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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

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Allergic asthma is thought to be regulated by Th2 cells, and inhibiting this response is a promising mode of intervention. Many studies have focused on differentiation of T cells to the Th1 or Th2 subset in vitro. IL-4 is essential for Th2 development, while IL-12 induces Th1 development, which can be enhanced by IL-18. In the present study, we investigated whether IL-12 and IL-18 were able to interfere in Th2 development and the associated airway symptoms in a mouse model of allergic asthma. Mice were sensitized with OVA using a protocol that induces IgE production. Repeated challenges by OVA inhalation induced elevated serum levels of IgE, airway hyperresponsiveness, and a predominantly eosinophilic infiltrate in the bronchoalveolar lavage concomitant with the appearance of Ag-specific Th2-like cells in lung tissue and lung-draining lymph nodes. Whereas treatments with neither IL-12 nor IL-18 during the challenge period were effective, combined treatment of IL-12 and IL-18 inhibited Ag-specific Th2-like cell development. This inhibition was associated with an absence of IgE up-regulation, airway hyperresponsiveness, and cellular infiltration in the lavage. These data show that, in vivo, the synergistic action of IL-12 and IL-18 is necessary to prevent Th2-like cell differentiation, and consequently inhibits the development of airway symptoms in a mouse model of allergic asthma. The Journal of Immunology, 1998, 161: 5054–5060.

Allergic asthmatic reactions follow from the activation of aberrant allergen-specific Th2 cells that induce excessive eosinophil and IgE production (1). During the last decade, research has focused on the development of T cells to IFN-γ- and IL-2-producing Th1 cells or to the IL-4- and IL-5-producing Th2 cells (2). APCs appear to be important in the skewing of Th cell differentiation. First, although still controversial, costimulatory molecules on the APCs are suggested to be associated with the development of Th1 (B7-1/CDS0) or Th2 (B7-2/CD86) cells (3, 4). Second, soluble factors such as cytokines derived from APCs are thought to play a role in the Th subset development. IL-12 promotes Th1 differentiation, enhances production of Th1 cytokines such as IL-2 and IFN-γ (5–7), and inhibits Th2 cytokine synthesis (8–10). IL-4 is essential for the development of Th2 cells and inhibits the proliferation of Th1 cells (2). Recently, a new cytokine IL-18, also called IFN-γ-inducing factor, has been cloned (11). IL-18 can be synthesized by Kupffer cells and activated macrophages (11), and is of particular interest, since it potentiates the IL-12-driven Th1 development in BALB/c mice (12), and in combination with IL-12 strongly induces IFN-γ production (11–14). These studies were performed in vitro, and not much is known about the effectiveness in vivo on the differentiation into either Th1 or Th2 cells.

In mouse models of Ag-induced airway inflammation, IL-4 and IL-5 have been shown to play a crucial role in the development of IgE, airway hyperresponsiveness, and eosinophilic infiltration (15–18), indicating a regulatory role for Th2-like cells. In previous studies, using a mouse model of allergic asthma, we have shown that after sensitization, Ag-specific T cells are present in the lung and lung-draining lymph nodes and display a typical Th0-like cytokine production pattern upon restimulation in vitro (19). The induction of airway symptoms by repeated Ag inhalation was associated with the appearance of Ag-specific Th2-like cells in lung and lymph nodes. This model can be used to investigate the in vivo differentiation of T cells into Th2-like cells upon Ag inhalation and relate this to disease symptoms.

Currently, we investigated whether IL-12 and IL-18 could interfere in the development of Th2-like cells and the implications for airway symptoms. Therefore, sensitized mice were treated during the challenge period with IL-12, IL-18, or a combination of IL-12 and IL-18. The cytokine production patterns were determined from Ag-stimulated T cells in the lymph nodes and lung tissue. In addition, these results were compared with allergic airway symptoms such as IgE, airway hyperresponsiveness, and cellular infiltration in the lungs.

The present study shows that only treatment with a combination of IL-12 and IL-18 could inhibit the development of Th2-like cells, which was associated with decreased IgE levels and inhibition of airway hyperresponsiveness and cellular inflammation. This effect...
was not observed after treatment with either IL-12 or IL-18, indicating a synergistic effect of IL-12 and IL-18.

Materials and Methods

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specific pathogen-free male BALB/c mice (6 wk) were obtained from the breeding center of Central Animal Laboratory (Utrecht, The Netherlands). The mice were housed in macronol cages and provided with food and water ad libitum.

