Interleukin-5-producing CD4$^+$ T Cells Play a Pivotal Role in Aeroallergen-induced Eosinophilia, Bronchial Hyperreactivity, and Lung Damage in Mice

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Although activated CD4$^+$ T cells have been implicated in the pathogenesis of asthma, the direct contribution of this leukocyte to the induction of aeroallergen-induced bronchial hyperreactivity and lung damage is unknown. In the present investigation, we have used a model of allergic airways inflammation, which displays certain phenotypic characteristics of late-phase asthmatic responses, together with interleukin-5-deficient (IL-5$^{-/-}$) mice and donor antigen-specific CD4$^+$ TH2-type cells to obtain unequivocal evidence for a role of this T lymphocyte in the pathophysiology of allergic airways inflammation. Antigen-primed CD4$^+$ T cells and CD4$^+$ cells (CD4$^+$-depleted population) were purified from the spleens of ovalbumin (OVA)-sensitized wild-type mice and adoptively transferred to OVA-sensitized and nonsensitized IL-5$^{-/-}$ mice. In vitro stimulation of the purified cell populations with OVA resulted in the secretion of IL-4 and IL-5, but not interferon-γ, from the CD4$^+$ T cells, indicating that they were of the TH2 type. In contrast, interferon-γ, but not IL-4 and IL-5, was produced by the CD4$^+$ T cells. The CD4$^+$ TH2-type cells (not the CD4$^+$ cells) reconstituted aeroallergen (OVA)-induced blood and airways eosinophilia, lung damage, and airways hyperreactivity to β-methacholine in IL-5$^{-/-}$ mice. The reconstitution did not require prior sensitization of the mice, but it did not occur if they were aerosolized with saline instead of OVA. The circulating levels of OVA-specific IgE and IgG, were not significantly altered by the adoptive transfer of either cell population. These investigations establish that IL-5-secreting CD4$^+$ TH2-type cells play a pivotal role in generating blood and airways eosinophilia and in the subsequent development of bronchial hyperreactivity and lung damage that occurs in response to aeroallergens. Hogan SP, Koskinen A, Matthaei KI, Young IG, Foster PS. Interleukin-5-producing CD4$^+$ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice.


Airways mucosal inflammation is thought to play a critical role in the clinical expression and pathogenesis of asthma (1, 2). Clinical investigations show a correlation between the presence of activated airways inflammatory cells, morphologic changes to airways tissue, and the development of enhanced bronchial responsiveness to spasmogens (airways hyperreactivity) (1-5). The cellular composition of the inflammatory infiltrate is complex, consisting of activated mast cells, eosinophils, neutrophils, and monocytes. Moreover, the recruitment and/or subsequent activation of these inflammatory cells appears to be regulated by the secretion of cytokines and inflammatory mediators from antigen-activated T lymphocytes (5-7). In a number of investigations on lung biopsies and respiratory secretions from patients with asthma, infiltration of the airways by CD4$^+$ T cells and eosinophils is a central feature of the inflammatory process, which often correlates with disease severity (6, 8-10). A nalysis of cytokine profiles in the airways suggests that the CD4$^+$ T cells are activated and of the TH2 phenotype, secreting interleukin-5 (IL-5) and several other cytokines that have the potential to augment the inflammatory response (8-12).

It has been proposed that IL-5 acts as a central mediator in the regulation of eosinophilic inflammation and in the etiology of asthma and allergic disease (9, 13-15). Elevated levels of IL-5 and cells expressing mRNA for IL-5 are found in the blood and lung secretions from patients with asthma or after allergen-induced late-phase asthmatic responses and correlate with the degree of eosinophilic inflammation of the airways (8, 9, 12). Investigations with animal models that mimic late-phase asthmatic responses also implicate CD4$^+$ T cells and IL-5 as mediating aeroallergen-induced eosinophilia and airways hyperreactivity (15-18). Depletion of either CD4$^+$ T cells or IL-5 with neutralizing monoclonal antibodies (mAbs) significantly reduces eosinophil recruitment into sites of allergen challenge in the skin and lung (16, 18, 19). Recently, adoptive transfer of antigen-primed CD4$^+$ T cells to naive rats followed by aerosolization of recipients with the corresponding antigen resulted in a concomitant increase in airways eosinophils and lung resistance (20). In a previous investigation using IL-5-
deficient (IL-5−/−) mice, we established that blood and airways eosinophilia and the subsequent development of lung damage and airways hyperreactivity, that occurs in response to aeroallergens, can be directly regulated by IL-5 (15). However, in this study, the role of IL-5 production from CD4+ T cells for the accumulation of airways eosinophils and the development of allergic disease was not investigated.

