

Vaccination with Allergen-IL-18 Fusion DNA Protects Against, and Reverses Established, Airway Hyperreactivity in a Murine Asthma Model¹

Holden T. Maecker,^{2*} Gesine Hansen,^{2†} David M. Walter,^{2†} Rosemarie H. DeKruyff,[†] Shoshana Levy,^{*} and Dale T. Umetsu^{3†}

Vaccination with naked DNA encoding a specific allergen has been shown previously to prevent, but not reverse, the development of allergen-induced airway hyperresponsiveness (AHR). To enhance the effectiveness of DNA vaccine therapies and make possible the treatment of established AHR, we developed a DNA vaccination plasmid containing OVA cDNA fused to IL-18 cDNA. Vaccination of naive mice either with this fusion DNA construct or with an OVA cDNA-containing plasmid protected the mice from the subsequent induction of AHR. Protection from AHR correlated with increased IFN- γ production and reduced OVA-specific IgE production. The protection appeared to be mediated by IFN- γ and CD8⁺ cells because treatment of mice with neutralizing anti-IFN- γ mAb or with depleting anti-CD8 mAb abolished the protective effect. Moreover, vaccination of mice with preexisting AHR with the OVA-IL-18 fusion DNA, but not with the OVA cDNA plasmid, reversed established AHR, reduced allergen-specific IL-4, and increased allergen-specific IFN- γ production. Thus, combining IL-18 cDNA with OVA cDNA resulted in a vaccine construct that protected against the development of AHR, and that was unique among cDNA constructs in its capacity to reverse established AHR. *The Journal of Immunology*, 2001, 166: 959–965.

The prevalence of allergic asthma has dramatically increased over the last two decades and is a major public health concern (1, 2). Asthma is characterized by airway hyperresponsiveness (AHR)⁴ to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, excessive mucus production, and high serum IgE levels. The pathology in asthma results from excessive production of IL-4, IL-5, and IL-13 by CD4⁺ Th2 cells (3, 4). Current treatments for asthma are not satisfactory, and disease prevention is not possible. Given the high prevalence of this disease, improved and more effective therapeutic strategies are needed.

One therapeutic approach for asthma might be to use DNA-based immunization to manipulate the immune system, and to alter the underlying Th2-biased allergic response in an allergen-specific

manner. Vaccination with allergen in the form of naked plasmid cDNA stimulates allergen-specific immune responses with a Th1 bias and amplifies the expansion of CD4⁺ T cells producing IFN- γ and of cytotoxic CD8⁺ T cells (5–9). The key feature of this strategy is that injection of plasmid DNA encoding a specific Ag produces an allergen-specific protective immune response that should be reinforced by natural exposure to the allergen, thus conferring long-lasting protection (10, 11). Previous studies with DNA immunization strategies demonstrated its success in preventing the development of Ag-specific IgE synthesis and AHR (6, 12, 13). However, although the genetic vaccination approach has succeeded in preventing allergic diseases and has been effective in models of infectious disease (14–16), cancer (17–19), and autoimmune disease (20), the efficacy of DNA vaccination has varied widely, and successful reversal of ongoing AHR with DNA vaccination has not been reported. Thus, improvement of gene vaccination methodologies is required for successful clinical application of DNA vaccination to symptomatic patients with allergic asthma.

To enhance the effectiveness of DNA vaccination and potentially treat patients with ongoing AHR, we constructed a DNA vaccination plasmid containing cDNA for a prototypic allergen, OVA, fused to the cDNA of a potent immune modulating cytokine, IL-18. This approach is based on the fact that IL-18, a product of activated macrophages and Kupffer cells (21–25), is very powerful in driving the production of Th1 cytokine synthesis in naive and memory T cells (26, 27). Furthermore, the rationale for the approach of fusing IL-18 cDNA with OVA cDNA was based on our previous findings with protein-based vaccines (28). In those experiments, an OVA-IL-12 fusion protein increased the immunogenicity of OVA, focused the effects of IL-12 on Ag-specific immune cells, and minimized the Ag-nonspecific effects of IL-12. In this study, we examined the efficacy of the OVA-IL-18 DNA fusion construct vector in a murine model of asthma and compared its efficacy with that of OVA DNA, IL-18 DNA, or a mixture of

*Division of Oncology, Department of Medicine, and the [†]Division of Immunology and Transplantation Biology, Department of Pediatrics, Stanford University Medical Center, Stanford, CA 94305

Received for publication January 10, 2000. Accepted for publication October 25, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Grants AI37219 and AI45900 from the U.S. Public Health Service, National Institutes of Health, and gift funds from Howard B. and Susan F. Sosin. G.H. was a fellow of the Deutsche Forschungsgemeinschaft (Ha2799/1-1). D.M.W. was recipient of a U.S. Public Health Service, National Research Training Award AI-07290-14, and is a current fellow of the American Heart Association, Western States Affiliate (9920076Y).

² H.T.M., G.H., and D.M.W. contributed equally to this work. Current addresses: (H.T.M.) BD Biosciences, 2350 Qume Drive, San Jose, CA 95131; and (G.H.) Martin-Luther University, Department of Pediatrics and Biocenter, 06120, Halle/Saale, Germany.

³ Address correspondence and reprint requests to Dr. Dale T. Umetsu, Department of Pediatrics/Immunology, Stanford University Medical Center, Stanford, CA 94305-5208. E-mail address: umetsu@stanford.edu

⁴ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; LN, lymph node(s); i.n., intranasal(ly); KLH, keyhole limpet hemocyanin; alum, aluminum potassium sulfate.

OVA DNA and IL-18 DNA on separate plasmids. We found that the OVA-IL-18 cDNA construct was more potent in protecting against the development of AHR than the other constructs, and that the OVA-IL-18 cDNA construct was unique in its capacity among cDNA constructs to effectively reverse established AHR. These studies suggest that modification of vaccination plasmids with IL-18 cDNA can greatly enhance the immunogenicity of Ag cDNA constructs, and generate constructs that may be clinically effective in treating patients with established allergic pulmonary disease.

Materials and Methods

Animals

BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were used between 6 and 10 wk of age and were age and sex matched within each experiment. The Stanford University Committee on Animal Welfare approved all animal protocols.

mAbs and reagents

mAbs were purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography. We used the following hybridomas: R46A2 (anti-IFN- γ ; obtained from American Type Culture Collection, Manassas, VA); XMG1.2 (anti-IFN- γ ; provided by Dr. T. Mosmann, University of Alberta, Edmonton, Canada); BVD4-1D11 (anti-IL-4) and BVD6-24G2 (anti-IL-4; obtained from Dr. M. Howard, DNAX Research Institute, Palo Alto, CA); 53.6.7 (anti-CD8; provided by Dr. Irving Weissman, Stanford University); and EM95 (rat anti-mouse IgE; generated by Z. Eshhar, Weizman Institute, Rehovot, Israel and provided by Dr. R. Coffman, DNAX Research Institute). Anti-OVA mAbs and biotinylated anti-OVA mAb were produced as described previously (28).

