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Involvement of IL-16 in the Induction of Airway Hyper-Responsiveness and Up-Regulation of IgE in a Murine Model of Allergic Asthma[1]

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Experiments were designed to investigate the role of IL-16 in a mouse model of allergic asthma. OVA-sensitized mice were repeatedly exposed to OVA or saline aerosols. Bronchoalveolar lavage fluid (BALF) was collected after the last aerosol, and the presence of IL-16 was evaluated using a migration assay with human lymphocytes. Migration of lymphocytes was significantly increased in the presence of cell-free BALF from OVA-challenged mice compared with BALF from saline-challenged controls. This response was significantly inhibited after addition of antibodies to IL-16, demonstrating the presence of IL-16 in BALF of OVA-challenged animals. Immunohistochemistry was performed and revealed IL-16 immunoreactivity particularly in airway epithelial cells but also in cellular infiltrates in OVA-challenged mice. IL-16 immunoreactivity was absent in nonsensitized animals; however, some reactivity was detected in epithelial cells of sensitized but saline-challenged mice, suggesting that sensitization induced IL-16 expression in airway epithelium. Treatment of mice with antibodies to IL-16 during the challenge period significantly suppressed up-regulation of OVA-specific IgE in OVA-challenged animals. Furthermore, antibodies to IL-16 significantly inhibited the development of airway hyper-responsiveness after repeated OVA inhalations, whereas the number of eosinophils in bronchoalveolar lavage or airway tissue was not affected. In conclusion, IL-16 immunoreactivity is present in the airways after sensitization. After repeated OVA inhalation, IL-16 immunoreactivity is markedly increased and IL-16 is detectable in BALF. Furthermore, IL-16 plays an important role in airway hyper-responsiveness and up-regulation of IgE but is not important for eosinophil accumulation in a mouse model of allergic asthma. ***The Journal of Immunology*****, 1998, 160: 2998–3005.**

In 1982, a potent chemotactic factor for T lymphocytes named lymphocyte chemoattractant factor or LCF was described (1, 2). This cytokine, which is now identified as IL-16, appears to be uniquely distinguished from other cytokines with respect to its chemical and biologic functions (3, 4). After it had been discovered that IL-16 utilizes the CD4 molecule as its receptor (5), functional chemotactic responses on other $CD4^+$ leukocytes were investigated, and IL-16 was subsequently shown to be a potent monocyte and eosinophil chemoattractant as well (5, 6). Migration of human eosinophils is elicited by IL-16 at concentrations of 10^{-12} to 10^{-11} M, indicating that IL-16 is more potent than platelet-activating factor PAF or C5a, two recognized eosinophil chemoattractants (6). Chemotactic responses evoked by IL-16 are proportional to the amount of cell surface-expressed CD4 (5). In addition to its chemotactic properties, IL-16 induces expression of the IL-2 receptor on T lymphocytes and expression of MHC class II molecules on both T lymphocytes and monocytes (5). Effects on eosinophil activation or priming, however, could not be found (6). Although IL-16 mRNA is constitutively expressed in both human $CD4^+$ and $CD8^+$ T lymphocytes, it has thus far been reported to be secreted from $CD8^+$ T lymphocytes only (7). Interestingly, IL-16 is produced by $CD8^+$ T lymphocytes and $CD8^+$ T cell clones after stimulation with histamine or serotonin (7–9), mediators released by human and murine mast cells after IgE cross-linking. Furthermore, airway epithelial cells of asthmatics but not normals or atopic nonasthmatics contain both IL-16 mRNA and protein (10). Although not rigorously proved, data from Bellini et al. (11) indicate that epithelial cells from asthmatics release IL-16 after stimulation with histamine in vitro. Since histamine and serotonin induce release of IL-16 and since IL-16 is a potent eosinophil and lymphocyte chemoattractant, IL-16 may be a likely candidate to play an important role in airway inflammation observed in patients with allergic asthma. This hypothesis is supported by the recent finding that IL-16 is present in bronchoalveolar lavage fluid (BALF)[3] of allergic asthmatics at 6 h after antigen provocation but not in Ag-challenged normal subjects (12). In our laboratory, we have developed a murine model of allergic asthma (13–15). Sensitization of BALB/c mice with OVA (without adjuvant) induces a high level of OVA-specific IgE in serum (14). Inhalation of OVA in sensitized mice causes an immediate bronchoconstrictive response that lasts for at least 15 min (15). Using electron microscopy, we have observed mast cell degranulation within the

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[3] Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; BAL, bronchoalveolar lavage; hpf, high power field; MIP1α, macrophage-inflammatory protein-1α.

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first 30 min after OVA inhalation in sensitized mice (14). Furthermore, repeated inhalation of OVA in sensitized animals induces nonspecific airway hyper-responsiveness in vivo, and infiltration of both eosinophils and $CD4^+$ cells in BAL and airway tissue (13, 16). In addition, the Th2-type cytokines IL4 and IL5 are detectable in BALF (17). In the present study, we investigated whether IL-16 was present in BALF after antigen challenge in sensitized animals, and we performed immunohistochemistry to localize IL-16 immunoreactivity in the airways. Furthermore, the role of IL-16 in upregulation of IgE, eosinophil infiltration, and development of airway hyper-responsiveness was investigated using mAbs to IL-16.

Materials and Methods

Sensitization and challenge

Specified pathogen-free male BALB/c mice (6–8 wk) were obtained from the breeding colony of the National Institute for Public Health and the Environment, Bilthoven, The Netherlands. The mice were housed in Macrolon cages and provided with food and water ad libitum. Active sensitization was performed by seven i.p. injections of 10 μg of OVA (grade II) in 0.5 ml of pyrogen-free saline on alternate days (one injection per day). This sensitization procedure induces high titers of total IgE Abs in serum of which 80% was OVA-specific IgE (14). Four weeks after the last injection, mice were exposed either to eight OVA (2 mg/ml) or eight saline aerosols, on consecutive days (one challenge per day). The aerosol was generated with an ultrasonic nebulizer (Medix 8001; particle size, 3–5 μm) connected to a Plexiglas exposure chamber (5 liters). Animals were exposed for 5 min in maximal groups of six. All experimental procedures were approved by the Dutch Committee of Animal Experiments.

