T Cell–dependent Regulation of Eotaxin in Antigen-induced Pulmonary Eosinophila

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Summary

T lymphocytes have been implicated in controlling the recruitment of eosinophils into the lung in murine models of allergic asthma. The mechanism by which T cells assist in the recruitment of eosinophils to the lung in these models is not completely understood. We hypothesized that eosinophil-active chemokines might be regulated by antigen (Ag)-induced T cell activation in vivo and thereby mediate T cell–dependent eosinophil recruitment. To test this hypothesis, we examined the effect of an anti-CD3 mAb on Ag-induced pulmonary eosinophilia and correlated this with the expression of three eosinophil-active chemokines: eotaxin, macrophage inflammatory protein (MIP)-1α, and RANTES. We found that Ag-induced pulmonary eosinophilia was associated with the induction of eotaxin and MIP-1α, but not RANTES mRNA. Prechallenge treatment with anti-CD3 mAb inhibited eotaxin, but not MIP-1α and RANTES mRNA induction, and significantly reduced eosinophil accumulation in the lung. In addition, Ag-specific antibody responses and mast cell degranulation after Ag challenge in sensitized mice were not affected by T cell elimination, and were not sufficient to induce the expression of eotaxin and cause pulmonary eosinophilia. These findings suggest that eotaxin is one of the molecular links between Ag-specific T cell activation and the recruitment of eosinophils into the airways.

Bronchial inflammation is a characteristic pathologic feature of allergic asthma (1, 2). Activated CD4$^+$ T lymphocytes, eosinophils, and mast cells are present in the cellular infiltrate in asthma and each of these cells is believed to contribute to the pathogenesis of this disease (3–5). CD4$^+$ T lymphocytes play an important role in regulating the immune response to Ag, in part through the secretion of cytokines. CD4$^+$ lymphocytes can be segregated on the basis of cytokine secretion patterns into Th1 and Th2 cells (6). Th2 cells are important mediators of allergic inflammatory responses as they secrete cytokines that promote the maturation and activation of eosinophils (IL-3, IL-5, and GM-CSF) (7), the growth and differentiation of mast cells (IL-3, GM-CSF, and IL-10) (8), and regulate the Ig class switch to favor the development of Ag-specific IgE Ab responses (IL-4) (9).

Murine models of Ag-induced pulmonary eosinophilia and airway hyperreactivity have been developed to facilitate the study of cellular immune responses in asthma (10–15). Pulmonary eosinophilia and airway hyperresponsiveness can be attenuated in these models by eliminating T cells with either anti-CD4 (12, 16) or anti-Thy1 (15) mAb, by the administration of neutralizing Abs to IL-4 (14) or IL-5 (10, 16), and in IL-5–deficient mice (17), demonstrating the importance of Th2 cells and cytokines in these models.

The importance of tissue eosinophilia in allergic airway disease is inferred from studies linking eosinophil-derived granular proteins and lipid metabolites to airway hyperresponsiveness (4, 18), and from clinical studies correlating the remission of asthma symptoms with the resolution of airway eosinophilia (19–21). These observations implicating eosinophils in the pathogenesis of asthma make it likely that understanding the mechanisms of eosinophil recruitment to the airways will offer new therapeutic approaches for the treatment of this important human disease.

There is an emerging but incomplete body of knowledge defining the mechanisms that lead to the recruitment and retention of airway eosinophils. Leukocyte extravasation is exquisitely regulated in vivo by a series of coordinated leukocyte–endothelial cell interactions involving several families of molecular regulators, such as the selectins, the integrins, and the chemokines (22, 23). The chemokines are a large family of inducible cytokines that are potent chemoattractants of leukocytes (24). They are classified into three families (α, β, and γ) based on the sequence of conserved cysteine residues in their primary structure. Eotaxin is a recently described β-chemokine that has been shown to specifically chemoattract eosinophils in the guinea pig, mouse, and human (25–29). Other chemokines with chemotactic activity for eosinophils include monocyte chemoattractant proteins 2 and 3, macrophage inflammatory pro-

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tein-1α (MIP-1α)[1], RANTES, and IL-8 (30). However, of these chemokines, only eotaxin has specific chemoattractant activity for eosinophils.

While chemokines often have overlapping activities in vitro, it is their disease-specific expression and the timing and location of their production that dictate their role in a given inflammatory disease. This pattern of expression will be determined by the mechanisms of chemokine regulation in vivo. In an effort to understand the chemokines relevant to allergic pulmonary inflammation, we examined the expression and regulation of three eosinophil-active chemokines: MIP-1α, RANTES, and eotaxin, in a murine model of antigen-induced pulmonary eosinophilia. Our findings demonstrate that eotaxin is regulated in vivo by Ag–specific T cells, and not by mast cells and IgE, and support the hypothesis that eotaxin is an important regulator of pulmonary eosinophilia.

Materials and Methods

Mice. BALB/cJ (H-2^d) mice between 5 and 10 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in a pathogen-free animal facility and were given food and water ad lib.

Immunization and Challenge Protocol. Mice were immunized with 10 μg of OVA (Sigma Chemical Co., St. Louis, MO) and 1 mg aluminum hydroxide intraperitoneally on days 0, 7, and 14. Sham-immunized mice received aluminum hydroxide alone. Mice underwent aerosol challenge with OVA (50 mg/ml in sterile saline) 7–10 d after the final immunization. Ag challenge was performed by placing mice in a Plexiglas box (dimensions: 22 × 23 × 14 cm) and aerosolizing OVA using a nebulizer (DeVilbiss Co., Health Care Division, Somerset, PA), driven by compressed air, for 20 min. Mice were killed at 3, 6, 24, and, in some experiments, 48 h after challenge. A minimum of three mice were included in each group at each time point.