Aithough previous observations support an association between CD4+ T cells and the development of allergic inflammation, a definitive role for the CD4+ T cell in the pathogenesis of allergic disease has yet to be established. Investigations with inhibitory CD4 mAbs are not definitive, as the CD4 molecule is not only expressed on lymphocytes involved in helper function but also on other inflammatory cells, such as eosinophils and monocytes, which are found in airways infiltrates after allergen provocation (1, 2). Moreover, from previous investigations in which allergic airways responses were reconstituted in rats by adoptive transfer of CD4+ T cells, it remains unclear whether eosinophilia and the onset of airways constriction was directly mediated by the adoptively transferred T cells or indirectly via T cell–dependent activation of other inflammatory cells (20). Evidence is also accumulating that CD8+ T cells may participate in the pathogenesis of allergen-induced bronchial hyperreactivity (21–25). Recently, investigations in mice demonstrated a critical role for the CD8+ T cell subset in the regulation of airways hyperreactivity, IL-5 production, and airways eosinophilia in response to antigen inhalation via the airways (21). Furthermore, while CD4+ TH2-type cells are thought to be the primary source of IL-5 and play a central role in regulating eosinophilia, IL-5 can also be secreted by activated mast cells and eosinophils. Circulating eosinophils may also be recruited to sites of allergen provocation by coordinate expression of a range of inflammatory cytokines and chemokines that are secreted not only from T lymphocytes but also from other cells at the site of inflammation (26–28).

Thus, although there is circumstantial evidence that IL-5 and CD4+ T cells may regulate allergen-induced bronchial hyperreactivity, the direct contribution of CD4+ TH2-type cells and IL-5 production by this lymphocyte to the morphologic and functional changes of the allergic lung is unknown. The aim of this investigation was to use an allergic airways model, containing certain phenotypic characteristics of late-phase asthmatic responses (15), and IL-5−/− mice to determine direct evidence for a role of antigen-activated CD4+ TH2-type cells in the initiation of the events that induce bronchial hyperreactivity and morphologic changes to the airways in response to aeroallergen challenge. IL-5−/− mice are specifically affected in IL-5 production, with no evidence of abnormalities in the production of other cytokines or antibodies (14). IL-5−/− mice did not develop pronounced eosinophilia or bronchial hyperreactivity and lung damage in response to sensitization and aeroallergen challenge. However, adoptive transfer of CD4+ TH2-type cells from ovalbumin (OVA)-sensitized wild-type mice (IL-5+/+) to OVA-sensitized and nonsensitized IL-5−/− mice restored blood and airways eosinophilia, bronchial hyperreactivity, and lung damage in response to inhaled antigen. These investigations directly demonstrate that antigen-activated CD4+ TH2-type cells in the airways can elicit all of the required signals for the induction of the pathophysiologic features of allergic airways inflammation.

**METHODS**

**Preparation of CD4+ T Cells**

C57BL/6 mice (6 to 8 wk of age) were sensitized by intraperitoneal injection with 50 μg OVA/1 mg A hydrogel (CSL Ltd., Parkville, Australia) in 0.9% sterile saline on Days 0 and 12. Nonsensitized mice received 1 mg A hydrogel in 0.9% saline. On Day 24, mice were killed by cervical dislocation and spleens excised to obtain CD4+ T cells. Erythrocytes were lysed and splenocytes (∼1 × 108) were suspended in 90 μL Hank’s Balanced Salt Solution (HBSS)/5% fetal calf serum (FCS) with 10 μl anti-CD4 mAb (L3T4)-coated microbeads and incubated for 20 min at 6 to 12°C. Suspensions were then centrifuged at 500 × g for 5 min at 4°C, and the resultant cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS)/0.5% bovine serum albumin. Bead-bound CD4+ cells were then isolated using high-gradient magnetic MiniMACS separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer’s instructions, washed, and resuspended in HBSS/5% FCS. The CD4+−depleted cell population (CD4+ population, non-bound cells) was also retained. The purity of the enriched CD4+ cell fraction and the CD4+ population was then determined by flow cytometry.

