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An Adoptive Transfer Model of Allergic Lung Inflammation in Mice Is Mediated by CD4\(^+\)CD62L\(^{low}\)CD25\(^+\) T Cells

James T. Wise,* Theodore J. Baginski,† and James L. Mobley††

Animal models of allergic lung inflammation have provided important insight into the cellular and biochemical factors involved in the pathogenesis of human asthma. Herein, we describe an adoptive transfer model of OVA-specific eosinophilic lung inflammation in the mouse that is used to characterize the cells involved in mediating the pulmonary inflammatory response. We report that freshly isolated spleen cells from OVA-sensitized mice are unable to prime naive recipient mice to respond to a subsequent OVA aerosol challenge. Subjecting the spleen cells to short term restimulation with Ag in vitro, however, renders the cells competent to transfer activity. The magnitude and the kinetics of the eosinophilic pulmonary inflammation in the adoptive transfer recipients are nearly identical with those generated by a more conventional active sensitization/challenge protocol, with the notable exception of differential production of plasma IgE in the two models. Extensive negative and positive selection of splenocyte subtypes indicates that the transfer of Ag-primed CD4\(^+\) T cells is both necessary and sufficient to establish full responsiveness in the recipient mice. Additional phenotypic characterization of the transfer-reactive CD4\(^+\) T cells indicates that they are found within the CD62L\(^{low}\)CD25\(^+\) subset and secrete high levels of IL-5 in response to Ag stimulation. Limiting dilution analysis-derived minimal frequency estimates indicate that approximately 1 in 8500 of the sensitized, cultured spleen cells produces IL-5 in response to OVA stimulation in vitro, suggesting that eosinophilic lung inflammation can be induced in naive mice by the transfer of <1200 Ag-specific CD4\(^+\) T cells. The Journal of Immunology, 1999, 162: 5592–5600.

The key pathologic features of human asthma include airway obstruction, airways hyperresponsiveness (AHR), and an extensive cellular inflammation consisting of eosinophils, lymphocytes, mast cells, neutrophils, and monocytes (1). Analyses of bronchoalveolar lavage (BAL) fluid and lung biopsies from asthmatic patients have shown that CD4\(^+\) T cells are a major component of the pulmonary infiltrate (2–4). Furthermore, the pattern of cytokine expression in BAL fluid and cytokine mRNA expression in lung tissue biopsies have implicated the Th2 subset of CD4\(^+\) T cells in this inflammatory process (5–9). The recruitment and activation of inflammatory cells into the lung appear to be regulated by the secretion of the Th2 cytokines IL-4 and IL-5. IL-4 is important in multiple functions associated with allergic lung inflammation, including the induction of B cell isotype switching to IgE production, mast cell differentiation, production of eotaxin from fibroblasts, and expression of VCAM-1 by endothelial cells (10–13). IL-5 facilitates the maturation, activation, and survival of eosinophils and may play a role in eosinophil migration into the lung (14–16).

A number of animal models of allergic lung inflammation have been developed to characterize the cell types and mechanisms involved in the pathogenesis of allergic lung inflammation and bronchial hypersensitivity, with the ultimate objective of therapeutic intervention in the disease process. Previous attempts to identify the cell types required for the generation of allergic lung inflammation in animal models used Ab-mediated depletion of leukocyte subsets or mice lacking CD4\(^+\) or CD8\(^+\) T cells (17–20). These studies demonstrated that CD4\(^+\) T cells, but not CD8\(^+\) T cells, are required for allergic lung inflammation in rodents. Additionally, adoptive transfer systems in both the rat and the mouse have been used to confirm these depletion studies by demonstrating that Ag-specific CD4\(^+\) T cells can transfer to naive mice the ability to respond to an aerosol challenge with Ag. Transfer of purified CD4\(^+\) T cells from Ag-sensitized donors into naive rats renders the recipients susceptible to increased AHR after aerosol challenge with Ag, but results in relatively low levels of eosinophil infiltration into the challenged lung (21–24). In the mouse, transfer of the conalbumin-specific Th2 clone D10 into naive mice resulted in pulmonary eosinophilia, mucus goblet cells in the airways, and increased airway responsiveness in response to conalbumin challenge, but at lower levels than in mice sensitized and challenged with Ag (25). Transfer of Ag-specific TCR transgenic CD4\(^+\) cells cultured in vitro to produce a Th2 phenotype results in increased airway mucus production, significant eosinophilic infiltration into lung tissue, and eosinophil recovery in BAL fluid (26). Hogan et al. recently used an adoptive transfer system to induce both pulmonary eosinophilia and AHR in naive mice by the transfer of highly purified CD4\(^+\) cells isolated from the spleens of sensitized donor mice (27).