DNA constructs

A series of plasmids expressing OVA fused to various cytokines was produced in our laboratory and has been described previously (9). One of these plasmids, expressing OVA-IL-4, was digested with *Xho*I and *Bam*HI to excise the IL-4 portion of the insert. The remainder of the plasmid was ligated to a similarly digested PCR product encoding mature murine IL-18 (Fig. 1A). This sequence was isolated by PCR amplification of cDNA produced from RNA of C3H mouse splenocytes activated with Con A. The forward PCR primer, which incorporated an *Xho*I site, was 5'-CATCGC GAGCCCAACTTTGGCCGACTTCAC-3'. The reverse PCR primer, which incorporated a *Bam*HI site, a stop codon, and a hexahistidine tag, was 5'-GTTAGATCTCTAATGGTGATGATGGTGATGACTTTGATGT AAGTTAGT-3'. The ligated plasmid (Fig. 1A) was electroporated into *Escherichia coli* and purified from a large-scale culture by alkaline lysis and CsCl density gradient centrifugation (29). This preparation was then sequenced to verify the correct insertion and correct sequence of the IL-18 fusion construct. Control plasmids, expressing either OVA alone or an irrelevant sequence (single-chain Fv), have also been described previously (9). Finally, a plasmid expressing IL-18 alone was produced for this study. The IL-18 sequence was moved into another vector, pTCAE 5.3, by PCR amplification that added restriction sites *Dra*III (5') and *Hpa*I (3'). From this vector, the insert was excised with *Dra*III and *Hpa*I digestion. An OVA-IL-6 expressing plasmid, pOVA-IL-6 (9), was digested with *Dra*III and *Bam*HI to remove the entire OVA-IL-6 insert. The IL-18 insert described above was then ligated into this vector backbone via *Dra*III; the remaining sticky ends were blunted by use of T4 polymerase, and joined by blunt-end ligation (29). The final three plasmids (OVA, IL-18, and OVA-IL-18) were fully sequenced and were found not to contain amino-acid altering mutations.

Immunization protocols

Prevention of AHR (Fig. 1D, top). On day 0, BALB/c mice were injected i.m. in the quadriceps muscles with 100 μ g of each plasmid DNA in a final volume of 100 μ l 0.9% NaCl, divided bilaterally. On day 17, the mice were boosted i.m. with the same amount of plasmid DNA. The mice were then sensitized to OVA protein using an established protocol for the induction of AHR in BALB/c mice (30). OVA (50 μ g) adsorbed to 2 mg aluminum potassium sulfate (alum) was administered i.p. on days 24 and 38, followed by 50 μ g OVA in 50 μ l PBS given intranasally (i.n.) on days 38, 49, 50, and 51. Control mice received i.p. injections of alum alone and i.n. PBS. One day after the last i.n. challenge (day 52), AHR was measured in conscious mice after inhalation of increasing concentrations of methacholine

(see below). Within 5 days of the last challenge, blood was taken, mice were sacrificed, lungs were removed and fixed, and splenocytes were isolated for in vitro culture.

Ag specificity test. In experiments to determine whether the effects of the different DNA constructs on the immune response of BALB/c mice were Ag specific, mice were first injected with the different OVA DNA constructs i.m. (see above). One week later, the mice were immunized in the footpads either with the relevant Ag, OVA (200 μ g/mouse), or an irrelevant Ag, keyhole limpet hemocyanin (KLH, 100 μ g/mouse), each emulsified in IFA. After 7 days the mice were sacrificed, and lymphocytes were isolated from the draining lymph nodes (LN) for in vitro culture.

Treatment of mice with anti-cytokine and depletion mAb. BALB/c mice were injected i.p. with 1 mg of mAb XMG1.2 (for IFN- γ depletion), 200 μ g of mAb 53.6.7 (for CD8 depletion), or 1 mg of mAb LC4 (control mAb) every other day for 6 days, then every fifth day thereafter, starting 5 days before immunization with DNA. Ab injection was continued until the immunization protocol was finished. Blood was collected on the day of sacrifice and stained with anti-mouse CD8-PE and anti-mouse CD4-FITC mAb (PharMingen, San Diego, CA). FACS analysis revealed a $\geq 90\%$ depletion of CD8⁺ cells in anti-CD8 mAb-treated mice in each of two replicate experiments.

Reversal of established AHR (Fig. 1D, bottom). To investigate whether DNA immunization can reverse established AHR rather than inhibit the development of AHR, BALB/c mice were first sensitized with OVA before vaccination with the DNA plasmids. OVA (50 μ g) adsorbed to alum was administered i.p. once on day 0. OVA (50 μ g) in 50 μ l PBS was administered i.n. on days 8 and 9. On days 10 and 25 the different DNA constructs were injected i.m. in the quadriceps muscles (100 μ g in 100 μ l 0.9% NaCl). On day 39 the mice were boosted again with OVA i.n., and AHR was measured 1 day later (day 40). Mice were sacrificed, and bronchial LN cells were isolated for in vitro culture within 5 days of the last OVA challenge.

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211; Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by P_{enh} using the following formula: $P_{\text{enh}} = ((Te/RT - 1) \times (PEF/PIF))$, where P_{enh} = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s) (31). Enhanced pause (P_{enh}), minute volume, tidal volume, and breathing frequency were obtained from chamber pressure, measured with a transducer (model TRD5100) connected to preamplifier modules (model MAX2270), and analyzed by system XA software (model SFT 1810). Measurements of methacholine responsiveness were obtained by exposing mice for 2 min to aerosolized 0.9% NaCl produced by a sonicator (Portable Ultrasonic, 5500D; DeVilbiss Health Care, Somerset, PA), followed by incremental doses (2.5–20 mg/ml) of aerosolized methacholine. Results were expressed for each methacholine concentration as the percentage of baseline P_{enh} values after 0.9% NaCl exposure.

OVA-specific IgE assay

Mice were bled at the time of sacrifice, and OVA-specific IgE was determined using a modified Ag-specific ELISA. Plates were coated overnight with rat anti-mouse IgE mAb EM95 (5.0 μ g/ml). After washing and blocking, samples were applied and incubated overnight. Plates were again washed, and biotinylated OVA (10 μ g/ml) was added. Two hours later, plates were washed and HRP-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL) was added. Plates were developed with o-phenylenediamine substrate, and the OD was determined at 492 nm. Serum from mice hyperimmunized with OVA in alum was standardized for IgE levels against an anti-OVA IgE mAb provided by E. Gelfand (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). This serum was used as a standard in the OVA-specific IgE ELISA.