**Flow Cytometry**

Briefly, 103 cells (MACS-purified CD4+ cells or whole splenocytes) were washed in HBSS/0.02% sodium azide and then incubated with phycoerythrin (PE)-conjugated anti-mouse CD4 (clone L3T4) (CD4+ PE) and Cy-Chrome™ (CyC)-conjugated anti-mouse CD3e (CD3e-CyC) mAbs (PharMingen, San Diego, CA) for 30 min on ice and FCS underlaid. The cells were then centrifuged at 500 × g for 5 min at 4°C. The supernatant was then removed and the cell pellet resuspended in HBSS/0.02% sodium azide at 4°C before being analyzed by flow cytometry. A lysing was performed on a Becton and Dickinson FACScan flow cytometer using CellQuest (Becton and Dickinson, San Jose, CA) and WinMDI software packages (kindly provided by J. Tetter, Scripps Research Institute, La Jolla, CA). The lymphocyte-gated region was determined based on forward- and side-scatter signals on control splenocytes stained with Thy 1.2−fluorescein isothiocyanate. Approximately 10,000 cells in the lymphocyte-gated region were analyzed for each sample. CD3e-CyC and CD4+−PE were used for optimal staining with minimal signal overlap before use in flow cytometry experiments.

**Characterization of Cytokine Profile of MACS-purified CD4+ T Cells**

Whole splenocytes, CD4+ T cells, and CD4− cells (2 × 106 cells per preparation), which were purified from OVA-sensitized IL-5−/− mice, were incubated with complete medium (consisting of Eagle’s medium supplemented with glucose [0.2 M], L-asparagine [2 mM], L-arginine [8.8 mM], L-glutamine [2 mM], HEPES [10 mM], pH 7.4), sodium pyruvate [1 mM], β-mercaptoethanol [50 μM], penicillin [30 mg/L], streptomycin sulfate [50 mg/L], neomycin sulfate [50 mg/L], folic acid [0.14 mM], and heat-inactivated FCS (10%) in OVA (100 μg/well)-coated, 96-well plates (100 μl/well) for 72 h. Cell-free culture supernatants were then collected and stored in aliquots at −70°C until analyzed for cytokines.

**Induction of Allergic Airways Inflammation**

IL-5−/− mice (C57BL/6, 6 to 8 wk of age) (14) were sensitized by intraperitoneal injection with 50 μg OVA/1 mg A hydrogel in 0.9% sterile saline on Days 0 and 12. Nonsensitized mice received 1 mg A hydrogel in 0.9% saline. On Day 23, CD4+ T cells (2 × 106 cells) were adoptively transferred to OVA-sensitized and nonsensitized mice. CD4− cells (2 × 106 cells) were adoptively transferred only to OVA-sensitized mice. On Day 25, the appropriate groups of mice were exposed three times at hourly intervals to an aerosol of OVA (10 mg/mL) in 0.9% saline or saline alone for 30 min and then every second day thereafter, for 8 d (23). On Day 32, 24 h after the last aerosolization, lungs were removed and analyzed for eosinophils and other inflammatory cells. The lungs were removed and analyzed for eosinophils and other inflammatory cells.
Mice were treated according to Australian National University Animal Welfare guidelines and were housed in an approved containment facility.

Measurement of Airways Hyperreactivity
A baseline constriction was measured with a bronchopasm transducer (Ugo Basile 7020) coupled to a Lab MacB analysis station (A di-instruments, Sydney, Australia). Changes in respiratory overflow volume were determined during cumulative intravenous administration of β-methacholine as previously described (15). The increase in respiratory overflow volume provoked by β-methacholine was represented as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula.

Characterization of Lung Morphology and Eosinophils in Blood, Tissue, and Bronchoalveolar Lavage Fluid
Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with May-Grünwald-Giemsa solution or hematoxylin-eosin. Leukocytes in the blood, bronchoalveolar lavage fluid (BALF), and lung were identified by morphologic criteria and quantified as previously described (15).