We have developed an adoptive transfer system in the mouse that has allowed us to further characterize the CD4\(^+\) T cells that are involved in the induction of pulmonary inflammation. In this model, spleen cells from OVA-sensitized mice are cultured in vitro for 3 days in the presence of OVA. Cultured spleen cells, but not freshly isolated spleen cells, when transferred into naive recipient mice promote eosinophilic lung inflammation in response to a single aerosol challenge with OVA. The kinetics and magnitude of this inflammatory response in the adoptive transfer system are

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†Abbreviations used in this paper: AHR, airways hyperresponsiveness; BAL, bronchoalveolar lavage; PE, phycoerythrin.
nearly identical with the response in mice receiving the conventional OVA sensitization and challenge. As previously described in other adoptive transfer models in mouse and rat, this Ag-specific pulmonary inflammation occurs in the absence of the increased plasma IgE levels found when the mice are directly sensitized (22, 28). Extensive negative and positive selection analysis indicates that the cells responsible for the transfer are found within the CD4<sup>+</sup>CD62L<sup>low</sup> CD25<sup>+</sup> phenotypic subset, which accounts for <1% of the cultured spleen cells. Thus, extensive lung inflammation can be induced in naive mice by the transfer of <100,000 cells. The acquisition of the capacity to promote eosinophilic lung inflammation that occurs during the 3-day culture period is associated with the initiation of IL-5 production by these cells, suggesting that the capacity to produce IL-5 may be the critical factor that distinguishes the cultured cells from freshly isolated splenocytes.

Materials and Methods

Animals

Female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained in the animal care facility at Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI) and were given food and water ad libitum. All protocols for animal use were approved by the Parke-Davis institutional animal care and use committee.

Monoclonal Abs

The following mAbs were purified from hybridoma supernatants obtained from the American Type Culture Collection (Manassas, VA): GK1.5, anti-CD4; M1/70, anti-Mac-1; 53-6.72, anti-CD8<sup>+</sup>; RA3-6B2, anti-B220; and 7D4, anti-CD25. mAbs conjugated to FITC, phycoerythrin (PE), or biotin from PharMingen (San Diego, CA) include: RM4-4, anti-CD4; MEL-14, anti-CD62L; S7, anti-CD43; IM47, anti-CD44; R1-2, anti-CD49d; H1.2F3, anti-CD69; M293, anti-β<sub>2</sub> integrin; and 53-5.8, anti-CD8<sup>−</sup>. FITC-conjugated anti-CD4 and anti-CD25, and PE-conjugated anti-CD71 were obtained from Caltag (Burlingame, CA). Hybridoma supernatants obtained from the American Type Culture Collection were used for cell staining in magnetic bead separation procedures. For ELISAs, mAbs include purified rat anti-mouse/human IL-5 mAb TRFK5 (PharMingen), biotinylated rat anti-mouse IL-5 mAb TRFK4 (PharMingen), purified anti-mouse IgE mAb (The Binding Site, Birmingham, U.K.), and horseradish peroxidase-conjugated rat anti-mouse IgE (Southern Biotechnology Associates, Birmingham, AL).

Conventional model of Ag sensitization and challenge

Mice were sensitized with a single i.p. injection of OVA (grade V, Sigma, St. Louis, MO) adsorbed to alum (10 µg of OVA and 9 mg of alum in 200 µl of saline) or vehicle control (9 mg of alum in 200 µl of saline). Two weeks later, all mice were challenged by a single 12-min inhalation of an aerosol consisting of 1.5% OVA (w/v) in saline produced by a nebulizer (Birmingham, AL). The aerosol consisted of 1.5% OVA (w/v) in saline produced by a nebulizer (Birmingham, AL). The cultured splenocytes were washed in complete medium by centrifugation, the spleens were excised, and the splenocytes were disaggregated into a single cell suspension. The suspension was injected i.p. into recipient mice. After 3 days, recipient mice were aerosol challenged as described above.

Negative and positive selection of splenocyte populations

CD4<sup>+</sup>, CD8<sup>+</sup>, Mac-1<sup>+</sup>, CD62L<sup>+</sup>, and/or CD25<sup>+</sup> cell populations were removed from the cultured splenocytes by negative selection with Dynal sheep anti-rat Ig beads (Dynal, Lake Success, NY) that had been incubated overnight at 4°C on a rotator with saturating amounts of GK1.5 mAb, 53-6.72 mAb, M1/70 mAb, MEL-14 mAb, or 7D4 mAb, respectively. The beads were washed to remove unbound mAb and then added to the cultured splenocytes at a concentration of five beads per target cell according to the manufacturer’s instructions. Bead-bound cells were magnetically removed using Dynal Magnetic Particle Concentrator-2 (MPC-2, Dynal), and the purity of each population was determined by flow cytometric analysis. BeD<sup>20</sup> cells were removed from cultured splenocytes by directly staining cells for 30 min on a 4°C rotator with saturating amounts of RA3-6B2 mAb. Stained cells were incubated with the sheep anti-rat Ig beads (five beads per target cell), bead-bound cells were removed as described above, and purity was assessed by flow cytometric analysis.

CD4<sup>+</sup> cells were positively selected using Dynal sheep anti-mouse CD4 beads according to the manufacturer’s instructions. Cultured cells were incubated with the beads at a concentration of five beads per target cell for 40 min on a 4°C rotator. Bead-bound and unbound cells were separated using a Dynal MPC-2, and bead-bound cells were detached and isolated from the beads using Dynal DETACHaBEAD. The purity of selection was determined by flow cytometric analysis.

