Interleukin 12 Inhibits Antigen-induced Airway Hyperresponsiveness, Inflammation, and Th2 Cytokine Expression in Mice

By Stephen H. Gavett,* Daniel J. O'Hearn,‡ Xiumin Li,§ Shau-Ku Huang,§ Fred D. Finkelman,‖ and Marsha Wills-Karp*

From the Departments of *Environmental Health Sciences and ‡Medicine, The Johns Hopkins University, Baltimore, Maryland 21205; the §Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland 21224; and the ‖Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

Summary

Allergic asthma is characterized by airway hyperresponsiveness and pulmonary eosinophilia, and may be mediated by T helper (Th) lymphocytes expressing a Th2 cytokine pattern. Interleukin (IL) 12 suppresses the expression of Th2 cytokines and their associated responses, including eosinophilia, serum immunoglobulin E, and mucosal mastocytosis. We have previously shown in a murine model that antigen-induced increases in airway hyperresponsiveness and pulmonary eosinophilia are CD4+ T cell dependent. We used this model to determine the ability of IL-12 to prevent antigen-induced increases in airway hyperresponsiveness, bronchoalveolar lavage (BAL) eosinophils, and lung Th2 cytokine expression. Sensitized A/J mice developed airway hyperresponsiveness and increased numbers of BAL eosinophils and other inflammatory cells after single or repeated intratracheal challenges with sheep red blood cell antigen. Pulmonary mRNA and protein levels of the Th2 cytokines IL-4 and IL-5 were increased after antigen challenge. Administration of IL-12 (1 μg/d × 5 d) at the time of a single antigen challenge abolished the airway hyperresponsiveness and pulmonary eosinophilia and promoted an increase in interferon (IFN) γ and decreases in IL-4 and IL-5 expression. The effects of IL-12 were partially dependent on IFN-γ, because concurrent treatment with IL-12 and anti-IFN-γ monoclonal antibody partially reversed the inhibition of airway hyperresponsiveness and eosinophilia by IL-12. Treatment of mice with IL-12 at the time of a second antigen challenge also prevented airway hyperresponsiveness and significantly reduced numbers of BAL inflammatory cells, reflecting the ability of IL-12 to inhibit responses associated with ongoing antigen-induced pulmonary inflammation. These data show that antigen-induced airway hyperresponsiveness and inflammation can be blocked by IL-12, which suppresses Th2 cytokine expression. Local administration of IL-12 may provide a novel immunotherapy for the treatment of pulmonary allergic disorders such as atopic asthma.

Airway hyperresponsiveness and chronic airway inflammation are fundamental traits of allergic asthma. The inflammatory component of this disease is characterized by increased numbers of activated Th lymphocytes, mast cells, and eosinophils within the airway lumen and bronchial submucosa (1–4). Recent studies have shown a strong correlation between the level of activated CD4+ T cells and disease severity (2, 5–7). Activated CD4+ T cells release a variety of cytokines that have been proposed to contribute to inflammation and airway hyperresponsiveness. The types of cytokines released by activated CD4+ T cells from allergic asthmatic individuals appear to fit one of the two basic patterns of cytokine expression described in human and murine CD4+ Th cell clones. Th1 cells are characterized

by elevated secretion of IL-2 and IFN-γ, whereas the Th2 subset of CD4+ T cells preferentially secrete IL-4, IL-5, IL-6, IL-9, and IL-10 (8–10). Examination of bronchoalveolar lavage (BAL)[1] fluid cells and supernatants from allergic asthmatics has revealed the existence of Th2-like cytokine patterns (11, 12). The relative proportions of cells expressing Th2 cytokines in BAL fluid or biopsies are increased in symptomatic compared with asymptomatic asthmatic individuals (13) and in allergic asthmatic patients after segmental allergen challenge (14, 15), suggesting that Th2 cytokine

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[1]Abbreviations used in this paper: BAL, bronchoalveolar lavage; HPRT, hypoxanthine–guanine phosphoribosyl transferase; RT, reverse transcription.

release contributes to airflow obstruction. Th2 cytokines may contribute to allergic reactions and therefore may play an important role in the inflammatory reactions and airway hyperresponsiveness observed in allergic asthma. In particular, IL-4 modulates the growth and differentiation of mast cells and regulates IgE synthesis (16, 17), whereas IL-5 controls the differentiation, recruitment, and activation of eosinophils (18–20). We and others have demonstrated the functional significance of $CD4^+$ T cells (21, 22) and IL-5 (22) in the recruitment of eosinophils into the murine lung after antigen challenge and in the development of airway hyperresponsiveness (21), suggesting that $CD4^+$ T cell cytokine production may play an important role in the pathogenesis of allergic asthma. Therefore, an effective therapy for allergic airway reactions may be to prevent the development of the Th2 cytokine pattern.

IL-12 is a recently characterized heterodimeric cytokine (23, 24) that is primarily produced by monocytes and macrophages (25) in response to infections. IL-12 induces cell-mediated immune functions, upregulates Th1 cytokines, especially IFN-γ (for review, see reference 26), and inhibits or downregulates Th2 cytokines. IL-12 inhibits the development of IL-4–producing Th2 cells in response to antigens such as *Dermatophagoides pteronyssinus* (27) and *Leishmania major* (28, 29). In addition, IL-12 inhibits IL-4 production in bulk cultures of peripheral blood leukocytes from allergic patients (27) and also markedly suppresses IL-4–mediated IgE production by human PBMC in vitro (30). In murine models of immune activation (31) and parasite infection (32, 33), administration of IL-12 in vivo suppresses the expression of Th2 cytokines and their associated responses, including eosinophilia, serum IgE, and mucosal mastocytosis. These studies suggest that IL-12 may be an effective suppressor of allergen-induced airway hyperresponsiveness and inflammation associated with atopic asthma.