Preparation of Whole Spleen, Lung, and Peribronchial Lymph Node Homogenates
To prepare homogenates, spleen and draining peribronchial lymph nodes (PBLN) were excised and homogenized in HBS/10% FCS at 4°C. The resulting cell suspension was filtered through nylon mesh (70 μm) and centrifuged at 500 × g for 5 min at 4°C. The cell pellet was then resuspended in red blood cell lysis solution and centrifuged at 500 × g for 5 min at 4°C. The resulting cell pellet was resuspended in complete medium, and cells per milliliter were quantified and used in OVA-stimulation assays. Lung homogenates were prepared after cannu-lating the trachea and perfusing the airways with a 1-ml solution of 1% collagenase/PBS at 4°C. The lungs were then removed, resuspended in HBS/10% FCS, and vigorously vortexed, before being incubated at 37°C for 30 min. The resulting cell suspension was homogenized and filtered through a 70-μm nylon mesh and centrifuged at 500 × g for 5 min at 4°C. The resulting cell pellet was then resuspended in ML C (mixed lymphocyte culture medium)/10% FCS, filtered through FACS gauze, and centrifuged at 500 × g for 5 min at 4°C. Cells were then resuspended in ML C/10% FCS and used for OVA-stimulation assays.

Cytokine Production by Spleen, Lung, and PBLN Cells in Whole Homogenates after Stimulation with OVA
Spleen, lung, and PBLN homogenates were cultured (10⁶ cells/ml) in complete medium on OVA (100 μg/ml)-coated, U-bottom, 96-well plates (100 μl/well) for 72 h. Cell-free culture supernatants were then collected and stored in aliquots at −70°C until cytokine analyses were carried out.

Analysis of Cytokines
Interferon-γ (IFN-γ), IL-5, and IL-4 concentrations were determined in the supernatants from OVA-stimulated CD4⁺ T cells, CD4⁻ T cells, and spleen, lung, and PBLN cell homogenates. IFN-γ concentrations were determined by ELISA. Briefly, 96-well, round-bottom immunonassay plates were coated with rat anti-mouse IFN-γ mAb (5 μg/ml, clone: RA-6A2). Plates were blocked with 5% FCS/PBS for 1 h at 37°C and incubated with serial dilutions of cultured supernatants or standard murine IFN-γ. Murine IFN-γ was detected with a rabbit anti-mouse IFN-γ antiserum. Plates were incubated with peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at 37°C and washed, and A B T S substrate solution (Kirk & Kregg & Perry Laboratories, Gathersburg, MD) was added. After an appropriate incubation time, the reaction was stopped with 1 M citric acid. Plates were read in a M icroplate reader (B io-Tek Instruments, Winooski, VT) at 490 nm with reference to 405 nm. The sensitivity of the ELISA system was 0.5 ng/ml of IFN-γ. The reagents used in ELISA assays were kindly provided by Dr. G.K. Arupiah (John Curtin School of Medical Research, Australian National University).

RESULTS

Phenotypic Characterization of MACS-purified CD4⁺ Splenocytes
Flow cytometry analysis of the CD4⁺-enriched splenocyte population (head-bound cells) isolated from OVA-immunized IL-5⁻/⁻ mice by MACS separation indicated that this population consisted of approximately 95% CD4⁺ cells (Figure 1A). The CD4⁺ population (CD4⁻ depleted cell population, non-bound cells) was routinely shown to consist of <5% CD4⁺ cells (results not shown). CD4⁺ cells were approximately 15% of the total splenocyte population (Figure 1A). To demonstrate that the MACS-purified CD4⁺ cell population consisted primarily of CD4⁺ T cells, this population was further characterized by flow cytometry using a CyC-conjugated rat antimouse CD3 mAb. The purified CD4⁺ cell population routinely consisted of >95% CD3⁺ CD4⁺ T cells (Figure 1B).

