

T Helper-2 Immunity Regulates Bronchial Hyperresponsiveness in Eosinophil-Associated Gastrointestinal Disease in Mice

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Background & Aims: Eosinophil-associated gastrointestinal diseases are frequently associated with extraintestinal features, including bronchopulmonary manifestations. The factors predisposing to bronchial hyperresponsiveness in eosinophil-associated gastrointestinal diseases are unknown. To elucidate the mechanistic link between eosinophil-associated gastrointestinal diseases and bronchial hyperresponsiveness, we used murine models of eosinophil-associated gastrointestinal diseases and eotaxin-1/transgene-induced eosinophil-associated gastrointestinal diseases. **Methods:** Mice were sensitized and orally challenged with ovalbumin-coated encapsulated particles to induce eosinophil-associated gastrointestinal disease, and bronchial responsiveness was examined. Furthermore, transgenic mice expressing eotaxin in the intestine (with the rat fatty acid-binding promoter) were used to specifically elucidate the contribution of this chemokine in eosinophil-associated gastrointestinal disease-associated bronchial hyperresponsiveness. **Results:** The induction of allergen-induced eosinophil-associated gastrointestinal disease was directly correlated with the development of bronchial hyperresponsiveness. The development of bronchial hyperresponsiveness in mice with allergen-induced eosinophil-associated gastrointestinal disease was dependent on eotaxin expression in the gastrointestinal tract. Expression of eotaxin in the gastrointestinal tract of transgenic mice was sufficient to promote bronchial hyperresponsiveness. Bronchial hyperresponsiveness was shown to be directly linked to the aberrant CD4⁺ T helper 2 lymphocyte production of interleukin-13. It is interesting to note that transgenic expression of eotaxin was linked with enhanced T helper 2 lymphocyte/cytokine synthesis (interleukin-4, -5, and -13) and the production of mucosal immunoglobulin G1 in the gastrointestinal lumen. We also showed that eotaxin treatment of CD4⁺ T cells enhanced interleukin-13 production in vitro. **Conclusions:** These studies suggest that increased expression of eotaxin in the gastrointestinal compartment can lead to increased CD4⁺ T cell-derived T helper 2 lymphocyte-cytokine production that drives aberrant immunophysiological responses in distant non-

inflamed mucosal tissue (the lung). These results provide a possible explanation for the altered lung function seen in some patients with inflammatory gastrointestinal disorders.

A variety of eosinophil-associated gastrointestinal diseases (EGID), including food allergy, eosinophilic esophagitis, eosinophilic gastroenteritis, and ulcerative colitis (UC), are frequently associated with extraintestinal features, including bronchopulmonary manifestations.^{1–4} The bronchopulmonary abnormalities can include asthma-like clinical manifestations such as obstruction of the upper airways, small airway dysfunction, pulmonary vasculitis, eosinophilic inflammation, and nonspecific bronchial hyperresponsiveness (BHR).^{5–9} Although the airways are not generally considered a common site of involvement, immunologic and bronchopulmonary abnormalities may be present in 40%–60% of patients with these disorders.

EGID, including food allergy and UC, are primarily associated with aberrant CD4⁺ T helper 2 lymphocyte (Th₂) responses within the intestinal mucosa.^{2,3} The type 2 cytokines interleukin (IL)-4, IL-5, and IL-13 have been shown to be up-regulated in biopsy samples from patients with UC.¹⁰ Furthermore, antigen-specific T cells from food allergy patients produce high levels of IL-4, IL-5, and IL-13 after in vitro stimulation.^{11–14} Indeed,

Abbreviations used in this paper: APC, allophycocyanin; BHR, bronchial hyperresponsiveness; CCR3, chemokine receptor-3; EGID, eosinophil-associated gastrointestinal diseases; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GI, gastrointestinal; IFABPp, intestinal rat fatty acid-binding protein promoter; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MBP, major basic protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OVA, ovalbumin; PBST, Tween-20 in PBS; sIL, soluble interleukin; Tg, transgenic; Th₂, T helper 2 lymphocyte; WT, wild type.

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experimental models of EGID have provided corroborative evidence of the importance of Th₂ cytokines as central regulators of disease pathogenesis.^{15–17}

Recently, we developed a mouse model of human EGID.^{15,16} This model mimics certain pathophysiological features of the human disease and is characterized by an allergen-induced Th₂ immune response, including peripheral blood eosinophilia; antigen-specific immunoglobulin (Ig)E and IgG1 production; CD4⁺ T cell–derived IL-4, IL-5, and IL-13 production; pathophysiological changes to the gastrointestinal (GI) tract (vascular congestion and edema); a prominent eosinophilic infiltrate; and GI dysfunction.¹⁶ We have further shown that GI dysfunction (villus/crypt shortening, gastric motility, gastromegaly, and axonal necrosis) is associated with GI eosinophilia and evidence of eosinophil degranulation (presence of extracellular major basic protein [MBP]–positive eosinophilic granules).^{15,16} The eosinophil-selective chemokine eotaxin was shown to be critical for the recruitment of eosinophils into the GI tract and the subsequent development of disease.

