IL-2 than either of the cytokine plasmids alone. Analysis of the IL-4 expression from the splenocytes of different groups of mice revealed no significant difference among the groups of mice vaccinated with pIFN-γ, pIL-12, or a combination of the two. A predominance of the T_H1-like response seen with cytokine adjuvants is consistent with the finding of Hogan et al,[12] who showed, using the vaccinia virus as a gene delivery vehicle, that IL-12 gene transfer to murine lung inhibited T_H2 cytokine production, which was mediated through the production of IFN-γ. Because allergic individuals produce limited amounts of IFN-γ, an adjuvant providing the expression of both IFN-γ and IL-12 induces a more effective T_H1-like response.

In patients with allergic asthma, it was shown that airway hyperresponsiveness correlates with the severity of disease and is therefore a major clinical feature of allergic asthma. A direct instillation into the respiratory tract or an intraperitoneal administration of either rIL-12 or rIFN-γ inhibits allergic airway inflammation and in some cases suppresses airway hyperresponsiveness.[24,25] However, these protocols require a regular or daily dosing over periods ranging from 5 to 13 days to produce significant protective effects. Furthermore, gene transfer studies using IFN-γ and IL-12 have been shown to suppress airway hyperreactivity.[11,12] The results of this investigation indicate that allergen vaccine formulations that include allergens and pIFN-γ, pIL-12, or a combination of both cytokines as an adjuvant significantly decrease airway hyperresponsiveness and that a combination of pIFN-γ and pIL-12 induces the highest reduction in airway responsiveness. There appears to be a synergistic effect when both cytokine plasmids (pIFN-γ and pIL-12) are administered to the mice. A parenteral administration of only 50 μg of allergen-induced massive cellular infiltration and airway obstruction in the lung tissue of mice in the present study. However, allergens given with pIFN-γ, pIL-12, or a combination as an adjuvant limited not only the cellular infiltration but also epithelial cell damage. Consistent with previous observations, either pIFN-γ or pIL-12 as an adjuvant decreased eosinophilic cellular infiltration,[11,12] whereas treatment with a combination of pIFN-γ and pIL-12 as an adjuvant was most effective.

The mechanism underlying the effectiveness of pIFN-γ and pIL-12 as an adjuvant is unknown. One possibility is that CpG sequences present in the backbone plasmid, pcDNA3.1, nonspecifically contribute to the endogenous IL-12/IFN-γ production.[4] However, this is unlikely, because all groups received an equal dose of plasmid and mice receiving both pIFN-γ and pIL-12 received an adjusted dose (50 μg each, for a total of 100 μg). The empty vector, which also contained CpGs, had no cytokine-inducing or immunomodulatory effect. Furthermore, the higher effectiveness of combined pIFN-γ and pIL-12 in comparison with either of the plasmids alone (100 μg) might be attributed to the CpG motifs in the coding sequences; however, the cDNA inserts are mammalian sequences, which have a low frequency of CpG dinucleotides, are mostly methylated, and do not have immunostimulatory activity.[26] Nonetheless, a search for a canonical hexamer purine-purine-CG-pyrimidine-pyrimidine as a possible core CpG motif led to the identification of GACGTT, GGCGTC, GGCGTT sequences in IL-12, and AACGCT, AGCGCT sequences in IFN-γ. However, whether these sequences in the mouse cDNA are capable of binding to the mouse Toll-like receptor 9 and cause immune stimulation, like the CpG motifs present in the bacterial DNA, remains to be investigated.[26]

Alternatively, the synergy between pIFN-γ and pIL-12 on the induction of a T_H1-dominant state and the inhibition of airway inflammation is inherent in their cellular mechanism of action. First, although IL-12 is the primary determinant of the T_H1 differentiation, IFN-γ endogenously synthesized during the priming of naive CD4-positive T cells both accelerates and enhances the T_H1 differentiating effects of IL-12.[27,28] Thus, IFN-γ presumably synergizes with T-cell receptor engagement and induces the expression of functional IL-12 receptors on naive T cells. This idea is consistent with the report that the presence of IFN-γ and IFN-α in cultures enhanced IL-12Rβ2 expression on CD4-positive and CD8-positive T cells and that the enhancing effect of IFN-γ was independent of endogenous IL-12 or IFN-α.[29] Second, the highest amount of IL-2 produced by a combined vaccination with pIFN-γ and pIL-12 might synergize with IFN-γ effects and contribute to a more effective T_H1-like response. IL-2 regulates expression of the IL-12β2

receptor on natural killer cells.[29] It is to be noted however, that these 2 possibilities are not mutually exclusive. Irrespective of the mechanism involved, the results lead to the conclusion that in a mouse model of grass allergy, the administration of cytokine plasmids encoding IFN-γ and IL-12 as an adjuvant enhances the therapeutic effectiveness of grass allergen vaccines.

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