

Tumor Necrosis Factor- α –Induced Synthesis of Interleukin-16 in Airway Epithelial Cells

Priming for Serotonin Stimulation

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Epithelial cells from individuals with asthma or from allergen-sensitized mice contain intracellular interleukin (IL)-16 protein, not present in epithelial cells from individuals without asthma or unsensitized mice. IL-16 is only present in the bronchoalveolar lavage (BAL) fluid following airway challenge with either allergen or vasoactive amine. This suggests that the initial response to allergen (sensitization) results in synthesis but not secretion of IL-16. In this study, we investigated what factors produced during the sensitization phase are responsible for epithelial cell priming for IL-16 production. We determined that ovalbumin (OVA)-sensitized mice have an increase in systemic tumor necrosis factor- α levels, and that serum or BAL fluid stimulation of bronchial epithelial cells results in production of IL-16 that is subsequently secreted only following serotonin stimulation. The mechanism for IL-16 production was shown to be caspase-3–dependent, and serotonin-induced secretion of IL-16 required binding of the serotonin type 2 receptor. The relevance of the priming effect associated with sensitization for IL-16 production and storage was confirmed *in vivo* by serotonin airway challenge of OVA-sensitized mice, resulting in rapid secretion of IL-16 into BAL fluid. As IL-16 has been shown to regulate CD4+ cell recruitment and activation, and is detected early following airway challenge of individuals with asthma, this two-step process for IL-16 production by epithelial cells may represent a rapid response mechanism in the orchestration of allergic airway inflammation.

The cellular components of allergic airway inflammation are primarily lymphocytes, eosinophils, and mast cells (1–3). Influx of these cells to the bronchial epithelium and subepithelium likely occurs due to chemoattractant factors generated by both resident epithelial cells and recruited effector cells responsible for perpetuating the immune response. Mast cells and eosinophils, through the release of vasoactive amines, tryptases, and leukotrienes, are directly responsible for several of the physiologic alterations consequent to allergic inflammation, such as bronchoconstriction and mucosal edema. CD4+ lymphocytes of the T-helper 2 (T_H2) subtype

have been implicated in both initiation and perpetuation of the immune response to antigen (4), and therefore play a central role in allergic airway inflammation.

Establishment of the allergic airway response can be broadly segregated into two phases: a sensitization phase, in which initial interaction with allergen induces a systemic immune response resulting in the generation of IgE antibody as well as production of selected cytokines; and a second, challenge phase, which is then triggered by subsequent local exposure to the allergen, resulting in an amplification of cytokine production which drives immune cell recruitment and activation in the lung. In association with sensitization, several cellular responses have been reported. There is a significant expansion of both CD4+ and CD8+ T cells, as well as B cells in the thoracic lymph nodes (5). This expanded T cell population expresses elevated levels of the transcription factors GATA-3 and STAT-6. Despite activation of these transcription factors, detection of T_H2 cytokines interleukin (IL)-4, IL-5, and IL-13 does not occur until further induction in association with the challenge phase. Sensitization alone does not appear to affect immune cell numbers in the lung (5). There is, however, a detectable change in lung epithelium. Sensitization results in synthesis and intracellular storage of IL-16 as determined by immunohistochemical staining (6). Although IL-16 is not detected in the bronchoalveolar lavage (BAL) fluid of individuals with stable atopic asthma, it is rapidly released following airway challenge with either allergen or histamine (7, 8). These data indicate that sensitization alone primes for the rapid expression of T_H2 cytokines by T cells and IL-16 by the epithelium.

IL-16 is a protein initially characterized as an *in vitro* chemoattractant factor for CD4+ T cells (9); it is now known that other CD4+ cells are also responsive. IL-16 stimulation of CD4+ T cells results in upregulation of IL-2R α (CD25), facilitating a proliferative response to either IL-2 or IL-15 (10). In addition, IL-16 has been reported to regulate the response of T cells to antigenic stimulation (11, 12) as well as to certain chemokines (13). By immunohistochemical staining, the major cell source of IL-16 in the lung are bronchial epithelial cells, although CD4+ and CD8+ T cells (14), eosinophils (15), mast cells (16), and dendritic cells (17) also are capable of producing IL-16. In T cells, IL-16 is generated as a 631 amino acid precursor protein that is cleaved by caspase-3 to yield the 121 amino acid bioactive protein (28). The receptor for IL-16 in T cells is CD4, demonstrated by both physical association and abrogation of cellular responses in which CD4 is absent or mutated (18–20).

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Abbreviations: serotonin, 5-HT; antibody, Ab; bronchoalveolar lavage, BAL; bronchial epithelial cell, BEC; enzyme-linked immunosorbent assay, ELISA; interleukin, IL; ovalbumin, OVA; phosphate-buffered saline, PBS; regulated on activation, normal T-cell expressed and secreted, RANTES; T-helper 2, T_H2; tumor necrosis factor- α , TNF- α .