Although eotaxin-regulated eosinophil accumulation plays a key role in EGID, we do not know whether inflammation at this mucosal surface predisposes to BHR and to what extent eotaxin affects the development of such manifestations. In this study, we investigated the role of eosinophil-associated GI inflammation in the development of nonspecific BHR. By using our model of EGID, we showed that allergen-induced allergic inflammation of the GI tract predisposes to the development of BHR. Furthermore, in this model we show a critical role of eotaxin and eosinophils in the development of BHR. To further elucidate the mechanism regulating EGID-associated BHR and the requirement for eotaxin and eosinophils in this process, we examined transgenic (Tg) mice that overexpressed eotaxin specifically in the GI tract by using the intestine-specific promoter of the rat fatty acid-binding protein promoter (IFABPp) gene.^{18–20} Overexpression of eotaxin specifically in the GI tract promoted altered IgG1 and IgA production and increased levels of CD4⁺ T cell–derived Th₂ cytokine production, GI eosinophilia, and BHR. BHR was not associated with increased levels of pulmonary eosinophils but was dependent on CD4⁺ T cells and IL-13. Furthermore, we showed that treatment of splenic-naïve T cells with eotaxin *in vitro* enhanced IL-13 production. Collectively, these studies suggest that expression of eotaxin in the GI compartment can lead to altered Th₂ immunity that not only may regulate disease at this mucosal surface, but also may be linked to the nonspecific BHR that is observed in

some patients with eosinophil-associated GI inflammatory conditions.

Materials and Methods

Mice

Six- to 8-week-old BALB/c mice were obtained from the specific pathogen-free facility or the gene-targeting facility of the John Curtin School of Medical Research (Australian National University, Canberra, Australia). Eotaxin^{−/−} mice were backcrossed for 12 generations with the BALB/c strain. Tg mice constitutively expressing murine eotaxin from the gut epithelium (IFABPp/eotaxin) were generated as previously described and were maintained by continual backcross to FVB/n.²² In some experiments, splenocytes from wild-type (WT) or IFABPp-eotaxin Tg mice (5×10^6 cells per mouse) were injected intravenously to syngeneic-naïve FVB/n mice. Six days after the adoptive transfer of splenocytes, bronchial reactivity was measured. IFABPp-eotaxin Tg IL-13^{−/−} mice were generated by crossing IFABPp-eotaxin Tg (FVB/n) mice with IL-13^{−/−} (N₁₂ BALB/c) mice. The transgene-positive offspring (N₁) were subsequently mated with IL-13^{−/−} (N₁₂ BALB/c) to generate IFABPp/eotaxin IL-13^{−/−} mice (N₂). The (N₂) IFABPp-eotaxin Tg IL-13^{−/−} (FVB/n $\times$ N₁₂ BALB/c) mice were crossed with IL-13^{−/−} (N₁₂ BALB/c) mice, thus generating chimeric transgene-positive and -negative IL-13^{−/−} mice. To generate background control mice, the IFABPp-eotaxin Tg mice were crossed with IL-13^{+/+} (N₁₂ BALB/c) mice, and the transgene-positive offspring (N₁) were subsequently backcrossed with WT N₁₀ BALB/c mice, thus generating background-matched IFABPp-eotaxin Tg IL-13^{+/+} mice. Mice were treated according to Australian National University Animal Welfare guidelines and were housed in an approved containment facility.

Experimental Model of Oral Antigen-Induced Eosinophil-Associated Gastrointestinal Diseases

Mice (BALB/c and eotaxin^{−/−}) were sensitized by an intraperitoneal injection of 50 μ g of ovalbumin (OVA)/1 mg of alum in 200 μ L of 0.9% sterile saline on day 0. On days 12, 14, and 16, mice were lightly anesthetized with methoxyflurane inhalation (Metofane; Pittman-Moore, Mundelein, IL) and orally administered 20 mg of either encapsulated OVA enteric-coated beads or placebo-encapsulated enteric-coated beads, followed by 200 μ L of acidified H₂O (pH 2.0).^{15,16} Seventy-two hours after the last antigen challenge, mice were killed by cervical dislocation, and disease parameters were measured. In some experiments, mice were intraperitoneally injected on days 0, 1, and 3 with either rat IgG2b-depleting anti-CD4 monoclonal antibody (mAb; 200 μ L of GK1.5 mAb 1 mg/mL in saline) or rat IgG control antibody (200 μ L of 1 mg/mL of β GL113 mAb in saline). Methacholine responsiveness was determined on day 4. To neutralize IL-13 activity, mice were intraperitoneally injected (200 μ g/200 μ L) with

IL-13R α_2 -Fc²³ (a gift from D. Donaldson Wyeth, Cambridge, MA) or Ig control protein (IgG control) in phosphate-buffered saline (PBS) on days 12, 14, 16, and 17. Three hours after the final intraperitoneal injection, bronchial responsiveness was determined.

Serum and Fecal Immunoglobulin Measurements

Serum and fecal total IgG1 and IgA concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Blood was collected from IFABPp-eotaxin Tg and WT mice, allowed to clot, and centrifuged at 12,000g for 10 minutes at room temperature. Serum was aliquoted and stored at -70°C until analysis. For fecal samples, the mice were killed and the colon was removed. Colon contents were removed and resuspended in 1 mL of PBS. The fecal pellet suspension was vigorously vortexed for 30 minutes at 4°C . This suspension was centrifuged at 12,000g for 10 minutes at 4°C , and the supernatant was removed, aliquoted, and stored at -70°C until analysis. To determine IgG1 and IgA levels, sample wells were coated with 100 μL of goat anti-mouse IgG1 or IgA (1 $\mu\text{g}/\text{mL}$; Southern Biotechnology Associates, Birmingham, AL) overnight at 4°C , blocked with 10% fetal calf serum (FCS) in PBS, and washed with 0.05% Tween-20 in PBS (PBST). Serum samples were diluted 1:1000 for IgG1 and 1:100 for IgA with 10% FCS in PBS and then serially diluted (1:10). After a 2-hour incubation at 37°C , plates were washed with PBST, and 100 μL of biotin-conjugated goat anti-mouse IgG1 or IgA (1 $\mu\text{g}/\text{mL}$; Southern Biotechnology Associates) was added. After a 1-hour incubation at 37°C , the sample wells were washed, and streptavidin-conjugated horseradish peroxidase (100 μL per well; 2 mg/mL; ImmunoTech, Mar-seilles, France) was added for 1 hour at 37°C . The assay was developed by using 100 μL of TMB Substrate Reagent (Pharmingen, Palo Alto, CA). The substrate reaction was stopped by adding 2N HCl 100 μL per well and was quantified by measuring optical density with an ELISA plate reader at 450 nm.