We have recently demonstrated that the development of antigen-induced airway hyperresponsiveness and pulmonary eosinophilia in a murine model is $CD4^+$ T cell dependent (21), suggesting that a Th2 pattern of cytokine production may be associated with the development of these allergic responses. In this study, we have examined the following: (a) whether IL-12 treatment could prevent airway hyperresponsiveness induced by a single antigen challenge; (b) whether IL-12 inhibits pulmonary cellular inflammation induced by such antigen challenge; (c) whether IL-12 treatment alters the pattern of cytokine expression induced by a single antigen challenge; (d) whether the effects of IL-12 in this model are dependent on IFN-γ; and (e) whether IL-12 treatment could prevent airway hyperresponsiveness and inflammation induced by repeated antigen challenge. Our results demonstrate that IL-12 was able to abrogate antigen-induced airway hyperresponsiveness and pulmonary eosinophilia, concomitant with suppression of Th2 cytokine expression, suggesting that these cytokines play an important role in the development of allergic airway responses. Furthermore, we demonstrate that IL-12 inhibits airway hyperresponsiveness and eosinophilia after repeated antigen challenge, suggesting that IL-12 administration may be use-

ful therapeutically for the modulation of chronic inflammation in allergic disorders.

Materials and Methods

Animals. Male A/J mice, 6–7-wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a laminar flow hood in a virus-free animal facility for the duration of the experiments. The studies reported here conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the Department of Health, Education, and Welfare (National Institutes of Health) guidelines for the experimental use of animals.

Effect of IL-12 Administration on Responses after a Single Antigen Challenge. Mice were sensitized and challenged with SRBC antigen as previously described (21). Briefly, mice were sensitized by i.p. injection of 10^8 SRBC in 0.5 ml PBS. 2 wk later, mice were anesthetized by i.p. injection with a mixture of ketamine and xylazine (45 and 8 mg/kg, respectively) and challenged intratracheally with 6×10^8 SRBC in 0.05 ml PBS. The effect of murine rIL-12 on airway responsiveness, pulmonary inflammation, and cytokine expression was compared in the following groups of mice: naive (untreated) mice, sham (PBS)- or antigen (SRBC)-sensitized, and -challenged mice treated with IL-12 vehicle (0.1% mouse serum in PBS), antigen-challenged mice treated with low-dose IL-12 (0.1 μg/d $\times$ 5 d), and sham- or antigen-challenged mice treated with high dose IL-12 (1 μg/d $\times$ 5 d). IL-12 or vehicle (0.2 ml) was injected i.p. -2, -1, $+1$, and $+2$ d relative to antigen or PBS challenge. On the day of challenge (day 0), IL-12 or vehicle was administered with PBS or antigen intratracheally (total volume = 0.05–0.06 ml). 3 d after challenge, airway responsiveness and numbers of BAL inflammatory cells were determined, and lungs were saved for measurement of cytokine expression.

Dependence of IL-12 Effects on IFN-γ. In a separate group of mice, the dependence of IL-12 effects on IFN-γ activity was determined. Mice were sensitized and challenged with antigen, treated with vehicle or IL-12 (1 μg/d $\times$ 5 d) as described above, and also injected i.p. with mAb to murine IFN-γ (XMG-6) or an irrelevant isotype-matched control mAb (GL113; IgG1). Antibodies were administered -2 and $+1$ d relative to antigen challenge (2 mg/0.2 ml PBS per injection).

Effect of IL-12 Administration on Responses after Repeated Antigen Challenges. To determine if IL-12 diminishes airway hyperresponsiveness associated with established antigen-induced pulmonary inflammation, sensitized mice were given two intratracheal challenges with SRBC (6×10^8 SRBC each) separated by a 7-d period. Preliminary experiments showed that antigen-induced hyperresponsiveness and inflammation were enhanced after this repeated antigen challenge protocol (data not shown). Sham groups were sensitized and challenged with PBS only. IL-12 (1 μg/d) or serum–PBS vehicle was administered i.p. -2, -1, $+1$, and $+2$ d relative to the second challenge and intratracheally on day 0 of the second challenge. Airway responsiveness to acetylcholine challenge and numbers of BAL leukocytes were measured on the 3rd d after the second challenge.

Airway Responsiveness Measurements. Airway responsiveness to i.v. acetylcholine challenge was measured as previously described (21). Briefly, mice were anesthetized with halothane, intubated with a 20-gauge tracheal cannula, and ventilated at a rate of 120 breaths per min with a constant tidal volume of air (0.2 ml). Airway pressure was measured with a pressure transducer via a port of the tracheal cannula. Further anesthesia and muscle paralysis

were provided by i.v. administration of a mixture of ketamine and decamethonium bromide (50 and 25 mg/kg, respectively) via the vena cava. After establishment of a stable airway pressure recording, acetylcholine was injected i.v. (25 μg/kg), and the changes in airway pressure were recorded. Airway responsiveness was defined by the time-integrated change in peak airway pressure (airway pressure–time index; centimeters H$_2$O per s).

Assessment of Airway Inflammation. After airway responsiveness measurements, lungs were lavaged with five 0.7-ml aliquots of HBSS solution without calcium or magnesium. The lavage fluid was centrifuged 300 g, 10 min, resuspended in 1 ml HBSS, and counted with a hemacytometer. Slide preparations were stained with Diff-Quik (Baxter, McGaw Park, IL), and BAL cell differential percentages were determined based on light microscopic evaluation of ≥500 cells/slide.

