

Induction of Airway Mucus Production By T Helper 2 (Th2) Cells: A Critical Role For Interleukin 4 In Cell Recruitment But Not Mucus Production

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Summary

Airway inflammation is believed to stimulate mucus production in asthmatic patients. Increased mucus secretion is an important clinical symptom and contributes to airway obstruction in asthma. Activated CD4 Th1 and Th2 cells have both been identified in airway biopsies of asthmatics but their role in mucus production is not clear. Using CD4 T cells from mice transgenic for the OVA-specific TCR, we studied the role of Th1 and Th2 cells in airway inflammation and mucus production. Airway inflammation induced by Th2 cells was comprised of eosinophils and lymphocytes; features found in asthmatic patients. Additionally, there was a marked increase in mucus production in mice that received Th2 cells and inhaled OVA, but not in mice that received Th1 cells. However, OVA-specific Th2 cells from IL-4-deficient mice were not recruited to the lung and did not induce mucus production. When this defect in homing was overcome by administration of TNF- α , IL-4 $-/-$ Th2 cells induced mucus as effectively as IL-4 $+/+$ Th2 cells. These studies establish a role for Th2 cells in mucus production and dissect the effector functions of IL-4 in these processes. These data suggest that IL-4 is crucial for Th2 cell recruitment to the lung and for induction of inflammation, but has no direct role in mucus production.

Asthma is a chronic inflammatory disease of the bronchial airways defined by intermittent episodes of airway obstruction. Patients with asthma present with wheezing and cough productive of mucous secretions. Mucus hypersecretion is an important cause of airway obstruction in asthma (1, 2). Airway biopsies show infiltration of the mucosa and submucosa with lymphocytes and eosinophils and hyperplasia of goblet cells and submucosal glands. In autopsy specimens from patients who died in status asthmaticus, obstructing plugs of mucus and cellular debris have been identified in the small airways.

In asthma, mucus hypersecretion is believed to result, at least in part, from inflammation, although the mechanisms by which mucus is induced have not been detailed. Studies have shown that the volume of expectorated sputum in asthmatics correlates with the number of eosinophils in the sputum (3). In addition, sputum DNA levels correlate with mucus production (4). A variety of inflammatory mediators have been shown to stimulate mucus secretion including histamine, prostaglandins, leukotrienes, platelet activating factor, and eosinophil cationic protein (5, 6). The cytokines

IL-4 and IL-5 have recently been shown to effect airway epithelial mucus production. Transgenic mice that overexpress IL-4 or IL-5 in the lung each showed a marked increase in mucus in the airway epithelium (7, 8). It is unclear if IL-4 and/or IL-5 act directly on the airway epithelium or if these cytokines are some of many inflammatory mediators involved in mucus production.

The regulation of airway epithelial cell mucus production by CD4 T cells has not been studied, although CD4 T cells are thought to play a primary role in initiating the inflammatory cascade that leads to asthma. Activated CD4 T cells have been identified in bronchial biopsies and bronchoalveolar lavage fluid of asthmatic patients (9–11), and animal models have shown that depletion of CD4 T cells inhibits airway inflammation (12, 13). Antigen-activated CD4 T cells can differentiate into effector cells with distinct functional properties (14–16). Th1 cells are a subset of CD4 T cells which secrete the potent macrophage activating cytokine, IFN- γ . CD4 Th2 cells make a different panel of cytokines, including IL-4, IL-5, and IL-10 (17, 18). Th2 cells are potent in activating B cells to secrete antibody,

particularly IgE (19–21). IL-5 secretion by Th2 cells also influences eosinophil differentiation, maturation and endothelial adherence (22).

Th2 cells secreting IL-4 and IL-5 have been shown to be present and activated in the bronchial wall of asthmatic individuals (9, 23). The presence of activated Th2 cells and eosinophils in the airways of asthmatics have been associated with more severe airway hyperresponsiveness (24, 25). Despite considerable correlative evidence supporting a role for Th2 cells in the pathogenesis of asthma, a cause and effect relationship has not been shown. Furthermore, IFN- γ has been identified in bronchoalveolar lavage (BAL)¹ fluid and serum of asthmatic patients suggesting that Th1-like cells may contribute to pathology in this disease (26–29).

To better understand the role of CD4 T cells in the development of airway pathology in asthma, we developed an *in vivo* system in which antigen-specific Th1 or Th2 cells were produced, and their ability to influence airway inflammation and airway epithelial mucus production was investigated. From these studies we show that Th2, but not Th1 cells, induce an inflammatory response which is strikingly similar to the pathologic features observed in human asthmatics. Th2 cells, but not Th1 cells induce mucus hypersecretion in the bronchial epithelium. Furthermore, using Th2 cells deficient in IL-4, we show that IL-4 is necessary for Th2-induced airway inflammation. Although current evidence suggests that IL-4 has a role in inducing mucus production by bronchial epithelial cells, we show that Th2-like cells can stimulate mucus production in the absence of IL-4.

Materials and Methods

Mice. DO11.10 mice, which are transgenic for the TCR recognizing OVA peptide 323–339 (pOVA^{323–339}) (30), were provided to us on BALB/c background by Ken Murphy (Washington University, St. Louis, MO) and were bred in our facilities. IL-4-deficient BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were bred in our facilities. Transfer recipients and immunizations were performed on 6–12-wk-old BALB/c mice (Jackson ImmunoResearch Labs., West Grove, PA).

Generation of Th1 or Th2 Cells. To generate Th1 or Th2 cells from DO11.10 mice, CD4 T cells were isolated by negative selection as previously described (31) using mAbs to CD8 (clone 53-6.72, clone 2.43 [32]), Class II MHC I-A^d (212.A1 [33]) and anti-Ig-coated magnetic beads (Advanced Magnetics, Inc. Cambridge, MA). Syngeneic T-depleted splenocytes were used as APC and prepared by negative selection using antibodies to CD4 (GK1.5 [34]), anti-CD8, anti-Thy1 (35) and treatment with rabbit complement. APCs were mitomycin-C treated. To induce Th2 cells from nontransgenic mice, IL-4-deficient mice and BALB/c mice were injected intraperitoneally with 100 μ g of OVA (Sigma Chemical Co., St. Louis, MO) in 4.5 mg of alum (Immuject; Sigma). 4 d after immunization, spleens and local draining lymph nodes were harvested and CD4 T cells were iso-

lated. All cultures were set up in flasks containing equal numbers of CD4 T cells and APCs at a concentration of $2\text{--}4 \times 10^6$ cells/ml. To generate Th1 cells, cultures contained pOVA^{323–339} at 5 μ g/ml, IL-12 at 5 ng/ml (Genetics Institute, Cambridge, MA), IL-2 at 10 U/ml (Collaborative Research Inc., Waltham, MA), and anti-IL-4 (11B11 [36]) at inhibitory concentration. To generate Th2 cells, cultures contained pOVA^{323–339} at 5 μ g/ml for DO11.10 cells or OVA at 50 μ g/ml for nontransgenic CD4 T cells, IL-4 at 200 U/ml (Collaborative Research, Inc.), IL-2 at 10 U/ml, and anti-IFN- γ (XMG1.2 [37]) at inhibitory concentration. Cultures were maintained for 4 d.

