The Th2 Lymphocyte Products IL-4 and IL-13 Rapidly Induce Airway Hyperresponsiveness Through Direct Effects on Resident Airway Cells

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Airway inflammation and airway hyperresponsiveness (AHR) are hallmarks of asthma. Cytokines produced by T helper type 2 (Th2) lymphocytes have been implicated in both processes. There is strong support for the idea that Th2 cytokines can produce AHR indirectly by promoting the recruitment of inflammatory cells. Less attention has been given to the possibility that Th2 cytokines might induce AHR by acting directly on resident airway cells. To investigate this, we polarized and activated $CD4^+$ T cells *in vitro* and analyzed airway function after administration of lymphocyte-conditioned media to the airways of naive mice. Th2-lymphocyte–conditioned medium induced AHR within 6 h. This finding was reproduced in mast-cell–deficient and in T- and B-lymphocyte–deficient mice. AHR did not occur when Th2-lymphocyte–conditioned medium was administered to mice lacking the IL-4 receptor α subunit or Stat6, suggesting a critical role for interleukin (IL)-4 and/or IL-13. This was confirmed by the finding that recombinant IL-4 and IL-13 both induced AHR within 6 h. The induction of AHR occurred in the absence of inflammatory cell recruitment or mucus production. These results strongly suggest that products of activated Th2 lymphocytes can rapidly perturb airway function through direct effects on resident airway cells.

Asthma is increasing in prevalence around the world. Recent estimates suggest that over 17 million persons in the United States alone have asthma (1). The hallmarks of asthma include reversible airways obstruction, airway inflammation, and exaggerated airway narrowing in response to a variety of stimuli, also known as airway hyperresponsiveness (AHR) (2). The symptoms of asthma, including cough, wheezing, and shortness of breath, are thought to be consequences of airway narrowing. Airway narrowing may result from a number of underlying processes, including smooth muscle contraction, inflammatory cell infiltration, mucus hypersecretion, epithelial cell sloughing, and airway remodeling.

The $CD4^+$ T lymphocyte is now recognized as a critical participant in the events leading to asthma. Histopathologic sections from patients with allergic asthma demonstrate peribronchial infiltration of $CD4^+$ T lymphocytes, and bronchoalveolar lavage (BAL) samples from patients with asthma contain increased numbers of activated $CD4^+$ T lymphocytes after antigen exposure (3, 4). These cells are required for the development of eosinophil-rich airway inflammation, mucus production, and AHR seen in murine models of allergic asthma. Although several cell types participate in the airway inflammation seen in asthma, the $CD4^+$ T lymphocyte appears to be critically important for the events resulting in AHR (5, 6).

$CD4^+$ T lymphocytes can differentiate toward a T helper type 1 (Th1) or Th2 phenotype (7, 8). Th2 lymphocytes are important for the host response to helminth infections and are important in the pathogenesis of asthma and other allergic diseases. These cells secrete the cytokines IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor. Activated Th2 lymphocytes are found in increased numbers in the airways of persons with asthma (4) and are important for the coordination of allergic airway inflammation.

Recent studies have highlighted the importance of two structurally related Th2 cytokines, interleukin (IL)-4 and IL-13, in the events leading to allergen-induced AHR. IL-4 is required for the differentiation of T lymphocytes to a Th2 phenotype (8). Once a Th2 response has been established in the lung, IL-13 appears to play a role in the downstream events leading to AHR. Sustained inhibition of IL-13 throughout the period of allergen challenge reduces airway inflammation and mucus overproduction and abrogates AHR (9, 10). Repeated administration of IL-13 to the airways of naive mice induces airway inflammation, mucus production, and AHR (9, 10). Overexpression of IL-13 in the airways of transgenic mice also results in inflammation and mucus overproduction and leads to marked fibrosis, airway remodeling, and increased airways resistance (11). Taken together, these studies suggest that IL-13 produced by Th2 cells may help to account for many of the airway abnormalities seen in asthma.