Serum and Lung Eotaxin and IL-5 Measurements

Blood was collected from IFABPp-eotaxin Tg and WT mice, allowed to clot, and centrifuged at 12,000g for 10 minutes at room temperature. Serum was aliquoted and stored at -70°C until analysis. For lung measurements, lungs were excised and frozen in liquid nitrogen. The lungs were then ground up and resuspended in PBS/0.1% FCS, vortexed vigorously for 5 minutes, and centrifuged at 12,000g for 10 minutes. The supernatant was aliquoted and stored at -70°C until analysis. IL-5 levels in serum were determined by using the OptEIA Mouse IL-5 kit (Pharmingen). Serum, lung eotaxin, and IL-13 levels were determined by sandwich ELISA. Sample wells were coated with 100 μL of anti-mouse eotaxin-specific goat IgG polyclonal antibody (200 ng/mL; AF-420NA; R&D Systems, Minneapolis, MN) or rat anti-mouse IL-13 mAb (R&D Systems; clone 38213.11) in carbonate

buffer (pH 9.6) and incubated overnight at 4°C . After incubation, plates were washed with PBST. Nonspecific binding sites were blocked with 250 μL of 10% FCS in PBS and incubated at 37°C for 2 hours. Mouse eotaxin (PeproTech, Rocky Hill, NJ) or IL-13 was used as the quantification standard. Eotaxin and IL-13 standard and serum/lung homogenate samples were diluted in blocking solution, added to sample wells, and incubated at 37°C for 2 hours. Wells were washed 3 times with PBST and then incubated with 100 μL of biotinylated, affinity-purified, polyclonal secondary antibody to mouse eotaxin (4 ng/mL; BAF-420; R&D Systems) or biotinylated rat anti-mouse IL-13 mAb (R&D Systems) for 2 hours at 37°C , followed by 3 washes with PBST. Streptavidin-conjugated horseradish peroxidase (100 μL per well; 2 $\mu\text{g}/\text{mL}$; ImmunoTech) was added for 1 hour at 37°C . The assay was developed by using 100 μL of TMB Substrate Reagent (Pharmingen). The substrate reaction was stopped by adding 2N HCl 100 μL per well and was quantified by measuring optical density with an ELISA plate reader at 450 nm.

Measurement of Airway Reactivity to Spasmogens

Bronchial reactivity to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography by using apparatus and software supplied by Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause that reflects changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration. This can be used to empirically monitor airway function. Measurement was performed as previously described.²⁴ Briefly, mice were placed in the chamber, and baseline readings were taken and averaged for 2–5 minutes. Aerosolized methacholine (concentrations in solution ranging from 3.125 to 50 mg/mL) was then delivered through an inlet into the chamber for 2 minutes, and readings were averaged over 2–5 minutes after each dose was administered.

Immunohistochemistry Detection and Quantification of Mast Cells and Eosinophils

The jejunum segment of the GI tract was fixed with 4% paraformaldehyde, processed with standard histological techniques, and immunostained with antiserum against mouse MBP as previously described.²⁵ Briefly, 5- μm sections were quenched with H_2O_2 , blocked with normal goat serum, and stained with rabbit anti-murine eosinophil MBP antiserum (a gift of J. and N. Lee, Mayo Clinic, Scottsdale, AZ). The slides were washed and incubated with biotinylated goat anti-rabbit antibody and avidin-peroxidase complex (Vectastain ABC Peroxidase Elite kit; Vector Laboratories, Burlingame, CA). The slides were then developed by nickel diaminobenzidine, enhanced with cobalt chloride to form a black precipitate, and counterstained with nuclear fast red. The sections were taken from the same position in the jejunum (3–5 cm distal to the stomach), and at least 4–5 random sections per mouse were

analyzed. Values were determined by quantifying the total number of eosinophils per square millimeter of tissue. Fifteen to 25 fields of view (magnification 40 $\times$) were counted per jejunum section in duplicate. Mast cells were stained for chloroacetate esterase activity as previously described.^{26,27}

Characterization of Eosinophils in Lung Tissue

Lung tissue representing the central (bronchi/bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin with standard histological techniques and stained with Charbol's chromotrope-hematoxylin for identification of eosinophils. Eosinophils in the central bronchi/bronchiole area were identified by morphological criteria and quantified as previously described.²⁴

Polyclonal Activated CD4⁺ T-Cell Response

Splenocytes were subjected to α CD3/ α CD28 stimulation as previously described.²⁸ In brief, 5×10^5 splenocytes per milliliter were cultured with α CD3 (5 μ g/mL) and α CD28 (1 μ g/mL) for 96 hours. IL-4, IL-5, and interferon (IFN)- γ levels were determined in supernatants from stimulated splenocyte homogenates by using the OptEIA Mouse IL-4, IL-5, and IFN- γ kits (Pharmingen). IL-13 levels were measured by using rat anti-mouse IL-13 mAb (R&D Systems; clone 38213.11) and biotinylated rat anti-mouse IL-13 mAb (R&D Systems).