Immunization protocol

Active sensitization was performed without an adjuvant by giving seven i.p. injections of 10 μg OVA (grade V) in 0.5 ml pyrogen-free saline on alternate days (one injection per day). Three weeks after the last injection, the mice were exposed to eight OVA (2 mg/ml) or eight saline aerosol challenges for 5 min on consecutive days (one aerosol per day). The aerosol was performed in a plexiglas exposure chamber (5 L) coupled to a Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment, Richmond, VA; particle size 2–3 μm) driven by compressed air at a flow rate of 6 L/min. Aerosol was given in groups composed maximally of six animals.

Treatment with IL-12 and/or IL-18 in vivo

OVA-sensitized BALB/c mice were divided in four groups of 16 mice; 8 mice per group received saline challenge and 8 mice per group received OVA challenge. Aerosol was given as described above. One group of mice received i.p. 0.25 ml saline (vehicle group); a second group received i.p. murine rIL-12 (100 ng in 0.25 ml saline); a third group received i.p. murine rIL-18 (500 ng in 0.25 ml saline); and a fourth group received i.p. a combination of murine rIL-12 and murine rIL-18 (respectively, 100 ng and 500 ng in 0.25 ml saline). Treatment started 30 min before the first aerosol and was given once per day before the OVA or saline challenge. Murine rIL-12 and murine rIL-18 were purchased by Sanvertech (Heerhugowaard, The Netherlands). The endotoxin levels were less than 0.1 ng/μg IL-12 or IL-18. Based on a study by Yoshimoto and colleagues (14), we chose the combination of murine rIL-12 and murine rIL-18 (respectively, 100 ng and 500 ng/ml peritoxinethylanol), and total cell number was counted. Cells (viability >95%, 2 × 10³ lymph node cells/well and 8 × 10³ lung cells/well) were plated in round-bottom 96-well plates (Costar, Badhoevedorp, The Netherlands) in a volume of 200 μl. The cells were cultured for 5 days with different stimuli: in the presence of OVA (10 μg/ml), or medium only. The cells were cultured at 37°C with 5% CO₂ in humidified air. Each in vitro stimulation was performed in triplicate. Supernatants were harvested, pooled per stimulation, and kept at −20°C until cytokine levels were determined by ELISA.

The IFN-γ, IL-4, and IL-5 ELISAs were performed according to the instructions of the manufacturer (PharMingen, San Diego, CA). The detection limits of the ELISAs were 156 pg/ml for IFN-γ, 15.6 pg/ml for IL-4, and 31.3 pg/ml for IL-5.

Stimulation of thoracic lymph nodes and lung cells in the presence of IL-12 and/or IL-18

In a different set of experiments, thoracic lymph node and lung cells were isolated from OVA-sensitized and OVA-challenged mice. The cells from different mice (3–6) were pooled and cultured, as described above, in the presence (5 ng/ml) or absence of IL-12, IL-18, or a combination of IL-12 and IL-18.

Data analysis

Unless stated otherwise, data are expressed as mean ± SEM and evaluated using an analysis of variance, followed by a post hoc comparison between groups. A probability value of p < 0.05 was considered statistically significant. Statistical analyses were conducted using Systat, version 5.03 (NaG, Oxford, U.K.).

Chemicals

OVA (chicken egg albumin, crude grade V) and o-phenylenediamine-dihydrochloride were purchased from Sigma (St. Louis, MO); PBS, RPMI, FCS, gentamicin, and glutamatan were purchased from Life Technologies (Merelbeke, Belgium); pentobarbitone (nembutal) was purchased from Sanofi Santé B.V. (Maassluis, The Netherlands); heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands); collagenease and Dnase were purchased from Boehringer Mannheim (Mannheim, Germany); saline was from B. Braun Medical B.V. (Oss, The Netherlands); and methacholine (acetyl-β-methylcholine) was purchased from Janssen Chimica (Beerse, Belgium). Mouse IgE reference serum was purchased from ICN Biomedicals (Aurora, OH); anti-mouse IgE and biotinylated anti-mouse IgE were purchased from Pharmingen (San Diego, CA); and Dif-Quick was purchased from Merz and Dade.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as described previously (17). The airways of the mice were cannulated and 5 times lavaged through this cannula with 1-ml aliquots of pyrogen-free saline warmed to 37°C. The total numbers of lavage cells were determined, and cells in cytospin preparations were differentiated (Dif-Quick; Merz and Dade, Düdingen, Switzerland) into mononuclear cells, eosinophils, and neutrophils by standard morphology. Per cytospin preparation, at least 200 cells were counted and the absolute number of each cell type was calculated. To evaluate differences between OVA-challenged mice and saline-challenged mice for the different treatments, the numbers of the various cell types were tested with an analysis of variance. For the very low number of eosinophils and neutrophils in saline-challenged mice, a Poisson distribution was assumed, and for differences between treatment groups, Fisher’s exact test was used.