Cytokine Production of MACS-purified CD4⁺ T Cells
An equal number of whole splenocytes, CD4⁺ cells, and purified CD4⁺ T cells were isolated from IL-5⁻/⁻ mice and stimulated with immobilized OVA for 72 h and the levels of IL-4, IL-5, and IFN-γ production determined (Table 1). Only the purified CD4⁺ T-cell population produced IL-4 and IL-5, with no IFN-γ being detected after stimulation, indicating that these OVA-specific CD4⁺ T cells were of the Th2 phenotype. Whole splenocytes and CD4⁺ populations produced detectable levels of IFN-γ but no IL-4 or IL-5 in response to OVA stimulation. Notably, detectable levels of IL-5 were only found in the enriched CD4⁺ T-cell population.
shows that the MACS-purified population of CD4 T cells tentatively 15% of the total splenocyte population. Open histogram shows that OVA-immunized IL-5/−/− mice, phenotypic characterization using the T-cell marker CD3−/− was performed. The MACS-purified CD4+ cell population consisted of > 95% CD3+ CD4+ T cells. (A) Representative histogram of CD4+ splenocytes from OVA-immunized IL-5/−/− mice stained with PE-conjugated anti-mouse CD4. Filled histogram shows that CD4+ cells were consistently 15% of the total splenocyte population. Open histogram shows that the MACS-purified population of CD4+ splenocytes were consistently > 95% of total cells. (B) Representative dot plot of dual-color immunofluorescence staining of MACS-purified CD4+ splenocytes with PE-conjugated anti-mouse CD4 and Cy5-conjugated anti-mouse CD3ε. To determine the percentage of T-cell populations obtained after MACS separation. A total of 2 × 10⁶ cells from each preparation was incubated with complete medium in OVA (100 μg/well)-coated, 96-well plates for 72 h. Supernatants were removed and analyzed for cytokines. IFNγ levels were determined by ELISA, and IL-4 and IL-5 levels by bioassays using the CT4.S and BCL1 cell lines, as described in Methods. Data are shown as mean ± SEM of two to three separate cultures with four to five mice per culture group. ND = below detectable limits.

Figure 1. Flow cytometric analysis of CD4+ splenocytes purified by high-gradient magnetic MiniMACS separation columns (MACS purified). (A) Representative histogram of CD4+ splenocytes from OVA-immunized IL-5/−/− mice stained with PE-conjugated anti-mouse CD4. Filled histogram shows that CD4+ cells were consistently 15% of the total splenocyte population. Open histogram shows that the MACS-purified population of CD4+ splenocytes were consistently > 95% of total cells. (B) Representative dot plot of dual-color immunofluorescence staining of MACS-purified CD4+ splenocytes with PE-conjugated anti-mouse CD4 and Cy5-conjugated anti-mouse CD3ε. To determine the percentage of T cells present in the MACS-purified CD4+ splenocytes, phenotypic characterization using the T-cell marker CD3ε was performed. The MACS-purified CD4+ cell population consisted of > 95% CD3+ CD4+ T cells. The lymphocyte-gated region was determined based on forward- and side-scatter signals on control splenocytes stained with Thy-1.2- fluorescein isothiocyanate. Approximately 10,000 cells in the lymphocyte-gated region were analyzed for each sample.

Adoptive Transfer of OVA-primed CD4+ T Cells Induces Blood and Airways Eosinophilia, Airways Hyperreactivity, and Lung Damage in IL-5−/− Mice

On Day 24, CD4+ T cells or CD4− cells were isolated from IL-5−/− mice and adoptively transferred to OVA-sensitized or nonsensitized IL-5−/− mice. Mice were then aerosolized with OVA or saline and, on Day 32, the airways were examined for changes in structure and function. No eosinophils were detected in the blood (Figure 2A) or BALF (Figure 2B) from OVA-sensitized and -aerosolized (OVA/OVA) CD4−-treated or OVA-sensitized and saline-aerosolized (OVA/Sal) CD4−-treated animals. In contrast, in the OVA/OVA-treated and Sal/OVA-treated mice that received CD4+ T cells, significantly higher levels of eosinophils were detected in the blood (Figure 2A) and BALF (Figure 2B). A significant blood eosinophilia was observed from Days 26 to 32; circulating and BALF eosinophil numbers were similar in both the OVA/OVA and Sal/OVA CD4+ T cell–treated mice. No significant difference in numbers of lymphocytes, neutrophils, or macrophages in BALF were observed between all groups that were OVA-sensitized and/or OVA-aerosolized (results not shown). Histologic sections of lung tissue from OVA/OVA- and Sal/OVA-treated (Figure 2D) and Sal/OVA-treatment (results not shown) mice, which received CD4+ T cells, showed a severe pulmonary eosinophilic inflammation that was widespread. Infiltrating eosinophils were primarily seen in the perivascular and peribronchial regions of the lungs, aggregating and marginating around the airways (Figure 2D). In some sections, eosinophils were also observed in the lumen of the airways. Furthermore, after aeroallergen challenge in OVA/OVA or Sal/OVA CD4+ T cell–treated mice, gross morphologic changes in the airway walls (bronchi and bronchioi) with epithelial cell shedding and extensive mucosal edema were seen. Increased tissue cellularity and particulate exudates in the airways lumina and plugging of the airways were also common features of sections from these mice. Neither airways eosinophilia nor alterations in the structural integrity of the airways walls were observed in lung sections from OVA/Sal CD4+ T cell–treated (Figure 2C) or OVA/OVA CD4−-treated (results not shown) mice. Eosinophilia and allergen-induced bronchial hyperreactivity and lung damage were not induced when the donor CD4+ T cells were adoptively transferred to Sal/Sal-treated mice (results not shown).