Proliferation Assay

In brief, 5×10^5 splenocytes per milliliter were cultured with α CD3 (5 μ g/mL) and α CD28 (1 μ g/mL) in 96-well round-bottomed plates for 96 hours. Six hours before completion of the assay, 20 μ L of 5 mg/mL 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to each well and incubated in 5% CO₂ at 37°C for a further 6 hours. After the incubation, 200 μ L of dimethyl sulfoxide was added to each well, and supernatants were pipetted up and down to dissolve crystals and placed at 37°C for 5 minutes. The absorbance in the supernatants was measured at 550 nm.

Quantification of CD4⁺ T Cells in the Splenocyte Population by Fluorescence-Activated Cell Sorting Analysis

A total of 10^6 splenocytes were incubated with 1 μ g of phycoerythrin-conjugated anti-mouse CD4 (Pharmingen) or the isotype-matched control antibodies at 4°C for 30 minutes. Cells were washed with 1% FCS/PBS, centrifuged, and resuspended in 1% FCS/PBS. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) by using CellQuest software (Becton Dickinson).

Anti-CD4 Monoclonal Antibody and IL-13R α_2 -Fc Treatment

In some experiments, IFABPp-eotaxin Tg and WT mice were intraperitoneally injected on days 0, 1, and 3 with

either rat IgG2b-depleting anti-CD4 mAb (200 μ L of GK1.5 mAb 1 mg/mL in saline) or rat IgG control antibody (200 μ L of β G113 mAb 1 mg/mL in saline). Methacholine responsiveness was determined on day 4. To neutralize IL-13 activity, mice were intraperitoneally injected (200 μ g/200 μ L) and intratracheally challenged (100 μ g/25 μ L) with sIL-13R α_2 -Fc fusion protein (sIL-13R 2-Fc)²³ (a gift from D. Donaldson Wyeth, Cambridge, MA) or Ig control protein in PBS on day 0 and were intraperitoneally injected (200 μ g/200 μ L) on day 1. Three hours after the secondary intraperitoneal injection, bronchial responsiveness was determined.

Identification of CD4 and Chemokine Receptor-3 Expression on T Cells by Fluorescence-Activated Cell Sorting Analysis

To examine the level of CD4 and chemokine receptor-3 (CCR3) expression, splenocytes were incubated with APC-conjugated anti-CD4 (clone GK1.5; BD Pharmingen; 1 μ g/10⁶ cells) and Alexa-488-conjugated anti-CCR3 (clone 6SH2-59; 1 μ g/10⁶ cells) in PBS/1% FCS on ice for 30 minutes and washed twice in PBS/1% FCS. Cells were sorted by flow cytometry on a FACSVantage SE cell sorter (Becton Dickinson).

Eotaxin Stimulation of Splenic CD4⁺ T Cells

A total of 10^6 splenocytes were incubated with 0, 0.01, or 0.1 ng/mL of eotaxin (PeproTech) in 5% CO₂ at 37°C for 24 hours and cultured with α CD3 (5 μ g/mL) and α CD28 (1 μ g/mL) for 96 hours. The IL-13 levels were measured as described previously.

Statistical Analysis

The significance of differences between the means of experimental groups was analyzed with the Student unpaired *t* test. Values are reported as the mean $\pm$ SEM. Differences in mean values were considered significant if *P* < 0.05.

Results

Bronchial Hyperresponsiveness in Eosinophil-Associated Gastrointestinal Diseases

To determine whether EGID predisposes to alterations in bronchial reactivity, we used our established model of OVA-induced EGID.¹⁶ OVA-sensitized mice receiving OVA-coated encapsulated particles developed the characteristic inflammatory response and disease in the GI tract, as previously described.¹⁶ Notably, the development of inflammation and disease directly correlated with the induction of BHR in the lung (Figure 1A). Pulmonary eosinophil levels and mucus secretion in the OVA-sensitized mice that received OVA-coated encapsulated particles were not significantly higher than in vehicle-treated or naive mice (results not shown). Col-

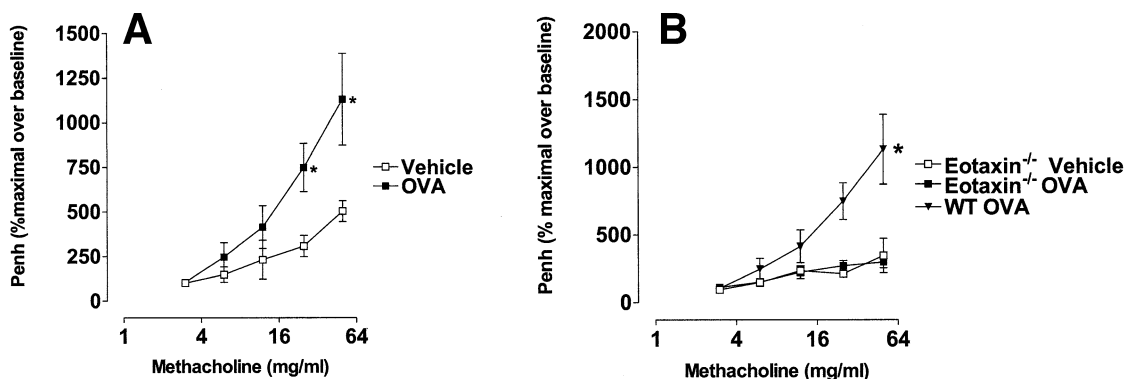


Figure 1. Characterization of bronchial hyperresponsiveness in OVA-sensitized and oral-challenged eotaxin^{-/-} and WT mice. Cumulative dose-response curves to methacholine in vehicle- or allergen-sensitized and oral-challenged (A) WT and (B) eotaxin^{-/-} mice. Experimental gastrointestinal allergy was induced as described in Methods. (A and B) BHR to methacholine challenge was measured by barometric plethysmography, and data represent the percentage increase in enhanced pause (Penh) over baseline reactivity. Data shown represent the mean Penh (percentage increase over baseline) $\pm$ SEM ($n = 8$ per group). The statistical significance of differences ($P < 0.005$ and $P < 0.01$) was determined with the Student unpaired t test.

lectively, these data suggest that allergic inflammation in the GI tract may predispose to BHR.