Stimulation of thoracic lymph nodes and lung cells in vitro

Cytokine production by Ag-stimulated T cells derived from both thoracic lymph nodes and lung tissue was determined, as described previously (19). Mice were injected with an overdose of pentobarbitone (0.5 g/kg body weight). The lungs were lavaged (as described below) and perfused via the right ventricle with 4 ml saline containing 100 U/ml heparin to remove any blood and intravascular leukocytes. The thoracic lymph nodes, derived from the paratracheal and parabronchial regions, and the lungs were removed. The lymph nodes were transferred to cold PBS and gently homogenized on a 70-μm cell strainer (Falcon, Lelystad, The Netherlands) to obtain a single cell suspension. The lungs were minced and digested with 3 ml RPMI containing 2.4 mg/ml collagenase, 1 mg/ml DNase, and 50 μg/ml gentamicin for 30 min at 37°C. The cell suspension was resuspended and filtered through a 70-μm cell strainer with 10 ml RPMI containing 20% heat-inactivated FCS. The lymph node and lung cell suspensions were washed and resuspended in culture medium (RPMI 1640 containing 10% FCS, 1% glutamax I, 50 μg/ml gentamicin, and 50 mM β-mercaptoethanol), and total cell number was counted. Cells (viability >95%, 2 × 10³ lymph node cells/well and 8 × 10³ lung cells/well) were plated in round-bottom 96-well plates (Costar, Badhoevedorp, The Netherlands) in a volume of 200 μl. The cells were cultured for 5 days with different stimuli: in the presence of OVA (10 μg/ml), or medium only. The cells were cultured at 37°C with 5% CO₂ in humidified air. Each in vitro stimulation was performed in triplicate. Supernatants were harvested, pooled per stimulation, and kept at −20°C until cytokine levels were determined by ELISA.

The IFN-γ, IL-4, and IL-5 ELISAs were performed according to the instructions of the manufacturer (PharMingen, San Diego, CA). The detection limits of the ELISAs were 156 pg/ml for IFN-γ, 15.6 pg/ml for IL-4, and 31.3 pg/ml for IL-5.
Results

Immunoglobulin E

IgE levels were determined in serum of all mice. No significant differences in IgE levels were observed between the groups of saline-challenged mice.

In the OVA-challenged vehicle-treated mice, the levels of IgE were significantly increased (388%, p < 0.01) compared with saline-challenged mice (Fig. 1). OVA-challenged mice treated with IL-12 or IL-18 also showed a significant increase (respectively 332% and 268%, p < 0.01) in IgE levels compared with the saline-challenged controls. Treatment with a combination of IL-12 and IL-18 in OVA-challenged mice significantly (p < 0.01) inhibited the IgE up-regulation back to the levels observed in saline-challenged mice. Therefore, the combination of IL-12 and IL-18 was able to inhibit the up-regulated IgE levels.

Airway responsiveness

Airway responsiveness in vivo to aerosolized methacholine was measured 24 h after the last challenge in conscious, unrestrained mice. For all mice, a complete dose-response curve to methacholine ranging from 1.6 to 50 mg/ml methacholine was constructed. In all groups of mice, no significant differences were observed in the basal Penh and saline aerosol-induced Penh values between OVA- and saline-challenged mice (Fig. 2). No significant differences in airway responses were observed between the saline-challenged mice of the different treatment groups.