Ab Administration. The rat anti–mouse CD3ε mAb was generously provided by K. Bottomly (Yale University School of Medicine, New Haven, CT) (clone YCD3, isotype IgG_{2b}) (31). The Ab was raised as ascites, subjected to a 50% ammonium sulfate precipitation, dialyzed extensively against normal saline, and sterile filtered (0.2 μm filter) before use. An isotype-matched Ab (rat IgG_{2b}; ICN Biomedicals, Inc., Costa Mesa, CA) was used as a control Ab. Mice were injected intravenously with either anti-CD3 mAb or control Ab (∼100 μg/dose) i.v. on each of the two days preceding secondary challenge with OVA by aerosol.

Bronchoalveolar lavage (BAL). BAL was performed at 3, 6, 24, and 48 h after aerosol challenge. Mice were anesthetized with chloral hydrate (400 μg/g). The lungs and heart were surgically exposed. The lungs were perfused with normal saline to remove blood. The trachea was canulated with polyethylene tubing and the lungs were lavaged with eight 0.5-ml aliquots of PBS containing 0.6 mM EDTA. Recovered live cells (trypan blue exclusion) were enumerated in a hemocytometer. Cell differential counts were determined by enumerating macrophages, neutrophils, eosinophils, and lymphocytes on Wright-stained (LEUKOSTAT; Fisher Scientific Co., Pittsburgh, PA) cytocentrifuge preparations of cells recovered by BAL.

Histology. Lungs were harvested at 3, 6, 24, and 48 h after aerosol challenge, fixed in 10% formalin, and embedded in paraffin blocks. Lung sections (5 μm) were stained with hematoxylin and eosin using standard protocols. Peribronchial and perivascular eosinophils were counted in a minimum of five high-powered fields (magnification, ×40) and expressed as the number of eosinophils per length of basement membrane.

RNA Extraction and Northern Blotting. Lungs excised for RNA extraction were perfused with sterile saline to remove blood and then frozen in liquid nitrogen and stored at −70°C before processing. Total cellular RNA was isolated from the lungs by homogenizing the tissue with a Polytron (Brinkmann Instruments, Inc., Westbury, NY) in 4 M guanidine hydrochloride and pelleting the RNA through a 5.7-M $CsCl_2$ cushion (32). 10 μg of total RNA was fractionated on a 1.2% agarose gel containing 0.7% formaldehyde and then transferred to GeneScreen (DuPont, Wilmington, DE) and hybridized with ^{32}P[dCTP] Klenow-labeled random-primed mouse eotaxin, mouse MIP-1α, mouse RANTES, and mouse ribosomal protein (rp)L32 cDNA probes. The eotaxin probe was a full-length mouse eotaxin cDNA (26) and the MIP-1α and RANTES probes were ∼300-bp coding fragments, kindly provided by Drs. Richard Young (Massachusetts Institute of Technology, Cambridge, MA) and Glenn Dranoff (Dana Farber Cancer Institute, Boston, MA), respectively. The ribosomal protein (rb) L32 probe is a 279-bp fragment encoding the ribosomal protein rpL32 and was used to control for RNA loading (33). Hybridization was performed in 50% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt's solution (0.0002% [wt/vol] polyvinylpyrrolidone, 0.0002% [wt/vol] BSA, 0.0002% [wt/vol] Ficoll 400), and 100 μg/ml of heat-denatured herring sperm DNA solution, 1% (wt/vol) SDS, and 20 mM Tris pH 7.5 at 42°C for 18 h. Blots were washed with 2× SSC/0.1% SDS at 42°C for 40 min and then in 0.2× SSC/0.1% SDS at 65°C for 40 min.

Lung Digests. Lung digests were performed as previously described (34). Briefly, lungs were perfused with sterile saline via the pulmonary artery to remove blood. Excised lung tissue was minced with scissors, resuspended in digest medium containing RPMI 1640, 10% FCS, DNAse (50 U/ml; Sigma Chemical Co.), and collagenase (150 U/ml; Worthington Biochemical Corp., Freehold, NJ), and placed in a shaker bath at 200 rpm for 90 min at 37°C. After digestion, the tissue was dispersed through an 80-μm mesh steel screen and passed through sterile gauze to remove debris and to produce a single cell suspension. The cells were washed three times in RPMI 1640 and counted in a hemocytometer. The cells were further purified by density gradient centrifugation using Lympholyte-M (Accurate Chemical and Scientific Corp., Westbury, NY).

Flow Cytometry. Three-color flow cytometry was performed as previously described (35). Commercial conjugated Abs used for cell staining included: anti–murine CD4-PE (clone GK1.5) (Becton Dickinson and Co., San Jose, CA), anti–murine CD8-red 613 (clone 53-6.7) (GIBCO BRL, Grand Island, NY), goat anti–rat IgG-FITC (mouse serum adsorbed) (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD). Protein G–purified anti-CD3 mAb (clone YCD3) was conjugated using the Quick Tag FITC conjugation kit (Boehringer Mannheim Corp., Indianapolis, IN), as per instructions provided by the manufacturer. Cell suspensions from lung digests were resuspended in staining buffer (PBS with 10% mouse serum) before fluorescence staining. Samples of 10^6 cells were stained with the conjugated Abs at 4°C for 30 min. After washing with PBS, the cells were fixed with 1% paraformaldehyde. Single and three-color flow cytometry was performed on a FACScan® (Becton Dickinson and Co.) cytofluorimeter and

[1]*Abbreviations used in this paper:* BAL, bronchoalveolar lavage; MIP, macrophage inflammatory protein; rp, ribosomal protein.