In asthma and in animal models of this disease, AHR is generally seen in the setting of airway inflammation. The relationship between airway inflammation and AHR is not clear, although Th2 cytokines have been implicated in both phenomena. One widely accepted possibility is that Th2 cytokines can produce AHR indirectly by promoting the recruitment and subsequent activation of eosinophils and other inflammatory cells. Inflammatory cells may then act on resident airway cells to produce AHR. For example, the release of eosinophil granule contents may result in smooth muscle contraction or in the development of AHR (12–14). Less attention has been given to the possibility that Th2 cytokines may induce AHR more directly by binding to cytokine receptors on resident airway cells, without a requirement for inflammatory cell recruitment. Recruitment-dependent ("indirect") and recruitment-independent ("direct") pathways are difficult to distinguish in

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Abbreviations: airway hyperresponsiveness, AHR; bronchoalveolar lavage, BAL; bronchoalveolar lavage fluid, BALF; interleukin, IL; IL-4 receptor α subunit, IL-4Rα; major basic protein, MBP; Periodic Acid-Schiff, PAS; phosphate-buffered saline, PBS; concentration of acetylcholine required to produce a 200% increase in resistance, PC_{200}; T helper type 1, Th1; T helper type 2, Th2.

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conventional models because inflammation generally precedes the development of AHR. We hypothesized that one or more $CD4^+$ T lymphocyte products could act rapidly on the airways to induce AHR without a requirement for inflammatory cell recruitment. To address this hypothesis, we developed a model to study the effects of lymphocyte products on airway responsiveness *in vivo*. We examined airway responsiveness after a single administration of Th2 lymphocyte products to airways of naive mice. We focused on early time points, reasoning that direct effects of Th2 lymphocyte products on resident cells might be apparent before the development of airway inflammation. These studies showed that Th2 cytokines can act rapidly and directly on the airway to induce AHR, a finding that may have significant implications for our understanding of the role of the $CD4^+$ T lymphocyte in asthma.

Materials and Methods

Mice

Splenocytes were obtained from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) housed in the transgenic barrier facility at the UCSF/SFGH Animal Care Facility. C57BL/6 and BALB/c mice used for the testing of conditioned medium effects *in vivo* were purchased from B & K Universal (Fremont, CA) or Simonsen (Gilroy, CA). RAG1-deficient mice and mast-cell–deficient mice (W/W^v) were purchased from Jackson Laboratories and housed in the barrier facility. IL-4Rα–deficient mice on a BALB/c background (15, 16) were graciously provided by F. Brombacher (University of Capetown, South Africa) and M. Mohrs (University of California, San Francisco, CA). Stat6-deficient mice on a BALB/c background (17) were graciously provided by M. Grusby (Harvard Medical School, Boston, MA).

Lymphocyte Purification and Culture

$CD4^+$ splenocytes were isolated by positive immunomagnetic selection using a MACS Midi Column (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of the cell suspension after selection was $> 90\%$ as measured by flow cytometry. $CD4^+$ lymphocytes were suspended at 7.5×10^5 cells/ml in complete RPMI-1640 media (10% FCS, 100 μg/ml penicillin-streptomycin, 2 mM L-glutamine, and 5 μM 2-mercaptoethanol) and stimulated on 96-well plates coated with rat anti-mouse CD3 antibody clone 145-2C11 (10 μg/ml) (PharMingen, San Diego, CA) for 2 h at 37°C. In each well, 200 μl of cell suspension was incubated in the presence of anti-CD28 antibody clone 37.51 (1 μg/ml) (PharMingen) and IL-2 (20 ng/ml) (R&D Systems, Minneapolis, MN). For Th2 polarization, anti-IFN-γ antibody clone R4-6A2 (5 μg/ml) (PharMingen) and IL-4 (10 ng/ml) (R&D Systems) were added at the time of primary stimulation. The cells were cultured for 6 d, with the addition of fresh medium as needed. After 6 d, cells were washed twice and then resuspended at 7.5×10^5 cells/ml in serum-free medium (Hybridoma-SFM; Gibco BRL, Frederick, MD). Cell suspension (200 μl) was added to each well of 96-well plates coated with anti-CD3 (10 μg/ml). Unstimulated Th2-lymphocyte–conditioned medium was produced by plating Th2 cells on uncoated 96-well plates. For comparative purposes, Th1-lymphocyte–conditioned medium was produced by incubating lymphocytes with anti-IL-4 antibody clone 11B11 (10 μg/ml) (PharMingen) and IL-12 (3.5 ng/ml) (R&D Systems) at the time of primary stimulation. Cells were incubated at 37°C for 2 d before collection of conditioned media. The presence of endotoxin in the conditioned medium was excluded by assaying the conditioned medium for lipopolysaccharide (not shown).