BAL and plasma collection

Three days after OVA challenge, the mice were anesthetized with an i.p. injection of anesthetic (ketamine/acepromazine/xylazine), and the tracheae were exposed and cannulated. The lungs and upper airways were lavaged with 0.5 ml of cold PBS. A portion (200 µl) of the BAL fluid was enumerated using a Coulter counter (model ZBI, Coulter, Hialeah, FL). The remaining BAL fluid was centrifuged at 300 × g for 5 min, and the cells were resuspended in 1 ml of HBSS (Life Technologies) containing 0.5% FCS (HyClone) and 10 mM HEPES (Life Technologies). The cell suspension was centrifuged in a cytospin (Shandon Southern Instruments, Sewickley, PA) and stained by Diff Quick (American Scientific Products, Sewickley, PA) and stained by Diff Quick (American Scientific Products, Sewickley, PA) and stained by Diff Quick (American Scientific Products, Sewickley, PA) and stained by Diff Quick (American Scientific Products, Sewickley, PA) and stained by Diff Quick (American Scientific Products, Sewickley, PA) and stained by Diff Quick (American Scientific Products, Sewickley, PA). Blood samples were microcentrifuged at 13,000 rpm for 25 min at 4°C, and plasma was collected.

IL-5 limiting dilution analysis

Multiple 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) were prepared by aliquoting 100 µl of complete medium to the first 48 wells and 100 µl/well of complete medium with 400 µg/ml OVA to the last 48 wells. Splenocytes were added to the wells at various concentrations in 100-µl volumes, resulting in a total volume of 200 µl/well and a final OVA concentration of 200 µg/ml in the last 48 wells of each plate. Each well of a single 96-well plate contained a total of 5 × 10<sup>3</sup> spleen cells, consisting of a mixture of sensitized spleen cells (0–4 × 10<sup>3</sup>) and naive spleen cells (1–5 × 10<sup>3</sup>). The plates were incubated for 72 h at 37°C, then centrifuged at 500 × g for 5 min. IL-5 ELISA analysis of the supernatants was performed to detect wells containing IL-5-secreting cells. A threshold level was distinguished positive from negative wells was calculated as 3 SD above the mean OD<sub>450</sub> reading for wells containing 5 × 10<sup>3</sup> naive spleen cells only, with no OVA stimulus. Minimal frequency estimates of OVA-specific IL-5-secreting cells were calculated using the weighted mean statistical analysis method (29).

Assay for IL-5 levels

BAL fluid or tissue culture supernatants were analyzed by sandwich ELISA to quantify IL-5 concentrations. Wells of half-area 96-well plates were incubated with 50 µl of 1/µg/ml anti-IL-5 mAb TRFK5 (PharMingen) in 0.1 M carbonate buffer (pH 8.2) overnight at 4°C. Unbound mAb was removed by extensive washing with PBS containing 0.05% Tween 20, a portion (200 µl) of the plate washer (96PW, Tecan U.S., Research Triangle Park, NC). The wells were blocked for 1 h at room temperature with 150 µl of blocking buffer (PBS/Tween-20/20% BSA). The plates were washed again, and 50-µl samples of BAL fluid, tissue culture supernatant, or recombinant mouse IL-5 standard (PharMingen) diluted in blocking buffer were added and incubated for 2 h at room temperature. The wells were washed and incubated at room temperature for 1 h with 50 µl of 2/µg/ml biotin-conjugated anti-IL-5 mAb TRFK4 (PharMingen) diluted in blocking buffer. After washing, 50 µl of a 1/2000 dilution of streptavidin-peroxidase (Southern Biotechnology Associates) was added and incubated at room temperature for 30 min. ABTS peroxidase substrate in hydrogen peroxidase (Southern Biotechnology Associates) was added and incubated overnight with saturating amounts of GK1.5 mAb, 53-6.72 mAb, M1/70 mAb, MEL-14 mAb, or 7D4 mAb, respectively. The beads were washed to remove unbound mAb and then added to the cultured splenocytes at a concentration of five beads per target cell according to the manufacturer’s instructions. Bead-bound cells were magnetically removed using Dynal sheep anti-mouse CD4 beads according to the manufacturer’s instructions. Cultured cells were incubated with the beads at a concentration of five beads per target cell for 40 min on a 4°C rotator. Bead-bound and unbound cells were separated using a Dynal MPC-2, and bead-bound cells were detached and isolated from the beads using Dynal DETACHaBEAD. The purity of selection was determined by flow cytometric analysis.

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a SoftMax Pro curve-fitting program (Molecular Devices) was used to calculate the sample IL-5 concentrations.