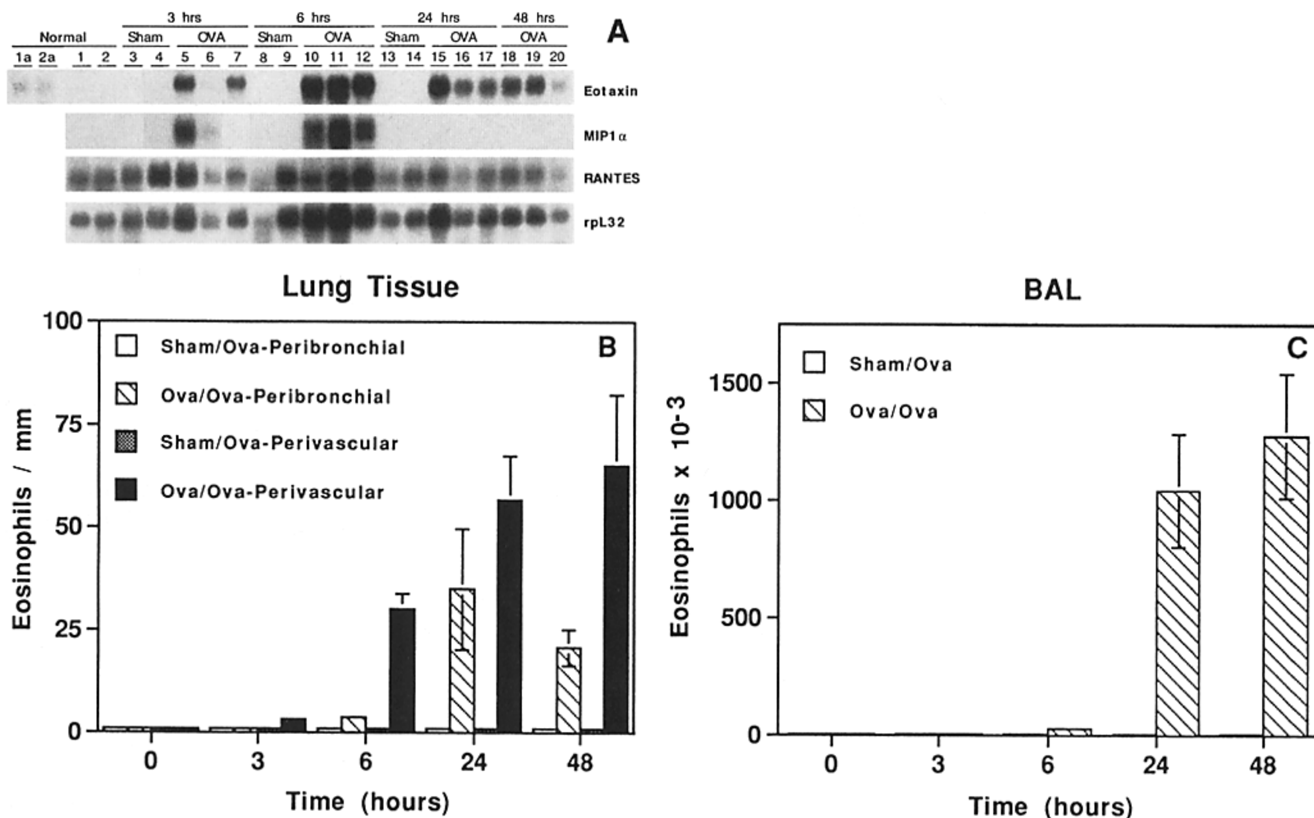


Figure 1. Kinetics of pulmonary chemokine mRNA expression and eosinophil accumulation in lung and BAL after Ag challenge. (*A*) Northern blot analysis of 10 μg of total lung RNA extracted from nonimmunized, nonchallenged mice (*Normal*) (lanes *1* and *2*); sham-immunized mice (lanes *3*, *4*, *8*, *9*, *13*, and *14*); or OVA-immunized mice (lanes *5–7*, *10–12*, *15–20*) after challenge with aerosolized OVA. Tissue was harvested at 3, 6, 24, or 48 h. The blot was sequentially hybridized with eotaxin, MIP-1α, RANTES, and rpL32 ^{32}P-labeled cDNA probes and exposed for 1 d (except lanes *1 a* and *2 a*, which were exposed for 4 d to demonstrate baseline lung eotaxin expression in normal mice), 1 d, 10 d, and 4 d, respectively. Each lane represents an individual mouse from a representative experiment ($n = 3$). (*B*) Quantification of peribronchial and perivascular eosinophils from sham- and OVA-immunized mice at sequential time points after aerosol OVA challenge. Data represent the mean ± SEM of eosinophils per millimeter basement membrane from five fields (× 40) from a representative experiment. (*C*) Quantification of eosinophils recovered from BAL from sham- and OVA-immunized mice at sequential time points after aerosol challenge. Data represent mean ± SEM of eosinophils; minimum of three mice per group.

analysis was performed using Lysys software (Hewlett-Packard Co., Palo Alto, CA).

OVA-specific IgG and IgE Levels. OVA-specific IgG levels were measured by ELISA using 96-well microtiter plates (Costar Corp., Cambridge, MA). The plates were coated with OVA in PBS overnight at 4°C and then washed three times with PBS-Tween (0.5 ml Tween-20 [Sigma Chemical Co.] in 1L PBS). The plates were blocked with PBS-10% FCS (Hazelton Biologicals Inc., Lenexa, KS) at room temperature for 2 h and washed three times in PBS-Tween. Serum samples were added to the wells after serial dilution in PBS-10% FCS and were allowed to incubate for 1 h at room temperature. The plates were washed five times with PBS-Tween before the addition of the secondary Ab, a goat anti–mouse IgG alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 45 min at room temperature. The plates were washed 5 times with PBS-Tween and 10 times with distilled water before development with phosphatase substrate (Kirkegaard & Perry Laboratories, Inc.). The reaction was allowed to proceed for 20 min and was stopped by the addition of dilute sulfuric acid. The plates were read in an ELISA plate reader at OD 405 nm.