In vehicle-, IL-12-, or IL-18-treated mice, repeated OVA challenge induced a significant increase (p < 0.05) in airway responsiveness to methacholine compared with the responding saline-challenged mice (Fig. 2, A, B, and C), at doses ranging from 12.5 to 50 mg/ml methacholine. Interestingly, treatment with a combination of IL-12 and IL-18 significantly (p < 0.05) inhibited this airway hyperresponsiveness, resulting in similar Penh values for both OVA- and saline-challenged mice (Fig. 2D). Thus, OVA-challenged mice demonstrate airway hyperresponsiveness compared with saline-challenged mice, which can

![Figure 1](http://www.jimmunol.org/) IgE levels in serum of OVA-sensitized BALB/c mice challenged with saline (white bars) or OVA (black bars) and treated with vehicle (VEH), IL-12, IL-18, or a combination of IL-12 and IL-18. Values expressed are mean ± SEM. Per experimental group, seven to eight mice were used. **, p < 0.01 as compared with saline-challenged mice; ##, p < 0.01 as compared with vehicle-treated OVA-challenged mice.

![Figure 2](http://www.jimmunol.org/) Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. BALB/c mice were sensitized with OVA, and later on, challenged with saline (white bars) or OVA (black bars) aerosol and treated with vehicle (A), IL-12 (B), IL-18 (C), or a combination of IL-12 and IL-18 (D). Basal values were measured, followed by measuring the response to nebulized saline (0) and to increasing concentrations of methacholine (1.6–50 mg/ml). Values expressed are mean ± SEM. Per experimental group, seven to eight mice were used. *, p < 0.05; **, p < 0.01 as compared with saline-challenged mice; #, p < 0.05 as compared with vehicle-treated OVA-challenged mice.
be completely inhibited by a combination of IL-12 and IL-18 treatment, but not by treatment with IL-12 or IL-18 only.

Bronchoalveolar lavage

The number of cells in the bronchoalveolar lavage was used as a measure for the infiltration of cells in the airways.

In mice treated with vehicle, IL-12, and the combination of IL-12 and IL-18, the numbers of mononuclear cells in the bronchoalveolar lavage were increased, although not statistically significant, in OVA- versus saline-challenged mice. In IL-18-treated mice, the numbers of mononuclear cells were significantly \((p < 0.05)\) increased in OVA-challenged mice compared with vehicle-treated OVA-challenged mice (Fig. 3C).

Low numbers of eosinophils and neutrophils were observed in the bronchoalveolar lavage fluid of saline-challenged mice. The numbers of eosinophils in the bronchoalveolar lavage of OVA-challenged mice in all treatment groups were significantly \((p < 0.01)\) increased when compared with the corresponding saline-challenged mice (Fig. 3A). The numbers of eosinophils between vehicle-, IL-12-, or IL-18-treated OVA-challenged mice were not significantly different. However, treatment with a combination of IL-12 and IL-18 almost completely inhibited (88%, \(p < 0.05\)) the bronchoalveolar lavage eosinophilia in OVA-challenged mice (Fig. 3A) compared with vehicle-treated OVA-challenged mice.

The numbers of neutrophils in the bronchoalveolar lavage of OVA-challenged mice treated with vehicle, IL-12, or IL-18 were significantly \((p < 0.01)\) increased when compared with the corresponding saline-challenged mice (Fig. 3B). Treatment with a combination of IL-12 and IL-18 almost completely inhibited (80%, \(p < 0.01\)) the neutrophils in the lavage of OVA-challenged mice (Fig. 3B) compared with vehicle-treated OVA-challenged mice. Therefore, OVA challenge induced an eosinophilic and neutrophilic cellular infiltrate in the bronchoalveolar lavage. This inflammation was almost completely inhibited by treatment with a combination of IL-12 and IL-18, but not by treatment with IL-12 or IL-18 only.

Cytokine production by Ag-stimulated thoracic lymph node and lung tissue cells

From each animal, the lung-draining lymph node cells and lung tissue cells were isolated and stimulated with Ag in vitro for 5 days. In previous studies, we have determined the optimal conditions for Ag-stimulated cytokine production.\(^3\) In the supernatants of these cultures, IFN-\(\gamma\), IL-4, and IL-5 levels were measured.