Isolation of RNA. After airway responsiveness measurements, the left lung of each mouse was placed in 1 ml RNAzol B (Tel-Test, Inc., Friendswood, TX) in a sterile cryovial frozen in liquid nitrogen, and stored at −80°C. The tissue was homogenized and RNA was isolated as described in the RNAzol B protocol. RNA was resuspended in sterile 10 mM Tris, 1 mM EDTA buffer, pH 7.5, made with diethylpyrocarbonate-treated water, and stored with ethanol at −70°C. RNA was subsequently recovered and quantitated spectrophotometrically. The integrity of individual RNA samples was confirmed by electrophoresing aliquots (0.5–1 μg) on a 2% agarose gel containing ethidium bromide and observing 28- and 18-s rRNA bands.

Reverse Transcription–PCR Detection of mRNA. The procedures for reverse transcription (RT) and PCR were previously reported (33–35) and are briefly described here. 1 μg of total cellular RNA was mixed with avian myeloblastosis virus reverse transcriptase (2.5 U), dNTPs (dATP, dCTP, dGTP, and dTTP; 1 mM each), RNase inhibitor (1 U), oligo(dT)$_{16}$ (2.5 μM), MgCl$_2$ (5 mM) in RT-PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) in a total volume of 20 μl. The mixture was incubated at 42°C for 20 min in a thermocycler (Perkin Elmer-Cetus Instruments, Norwalk, CT) for RT, followed by incubation at 99°C for 5 min to inactivate the transcriptase. The cDNAs were stored at −20°C. For PCR, 2 μl of the cDNA preparation was mixed with dNTPs (0.2 mM each), paired primers for IFN-γ, IL-4, IL-5, or hypoxanthine–guanine phosphoribosyl transferase (HPRT; 0.5 μM each primer), MgCl$_2$ (1.5 mM), and AmpliTaq polymerase (1 U; Perkin Elmer-Cetus Instruments) in RT-PCR buffer in a total volume of 50 μl. The sequences of cytokine- and HPRT-specific primer pairs and probes were previously reported (35). After initial incubation at 95°C for 3 min, PCR was carried out under the following conditions: denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. After the last PCR cycle, samples were incubated an additional 10 min at 72°C for final extension. The numbers of PCR cycles used for each primer pair were as follows: IL-4 (33), IL-5 (32), IFN-γ (30), and HPRT (23). In separate experiments, we confirmed that this number of PCR cycles was optimal for producing a linear relationship between initial quantity of mouse lung RNA and final PCR product, as previously described (34). As negative controls, aliquots of RNA samples were subjected to PCR amplification without the RT step. The reaction products were electrophoresed and visualized on 2% agarose gel (1% NuSieve GTG [FMC Corp., Rockland, ME] and 1% agarose [Sigma Chemical Co., St. Louis, MO]) containing 0.5 μg/ml ethidium bromide.

To estimate the relative levels of cytokine gene expression, ethidium bromide–stained gels were photographed with type 55 film (Polaroid Corp., Cambridge, MA). Negatives were scanned

and band intensities were digitized with a gel scanner (Molecular Dynamics, Sunnyvale, CA), and the digital data were analyzed (ImageQuant version 3.2; Molecular Dynamics). A one-dimensional plot was computed by averaging the horizontal pixels across each band (plot profile option). The integrated band intensities were taken as the OD of the sample (36). Background densities of the gel were computed and subtracted from cytokine and HPRT band densities, and the expression of each cytokine was calculated relative to the intensity of HPRT in each sample (OD ratio). These results were confirmed by comparison with Southern blots, which were hybridized with fluorescein-conjugated, cytokine-specific probes and developed using a chemiluminescence detection system (Amersham Corp., Arlington Heights, IL), as described previously (34). Chemiluminescent signals on ECL hyperfilm (Amersham Corp.) were quantified using a OneScanner (Apple Computer, Inc., Cupertino, CA) using National Institutes of Health Image.

Quantitation of Cytokine Protein Levels in BAL Fluid. In a separate experiment, groups of mice were sensitized and challenged with antigen or vehicle and treated with IL-12 or vehicle, as described above in the effects of IL-12 administration on responses after a single antigen challenge. The following groups were examined: sham challenged + vehicle treated; sham challenged + IL-12 treated (1 μg/d × 5 d); antigen challenged + vehicle treated; antigen challenged + IL-12 treated (0.1 μg/d × 5 d); antigen challenged + IL-12 treated (1 μg/d × 5 d). 3 d after challenge, mice were lavaged three times with one 1-ml aliquot of HBSS. The lavage fluid was centrifuged, and aliquots of the supernatant were stored without further treatment at −80°C until analyzed by ELISA. ELISA for IL-4 and IL-5 were conducted using matching antibody pairs obtained from PharMingen (San Diego, CA) according to the manufacturer's instructions. The following antibody pairs were used for ELISA detection of IL-4 and IL-5, respectively: BVD4-1D11 and BVD6-24G2; TRFK5 and JES1-39D10. IFN-γ protein levels were measured by a commercially available ELISA kit (Genzyme Corp., Cambridge, MA). OD readings of samples were converted to picograms per milliliter using values obtained from standard curves generated with varying concentrations of recombinant IL-4, IL-5, and IFN-γ (2000–5 pg/ml). The limit of detection in each assay was 5 pg/ml.

Statistical Analysis. Differences in airway responsiveness, inflammatory cell numbers, and cytokine levels among groups of mice were determined using analysis of variance (Statview; Abacus Concepts, Inc., Berkeley, CA). If differences among groups were significant (P <0.05), Fisher's protected least significant difference test was used to distinguish between pairs of groups.