OVA-specific IgE levels were measured by capture-ELISA. ELISA microtiter plates (Nunc Inc., Naperville, IL) were coated with a purified anti–mouse IgE mAb (Pharmingen, San Diego, CA) at a concentration of 2 μg/ml in 0.1 M $NaHCO_3$ overnight at 4°C. The plates were washed with PBS-Tween and blocked with PBS-10% FCS. Serum samples were diluted in PBS-10% FCS and incubated in the wells for 2 h. After washing with PBS-Tween, biotinylated-OVA (10 μg/ml) was added to the wells for 1 h. The plates were washed with PBS-Tween followed by the addition of avidin alkaline phosphatase (Vector Laboratories) for 1 h. The plates were washed with PBS-Tween and distilled water, before the addition of the phosphatase substrate. The plates were allowed to develop overnight in the dark. The plates were read in an ELISA plate reader at OD 405 nm.

BAL Histamine Determination. BAL was performed on groups of four mice, 30 min after aerosol challenge with either saline or OVA (50 mg/ml). BAL was performed with two 1-ml washes with ice-cold saline. The collected fluid was centrifuged to remove cells and histamine was measured by enzyme immunoassay (Immunotech, Marseille, France) as per the instructions provided by the manufacturer.

Results

Induction of Eotaxin and MIP-1α but not RANTES mRNA after Airway Ag Challenge in Sensitized Mice. The kinetics

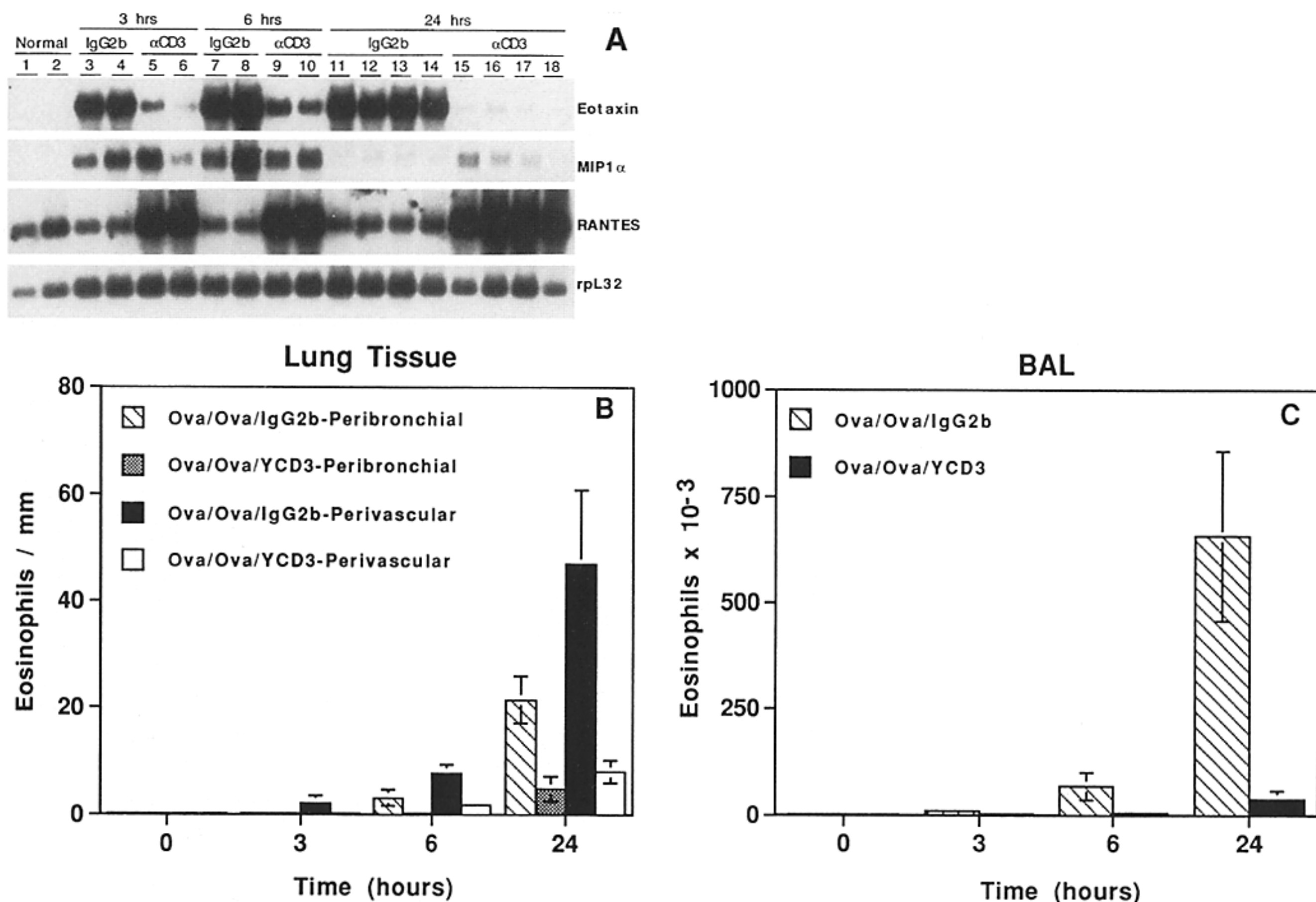


Figure 2. Effect of anti-CD3 mAb on pulmonary chemokine mRNA and eosinophil accumulation in OVA-immunized mice after challenge. (*A*) Northern blot analysis of 10 μg of total lung RNA from OVA-immunized mice pretreated with the control Ab (IgG2b) (lanes *3, 4, 7, 8, 11–14*) or anti-CD3 mAb (αCD3) (lanes *5, 6, 9, 10, 15–18*) and then challenged with aerosolized OVA. Lung tissue was harvested 3, 6, and 24 h after challenge. Also included are lungs from nonimmunized, nonchallenged control mice (lanes *1* and *2*). The blot was sequentially hybridized with eotaxin, MIP-1α, RANTES, and rpL32 ^{32}P-labeled cDNA probes and exposed for 4, 10, 3, and 4 d, respectively. Each lane represents an individual mouse from a representative experiment ($n = 3$). (*B*) Quantification of peribronchial and perivascular eosinophils from OVA-immunized mice treated with either the isotype control Ab or anti-CD3 mAb, at sequential time points after aerosol challenge. Data represent the mean ± SEM of eosinophils per millimeter basement membrane from five fields (× 40) from a representative experiment. (*C*) Quantification of eosinophils recovered from BAL of OVA-immunized mice treated with either the isotype control Ab or anti-CD3 mAb, at sequential time points after aerosol challenge. Data represent mean ± SEM of eosinophils; minimum of three mice per group.