Treatment with Abs to IL-16

To verify the effectiveness of neutralizing anti-human IL-16 Abs for mouse IL-16, human lymphocytes were incubated with supernatant of Con A-stimulated (3 μg/ml, 37°C, 24 h) mouse splenocytes in the presence of various anti-human IL-16 Abs or control Abs. In a migration assay (described below), it appeared that the migratory response of lymphocytes was significantly inhibited by all anti-IL-16 Abs tested (Table I), indicating that anti-human IL-16 Abs are capable of neutralizing mouse IL-16.

Abs to IL-16 were given to the mice only during the challenge period. This approach was chosen so as not to interfere with the induction of IgE during the OVA sensitization period. Thirty minutes before the first and fifth OVA or saline inhalation, OVA-sensitized mice were injected i.v. with 500 μg (injection volume, 80 μl) of a mouse anti-human IL-16 mAb (clone 14.1, IgG2a) or with an equal amount of isotype control Ab. The isotype control Ab applied was a mouse mAb directed against Semliki Forest virus (clone UM 8-139), an Ag that is normally not present in mice. This Ab was generously provided by M. Harmssen of the Eijkman-Winkler Laboratory for Medical Microbiology, University Hospital Utrecht, Utrecht, The Netherlands. Both control and anti-IL-16 Abs were shown to be LPS negative (<0.125 U/ml). Ab solutions were freshly prepared and diluted in PBS (pH 7.4).

Table I. *Migration of human lymphocytes induced by the supernatant of Con A-stimulated mouse splenocytes in the presence of Abs to IL-16 or control Abs*[a]

Ab	Migration (Cells/hpf)
No Ab	29.7 ± 1
Polyclonal	
Preimmune serum	29.2 ± 1
Rabbit anti-human IL-16	18.5 ± 1[b]
Monoclonal	
Isotype control Ab	29.7 ± 1
Mouse anti-human IL-16	21.0 ± 1[b]

[a] Both the polyclonal rabbit anti-human and the monoclonal mouse anti-human Abs were used at a concentration of 5 μg/ml. This concentration is sufficient to block migration induced by 0.1 nM recombinant human IL-16, which induces maximal migration of human lymphocytes. Samples were tested in triplicate, and values are expressed as mean ± SD. *, Significantly different from the migratory response in presence of preimmune serum or control Ab, respectively ($p < 0.05$).

Cell preparation and migration assay

Human lymphocytes were isolated from heparinized venous blood samples of healthy normal volunteers by density centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The resulting cell layer containing PBMC was recovered and washed three times in Medium 199 supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated on a nylon wool column at 37°C and 5% CO_2 for 45 min. The cell population eluted from the column contained >97% T lymphocytes as determined by fluorescent staining with anti-CD3 mAb (Becton Dickinson, Mountain View, CA). Migration of lymphocytes was assessed using a modified Boyden chemotaxis chamber as described by Cruikshank et al. (5). The lymphocytes, 1×10^7 cells in 50 μl of Medium 199 enriched with 0.4% BSA, were loaded into the upper well of the chamber and 30 μl of cell-free BALF derived from OVA or saline-challenged mice were placed in the lower well. For blocking experiments, rabbit anti-human IL-16 polyclonal Ab was added to the lower well as well. In previous experiments, it was shown that 5 μg/ml anti-IL-16 Ab neutralizes 0.1 nM recombinant human IL-16 protein. The upper and lower well were separated by a nitrocellulose filter with a pore size of 8 μm. The chamber was incubated for 3 h, and afterward the filter was fixed and stained with hematoxylin. Migration was quantified by counting the number of cells that migrated beyond a depth of 50 μm utilizing an Optomax automated image analyzer (Burlington, MA). All migration data are expressed as the number of cells per high power field (hpf). All samples were performed in triplicate. On average 14 to16 cells/hpf were counted under control conditions.

Immunohistochemistry

Nonsensitized mice and OVA-sensitized animals challenged with OVA or saline were anesthetized with a mixture of Ketalar (35 mg/ml), xylazine (0.6%/ml), and atropine (0.1 mg/ml), of which 0.2 ml was injected i.m. Thereafter, abdomen and chest were opened, and the abdominal aorta was incised. The vascular bed of the lungs was perfused with PBS (37°C), through injection via the right heart ventricle. The lungs were removed and filled intratracheally with 1 to 2 ml of fixation solution (0.8% formalin, 4% acetic acid). Subsequently, the trachea was tied off with a ligature, and the lungs were immersed in the fixative for at least 24 h. Then the tissues were dehydrated and embedded in Paraplast (Monoject, Kildare, Ireland). Transverse sections of 5 μm were prepared, subsequently deparaffinized, and hydrated by submerging in xylenes followed by reagent grade alcohol. The sections were rinsed for 5 min and incubated with 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. After a washing in PBS for 5 min, the sections were incubated with goat serum (1:100 diluted in PBS) for 20 min, and afterward the excess of serum was blotted from the sections. Then the sections were incubated with mouse anti-human IL-16 mAb (clone 14.1, IgG2a, 20 μg/ml) or an isotype control Ab for 30 min. The sections were washed in PBS for 5 min, and a goat anti-mouse Ig secondary Ab (1:100 diluted in PBS) was applied for 30 min. After a washing, sections were incubated with Vectastain ABC reagent (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 min. The sections were washed again and stained with peroxidase substrate solution until the desired intensity was reached. After a rinsing in running water, the sections were counterstained with hematoxylin. The used reagents and protocol were derived from the commercially available Vectastain Elite ABC kit. At least five mice per group were examined.

OVA-specific IgE ELISA

Using an ELISA as described by De Bie et al. (16), the presence of OVA-specific IgE was measured in serum samples derived from OVA or saline-challenged mice at 24 h after the last challenge. In short, 96-well microtiter plates were coated with recombinant human FcϵR1-IgG fusion protein (2 μg/ml, 4°C, 24 h). After a washing, plates were blocked with ELISA buffer, which contained 2 mM EDTA, 137 mM NaCl, 50 mM Tris, 0.5% BSA, and 0.05% Tween-20 (pH 7.2) (room temperature, 1 h). After removal of the ELISA buffer, plates were incubated with serum samples and duplicate dilution series of an OVA-specific IgE reference standard (room temperature, 2 h). The standard was obtained by i.p. immunization of mice with OVA according to previously published methods (18) and arbitrarily assigned a value of 10,000 U/ml OVA-specific IgE. After washing, OVA (10 μg/ml) was added to each well and the mixture was incubated at room temperature for 1 h. This step was followed by washing and incubation with horseradish peroxidase-conjugated goat anti-OVA mAb (room temperature, 1 h). Then, *o*-phenylenediamine (10 mM) was added, and after 15 min at room temperature the reaction was stopped by addition of 4 M H_2SO_4. The optical density was read at 492 nm using a Titertek Multiskan (Flow Laboratories, Irvine, U.K.). Serum samples were compared with the

OVA-specific IgE reference standard, and values were expressed in units per milliliter. FcεR1-IgG, OVA-specific IgE reference standard, and horseradish peroxidase-conjugated goat anti-OVA mAb were generously provided by Dr. P. M. Jardieu (Genentech Inc., South San Francisco, CA).