Transfer of Cells and Aerosol Administration of OVA. Cultured Th1 or Th2-like cells were harvested after 4 d, washed with PBS and 5×10^6 cells were injected intravenously into syngeneic BALB/c recipients. Control mice received freshly isolated CD4 T cells from naive BALB/c mice or in some experiments no cells were transferred. In experiments in which mice received TNF- α , 2 μ g of TNF- α (R&D Systems, Minneapolis, MN) was administered intranasally in 50 μ l PBS to anesthetized animals at the time of cell transfer. 1 d after transfer of cells, mice were challenged with inhaled 1% OVA in PBS or PBS alone for 20 min daily for 7–10 d. Mice were placed in a plastic chamber (27 \times 20 \times 10 cm) fitted with an attachment to allow entry of the aerosol from an ultrasonic nebulizer (1–5- μ m particles by manufacturer's specifications, OMRON; UltraAir NE-U07, Vernon Hills, IL). A small hole on the opposite end of the chamber ensured continuous airflow.

Lymph Node Isolation and Bronchoalveolar Lavage. Lung draining lymph nodes (peribronchial and parathymic), were isolated and single cell suspensions prepared for FACS[®]. BAL was performed by cannulation of the trachea and lavage with 1 ml of PBS. Cytospin preparations of BAL cells were stained with Dif-Quik (Baxter Healthcare Corp., Miami, FL) and differentials were performed based on morphology and staining characteristics. Isolation of lung lymphocytes was performed after BAL and perfusion of blood from lungs. Lung tissue was passed through a wire mesh, digested with collagenase Type IV 150 U/ml (Worthington Biochemical Corp., Freehold, NJ), elastase 10 U/ml (Sigma), and DNase 10 U/ml (Sigma) for 1 h at 37°C and passed again through a wire mesh to dissociate cells. Cell preparations were subjected to FACS[®] analysis.

Cytokine Production by Th1 or Th2 Cells. At the time of transfer, an aliquot of Th1- or Th2-like cells or naive CD4 T cells were retained for restimulation. 1×10^6 CD4 T cells/ml and 1×10^6 /ml freshly isolated BALB/c APCs were cultured with OVA (50 μ g/ml) or pOVA (5 μ g/ml). Supernatants were collected at 48 h. BAL cells obtained from individual mice were restimulated *in vitro* at 2×10^6 cells/ml in the presence of pOVA (5 μ g/ml). FACS[®] analysis was performed on BAL cells to determine the percentage of DO11.10 transgenic CD4 T cells and the amount of cytokine per ml per 10^6 transgenic T cells was calculated.

Cytokine Assays. IFN- γ , IL-4, IL-5, and IL-10 levels from cell supernatants were determined by ELISA (Endogen, Cambridge, MA). Assays were standardized with recombinant IFN- γ , IL-5, IL-10 (Endogen), and IL-4 (Collaborative Research, Inc.). The lower limit of sensitivity for each of the ELISAs was 0.6 ng/ml (IFN- γ), 5 pg/ml (IL-4), 0.010 ng/ml (IL-5), and 200 pg/ml (IL-10).

FACS[®] Analysis. At the time of transfer, FACS[®] analysis was performed on Th1, Th2, and naive CD4 T cell preparations to determine the purity of transferred cell populations. Cells were stained with anti-CD4 (Quantum Red-L3T4; Sigma) and in mice that received DO11.10 transgenic CD4 cells, the biotinylated anticolonotypic antibody, KJ1-26 (38), and fluorescein isothiocyanate-

¹Abbreviations used in this paper: BAL, bronchoalveolar lavage; DPAS, diastase-periodic acid-Schiff; HMI, histologic mucus index; pOVA^{323–339}, OVA peptide 323–339.

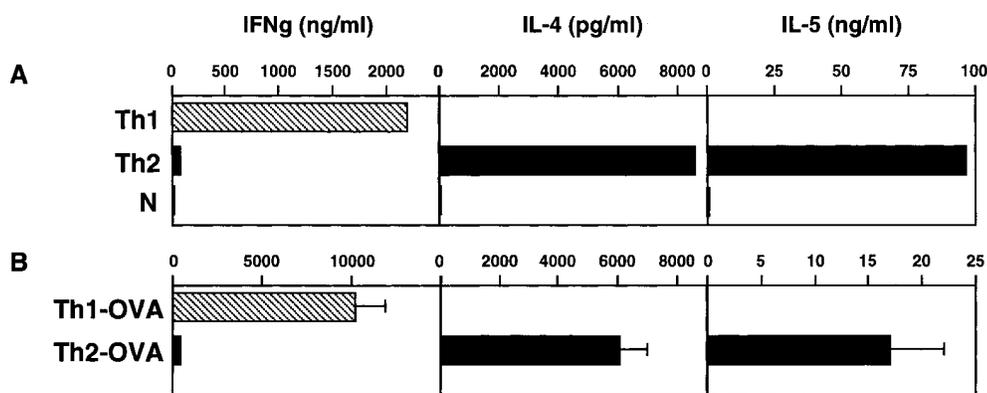


Figure 1. Cytokine production by Th1 or Th2-like cells before and after transfer. (A) At the time of transfer into recipient mice in vitro generated DO11.10 CD4 Th1, Th2 or freshly isolated CD4 T cells from naive BALB/c mice (N) were cultured with antigen presenting cells (2×10^6 cells/ml) in the presence of pOVA³²³⁻³³⁹. (B) After 7 d of exposure to inhaled OVA, BAL was performed on individual mice that received Th1 (Th1-OVA), Th2 (Th2-OVA), or naive (N) CD4 T cells. Total leukocytes recovered

from BAL were restimulated in vitro with pOVA³²³⁻³³⁹. BAL cells from mice that received naive CD4 T cells (N) and inhaled OVA contained <3% lymphocytes and were insufficient for cytokine analysis. Supernatants were collected at 48 h and cytokine ELISAs were performed. Cytokines production in BAL was adjusted for 10^6 DO11.10 CD4 T cells per ml as determined by FACS[®] analysis. Mean cytokine levels (\pm SEM) are shown ($n = 4-5$ mice per group). One experiment is shown and is representative of three experiments.

avidin D (Vector Laboratories, Burlingame, CA). KJ1-26 is specific for the transgenic T cell receptor in the DO11.10 mice. After a period of inhalational exposure, PBMC, LN, lung, and BAL cells were also analyzed by FACS[®] using these antibodies.