Aeroallergen-induced blood and airways eosinophilia and morphologic changes to the airways were correlated directly with the induction of airways hyperreactivity to β-methachoo-
OVA-specific Immunoglobulins in OVA-immunized and Aerosolized IL-5−/− Mice
The levels of OVA-specific IgE and IgG1 were determined in serum samples from all groups of mice on Day 32. OVA-spe-
cific IgG1 and IgE were detected in all groups on Day 32 after OVA aerosolization treatment. Levels of IgG1 and IgE in OVA/OVA CD4+ and Sal/OVA CD4+ treated mice were significantly elevated in comparison with OVA-sensitized and Sal-aerosolized (OVA/Sal) CD4+ T cell–treated mice, indicating that direct exposure of the airways with allergen induces isotype switching (Table 2). The levels of OVA-specific IgG1 and IgE in OVA/Sal CD4+ T cell–treated mice (Day 32) were comparable to levels seen on Day 23 in OVA/OVA CD4+ T cell–treated mice (results not shown). No significant differences were observed in OVA-specific immunoglobulins between OVA/OVA CD4+ T cell–treated and OVA/OVA CD4+–treated mice, indicating that adoptively transferred cells did not significantly affect antibody production. Interestingly, Sal/OVA CD4+ T cell–treated mice produced OVA-specific immunoglobulins on Day 32, suggesting that passive immunization of the airways can induce allergen-specific antibody production. The levels of OVA-specific IgG1 were similar to that obtained from OVA/OVA CD4+ T-cell group (Table 2). However, direct challenge of the airways with OVA in the absence of sensitization resulted in significantly greater production of IgE in comparison to OVA/OVA CD4+–treated mice. On Day 23, prior to aerosolization, no OVA-specific immunoglobulins were detected in Sal/OVA CD4+ T cell–treated mice (results not shown).

Cytokine Production by Whole Homogenates of Spleen, PBLN, and Lung from OVA/OVA CD4+ T Cell–Treated IL-5−/− Mice

Cytokine production by splenocytes, draining PBLN, and homogenized lung from OVA/OVA CD4+–treated IL-5−/− mice was determined after stimulation with OVA for 72 h (Table 3). IL-5 was detected only in the PBLN and lung homogenates (Table 3) in response to OVA stimulation. In contrast, IL-4 and IFN-γ production was observed in all extracts.

DISCUSSION

The role of T cells in the cellular and molecular mechanisms underlying the pathogenesis of airways disease in patients with asthma is unknown. In the present investigation, we have used a model of allergic airways inflammation and IL-5−/−

<table>
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<th>OVA-specific Immunoglobulins</th>
<th>Adoptively Transferred T-cell Population</th>
<th>IgG1 (×10^6 ng/ml)</th>
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<td>OVA/OVA CD4+</td>
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<tr>
<td>Sal/OVA CD4+</td>
<td>30.1 ± 7.3</td>
<td>4,633.8 ± 549.0*</td>
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Mice that received CD4+ T cells were (1) OVA-sensitized and aerosolized with OVA (OVA/OVA), (2) OVA-sensitized and aerosolized with saline (OVA/Sal), or (3) nonsensitized and aerosolized with OVA (Sal/OVA). Mice that received CD4+ cells were OVA-sensitized and aerosolized with OVA (OVA/OVA). Airways constriction was measured by determining changes in respiratory overflow volume during cumulative intravenous administration of β-methacholine and is represented as a percentage of the maximal overflow volume (100%) obtained by total occlusion of tracheal cannula. Data represent mean ± SEM for groups of four to six mice. The significance of differences between experimental groups was analyzed using the unpaired Student’s t test. Differences were considered significant if p < 0.05. *p < 0.01 compared with OVA/Sal CD4+–treated mice.