Airway responsiveness in vivo

Airway responsiveness to methacholine was measured in vivo 24 h after the last aerosol exposure using the air-overflow pressure method as previously described (19). With this method, bronchial resistance to inflation is measured. Mice were anesthetized by i.p. injection of urethan (2 g/kg) and placed on a heated blanket (30°C). Then the trachea was cannulated, and a small polyethylene catheter was placed in the jugular vein for i.v. administrations. Spontaneous breathing was suppressed by i.v. injection of tubocurarine chloride (3.3 mg/kg). When it stopped, the tracheal cannula was attached to a respiration pump (C. F. Palmer, London, U.K.). The inflation volume of the pump was 0.8 ml of which the mouse inhales ~0.1 ml per breath with a rate of 190 breaths per min. The ventilation circuit contained a pressure transducer (MPB-6207, Depex, Bilthoven, The Netherlands). Any increase in airway tone causes a reduction of the amount of air floating into the lungs, and subsequently the remainder overflows, resulting in an increase in air-overflow pressure. Pressure signals were recorded breath by breath on a Graphtex thermal arraycorder (Ankersmit, Breda, The Netherlands). At intervals of at least 4 min and after the response had returned to baseline level, doubling doses of methacholine ranging from 40 to 1280 μg/kg were administered. Concentrations of methacholine were prepared in saline and kept on ice for the duration of the experiment. At the end of the dose-response curve, the maximal response was determined by clamping the tracheal cannula. The increase in air-overflow pressure was measured at its peak and expressed as percentage increase of the maximal response. At least six mice were evaluated per group.

Bronchoalveolar lavage

Three and 24 hours after the last aerosol, mice were anesthetized by i.p. injection of 0.25 ml of sodium pentobarbitone (60 mg/ml). The abdomen and chest were opened, and the abdominal aorta was incised. Below the larynx, a small incision was made, and a flexible polyethylene cannula (PE 50, Intramedic, Clay Adams, NJ) was inserted into the trachea and fixed with a ligature. Subsequently, the mice were lavaged five times with 1-ml aliquots of pyrogen-free saline warmed to 37°C. The first milliliter of saline was supplemented with aprotinine (2 μg/ml) and was centrifuged after withdrawal (400 × *g*, 4°C, 5 min). The supernatant was immediately frozen at −70°C. Cells derived from this first milliliter of BALF were pooled with the other BAL cells collected. Then all cells were washed with cold PBS (400 × *g*, 4°C, 5 min), and the pellet was resuspended in 200 μl of cold PBS. Total numbers of BAL cells were counted in a Bürker-Türk chamber. For differential BAL cell counts, cytospin preparations were made and stained with Diff-Quick (Merz and Dade AG, Düdingen, Switzerland). After coding, all cytospin preparations were evaluated by one observer using oil immersion microscopy. Cells were identified and differentiated into mononuclear cells, neutrophils, and eosinophils by standard morphology. Per cytospin preparation, at least 400 cells were counted and the absolute number of each cell type was calculated. To evaluate differences between OVA and saline-challenged groups and to evaluate the effects of treatment with Abs to IL-16, total BAL cell numbers and the numbers of the various BAL cell types were tested with an analysis of variance. For cell types with a very low number in control animals (i.e., eosinophils or neutrophils) a Poisson distribution was assumed.

Eosinophil counts in tracheal tissue

Twenty-four hours after the last aerosol exposure, mice were anesthetized by i.p. injection of 0.25 ml of sodium pentobarbitone (60 mg/ml). The abdomen and chest were opened, and the abdominal aorta was incised. The vasculature of the lungs was perfused with a mixture of paraformaldehyde (2%) and glutaraldehyde (2.5%) in cacodylate buffer (0.1 M, pH 7.4) injected via the right heart ventricle. The trachea was isolated and immersed in the same paraformaldehyde-glutaraldehyde mixture as used for perfusion. After 2 wk, the trachea was cut into pieces and embedded in Paraplast. Before embedding in Paraplast, the tissues were thoroughly rinsed in tap water and routinely processed. Transverse sections of 5 μm were prepared and stained according to Luna's method for eosinophil staining (20). Using light microscopy, the sections were examined for the number of eosinophils. Per mouse two to three tracheal sections were evaluated, and a mean number per section was calculated. Differences in eosinophil numbers were tested with a nonparametric Mann-Whitney *U* test. Four to six mice were examined per group.

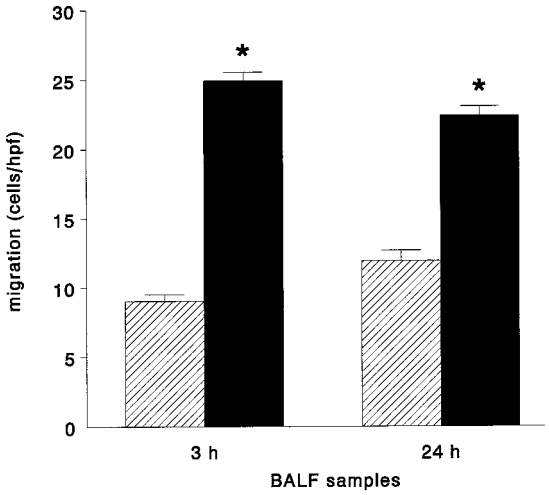

FIGURE 1. Migration of human lymphocytes induced by BALF samples derived from OVA-sensitized mice challenged either with saline (▨) or OVA (■) at 3 or 24 h after the last aerosol exposure. Migration is expressed as the number of cells per hpf. Samples were tested in triplicate, and values are expressed as mean ± SEM. *, significantly different from the response in OVA-sensitized and saline-challenged controls ($p < 0.05$).