Thoracic lymph node cells

Thoracic lymph node cells cultured in medium only produced undetectable levels of cytokines (data not presented). In all treatment groups, thoracic lymph node cells from saline-challenged mice produced low levels of cytokines upon Ag-specific stimulation (Fig. 4). After repeated OVA inhalation, thoracic lymph node cells from vehicle-treated mice produced low levels of IFN-\(\gamma\) and significantly \((p < 0.01)\) higher levels of IL-4 and IL-5 compared with cultures of saline-challenged mice (Fig. 4), indicating differentiation into the Th2-like cell population. Compared with these cultures, Ag-stimulated thoracic lymph node cells of IL-12-treated mice produced comparable IFN-\(\gamma\) and IL-4 levels, but significantly \((p < 0.05)\) increased IL-5 levels. In lymph node cell cultures from IL-18-treated mice, comparable levels of IL-4, IL-5, and IFN-\(\gamma\) were detected compared with similar cultures of vehicle-treated mice. Treatment with a combination of IL-12 and IL-18 resulted in significantly lower levels of IL-4 (71% inhibition, \(p < 0.05\)) decreased levels of IL-5 (43% inhibition), and increased levels of IFN-\(\gamma\) (187% increase) compared with similar cultures of vehicle-treated mice, indicating the presence of Th0-like cells.

An Ag-specific Th2-like cytokine profile was observed in the OVA-challenged vehicle-treated mice. Treatment with a combination of IL-12 and IL-18 can modulate the differentiation to Ag-specific Th2-like profile in OVA-challenged mice, to a Th0/Th1-like cytokine profile.

Lung tissue cells

In agreement with previous studies,\(^3\) only IL-5, but not IFN-\(\gamma\) and IL-4, levels were detectable in Ag-stimulated lung cell cultures.

In Ag-stimulated lung cell cultures from saline-challenged mice, only low levels of IL-5 could be measured (Fig. 5); these levels did not differ between the treatment groups. In all treatment groups, lung cell cultures from OVA-challenged mice showed significantly increased IL-5 levels \((p < 0.05)\) compared with cultures from saline-challenged mice. Treatment of mice with either IL-12 or IL-18 during OVA challenge did not alter the IL-5 levels in Ag-stimulated lung cell cultures. However, Ag-stimulated lung cell cultures from OVA-challenged mice treated with a combination of IL-12 and IL-18 showed a significantly decreased (76%, \(p < 0.05\)) IL-5 production.

Thus, IL-5 production was observed by Ag-stimulated lung tissue cells derived from OVA-challenged mice. This Ag-induced IL-5 production by lung cells could only be down-regulated by treatment with a combination of IL-12 and IL-18.

Stimulation of thoracic lymph nodes and lung cells in the presence of IL-12 and/or IL-18

To study the direct effect of IL-12 and IL-18 on the cytokine production by Th2-like cells, thoracic lymph node and lung cells from OVA-sensitized and OVA-challenged mice were Ag stimulated in the presence of IL-12 and/or IL-18. Different concentrations of IL-12 and IL-18 were used, and shown are the doses of IL-12 and IL-18, resulting in the maximal effects.

In both non- and Ag-stimulated thoracic lymph node and lung cell cultures, the addition of IL-12 induced dose dependently low levels of IFN-\(\gamma\) (respectively 4.1 ± 0.2 ng/ml and 0.9 ± 0.09 ng/ml).
ng/ml) (Fig. 6A) as compared with polyclonal-stimulated cell cultures (respectively 8 ng/ml and 2.5 ± 0.05 ng/ml). Although IL-18 itself did not induce IFN-γ production, it enhanced dose dependently the IL-12-induced IFN-γ production in thoracic lymph node and lung cell cultures irrespective of the stimulation. Similar to our previous experiments, no IL-4 production was observed in Ag-stimulated lung cell cultures (Fig. 6B). In OVA-stimulated thoracic lymph node cell cultures, addition of IL-18 slightly reduced the IL-4 levels (28% inhibition). Addition of IL-12 alone did not alter the IL-4 levels in these cultures.

The IL-5 levels (Fig. 6C) in OVA-stimulated lung cell cultures were slightly reduced by the addition of IL-12 (28% inhibition). Addition of IL-18 did not alter the IL-5 levels in these cultures. In contrast, the IL-5 levels in Ag-stimulated thoracic lymph node cell cultures were not influenced by the addition of IL-12, but slightly decreased by the addition of IL-18 (35% inhibition). No synergistic effects of IL-12 and IL-18 were observed on the IL-4 and IL-5 levels by Ag-stimulated thoracic lymph node and lung cell cultures. Thus, a combination of IL-12 and IL-18 synergistically induced IFN-γ production in an Ag-independent manner, whereas no synergistic effect on IL-4 and IL-5 levels was observed.