Restimulation of spleen and LN cells in vitro

Spleens or bronchial LN were removed, depleted of resting B cells by adherence to goat anti-mouse Ig-coated plates (32), and 4×10^5 cells were restimulated in vitro with OVA (100 μ g/ml) or KLH (10 μ g/ml). Cells were cultured in 96-well microtiter plates in 150 μ l medium. Supernatants were harvested after 4 days for determination of IL-4 and IFN- γ levels. Cytokine content in each sample was measured in triplicate by ELISA.

IL-18 assays

IL-18 activity was examined by ELISA and by bioassay. Plasmids expressing IL-18, OVA-IL-18 or, as a control, OVA-IL-4, were transfected into COS-7 cells using DEAE-dextran in a standard method (29). Supernatants from the transfected cells were harvested after 3 days and stored at 4°C. IL-18 ELISA was performed using reagents and protocols from PharMingen. To determine IL-18 bioactivity of the OVA-IL-18 fusion construct, the recombinant protein was tested for induction of IFN- γ production by a murine Th1 cell line, DOH2. The murine Th1 cell line, DOH2, was produced and maintained as described previously (33). DOH2 cells were resuspended at 5×10^5 per ml in DMEM with 10% FBS. Cell suspension (100 μ l per well of a microtiter plate) was plated, along with 100 μ l of media or COS-7 supernatant at dilutions of 1:2, 1:4, 1:8, or 1:16. The cells were incubated at 37°C for 48 h, then supernatants were harvested from each well and tested for IFN- γ production by ELISA as described (9).

Cytokine ELISA

ELISA for IL-4 and IFN- γ were performed as previously described (32). The mAb pairs used were as follows, listed by capture/biotinylated detection mAb: IFN- γ , R4-6A2/XMG1.2; IL-4, 11B11/BVD6-24G2. A standard curve using recombinant cytokine in 1:2 dilutions from 20 to 0.156 ng/ml for IFN- γ , or from 500 to 7.5 pg/ml for IL-4, was used for quantitation.

Collection of bronchoalveolar lavage (BAL) fluid

Animals were sacrificed by CO₂ asphyxiation. The trachea was cannulated, and the lungs were lavaged four times with 300 μ l of 1% BSA in 1 $\times$ PBS. Cells in the lavage fluid were then counted using a hemocytometer, and BAL cell differentials were determined on slide preparations stained with Hansel stain (Lide Laboratories, Florissant, MO). At least 200 cells were differentiated by light microscopy based on conventional morphogenic criteria.

Results

IL-18 bioactivity of fusion construct

To test the bioactivity of the OVA-IL-18 and the IL-18 plasmids, COS-7 cells were transiently transfected with plasmid DNA, and the cells were cultured for 4 d. We then examined the supernatants from these cell cultures for IL-18 bioactivity, via the ability to induce IFN- γ production in a Th1 cell line, and for IL-18 protein content by ELISA. Fig. 1B shows that supernatant from OVA-IL-18-transfected COS-7 cells induced IFN- γ production from the established Th1 cell line, DOH2, whereas medium or supernatant from control OVA-IL-4-transfected cells did not, indicating that protein produced from the IL-18 plasmid had biological activity. The COS-7 supernatants generated with both the IL-18 and OVA-IL-18 plasmids had comparable IL-18 activity (ranges of 0.3–6.1 ng/ml in independent transfection experiments). In addition, ELISA of the COS-7 supernatants indicated that both the IL-18 and the OVA-IL-18 constructs induced the production of IL-18 protein (Fig. 1C).

Inhibition of AHR by vaccination with different DNA vectors

Having established that the IL-18 and OVA-IL-18 fusion constructs had significant IL-18 bioactivity, the plasmids were next tested *in vivo* for their ability to inhibit AHR in a murine asthma model. BALB/c mice were vaccinated i.m. with irrelevant DNA, OVA DNA, IL-18 DNA, a mixture of OVA DNA and IL-18 DNA, or the OVA-IL-18 DNA fusion construct. The mice were then sensitized for AHR with i.p. and i.n. administrations of OVA (Fig. 1D, prevention model). Fig. 2A demonstrates that sensitization of mice with OVA resulted in the development of significant AHR when the mice were challenged with methacholine. Vaccination of mice with the OVA-IL-18 DNA fusion construct dramatically inhibited the development of AHR. Vaccination with OVA DNA, OVA DNA + IL-18 DNA, or IL-18 DNA also inhibited development of AHR, although to a lesser extent. Injection of irrelevant DNA had no effect on the OVA-induced AHR.

Effects of DNA vaccination on cytokine production

A known property of both IL-18 and DNA vaccination in general is the ability to induce IFN- γ production *in vivo*. To determine whether the reduced AHR in mice vaccinated with the OVA-IL-18 plasmid correlated with alteration of cytokine profiles in CD4⁺ T cells, mice were sacrificed after measurement of airway reactivity. Spleen cells were removed and stimulated with OVA *in vitro*. Fig. 2B shows that DNA vaccination significantly increased OVA-specific IFN- γ production in OVA-immunized mice. The strongest IFN- γ increase was induced by the OVA-IL-18 DNA fusion construct. The increase of IFN- γ production was comparable in the

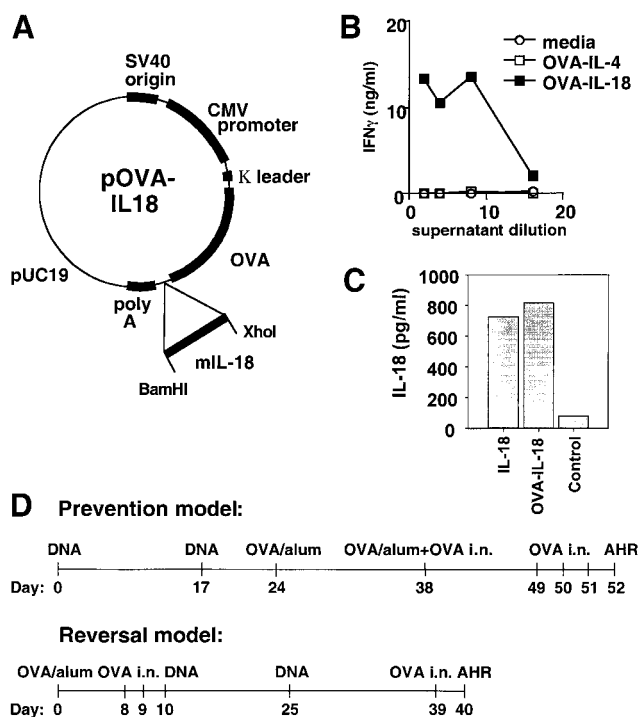


FIGURE 1. A, Immunization vector expressing an OVA-IL-18 fusion construct. See *Materials and Methods* for construction details. B, Bioactivity of the *in vitro* secreted OVA-IL-18 fusion protein. COS-7 cells were transfected with plasmids expressing OVA-IL-4 or OVA-IL-18 fusion constructs, and supernatants were tested for the ability to induce IFN- γ secretion from a cloned Th1 cell line (DOH2). Only OVA-IL-18 containing supernatant generated IFN- γ production from DOH2 cells, in a dose-dependent manner. C, COS-7 supernatants generated with the IL-18 and the OVA-IL-18 plasmids contain IL-18 protein as detected by an IL-18 ELISA. D, Immunization scheme for prevention of AHR by DNA vaccination (*top*) or reversal of established AHR by DNA vaccination (*bottom*). See *Materials and Methods* for details.