Enzyme-Linked Immunosorbent Assays

Capture and detection antibodies for the IL-4, IL-5, IL-9, and IFN-γ enzyme-linked immunosorbent assays (ELISAs) were purchased from PharMingen, and the reagents for the IL-2 and IL-13 ELISAs were purchased from Caltag Laboratories (Burlingame, CA) and R&D Systems, respectively. ELISAs were performed according to the manufacturer's instructions. The lower limits of detection for each of the ELISAs were 1.25 ng/ml (IL-2), 3.13 ng/ml (IL-4), 1.56 ng/ml (IL-5), 3.13 ng/ml (IL-9), 3.13 ng/ml (IL-13), and 3.13 ng/ml (IFN-γ).

Intranasal Administration of Conditioned Medium and Recombinant Cytokine

Mice were anesthetized with methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL). Activity and respiratory rate were closely monitored. When the mice were unresponsive but breathing comfortably, a Pipetman (Rainin, Emeryville, CA) was used to administer 50 μl of culture medium or recombinant cytokine diluted in fresh culture medium via the nostrils. The animals were allowed to slowly inhale the liquid and were then recovered in a supine position. rmIL-4 was obtained from PeproTech (Rocky Hill, NJ), and rmIL-13 was graciously provided by Deb Donaldson (Genetics Institute).

Induction of Allergic Airway Inflammation

For comparative purposes, separate experiments were conducted using a traditional protocol of antigen sensitization and challenge as previously described (6). Naive mice were immunized with ovalbumin (OVA)-alum 100 μg by intraperitoneal injection on Days 1 and 14 and received 100 μg OVA intranasally on Days 14, 25, 26, and 27. BAL and collection of lungs for histology were performed on Day 28.

Airway Physiology

At 6 and 12 h after medium administration, invasive measurements of airway physiology were performed as previously described (6, 18). Animals were anesthetized with etomidate (14 μg/g). The trachea was cannulated with a 20-gauge catheter (Becton-Dickinson, Franklin Lakes, NJ), and the mouse was connected to a rodent ventilator (Harvard Apparatus, Holliston, MA). Ventilation was delivered with 100% O_2 (9 μl/g body weight) at 150 breaths/min. The mouse was pharmacologically paralyzed with pancuronium bromide. The tail vein was cannulated with a 27-gauge needle and flushed with normal saline. Mice were then placed into a plethysmograph, and continuous measurements of box volume and airway pressure were made. Lung resistance was calculated on a continuous basis from these measurements. After establishing a stable baseline lung resistance, increasing doses of acetylcholine (0.032, 0.10, 0.32, 1.0, and 3.2 μg/g) were administered intravenously over 1 s until resistance increased by at least 200%. The concentration of acetylcholine required to produce a 200% increase in resistance (PC_{200}) was calculated by log-linear interpolation. In OVA-treated animals, all measurements were performed 24 h after the final intranasal challenge. Statistical analysis was performed with the InStat computer program (GraphPad Software, San Diego, CA), using analysis of variance for comparisons of three or more groups, and by two-tail Student's *t* test for comparisons of two groups.

Collection of BAL Fluid and Preparation of Lungs for Histology

After measurements of airway physiology, BAL was performed via the tracheal catheter using five aliquots of 0.6 ml phosphate-buffered saline (PBS). Cell counts were determined by hemacytometer, and differential cell counts were obtained by cytospin as

previously described (6). Following the collection of BAL fluid (BALF), the lungs were surgically removed from the mice. Formaldehyde (7.5%; 1 ml) was injected via the tracheal catheter, and the lungs were then placed in 7.5% formaldehyde. The lungs were embedded in paraffin and cut into 2- to 3-μm sections. Sections were stained with hematoxylin and eosin to assess airway inflammation and Periodic Acid-Schiff (PAS) solution to determine the presence of mucus production. For immunofluorescence, lungs were isolated, inflated with 10% sucrose/Tissue-Tek O.C.T. compound (VWR, Brisbane, CA) mixture, placed in embedding molds with O.C.T. compound, snap frozen in liquid nitrogen, and stored at −70°C. Tissue sections (5 mm thick) were cut, mounted on slides, and fixed for 10 min in acetone. Slides were air dried for at least 2 h and rehydrated in PBS before staining.

Eosinophil Staining

Major basic protein (MBP) was detected as previously described using rabbit anti-mouse MBP (a kind gift from J. Lee, Mayo Clinic, Scottsdale, AZ) (19). Acetone-fixed lung sections were obtained as described above. These were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA) overnight at 4°C, washed with PBS, and incubated with rabbit anti-mouse MBP for 1 h at 37°C. After washing, lung sections were incubated in 1% Chromotrope 2R (Sigma, St. Louis, MO) for 30 min at room temperature and placed in fluorescein-labeled goat anti-rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA) for 40 min at 37°C. Eosinophils were quantified in a blinded fashion by averaging the number of MBP-positive cells in five consecutive random fields at a magnification of 10×.