Assay for plasma IgE levels

Plasma IgE concentrations were measured using another ELISA, in which the wash medium, blocking buffer, ABTS substrate, and volumes used were identical with those described for the IL-5 ELISA. The plates were coated overnight with sheep anti-mouse IgE (The Binding Site) in 0.02 M carbonate buffer (pH 9.6) and blocked as described above. A standard curve was constructed using threefold dilutions of mouse IgE (mAb anti-DNP from Sigma) from 250 μg/ml to 1.4 ng/ml PBS/Tween-20/BSA. Plasma samples were tested at 1/2 or 1/5 dilution in blocking buffer. Standards and unknown plasma samples were incubated for 2 h at room temperature, and the plates were washed. A 1/2000 dilution of peroxidase-labeled anti-mouse IgE (Southern Biotechnology Associates) was added and incubated for 1 h at room temperature. After washing, the substrate was added, and the data were analyzed as described for the IL-5 ELISA above. Total plasma IgE levels, rather than OVA-specific IgE levels, were determined for several reasons. First, in specific human studies, total serum IgE was found to be a sufficient predictor of asthma (30). Second, an OVA-specific IgE standard was not available for the ELISA. Third, careful analysis of commercially available Abs specific for IgE revealed a significant level of cross-reactivity to other Ig isotypes.

Flow cytometric analysis

Dead cells were removed from cultured splenocytes by preparative centrifugation over Lympholyte-M (Accurate Chemical & Scientific, Westbury, NY). One million cells were added to individual wells of a 96-well V-bottom microtiter plate (Costar) and centrifuged at 500 × g for 3 min. The supernatant was removed, and the pellet was resuspended in 50 μl of FACS buffer (HBSS with 5% FCS, 10 mM HEPES, and 0.02% sodium azide) containing saturating amounts of purified anti-mouse CD16/CD32 (Fcγ III/II receptor; Pharmingen) for 10 min at 4°C. A 50-μl volume of FACS buffer was added containing saturating amounts of various combinations of Abs directly conjugated to FITC, PE, or biotin and incubated for 30 min at 4°C. The cells were washed twice with FACS buffer and resuspended in 100 μl of FACS buffer containing saturating amounts of avidin-conjugated Cy-Chrome (Pharmingen) for 20 min at 4°C if biotinylated Abs were used. The cells were washed twice more and resuspended in 700 μl of phenol red-free buffer (PBS with 0.02% sodium azide) for flow cytometric analysis. Stained cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer. Fluorescence and forward light scatter signals were collected on 20,000 or 200,000 cells and were analyzed using the CellQuest software program (Becton Dickinson). Forward angle and orthogonal light scatter was used to exclude dead cells from analysis. The percentage of cells with a particular fluorescence pattern was determined by integration of the corresponding populations. All fluorescence profiles are plotted on a four-decade log scale.

Statistics

All data were compared using one-way analysis of variance. Means that are significantly (p < 0.05) different in the figures are denoted by asterisks.

Results

Kinetics of pulmonary cellular influx after OVA aerosol challenge

Groups of four C57BL/6 mice actively sensitized by i.p. administration of OVA/alum (Fig. 1A) or naive recipients of OVA-cultured, sensitized spleen cells (Fig. 1B) were subjected to BAL at various times after a single aerosol challenge with OVA to determine the kinetics of cellular infiltration into the airways. Both the conventional sensitization protocol and the adoptive transfer protocol resulted in a similar course of leukocyte accumulation within the airways after aerosol challenge. In each model, a small accumulation of eosinophils in the BAL fluid was observed 24 h after challenge that increased dramatically over the next 48 h until by 72 h postaerosol challenge the majority of cells within the BAL fluid were eosinophils. The accumulation of eosinophils continued to increase, peaked at 7 days postaerosol, and declined to near baseline levels by 14 days postaerosol in each model (data not shown). Neutrophil accumulation in the BAL fluid was low in either protocol, with peak levels (at 48 h postaerosol challenge) accounting for only 9.8 and 13.4% of BAL fluid leukocytes in the conventional and adoptive transfer models, respectively. The kinetics of monocyte and lymphocyte accumulation within the lungs were nearly identical for the two models.

Previous histopathologic analysis of fixed lung sections from OVA-sensitized and challenged mice has revealed extensive perivascular and peribronchial infiltration of eosinophils and mononuclear cells (31, 32). Analyses of fixed lungs from OVA-challenged recipients of adoptively transferred cells demonstrated a similar pattern of perivascular and peribronchial leukocyte infiltration (data not shown).

Kinetics of BAL fluid IL-5 and plasma IgE

The BAL fluid from these kinetic analyses was also assayed for the presence of IL-5. As shown in Fig. 2A, the kinetics of IL-5 accumulation in the BAL fluid were similar for the conventional and adoptive transfer models. Substantial IL-5 levels were detected as early as 8 h, peaked at 24 h, and decreased to nearly basal levels by 72 h after aerosol challenge. Thus, levels of IL-5 peaked and subsided well before the time of maximum eosinophil accumulation. Although the kinetics of IL-5 production were similar in the two models, the conventional sensitization/challenge model routinely demonstrated two- to fourfold higher levels of maximum IL-5 production than the adoptive transfer model.

Sensitization with OVA/alum has previously been shown to result in the production of high levels of OVA-specific plasma IgE
pressing CD4 and a slight increase in the percentages of CD8 period resulted in a slight reduction in the percentage of cells ex-
analysis of fresh and cultured splenocytes revealed that the culture account for this difference in functional activity. Flow cytometric between fresh spleen cells and the cultured splenocytes that could for 3 days in the presence of Ag. We sought to identify differences when transferred into naive recipient mice, but only after culture 3 days later. The number of BAL fluid leukocytes was enumerated by Coulter counter, and the percentage of eosinophils was determined by differential counting.