of eosinophil chemokine mRNA expression was examined in sham- and OVA-immunized mice at sequential time points after aerosol challenge with OVA. Low levels of constitutive expression of eotaxin mRNA were detected in the lungs of nonimmunized, nonchallenged mice (Fig. 1 *A*, lanes *1a* and *2a*). An increase in eotaxin mRNA was detected in the OVA-immunized mice 3 h after challenge, reached a peak at 6 h after challenge, and remained elevated at 24 and 48 h (Fig. 1 *A*). Eotaxin mRNA remained at prechallenge levels in the sham-immunized mice. MIP-1α mRNA was also increased 3 h after challenge in the OVA-immunized mice, peaked at 6 h, but returned to undetectable levels by 24 h (Fig. 1 *A*). MIP-1α mRNA levels did not change in the sham-immunized mice. Constitutive expression of RANTES mRNA was detected in nonimmunized mice and did not change significantly in either the sham- or OVA-immunized mice after challenge over the time period studied when compared with levels of mRNA for the rpL32, used as a control for RNA loading (Fig. 1 *A*).

Ag-induced Pulmonary Eosinophilia Correlates with Induction of Eotaxin and MIP-1α mRNA. The kinetics of eosinophil accumulation into lung tissue and into the airway lumen of sham- and OVA-immunized mice was examined in parallel to the expression of eotaxin, MIP-1α, and RANTES mRNA. No eosinophils were present in the lung (Fig. 1 *B*) or recovered from BAL (Fig. 1 *C*) before challenge (time 0 h) in either group. 3 h after challenge, a perivascular accumulation of inflammatory cells could be seen in the lungs of OVA-immunized mice that consisted of neutrophils, mononuclear cells, and rare eosinophils (Fig. 1 *B*) and a small number of eosinophils were recovered from BAL (Fig. 1 *C*). By 6 h after challenge, the perivascular accumulation of eosinophils had progressed in the OVA-immunized mice, and eosinophils were now also identified peribronchially (Fig. 1 *B*). Eosinophils constituted ~5% of the cells recovered from the BAL of OVA-

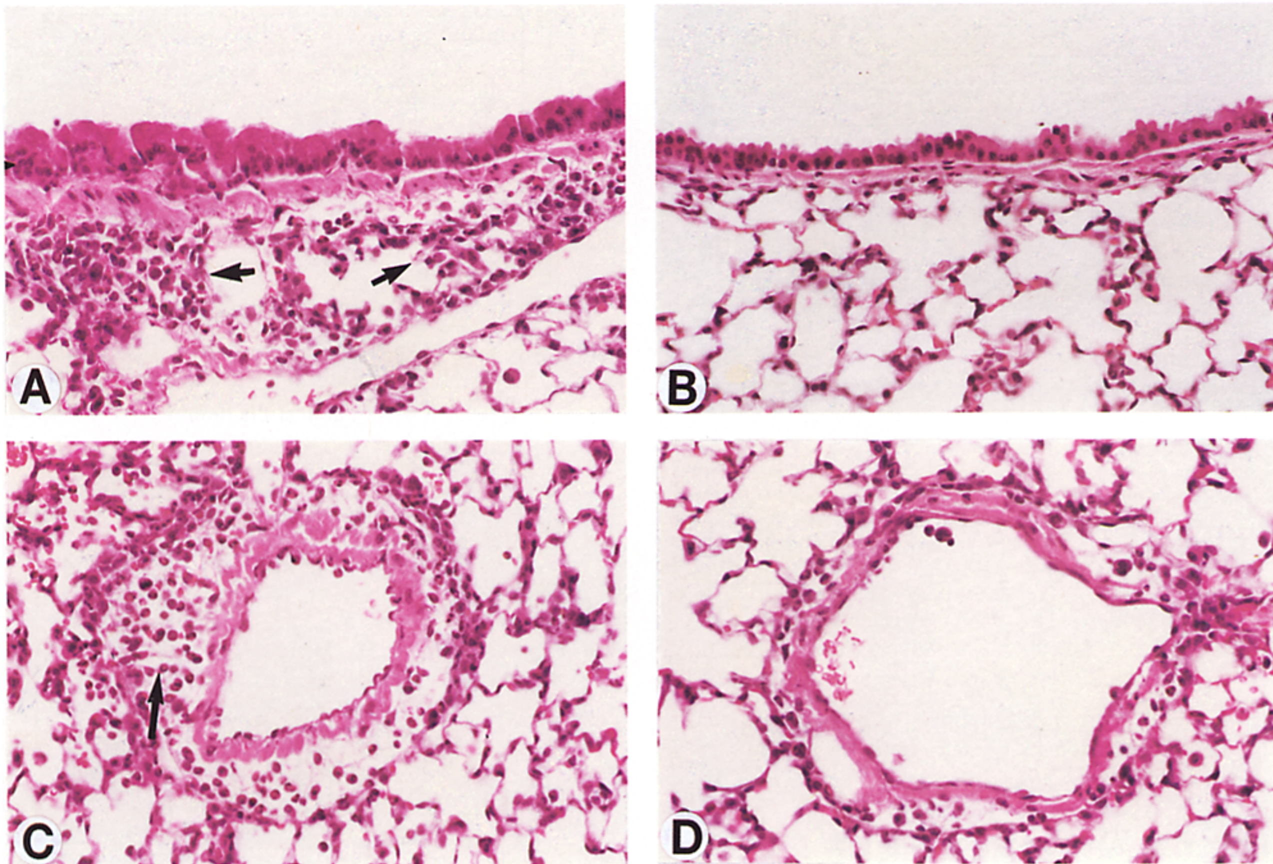


Figure 3. Reduction in peribronchial and perivascular inflammation after treatment with anti-CD3 mAb. Histologic analysis of lungs from OVA-immunized mice treated with either the isotype control Ab (*A*, *C*) or anti-CD3 mAb (*B*, *D*), 24 h after aerosol challenge. Note the peribronchial (*A*) and perivascular (*C*) inflammation with eosinophils (*arrows*) in the mice treated with control Ab compared with the lung from the anti-CD3–treated mice (*B*, peribronchial; *D*, perivascular). Lung tissue was stained with hematoxylin and eosin. (× 350). Sections shown are from a representative experiment.