Results

Lymphocyte Activation and Culture

To identify lymphocyte-mediated mechanisms of AHR, we first generated products of activated lymphocytes *in vitro*. Spleen-derived mouse $CD4^+$ T cells were polarized toward a Th2 phenotype *in vitro* and restimulated with plate-bound antibody against CD3 after 6 d. As a control, cells were cultured under Th2 conditions but not restimulated with anti-CD3. Two days after restimulation, analysis of the culture media showed the expected Th2 cytokine profiles and absence of measurable cytokine in the medium from cells that were not restimulated. Measured cytokine levels in the Th2-lymphocyte–conditioned medium were as follows (averages from three separate cultures, in ng/ml ± SEM): IFN-γ (23.0 ± 9.0), IL-2 (3.6 ± 1.9), IL-4 (240.0 ± 31.2), IL-5 (226.3 ± 117.5), IL-9 (8.1 ± 1.0), and IL-13 (298.0 ± 82.9). For comparative purposes, cytokine levels were measured in Th1-lymphocyte–conditioned medium: IFN-γ (506.4 ± 186.6), IL-2 (17.2 ± 12.2), IL-4 (5.6 ± 5.5), IL-5 (5.5 ± 3.2), IL-9 (0.4 ± 0.4), and IL-13 (23.6 ± 11.1). Cytokine levels in medium taken from cells that were cultured under Th2 polarization conditions but not restimulated were all below the level of detection of the ELISA.

Airway Responsiveness in Naive Mice Following Intratracheal Administration of Th2-Lymphocyte–Conditioned Medium

After generating products of activated lymphocytes *in vitro*, conditioned medium was tested for its ability to induce changes in airway responsiveness *in vivo*. A single 50-μl dose of Th2 lymphocyte-conditioned medium was administered intranasally to naive mice, and airway physiology was characterized 6 h later. Both BALB/c and C57BL/6 mice treated with Th2-lymphocyte–conditioned medium developed AHR within 6 h (Figures 1A and 1B). The magnitude of AHR was similar to that seen in models of ovalbumin-induced AHR used in our laboratory (10). This effect was not seen when unconditioned medium or conditioned medium from unstimulated lymphocytes was tested. The baseline airway resistance was similar in all groups (not shown). These results show that products of activated Th2 lymphocytes alone can rapidly induce AHR in naive mice.

To characterize the time course of the AHR induced by Th2-lymphocyte–conditioned medium, airway responsiveness was determined at 6 and 12 h after treatment with Th2-lymphocyte–conditioned medium. Animals developed AHR within 6 h of medium administration, and this effect was still present at 12 h (Figure 1C).

Additional studies were performed to characterize the nature of the substance(s) responsible for the rapid development of AHR. Studies of Th2-lymphocyte–conditioned medium following ultrafiltration (Centricon 3; Amicon, Beverly, MA) or heating (120°C for 3 min) showed that AHR was induced by substances that were > 3 kD and heat labile (not shown).

Importance of Airway Mast Cells and Lymphocyte Recruitment

Mast cells reside in the airways, and mast-cell–derived mediators are known to induce bronchoconstriction in hu-

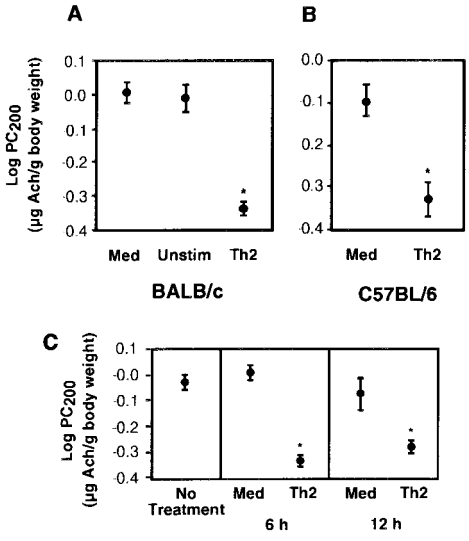

Figure 1. Th2-lymphocyte–conditioned media rapidly induces AHR. BALB/c (*A*) or C57BL/6 (*B*) mice received a single intranasal 50-μl dose of unconditioned medium (Med), conditioned medium from unstimulated Th2 lymphocytes, or conditioned medium from Th2 lymphocytes that had been restimulated after polarization. Airway responsiveness was measured 6 h later. A lower value for log PC_{200} represents increased airway responsiveness. (*C*) Airway responsiveness was measured at 6 and 12 h after treatment of BALB/c mice with unconditioned medium or Th2-lymphocyte–conditioned medium. Each group contained 4–6 mice. Data represent mean ± standard error of the mean. *Statistically significant ($P < 0.05$) differences compared with unconditioned medium controls.