Results

IL-12 Inhibits Airway Responsiveness and Pulmonary Inflammation after a Single Antigen Challenge. To determine if IL-12 could reverse antigen-induced airway hyperresponsiveness and inflammation observed 3 d after a single challenge, antigen-challenged mice were administered two different doses of IL-12 or vehicle for 5 consecutive days beginning 2 d before challenge and ending the day before response measurements. As previously reported (21), airway reactivity to i.v. acetylcholine challenge in antigen-challenged mice was significantly increased compared with that in untreated or sham-challenged control mice (Fig. 1). In antigen-sensitized and -challenged animals, IL-12 caused a dose-dependent inhibition of antigen-induced airway hy-
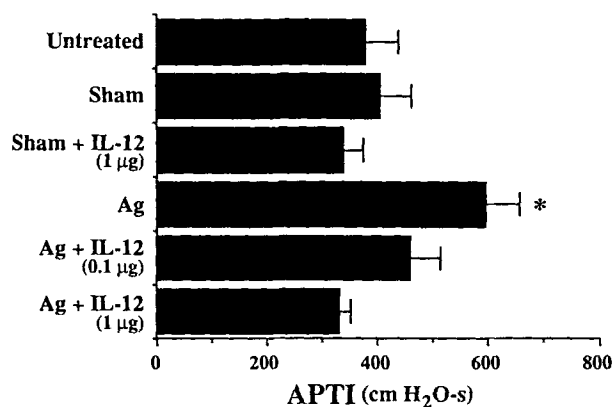
Figure 1. Effect of IL-12 on airway responsiveness to i.v. acetylcholine (25 μg/kg) in mice 3 d after a single intratracheal challenge with antigen or PBS (sham). Values shown are mean and SE of 4–15 mice per group. Sham- or antigen-challenged mice were injected with vehicle, 0.1 μg IL-12, or 1 μg IL-12 i.p. for 5 consecutive days beginning 2 d before challenge; another group of mice remained untreated. *P <0.05 compared with all other groups except antigen + 0.1 μg IL-12. *APTI*, airway pressure–time index.

perresponsiveness. Treatment of mice with 0.1 μg/d IL-12 for 5 d decreased mean airway responsiveness, but this effect was not statistically significant. A dose of 1 μg/d for 5 d completely inhibited the antigen-induced increases in airway responsiveness. IL-12 (1 μg/d for 5 d) tended to reduce airway responsiveness in sham-challenged controls, but this effect was not statistically significant.

Consistent with our previous findings (21), numbers of macrophages, neutrophils, lymphocytes, and eosinophils were all significantly increased in antigen-challenged mice compared with untreated and sham-challenged control groups (Fig. 2). Treatment with IL-12 at either 0.1 or 1 μg/d for 5 d resulted in a significant decrease in neutrophils and lymphocytes, whereas macrophage numbers were not significantly affected. Eosinophils were virtually ablated with
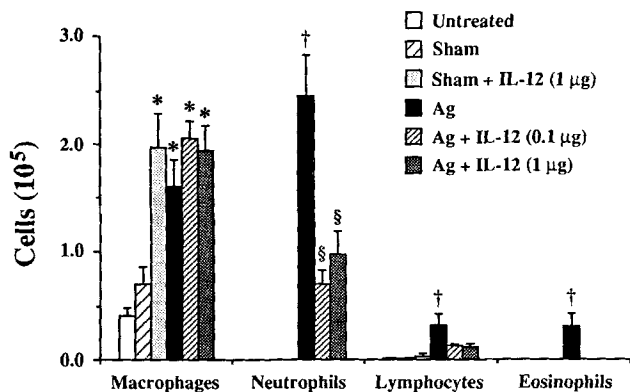
either dose of IL-12. The lack of a statistically significant effect of 0.1 μg/d IL-12 on airway responsiveness after antigen challenge might have been caused by incomplete prevention of eosinophilia in lung tissue, which might not be reflected in BAL fluids. To assess this possibility, pulmonary leukocytes were isolated after lung perfusion, mincing, and enzymatic digestion (21). Treatment with 0.1 μg/d IL-12 for 5 d prevented the antigen-induced increases in eosinophils observed in lung tissue (data not shown). Therefore, unlike the effect of IL-12 on airway responsiveness, a dose-dependent effect of IL-12 on airway eosinophilia after antigen challenge was not observed. IL-12 administration in sham-challenged mice induced a significant increase in numbers of BAL macrophages, but no other changes, compared with untreated or sham-challenged controls.

IL-12 Inhibits Antigen-induced Increases in Lung Th2 Cytokine Expression. The profiles of cytokine gene expression in lung tissues were examined using the RT-PCR technique (Fig. 3). Whereas lungs from untreated and sham-challenged mice showed extremely low levels of IL-4, there were clearly elevated levels of IL-4 mRNA expression in lungs from all antigen-challenged mice. The results were confirmed by measuring protein cytokine levels in BAL supernatants (Fig. 4 *A*). Similarly, very low levels of IL-5 mRNA were found in lung tissues from untreated and sham-challenged mice, whereas antigen challenge resulted in readily detectable levels of IL-5 transcripts (Fig. 3). An increase in IL-5 protein levels in BAL fluid of antigen-challenged mice was also detected, although the overall difference among groups was not significant (Fig. 4 *B*). In con-