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The association of IL-16 with bronchial epithelial cells (BEC) was first reported by Bellini and coworkers (21), who described the release of IL-16 by BEC derived from individuals with asthma following *in vitro* stimulation with histamine. Histamine-stimulated epithelial cells from individuals without asthma did not release IL-16. Subsequent studies identified IL-16 mRNA and protein in the bronchial epithelium and subepithelium of biopsies from individuals with asthma, which were absent in those without asthma (6). The analogous *in vivo* experiment demonstrated the presence of IL-16 in the BAL fluid obtained from individuals with asthma following segmental bronchoprovocation with histamine (8). IL-16 was not detected in the BAL fluid from individuals without asthma, nor from saline-challenged individuals with asthma. There was a similar pattern of pulmonary IL-16 expression in a murine model of allergic airway inflammation. IL-16 protein was present in the epithelium and BAL fluid of ovalbumin (OVA)-sensitized and aerosol-challenged mice and absent in control mice (22). Interestingly, IL-16 immunoreactivity was also detected in the epithelium, but not in the BAL fluid, of systemically sensitized mice challenged with saline. The data from bronchial biopsies and BAL of allergic airway inflammation in humans and mice and bronchoprovocation experiments with histamine suggest that epithelium can be stimulated to synthesize and store precursor IL-16 protein, which is thereafter secreted following a secondary stimulus. The presence of IL-16 mRNA and protein in the epithelium of asymptomatic individuals with asthma or sensitized mice before allergen challenge further indicates that IL-16 synthesis is induced during allergen sensitization. Although cytokine factors that elicit epithelial cell IL-16 synthesis *in vitro* have been described (23, 24), a direct effect of these cytokines on *in vivo* synthesis of IL-16 has not been reported.

In this study, we investigated the mechanism by which OVA sensitization in a mouse model of allergic inflammation induces epithelial cell production of IL-16. We demonstrate that the serum and BAL fluid of OVA-sensitized mice, although unable to directly elicit IL-16 synthesis and release, are able to prime LA-4 cells (murine BEC) to synthesize IL-16, which is secreted with subsequent stimulation by serotonin (5-HT). The majority of this effect is attributable to tumor necrosis factor (TNF)- α . We further demonstrate that the effects of 5-HT occur via interaction with the S2 receptor, and that secretion is independent of *de novo* synthesis of IL-16 protein. These *in vitro* observations of epithelial cell priming were correlated *in vivo* by intratracheal airway challenge with 5-HT in sensitized mice, which elicits secretion of IL-16 bioactivity detected in BAL fluid. This model for IL-16 generation by airway epithelial cells represents a potential rapid response mechanism associated with a secondary exposure to allergen in the sensitized individual, similar to the mechanism described for T_H2 cytokine generation by primed T cells (5).

Materials and Methods

Reagents

Murine tumor necrosis factor α (mTNF- α) was obtained from R&D Systems (Minneapolis, MN). Serotonin (5-HT) was pur-

chased from Sigma Chemical Co. (St. Louis, MO) and used at 10^{-6} M for all experiments. Serotonin receptor inhibitors were obtained from the following sources: LY53857 (10^{-6} M), Eli Lilly and Co. (Indianapolis, IN); ketanserin (10^{-8} M), Janssen Pharmaceutical (Piscataway, NJ); pindopind-5-HT_{1A} (5×10^{-6} M), Research Biochemicals, Inc. (Natick, MA); and methiopropin (10^{-6} M), Hoffmann-LaRoche (Nutley, NJ). The specific peptide inhibitors of caspase-1 and -3 Ac-YVAD-CHO (acetyl-Try-Val-Ala-Asp-aldehyde) and Ac-DEVD-CHO (acetyl-Asp-Glu-Val-Asp-aldehyde), respectively, were purchased from Bachem (Torrance, CA).

Anti-IL-16 monoclonal antibody was isolated from clone 14.1 and purified by protein A affinity chromatography, and used at a concentration of 5 μ g/ml, a concentration sufficient to neutralize the bioactivity of 10^{-10} M recombinant human IL-16 as well as murine IL-16 (18, 25). Anti-murine TNF- α , IL-9, and regulated on activation, normal T cells expressed and secreted (RANTES) antibodies (R&D Systems) were used at the neutralizing concentrations of 10 μ g/ml in BAL fluid, serum, or cell culture supernatants as noted.

Mice Sensitization

Male BALB/c mice (aged 6–8 wk) were obtained from Jackson Laboratory (Jackson, ME). The mice received two intraperitoneal injections of 25 μ g ovalbumin with aluminum hydroxide (Imject alum; Pierce, Rockford, IL) in sterile phosphate-buffered saline (PBS) on Days 1 and 14. Serum and BAL fluid was obtained at Day 28. BAL fluid was obtained by tracheal cannulation, followed by two sequential lavages using 1 ml of sterile PBS in each lavage. The fluid was centrifuged to remove cells and debris and added to cell cultures at a 1:10 dilution. Sensitization of the mice was confirmed by the presence of elevated IgE antibody levels contained in the serum, as determined by enzyme-linked immunosorbent assay (ELISA) (22).

For some experiments, sensitized and unsensitized mice were subjected to isoflurane anesthesia (Abbott, Chicago, IL) and intratracheally challenged with 100 μ l (10^{-7} M) 5-HT contained in sterile PBS. One hour following 5-HT instillation, lungs were lavaged with 1 ml sterile PBS. The BAL fluid was centrifuged to remove cellular debris, and then concentrated 10-fold using Centricon centrifugation (Millipore, Bedford, MA) before assessment for induced chemoattraction and IL-16 ELISA. The mice were housed according to guidelines established by the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. The Institutional Animal Care and Use Committee at the Boston Medical Center have approved the protocol for animal usage described in these studies.

Cells and Culture Conditions

The immortalized murine airway epithelial LA-4 cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured on tissue culture treated dishes in F-12 (HAM; Gibco-BRL, Grand Island, NY) media supplemented with HEPES, penicillin/streptomycin, and 10% fetal bovine serum, and passaged weekly. All experiments were conducted at 90–95% confluence. Following stimulation for designated time periods, supernatants were harvested, centrifuged to remove any cellular debris, and frozen at -80° C before assay. For cell lysis, cells were detached by incubation with EDTA in cold media. Cell suspensions were sonicated at 4° C in PBS containing the protease inhibitors PMSF, aprotinin, and leupeptin (all obtained from Sigma Chemicals) as previously described (26, 27).