Chemicals

OVA, aprotinine, ExtrAvidin peroxidase, 2,2′-azinobis(3-ethylbenzthiazoline 6-sulfuric acid) and *o*-phenylenediamine were purchased from Sigma Chemical Company (St. Louis, MO), urethan and methacholine from Janssen Chimica (Beerse, Belgium), Ketalar from Aesculaap (Boxtel, The Netherlands), xylazine from Bayer (Leverkusen, Germany), atropine from Brocacef (Utrecht, The Netherlands), tubocurarine chloride from Nogepha (Alkmaar, The Netherlands), sodium pentobarbitone (Nembutal) from Abbott Laboratories (North Chicago, IL), and Medium 199 from M.A. Bioproducts (Wakersville, MD).

Data analysis

Unless stated otherwise, data are expressed as mean ± SEM and evaluated using an analysis of variance followed by post hoc comparison between groups. A difference was considered to be significant when $p < 0.05$. Statistical analyses were conducted using SYSTAT, version 5.03 (SYSTAT Inc., Evanston, IL) or GLIM, version 4.0 (NaG Inc., Oxford, U.K.).

Results

Presence of IL-16 bioactivity in BALF

When BALF samples were tested in a migration assay, it was observed that BALF derived from OVA-sensitized and OVA-challenged mice induced a significantly enhanced migration of lymphocytes when compared with the migratory response induced by BALF from OVA-sensitized and saline-challenged mice (Fig. 1). This was observed both with BALF collected at 3 and 24 h after the last aerosol exposure. BALF derived from saline-challenged mice inhibited migration in comparison with the migratory response observed in the presence of medium, which was 15.3 cells per high power field. When BALF samples of OVA-challenged mice were incubated with rabbit anti-human IL-16 polyclonal Ab, a significant inhibition of migration was observed (Fig. 2), demonstrating the presence of IL-16 in these samples. Inhibition of migration was less at 24 h than at 3 h. This observation indicates that the IL-16-induced lymphocyte motility is transiently expressed at early time points. Incubation of BALF samples of saline-challenged mice with Abs to IL-16 had no effect on the migratory response. Neither did Abs to IL-16 affect migration in response to medium alone (data not shown).

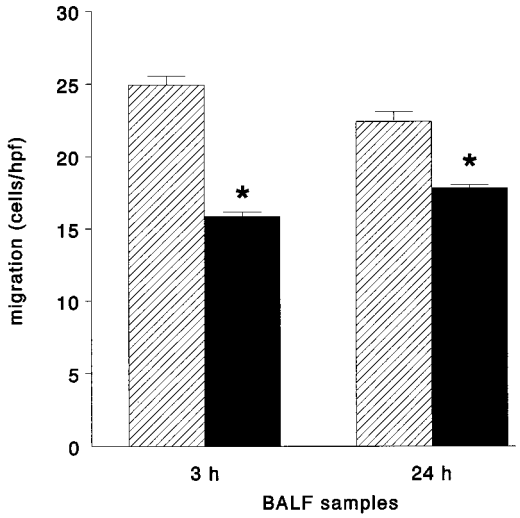

FIGURE 2. Migration of human lymphocytes induced by BALF samples derived from OVA-sensitized and OVA-challenged mice at 3 or 24 h after the last aerosol exposure in absence (▨) or presence (■) of a rabbit anti-human anti-IL-16 Ab (5 μg/ml). There was no effect of the anti-IL-16 Ab on the migratory response to medium alone. Nor did incubation with Abs to IL-16 affect the migratory response induced by BALF samples of saline-challenged mice (data not shown). Migration is expressed as the number of cells per hpf. Samples were tested in triplicate, and values are expressed as mean ± SEM. *, significantly different from migration in the absence of Abs to IL-16 ($p < 0.05$).

BALF collected from mice at 3 and 24 h after the last OVA challenge contained a significant number of eosinophils, whereas these cells were not observed in BALF of saline-challenged control mice (Table II). At 24 h, the number of eosinophils was 3.5 times higher then at 3 h. When the absolute numbers of eosinophils in BALF of OVA-challenged mice were compared with the percentage of lymphocyte migration induced in vitro by BALF samples derived from the same mice, no significant correlation was observed ($r = 0.529$, $p = 0.178$).

Presence of IL-16 immunoreactivity in the airways

IL-16 immunoreactivity was identified in airway sections of OVA-sensitized and OVA-challenged mice (Fig. 3*A*). In these mice, IL-16 was localized predominantly in the epithelium (Fig. 3*A*), but also IL-16-positive cells were detected in the cellular infiltrates surrounding bronchi and blood vessels (Fig. 3*C*). In sections of OVA-sensitized and saline-challenged mice but not in sections of nonsensitized mice, some IL-16 immunoreactivity was found in epithelial cells, suggesting that the sensitization procedure already induced the expression of IL-16 (Fig. 3, *E* and *F*, respectively). No staining could be detected in sections of OVA-sensitized and OVA-challenged mice incubated with control Ab (Fig. 3, *B* and *D*).

Table II. *Absolute numbers of total cells, mononuclear cells, neutrophils, and eosinophils in BALF of OVA-sensitized mice at 3 or 24 h after the last OVA or saline aerosol exposure*[a]

Time Point	Group	Total Cells ($\times 10^{-5}$)	Mononuclear Cells ($\times 10^{-5}$)	Neutrophils ($\times 10^{-3}$)	Eosinophils ($\times 10^{-3}$)
3	OA	3.5 ± 0.3	3.5 ± 0.3	1.2 ± 0.7	4.0 ± 1.2*
	SAL	4.1 ± 0.7	4.1 ± 0.6	1.6 ± 0.8	0.0 ± 0.0
24	OA	2.7 ± 0.5	2.5 ± 0.4	2.0 ± 1.1	14.4 ± 6.1*
	SAL	2.5 ± 0.5	2.4 ± 0.5	0.5 ± 0.3	0.0 ± 0.0

[a] Values are expressed as mean ± SEM. OA, OVA-challenged mice; SAL, saline-challenged mice. *, Significantly different from saline-challenged mice. Among OVA- and saline-challenged groups, no significant difference was observed in the number of total cells, the number of mononuclear cells, or the number of neutrophils.