Figure 2. Effect of IL-12 on numbers of BAL inflammatory cells recovered from mice 3 d after a single antigen or sham challenge. Values shown are mean and SE of 4–15 mice per group. Mice were treated as described in Fig. 1. *P <0.05 compared with untreated and sham groups; †0.05 compared with all other groups; §0.05 compared with untreated, sham, and sham + 1 μg IL-12 groups.
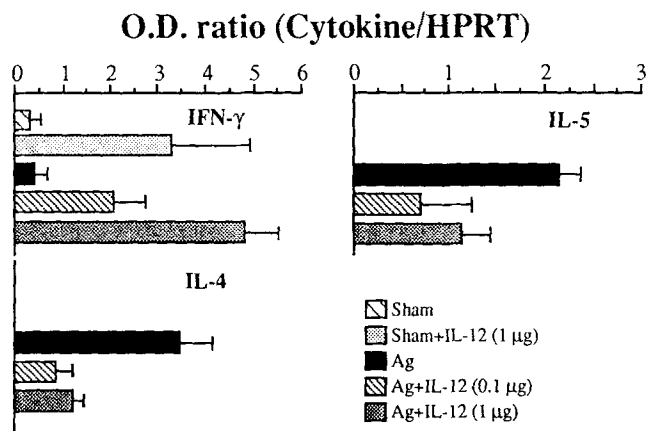
O.D. ratio (Cytokine/HPRT)

Figure 3. Effect of IL-12 on IFN-γ, IL-4, and IL-5 mRNA expression in lungs of mice 3 d after a single antigen or sham challenge. Sham- or antigen-challenged mice were treated with vehicle or IL-12 as described in Fig. 1. RNA was extracted from tissues and purified using the RNAzol B method, reverse-transcribed into cDNA in the presence of oligo-d(T) as a primer, and then subjected to varying cycles of PCR as described in Materials and Methods. Amplification of HPRT was performed as a control for the total amount of cDNA used in PCR. PCR-amplified products were electrophoresed in 2% agarose gel containing 0.5 μg/ml ethidium bromide. After subtracting background absorbance, OD ratios of cytokines to HPRT-amplified products were calculated. Results shown are mean + SE of OD ratios for IFN-γ, IL-4, and IL-5 in each experimental group (n = 5, 2, 7, 3, and 3, respectively, for the five groups listed in the figure).
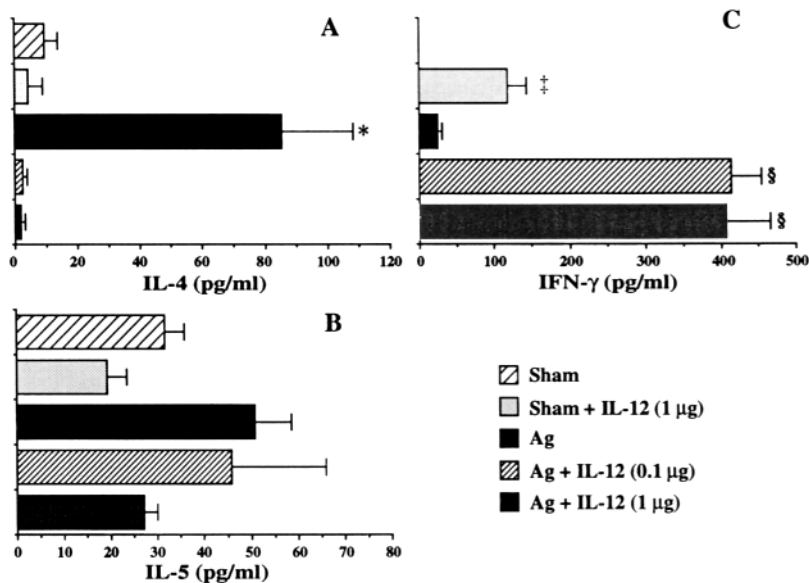
Figure 4. Effect of IL-12 on IL-4 (*A*), IL-5 (*B*), and IFN-γ (*C*) protein levels in BAL supernatants of mice 3 d after a single antigen or sham challenge. Sham- or antigen-challenged mice were treated with vehicle or IL-12 as described in Fig. 1. Protein levels were analyzed using ELISAs as described in Materials and Methods. After subtracting background absorbance, OD readings were converted to picograms per milliliter by comparison with standard curves. Results shown are mean + SE of cytokine protein levels for four to seven mice in each group. *P <0.05 compared with all other groups; ‡P <0.05 compared with sham group; §P <0.05 compared with antigen only group.

trast, there was no appreciable increase in the intensity of IFN-γ mRNA expression or BAL protein levels in the antigen-challenged lungs, suggesting that there is a selective induction of a Th2-like cytokine profile in lungs of mice challenged with SRBC antigen. Because IL-12 inhibited antigen-induced airway hyperresponsiveness and pulmonary inflammation, we examined whether this treatment inhibited some of the Th2 cytokines considered to be responsible for allergic reactions. Treatment with IL-12 significantly increased IFN-γ mRNA expression and BAL protein levels in sham-challenged mice (Figs. 3 and 4 C). IFN-γ mRNA expression and BAL protein levels were also significantly increased in antigen-challenged mice after treatment with two different doses of IL-12. A dose-dependent increase in mRNA levels was found, whereas no such dependence was found with IFN-γ protein levels; a >15-fold increase in protein levels was found with either dose of IL-12 compared with mice challenged with antigen only. Both doses of IL-12 suppressed IL-4 mRNA expression in antigen-challenged mice to a similar degree, which was significant but incomplete in comparison with sham-challenged mice (Fig. 3). Protein levels of IL-4 in antigen-challenged mice, however, were completely suppressed by both doses of IL-12, compared with sham-challenged mice (Fig. 4). Analogous to its effect on IL-4, both doses of IL-12 inhibited IL-5 transcript expression in antigen-challenged mice to a comparable degree. The higher dose of IL-12 inhibited IL-5 protein levels to a level similar to that in sham-challenged controls, but the differences among groups was not significant overall. In general the results obtained with mRNA and protein levels were in agreement, as both demonstrated antigen-induced increases in Th2 cytokines, IL-12 inhibition of Th2 cytokines, and increases in the Th1 cytokine; however, there were some differences. The reasons for these discrepancies may be the result of inherent differences in the kinetics of mRNA and protein production and release, or they may result from the differ-