In some experiments, caspase-specific peptides were used to demonstrate caspase-3 enzymatic activity in IL-16 processing (28).

For those experiments, peptides Ac-DEVD-CHO or Ac-YVAD-CHO (100 μ M each) in PBS were added to the cell cultures 5 min before the addition of TNF- α , and remained in the cultures throughout the experiment. In some experiments, cycloheximide (20 μ g/ml) was added 2 h before cytokine stimulation to prevent *de novo* protein synthesis.

Chemotaxis

The presence of bioactive IL-16 in epithelial cell supernatants was determined by the induction of human T lymphocyte migration using a modified Boyden chemotaxis chamber technique, as described below and previously (23, 27). T cells were isolated from healthy volunteers by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, followed by passage through a sterile nylon wool column to purify nonadherent T cells. This method yields a > 95% pure T cell population. After isolation, cells were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) with 1% bovine serum albumin, 200 U/ml penicillin G, 0.2 mg/ml streptomycin, and 24 mM HEPES (Gibco-BRL) in standard tissue culture conditions. Fifty microliters of the migrating cell suspension (6×10^6 /ml) was placed in the upper compartments of 48-well microchemotaxis chambers separated from 32 μ L of test samples by 8- μ m micropore nitrocellulose filters (Neuroprobe, Cabin John, MD) and were incubated in tissue culture conditions for 2 h. After migration, filters were fixed, stained with hematoxylin, dehydrated, and then mounted on glass slides. Cell migration was quantified by counting the total number of cells migrating beyond a certain depth, using light microscopy. The depth was set to routinely give a baseline migration under control conditions of 10–15 cells/hpf. To assess specificity for IL-16 and RANTES, supernatants were incubated for 20 min with anti-IL-16 or anti-RANTES Ab before chemotaxis assay. There is no detectable cross-neutralization between anti-IL-16 and anti-RANTES Ab (22, 29). Five high-power fields were counted in duplicate for each sample, means determined, and expressed as a percent of cell migration elicited by unconditioned media alone.

Experiments were performed in triplicate or quadruplicate, and results expressed as mean \pm SEM. Two-tailed unpaired Student's *t* tests were performed using StatviewSE + Graphics software (Abacus Concepts, Berkeley, CA), with Type I error probabilities (*P*) noted in figure legends.

ELISAs

IL-16 ELISAs were conducted as previously described (30). On a 96-well microtitre plate (Nunc, Naperville, IL), Ab 14.1 was plated as capture Ab followed by blocking with 1% bovine serum albumin (Intergen) in PBS. Standards were generated by serial dilutions of rIL-16 in PBS. Samples and standards were plated in duplicate and incubated at 37°C for 1 h. Between each step, wells were washed five times with PBS/0.05% Tween-20. Biotinylated polyclonal anti-IL-16 Ab (10 μ g/ml; R&D Systems) diluted in PBS containing 0.05% Tween-20 was added as detection Ab. The presence of an IL-16/anti-IL-16 complex was detected by incubation for 1 h with peroxidase-labeled streptavidin, development with peroxide-based chromogen, and stopped with 1 M phosphoric acid. The lower limit of detection for IL-16 was 15 pg/ml. TNF- α ELISA (BioSource International, Camarillo, CA) was performed according to the manufacturer's instructions. The lower limit of detection for TNF- α was 10 pg/ml.

Determination of Cellular Caspase-3 Activity

LA-4 cell caspase-3 activity following TNF- α stimulation was determined by cell lysates' ability to release a fluorescent marker

attached to substrate peptide sequence. Cell lysates were assayed for caspase-3 activity per manufacturer's instructions (Medical and Biological Laboratories Co., Ltd, Nagoya, Japan). Briefly, after rinsing twice with PBS, cells are harvested and subjected to a detergent-based lysis buffer from the manufacturer. Cell lysates are incubated with an equal volume of 2 \times reaction buffer and DEVD-AFC in the dark at 37°C. Fluorescence with 350 nm excitation and 510 nm emission wavelengths were assayed at 2–3 h of incubation. Caspase-3 activity is expressed as fold increase of diluent-treated control dishes.

Results

Epithelial Cell Priming by Serum and BAL Fluid Obtained from OVA-Sensitized Mice

To determine whether a soluble factor generated during the sensitization phase was responsible for IL-16 induction in airway epithelial cells, LA-4 cells were stimulated with 5% serum or 10% BAL fluid obtained from OVA-sensitized mice. After 24 h, the supernatants were assessed for increased T cell chemoattractant activity. As shown in Figures 1A and 1C, there was no detectable increase in chemoattractant activity. Because vasoactive amine challenge of epithelial cells derived from individuals with asthma had been shown to induce secretion of IL-16 (21), the cells were then cultured in the presence of serum or BAL for 24 h, followed by a 2-h stimulation by 5-HT (10^{-6} M). Under these conditions LA-4 cells cultured with serum or BAL from sensitized mice followed by 5-HT stimulation generated significant chemoattractant activity. There was no increase in chemoattractant activity in cultures treated with BAL or serum from unsensitized (control) mice. As airway epithelial cells can generate a number of chemoattractant cytokines, activity attributable to IL-16 was established by co-incubation with neutralizing concentrations of anti-IL-16 antibody in the chemotaxis assay. As depicted in Figures 1A and 1C for serum and BAL, respectively, the presence of anti-IL-16 antibody reduced the overall migratory effect by \sim 50%, indicating that IL-16 was inducing about half of the total migratory effect seen in the supernatants. The majority of the residual activity was attributable to RANTES, as determined by the addition of anti-RANTES antibody to the chemotaxis assay (data not shown).