Lung Histology. Lungs were prepared for histology by perfusing the animal via the right ventricle with 20 ml of PBS. Lungs were then inflated with 1.0 ml of fixative instilled through a tracheostomy tube. Samples for paraffin sectioning were formalin fixed and stained with hematoxylin and eosin (H & E), Giemsa, diastase-periodic acid-Schiff (DPAS), mucicarmine, and alcian blue. Immunohistochemistry was performed on lungs perfused and fixed with periodate-lysine-pyrophosphate as previously described (39). Sections were stained with antibodies to MHC Class II, CD4, and the transgenic TCR using KJ1-26.

Histological Mucus Index. Formalin-fixed, paraffin embedded lungs were sectioned in the coronal plane at 5 microns. Lungs were sectioned to ensure that central airways were visible. After staining with DPAS, marker dots in a grid with 2-mm spacing were placed over the entire lung section. The slide was examined at $100\times$ final magnification on an Olympus BH-2 microscope (Olympus, Tokyo, Japan) with a rectangular 10-mm square reticule grid (American Optical Corp. Buffalo, NY) inserted in one eyepiece. Each marker dot was placed in the lower left corner of the field, and all intersections of airway epithelium with the reticule grid were counted in that field, distinguishing mucus containing or normal epithelium. Approximately 25% of the total lung section was scored. The ratio of total number of mucus positive intersections and the total of all intersections, which we call the histologic mucus index (HMI), is equivalent to the linear percent of epithelium positive for mucus (40). This index was calculated for each mouse lung and then the mean of the HMI was calculated for each experimental group.

Results

Generation and Characterization of OVA-specific Th1 and Th2 Cells. To investigate the effects of different T cell subsets on airway pathology, Th1 and Th2 cells were generated from CD4 T cells isolated from DO11.10 mice which are transgenic for the TCR recognizing pOVA³²³⁻³³⁹. Using a standard procedure to polarize CD4 T cell re-

sponses (41), splenic CD4 T cells were stimulated by pOVA³²³⁻³³⁹ in the presence of IL-12, IL-2, and anti-IL-4 to induce Th1-like cells or IL-4, IL-2, and anti-IFN- γ to induce Th2-like cells. 99% of the resulting activated Th1 or Th2 effector cells expressed CD4 and the DO11.10 TCR, recognized by the clonotypic monoclonal antibody, KJ1-26. An aliquot of cells from each culture was restimulated in vitro in the presence of pOVA³²³⁻³³⁹ and APCs, and supernatants were assayed for IFN- γ , IL-4, IL-5 (Fig. 1 A), and IL-10 (data not shown). The CD4 T cells stimulated to differentiate into Th1 cells produced high levels of IFN- γ and undetectable IL-4, IL-5, and low levels of IL-10, while the cells stimulated to differentiate into Th2 cells secreted high levels of IL-4, IL-5, IL-10, and minimal IFN- γ . Thus, we generated CD4 Th1 and Th2 effector cells responsive to OVA peptide. Naive CD4 T cells (N) freshly isolated from BALB/c mice did not secrete cytokines in response to OVA.

In Vivo Transfer and Recruitment of OVA-specific T Cells to the Lung. To determine whether Th1 or Th2 CD4 T cells could be recruited to the lung as committed effector cells, DO11.10 Th1 or Th2 cells were transferred intravenously into syngeneic BALB/c mice. A control group of mice received unprimed naive CD4 T cells. OVA was then administered to the mice by aerosol inhalation to recruit OVA-specific cells to the respiratory tract and to activate the cells locally. A control group of mice received aerosolized PBS. After 7 d of aerosol exposure, leukocytes from peripheral blood, lung-draining lymph nodes, BAL, and lung tissue were isolated and analyzed by FACS[®] to determine the contribution to inflammation of the transferred population of transgenic DO11.10 CD4 T cells (Table 1). While the donor TCR transgenic cells were not detectable in PBMC or uninvolved lymph nodes after aerosolized OVA, the transgenic TCR was expressed on CD4 T cells of the draining LN (8 and 13%, Th1 and Th2). Even more notable were the cells recovered from BAL and lung which expressed the donor transgenic TCR on a majority of CD4 T cells after aerosolized OVA administration. However,

Table 1. FACS[®] Staining of Mononuclear Cells from Mice that Received TCR Transgenic CD4 T cells

Aerosol exposure	Source of cells	Donor cells*		
		Th1	Th2	N
—	In vitro primed cells at time of transfer	99	99	—
PBS	Lung-draining LN	<1	<1	<1
PBS	BAL	‡	‡	‡
OVA	PBMC	<1	<1	<1
OVA	Inguinal, mesenteric LN	Not tested	<1	<1
OVA	Lung-draining LN	13 (8–16)	8 (4–10)	<1
OVA	BAL	69 (61–77)	57 (50–60)	<1
OVA	Lung	74	73	<1

*DO11.10 TCR transgenic Th1, Th2, or naive (N) CD4 T cells were transferred into BALB/c mice, and the mice were exposed to 7 d of aerosolized OVA or PBS. FACS[®] analysis was performed after aerosol exposure to PBS or OVA, as denoted. Cells were stained with anti-CD4 and KJ1-26 antibodies. Values are the mean percent (range) for individual Th1 or Th2 mice (3–5 mice/group). Lung lymphocytes were isolated and pooled from three mice.

‡After transfer of CD4 Th1, Th2, or naive cells and administration of PBS, BAL fluid contained too few CD4 T cells for these studies.

TCR transgenic cells were not seen in the lung, BAL, or lung draining LN after PBS administration. Thus, in the mice that received donor DO11.10 CD4 T cells and exposure to inhaled OVA, Th1 and Th2 cells were both recruited selectively to the lung and local LN with similar efficiency.

To determine if the donor cells retained the polarized cytokine profiles they exhibited at the time of transfer, BAL was performed on mice after 7 d of exposure to aerosolized OVA, and recovered cells were restimulated with OVA. As seen in Fig. 1 B, BAL cells from the primed mice that received Th1 cells (Th1-OVA) produced high levels of IFN- γ and minimal IL-4, IL-5, and IL-10 (data not shown) upon restimulation, while BAL cells from OVA-primed mice that received Th2 donor cells (Th2-OVA) produced IL-4, IL-5, and IL-10. BAL cells from control mice that received naive CD4 T cells and were exposed to inhaled OVA contained too few lymphocytes for restimulation.

Thus, after transfer and exposure to aerosolized OVA, OVA-reactive CD4 Th1 or Th2 cells were recruited to the respiratory tract and were reactivated to secrete the cytokines they exhibited at the time of transfer. The transferred cells maintained their commitment to the secretion of Th1 or Th2 cytokines in response to OVA as has been described previously (41).