groups that received either OVA DNA or IL-18 DNA alone, and was slightly higher after injection of the mixture of OVA DNA and IL-18 DNA. Although vaccination with OVA DNA, IL-18 DNA, or a mixture of OVA DNA and IL-18 DNA also induced IL-4 production, the OVA-IL-18 DNA fusion construct did not significantly reduce IL-4 levels in splenic T cells from OVA-immunized mice (data not shown). (Bronchial LN T cells were examined for cytokine production in subsequent experiments, see Fig. 5C.) Irrelevant DNA had no significant effect on IFN- γ or IL-4 production.

Inhibition of IgE synthesis by DNA vaccination

We also analyzed the levels of anti-OVA IgE Ab responses in serum collected from these mice. OVA-specific IgE was very high in OVA-immunized BALB/c mice (Fig. 2C). Vaccination with the different DNA vectors before immunization with OVA significantly reduced the level of OVA-specific IgE. The inhibitory effect on IgE production was strongest with the OVA-IL-18 fusion construct and did not differ significantly between OVA DNA, IL-18 DNA, or the mixture of OVA-DNA and IL-18 DNA. In contrast, irrelevant DNA had little effect on IgE production.

Specificity of DNA vaccination

To test whether the effects of immunization with OVA-IL-18 DNA were Ag specific, we boosted DNA-vaccinated mice after 1 wk with either the relevant protein (OVA) or with an irrelevant protein, KLH. After 7 days, spleens were removed and splenocytes were cultured with the Ag used for boosting. Fig. 3, *left*, shows that in OVA protein-boosted mice, the increase in IFN- γ was most notable in the group that received the OVA-IL-18 DNA fusion construct. In contrast, in mice boosted with the irrelevant Ag KLH, IFN- γ production was not increased by vaccination with OVA-IL-18 DNA, and was similar in all groups receiving the various OVA DNA constructs (Fig. 3, *right*). These results indicated that vaccination with the OVA-IL-18

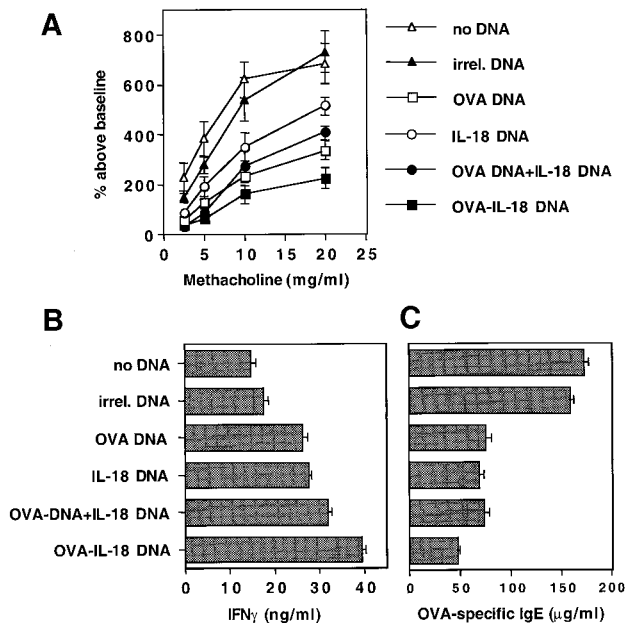


FIGURE 2. A, DNA vaccination prevents induction of AHR in BALB/c mice. Mice were vaccinated twice with the indicated DNA plasmids, then sensitized to OVA by i.p. and i.n. injection (see *Materials and Methods*). One day after the last OVA challenge, AHR was measured in response to increasing concentrations of methacholine in conscious mice placed in a whole body plethysmograph. Mean P_{enh} values were calculated, and data was expressed as a percent above baseline (NaCl-induced AHR). Error bars represent SEM of five animals per group. B, Mice were sacrificed within 5 days of the last challenge, and spleen cells were removed and cultured with 100 μ g/ml OVA. IFN- γ levels in the supernatants were determined by ELISA. C, OVA-specific IgE production from sera of the mice was collected within 5 days of the last challenge. Error bars represent SD of triplicate ELISA wells. All results are representative of three different experiments.

DNA construct greatly enhanced IFN- γ production, but the effect on IFN- γ production was confined to the OVA-specific response.

Inhibition of AHR depends upon CD8 $^{+}$ T cells and IFN- γ

To investigate the mechanisms by which vaccination with OVA-IL-18 affected OVA-specific responses, we administered blocking Ab to IFN- γ or Ab to CD8 $^{+}$ T cells during the immunization protocol. As expected, mice that received irrelevant DNA and control mAb developed strong AHR, which was significantly reduced in mice vaccinated with OVA-IL-18 DNA in the presence of control mAb (Fig. 4). Treatment with anti-CD8 mAb largely restored AHR in the OVA-IL-18 DNA-immunized animals. Anti-IFN- γ mAb also restored AHR, although to a lesser extent than that seen with CD8 depletion. Treatment of mice sensitized and challenged with OVA with either the anti-IFN- γ or the anti-CD8 mAb had no significant effect on AHR (data not shown). Thus, inhibition of AHR by OVA-IL-18 DNA was dependent upon both IFN- γ production and the presence of CD8 $^{+}$ cells.