A limited number of cytokines (IL-9 and TNF- α) have been reported to induce IL-16 production from resting epithelial cells. To initially characterize whether either of these cytokines were contributing to IL-16 production, serum and BAL fluid from sensitized mice were combined with neutralizing concentrations of either anti-IL-9 or anti-TNF- α Ab. After 24 h, 5-HT was added to the cultures for 2 h before harvesting supernatants for chemotaxis assay. As shown in Figures 1B and 1D, the addition of anti-TNF- α antibody resulted in a 60% reduction in total T cell chemoattractant activity for both serum and BAL. The addition of anti-IL-16 antibody in the chemotaxis assay further reduced the migratory response, indicating that although TNF- α appeared to be the major component, other factor(s) were also contributing to the production of IL-16. The addition of anti-IL-9 antibody did not alter the migratory effect (data not shown), indicating that IL-9 was not involved in the priming effect.

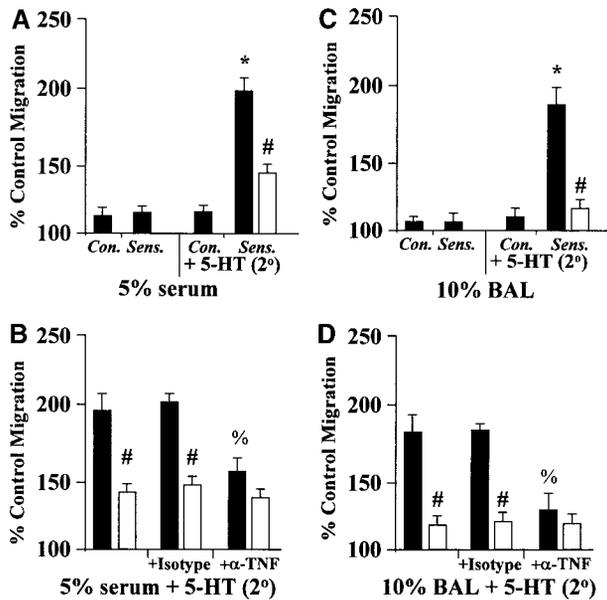


Figure 1. Priming of LA-4 cells by serum and BAL from OVA-sensitized mice for serotonin-stimulated release of T cell chemoattractant activity. Serum and BAL fluid were harvested from BALB/c mice intraperitoneally injected with OVA-alum (Sens) or saline (Con). LA-4 cells were stimulated with 5% serum or 10% BAL for 24 h with or without 5-HT added for the last 2 h as noted. Cell supernatants from serum-stimulated cells (A, dark bars) or BAL fluid-stimulated cells (C, dark bars) were collected and subjected to T cell chemotaxis assay. Contribution of TNF- α to LA-4 cell activation was determined by incubation of serum (B) or BAL fluid (D) from sensitized mice with vehicle, anti-TNF- α Ab, or isotype control Ab before LA-4 cell treatment and 5-HT stimulation. In all experiments, contribution of IL-16 to total migratory response was determined by the addition of anti-IL-16 Ab during the chemotaxis assay (open bars). Data represented are means \pm SEM from triplicate experiments. * $P < .05$ versus control migration (media alone, designated as 100%); % $P < .05$ versus isotype control Ab-incubated serum/BAL; # $P < .05$ versus without IL-16 neutralizing Ab.

We next determined whether TNF- α was elevated systemically and in the BAL fluid of OVA-sensitized mice. TNF- α was detected in the sera of sensitized mice at a concentration of 23 ± 8 pg/ml. TNF- α was not detected by ELISA in either BAL fluid from sensitized mice or in the serum or BAL fluid from unsensitized mice.

Induction of IL-16 Generation by TNF- α in LA-4 Cells

There appeared to be a mechanistic difference in the generation of IL-16 between high doses of TNF- α (100 ng/ml) (24) and lower concentrations found in serum (20 pg/ml) in the current study. To further characterize the response of murine BEC to direct stimulation with TNF- α , LA-4 cells were stimulated in a dose-dependent fashion with recombinant murine TNF- α for 24 h. The supernatant was assessed for chemoattractant activity of human T cells. As shown in Figure 2A, TNF- α alone induced an increase in chemoattractant activity from LA-4 cells at concentrations of 50 pg/ml or greater. IL-16 represented at least 50% of

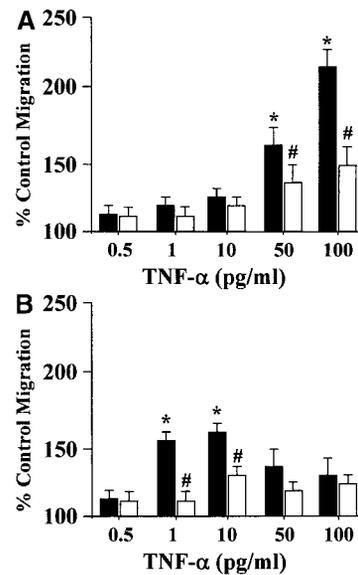


Figure 2. Synthesis and secretion of IL-16 by TNF- α -stimulated LA-4 cells. LA-4 cells were stimulated with a dose range of TNF- α for 24 h, supernatants harvested, and cells lysed by sonication in media containing protease inhibitors. Supernatants (A) and a 1:10 cell lysate diluted with media (B) were subjected to T cell chemotaxis in the absence (solid bars) or presence (open bars) or anti-IL-16 Ab. Data represented are means of four separate experiments. * $P < .05$ versus control migration (designated as 100%); # $P < .05$ versus supernatant or lysate without addition of neutralizing anti-IL-16 Ab.