Airway Inflammation in Mice that Received Th1 or Th2 Cells. To investigate how different effector CD4 T cells

influence inflammation in the respiratory tract, mice that received Th1, Th2, or unprimed naive CD4 T cells were compared. Mice that received Th1 or Th2 cells and were exposed to aerosolized OVA had moderate inflammation in the respiratory tract (Fig. 2, A1 and B1). The lungs from both groups of mice showed inflammation in a predominantly peribronchial and perivascular pattern. Despite similar degrees of inflammation, the two groups of mice had strikingly different inflammatory processes as determined by lung histology and analysis of BAL cells. The inflammatory infiltrates in the lungs of mice that received Th1 cells consisted of neutrophils, small mononuclear cells and macrophages (Fig. 2 B1). Differential counts performed on cells recovered from BAL confirmed these findings (Fig. 3). Immunohistochemical analysis of lung tissue showed that many infiltrating inflammatory cells stained with an anti-CD4 antibody (Fig. 4 A), the majority of these cells also stained with the TCR anticonotypic antibody, KJ1-26 (Fig. 4 B). MHC Class II expression in the lungs of these mice was increased on bronchial epithelial cells (Fig. 4 C) when compared to mice that received Th2 cells (Fig. 4 D), as expected from the effects of IFN- γ on airway epithelial cells (42).

In mice that received Th2 cells, the inflammation showed a large proportion of infiltrating eosinophils (Fig. 2 B2). By immunohistochemistry, CD4 positive cells were seen as the other predominant cell population in the inflammatory infiltrate, and a majority of CD4 expressing cells stained with the KJ1-26 antibody (data not shown). BAL cell counts show that eosinophils and lymphocytes were the predominant inflammatory cells present (Fig. 3). A minority of cells in the BAL fluid were neutrophils after 7 d of exposure to inhaled OVA. When exposures were carried out for 10 d, the percentage of neutrophils in BAL fluid decreased to zero, while mice that received Th1 cells and inhaled OVA had persistence of neutrophilia (data not shown).

Mice that received Th1 or Th2 cells and were exposed to aerosolized PBS had no lung pathology (data not shown). Mice that received naive CD4 T cells and aerosolized OVA had no significant lung inflammation (Fig. 2 D). Mice that received Th1 or Th2 cells and inhaled OVA did not exhibit histopathologic evidence of inflammation in other organs (data not shown).

Increased Mucus Staining and Secretion in Lungs of Mice that Received Th2 but Not Th1 Cells. Bronchial epithelial cells in mice that received OVA-specific Th2 cells and inhaled OVA showed hyperplasia and extensive DPAS positive staining indicating the presence of mucin collections (Fig. 2 B3). These findings were most striking in the central airways, although peripheral airways were also involved. Histological sections also showed increased amounts of mucus within the airway lumina of mice that received Th2 cells and aerosolized OVA. The material also stained positive for mucicarmine and alcian blue (data not shown). Mice that received Th1 cells and inhaled OVA had minimal to absent mucus staining (Fig. 2 A3). An HMI performed on lung sections from mice that received Th2 cells and inhaled OVA showed that 65% of airway epithelial cells were mucinous,