Presence of IL-16 bioactivity in BALF after in vivo treatment with Abs

BALF derived from OVA-challenged mice treated with control Ab induced an increased migratory response compared with the response induced by BALF from control Ab-treated saline-challenged mice ($p < 0.001$, Fig. 4). After in vivo treatment with Abs to IL-16, this enhanced migratory response toward BALF of OVA-challenged mice was significantly ($p < 0.01$, Fig. 4), although not completely, inhibited. When Abs to IL-16 were added in vitro, no additional blocking of migration was observed, indicating that IL-16 generated in vivo was completely inhibited (data not shown). Migration induced by BALF from saline-challenged mice was not affected by in vivo treatment with Abs to IL-16.

Effect of treatment with Abs to IL-16 on IgE up-regulation

In control Ab-treated mice, repeated inhalation of OVA induced a marked up-regulation of the amount of OVA-specific IgE in serum compared with their saline-challenged controls ($p < 0.001$, Fig. 5). Interestingly, when mice were treated with Abs to IL-16 and challenged with OVA this up-regulation was significantly decreased (55%, $p < 0.01$). OVA-specific IgE levels measured in the anti-IL-16-treated OVA-challenged mice were still increased compared with anti-IL-16-treated saline-challenged mice; however, this did not reach the level of significance ($p = 0.06$). No effect of treatment with Abs to IL-16 on the amount of OVA-specific IgE detected in saline-challenged mice was observed.

Effect of treatment with Abs to IL-16 on airway hyperresponsiveness

The responsiveness to methacholine was significantly increased in control Ab-treated OVA-challenged mice compared with control Ab-treated saline-challenged mice (Fig. 6*A*). The maximal potentiation in responsiveness observed in OVA-challenged animals was 44% at a dose of 320 μg/kg methacholine. After treatment with Abs to IL-16, the responsiveness to methacholine measured in OVA-challenged mice was not significantly different from the responsiveness measured in saline-challenged mice, indicating that the development of airway hyper-responsiveness in the OVA-challenged group was prevented (Fig. 6*B*). When anti-IL-16-treated OVA-challenged mice were compared with control Ab-treated OVA-challenged mice the responsiveness in anti-IL-16-treated mice was significantly decreased at doses of 320 and 640 μg/kg methacholine. Methacholine dose-response curves measured in the saline-challenged groups were not significantly different among the different treatment groups. Irrespective of the treatment, no changes in ED_{50} were observed and there was no difference in basal bronchial resistance to inflation between the various groups (data not shown).

Effect of treatment with Abs to IL-16 on eosinophils in BAL and trachea

No eosinophils were observed in BALF of both control Ab and anti-IL-16-treated mice that were exposed to saline aerosols. In contrast, after repeated OVA inhalations in control Ab-treated mice, a significant number of eosinophils was present ($p < 0.05$, Fig. 7*A*). Treatment with Abs to IL-16 did not affect the number of eosinophils present in the BALF after repeated OVA exposure (Fig. 7*A*). In addition, no effects of treatment with Abs to IL-16 were observed on the total amount of BAL cells, the number of

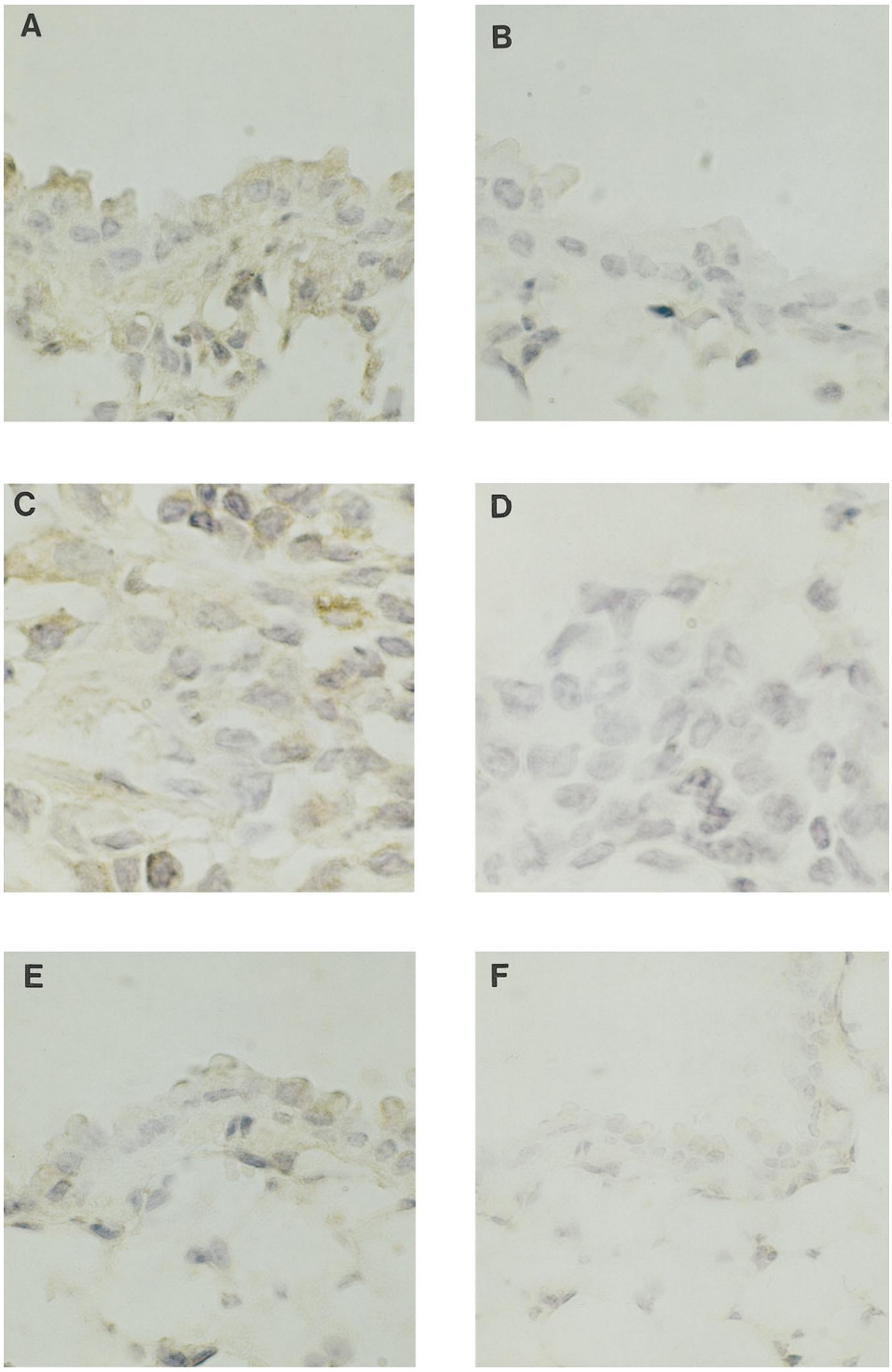

FIGURE 3. Localization of IL-16 immunoreactivity in airway sections of mice sensitized with OVA and exposed to repeated OVA inhalations demonstrating staining in the epithelium (*A*) and in the cellular infiltrates detected in the airways of these mice (*C*). No staining was observed after incubation with an isotype control Ab (*B* and *D*). Some IL-16 immunoreactivity was also detectable in sections of OVA-sensitized and saline-challenged mice (*E*) but not in sections of nonsensitized mice incubated with Abs to IL-16 (*F*).