Discussion

In the present study, repeated OVA inhalation induced IgE production, airway hyperresponsiveness, and eosinophilic and neutrophilic infiltration in the bronchoalveolar lavage, concomitant with the appearance of Ag-specific Th2-like cells in the lung-draining lymph nodes and IL-5-producing lung cells. In vivo treatment with a combination of IL-12 and IL-18, but neither treatment with IL-12 nor IL-18, prevented Ag-induced Th2-like development concurrent with the inhibition of airway symptoms and serum IgE levels.

In literature, conflicting data exist on the role of IL-12 in secondary IgE responses in mice. Similar to the present study, unaltered IgE levels have been observed after treatment with IL-12 during the challenge period (21–23), but others have demonstrated that IgE responses could be inhibited using a similar dose of IL-12 (14). The difference in Ag used in the latter study compared with the other studies may account for this discrepancy. Interestingly, in our experiments, the potentiation of the IgE levels could almost completely be inhibited by treatment with a combination of IL-12 and IL-18. These data are in accordance with a study by Yoshimoto and colleagues (14), who suggested that the inhibition of IgE levels by IL-12 and IL-18 was mediated via an IFN-γ-dependent mechanism. In line with these data, we and others have shown that treatment with IFN-γ during Ag inhalation or during sensitization can inhibit the production of IgE in mice (24–26). Furthermore, IFN-γ can also inhibit IgE production by B cells in vitro (27).

In the present study, eosinophil and neutrophil infiltration was observed in the bronchoalveolar lavage after repeated OVA challenge.
Infiltration of eosinophils in the airways is one of the characteristic features of allergic asthma. The eosinophils are thought to be responsible for the airway epithelial damage in patients with allergic asthma, which may result in the induction of airway hyperresponsiveness (28). In the present study, treatment with a combination of IL-12 and IL-18 during the challenge period was effective in almost completely inhibiting the eosinophilic and neutrophilic inflammation after OVA challenge. It is generally accepted that IL-5 is essential for the eosinophilic infiltration in the airways (16). In addition, depletion of CD4⁺ cells in mice could also prevent allergen-induced eosinophilic infiltration (29), indicating the role of Th2-like cells in this response. Interestingly, in our experiments, eosinophilic infiltration in OVA-challenged mice was accompanied with IL-5 production by Ag-stimulated lung cells, and both were inhibited in mice treated with a combination of IL-12 and IL-18. It can be speculated that IL-5 production by Ag-specific lung cells is responsible for the eosinophilic infiltration. Similarly, lung CD4⁺ cells from atopic patients produced high levels of IL-5 after allergen challenge (30). No altered eosinophilic and neutrophilic infiltration was observed after treatment with either IL-12 or IL-18 during the challenge period. This is in contrast with other studies, which showed that IL-12 treatment during the challenge period can inhibit airway eosinophilia (9, 21, 31, 32). In these studies, mice were treated with a 10 times higher dose of IL-12 than the present study, which probably explains the different results.

In patients with allergic asthma, it was shown that airway hyperresponsiveness correlates with disease severity (33), and is therefore a major feature of allergic asthma. In our experiments, OVA-challenged mice showed airway hyperresponsiveness that was effectively inhibited by treatment with a combination of IL-12 and IL-18, but not by treatment with either IL-12 or IL-18. Contrasting data exist on the effect of IL-12 on airway hyperresponsiveness during the challenge period. Similar to the present study, Sur and colleagues demonstrated that treatment with IL-12 during the challenge period resulted in unaltered airway hyperresponsiveness (23). In contrast, inhibition of the airway hyperresponsiveness was observed by others using the same dose of IL-12 (9, 21). As mentioned before, the dose of IL-12 in these studies was higher compared with the present study, which can partially explain the differences.