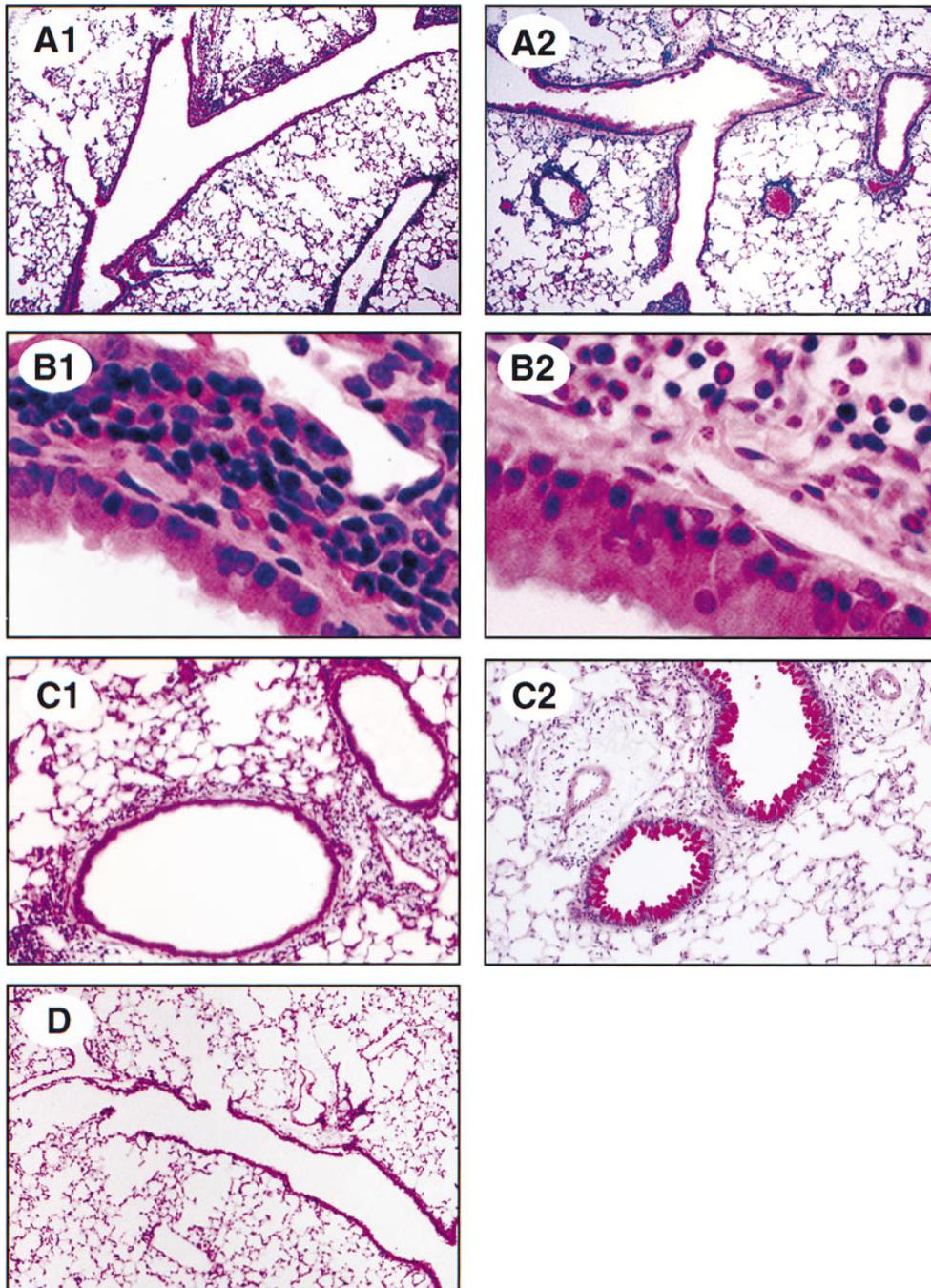


Figure 2. Lung histology in mice that received transfer of OVA-specific Th1, Th2, or naive CD4 T cells and 7 d of aerosolized OVA. (A1, B1, C1) = Th1 cell recipient. (A1) Lung showing moderate inflammation in peribronchial and perivascular location; H & E, 50 \times . (B1) Small airway showing neutrophil and mononuclear cellular infiltrate; H & E, 500 \times . (C1) Airways showing no staining for mucus; DPAS, 100 \times . (A2, B2, C2) = Th2 cell recipient. (A2) Lung showing moderate inflammation in peribronchial and perivascular location; H & E, 50 \times . (B2) Small airway showing infiltrate of eosinophils and small mononuclear cells; H & E, 500 \times . (C2) Airways showing increased purple-staining mucus in bronchial epithelium; DPAS, 100 \times . (D) Mouse that received transfer of naive BALB/c CD4 T cells; H & E 50 \times .

while mice that received Th1 cells and inhaled OVA or naive CD4 T cells and inhaled OVA had <5% of mucinous cells in the airways (Fig. 5).

Thus, mice that received transfer of OVA-specific Th2 cells and exposure to inhaled OVA had markedly increased mucus staining in the bronchial epithelium. Transfer of Th1 or Th2 cells and exposure to inhaled OVA resulted in a comparable level of inflammation, albeit comprising different cell populations, but only Th2 cells stimulated bronchial epithelial mucus production.

IL-4 Production by Donor T Cells Is Critical for Th2 Cell-induced Lung Inflammation. Previous studies had shown

that IL-4 overexpression in the airways resulted in mucus hypersecretion (7). To investigate the precise mechanism by which Th2 cells induced increased mucus staining, we began by studying the role of IL-4 in these processes. OVA-specific Th2 cells from IL-4-deficient (IL-4 $-/-$) mice or wild-type (IL-4 $+/+$) BALB/c mice were generated as has been described previously (41, 43). Mice were immunized with OVA in alum, and primed CD4 T cells were isolated and stimulated *in vitro* for 4 d with OVA in the presence of IL-4 and anti-IFN- γ . An aliquot of cells from each culture was restimulated *in vitro* in the presence of OVA and APCs, and supernatants were assayed for IFN- γ ,

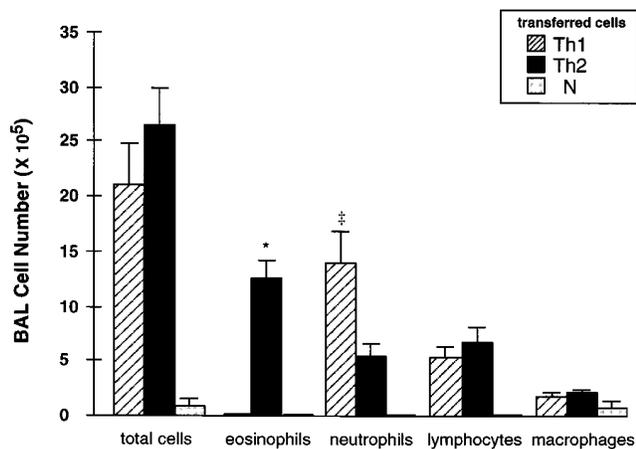


Figure 3. Bronchoalveolar lavage cell recovery in mice after transfer of cells and exposure to inhaled OVA. Differential counts were performed on cytospins of cells recovered from BAL from individual mice. Mean cell counts (\pm SEM) are shown ($n = 5$ mice per group). One experiment is shown and is representative of three experiments. Statistical significance was determined by unpaired Student's *t* test. * $P < 0.0001$ Th2 vs. Th1; [‡] $P < 0.02$ Th1 vs. Th2.

IL-4, IL-5, and IL-10 (Table 2). IL-4 $-/-$ OVA-specific Th2 cells produced comparable levels of IL-5 and IL-10 when compared to IL-4 $+/+$ OVA-specific Th2 cells, but IL-4 was produced only by IL-4 $+/+$ Th2 cells. CD4 Th2 cells were then transferred into BALB/c IL-4 $+/+$ recipients and the mice were exposed to inhaled OVA for 7 d. Control mice received no transferred cells and were exposed to inhaled OVA. In contrast to mice that received

IL-4 $+/+$ Th2 cells, IL-4 $-/-$ Th2 cells did not induce significant lung inflammation or mucus production (Fig. 6). Mice that received IL-4 $-/-$ Th2 cells and inhaled OVA had a 10-fold reduction in total BAL cell recovery compared to mice that received IL-4 $+/+$ Th2 cells. Furthermore, eosinophils and lymphocytes were strikingly reduced in the BAL fluid. These data suggested that IL-4 $-/-$ Th2 cells had either died or that production of IL-4 by the donor Th2 cell was necessary for its entry into the lung. Since staining of tissue sections revealed that VCAM-1 expression was markedly reduced in mice that received IL-4 $-/-$ Th2 cells and inhaled OVA (data not shown; Cohn, L., manuscript in preparation) and since VCAM-1 expression on lung endothelium has previously been shown to be critical for both lymphocyte and eosinophil recruitment to the lung (44), these experiments suggested that the transferred IL-4 $-/-$ Th2 cells were not recruited to the lung because of a defect in lymphocyte-endothelial adhesion.

IL-4 Is not Critical for Mucus Induction Once Cells Have Been Recruited to the Lung. To test if induction of adhesion molecules on the vascular endothelium would facilitate recruitment of OVA-specific IL-4 $-/-$ Th2 cells to the lung, we transferred OVA-specific IL-4 $+/+$, IL-4 $-/-$ Th2 cells or naive CD4 T cells into mice and treated the mice with inhaled TNF- α . TNF- α has previously been shown to increase both VCAM-1 and E-selectin expression on lung endothelium (45). Mice were then exposed to 7 d of inhaled OVA. As seen in Fig. 7 B, inflammation, as indicated by increased numbers of cells in the BAL, was observed in the mice that received IL-4 $+/+$ and IL-4 $-/-$ Th2 cells, whereas mice that received naive CD4 T cells