**Table 3**

| CYTOKINE PRODUCTION BY OVA-STIMULATED CELLS FROM HOMOGENATES OF SPLEEN, LUNG, AND PERIBRONCHIAL LYMPH NODES ISOLATED FROM OVA/OVA CD4+ T CELL–TREATED IL-5−/− MICE |
|-----------------|----------------|----------------|
|                  | IL-5 (U/ml) | IL-4 (ng/ml) | IFN-γ (U/ml) |
| Spleen          | ND           | 11.6 ± 16.5   | 21.1 ± 3.0    |
| Lung            | 0.36 ± 0.01  | 5.1 ± 0.6     | 32.1 ± 3.8    |
| Lymph node      | 0.11 ± 0.01  | 2.2 ± 2.2     | 10.5 ± 7.4    |

Spleen, lung, and PBLN homogenates were prepared from OVA/OVA CD4+ T cell–treated IL-5−/− mice on Day 32. A total of 2 × 10^6 cells were incubated in OVA-coated, 96-well plates for 72 h. Supernatants were removed and analyzed for cytokines. IFN-γ levels were analyzed by ELISA, and IL-4 and IL-5 levels by biosays, using the CT4.5 and 3E10 cell lines, as described in Materials. Data are shown as mean ± SEM of two to three separate cultures with four to five mice per culture group. ND = below detectable limits.
mice to demonstrate that CD4+ TH2-type cells, through the secretion of IL-5, orchestrate the recruitment of eosinophils to the airways and the subsequent induction of allergic airways inflammation. A adoptive transfer of CD4+ TH2-type cells to aeroallergen-challenged OVA-sensitized IL-5−/− mice induced blood and airways eosinophilia, airways hyperreactivity to β-methacholine, and morphologic changes to the airways. In contrast, transfer of a CD4− cell population to aeroallergen-challenged OVA-sensitized IL-5−/− mice did not promote eosinophilia or allergic airways inflammation. The CD4+ T-cell-induced airways inflammation and changes in lung function did not require prior sensitization of mice with OVA but was dependent on aeroallergen challenge. The location of CD4+ T cells during allergic inflammation in IL-5−/− mice was identified by assessing the secretion of IL-5. IL-5 was detected in the lung and PBLN and not in the spleen of OVA-sensitized and aerosolized CD4+ T cell–treated IL-5−/− mice. Thus, a direct association was observed between the presence of antigen-activated CD4+ TH2-type cells in the lungs of IL-5−/− mice and the induction of blood eosinophilia, the accumulation of eosinophils in the airways, and the development of characteristics of allergic lung inflammation. Interestingly, in other investigations, adoptively transferred CD8+ T cells were also found to preferentially home to PBLN in response to OVA sensitization via the airways (21), suggesting that chemokine signals for the recruitment of both CD4+ and CD8+ T-cell subsets may be elicited from the lung in response to a specific antigen.

Collectively, our experiments show a critical role for signals elicited from the lung, in response to the processing of inhaled antigen, for the subsequent recruitment and activation of airways CD4+ T cells and the induction of bronchial hyperreactivity and morphologic changes to the airways. The data also indicate that OVA sensitization stimulates the expansion of OVA-reactive, IL-5-producing CD4+ T cells and it is these cells that are essential for the induction of allergic airways inflammation in response to allergen provocation. Interestingly, OVA sensitization also stimulated the development of OVA-reactive CD4+ T cells that secrete IFNγ, but not IL-4 or IL-5, which did not mediate disease.

Moreover, our results clearly demonstrate that CD4+ TH2-type cells can provide all the signals essential for the induction of allergen-induced bronchial hyperreactivity and lung damage and support the concept that the accumulation and activation of airways eosinophils during the asthmatic response may be initiated and sustained by cytokines released from this lymphocyte. A IgE-dependent bronchial hyperreactivity may also be regulated in parallel by IgE-dependent activation of mast cells. Recently, other investigators have characterized the phenotype of airways T-cell populations during OVA-induced allergic inflammation in mice (29, 30). In support of our observations, the majority of infiltrating T cells were CD4+ expressing TH2 cytokines as well as surface markers indicative of memory T cells (29, 30). A nigen-activated T cells have also been directly implicated in the induction of the expression of eotaxin, a chemokine that selectively mediates eosinophil recruitment to the lung (31). Eotaxin has been shown to play an important role in the accumulation of eosinophils to tissues in the early phases of inflammatory responses (26, 27). Thus, antigen-activated T cells may play a pivotal role in the regulation of airways eosinophilia by inducing the expression of local eosinophil-specific chemokine signals as well as by providing the critical signal for eosinophil mobilization from the bone marrow (15, 31). Interestingly, no role for mast cell factors or IgE in the regulation of pulmonary eosinophilia was observed (31).