immunized mice at 6 h (Fig. 1 *C*). At 24 and 48 h, a marked perivascular and peribronchial accumulation of eosinophils was present in the OVA-immunized mice (Fig. 1 *B*) and >35% of cells recovered from BAL were eosinophils (Fig. 1 *C*). In contrast, lungs from sham-immunized mice showed no perivascular or peribronchial inflammation over the time period studied (Fig. 1 *B*) and no eosinophils were recovered from BAL (Fig. 1 *C*).

Anti-CD3 Inhibits Eotaxin but not MIP-1α and RANTES mRNA Expression while Eliminating Ag-induced Pulmonary eosinophilia. It is known that T cell elimination can inhibit Ag-induced pulmonary eosinophilia in the mouse (10, 12, 15, 16). We hypothesized that T cell elimination may diminish pulmonary eosinophilia by modulating eosinophil chemokine mRNA expression. To test this hypothesis, a rat anti–mouse CD3 mAb was administered to OVA-immunized mice on two consecutive days before challenge; OVA-immunized control mice received an isotype-matched Ab (IgG2b). Anti-CD3 mAb effectively reduced both $CD4^+$ and $CD8^+$ lymphocytes from the lung compared with mice receiving the control Ab, as assessed by flow cytometric analysis of cells recovered from enzymatic lung digests, resulting in a >95% reduction in total T lymphocytes recovered from lung digests, compared with controls (data not shown).

OVA-immunized mice treated with anti-CD3 mAb showed diminished eotaxin mRNA induction at 3, 6, and 24 h after challenge compared with immunized mice treated with an isotype-matched control Ab (Fig. 2 *A*). In marked contrast, anti-CD3 actually prolonged MIP-1 α induction until 24 h and induced RANTES mRNA at 3, 6, and 24 h (Fig. 2 *A*). The diminished expression of eotaxin mRNA in the anti-CD3–treated mice was accompanied by a significant reduction in tissue eosinophilia (Fig. 2 *B*, and Fig. 3, *B* and *D*) and the number of eosinophils recovered by BAL (Fig. 2 *C*). OVA-immunized mice treated with the control Ab showed an increase in eotaxin and MIP-1α mRNA (Fig. 2 *A*) that was similar to that seen in untreated immunized mice after challenge (Fig. 1 *A*), demonstrating that the control Ab did not significantly affect the induction of chemokine mRNA. Likewise, treatment with the control Ab did not affect the development of peribronchial and perivascular inflammation with eosinophils (Fig. 2 *B*, and Fig. 3, *A* and *C*) or the recruitment of eosinophils to the airway lumen (Fig. 2 *C*).

Anti-CD3 Induces MIP-1α and RANTES mRNA but not Eotaxin or Pulmonary Eosinophilia in Sham-immunized Mice. The divergent expression of eotaxin versus MIP-1α and RANTES mRNA expression in response to anti-CD3

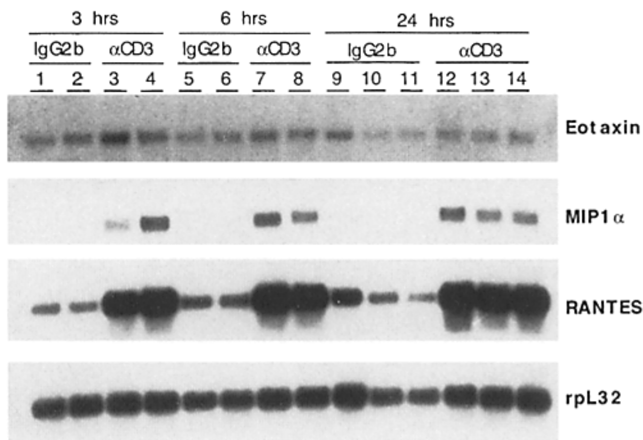
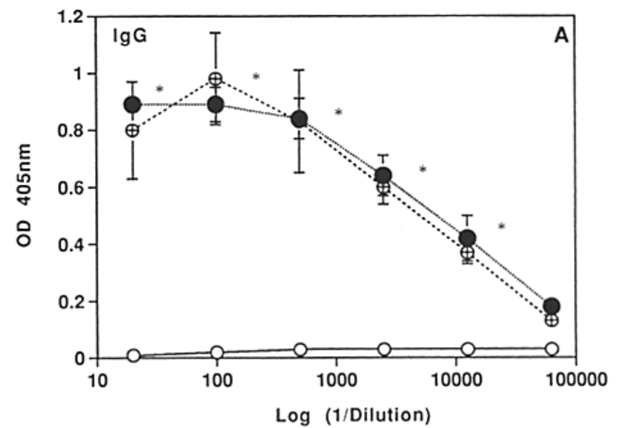


Figure 4. Induction of MIP-1α and RANTES but not eotaxin mRNA in sham-immunized mice. Northern analysis of 10 μg of total lung RNA isolated at 3, 6, and 24 h from sham-immunized mice after control Ab treatment (IgG2b) (lanes *1, 2, 5, 6, 9–11*) or anti-CD3 mAb treatment (lanes *3, 4, 7, 8, 12–14*). The blot was sequentially hybridized with eotaxin, MIP-1α, RANTES, and rpL32 ^{32}P-labeled cDNA probes and exposed for 3 days, 3 d, 10 d, and 4 d, respectively. Each lane represents an individual mouse from a representative experiment (n = 2).