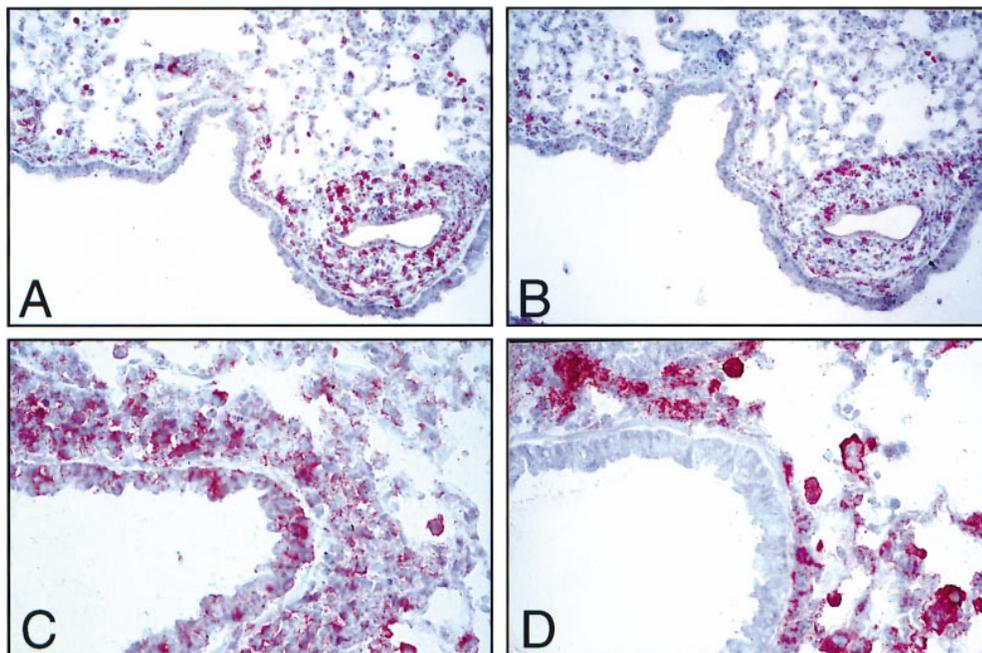


Figure 4. Immunohistochemical analysis of lungs from mice that received transfer of DO11.10 TCR transgenic cells. Localization in the lung of CD4 and donor TCR transgenic cells (A, B). Sequential lung sections from mice that received Th1 cells and exposure to aerosolized OVA were stained with (A) anti-CD4 and (B) KJ1-26 antibodies. KJ1-26 recognizes the DO11.10 TCR and was present on $>99\%$ of Th1 cells at the time of transfer. An airway and small vessel are shown with surrounding inflammatory cells ($100\times$). A majority of the infiltrating cells stain positively for both CD4 and the transgenic TCR. (C, D) MHC Class II expression in the lung after transfer of Th1 or Th2 cells. Lung sections from mice that received (C) Th1 or (D) Th2 cells and exposure to inhaled OVA were stained with an anti-I-A antibody (212.A1). A small bronchiole is shown. Both mice

that received transfer of Th1 or Th2 cells and aerosolized OVA exhibited increased MHC Class II expression on cells in the inflammatory infiltrates (red-stained cells) ($200\times$). Mice that received transfer of Th1 cells had increased red-staining of bronchial epithelium, whereas mice that received Th2 cells had no evidence of Class II MHC expression on bronchial epithelial cells.

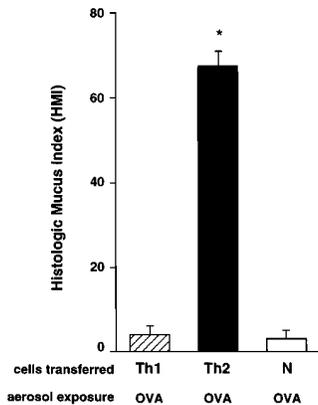


Figure 5. Mucus staining in airways of mice after transfer of Th1, Th2, or naive (N) CD4 T cells and exposure to inhaled OVA. An Histologic Mucus Index (HMI) was performed on lung sections stained with DPAS. Mean HMI (\pm SEM) is shown ($n = 4$ mice per group). One experiment is shown and is representative of three experiments. Statistical significance was determined by unpaired Student's *t* test. * $P < 0.0001$, Th1 vs. Th2.

and TNF- α had minimal inflammation. Mucus staining was increased in the bronchial epithelium of mice that received IL-4 $+/+$ or IL-4 $-/-$ Th2 cells and exposure to inhaled OVA (Fig. 7 A). Mice that received transfer of naive CD4 T cells, TNF- α and inhaled OVA did not show mucus staining in the bronchial epithelium.

OVA-specific IL-4 $+/+$ Th2 cells and IL-4 $-/-$ Th2 cells both induced eosinophilic inflammation, although IL-4 $+/+$ Th2 cells induced a greater degree of airway eosinophilia (Fig. 7 B). This may be the result of more effective entry into the lung of IL-4- and IL-5-producing Th2 cells or to a greater stability of this Th2 population compared to IL-4 $-/-$ Th2 cells, since it is known that IL-4 is important for persistence of Th2 cell populations early after polarization (46).

These results show clearly that donor IL-4-deficient Th2 cells can stimulate mucus production in the airway. However, to rule out a possible role of endogenous IL-4 secretion in mucus production, we transferred IL-4 $+/+$ and IL-4 $-/-$ Th2 cells into IL-4-deficient recipient mice. Mice were exposed to TNF- α and then aerosolized OVA for 7 d. As seen in Fig. 8, there was persistent induction of

Table 2. Cytokine Production by Transferred OVA-specific IL-4 $+/+$ and IL-4 $-/-$ Th2 Cells

Transferred cell population	IFN- γ	IL-4	IL-5	IL-10
	ng/ml	pg/ml	ng/ml	pg/ml
IL-4 $+/+$ Th2	nd	100	0.9	960
IL-4 $-/-$ Th2	nd	nd	2.0	440

At the time of transfer, OVA-specific CD4 Th2 cells generated from BALB/c (IL-4 $+/+$) or IL-4 $-/-$ mice were restimulated in vitro with OVA. Supernatants were collected at 48 h and cytokine ELISAs were performed. One experiment is shown and is representative of three experiments. nd, not detectable.

mucus in the airway epithelium in mice that received IL-4 $-/-$ Th2 cells in the absence of any recipient IL-4. Clearly, IL-4 secreted by CD4 T cells or by other cells recruited in the inflammatory process is not necessary to stimulate mucus production in the airway epithelium.