ences in the kinetics between the two compartments from which these levels were measured. The mRNA levels were derived from cells in the lung interstitium and the protein levels from fluids and/or cells in the airway spaces. Cells that have migrated into the alveolar spaces may represent a different stage of the inflammatory response than cells that still remain in the interstitium. In any case, the use of both techniques has produced results that are in firm agreement with respect to overall trends.

The Effects of IL-12 Are Partially Dependent on IFN-γ. Measurement of cytokine levels in lungs of antigen-challenged mice treated with IL-12 suggested that the effects of this cytokine may be mediated through IFN-γ release. To investigate this possibility, groups of vehicle- or IL-12–treated mice were treated concurrently with anti–IFN-γ mAb or an isotype-matched control antibody. Treatment with isotype-matched control antibody had no significant effect on airway hyperresponsiveness in vehicle- or IL-12–treated, antigen-challenged mice (Fig. 5). Numbers of eosinophils were reduced by ~50% in isotype-treated, antigen-challenged mice. Treatment with anti–IFN-γ mAb had no significant effect on airway hyperresponsiveness or pulmonary eosinophilia in vehicle-treated antigen-challenged mice. Anti–IFN-γ mAb partially reversed the IL-12–induced inhibition of airway hyperresponsiveness in antigen-challenged mice. Airway responsiveness in this group of mice was slightly but not significantly lower than that of mice only challenged with antigen. The effects of IL-12, however, were only partially dependent on IFN-γ; treatment with anti–IFN-γ mAb did not significantly reverse the IL-12–induced depletion of eosinophils in antigen-challenged mice. Numbers of eosinophils in this group, though not completely ablated, comprised only 13% of the number recovered from mice challenged with antigen only.

IL-12 Prevents Airway Hyperresponsiveness and Pulmonary Eosinophilia Induced by Repeated Antigen Challenge. Because clinical asthma is characterized by chronic pulmonary inflam-
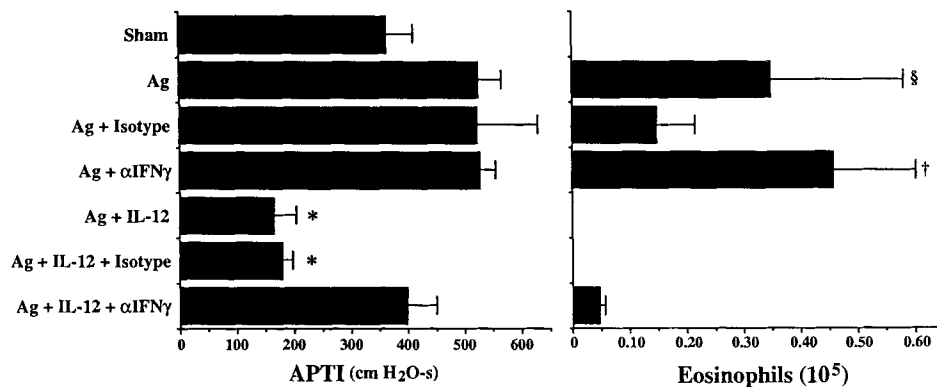
Sham

Ag

Ag + Isotype

Ag + αIFNγ

Ag + IL-12   ⊣ *

Ag + IL-12 + Isotype   ⊣ *

Ag + IL-12 + αIFNγ

0   100   200   300   400   500   600
APTI (cm H2O-s)

§

†

0   0.10   0.20   0.30   0.40   0.50   0.60
Eosinophils (10⁵)

Figure 5. Dependence of IL-12 effects on IFN-γ. Antigen-challenged mice were injected with vehicle or 1 μg IL-12 i.p. for 5 consecutive days beginning 2 d before challenge, and measurements were determined 3 d after challenge. Some groups were injected i.p. with 2 mg isotype-matched control mAb or anti–IFN-γ mAb −2 d and +1 d relative to challenge. Results shown are mean + SE of three to six mice per group. *P <0.05 compared with antigen-challenged non–IL-12–treated groups and IL-12 + anti–IFN-γ–treated group; †0.05 compared with all except antigen-only group; §0.05 compared with all except antigen + isotype and antigen + αIFN-γ groups.

mation, we examined whether IL-12 could suppress these responses induced by repeated antigen challenge in our murine model. 3 d after a second antigen challenge (10 d after the first challenge), airway responsiveness to acetylcholine was significantly greater compared with that in mice given repeated sham challenges (Fig. 6). The increase in airway responsiveness observed after the second challenge tended to be greater than that observed after the first challenge (Fig. 1). IL-12 administration in sham-challenged mice (1 μg/d for 5 d) reduced airway responsiveness, but this reduction did not reach significance. In contrast, IL-12 administration at the time of a second antigen challenge resulted in complete reduction of airway responsiveness to control levels.

Repeated antigen challenge resulted in significant increases in all bronchoalveolar lavage cell types (Fig. 7), with eosinophils comprising 25–44% of all cells. IL-12 administration in sham-challenged mice caused significant increases in macrophages but not in other cell types. IL-12 administration in antigen-challenged mice significantly reduced all cell types except macrophages. Unlike the inflammatory response observed after a single challenge, IL-12 adminis-

tration did not completely reduce eosinophil numbers to control levels, although there was a 12-fold reduction in these cell numbers compared with vehicle-treated antigen-challenged mice.