total chemoattractant activity for both the 50 and 100 pg/ml conditions, as determined by the addition of anti-IL-16 antibody (Figure 2A). The addition of anti-RANTES antibody to the chemotaxis assay identified that the residual chemoattractant activity was attributable to RANTES, a chemokine that has been previously identified in bronchial epithelium (23, 31) (data not shown).

The discrepancy in the dose response for IL-16 production, in combination with the observation that 5-HT was required to induce secretion of IL-16 following low doses of TNF- α , suggested that under certain circumstances airway epithelial cells can generate IL-16 without concomitant secretion (6, 8, 21). To determine whether the low concentrations of TNF- α observed in the serum and BAL fluid of sensitized mice could induce the synthesis and storage of IL-16, cell lysates were collected in parallel with the supernatants described in Figure 2A and were assessed for IL-16 bioactivity following 24 h of TNF- α stimulation. In contrast to supernatants, cell lysates contained IL-16 bioactivity resulting from 1 pg/ml TNF- α (Figure 2B). The amount of IL-16 bioactivity and protein, detected in association with cell lysates, peaked with the 10 pg/ml concentration of TNF- α and decreased with higher concentrations. This correlates with an increase in detectable chemoattraction observed in the supernatants with higher concentrations of TNF- α stimulation, suggesting that lower concentrations of TNF- α can stimulate synthesis of IL-16 without detectable release. Higher concentrations induce synthesis and release, resulting in less intracellular IL-16. Quantification of both secreted and intracellular IL-16 is depicted in Table 1. The cause of slightly lower levels of T cell chemoattractant activity in cell lysates compared with supernatants is unclear, but is presumably due to the presence of inhibitory proteins and/or proteases in whole-cell sonicates.

TABLE 1
*Generation of IL-16 by LA-4 cells stimulated with TNF- α **

		Concentration of TNF- α (pg/ml)				
		0	1	10	50	100
IL-16 pg/ml	Intracellular [†]	ND	63 \pm 12	117 \pm 34	31 \pm 17	20 \pm 18
	Secreted [‡]	ND	ND	ND	77+/-23	138 \pm 41

Definition of abbreviations: IL, interleukin; ND, none detected; TNF- α , tumor necrosis factor- α .

* LA-4 cells were stimulated with varying concentrations of TNF- α for 24 h before assessing for IL-16 or RANTES protein by ELISA.

[†] Intracellular proteins were obtained by sonication at 4°C in the presence of protease inhibitors.

[‡] Cell supernatants were obtained by aspiration of culture wells and centrifugation (500 \times g for 10 min) to remove all cells and cellular debris.

Requirement of Caspase-3 Activity for Generation of Bioactive IL-16

In T cells and fibroblasts, IL-16 processing by caspase-3 is required for generation of bioactive IL-16 (28, 30). To assess whether the intracellular generation of IL-16 at TNF- α doses that were insufficient to elicit secretion (depicted in Figure 2B) was dependent on caspase-3, cells were incubated with either Ac-DEVD-CHO (100 μ M), a specific caspase-3 inhibitor, or Ac-YVAD-CHO (100 μ M), a specific caspase-1 inhibitor, for 24 h before stimulation. Cells were then stimulated for 24 h with TNF- α 1 pg/ml in the presence or absence of caspase inhibitors, and sonicates harvested and subjected to T cell chemotaxis assay. As depicted in Figure 3A, the caspase-3 inhibitor peptide Ac-DEVD-CHO abrogated generation of intracellular IL-16. Peptide Ac-DEVD-CHO alone had no effect on the cell migration assay, and higher doses of TNF- α (50 pg/ml) did not override caspase-3 inhibition with respect to secretion of IL-16 (data not shown). To confirm that low-dose TNF- α stimulation of LA-4 cells induces increase in caspase-3 activity, caspase-3 activity was measured directly in cell lysates after 3 h of a dose range of stimulation with TNF- α (Figure 3B).

As a control for nonspecific inhibitory effect of anti-caspase peptides, supernatants were assayed for chemoattractant activity attributable to RANTES, a product of airway epithelial cell stimulation (23). Incubation of supernatants with anti-RANTES antibody 15 min before the assay significantly reduced T cell chemotaxis (not depicted), confirming that the inhibition by caspase inhibitors was specific to IL-16.