Reversal of AHR by DNA vaccination

To determine whether DNA vaccination could reverse established AHR in addition to inhibiting the development of AHR, mice were first sensitized with OVA protein by administering OVA i.p. in alum, and OVA i.n. to establish AHR in these mice. The mice were then vaccinated either with OVA-IL-18 DNA, OVA DNA, IL-18 DNA, OVA DNA + IL-18 DNA, or irrelevant DNA (as indicated in Fig. 5A), and AHR was measured after a final i.n. OVA boost. Mice that received irrelevant DNA developed strong AHR. In contrast, vaccination of the mice with the OVA-IL-18 DNA construct greatly reduced AHR and was significantly more effective than OVA DNA + IL-18 DNA, IL-18 DNA alone, or OVA DNA alone. The reduction of AHR was consistent with the examination of BAL fluid, in which OVA-IL-18 DNA, but not OVA DNA, greatly reduced the percentage of eosinophils in BAL fluid (Fig. 5B). Eosinophils were still present in the

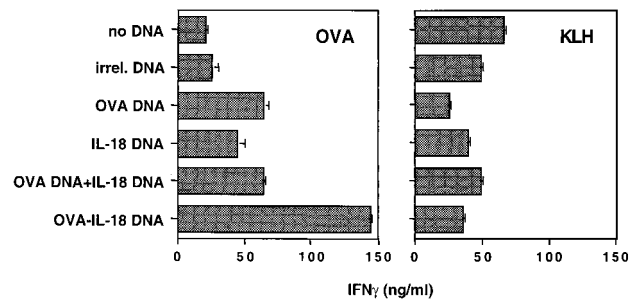


FIGURE 3. Ag specificity of IFN- γ production in response to OVA-IL-18 DNA vaccination. Mice were vaccinated twice with the indicated DNA plasmid, then challenged with either OVA protein or KLH in IFA. After 7 days, mice were sacrificed and splenocytes were restimulated in vitro with the same Ag (OVA or KLH). IFN- γ levels in the culture supernatants were measured by ELISA. The increased IFN- γ production by OVA-IL-18 DNA-immunized mice is specific for OVA. Error bars represent SD of triplicate ELISA wells.

lungs of mice treated with OVA-IL-18 DNA, perhaps consistent with the fact that some degree of AHR was present in these mice, and with the observation that IL-18 can recruit eosinophils into the airways (34). Nevertheless, these data demonstrate that 1) OVA-IL-18 cDNA but not OVA cDNA reverses ongoing AHR in previously sensitized mice; and 2) the activity of OVA-IL-18 DNA is clearly superior to that of OVA DNA in such sensitized animals.

IL-4 and IFN- γ measurements in OVA-immunized mice before and after DNA vaccination

Mice, vaccinated with DNA constructs after the establishment of OVA-induced AHR, were sacrificed and analyzed for cytokine production. Vaccination with OVA-IL-18 DNA resulted in a dramatic increase of IFN- γ production in bronchial LN cells as compared with that of animals receiving irrelevant DNA or OVA DNA alone (Fig. 5C). Vaccination with OVA-IL-18 DNA also reduced OVA-specific IL-4 production compared with the other DNA constructs (Fig. 5C). In addition, the OVA-IL-18 plasmid was much more effective than the OVA plasmid in reducing OVA-specific IgE production (OVA plasmid-treated group, 5690 ± 800 ; OVA-IL-18 plasmid treated group, 2968 ± 81 ng/ml). These experiments demonstrated that OVA-IL-18 DNA, but not OVA DNA, could boost OVA-specific IFN- γ production and reduce IL-4 and IgE production, even when given in the context of ongoing AHR.

Discussion

In this study, we demonstrated that an OVA-IL-18 fusion DNA construct was highly effective in preventing and reversing allergen-induced AHR in a murine asthma model. Previous studies have demonstrated the usefulness of allergen DNA immunization in the prevention of allergic diseases and AHR (6, 12, 13, 35, 36), but allergen DNA vaccination has not been previously reported to successfully reverse ongoing AHR, a cardinal feature of asthma. We now describe an OVA-IL-18 DNA construct that effectively corrected established AHR in an allergen-specific fashion when administered only twice. Although both the OVA-IL-18 and the OVA DNA constructs, when administered to naive mice, prevented the subsequent induction of AHR and reduced allergen-specific IgE production, the OVA-IL-18 DNA was unique among the cDNA constructs in its capacity to reverse established AHR. The protective effects of OVA-IL-18 appeared to be mediated by IFN- γ and CD8 cells, and because the OVA plasmid was not capable of reversing established AHR, we conclude that the addition of IL-18 as a fusion construct greatly enhanced the immunogenicity and effectiveness of plasmid vaccination. These results indicate that the addition of IL-18 to allergen DNA constructs may substantially improve the immunogenicity and efficacy of allergen cDNA vaccines, and suggest that vaccination with allergen-IL-18

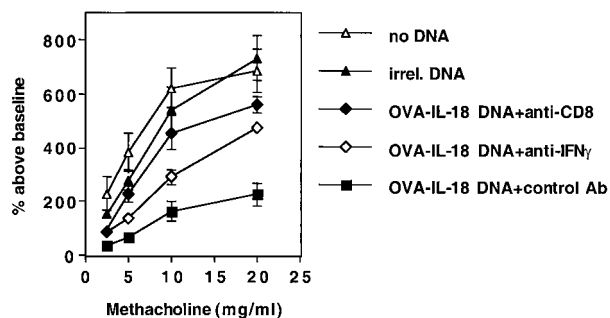


FIGURE 4. Inhibition of AHR development by OVA-IL-18 DNA vaccination depends upon CD8⁺ cells and IFN- γ . Mice were immunized with the indicated plasmid DNA and sensitized to OVA as in Fig. 2A. To determine mechanism of protection, mice were injected repeatedly with mAb 53.6.7 (for CD8 depletion), XMG1.2 (for IFN- γ blocking), or LC4 (control mAb). One day after the last i.n. challenge with OVA, AHR was measured as in Fig. 2A. Data are expressed as mean percent above baseline $\pm$ SEM of five mice. Treatment with mAb to either CD8 or IFN- γ partially reversed the DNA-induced protection from AHR.

cDNA may be clinically effective in the treatment of patients with ongoing chronic allergic asthma.

The potent inhibitory effects of OVA-IL-18 DNA vaccination on AHR and IgE production was dependent on the fusion of the cytokine and allergen. Thus, vaccination with the OVA plasmid alone or with the IL-18 plasmid alone was less effective than the OVA-IL-18 fusion plasmid in inducing IFN- γ production, reducing IgE production, and preventing the development of AHR. In addition, codelivery of nonfused OVA DNA together with IL-18 DNA was much less effective compared with the fusion construct vector in reducing IgE production and preventing the development of AHR. This indicated that the presence of the IL-18 cDNA fused to the OVA cDNA was crucial for protection in this model, and was entirely consistent with our previous findings with OVA and IL-12 fusion proteins (28). IL-12, like IL-18, potently induces IFN- γ production and enhances Th1-biased immune responses, but IL-12 has significant Ag-nonspecific effects when administered *in vivo*. For example, *in vivo* administration of IL-12 has been associated in clinical trials with substantial morbidity and mortality (37), has been ineffective in suppressing Th2 recall responses (38, 39), and, when administered in high doses to mice, paradoxically enhances IgE synthesis (40). In contrast, we previously demonstrated that administration of the IL-12 when fused to OVA protein was associated with minimal Ag-nonspecific IL-12 effects and with maximized induction of IFN- γ production in an Ag-specific fashion. In addition, the fusion of cytokine and Ag has been shown to be effective in other models, for example, with idiotype Ag of B cell lymphoma conjugated to GM-CSF, which enhanced the induction of protective immunity against a subsequent lethal tumor challenge (18, 19).