Discussion

In this study, we demonstrate that airway hyperresponsiveness and pulmonary inflammation induced by antigen challenge are associated with a Th2 pattern of cytokine expression in the murine lung. IL-12 administration prevented the development of these responses in conjunction with suppressing the antigen-induced increases in expression of the Th2 cytokines IL-4 and IL-5. Suppression of these two cytokines is particularly important because they have been implicated in the pathogenesis of allergic responses via their role in mediating IgE production, mast cell activation, and eosinophilia. Inhibition of antigen-induced airway hyperresponsiveness by IL-12 was dependent on IFN-γ activity, but depletion of eosinophils was only partially IFN-γ dependent. IL-12 was equally effective in suppressing airway hyperresponsiveness associated
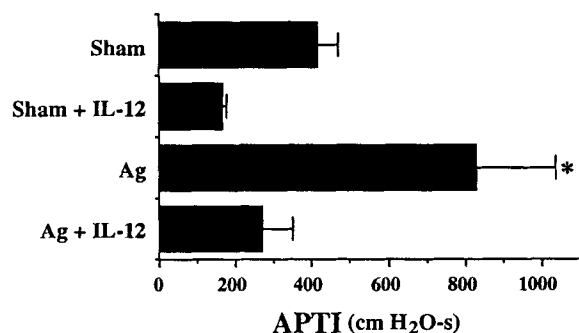
Sham

Sham + IL-12

Ag

Ag + IL-12   *

0   200   400   600   800   1000
APTI (cm H2O-s)

Figure 6. Effect of IL-12 on airway responsiveness to i.v. acetylcholine (25 μg/kg) in mice 3 d after a second intratracheal challenge with antigen or PBS (sham). Intratracheal challenge was repeated 7 d after first challenge. Values shown are mean and SE of five to six mice per group. Sham- or antigen-challenged mice were treated with vehicle or 1 μg IL-12 for 5 consecutive days beginning 2 d before the second challenge. *P <0.05 compared with all other groups.
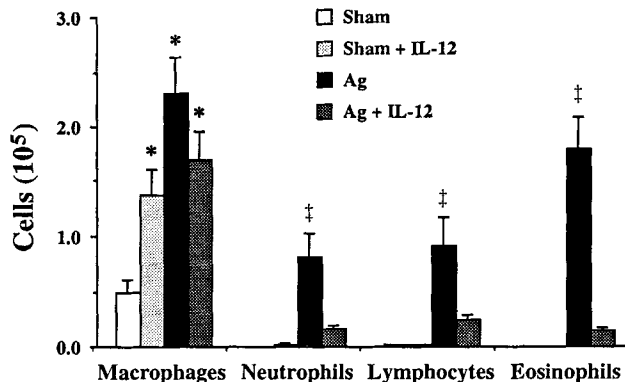
3.0

2.0

1.0

0.0

Cells (10⁵)

□ Sham
▨ Sham + IL-12
■ Ag
▩ Ag + IL-12

Macrophages   Neutrophils   Lymphocytes   Eosinophils

*   *   *   ‡   ‡   ‡

Figure 7. Effect of IL-12 on numbers of BAL inflammatory cells recovered from mice 3 d after a second antigen or sham challenge. Values shown are mean and SE of five to six mice per group. Mice were challenged and treated as described in Fig. 6. *P <0.05 compared with sham group; ‡P <0.05 compared with all other groups.

with established inflammatory responses induced by repeated antigen challenge. These results support the concept that Th2 cytokines play a central role in antigen-induced allergic responses and provide the first in vivo evidence that IL-12 can suppress responses associated with allergic asthma.

Antigen challenge induced increases in pulmonary IL-4 and IL-5 gene expression and protein levels, but not significant increases in IFN-γ, concomitant with the development of antigen-induced airway hyperresponsiveness and pulmonary eosinophilia, thus supporting a role for Th2-type cytokines in the development of these responses. To our knowledge, this is the first demonstration of antigen-induced increases in cytokine proteins in murine BAL fluid. The association of antigen-induced airway hyperresponsiveness and increased Th2-type cytokine expression is also consistent with our previous demonstration that these functional responses are dependent on $CD4^+$ Th cells, as administration of a monoclonal anti-CD4 antibody before antigen challenge completely prevented these responses (21). Further support for a role for Th2 cytokines in these responses is the demonstration that BAL and bronchial mucosal T cells from allergic asthmatic patients produce higher levels of IL-4 and IL-5 than do cells from normal controls (11, 13–15). Our results are also consistent with previous reports examining cytokine expression in bronchial mucosa induced by allergen challenge (6). Several other lines of evidence indicate that IL-4 and IL-5 may play important immunopathogenic roles in the development of allergic asthma. IL-4 has particularly been shown to be a primary determinant of Th2 cell differentiation (37) and is also essential for the production of IgE (17), which can mediate antigen-induced mast cell degranulation. Elevated serum levels of IgE are characteristic of allergic asthmatic individuals (11, 12), indicating a likely role for mast cell mediator release as a significant component of allergen-induced airway constriction. Mast cells release a variety of preformed and newly synthesized mediators, which are potent bronchoconstrictors, in response to antigen stimulation (38). IL-4 may also contribute to eosinophilia by promoting the upregulation of endothelial vascular cell adhesion molecule-1, which controls the attachment and migration of eosinophils (39). IL-5 promotes the differentiation, recruitment, activation, and survival of eosinophils (18–20, 40), which are consistently elevated in blood and airways of allergic and nonallergic asthmatic patients compared with normal subjects. Eosinophils may contribute to airway hyperresponsiveness by releasing mediators that induce epithelial injury and bronchoconstriction, including major basic protein, leukotrienes, and platelet-activating factor (41–43). Our finding that expression of IL-5 mRNA was significantly increased in the lungs of antigen-challenged mice is consistent with the elevation in eosinophil numbers. Other studies have also found increases in IL-5 levels after antigen challenge in animal models and human asthmatic individuals. For example, IL-5 induced in vitro airway hyperreactivity in guinea pigs, whereas anti–IL-5 treatment completely inhibits this response in antigen-challenged animals (44). Anti–IL-5 treatment also inhibits eosinophil recruitment into tracheal