mononuclear cells, or the number of neutrophils (Table III). Eosinophils were also counted in tracheal tissue. After exposure to repeated OVA aerosols, the number of eosinophils observed in the trachea was significantly increased in control Ab-treated mice compared with their saline-challenged controls ($p < 0.05$, Fig. 7*B*). In OVA-challenged animals treated with Abs to IL-16, the eosinophil number was also increased in comparison with their saline-challenged controls, indicating that treatment with Abs to IL-16 had no effect on the number of eosinophils in the trachea. Compared with OVA-challenged animals treated with control Ab, the number of eosinophils in the anti-IL-16-treated OVA-challenged group was higher; however, this was not significant.

Discussion

The study described in this report demonstrates the presence of IL-16 immunoreactivity in airway epithelium of mice after OVA sensitization. When mice are exposed to repeated OVA inhalations, the presence of IL-16 immunoreactivity in the airway tissue is markedly increased and IL-16 is detectable in BALF. Treatment of sensitized mice with Abs to IL-16 during the challenge period

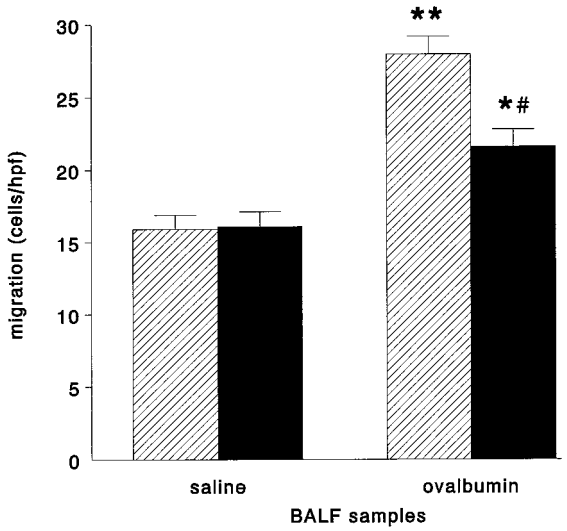

FIGURE 4. Migration of human lymphocytes induced by BALF samples derived from OVA-sensitized mice repeatedly challenged either with saline or OVA. Mice were treated during the challenge period with control Ab (▨) or Abs to IL-16 (■). Mean response ± SEM is shown. Migration is expressed as the number of cells per hpf. * and **, significantly different from the response in OVA-sensitized and saline-challenged controls ($p < 0.05$ and $p < 0.001$, respectively); #, significantly different from the response in control Ab-treated OVA-sensitized and OVA-challenged mice ($p < 0.01$). After addition of anti-IL-16 Ab in vitro, no additional blocking of the migratory response was observed, indicating that IL-16 generated in vivo was completely inhibited (data not shown).

suppresses the up-regulation of OVA-specific IgE observed in OVA-challenged mice. Furthermore, Abs to IL-16 markedly inhibit the induction of airway hyper-responsiveness in OVA-challenged mice, whereas there is no effect on the number of eosinophils in BAL or airway tissue.

BALF derived from OVA-sensitized and challenged mice at 3 and 24 h after the last OVA inhalation stimulated human lymphocyte migration in vitro. This migratory response was inhibited by Abs to IL-16, clearly demonstrating the presence of IL-16 in

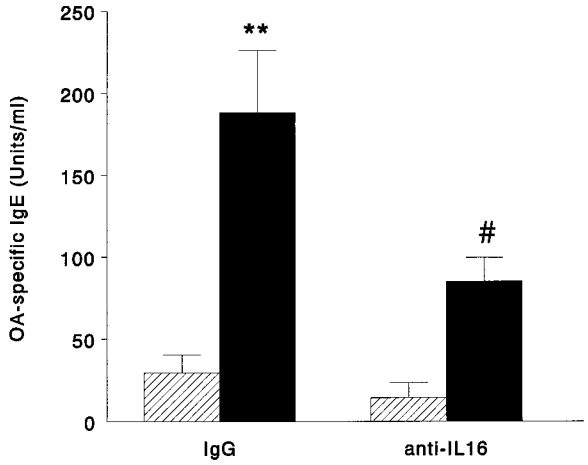

FIGURE 5. OVA-specific IgE measured in serum after repeated saline (▨) or OVA (■) inhalations in OVA-sensitized mice. During the challenge period, mice were treated with control Ab (IgG) or Abs to IL-16 (anti-IL-16). Data are expressed as mean ± SEM. **, significantly different from values measured in control Ab-treated saline-challenged mice ($p < 0.001$); #, significantly different from control Ab-treated OVA-challenged mice ($p < 0.01$).