Our current studies suggest that the deleterious effects of IL-18 may be similarly minimized by fusion of IL-18 with OVA. Administration of high doses of IL-18 protein alone *in vivo* (7–20 μ g/mouse, i.p.) appears to accentuate the undesirable effects of the cytokine, which include paradoxical increase in the recruitment of eosinophils into the airways (34), and enhanced allergic sensitization and IgE production (41). In contrast, we showed that vaccination with the fusion OVA-IL-18 cDNA construct, which more effectively reduced Th2-biased immune responses than did an OVA-IL-12 fusion cDNA construct (9), maximized the salutary IL-18 effects for asthma, presumably by focusing the activity of IL-18 onto OVA-specific T cells and B cells. These results, dem-

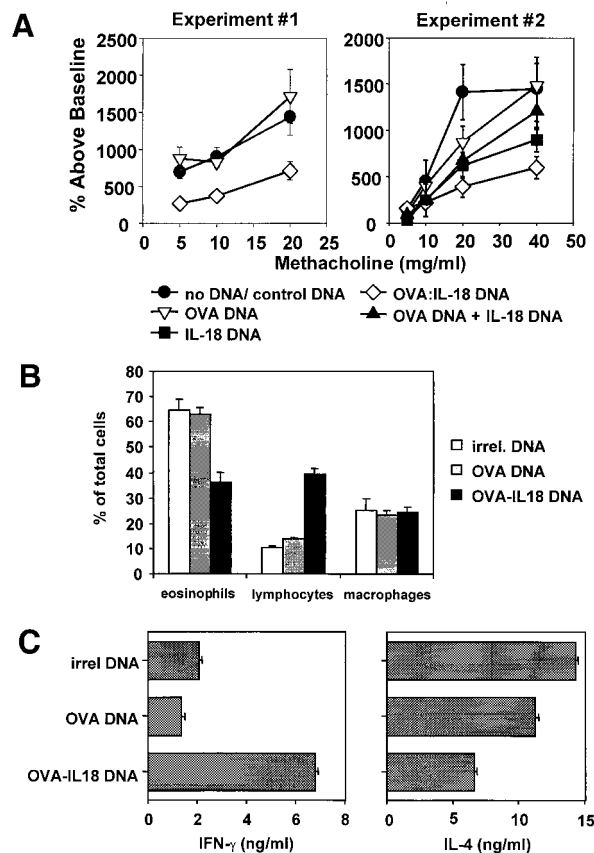


FIGURE 5. A, Reversal of AHR by OVA-IL-18 DNA vaccination. To determine whether OVA-IL-18 DNA vaccination could reverse established AHR, mice were first sensitized to OVA (see *Materials and Methods*), and then immunized twice with either OVA DNA or OVA-IL-18 DNA (left) or OVA DNA, IL-18 DNA, OVA-IL-18 DNA, or a combination of OVA DNA + IL-18 DNA (right). AHR in response to methacholine challenge was measured as above. Only OVA-IL-18 DNA, and not OVA DNA alone, reversed established AHR under these conditions. Data are expressed as mean percent above baseline $\pm$ SEM of five mice. B, Recruitment of specific cell types into the airways of immunized mice. BAL was performed on the above mice just before sacrifice, and the percentages of eosinophils, lymphocytes, and macrophages (identified morphologically) were tabulated. Only OVA-IL-18 DNA vaccinated mice showed a decrease in eosinophil proportion, along with a rise in lymphocyte proportion of total BAL cells. Data are expressed as mean $\pm$ SD of five mice. C, IFN- γ and IL-4 secretion by bronchial LN cells from the vaccinated mice. Within 5 days of the last challenge, mice were sacrificed and bronchial LN cells were restimulated *in vitro* with OVA (100 μ g/ml). IFN- γ and IL-4 levels in the culture supernatants were measured by ELISA. Only OVA-IL-18 DNA-immunized mice showed an appreciable increase in IFN- γ production and decrease in IL-4 production. Error bars represent SD of triplicate ELISA wells. All results are representative of two different experiments.

onstrating protective effects of IL-18 on allergic inflammation, are consistent with the observation that IL-18 knockout mice developed increased allergen-induced eosinophilia (42). Moreover, administration of OVA-IL-18 as a plasmid rather than as protein may enhance the allergy-suppressive effects of IL-18 because the plasmid contains CpG motifs that induce the production of IL-12, which up-regulates IL-18 receptors on T cells (43) and synergistically down-modulates airway inflammation (44). The effects of IL-18 (and IL-12) may have been further augmented by fusion of the OVA and IL-18 in a plasmid because the OVA-IL-18 plasmid had the capacity to reverse established AHR, whereas administration of IL-18 and IL-12 together as proteins, did not (44). These

studies thus suggest that the strategy of delivering IL-18 conjugated with Ag as a plasmid vaccine may be applicable to treatment of allergic disease, particularly because the major allergens (and in many instances the major allergenic proteins) have been identified.

The inhibitory effect of OVA-IL-18 DNA on AHR was dependent on the presence of CD8⁺ T cells because the protective effects of OVA-IL-18 DNA could be almost completely reversed by depletion of CD8⁺ T cells. This observation supports other studies demonstrating the important role of CD8⁺ T cells in asthma. For example, Hsu et al. demonstrated that the protective effect of allergen DNA vaccination could be transferred with CD8⁺ T cells (12). Furthermore, animal experiments have revealed that CD8⁺ T cells regulate IgE production and allergen-induced AHR (45–48). In our model, the induction of regulatory CD8 cells may have been enhanced by the potent capacity of IL-18 to induce CD8 T cells (49), and by the administration of OVA as cDNA, which may skew Ag presentation through an endogenous pathway. It is well established that peptides derived from intracellular Ags are generally presented to CD8⁺ T cells by MHC class I molecules (50), and this Ag presenting pathway may be important in the induction of regulatory CD8 cells when allergen cDNA is administered i.m.