Effect of Serotonin Stimulation on TNF- α -Treated Cells

Data presented in Figure 1 demonstrated that 5-HT stimulation induced secretion of IL-16 following exposure to serum or BAL fluid containing TNF- α . We next wanted to characterize the relationship between the priming effect of TNF- α and the secretagogue effect of 5-HT. For these studies, cells were incubated with TNF- α (1 pg/ml) or 5-HT (10⁻⁶ M) for 24 h or with TNF- α for 24 h before the addition of 5-HT for an additional 2 h. Supernatants were assessed for chemoattractant bioactivity. As shown in Figure 4, stimulation by TNF- α or 5-HT for 24 h did not result in the release of any detectable T cell chemoattractant activity. However, cells incubated for 24 h with TNF- α followed by 2 h of

5-HT did result in significant levels of T cell chemoattractant activity in the cell supernatant. Co-incubation of supernatant with neutralizing anti-IL-16 antibody resulted in a 50–60% loss of chemoattractant activity, indicating that a majority of the activity was attributable to IL-16. To determine whether 5-HT was inducing *de novo* synthesis of protein or acting solely as a secretagogue for preformed protein, LA-4 cells were stimulated with TNF- α for 24 h and then incubated with cycloheximide (20 μ g/ml for 2 h) before 5-HT stimulation. Cycloheximide-treated cells secreted comparable levels of bioactivity as untreated cells when stimulated with TNF- α followed by 5-HT stimulation, indicating that *de novo* protein synthesis was not induced by 5-HT stimulation. To demonstrate that the cycloheximide was efficiently blocking protein synthesis, cells were treated with

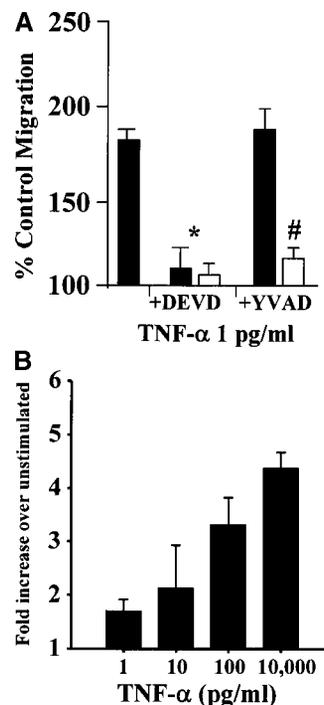


Figure 3. Contribution of caspase-3 to epithelial cell priming by TNF- α . (A) Effect of caspase-specific inhibitor peptides on the synthesis of IL-16 by low-dose TNF- α -stimulated epithelial cells. LA-4 cells were stimulated for 24 h with either TNF- α alone, or in the presence of 100 μ M caspase-3- and -1-specific peptides DEVD or YVAD, respectively. The sonicates were then assessed for T cell migration either alone (solid bars) or in the presence of anti-IL-16 antibody (open bars). (B) Dose response to TNF- α on caspase-3 activity in LA-4 cell. Cells were stimulated for 3 h with diluent and a dose range of TNF- α before cell lysis and determination of caspase-3 activity as described in MATERIALS AND METHODS. Data represented are means of three or more separate experiments. * P < .05 versus no inhibitor; # P < .05 versus supernatant without addition of neutralizing anti-IL-16 Ab.

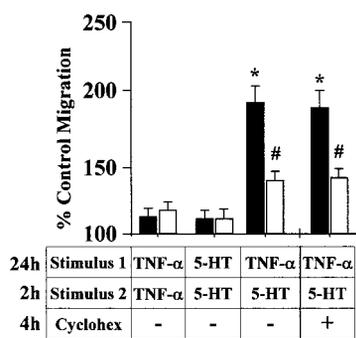


Figure 4. Priming effect of TNF- α for serotonin stimulation. LA-4 cells were stimulated with TNF- α (1 pg/ml) or serotonin (5-HT, 10^{-6} M) for 24 h (Stimulus 1) followed by 2 h of additional TNF- α (1 pg/ml) or 5-HT (10^{-6} M) (Stimulus 2), as indicated. In separate studies LA-4 cells were stimulated with TNF- α for 24 h, with cycloheximide

(20 μ g/ml) added for the last 4 h and 5-HT 2 h before harvest. Supernatants were harvested and assessed for T cell chemoattractant activity either alone (*solid bars*) or in the presence of anti-IL-16 antibody (*open bars*). Data represented are means of four separate experiments. * $P < .05$ versus control cells (designated as 100%); # $P < .05$ versus supernatant without addition of neutralizing anti-IL-16 Ab.

cycloheximide before stimulation with TNF- α (50 pg/ml) for 24 h. Under this condition, no chemoattractant activity was detected in the cell supernatant (data not shown). These studies indicate that 5-HT is acting solely as a secretagogue for IL-16 secretion.

Previous studies demonstrating secretion of preformed, bioactive IL-16 from human CD8 $^{+}$ T cells identified that the serotonin S2 receptor was required (27). To determine whether 5-HT was interacting with the same subclass of receptor, a panel of receptor antagonists was added to the cultures before the addition of 5-HT. Epithelial cells were incubated with 5-HT in the presence of the nonspecific 5-HT receptor antagonist methiothepin (10^{-6} M), the type 2 receptor antagonists LY53857 (10^{-6} M) or ketanserin (10^{-8} M), or with the 5-HT type 1a receptor antagonist pindopind-5-HT1a (5×10^{-6} M) for 24 h. None of the antagonists had an effect on IL-16 generation, either alone or in combination with TNF- α (50 pg/ml) stimulation (data not shown). In the presence of TNF- α (1 pg/ml) in combination with 5-HT stimulation, release of all chemoattractant activity was completely blocked by methiothepin, LY53857, and by ketanserin (Figure 5). In the presence of pindobind, the T cell migratory response was identical to that induced by serotonin alone. Taken together, this data indicates that stimulation of epithelial cells with lower concentrations of TNF- α results in generation of processed and stored IL-16, which is secreted following interaction of serotonin with its type 2 (S2) receptor.