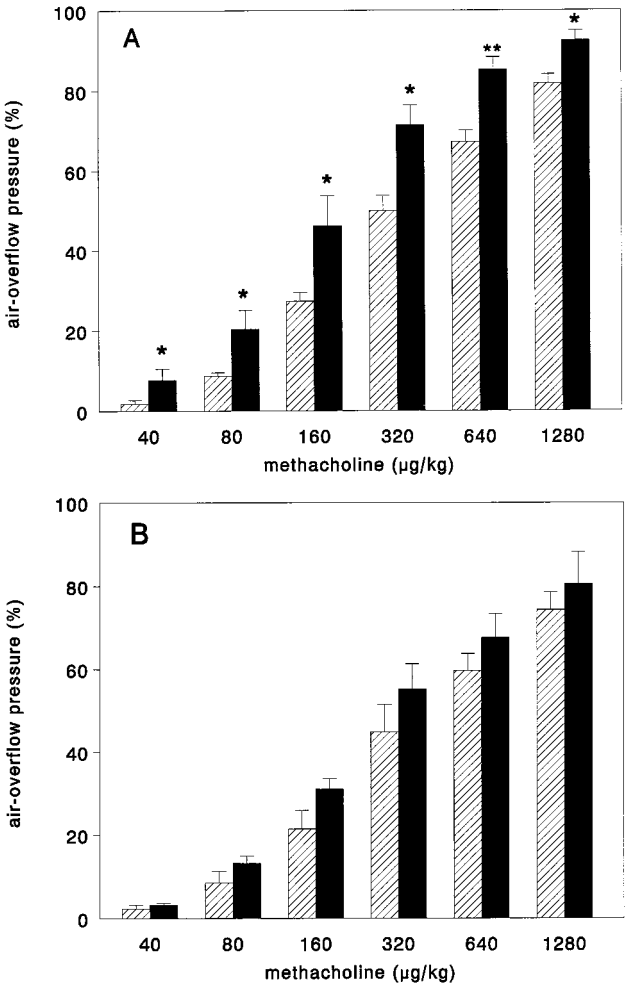

FIGURE 6. Airway responsiveness to methacholine measured in vivo in OVA-sensitized mice exposed to either repeated saline (▨) or OVA (■) aerosols. The increase in air overflow pressure (%) is shown in mice treated during the challenge period with control Ab (*A*) or Abs to IL-16 (*B*). Results are expressed as mean ± SEM. * and **, significantly different from value measured in corresponding OVA-sensitized and saline-challenged mice ($p < 0.05$ and $p < 0.01$).

BALF of the OVA-challenged animals. From our experiments, it appeared that the amount of IL-16 present in BALF was higher at 3 h than at 24 h, suggesting that IL-16 is transiently expressed at early time points. BALF obtained from OVA-sensitized and saline-challenged mice inhibited lymphocyte migration in comparison with migration induced by medium, indicating that BALF contains factor(s) that are inhibitory for migration. BALF samples applied in our experiments were not placed over an IL-16 affinity column and therefore may contain many different factors that can affect overall migration. As a result of this, the observed increase in migration in the OVA group could be caused by production of chemoattractant factors or loss of inhibitory factors. This is why neutralization studies are performed to determine the presence of a specific chemoattractant, IL-16. Our data are very similar to those described for humans (12). BALF obtained from allergen-challenged atopic asthmatics, but not from saline-challenged asthmatics, stimulates migration of human lymphocytes in vitro, and a significant part of this chemoattractant activity is attributable to IL-16 (12). In humans, IL-16 is detected early after antigen challenge, i.e., at 6 h (12), which is comparable to our observations in mice. In addition, in humans it has also been shown that BALF

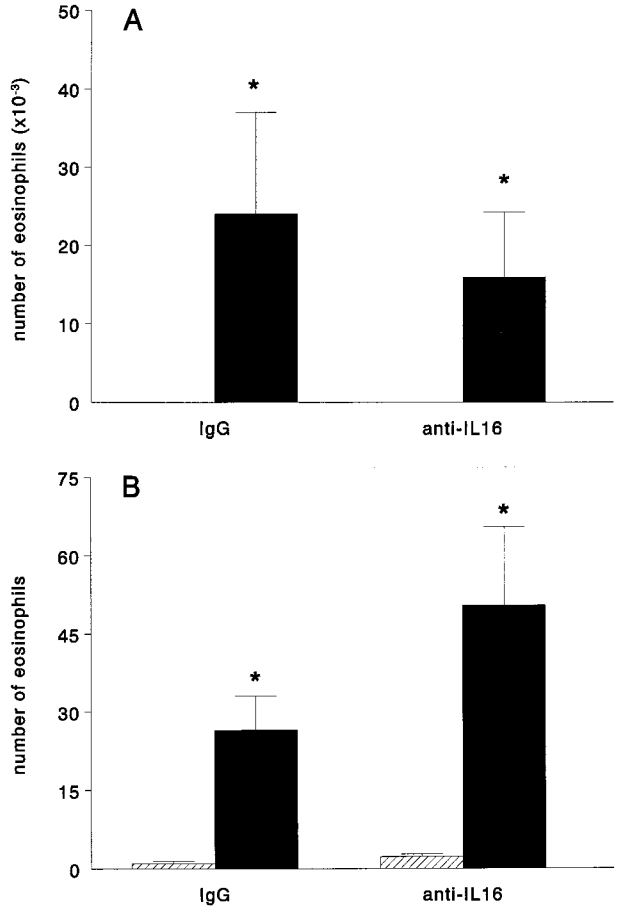

FIGURE 7. Number of eosinophils observed in BALF (*A*) and trachea (*B*) after repeated saline (▨) or OVA (■) aerosols in OVA-sensitized mice. Mice were treated during the challenge period with control Ab (IgG) or Abs to IL-16 (anti-IL-16). Values are expressed as mean ± SEM. *, significantly increased compared with the corresponding OVA-sensitized and saline-challenged control mice ($P < 0.05$). BALF of OVA-sensitized and saline-challenged mice did not contain eosinophils (*A*).

obtained after saline challenge (in both normal subjects and asthmatics) contains factors that inhibit migration of human lymphocytes (12).

Immunohistochemistry data demonstrate that IL-16 immunoreactivity was induced in the airway tissue through OVA sensitization and markedly increased after repeated OVA inhalations. Because the Ab applied in these studies detects both IL-16 and the IL-16 precursor protein, we hypothesize that OVA sensitization induces expression of IL-16 precursor protein in airway epithelial cells, which is cleaved and released into BALF after repeated exposure to OVA. Because we also found IL-16-positive cells within cellular infiltrates after repeated OVA exposure, it seems likely that these cells contribute to the release of IL-16. Our data are comparable with those found in humans. Laberge et al. (10) have shown that airway epithelial cells of asthmatics contain both IL-16 mRNA and protein. Interestingly, in that study, a significant association was demonstrated between epithelial IL-16 immunoreactivity and airway responsiveness to methacholine (10).

IgE cross-linking of human and murine mast cells induces the release of histamine and serotonin, mediators that stimulate IL-16 production by $CD8^+$ T lymphocytes (7, 8). Although not rigorously proved, other in vitro data suggest that epithelial cells also release IL-16 after stimulation with histamine (11). Previously, we have observed mast cell degranulation within 30 min after OVA challenge in sensitized mice (14). It may therefore be hypothesized that in our mouse model mast cell-derived mediators induce the secretion of IL-16 by $CD8^+$ T lymphocytes and/or epithelial cells after repeated OVA inhalations in sensitized mice.