Adoptive transfer dose response
To determine the number of cells required to promote eosinophilic lung inflammation in naive recipients, varying amounts of cultured splenocytes from OVA-sensitized donor mice were transferred to naive recipients. The number of eosinophils present in the BAL fluid 3 days after aerosol challenge, suggesting that accumulation of inflammatory cells and production of IL-5 within the first 72 h after aerosol challenge may not be dependent on the presence of Ag-specific IgE.

(31, 33). The transfer of cultured splenocytes into naive recipients, however, did not result in the immediate accumulation of IgE in the plasma of the recipient mice (Fig. 2B). Significant levels of IgE were not found within the plasma until >72 h after aerosol challenge, suggesting that accumulation of inflammatory cells and production of IL-5 within the first 72 h after aerosol challenge may not be dependent on the presence of Ag-specific IgE.

Adoptive transfer dose response
To determine the number of cells required to promote eosinophilic lung inflammation in naive mice, varying amounts of cultured splenocytes from OVA-sensitized donor mice were transferred to naive recipients. The number of eosinophils present in the BAL fluid 3 days after aerosol challenge was then determined. From these data, a dose-response curve was produced (Fig. 3), showing a direct correlation between the number of cells transferred and the level of BAL fluid eosinophilia. To standardize the adoptive trans-
protocol, a dose of 1 × 10^7 cells was used in subsequent in-
jections. Spleen cells freshly isolated from sensitized donors, in the absence of further culture in vitro, did not promote eosinophilic lung inflammation in naive recipients 3 days after OVA aerosol challenge, even when 2 × 10^7 cells were transferred.

The results depicted in Fig. 3 suggest that lymphocytes from sensitized spleen cells promote eosinophilic lung inflammation when transferred into naive recipient mice, but only after culture for 3 days in the presence of Ag. We sought to identify differences between fresh spleen cells and the cultured splenocytes that could account for this difference in functional activity. Flow cytometric analysis of fresh and cultured splenocytes revealed that the culture period resulted in a slight reduction in the percentage of cells expressing CD4 and a slight increase in the percentages of CD8^+ and B220^+ cells (Table I). The dramatic reduction in cells expressing Mac-1 after culture could be due to adherence of these cells to the plastic culture flask during culture, because no attempt was made to recover tightly adherent cells.

CD4^+ T cells, and not B cells or CD8^+ T cells, are responsible for promoting Ag-induced eosinophilic lung inflammation
To characterize lymphocytes that are required for the transfer of eosinophilic lung inflammation into naive mice, subpopulations of splenic lymphocytes were subjected to negative selection by magnetic bead separation. Depletion of >93% of B cells or >99% of CD8^+ T cells, as assessed by flow cytometric analysis, did not inhibit transfer of the inflammatory response (Fig. 4A). Conversely, depletion of >96% CD4^+ T cells completely prevented the eosinophilic response in the recipient mice (Fig. 4). Although the depletion studies suggest that CD4^+ T cells are responsible for the transfer of reactivity to naive mice, positive selection experiments were performed to verify this result. Highly purified (>95%) CD4^+ T cells were isolated by detachable magnetic bead isolation from spleen cell cultures and were transferred to naive recipients. The remaining CD4^- cells were injected into another naive recipient group. Three days after OVA aerosol challenge, eosinophils were detected in the lungs of the CD4^+ T cell recipients, but not in the CD4^- cell recipients (Fig. 4B).

Ag-reactive cells are found within the CD62L^lowCD25^+ subpopulation of CD4^+ T cells
The loss of surface expression of CD62L (L-selectin) is commonly used as a phenotypic marker for T cells that have previously encoun-
tered Ag (34, 35). Flow cytometric analysis of the cultured splenocytes revealed that the majority of the CD4^+ T cells (85.6%)
expressed high levels of CD62L (Fig. 5A, region 1). Those CD4+ cells with little or no surface expression of CD62L fell into two distinct subpopulations based upon expression of CD4 and CD62L. One group expressed slightly lower levels of both of these molecules (region 2) than the other population (region 3). Magnetic bead immunoselection with MEL-14 removed >99% of the cells expressing CD62L at levels greater than that indicated by the dotted line in Fig. 5A, including region 1 (CD62Lhigh), but did not remove cells below the dotted line, including regions 2 and 3 (CD62Llow; data not shown). To determine whether Ag-reactive CD4+ T cells resulting from the culture of OVA-sensitized spleen cells are included within the CD62Llow subset, we employed magnetic bead negative selection to remove splenocytes expressing CD8, B220, Mac-1, or CD62L. Upon adoptive transfer, the CD62L-depleted cells were still able to promote eosinophilic lung inflammation in response to OVA aerosol challenge to a degree comparable to that in unseparated cells (Fig. 5B), indicating that the CD4+CD62Llow population includes the effector cells of interest.