mans. Because some studies suggest a role for mast cells in the rapid generation of AHR in other models (20–22), Th2-lymphocyte–conditioned medium was administered to mast-cell–deficient (W/W^v) mice. Animals treated with Th2-lymphocyte–conditioned medium developed airway hyperresponsiveness compared with animals that received unconditioned medium (Figure 2A). Th2-lymphocyte–conditioned medium also induced AHR in RAG1-deficient mice, which lack T and B cells (Figure 2B). Taken together, these results demonstrate that Th2-lymphocyte–conditioned, medium-induced AHR does not depend on the release of mediators from mast cells or the presence of T or B lymphocytes in the treated animal.

Airway Responsiveness in IL-4Rα–Deficient and Stat6-Deficient Mice

IL-4 and IL-13 have been shown to be important in the development of the Th2 immune response and for the development of AHR after a Th2 response is established (8–10). Both cytokines bind to receptors containing the IL-4Rα subunit, and the IL-4Rα–Stat6 pathway is important for IL-4 and IL-13 signaling. To determine whether IL-4 and/or IL-13 were required for the induction of AHR in this model, we tested Th2-lymphocyte–conditioned medium in mice deficient in IL-4Rα or Stat6. IL-4Rα–deficient mice and Stat6-deficient mice did not develop AHR when treated with Th2-lymphocyte–conditioned medium (Figure 3). These results show that the AHR resulting from Th2-lymphocyte–conditioned medium was dependent on IL-4Rα–Stat6 signaling and implicate the cytokines IL-4 and IL-13 as necessary participants in this process.

Role of IL-4 and IL-13 in the Induction of AHR

We next sought to determine whether IL-4 and/or IL-13 were sufficient to reproduce the AHR induced by Th2-lymphocyte–conditioned medium. We administered recombinant IL-4 or IL-13 (5 μg per mouse) to naive C57BL/6 mice. This dose of cytokine was selected to duplicate the dose of cytokine used in previous studies (9, 10) and because it has been shown that the *in vivo* half-life of recombinant cytokine can be quite short (23, 24). Both cytokines induced a significant increase in airway responsiveness 6 h after treatment (Figure 4). This result demonstrates that the cytokines IL-4 and IL-13 are sufficient for the induction of AHR in naive mice.

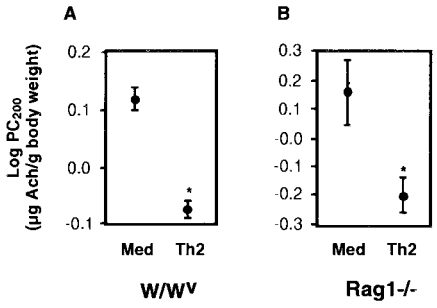

Figure 2. Th2-lymphocyte–conditioned medium induces AHR in mast-cell–deficient mice and in T- and B-lymphocyte–deficient mice. W/W^v mast-cell–deficient mice (*A*) and RAG1-deficient mice (*B*), which lack T and B lymphocytes, were treated with unconditioned medium (Med) or Th2-lymphocyte–conditioned medium as described in Figure 1, and airway physiology was measured 6 h later. Each group contained 4–5 mice. *Statistically significant ($P < 0.05$) differences compared with unconditioned medium controls.

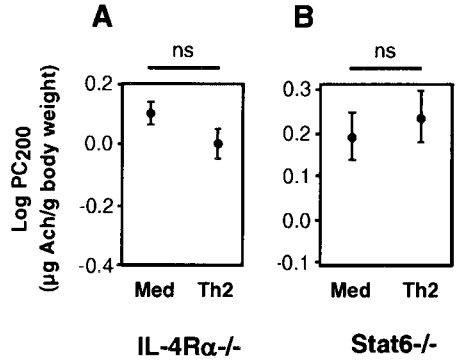

Figure 3. IL-4Rα and Stat6 are required for AHR induced by Th2-lymphocyte–conditioned medium. Mice deficient in IL-4Rα (*A*) or Stat6 (*B*) were treated with unconditioned medium (Med) or Th2-lymphocyte–conditioned medium as described in Figure 1, and airway physiology was measured 6 h later. Each group contained 4–5 mice. Results are representative of two separate experiments each for IL-4Rα- and Stat6-deficient mice. ns, not statistically significant.