In this study, sensitized mice were treated with Abs to IL-16 during the challenge period. The migratory response induced by BALF from these mice was clearly but not completely inhibited. Addition of Abs to IL-16 to the samples in vitro did not further inhibit the migratory activity. Therefore, it can be concluded that all IL-16 present in the BALF was blocked by in vivo treatment with Abs to IL-16. Furthermore, these results indicate that, besides IL-16, BALF obtained after repeated OVA inhalation also contains other factors that induce migration of lymphocytes. A possible candidate may be macrophage-inflammatory protein-1α (MIP1α), which is a chemoattractant for lymphocytes (21) and has been detected in lung tissue in a mouse model of airway inflammation (22). In humans, it has been shown that the remaining migration activity after addition of Abs to IL-16 is attributable to MIP-1α and not to RANTES (12).

Treatment with Abs to IL-16 decreased up-regulation of OVA-specific IgE during repeated OVA inhalation in sensitized mice. The production of IgE is dependent on $CD4^+$ T lymphocytes (23), and since IL-16 binds to the CD4 molecule (5) it is possible that it may act as a costimulus. In this way it may be involved in the process of IgE up-regulation. Alternatively, IL-16-induced production of IFN-γ may up-regulate OVA-specific IgE in previously sensitized mice. IL-16 has been found to induce the release of IFN-γ after incubation with T lymphocytes (W. W. Cruikshank, unpublished observation), and we have previously demonstrated that memory IgE responses can be up-regulated by IFN-γ (22). More experiments are needed, however, to elucidate the precise mechanism by which IL-16 stimulates up-regulation of IgE.

Interestingly, treatment of sensitized and OVA-challenged mice with Abs to IL-16 during the challenge period markedly inhibited the development of airway hyper-responsiveness. This observation demonstrates the importance of IL-16 in the induction of airway hyper-responsiveness. We can only speculate how IL-16 induces airway hyper-responsiveness in our murine model of allergic asthma. In the present study, treatment with Abs to IL-16 did not inhibit the process of eosinophil infiltration. In addition, there was no correlation between the amount of IL-16 in BALF and the number of eosinophils in BAL. These observations in itself do not rule out the involvement of eosinophils in the development of airway hyper-responsiveness because Abs to IL-16 could have acted at the level of eosinophil activation. However, since it has been described that IL-16 has no effect on eosinophil activation or priming (6), this explanation seems unlikely. An alternative explanation for

Table III. *Absolute numbers of total cells, mononuclear cells, neutrophils, and eosinophils in BALF of OVA-sensitized mice treated either with control Ab or with Abs to IL-16 during the OVA or saline challenge period*[a]

Treatment	Group	Total Cells ($\times 10^{-5}$)	Mononuclear Cells ($\times 10^{-5}$)	Neutrophils ($\times 10^{-3}$)	Eosinophils ($\times 10^{-3}$)
Control Ab	OA	5.7 ± 1.0	5.0 ± 0.6	3.5 ± 1.7	24.0 ± 13.0*
	SAL	7.1 ± 1.2	7.1 ± 1.2	1.8 ± 1.4	0.0 ± 0.0
Anti-IL-16	OA	4.6 ± 0.7	4.4 ± 0.7	2.7 ± 1.2	15.9 ± 8.4*
	SAL	4.4 ± 0.5	4.3 ± 0.6	4.7 ± 2.1	0.0 ± 0.0

[a] Values are expressed as mean ± SEM. OA, OVA-challenged mice, SAL, saline-challenged mice. *, Significantly different from saline-challenged mice. Among OVA- and saline-challenged groups, no significant difference was observed in the number of total cells, the number of mononuclear cells, or the number of neutrophils. Compared with control antibody, treatment with Abs to IL-16 had no significant effect on the absolute number of any cell type evaluated.

the importance of IL-16 in the induction of airway hyper-responsiveness may be that IL-16 acts via other cells. A likely candidate is the $CD4^+$ T lymphocyte, since depletion of these T cells inhibits the development of airway hyper-responsiveness in a mouse model of Ag-induced airway hyper-responsiveness (25). IL-16 activates T lymphocytes and induces expression of MHC class II molecules and the IL-2 receptor on human T lymphocytes (5). In addition, as mentioned above, incubation of T lymphocytes with IL-16 induces the release of IFN-γ. We have recently demonstrated that Abs to IFN-γ could inhibit airway hyper-responsiveness without any effect on the presence of eosinophils in BAL (19). It is tempting to speculate that Ag inhalation in sensitized animals causes mast cell degranulation, leading to the early release of IL-16 by $CD8^+$ T lymphocytes and/or epithelial cells, which subsequently induces IFN-γ production by lymphocytes. IFN-γ in its turn may be involved in the development of airway hyper-responsiveness without affecting eosinophil infiltration.

Although IL-16 has been described as a potent human eosinophil chemoattractant in vitro (6), treatment with Abs to IL-16 during the challenge period had no effect on the number of eosinophils appearing in BALF or airway tissue in our mouse model. These results indicate that IL-16 plays no crucial role in this process. It is known, however, that there is a great redundancy in cytokines capable of evoking eosinophil infiltration (26). Furthermore, it has been described that chemotactic responses induced by IL-16 are proportional to the amount of cell surface-expressed CD4 (5), and thus an alternative explanation for our findings may be that mouse eosinophils may not express high numbers of CD4 on their surface. We were not able to detect CD4 molecules on mouse eosinophils as determined by flow cytometry (data not shown). On the basis of our results, we conclude that IL-16 may not be a potent chemoattractant factor for mouse eosinophils in vivo.

In summary, IL-16 immunoreactivity is present in the airways after sensitization. After repeated OVA inhalation, IL-16 immunoreactivity is markedly increased and IL-16 is detectable in BALF. Treatment in vivo with Abs to IL-16 suppresses the upregulation of OVA-specific IgE during the OVA challenge period. Furthermore, Abs to IL-16 markedly inhibit the development of airway hyper-responsiveness without an effect on the infiltration of eosinophils. Altogether, these data suggest a prominent role for IL-16 in this mouse model of allergic asthma.

Acknowledgments

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