Although reduced CD62L expression is indicative of a previous encounter with Ag, both activated effector cells and resting memory CD4+ T cells lack expression of this adhesion molecule. Extensive flow cytometric analysis of the CD4+CD62Llow cells depicted in Fig. 5A suggested that the cells within region 2 (CD4+CD62Llow) are resting memory cells, whereas the cells within region 3 (CD4highCD62Llow) are activated lymphoblasts. The region 3 cells were significantly larger and expressed dramatically higher levels of the activation markers CD25, CD71, CD69, and CD43 as well as higher levels of the adhesion molecules CD44, CD49d, and β7 integrin (Table II). Immunomagnetic depletion of the CD25+ subset of CD4+CD62Llow T cells significantly inhibited the ability to transfer responsiveness to naive mice (Fig. 5C). Thus, cells with the phenotype CD4highCD62LlowCD25+ representing only 0.81% of the 1 × 10^7 cells normally transferred, contained the majority of functional activity.

**Pulmonary IL-5 production in response to aerosol challenge is dependent on CD4+ T cells**

The data in Fig. 2 indicate that in adoptive transfer recipients, OVA aerosol challenge induced a rapid accumulation of IL-5 in the lung that was measurable in the BAL fluid within 8 h of challenge. We hypothesized that the most likely source of the BAL...
fluid IL-5 was the transferred CD4$^+$ T cells. However, the rapidity of the IL-5 response after aerosol challenge suggested an alternative possibility that resident cells within the host lung, rather than transferred T cells, might have been responsible for the lung IL-5 production. To test this hypothesis, naive recipient mice were injected with cultured spleen cells, cultured spleen cells depleted of CD4$^+$ T cells by magnetic bead negative selection, or no cells at all. Three days later, the mice were challenged with an OVA aerosol. Twenty-four hours after challenge, BAL fluid was collected and tested for IL-5. Depletion of CD4$^+$ T cells from the transferred splenocyte cultures reduced the BAL IL-5 to levels exhibited in naive mice receiving no cells (Fig. 6). These results indicate that transferred CD4$^+$ T cells are required for the early spike of IL-5 production in the lung as well as the later influx of eosinophils.

**IL-5 secretion is induced during 3-day culture**

Although the depletion studies implicated CD4$^+$ T cells as the critical cell type required for the transfer of responsiveness to naive mice, the flow cytometric analysis described in Table I demonstrated no increase in the proportion of CD4$^+$ T cells after culture. In addition, no evidence for dramatic T cell proliferation was found after culture in the presence of OVA; viable cell recovery of cultured cells was usually from 40–60% of the number originally found after culture in the presence of OVA; viable cell recovery of 3-day cultures from OVA-sensitized donor mice was 40–60% of the number originally added to the culture flasks, and no significant differences in [H]thymidine incorporation were found between sensitized and naive spleen cells in response to culture with OVA (data not shown). There was, however, a dramatic increase in the ability of sensitized spleen cells to produce IL-5 after 3 days of culture with OVA. OVA-sensitized spleen cells, when cultured in the presence of OVA for 3 days, produced high levels of IL-5, whereas naïve spleen cells did not (Fig. 7A). Limiting dilution analysis was used to estimate the minimal frequency of cells responding to OVA and the relative ability of fresh spleen cells vs cultured spleen cells to produce IL-5 without additional OVA stimulation.

As shown in Fig. 7B, among freshly isolated sensitized spleen cells, approximately 1 in 94,594 cells was able to produce measurable IL-5 when stimulated with OVA, whereas no IL-5 production was determined in the absence of OVA stimulation. When these same spleen cells were cultured for 3 days with OVA and then subjected to limiting dilution analysis, a different result was obtained. The cultured cells when restimulated with OVA had a minimal frequency estimate for OVA-specific IL-5-producing cells of 1 in 8,767. Thus, the 3-day culture period with OVA increased the frequency of cells capable of producing IL-5 in response to Ag by approximately 10-fold. In addition, a significant number of these cultured cells produced IL-5 even in the absence of additional OVA stimulation (frequency = 1/61,425).

**Discussion**

The present study describes a protocol for the passive transfer of allergic lung inflammation responsiveness to naive mice. Spleenocytes from OVA-sensitized donor mice were cultured for 3 days in vitro in the presence of OVA and then transferred to naive mice. A subsequent single aerosol challenge resulted in an intense eosinophilic pulmonary inflammation that was nearly identical with that obtained in mice directly sensitized with OVA. Extensive phenotypic analysis of the transferred cells indicated that CD4$^{high}$CD62L$^{low}$CD25$^+$ T lymphocytes, representing <1% of total splenocytes, were both necessary and sufficient to transfer the potential to respond to the aerosol challenge to naive mice. These effector cells expressed high levels of many activation and adhesion molecules that have previously been identified on lung-infiltrating CD4$^+$ T cells (36). The relatively high level of CD4 expression on the activated effector cells is consistent with a recent report demonstrating increased CD4 expression following T cell activation by Ag in vitro or in vivo (37). These results support and extend previous observations implicating CD4$^+$ T cells in allergic lung inflammation.