In previous studies, we have characterized the cytokine production by Ag-stimulated T cells derived from the lungs and draining lymph nodes in mice with and without airway symptoms. No Ag-specific T cell responses were observed after sensitization in lung-draining lymph nodes or lung tissue in the present study. However, after repeated OVA challenges, Ag-stimulated lymph node cells produced IL-4 and IL-5. More importantly, concomitant with development of airway eosinophilia, Ag-stimulated lung cells produced only IL-5, but no IFN-γ or IL-4. In previous studies (19), we showed that CD4⁺ cells are responsible for the Ag-induced cytokine production in these cell cultures, implicating the presence of Th2-like cells. Thus, airway symptoms were observed concomitant with Th2-like cell development, indicating an important role for Th2-like cells and the cytokines they produce. In addition, in murine studies it was shown that IL-4 is essential for IgE production (15), and that IL-5 is necessary for eosinophilic infiltration in the airways (34). After treatment with a combination of IL-12 and IL-18 in vivo, decreased IL-4 and IL-5 levels and increased IFN-γ levels were observed by Ag-stimulated lymph node cells. Ag-stimulated lung cells produced decreased levels of IL-5, but still no IFN-γ. These data indicate that either the differentiation of Ag-specific T cells to the Th2-like phenotype is prevented or that the synthesis of Th2-like cytokines is inhibited. To examine the latter explanation, the direct effects of IL-12 and IL-18 on Th2-like cells derived from thoracic lymph node and lung cells were studied. Addition of IL-12 induced IFN-γ production by thoracic lymph node and lung cells from OVA-sensitized and OVA-challenged mice. The IL-12-induced IFN-γ production was further enhanced by the addition of IL-18. These results are in agreement with other in vitro studies (11–14) in which synergistic effects of IL-12 and IL-18 on IFN-γ production by T cells were observed. However, opposite to the effects of in vivo treatment, the combination of IL-12 and IL-18 in vitro does not synergistically reduce the cytokine production by Th2-like cells. Therefore, it can be suggested that in vivo treatment with a combination of IL-12 and IL-18 prevents the development of Th2-like cells rather than reducing the cytokine production by Th2-like cells.

Treatment with either IL-12 or IL-18 did not result in an altered cytokine profile by Ag-stimulated lymphocytes. This is in contrast with a murine study of airway inflammation in which IL-12 treatment inhibited the IL-4 and IL-5 production by Ag-stimulated

**FIGURE 6.** IFN-γ (A), IL-4 (B), and IL-5 (C) production by OVA (black bars) and unstimulated (white bars) lymphocytes isolated from the lung (left) and lung-draining lymph nodes (right) from OVA-sensitized and OVA-challenged BALB/c mice. The cells were cultured in the absence or presence (5 ng/ml) of IL-12, IL-18, or a combination of IL-12 and IL-18. Values expressed are mean ± SEM. Cells were pooled from mice and cultured at least in triplicate. Shown is a representative experiment of three different independent experiments.
splenocytes (32). The high dosage of IL-12 used in this study might have contributed to the decreased cytokine production.

In the present study, we observed that treatment with a combination of low doses of IL-12 and IL-18, but not treatment with either cytokine, can inhibit airway inflammatory symptoms. The mechanism of the synergistic effect of IL-12 and IL-18 on the inhibition of the airway inflammatory symptoms is still unclear, but several explanations can be postulated. First, although IL-18 itself is unable to induce differentiation to the Th1-like phenotype, it has been shown to enhance IL-12-induced Th1 differentiation (12). In our experiments, the relatively low dose of IL-12 used seems not sufficient to enhance the development to Th1-like cells or to inhibit the development of Th2-like cells. This could explain why treatments with either IL-12 or IL-18 were not capable of inhibiting the Th2-like cell development, while the combined treatment was effective. Second, it has been shown that IL-12 can enhance the IL-12-induced IFN-γ production (11–14). Previously, we (24) and others have shown that treatment with IFN-γ can inhibit IgE production (25, 26), and several airway inflammatory processes such as airway hyperresponsiveness (17, 26) and eosinophilic infiltration (31, 35, 36). Although IFN-γ has no effect on IL-4 and IL-5 production by T cells upon restimulation in vitro (24), it has been shown to inhibit the proliferation of Th2-like cells, resulting in a less pronounced Th2 skewing. However, using IFN-γ receptor-deficient mice, it was shown that the inhibition of eosinophil influx by treatment with IL-12 during the challenge period is independent of IFN-γ (22). Thus, it remains possible that IFN-γ-independent mechanisms are involved in the effects of IL-12 and IL-18 in vivo. Third, an explanation for the synergistic effect of IL-12 and IL-18 can be found in the fact that IL-12 can up-regulate the expression of IL-18R (13), and in this way stimulate the effects mediated by IL-18.

From the present study, we can conclude that the combination of low doses of IL-12 and IL-18 treatment can prevent Th2-like cell development concurrent with inhibition of airway symptoms. More knowledge is required to fully understand the synergistic mechanism of IL-12 and IL-18 in vivo.

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