Assessment of Airway Inflammation and Mucus Production

Airway inflammation and mucus production are prominent features of murine allergic asthma models and of models that utilize repeated dosing of cytokine to induce airway inflammation and AHR. To determine whether the AHR induced by lymphocyte products in these experiments was associated with airway inflammation, we examined BAL, histology, and immunofluorescence (Figures 5–7). Analysis of BALF obtained 6 h after treatment showed no differences in any cell type between the control, IL-13, and Th2 groups (Figure 5). The IL-4–treated animals showed an increase in BAL macrophages, but eosinophils and other cells were not changed. This was a contrast to

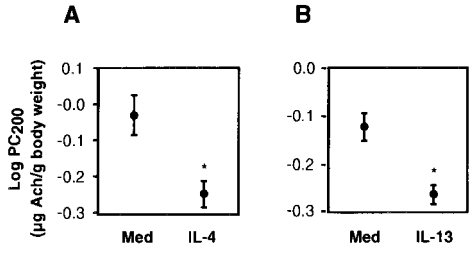

Figure 4. IL-4 and IL-13 treatment rapidly induces AHR. (*A*) BALB/c mice were treated intranasally with unconditioned media (Med) or medium supplemented with IL-4 (5 μg per mouse), and airway physiology was assessed 6 h later. Each group contained 5–11 mice. (*B*) BALB/c mice received unconditioned medium (Med) medium supplemented with IL-13 (5 μg per mouse), and airway physiology was measured 6 h later. Each group contained 4–5 mice. *Statistically significant ($P < 0.05$) differences.

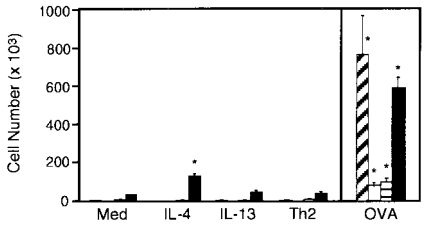

Figure 5. Quantification of inflammatory cells in BALF. BAL was performed 6 h after treatment of naive mice with control medium, IL-4, IL-13, or Th2-lymphocyte–conditioned medium or after allergen challenge of OVA-sensitized mice. Eosinophils (*diagonal lines*), lymphocytes (*open bars*), neutrophils (*horizontal lines*), and monocytes/macrophages (*solid bars*) were counted as described in MATERIALS AND METHODS. Each group contained 3–4 mice. *Statistically significant ($P < 0.05$) differences when compared with same cell type in medium-treated animals.

the marked eosinophil-rich inflammation seen in OVA-treated mice.

Because of the possibility that early airway inflammation might not be detected by BAL, we examined tissue sections for evidence of inflammation. Lungs were stained with H&E to assess airway inflammation (Figures 6A–6C) and with PAS for the identification of mucus overproduction (Figures 6D–6F), and with anti-MBP antibody to detect tissue eosinophils. Histologic examination showed no evidence of airway inflammation or mucus overproduction in any of the treatment groups at 6 h, in contrast to the intense inflammation and mucus production seen in OVA-treated animals. No significant increase in MBP-positive cells was present in the treatment groups when compared with the medium group (Figure 7). These results suggested that the rapid development of AHR induced by Th2-lymphocyte–conditioned medium or recombinant IL-4 or IL-13 did not result from the recruitment of inflammatory cells to the airways or from excessive mucus production.

Discussion

Murine models of AHR are useful for studying mechanisms that may be relevant to asthma. Much of the popularity of these models results from the availability of genetically engineered mice that can be used to study specific molecules or cell types that might be important in the cascade of events leading to AHR. These models have been used to characterize the contribution of specific inflammatory cells and their products to the development of AHR. Although more than one pathway may lead to AHR, these models have shown that the $CD4^+$ T lymphocyte is required for the generation of allergic airway inflammation and AHR (5, 6).

The studies described in this report were designed to identify Th2-cell–driven mechanisms of AHR. We found that the administration of activated Th2-cell–conditioned medium to the airways of naive mice rapidly induced AHR (Figure 1). The AHR seen in these experiments was similar in magnitude to that seen in traditional allergic models of AHR used in our laboratory (10). Changes in airway responsiveness developed as early as 6 h after administration of lymphocyte products and persisted for at least 12 h (Figure 1C). Using BAL, histologic examination of the airways, and immunofluorescence, we were unable to demonstrate the presence of airway inflammation at 6 h (Figures 5–7).