Recently we have shown that in the absence of eotaxin, OVA-induced GI eosinophilia and dysfunction was significantly attenuated.^{15,16} We were therefore interested in elucidating what contribution eotaxin and eosinophils played in the BHR associated with GI disease. Oral antigen challenge of OVA-sensitized eotaxin^{-/-} mice did not promote BHR (Figure 1B). The level of responsiveness to methacholine in OVA-sensitized eotaxin^{-/-} mice was not significantly different from that observed in OVA-sensitized eotaxin^{-/-} mice receiving vehicle (Figure 1B).

Bronchial Hyperresponsiveness in IFABPp Eotaxin Transgenic Mice

To dissect out the role of eotaxin and eosinophils in BHR in EGID, we examined Tg mice that overexpress eotaxin specifically in the GI tract, by using IFABPp.^{18–20} This promoter has been extensively used to direct the expression of genes specifically in enterocytes of the small intestine.^{18–20} First, we assessed the effect of ectopic expression of eotaxin in the GI tract on airway function and compared responses with WT. BHR in IFABPp-eotaxin Tg mice was significantly increased in comparison with WT mice (Figure 2). Notably, the BHR displayed by IFABPp-eotaxin Tg mice was achieved in the absence of an inflammatory response, because no eosinophils or mucus secretion was observed in the lung (results not shown). These data suggest that GI overexpression of eotaxin can enhance bronchial reactivity. BHR in mice has been shown to be linked to inflammatory cells and cytokines.²⁹ To examine whether BHR in IFABPp-eotaxin Tg mice was a direct conse-

quence of enhanced levels of expression of eotaxin in the GI tract or whether it was due to an altered immunologic phenotype, we adoptively transferred splenocytes from IFABPp-eotaxin Tg mice into WT mice and examined bronchial reactivity. Adoptive transfer of splenocytes from IFABPp-eotaxin Tg mice into syngeneic FVB/n mice enhanced bronchial reactivity as compared with FVB/n mice receiving WT FVB/n splenocytes. The demonstration that adoptive transfer of splenic cells from IFABPp-eotaxin Tg mice enhanced bronchial reactivity in WT mice suggests that BHR is not directly induced by increased levels of eotaxin but, rather, is an altered immunologic phenotype.

Role of CD4⁺ T Cells in the Regulation of Gastrointestinal Disease-Associated Bronchial Hyperresponsiveness

Previous studies have shown an important role for CD4⁺ T cells in the regulation of BHR.^{30,31} To determine whether CD4⁺ T cells were involved in mediating increased BHR in IFABPp-eotaxin Tg mice, WT and IFABPp-eotaxin Tg mice were treated with anti-CD4-depleting mAb, and bronchial reactivity to methacholine was measured (Figure 3). CD4⁺ T-cell numbers in the spleen of anti-CD4 mAb-treated mice were significantly lower (95% reduction) than those in control isotype-treated mice ($P < 0.005$; results not shown). Treatment of WT mice with anti-CD4 mAb did not reduce the baseline bronchial reactivity to methacholine as compared with the IgG isotype-treated mice (Figure 3A). In contrast, depletion of CD4⁺ T cells in IFABPp-eotaxin Tg mice significantly attenuated bronchial responsiveness to methacholine (Figure 3B). Notably, the level of bronchial responsiveness observed in anti-CD4 mAb-