Serotonin Airway Challenge of Sensitized Mice Results in IL-16 Secretion

Asthmatic epithelium in humans contains bioactive IL-16 that has been shown to be secreted following stimulation with vasoactive amines (8). Similar to humans, airway epithelium of antigen-sensitized mice express intracellular IL-16. To determine whether 5-HT alone could induce IL-16 secretion *in vivo*, OVA-sensitized mice were challenged directly with either 5-HT or with PBS. Mice were intratracheally challenged with 10^{-7} M 5-HT (100 μ l) for

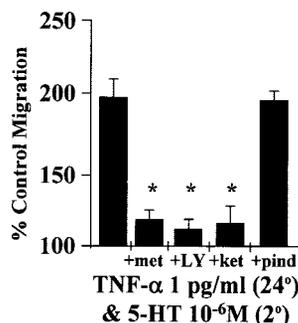


Figure 5. Identification of serotonin receptor subclass responsible for mediating LA-4 cell responsiveness. LA-4 cells were stimulated with TNF- α (1 pg/ml) for 24 h followed by serotonin (5-HT 10^{-6} M) for 2 h either alone or in the presence of methiothepin (10^{-6} M), LY53857 (10^{-6} M), ketanserin (10^{-8} M), or pindopind-5-HT1a (5×10^{-6} M). Supernatants were harvested and assessed for T cell chemoattractant activity. Data represented are means of three separate experiments. * $P < .05$ versus TNF- α /5-HT without receptor inhibitors.

1 h before BAL with PBS. The BAL fluid was concentrated 10-fold and then subjected to T cell chemotaxis assay. As demonstrated in Figure 6, the BAL fluid from OVA-sensitized mice challenged with 5-HT contained T cell chemoattractant bioactivity, the majority of which was attributable to IL-16. Neither 5-HT-challenged unsensitized nor saline-challenged sensitized mice had detectable IL-16 in 10-fold concentrated BAL fluid. This data supports the concept that sensitization alone results in generation and storage of bioactive IL-16 that is rapidly secreted following vasoactive amine stimulation.

Discussion

Selective recruitment and activation of CD4 $^{+}$ T cells at sites of asthmatic inflammation occurs as a result of lung cell-derived cytokines. A variety of cytokines are generated by lung cells; however, one cytokine in particular, IL-16, is a ligand for CD4 and therefore acts on CD4 $^{+}$ cells exclusively (18, 32). Bellini and coworkers first reported the presence of IL-16 bioactivity associated with asthmatic epithelium (21). They determined that primary epithelial cells derived from individuals with asthma cultured in the presence of histamine released IL-16; however, no IL-16 bioactivity was detected before histamine stimulation. Using immunohisto-

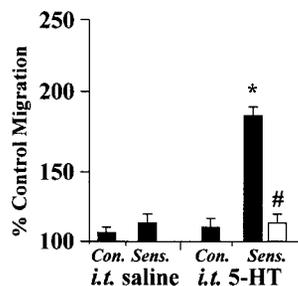


Figure 6. Effect of *in vivo* local serotonin challenge on airway secretion of IL-16. Unsensitized (Con.) and OVA-sensitized (Sens.) mice were intratracheally challenged with saline or 5-HT and BAL harvested 1 h later as described in MATERIALS AND METHODS. Ten-fold concentrated BAL fluid samples were subjected to T cell chemotaxis assay without (*dark bars*) or with (*open bar*) IL-16 neutralizing Ab. Data represented are means of two experiments. * $P < 0.05$ versus control migration (media alone, designated as 100%); # $P < .05$ versus without IL-16 neutralizing Ab.

chemical staining and *in situ* hybridization of lung tissue obtained from individuals with asthma, these initial observations identifying that airway epithelial cells contained high levels of IL-16 message and protein were confirmed (6). This study also highlighted the concept that elevated levels of IL-16 expression by airway epithelial cells *in vivo* were asthma-specific, as tissue obtained from normal or atopic individuals without asthma only expressed sporadic pockets of low levels of both IL-16 message and protein (6). This same specificity appears to exist in mice. IL-16 staining in the lungs of control, unsensitized mice had undetectable IL-16 protein, whereas the epithelium from OVA-sensitized mice contained detectable levels of IL-16 (22). However, whereas IL-16 is readily detected in the BAL fluid of individuals with asthma or that of sensitized mice as early as 2–6 h following antigenic challenge, IL-16 is not detected in the BAL fluid from either unchallenged individuals with asthma (7) or sensitized but unchallenged mice (22). These findings suggest that cytokines generated during the sensitization phase induce epithelial cell synthesis, but not secretion, of IL-16. IL-16 is then released from the epithelium following airway challenge with allergen or vasoactive amine stimulation.

Cytokines which induce epithelial cell-derived IL-16, and the mechanism by which epithelial cells synthesis and secrete IL-16, are currently not well understood. To better understand the process of IL-16 generation in the lung, we used a murine system to investigate the apparent two-step mechanism for IL-16 production and secretion *in vivo*. We chose to determine the effects of TNF- α and IL-9 on epithelial cell priming because reports have indicated that these cytokines can induce synthesis and secretion of IL-16 (23, 24). We detected elevated levels of TNF- α in the serum of sensitized mice. These levels, however, were far below those reported to induce IL-16 protein production and secretion *in vitro* (24). They were sufficient, however, to induce generation of bioactive IL-16. At this concentration of TNF- α , IL-16 was not secreted into the supernatant, but was detected upon cell lysis or stimulation with 5-HT. The presence of bioactive IL-16 contained within the cell with no detectable secretion is similar to what is observed in the epithelium of an asthmatic or sensitized but unchallenged mouse. IL-16 is then secreted following stimulation with 5-HT. Serotonin, acting through the 5-HT₂ receptor, appears to function in this system primarily as a secretagogue, as alone it was not sufficient to induce IL-16 production either *in vitro* in LA-4 cell cultures or *in vivo* as assessed in BAL fluid from unsensitized mice. In addition, inhibition of protein synthesis *in vitro* had no effect on 5-HT-induced IL-16 release. These findings have two implications. The first is that systemic sensitization alone is sufficient to induce generation of IL-16 in the airway epithelium, likely through the generation of immune-related cytokines, such as TNF- α . The second implication is that the epithelium is capable of synthesizing, processing, and storing bioactive IL-16 in response to an initial stimulation, which is then secreted with a secondary stimulus.