submucosa of allergen-challenged mice (22). Other cytokines may also be important in the development of allergen-induced airway hyperresponsiveness, including IL-3 (released by both Th1 and Th2 cells) and IL-9, which contribute to the growth and activation of mast cells (16).

IL-12 was highly effective in preventing the development of antigen-induced airway hyperresponsiveness and pulmonary inflammation in this murine model. The inhibition of pulmonary inflammation by IL-12 was most striking with eosinophils, which have been proposed to mediate tissue injury and airway hyperresponsiveness (45, 46). Concomitant with the prevention of these antigen-induced responses, IL-12 administration reduced antigen-induced increases in the levels of IL-4 and IL-5 protein and mRNA and increased the expression of the Th1 cytokine IFN-γ, giving further support for the concept that Th2 cytokines are important in the development of allergic responses. These results are consistent with the results of other studies of allergic models in which IL-12 suppresses Th2 cytokine expression and associated responses in vivo (31–33).

In our study, IL-12 inhibition of antigen-induced airway hyperresponsiveness and eosinophilia was partially dependent on IFN-γ activity since coadministration of anti–IFN-γ mAb partially prevented these effects of IL-12. IFN-γ may mediate IL-12–induced suppression of antigen-induced airway hyperresponsiveness and eosinophilia through its ability to inhibit Th2 cytokines because IL-12–induced increases in IFN-γ protein levels were correlated with inhibition of Th2 cytokines. In addition, IFN-γ may have direct effects on effector cells such as eosinophils, B cells, or mast cells. The partial dependence of antigen-induced eosinophilia on IFN-γ is consistent with studies showing that IFN-γ administration can block antigen-induced eosinophil influx into murine airways (22, 47). Because IFN-γ also suppressed antigen-induced infiltration of $CD4^+$ T cells in those studies, its effects on $CD4^+$ T cells cannot be separated from any direct effects it may have on eosinophils. The involvement of IFN-γ in IL-12 inhibition of antigen-induced airway hyperresponsiveness in our study is also consistent with the ability of rIFN-γ to reverse OVA-induced alterations in airway reactivity in mice (48).

The lack of complete reversal of IL-12 inhibition of allergic responses by anti–IFN-γ administration may also suggest that IL-12 directly suppresses airway hyperresponsiveness and eosinophil production or stimulates production of mediators other than IFN-γ that have these effects. Alternatively, IL-12 could directly inhibit Th2 cytokine functions, such as inhibiting IL-4–mediated IgE production by an IFN-γ–independent mechanism, as demonstrated in vitro (30). In vivo, however, this effect can be at least partially IFN-γ dependent because IFN-γ mAb considerably inhibits IL-12 suppression of IgE, mast cell, and eosinophil responses in worm-infected mice (32). The fact that airway hyperresponsiveness was dose-dependently suppressed by IL-12, whereas Th2 cytokines and eosinophils were virtually ablated at both doses, also supports the conclusion that IL-12 may directly inhibit effector cells such as mast cells and B cells. We also cannot rule out the possibility that

IL-12 or IL-12–induced IFN-γ may have direct effects on receptors or signal transduction mechanisms in airway smooth muscle that mediate airway responsiveness, because IL-12 tended to reduce airway responsiveness in sham-challenged mice, although this effect was not statistically significant. Alternatively, the lack of complete IFN-γ dependence of IL-12 effects on allergic responses may be caused by incomplete anti–IFN-γ neutralization of IL-12–induced IFN-γ, as has been suggested previously (31). Further studies with mice that lack functional IFN-γ (49) or IFN-γ receptor gene (50) may be needed to address this question definitively.

Novel therapeutic approaches for the treatment of allergic diseases such as allergic asthma are in demand. Such therapies must be able to inhibit the chronic inflammation and hyperresponsiveness of asthmatic individuals, and not merely prevent acute responses. Strikingly, administration of IL-12 concurrent with the time of a second antigen challenge completely inhibited the airway hyperresponsive-

ness associated with repeated antigen exposure. Eosinophil numbers were reduced by 80% in IL-12–treated animals after repeated antigen challenge compared with vehicle-treated challenged mice, and other inflammatory cells were also significantly reduced. These results are consistent with those of a study in which IL-12 inhibited established Th2 responses in a murine model of Schistosoma mansoni infection (33). In contrast, IL-12 was ineffective in preventing Th2 responses, with the exception of eosinophilia, after secondary infection with Nippostrongylus brasiliensis (32), indicating that the ability of IL-12 to inhibit Th2-associated responses effectively may be dependent on type or level of antigen exposure. The ability of IL-12 to prevent in vivo airway hyperresponsiveness associated with repeated antigen challenges strongly supports the idea that local administration of this cytokine or agents that induce production of this cytokine may be useful in the treatment of chronic allergic conditions such as atopic asthma.

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