Freshly isolated splenocytes from OVA-sensitized mice were unable to transfer activity to naive recipients, whereas restimulation of these cells for 3 days in vitro rendered them competent to transfer activity. When attempts to transfer reactivity using freshly isolated spleen cells failed repeatedly, the extra in vitro culture step

![FIGURE 6](image-url) CD4$^+$ cells are required for pulmonary IL-5 production. BAL fluid IL-5 levels were assessed by ELISA 24 h after aerosol challenge in mice that received a transfer of unseparated, CD4$^+$-depleted, or no OVA-sensitized splenocytes. Both the CD4$^+$-depleted and naive mice (no injection) exhibited significantly ($p < 0.05$) less pulmonary IL-5 production.
The simplest explanation is that the Ag-specific CD4\(^+\) T cells in the eosinophilic lung inflammation model is unresponsive (39). Previously reported Th1 dependence of the hypersensitivity pneumonitis (38) was not supported in our system when OVA splenocytes were cultured in the presence of Con A. After culturing naive or OVA-sensitized splenocytes for 3 days with or without 200 μg/ml OVA stimulus, IL-5 production in culture was determined by ELISA (A). Only OVA-sensitized splenocytes in the presence of OVA secreted IL-5, indicating that IL-5 production occurs in an OVA-specific manner. OVA-specific IL-5-secreting splenocyte frequency was determined by IL-5 limiting dilution analysis (B). IL-5 secretion was detected by ELISA after a 3-day limiting dilution microculture of fresh or cultured splenocytes with or without OVA stimulus. Results are presented as the number of cells per well vs the percentage of wells at each particular dilution that contained no IL-5. Minimal frequency estimates of OVA-specific IL-5-secreting cells within microcultures of fresh cells without OVA stimulus, fresh cells with OVA stimulus, cultured cells without OVA stimulus, and cultured cells with OVA stimulus were <1/1,000,000, 1/94,594, 1/61,425, and 1/8,767, respectively.

was added, based upon a published rat model of hypersensitivity pneumonitis (38). In this rat model, OVA-sensitized spleen cells were cultured for 72 h in vitro with OVA and Con A before transfer into naive recipients. Subsequent aerosol challenge with OVA resulted in a T cell-rich alveolitis. In our system, the addition of Con A was unnecessary. In fact, culturing the OVA/alum-sensitized spleen cells with a combination of OVA and Con A in vitro produced cells incapable of transferring the eosinophilic lung inflammatory response (our unpublished observations). The supernatants from these Con A-containing cultures contained high levels of IFN-γ and little or no IL-5. This is in keeping with the reported Th1 dependence of the hypersensitivity pneumonitis response (39).

The nature of the deficiency in the freshly isolated, sensitized splenocytes in the eosinophilic lung inflammation model is unclear. The simplest explanation is that the Ag-specific CD4\(^+\) T cell population expanded over the course of the culture period in vitro. However, this expansion was not obvious. Total cell counts at the end of the culture period were typically only 40–60% of input counts, and there was no increase in the percentage of CD4\(^+\) T cells at the end of the culture period that might indicate selective expansion of these cells. In addition, \(^{3}H\)thymidine incorporation assays demonstrated little or no detectable Ag-specific proliferation. The only assay that suggested an expansion of Ag-specific CD4\(^+\) T cells was the IL-5 limiting dilution analysis, in which the culture period resulted in a 10-fold increase in the number of cells capable of producing IL-5 in response to OVA. However, the limiting dilution assay results do not necessarily indicate the proliferation of Ag-specific cells. The 10-fold increase could also be the result of the differentiation of Ag-specific splenocytes that were incapable of secreting IL-5 when freshly isolated. In a direct comparison of freshly isolated, sensitized cells and cultured cells, 2 × 10⁶ fresh splenocytes were unable to transfer activity to naive mice, whereas 20-fold fewer cultured splenocytes (1 × 10⁵) were capable of transferring significant activity. These results suggest that the culture step functions not simply as a means of increasing the total number of OVA-specific CD4\(^+\) T cells, but instead (or in addition) provides a necessary differentiation event that allows these cells to function in vivo.

One possible consequence of CD4\(^+\) T cell differentiation in vitro could be an enhanced capacity to secrete cytokines, including IL-5, upon encountering Ag in the aerosol-challenged host animal. Resting OVA-specific T cells within the spleens of donor mice might have a significant lag time before being able to produce IL-5 in sufficient quantity to promote eosinophilic lung inflammation in the naive mice, whereas the restimulated cells from culture might be sufficiently activated to produce IL-5 in a shorter timeframe. Limiting dilution analysis supported this hypothesis; freshly isolated spleen cells contained no T cells actively secreting IL-5, whereas the cultured cells did. This suggests that once transferred, the cultured cells would be capable of immediate OVA-induced IL-5 secretion in vivo.