Although the presence of increased numbers of eosinophils in the airways mucosa is a characteristic feature of asthma (3, 8), there is conflicting evidence about the relative importance of this leukocyte and IL-5 to the mechanisms underlying the induction of allergen-induced bronchial hyperreactivity and lung damage (5, 6, 14, 16, 18, 29). It is clear from investigations with IL-5−/− mice that the absence of IL-5 alone can abolish tissue and blood eosinophilia and the characteristic pathology and changes in airways function generated by allergic airways responses (15). In marked contrast, a recent investigation using inhibitory mAbs suggests an essential role for IL-4, and no role for IL-5 and eosinophils, in the development of aeroallergen-induced airways hyperreactivity that was linked to CD4+ TH2-type cells (32). In the current investigation, we show a direct relationship between IL-5 production from antigen-activated CD4+ T cells, the accumulation of airways eosinophils, and the onset of bronchial hyperreactivity. Notably, the numbers of neutrophils, monocytes, and lymphocytes in the BALF were not significantly different between experimental groups sensitized and/or aerosolized with OVA (results not shown). A s previously shown (15), no increase in mast cell numbers after sensitization and aeroallergen challenge of IL-5−/− mice was observed (results not shown), supporting recent investigations that the release of mast cell factors is not essential for the induction of blood or airways eosinophilia (31, 33). Thus, our investigation not only shows a key role for CD4+ T-cell–dependent regulation of eosinophil trafficking but also further implicates the eosinophil as a mediator of bronchial hyperreactivity and changes in airways structure during aeroallergen provocation. While alternative pathways, which are intimately regulated by IL-4 and the effector functions of other inflammatory cells, may predispose to aeroallergen-induced airways hyperreactivity (32), IL-5-regulated eosinophilia and factors coupled to this pathway are also central to the development of allergic airways inflammation.

Although the onset of bronchial hyperreactivity and lung damage in the present study was critically linked to the presence of OVA-specific CD4+ TH2-type cells in the airways of aeroallergen-challenged mice and associated with the recruitment of eosinophils to the lung, the molecular mechanisms underlying the induction of airways dysfunction were not defined. In response to sensitization and aeroallergen challenge or aeroallergen alone, OVA-specific IgE and IgG1 isotypes were produced. OVA-specific IgE production was markedly upregulated by antigen inhalation. Recently, allergen-specific IgE and IgG1 were shown to have the potential to regulate components of allergic inflammation that are controlled by CD4+ T cells and eosinophils. IgE-dependent mechanisms were shown to be essential for the development of eosinophil-mediated airways hyperreactivity in response to antigen inhalation and to regulate cytokine production by CD4+ TH2-type cells (34, 35). In vitro studies have also shown that allergen-specific immunoglobulins may trigger the release of preformed inflammatory mediators from eosinophils (36). Thus, allergen-specific IgE and IgG1, which were generated in response to OVA inhalation may play a role in regulating eosinophilia and the induction of airways disease associated with this leukocyte. Alternatively, direct interactions between CD4+ T cells and eosinophils may mediate allergen-induced bronchial hyperreactivity and lung damage (20, 30).

In summary, we hypothesize that OVA-reactive CD4+ T cells are selectively recruited to the sites of inflammation by signals resulting from antigen delivery to the airways. Within the lung, CD4+ T cells are activated by antigen-presenting cells, inducing the secretion of an array of cytokines including IL-5. The secretion of IL-5 stimulates the recruitment of eosinophils...
from the bone marrow to the airways, where they become activated and degranulate to induce airways dysfunction.

While the chain of events leading to asthma involves a complex cascade of interacting cells and inflammatory mediators, our results clearly demonstrate that CD4+ T-cell and IL-5 secreted from this cell, in association with the influx of eosinophils to the site of inflammation, can provide the critical signals for the development of the pathophysiology of allergic airways inflammation. These results also support clinical investigations implicating CD4+ T-cell and eosinophils as key effector cells for the induction of bronchial hyperreactivity in late-phase asthmatic responses.

References