Th2 lymphocyte products induced AHR via the IL-4Rα–Stat6 pathway, which is important for IL-4 and IL-13 binding and signaling (17, 25, 26). IL-4Rα and Stat6 are both required for the development of the Th2 immune response and the asthma phenotype in allergic mouse models (27, 28). More recent studies show a requirement for

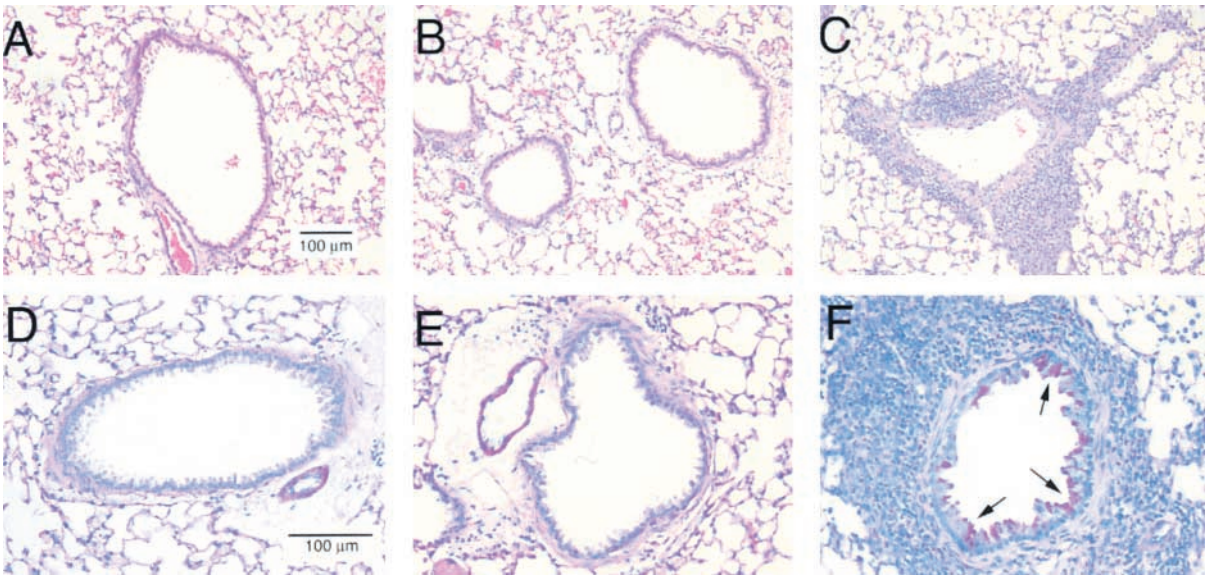

Figure 6. Absence of airway inflammation and mucus production 6 h after IL-13 treatment. Animals were treated intranasally with unconditioned medium (*A* and *D*) or IL-13 (*B* and *E*) (5 μg per mouse) or sensitized and challenged with OVA (*C* and *F*). Lungs were removed and prepared as described in MATERIALS AND METHODS. Sections were stained with H & E (*A–C*) and PAS (*D–F*). Representative sections are shown. *Arrows* indicate the purple staining of mucus.

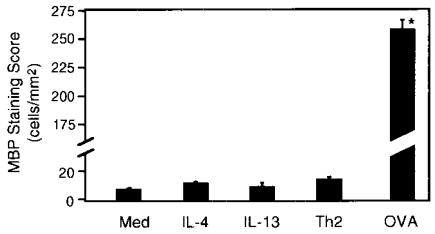

Figure 7. Treatment with IL-4–, IL-13–, or Th2-lymphocyte–conditioned medium does not induce significant increases in the numbers of lung eosinophils. Sections were prepared and stained as described in MATERIALS AND METHODS, using 5–6 sections from each group. *Statistically significant ($P < 0.05$) differences.

IL-4Rα for the development of mucus overproduction and AHR in response to the repeated administration of IL-13 to the airways (9, 10). In the studies described here, mice deficient in either of these molecules did not develop AHR after treatment with Th2-lymphocyte–conditioned medium (Figure 3). This finding suggests an important role for the cytokines IL-4 and IL-13 in the Th2-induced effect because these are the only two cytokines known to bind to receptors containing IL-4Rα. Moreover, Th2 lymphocyte products that act through IL-4Rα–Stat6-independent pathways were not sufficient to cause AHR in this model. We confirmed that the cytokines IL-4 and IL-13 could induce AHR by examining airway responsiveness 6 h after treatment with purified cytokine (Figure 4). Although previous studies have shown that the administration of IL-13 to the airways can induce AHR (9, 10), the experiments described here differ in important ways. In the previous studies, the development of AHR resulted from multiple doses of IL-13 given over a 3- to 5-d period. Following this extended dosing regimen, there was evidence of marked airway inflammation and mucus hypersecretion. Our laboratory has also observed airway inflammation and mucus production following multiple doses of Th2-lymphocyte–conditioned medium given over 3 d (data not shown). In the studies described here, we looked at a much earlier time point (6 h) and found AHR in the absence of detectable inflammation. This finding raises the novel possibility that a direct effect of IL-4 or IL-13 on airway cells can lead to AHR.