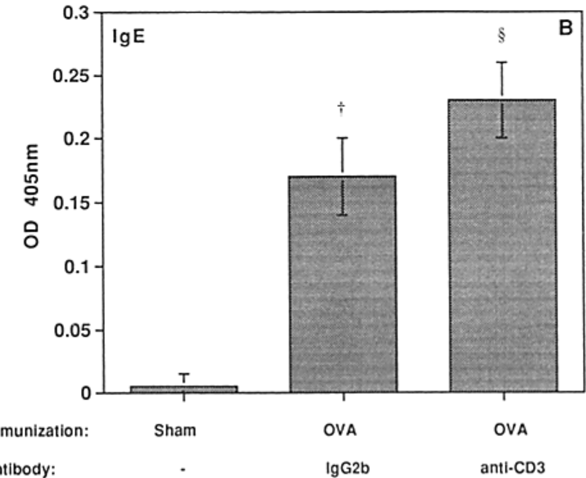


Figure 5. OVA-specific IgG and IgE titers are unchanged by treatment with anti-CD3 mAb. OVA-specific IgG and IgE were measured from serum obtained 24 h after aerosol challenge. (*A*) OVA-immunized mice treated with the isotype control mAb on the 2 d before challenge (*closed circles*) had elevated titers of OVA-specific IgG that were significantly greater than nonimmunized controls (*open circles*) (*P <0.01). Treatment of OVA-immunized mice with anti-CD3 mAb on the 2 d before secondary challenge did not significantly alter titers of OVA-specific IgG (*hatched circles*) compared with OVA-immunized mice treated with the control Ab. (*B*) OVA-immunized mice treated with the isotype control Ab had elevated titers of OVA-specific IgE compared with nonimmunized controls (†P < 0.01). Treatment of OVA-immunized mice with anti-CD3 mAb on the 2 d before secondary challenge did not significantly alter titers with OVA-specific IgE compared to OVA-immunized mice treated with the control Ab (§ P = 0.46). Data represent mean OD ± SEM of serum samples from at least three separate experiments (minimum three mice/group). OVA-specific IgG was assayed at fivefold serial dilutions from 1:20 to 1:62,500. OVA-specific IgE was assayed at a dilution of 1:3.

treatment in OVA-immunized mice was an unexpected finding. This was further investigated by comparing the effects of Ab treatment in sham-immunized mice. Anti-CD3 induced both MIP-1α and RANTES but not eotaxin mRNA in sham-immunized mice, while the control Ab had no effect on chemokine mRNA expression (Fig. 4). It is notable that the induction of MIP-1α and RANTES was not accompanied by significant tissue eosinophilia (0 eosinophils/mm in both perivascular and peribronchial areas for all time points studied) and no eosinophils were recovered from BAL.

OVA-specific IgG and IgE Titers and BAL Histamine Levels Are Unchanged by Treatment with anti-CD3 mAb in OVA-immunized Mice. Anti-CD3 effectively eliminated Ag-induced pulmonary eosinophilia and eotaxin induction in OVA-immunized mice, suggesting that eosinophil recruitment and eotaxin are T cell dependent in this model. To determine whether Ag-specific Ab responses and mast cell degranulation can be dissociated from eotaxin expression and eosinophil recruitment, we examined the effect of anti-CD3 treatment on OVA-specific IgG and IgE titers and BAL histamine levels in OVA-immunized mice. As expected, control animals developed significantly elevated titers of both OVA-specific IgG and IgE compared with sham-immunized controls (Fig. 5, *A* and *B*). Treatment of OVA-immunized mice with the anti-CD3 mAb did not significantly alter the titers of OVA-specific IgG or IgE compared with mice receiving the isotype control Ab (Fig. 5, *A* and *B*).

Mast cell degranulation was assessed by measuring BAL histamine levels in sham- and OVA-immunized mice after airway challenge with either saline or OVA. Sham-immunized mice, challenged with OVA, showed no significant elevation of BAL histamine level compared with saline-challenged mice (Fig. 6). In contrast, OVA-immunized mice, challenged with OVA, had an elevated BAL histamine level compared with saline-challenged mice and this was not affected by treatment with either the isotype control Ab or with the anti-CD3 mAb (Fig. 6). This demonstrates that Ag-induced mast cell degranulation can be dissociated from pulmonary eosinophilia and eotaxin induction in this model.

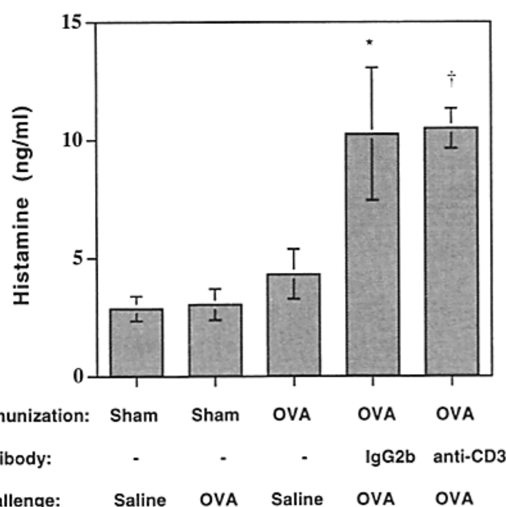


Figure 6. BAL histamine levels in sham- and OVA-immunized mice after challenge. BAL histamine levels were measured in sham- and OVA-immunized mice after aerosol challenge with either saline or OVA. Histamine levels in sham-immunized mice were similar after challenge with either saline or OVA. In contrast, OVA-immunized mice treated with either isotype-control Ab or anti-CD3 mAb showed a significant increase in BAL histamine after challenge with OVA compared with OVA-immunized mice challenged with saline (*$P < 0.048$ and † $P < 0.01$, respectively). Data represent the mean ± SEM BAL histamine; minimum of three mice per group.