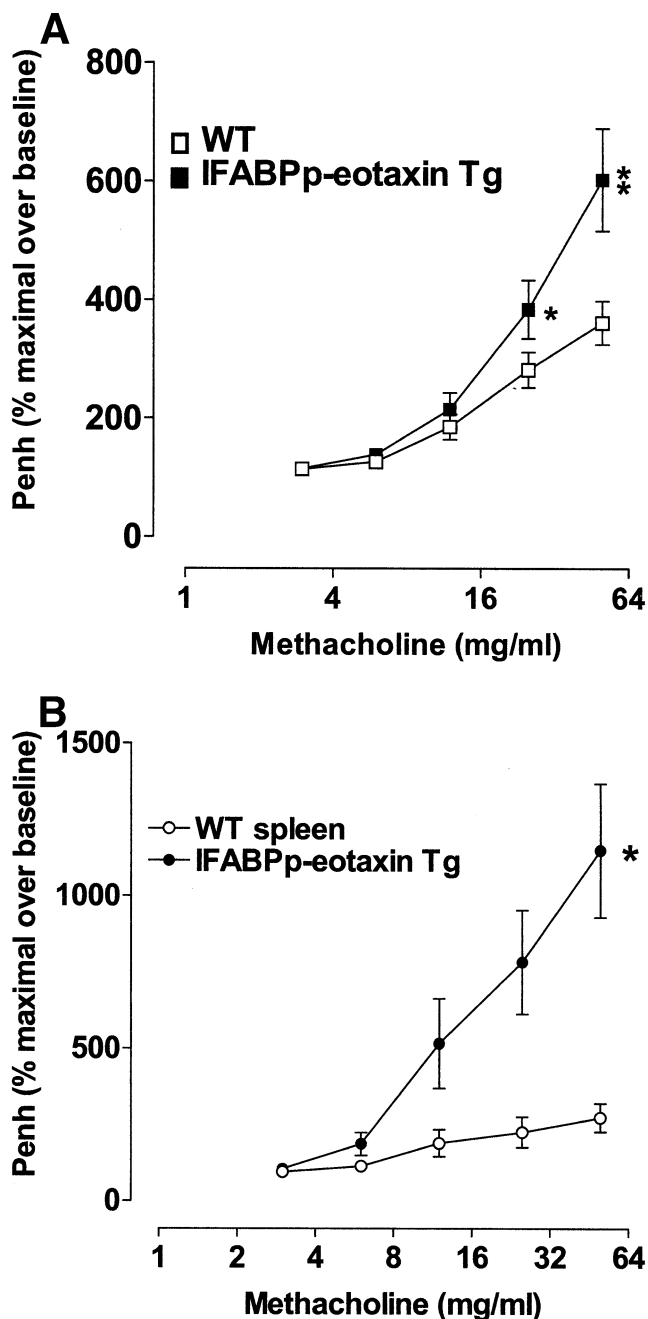


Figure 2. Bronchial hyperresponsiveness in IFABPp-eotaxin transgenic mice. (A) Cumulative dose-response curves to methacholine were constructed in IFABPp-eotaxin Tg and WT mice. (B) Cumulative dose-response curves to methacholine were constructed in WT mice receiving either WT or IFABPp-eotaxin Tg splenocytes. Data are expressed as the mean enhanced pause (Penh; percentage over baseline) $\pm$ SEM; (A) $n = 12$ –16 and (B) 4–5 mice per group. BHR to methacholine challenge was measured by barometric plethysmography, and the data represent the percentage increase in Penh over baseline reactivity. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired t test. Significant differences (* $P < 0.05$; ** $P < 0.01$) between groups are shown.

treated IFABPp-eotaxin Tg mice was similar to that observed in anti-CD4 mAb-treated or IgG isotype-treated WT mice (Figure 3A). Collectively, these findings suggest that increased airway reactivity in IFABPp-eotaxin mice is critically regulated by CD4⁺ T cells. We were also interested in elucidating whether the attenuated BHR observed in IFABPp-eotaxin Tg mice after depletion of CD4⁺ T cells was associated with any reduction in GI eosinophil levels. Eosinophil levels in the peripheral blood and jejunum of anti-CD4 mAb-treated IFABPp-eotaxin Tg mice were not significantly different from those observed in isotype-matched control antibody-treated IFABPp-eotaxin Tg mice (eosinophils: 54.44 ± 8.91 vs. $39.78 \pm 6.08 \times 10^4/\text{mL}$ of peripheral blood; $n = 4$ –6 mice per group; 12.11 ± 0.39 vs. $12.04 \pm 0.52/\text{mm}^2$ of lamina propria/jejunum; 4–5 random sections per mouse for 7 mice per group; mean $\pm$ SEM; + β GL113-treated vs. +GK1.5 mAb-treated IFABPp-eotaxin Tg mice, respectively). To determine whether CD4⁺ T cells regulated BHR in mice with OVA-induced EGID, OVA-sensitized mice receiving OVA-coated encapsulated particles were treated with anti-CD4 mAb. Treatment of OVA-sensitized mice receiving OVA-coated encapsulated particles with anti-CD4 mAb significantly suppressed bronchial reactivity to methacholine as compared with the IgG isotype-treated group (Figure 3C). The level of BHR in these mice was equivalent to that observed in OVA-sensitized mice receiving vehicle-coated encapsulated particles treated with IgG isotype mAb (Figure 3C).

Role of IL-13 in the Regulation of Gastrointestinal Disease-Associated Bronchial Hyperresponsiveness

Recent studies have shown a critical role for IL-13 in the induction of BHR in mouse asthma models.^{32–34} We therefore examined IL-13 levels in the serum and pulmonary compartment of IFABPp-eotaxin Tg and WT mice. IL-13 levels in the serum and pulmonary compartments were significantly increased as compared with WT mice (Figure 4). IFABPp-eotaxin Tg mice were also treated with sIL-13R α 2-Fc (specifically binds IL-13) or immunoglobulin control protein, and bronchial responsiveness to methacholine was measured. Bronchial responsiveness to methacholine in sIL-13R α 2-Fc-treated IFABPp-eotaxin Tg mice was significantly attenuated as compared with Ig control-treated mice (Figure 5A). The level of responsiveness in sIL-13R α 2-Fc-treated IFABPp-eotaxin mice was comparable to that observed in anti-CD4 mAb-treated IFABPp-eotaxin Tg mice (Figures 3B and 5A). To confirm a critical role for IL-13 in the BHR observed in IFABPp-eotaxin Tg mice, we