Discussion

Th2 cells, through the production of cytokines after specific antigen stimulation, have been hypothesized to initiate a cascade of events that leads to asthma. Although less widely acknowledged, BAL cells from asthmatic subjects have been shown to produce Th1-like cytokines (26–29). Our objective was to determine whether CD4 Th2 or Th1 cells in isolation could reproduce some of the inflammatory changes associated with asthma in a direct transfer model, and whether these inflammatory change would influence other important pathologic processes in asthma, specifically mucus hypersecretion. To do this we set up in vitro cultures designed to generate populations of cells containing a

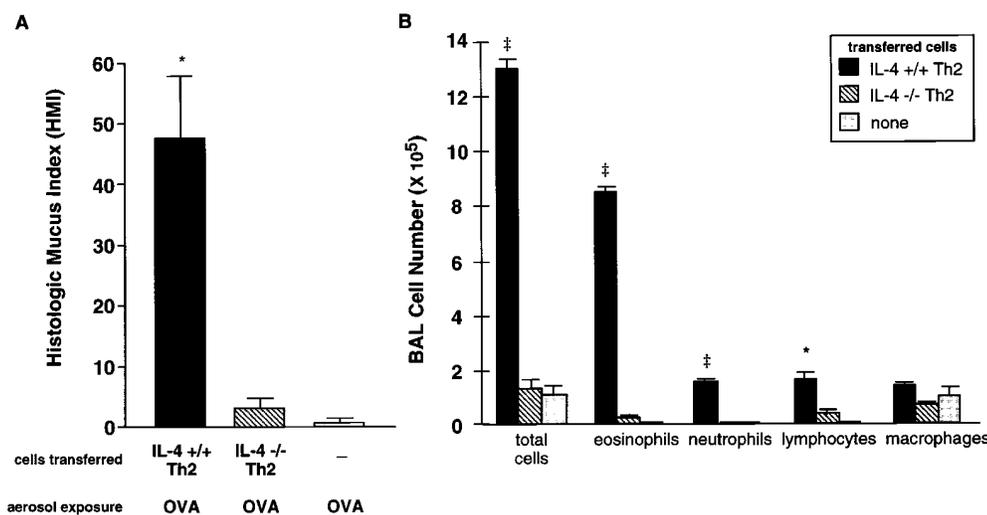


Figure 6. Mucus production and airway inflammation in mice after transfer of IL-4 $+/+$ or IL-4 $-/-$ Th2 cells and exposure to inhaled OVA. BALB/c mice received transfer of OVA-specific IL-4 $+/+$ or IL-4 $-/-$ Th2 cells and inhaled OVA. Controls received no cells and inhaled OVA. (A) HMI was performed on lung sections stained with DPAS. Mean HMI (\pm SEM) is shown. (B) BAL cells recovered from mice after exposure to 7 d of inhaled OVA. Differential counts were performed on cytopins of cells recovered from BAL of individual mice. Mean cell counts (\pm SEM) are shown ($n = 4$ mice per group). One experiment is shown and is representative of three experiments. Statistical significance was determined by unpaired Student's *t* test. * $P < 0.005$, IL-4 $+/+$ Th2 vs. IL-4 $-/-$ Th2. $\ddagger P < 0.0001$, IL-4 $+/+$ Th2 vs. IL-4 $-/-$ Th2.

representative of three experiments. Statistical significance was determined by unpaired Student's *t* test. * $P < 0.005$, IL-4 $+/+$ Th2 vs. IL-4 $-/-$ Th2. $\ddagger P < 0.0001$, IL-4 $+/+$ Th2 vs. IL-4 $-/-$ Th2.

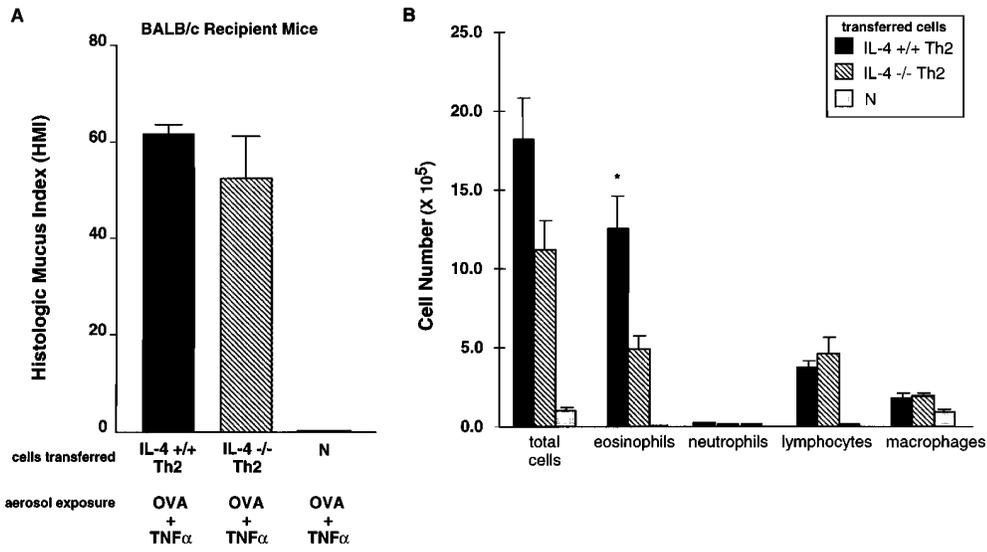


Figure 7. Mucus production and airway inflammation in mice after transfer of cells and exposure to TNF- α and inhaled OVA. BALB/c mice received transfer of OVA-specific IL-4 +/+, IL-4 -/- Th2 cells or naive CD4 T cells (N), then exposure to TNF- α and 7 d of inhaled OVA. (A) HMI was performed on lung sections stained with DPAS. Mean HMI (\pm SEM) is shown. (B) BAL cells recovered from mice. Differential counts were performed on cytopins of cells recovered from BAL of individual mice. Mean cell counts (\pm SEM) are shown ($n = 4-5$ mice per group). One experiment is shown and is representative of three experiments. Statistical significance was determined by unpaired Student's *t* test. * $P < 0.02$, IL-4 +/+ Th2 vs. IL-4 -/- Th2.

high frequency of antigen-specific Th1 or Th2 cells. The populations of CD4 effector cells we generated secreted cytokines in patterns that define them as Th1 or Th2. By biasing the generation towards Th1 or Th2 cells, this technique limits the potential for inducing mixed Th1 and Th2 populations typically generated by systemic immunization with antigen. Once CD4 Th1 or Th2 cells were generated, they were transferred into recipient mice that were then challenged with inhaled antigen.

We demonstrate that OVA-specific CD4 Th1 or Th2 cells can be recruited and activated in the respiratory tract and their activation results in different inflammatory pathology. Th2 cells activated in the respiratory tract result in pulmonary eosinophilia and mucus hypersecretion. Mice that received CD4 Th1 cells and inhaled OVA have comparable degrees of inflammation, but do not show significant changes in mucus production or eosinophilia. The histological findings in mice that received Th2 cells have a striking resemblance to human asthmatics.