The mechanisms responsible for the rapid generation of AHR in this model remain to be defined. We began to address this issue by examining the role of various cells and pathways in this model of AHR. We used mast-cell–deficient and T- and B-lymphocyte–deficient mice to show that the presence of these cells was not required for the development of AHR after Th2-lymphocyte–conditioned medium or IL-4/IL-13 treatment. We could not find evidence of mucus overproduction in the Th2 or cytokine-treated animals, making this an unlikely participant in this mechanism of AHR. This was in significant contrast to animals that were sensitized and treated with ovalbumin, which demonstrated marked airway inflammation and mucus production. Although eosinophils, mast cells, and mucus overproduction have been shown to play important roles in asthma and in other animal models, they seem unlikely to be involved in the events leading to AHR soon after the administration of Th2-cell–conditioned medium, IL-4, or IL-13. Instead, it is more likely that the cytokines IL-4 and IL-13 can rapidly induce AHR through actions on resident airway cells.

The suggestion that direct effects of IL-4 and IL-13 on airway cells contribute to AHR in persons with asthma is biologically plausible. Asthma is associated with greatly increased production of IL-4 and IL-13 by activated Th2 lymphocytes and other cells within the airway wall (4). Receptors for IL-4 and IL-13 are expressed by a wide variety of cell types in the airway, including epithelial cells and smooth muscle cells (29, 30). *In vitro* studies of the effects of IL-4 and IL-13 indicate that these cytokines affect epithelial cells and smooth muscle cells in several ways that might contribute to the development of AHR (31, 32). In both cell types, IL-4 and IL-13 inhibit the activity of inducible nitric oxide synthase and decrease production of nitric oxide, a bronchodilator (33). IL-4 also increases pulmonary epithelial cell production of the complement component C3 (34). The C3 cleavage product C3a causes smooth muscle contraction and has been implicated in asthma pathogenesis in recent studies (35–37). IL-4 affects the barrier function of intestinal epithelium (38), an effect that could contribute to airway narrowing if it occurred in airway epithelium. Further studies will be required to determine which effects of IL-4 and IL-13 are responsible for rapid induction of AHR *in vivo*. These studies are likely to be highly relevant given recent work indicating that the inhibition of airway IL-4 by inhalation of soluble IL-4 receptor leads to rapid improvement in pulmonary function in subjects with asthma (39).

The development of AHR *in vivo* is a complex process that is likely to result from multiple pathways. Other biological mediators such as IL-5 have been shown to be important in some animal models and almost certainly participate in the events leading to the eosinophilic inflammation and the AHR seen in asthma (19, 40, 41). Results of experiments in IL-4Rα–deficient and Stat6-deficient mice suggest that Th2 lymphocyte mediators other than IL-4 and IL-13 are not sufficient for the development of AHR in this model. We therefore chose to directly investigate the effects of purified IL-4 and IL-13 on the airway and did not assess the effects of administering other Th2 cell products, such as IL-5, in a purified form. These findings, however, do not preclude the importance of IL-5 and other mediators in the genesis of AHR and airway constriction in asthma.

The complexity of the airway inflammation found in asthma makes it difficult to identify specific mechanisms leading to AHR. We have described a new model for examining processes leading to AHR. This model highlights the contribution of lymphocyte-derived mediators and provides a system with which to examine lymphocyte-mediated pathways to AHR. Although this model bypasses the requirement for recruitment of inflammatory cells, it does not diminish the likelihood that other cell types and pathways are important in asthma and in animal models of this disease. A large body of evidence has shown that mast cells, eosinophils, and mucus overproduction are important contributors to the array of changes that lead to airway narrowing in asthma. Our observations show that $CD4^+$ T cells and the cytokines IL-4 and IL-13 may have a more direct role in the generation of AHR than previously appreciated. These results have im-

portant implications for understanding the mechanisms leading to airway dysfunction after allergen exposure or nonallergic stimuli.

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