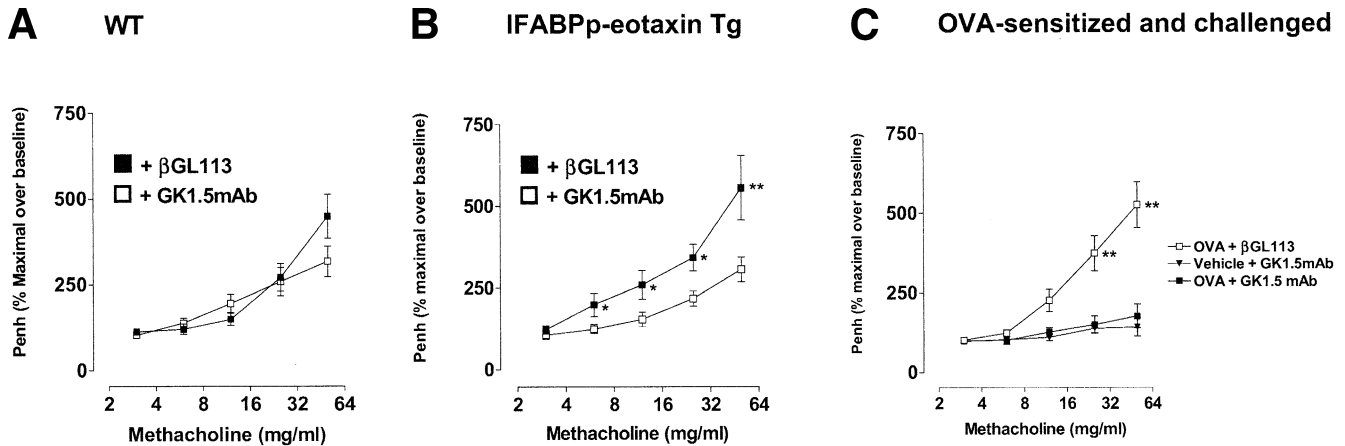


Figure 3. Bronchial hyperresponsiveness in IFABP-eotaxin transgenic and OVA-sensitized and oral-challenged mice is dependent on CD4⁺ T cells. Cumulative dose-response curves to methacholine are shown in (A) WT and (B) IFABP-eotaxin Tg mice and in (C) vehicle- or allergen-sensitized and oral-challenged mice treated with anti-CD4 neutralizing mAb. Data are expressed as the mean enhanced pause (Penh; percentage over baseline) $\pm$ SEM from 6–8 mice per group. BHR to methacholine challenge was measured by barometric plethysmography, and the data represent the percentage increase in Penh over baseline reactivity. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired *t* test. Significant differences (* $P < 0.05$ and ** $P < 0.01$) between groups are shown. (C) Significant differences (** $P < 0.01$) compared with vehicle plus GK1.5 mAb and OVA plus GK1.5 mAb.

generated IFABP-eotaxin Tg mice on the IL-13^{-/-} background (Figure 5B). Consistent with our antibody neutralization studies, BHR to methacholine in IFABP/eotaxin Tg IL-13^{-/-} mice was significantly attenuated as compared with control IFABP-eotaxin Tg mice (Figure 5B). To determine whether IL-13 regulated BHR in mice with OVA-induced EGID, OVA-sensitized mice receiving OVA-coated encapsulated particles were treated with sIL-13R α 2-Fc. Treatment of OVA-sensitized mice receiving OVA-coated encapsulated particles with sIL-13R α 2-Fc significantly suppressed bronchial reactivity to methacholine as compared with the IgG control-treated mice (Figure 5C). The level of BHR in these mice was equivalent to that observed in OVA-sensitized mice receiving vehicle-coated encapsulated particles treated with IgG control (Figure 5C). Collectively, these studies suggest that EGID-mediated BHR is dependent on CD4⁺ T cells and IL-13.

Eosinophil Levels in Peripheral Blood, Pulmonary Compartment, and Gastrointestinal Tract of IFABP/Eotaxin Mice

Previously, we have shown that OVA-induced EGID is associated with a CD4⁺ Th₂-type T-cell response.^{16,35} In light of these findings, the observations in this study with IFABP-eotaxin Tg mice suggest that expression of eotaxin in the GI tract may predispose to alterations in CD4⁺ Th₂ development and lead to the development of BHR. Therefore, to elucidate the effect of ectopic expression of eotaxin in the GI tract on CD4⁺

T-cell function, we characterized the immune profile in IFABP-eotaxin Tg mice. Initially, we examined eotaxin and eosinophil levels in IFABP-eotaxin Tg mice. The levels of serum eotaxin in IFABP-eotaxin Tg mice was significantly increased (~ 6 – 7 -fold) as compared with WT mice (Figure 6A). Furthermore, we observed no significant difference in pulmonary eotaxin levels between IFABP-eotaxin Tg mice and WT mice (eotaxin, 0.99 ± 0.11 ng/mL vs. 1.00 ± 0.21 ng/mL; mean $\pm$ SEM; $n = 4$ mice per group; WT vs. IFABP-eotaxin Tg mice, respectively). Next we were interested in elucidating the effect of eotaxin expression in the GI tract on the distribution of eosinophils in various tissue compartments. The level of eosinophils in the peripheral blood of IFABP-eotaxin Tg mice was comparable to that observed in WT mice (Figure 6B). By contrast, levels of GI eosinophils in IFABP-eotaxin Tg mice were significantly higher in the jejunum (8-fold) and ileum (5-fold) as compared with WT mice (Figure 6C). No differences in eosinophil levels were observed in the colon (Figure 6C) between IFABP-eotaxin Tg and WT mice. The increased level of eosinophils in the jejunum and ileum correlates spatially with the degree of expression of the intestinal fatty acid-binding protein and, hence, eotaxin levels.^{18–20} We also examined the effect of ectopic expression of eotaxin on the recruitment of mast cells (another eotaxin-sensitive granulocyte). We observed no significant difference in mast cell numbers in the jejunum or colon of IFABP-eotaxin Tg mice as compared with WT mice (number of mast cells per square milli-