We have reported previously that the T_H2 cytokine IL-9 stimulation results in epithelial cell generation of IL-16 (23). IL-9 does not appear to be involved with priming epithelial

cells, as neutralizing antibodies had no effect. This observation is consistent with the findings that T_H2 cytokine production in the lung is not detected until after antigenic challenge (5). This suggests that IL-9 generated in conjunction with secondary challenge is capable of further upregulating IL-16 production. It is conceivable that 5-HT produced by activated mast cells in response to antigenic challenge induces a rapid release of stored IL-16, and that IL-9 stimulation would function to further upregulate *de novo* synthesis of IL-16 protein.

This mechanism for IL-16 synthesis, processing, and release is similar to the mechanism identified in CD8⁺ T cells. CD8⁺ T cells express constitutive IL-16 mRNA and pro-IL-16 protein, as well as constitutively active caspase-3. The result is the constitutive expression and storage of bioactive IL-16 (26, 27). Vasoactive amine stimulation results in release of processed IL-16 independent of *de novo* protein synthesis. A human epithelial cell line, BEAS-2B, has been shown to have constitutive IL-16 message with histamine-inducible secretion of bioactive protein (24). Assessment of unstimulated cell lysates identified the presence of stored bioactive IL-16 in this cell line (unpublished observations). These observations are consistent with the findings that histamine challenge of individuals with asthma results in secretion of IL-16, as detected in the BAL fluid. Unlike the BEAS-2B cell line, which may display an asthmatic-like phenotype, the epithelial cell line used for our studies appears to be similar to primary epithelial cells found in individuals without asthma. These cells do not contain constitutively expressed IL-16, and are unresponsive to vasoactive amine stimulation unless primed with cytokines such as TNF- α .

Confirmation of the involvement of TNF- α in the induction of IL-16 *in vivo* was established using sera and BAL fluid isolated from OVA-sensitized mice. Stimulation of LA-4 cells by the OVA-sensitized sera or BAL fluid resulted in priming of the epithelial cells that were induced to release bioactive IL-16 with subsequent 5-HT stimulation. Inhibition of TNF- α did not completely eliminate generation of these cytokines, indicating that other factors contained within the serum or BAL fluid could also prime for IL-16 production and secretion. The ability of TNF- α to induce IL-16 priming is consistent with other studies identifying its presence in BAL fluid from individuals with asthma (33, 34) and its ability to induce synthesis of epithelial cell-derived IL-6 (24), eosinophil chemotactic activity (ECA) (35), and expression of ICAM-1 (36). Despite the similarities between *in vivo* and *in vitro* observations, our findings should be interpreted in light of the fact that LA-4 cells are from a cell line and may not respond identically to primary cells.

The primary source(s) of TNF- α affecting the airway epithelium has not been identified; B cells, T cells, and macrophages activated during the sensitization phase are likely candidates. Gajewska and coworkers report that there is a significant increase in B cell numbers in the thoracic lymph nodes following sensitization alone (5). It is possible that these B cells, activated in response to the sensitization phase, generate the increased TNF- α levels detected in the sera from these mice, which we demonstrate to be sufficient

to induce IL-16 production. The potential involvement of 5-HT in the *in vivo* release of IL-16 was also demonstrated. IL-16 bioactivity was detected in the BAL fluid of sensitized mice challenged with 5-HT for 60 min before BAL. The lack of IL-16 detected from sensitized mice challenged with saline, or from unsensitized mice challenged with 5-HT, indicates the requirement of both priming and secondary stimulation. A similar priming effect by OVA sensitization has been reported for generation T_H2 cytokines in the lymph nodes (5).

The importance of epithelial cell priming resulting in storage of IL-16 is not clearly understood. One might hypothesize that this would facilitate a rapid response mechanism induced by mast cell release of vasoactive amines following allergen provocation. Indeed, allergen challenge of individuals with asthma results in IL-16 protein, detected in the BAL fluid, as early as 4–6 h (7, 8). The response may be more rapid, but earlier time points have not as yet been reported. Histology studies have indicated that IL-16 protein levels in the epithelium are further upregulated following antigenic challenge (37). It is likely that other factors produced during antigenic stimulation, such as IL-9 (23) or increased levels of TNF- α , act to facilitate synthesis and secretion of IL-16.

Our understanding of the role of IL-16 in asthmatic inflammation is expanding but incomplete. *In vitro*, it has been shown to downregulate activation induced by T cell receptor signaling (11, 12, 18) as well as to alter the chemoattractant activity of certain chemokines (13, 38). Meanwhile, IL-16 is a potent chemoattractant for eosinophils (15, 39). The presence of IL-16 in asthmatic bronchi would therefore implicate the airway epithelial cell in the orchestration of CD4⁺ T cell-dependent allergic inflammation.

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