IL-5 has been shown to play a critical role in the generation of eosinophilic lung inflammation. Studies employing IL-5-blocking mAbs in vivo and IL-5-deficient mice demonstrate that pulmonary eosinophilia, AHR, and mucus production are all greatly reduced in the absence of this cytokine (27, 40–42). Although the majority of allergen-induced IL-5 secretion is thought to be attributed to Th2 cells, there is also evidence that mast cells produce and secrete IL-5 in response to IgE ligation (27, 43–45). In our adoptive transfer model, depleting CD4\(^+\) T cells from the cultured, sensitized splenocyte preparation resulted in an absence of detectable BAL fluid IL-5 after aerosol challenge. This suggests that no other cells in the splenocyte culture can produce IL-5 or provide the necessary factors to naive recipient mice to promote IL-5 production. Host mast cells also contribute little or no IL-5 production, perhaps due to the lack of OVA-specific IgE in the recipient mice.

In both the conventional (active sensitization) and adoptive transfer protocols used in this report, BAL fluid IL-5 protein levels increased by 8 h after aerosol challenge, peaked at 24 h, and declined to near baseline levels by 72 h after aerosol challenge. Despite the reduction in IL-5 levels at 72 h, eosinophil levels in the lung remained elevated for at least 4 days longer. This suggests that high IL-5 concentrations in the lung are not required to maintain eosinophil survival. It is possible that the initial spike in IL-5 production is required to initiate eosinophil maturation in the bone marrow, and perhaps contributes to the migration of these cells to the lung. Other cytokines, such as IL-3 or granulocyte-macrophage CSF, that have previously been reported to promote eosinophil survival, might contribute to the maintenance of chronic eosinophilia in this model. However, attempts to measure IL-3 and granulocyte-macrophage CSF in BAL fluid by ELISA resulted in inconsistent results.

In addition to enhanced cytokine secretion, another possible consequence of the differentiation of OVA-specific T cells in vitro is an alteration in adhesive or migratory function. Flow cytometric
analysis revealed that the CD4^{high}CD62L^{low} cells expressed higher levels of several important adhesion molecules, including CD44, CD49d, and the β₁ integrin. Low CD62L expression could reduce the capacity of T cells to recirculate through lymph nodes and redirect them toward the inflamed lung tissues (46). Culturing lymphocytes with Ag or IL-5 has been reported to induce CD44-mediated adhesion to hyaluronic acid, suggesting that both expression levels and functional adhesive capacity might be enhanced under the culture conditions employed (47, 48). The expression and adhesive function of other adhesion molecules, including the β₁ and β₂ integrins, are altered following T cell activation (49, 50). The combination of the β₁ or β₂ integrin chains with CD49d could be the key to CD4^{+} T cell migration to the lung by mediating adhesion to VCAM-1 (51). Alterations in the expression of chemokine receptors could also significantly alter the capacity of CD4^{+} T cells to migrate to the lung (52–55). Preliminary evidence suggests that the CD4^{high}CD62L^{low} T cells containing the transferable activity in our adoptive transfer model demonstrate selective migration to the lung following OVA aerosol challenge, whereas the remaining CD4^{+}CD62L^{high} T cells do not (our unpublished observation).

One of the more striking differences between actively sensitized mice and naive mice receiving Ag-specific T cells is that the latter are devoid of Ag-specific IgE (22, 25). The lack of IgE in the recipient animals is probably due to the fact that they were not actively immunized with OVA/alum. Mice injected with alum only (no OVA) and subsequently challenged with aerosolized OVA have similarly low plasma IgE levels (31). In actively sensitized mice, blocking serum IgE before aerosol challenge inhibits the production of IL-4 and IL-5 by lung T cells and greatly reduces pulmonary eosinophilia, suggesting that CD23-mediated focusing of Ag-loaded IgE is necessary for efficient Ag presentation to T cells (56). Sensitized splenocytes transferred to naive mice lacking OVA-specific IgE face a similar situation; CD23/IgE-dependent Ag focusing is unlikely to occur. If, however, the CD4 T cells had bound Ag-specific IgE within the donor mouse before spleen cell isolation or, alternatively, had bound IgE during the 3-day culture period, then the transferred cells might not have required Ag-specific IgE in the recipient animal. Analysis of transferred T cells by flow cytometry for surface-bound IgE was negative, further suggesting that no IgE-CD4 T cell interaction takes place in the recipient animals (data not shown). Thus, culturing sensitized splenocytes in vitro with Ag may provide the activation and differentiation signals necessary to overcome an IgE deficiency in the recipient mice. Future studies will seek to determine the result of passive IgE transfer in this model.

All experiments described here were conducted in C57BL/6 mice. There is ample evidence that pulmonary inflammation responses vary widely in different strains of mice, and C57BL/6 mice are considered hyporesponsive in several of these studies (57, 58). C57BL/6 mice are also genetically deficient in the expression of certain mediators associated with lung inflammation, including IL-9 and mast cell protease 7 (59, 60). To ensure that the results reported here were not specific to C57BL/6 mice, we analyzed the adoptive transfer protocol in BALB/c mice. The results of the experiments using BALB/c mice essentially mirrored those obtained using C57BL/6 mice; fresh spleen cells were unable to transfer activity to naive mice, whereas restimulation in culture rendered them competent to transfer activity. The kinetics and degree of activity of the transferred BALB/c spleen cells were nearly identical with those obtained using C57BL/6 mice (data not shown).

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References

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