Discussion

In this study, we examined the expression and regulation of eosinophil-active chemokines in vivo, in a murine model of allergic pulmonary inflammation. We found that eotaxin and MIP-1α mRNA were rapidly induced after Ag challenge in immunized mice with levels detectable by 3 h and peaking by 6 h. This initial increase in eotaxin and MIP-1α levels preceded the recruitment of eosinophils into the lung and airway lumen (BAL), suggesting that these chemokines may play an important role in recruiting eosinophils to the lung. However, by 24 to 48 h when eosinophil infiltration was peaking, eotaxin levels were still markedly elevated, while MIP-1α levels returned to baseline. In contrast, RANTES mRNA expression was unchanged after challenge, suggesting that the induction of this chemokine is not critical for the recruitment of eosinophils in this model.

T lymphocytes have been implicated in controlling the recruitment of eosinophils into the lung in murine models of allergic asthma. Ag-specific $CD4^+$ Th2 lymphocytes play a central role in eosinophil recruitment, as depletion of these cells (12, 15) or their secreted products (IL-4 and IL-5) (10, 14, 16, 17), significantly reduces pulmonary eosinophilia. The mechanism by which T cells assist in the recruitment of eosinophils to the lung in these models is not completely understood. We hypothesized that eosinophil active chemokines may be regulated by Ag-induced T cell activation in vivo and thereby mediate T cell–dependent eosinophil recruitment. To test this hypothesis, we examined the effect of an anti-CD3 mAb on Ag-induced pulmonary eosinophilia and correlated this with the expression of eotaxin, MIP-1α, and RANTES mRNA. Anti-CD3 effectively inhibited eotaxin mRNA expression in immunized mice after airway challenge and this was associated with a significant reduction in pulmonary eosinophilia. In contrast, anti-CD3 actually induced both MIP-1α and RANTES mRNA expression; this was observed in both sham- and OVA-immunized mice. Importantly, the induction of MIP-1α and RANTES mRNA was not associated with pulmonary eosinophilia. These findings demonstrate that the induction of eotaxin in vivo is dependent on Ag-specific T cells, and that its expression can be dissociated from MIP-1α and RANTES in anti-CD3 treated mice.

The correlation of diminished eotaxin, but not MIP-1α or RANTES mRNA expression, with reduced pulmonary eosinophilia in anti-CD3–treated mice, suggests that eotaxin may be a key regulator of pulmonary eosinophilia and the critical eosinophil chemoattractant in this model. Eotaxin was originally isolated as the predominant eosinophil chemoattractant in the lung lavage fluid of sensitized guinea pigs after allergic exposure (25). In contrast to other eosinophil-active chemoattractants, eotaxin is unique in that it specifically attracts eosinophils. This specificity has recently been demonstrated in vitro for both mouse and human eotaxin in chemotaxis and calcium flux assays (26–29, 36). The in vivo potency and specificity of eotaxin have been confirmed in studies demonstrating tissue eosinophil recruitment after instillation into the airways of rodents and after injection into the skin of rodents and rhesus monkeys (28, 29, 37). Furthermore, eotaxin acts synergistically with IL-5, another eosinophil-specific cytokine, to produce tissue eosinophilia in the skin of guinea pigs (38) and the skin and lungs of mice (36). The specificity and potency of eotaxin for eosinophils are the result of its high affinity interaction with recently described β-chemokine receptor, CKR3, that is highly expressed in human and mouse eosinophils (39, 40).

The major cellular sources of eotaxin are thought to be the epithelium, the endothelium, and activated infiltrating leukocytes, like eosinophils (27–29). We have found that TNF-α, IL-1, and IFN-γ regulate the expression of eotaxin in epithelial and endothelial cells in vitro (27). The inhibition of Ag-induced eotaxin mRNA expression by anti-CD3 may result from the elimination of T cell–derived factors that induce eotaxin mRNA expression in eotaxin-producing cells in the lung. Alternatively, if T lymphocytes are an important source of eotaxin in vivo, anti-CD3 may exert its effect directly, by eliminating these cells. Finally, T cell activation and cytokine release after the administration of anti-CD3 (41, 42), may result in the production of an inhibitory cytokine that is not produced by T cells after activation by Ag. In contrast to eotaxin, MIP-1α and RANTES were induced by anti-CD3 treatment, demonstrating that these chemokines are regulated by a fundamentally different mechanism than eotaxin in vivo.

Ag-specific Abs are thought to contribute to inflamma-

tory cell accumulation after Ag challenge (i.e., late-phase reactions) via IgE-mediated degranulation of mast cells (43). Administration of anti-CD3 after primary immunization allowed us to assess the role of T cell depletion on eotaxin induction and pulmonary eosinophilia in the presence of Ag-specific Abs and activated mast cells. We found that treatment with anti-CD3 did not significantly alter titers of Ag-specific IgG and IgE or the release of histamine from mast cell after Ag challenge in immunized mice. Yet, anti-CD3 significantly reduced eotaxin expression and pulmonary eosinophilia, suggesting that in the absence of T cells, Ag-specific IgE and IgG Abs and mast cell degranulation were not sufficient to induce eotaxin expression or to produce pulmonary eosinophilia after Ag challenge.

In summary, we have demonstrated that Ag-induced pulmonary eosinophilia was associated with the induction of eotaxin and MIP-1α but not RANTES mRNA. Prechallenge treatment with anti-CD3 mAb inhibited eotaxin but not MIP-1α and RANTES mRNA induction and significantly reduced eosinophil accumulation in the lung. In addition, Ag-specific Ab responses and mast cell degranulation after Ag challenge in sensitized mice were not affected by T cell elimination and were not sufficient to induce the expression of eotaxin and cause pulmonary eosinophilia. These findings suggest that eotaxin may be one of the molecular links between Ag-specific T cell activation and the recruitment of eosinophils into the airways.

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