The function of CD4 Th2 cells in modulating mucus production has not been studied previously. We show that Th2 cells specifically stimulate mucus hypersecretion. The striking difference in mucus induction by Th1 and Th2 cells and the previous finding that IL-4 over-expression induced mucus hypersecretion (7) suggested that this was due to IL-4. When Th2 cells from IL-4 -/- mice were transferred into mice and exposed to inhaled antigen there was no increase in mucus production, however, the cells were not recruited to the lung. These studies show the critical role of inflammation in mucus hypersecretion and confirms observations in a variety of human diseases, including asthma, that lung inflammation is necessary for mucus production (47). Others have investigated the role of IL-4 in lung inflammation using antigen immunized IL-4-deficient mice (48). They concluded that the lack of lymphocytes and

eosinophils in the lung after antigen challenge related to an inability to generate Th2 cells. Recent studies show that with prolonged antigen challenge, some airway inflammation can be induced in IL-4-deficient mice (49). In these systemically immunized IL-4-deficient mice, inflammation may result from activation of a mixed population of Th1 and Th2 cells, since IFN- γ produced by Th1 cells can activate different inflammatory pathways. Our work shows that IL-4 is required by Th2 cells to home to the lung and this function of IL-4 is distinct from its effects on Th2 cell development since in our study Th2 cells were generated in vitro. Thus, lung inflammation induced by activated CD4 Th2 cells is dependent on their secretion of IL-4.

The precise function of IL-4 in regulating Th2 cell-induced inflammation in the lung is not clear. It has been

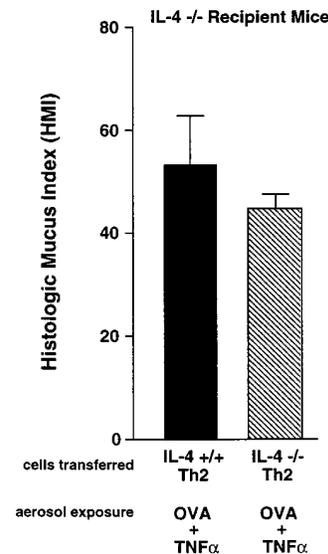


Figure 8. Mucus production and airway inflammation in mice after transfer of cells to IL-4 -/- recipient mice. IL-4 -/- mice received transfer of OVA-specific IL-4 +/+ or IL-4 -/- Th2 cells and exposure to TNF- α and 7 d of inhaled OVA. HMI was performed on lung sections stained with DPAS. Mean HMI (\pm SEM) is shown. ($n = 3-4$ mice per group). One experiment is shown and is representative of two experiments.

shown that IL-4 upregulates VCAM-1 expression on lung endothelial cells (45) and the interaction of VCAM-1 and VLA-4 on lymphocytes and eosinophils is critical for transendothelial migration of these cells to lung after antigen challenge in previously sensitized mice (44). In addition, chemokines including RANTES, MIP-1 α , MCP-1, and eotaxin, have been shown to have important roles in recruitment of lymphocytes and eosinophils to the lung (50, 51). The interplay of cytokines and chemokines in recruitment of leukocytes to the lung after antigen challenge is complex. Although our studies point to a reduction in VCAM-1 expression to explain the inhibition of inflammatory cell recruitment in mice that received IL-4-deficient Th2 cells, the precise effector function of IL-4 in these processes has not yet been detailed.

Once Th2 IL-4 $-/-$ cells were activated and recruited to the lung after administration of TNF- α to the recipient mice, we showed that mucus was still induced in the bronchial epithelium. Therefore, Th2 secretion of IL-4 did not directly induce airway epithelial mucus. Furthermore, IL-4 production by non-CD4 T cells was not directly responsible for induction of mucus.

Mucus hypersecretion was recently described in transgenic mice that overexpress IL-4 selectively in the lung (7). At an early age these mice had a marked peribronchial cellular infiltration with eosinophils, lymphocytes and neutrophils. In these transgenic mice, the precise effects of IL-4 could not be separated from the effects of other inflammatory cells found in the lungs. Others have shown a temporal correlation of mucus production with lung IL-4 mRNA levels (52). Our data show that IL-4 is crucial for mucus induction. IL-4 appears to function predominantly as an effector cytokine for recruitment of inflammatory cells to the lung to sites of antigen delivery.

The factors that directly induce mucus are still not clear. In our studies, airway eosinophilia is associated with increased airway epithelial mucus collections. Other studies suggest that eosinophils may have a role in mucus secretion. Recent studies of mice that overexpress IL-5 in the lung epithelium show increased mucus staining in the airways (8). These mice also have dramatic peribronchial infiltration with eosinophils. Furthermore, eosinophil cationic protein has been shown in the guinea pig to be a mucus secretagogue (5). Hogan et al. (49) recently showed that

mice can be induced to develop some peribronchial eosinophilic inflammation, along with airway epithelial cell damage and mucosal edema in the absence of IL-4. When these mice were treated with anti-IL-5, these pathological findings were inhibited, suggesting an important role for IL-5 and eosinophilia in some of the pathological changes associated with asthma. It has also been suggested that eosinophils, by damaging the bronchial epithelium and exposing the bronchial wall to more chemical stimuli, increase neural-mediated mechanisms of mucus secretion (47). We are currently studying the role of Th2 cell secretion of IL-5 in mucus production. CD4 Th2 cells also produce other known factors that are distinct from Th1 cells, such as IL-10 and IL-6, that may modulate mucus production. Henderson et al. (53) showed that leukotriene inhibition reduced mucus accumulation and eosinophilia in the airways of antigen stimulated mice. Leukotrienes are secreted predominantly by mast cells after IgE engagement with antigen. Thus, these findings suggest a Th2-mediated mechanism of mucus control. We measured equivalent levels of leukotrienes in the BAL fluid of both Th1 and Th2 mice after exposure to inhaled OVA despite the striking differences in mucus production (data not shown). Serum OVA-specific IgE levels were undetectable in mice that received Th2 cells and inhaled OVA at the time mice were sacrificed, also indicating that mast cells were not activated in our system. Thus, these differences suggest that mucus may be induced by a variety of inflammatory mediators. It is not clear at present if these mediators function through a final common pathway, perhaps the eosinophil.

In summary, these studies examine the direct effects of T cell subsets in airway inflammation. We have shown that Th2 cells influence some of the pathological processes that we associate with human asthma. Despite similar degrees and localization of lung inflammation, Th2 cells, and not Th1 cells, induce airway epithelial mucus production. We have further dissected the effector functions of CD4 Th2 cells using IL-4-deficient mice. Although some recent studies suggest that IL-4 mediates the induction of mucus, we have shown that IL-4 is critical only for the primary phase of mucus induction, lung inflammation. Once lung inflammation is initiated, CD4 Th2-like cells stimulate airway epithelial mucus production in the absence of IL-4.

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