The inhibitory effect of OVA-IL-18 DNA on AHR was also partially dependent on IFN- γ activity because coadministration of anti-IFN- γ mAb partially prevented the effects of OVA-IL-18 DNA. Both IL-18 as well as CpG motifs present on the vector backbone effectively induce IFN- γ production, which has been shown in studies with direct mucosal IFN- γ gene transfer to inhibit both the induction of Ag- and Th2-cell-induced pulmonary eosinophilia and AHR (51). In addition, CpG motifs induce IL-12 production, important not only in enhancing the induction of IFN- γ , but also in promoting the expression of IL-18 receptors on T cells, and in inhibiting Ag-induced airway eosinophilia and bronchial hyperreactivity in a murine model (52). Whether other regulatory cytokines or other cell types are involved in suppression of allergen-induced AHR by OVA-IL-18 is not yet clear.

In our studies, only two injections of OVA-IL-18 DNA were sufficient to reverse established AHR, suggesting that such an approach might be clinically useful for the treatment of chronic allergic disease and asthma. Currently, conventional allergen immunotherapy, performed by s.c. injection of increasing doses of allergen, is used to treat patients with allergic disease. However, such therapy is inefficient, requiring nearly 100 injections over a period of 3–5 years, and it is associated with frequent allergic reactions, including anaphylaxis (53). Nevertheless, conventional allergen immunotherapy is the only currently available therapy that, when successful, alters the underlying pathologic allergen-specific Th2 driven responses, resulting in clinical tolerance to subsequent allergen exposure (54, 55). DNA vaccination may be a safer form of allergen immunotherapy, particularly because DNA-based immunization provides prolonged, endogenous expression of Ag (56). Plasmids have been found to persist episomally in muscle cells, and gene expression in the skeletal muscle and persistent immunity to the Ag can be detected for more than a year after injection (10, 57, 58). Moreover, our studies, demonstrating that OVA-cytokine fusion constructs have much greater immunogenicity than allergen-only cDNA constructs, suggest that allergen-IL-18 DNA constructs may provide rapid, effective, and potentially curative therapy for allergic disease and asthma. However, rigorous studies of DNA-based immunization with respect to mechanism of action, safety, and delivery will be crucial before its ultimate application in human atopic disease.

Acknowledgments

We thank Dr. Carmen Wong for cloning IL-18 into the TCAE 5.3 vector.

References

- Shirakawa, T., T. Enomoto, S. Shimazu, and J. M. Hopkin. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* 275:77.
1998. Forecasted state-specific estimates of self-reported asthma prevalence—United States. *Morbidity Mortality Wkly. Rep.* 47:1022.
- Umetsu, D. T., and R. H. DeKruyff. 1997. Th1 and Th2 CD4⁺ cells in allergic diseases. *J. Allergy Clin. Immunol.* 100:1.
- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258.
- Manickan, E., R. J. Rouse, Z. Yu, W. S. Wire, and B. T. Rouse. 1995. Genetic immunization against herpes simplex virus: protection is mediated by CD4⁺ T lymphocytes. *J. Immunol.* 155:259.
- Raz, E., H. Tighe, Y. Sato, M. Corr, J. A. Dudley, R. Roman, S. L. Swain, H. L. Spiegelberg, and D. A. Carson. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* 93:5141.
- Roman, M., H. L. Spiegelberg, D. Broide, and E. Raz. 1997. Gene immunization for allergic disorders. *Springer Semin Immunopathol.* 19:223.
- Sato, Y., M. Roman, H. Tighe, D. Lee, M. Corr, M.-D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273:352.
- Maecker, H. T., D. T. Umetsu, R. H. DeKruyff, and S. Levy. 1997. DNA vaccination with cytokine fusion constructs biases the immune response to ovalbumin. *Vaccine* 15:1687.
- Wolff, J. A., J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani. 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* 1:363.
- Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu. Rev. Immunol.* 15:617.
- Hsu, C. H., K. Y. Chua, M. H. Tao, Y. L. Lai, H. D. Wu, S. K. Huang, and K. H. Hsieh. 1996. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat. Med.* 2:540.
- Hsu, C. H., K. Y. Chua, M. H. Tao, S. K. Huang, and K. H. Hsieh. 1996. Inhibition of specific IgE response in vivo by allergen-gene transfer. *Int. Immunol.* 8:1405.
- Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dworki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, et al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259:1745.
- Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* 2:1122.
- Tascon, R. E., M. J. Colston, S. Ragno, E. Stavropoulos, D. Gregory, and D. B. Lowrie. 1996. Vaccination against tuberculosis by DNA injection. *Nat. Med.* 2:888.
- Spooner, R. A., M. P. Deonarain, and A. A. Epenetos. 1995. DNA vaccination for cancer treatment. *Gene Ther.* 2:173.
- Syngelas, A. D., T. T. Chen, and R. Levy. 1996. DNA immunization induces protective immunity against B-cell lymphoma. *Nat. Med.* 2:1038.
- Hakim, I., S. Levy, and R. Levy. 1996. A nine-amino acid peptide from IL-1 β augments antitumor immune responses induced by protein and DNA vaccines. *J. Immunol.* 157:5503.
- Waisman, A., P. J. Ruiz, D. L. Hirschberg, A. Gelman, J. R. Oksenberg, S. Brocke, F. Mor, I. R. Cohen, and L. Steinman. 1996. Suppressive vaccination with DNA encoding a variable region gene of the T-cell receptor prevents autoimmune encephalomyelitis and activates Th2 immunity. *Nat. Med.* 2:899.
- Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori, et al. 1995. Cloning of a new cytokine that induces IFN- γ production by T cells. *Nature* 378:88.
- Gu, Y., K. Kuida, H. Tsutsui, G. Ku, K. Hsiao, M. A. Fleming, N. Hayashi, K. Higashino, H. Okamura, K. Nakanishi, et al. 1997. Activation of interferon- γ inducing factor mediated by interleukin-1 β converting enzyme. *Science* 275:206.
- Ghayur, T., S. Banerjee, M. Hugunin, D. Butler, L. Herzog, A. Carter, L. Quintal, L. Sekut, R. Talanian, M. Paskind, et al. 1997. Caspase-1 processes IFN- γ -inducing factor and regulates LPS-induced IFN- γ production. *Nature* 386:619.
- Yoshimoto, T., H. Okamura, Y.-I. Tagawa, Y. Iwakura, and K. Nakanishi. 1997. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon- γ production from activated B cells. *Proc. Natl. Acad. Sci. USA* 94:3948.
- Kohno, K., J. Kataoka, T. Ohtsuki, Y. Suemoto, I. Okamoto, M. Usui, M. Ikeda, and M. Kurimoto. 1997. IFN- γ -inducing factor (IGIF) is a costimulatory factor on the activation of Th1 but not Th2 cells and exerts its effect independently of IL-12. *J. Immunol.* 158:1541.
- Dao, T., K. Ohashi, T. Kayano, M. Kurimoto, and H. Okamura. 1996. Interferon- γ -inducing factor, a novel cytokine, enhances Fas ligand-mediated cytotoxicity of murine T helper 1 cells. *Cell. Immunol.* 173:230.
- Xu, D., W. L. Chan, B. P. Leung, D. Hunter, K. Schulz, R. W. Carter, I. B. McInnes, J. H. Robinson, and F. Y. Liew. 1998. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *J. Exp. Med.* 188:1485.
- Kim, T. S., R. H. DeKruyff, R. Rupper, H. T. Maecker, S. Levy, and D. T. Umetsu. 1997. An OVA-IL-12 fusion protein is more effective than OVA plus rIL-12 in inducing a Th1-dominated immune response and inhibiting antigen-specific IgE production. *J. Immunol.* 158:4137.

29. Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. 1995. Current protocols in immunology. In *Current Protocols*. R. Coico, ed. Wiley, London.
30. Hansen, G., G. Berry, R. H. DeKruyff, and D. T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 103:175.
31. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766.
32. Macaulay, A. E., R. H. DeKruyff, C. C. Goodnow, and D. T. Umetsu. 1997. Antigen-specific B cells preferentially induce CD4⁺ T cells to produce IL-4. *J. Immunol.* 158:4171.
33. DeKruyff, R. H., R. S. Gieni, and D. T. Umetsu. 1997. Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40. *J. Immunol.* 158:359.
34. Kumano, K., A. Nakao, H. Nakajima, F. Hayashi, M. Kurimoto, H. Okamura, Y. Saito, and I. Iwamoto. 1999. Interleukin-18 enhances antigen-induced eosinophil recruitment into the mouse airways. *Am. J. Respir. Crit. Care Med.* 160:873.
35. Roy, K., H. Q. Mao, S. K. Huang, and K. W. Leong. 1999. Oral gene delivery with chitosan—DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* 5:387.
36. Broide, D., J. Schwarze, H. Tighe, T. Gifford, M.-D. Nguyen, S. Malek, J. Van Uden, E. Martin-Orozco, E. W. Gelfand, and E. Raz. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161:7054.
37. Marshall, E. 1995. Cancer trial of interleukin-12 halted. *Science* 268:1555.
38. Bliss, J., C. V. Van, K. Murray, A. Wiencis, M. Ketchum, R. Maylor, T. Haire, C. Resmini, A. K. Abbas, and S. F. Wolf. 1996. IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J. Immunol.* 156:887.
39. Wynn, T. A., A. Reynolds, S. James, A. W. Cheever, P. Caspar, S. Hieny, D. Jankovic, M. Strand, and A. Sher. 1996. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. *J. Immunol.* 157:4068.
40. Germann, T., S. Guckes, M. Bongartz, H. Dlugonska, E. Schmitt, L. Kolbe, E. Kolsch, F. J. Podlaski, M. K. Gately, and E. Rude. 1995. Administration of IL-12 during ongoing immune responses fails to permanently suppress and can even enhance the synthesis of antigen-specific IgE. *Int. Immunol.* 7:1649.
41. Wild, J. S., A. Sigounas, N. Sur, M. S. Siddiqui, R. Alam, M. Kurimoto, and S. Sur. 2000. IFN- γ inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. *J. Immunol.* 164:2701.
42. Kodama, T., T. Matsuyama, K. Kuribayashi, Y. Nishioka, M. Sugita, S. Akira, K. Nakanishi, and H. Okamura. 2000. IL-18 deficiency selectively enhances allergen-induced eosinophilia in mice. *J. Allergy Clin. Immunol.* 105:45.
43. Yoshimoto, T., K. Takeda, T. Tanaka, K. Ohkusu, S. Kashiwamura, H. Okamura, S. Akira, and K. Nakanishi. 1998. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN- γ production. *J. Immunol.* 161:3400.
44. Hofstra, C. L., I. Van Ark, G. Hofman, M. Kool, F. P. Nijkamp, and A. J. Van Oosterhout. 1998. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsiveness, eosinophilia, and serum IgE levels. *J. Immunol.* 161:5054.
45. Sedgwick, J. D., and P. G. Holt. 1985. Induction of IgE-secreting cells and IgE isotype-specific suppressor T cells in the respiratory lymph nodes of rats in response to antigen inhalation. *Cell. Immunol.* 94:182.
46. Kemeny, D. M., and D. Diaz-Sanchez. 1991. Role of CD8 T cells in rat IgE responses. *Int. Arch. Allergy Appl. Immunol.* 94:99.
47. McMenamin, C., and P. G. Holt. 1993. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8⁺ T cell-mediated but MHC class II-restricted CD4⁺ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J. Exp. Med.* 178:889.
48. Renz, H., G. Lack, J. Saloga, R. Schwinzer, K. Bradley, J. Loader, A. Kupfer, G. L. Larsen, and E. W. Gelfand. 1994. Inhibition of IgE production and normalization of airways responsiveness by sensitized CD8 T cells in a mouse model of allergen-induced sensitization. *J. Immunol.* 152:351.
49. Okamoto, I., K. Kohno, T. Tanimoto, H. Ikegami, and M. Kurimoto. 1999. Development of CD8⁺ effector T cells is differentially regulated by IL-18 and IL-12. *J. Immunol.* 162:3202.
50. Brodsky, F. M., and L. E. Guagliardi. 1991. The cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 9:707.
51. Li, X. M., R. K. Chopra, T. Y. Chou, B. H. Schofield, M. Wills-Karp, and S. K. Huang. 1996. Mucosal IFN- γ gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 157:3216.
52. Gavett, S. H., D. J. O'Hearn, X. Li, S. K. Huang, F. D. Finkelman, and M. Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* 182:1527.
53. Durham, S. R., S. M. Walker, E. M. Varga, M. R. Jacobson, F. O'Brien, W. Noble, S. J. Till, Q. A. Hamid, and K. T. Nouri-Aria. 1999. Long-term clinical efficacy of grass-pollen immunotherapy. *N. Engl. J. Med.* 341:468.
54. Secrist, H., C. J. Chelen, Y. Wen, J. D. Marshall, and D. T. Umetsu. 1993. Allergen immunotherapy decreases interleukin 4 production in CD4⁺ T cells from allergic individuals. *J. Exp. Med.* 178:2123.
55. Akoum, H., A. Tsicopoulos, H. Vorng, B. Wallaert, J. P. Dessaint, M. Joseph, Q. Hamid, and A. B. Tonnel. 1996. Venom immunotherapy modulates interleukin-4 and interferon- γ messenger RNA expression of peripheral T lymphocytes. *Immunology* 87:593.
56. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. *Science* 247:1465.
57. Davis, H. L., M. Mancini, M. L. Michel, and R. G. Whalen. 1996. DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. *Vaccine* 14:910.
58. Deck, R. R., C. M. DeWitt, J. J. Donnelly, M. A. Liu, and J. B. Ulmer. 1997. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* 15:71.