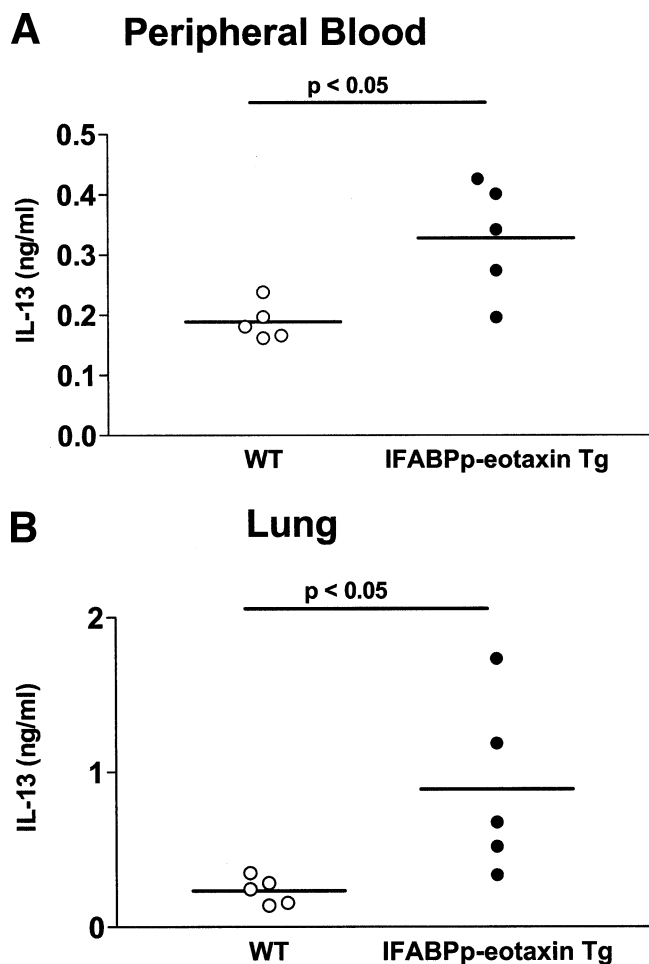


Figure 4. IL-13 levels in the periphery and the pulmonary compartments in IFABPp-eotaxin Tg mice. IL-13 levels are shown in (A) serum and (B) the pulmonary compartment of IFABPp-eotaxin Tg mice and wild-type (WT) mice. IL-13 levels were determined by ELISA as described in Materials and Methods. Data represent the mean $\pm$ SEM of 5 mice per group from duplicate experiments. The statistical significance of differences ($P < 0.05$) was determined with the Student *t* test.

meter: jejunum, 0.57 ± 0.08 vs. 0.44 ± 0.08 ; colon, 1.29 ± 0.25 vs. 1.18 ± 0.21 ; mean $\pm$ SEM; $n = 4$ mice per group; WT vs. IFABPp-eotaxin Tg mice, respectively). These data suggest that chronic expression of eotaxin promotes a GI eosinophilia but has no effect on systemic eosinophil levels. Furthermore, overexpression of eotaxin does not affect mast cell accumulation in the GI tract.

Characterization of Mucosal Ig Levels and CD4⁺ T Cells in IFABPp/Eotaxin Transgenic and Wild-type Mice

We next examined levels of fecal total IgG1 and IgA in IFABPp-eotaxin Tg mice. It is interesting to note that fecal total IgG1 and IgA in IFABPp-eotaxin Tg mice was significantly higher than that observed in WT

mice (Figure 7). These findings suggest that the ectopic expression of eotaxin markedly alters the secretion of immunoglobulin isotypes into the GI lumen. Immunoglobulin class switching to the IgG1 isotype primarily

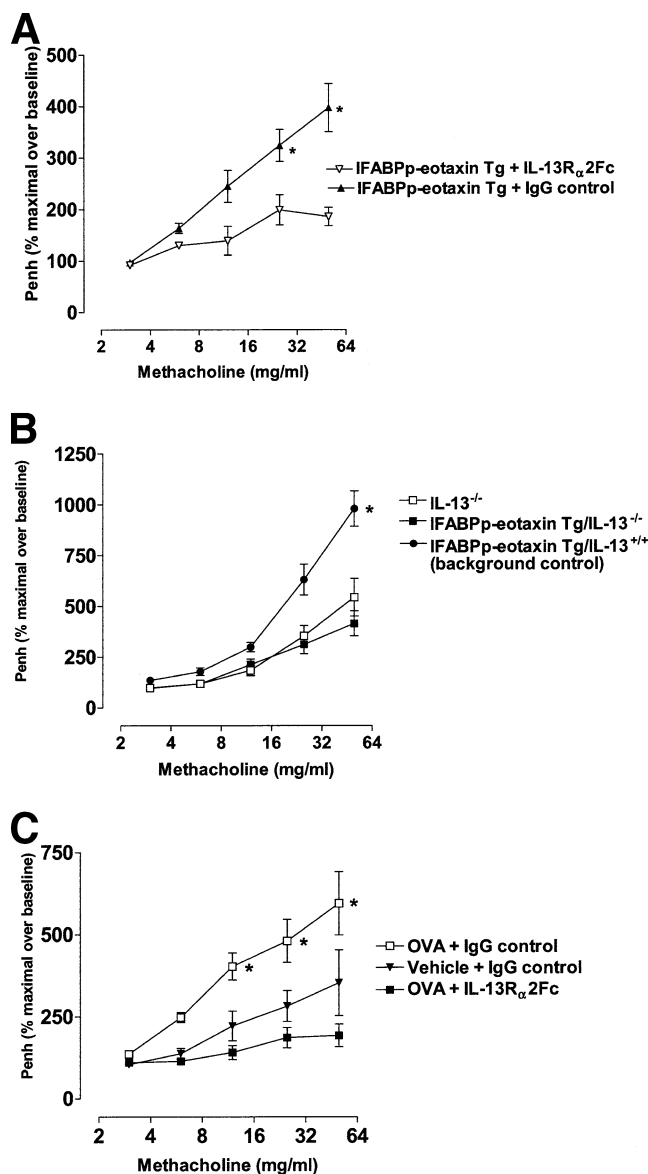


Figure 5. Bronchial hyperresponsiveness in IFABPp-eotaxin transgenic and OVA-sensitized and oral-challenged mice is dependent on IL-13. Cumulative dose-response curves to methacholine were constructed in (A) IFABPp-eotaxin Tg mice treated with IL-13R α 2Fc or IgG control, (B) IFABPp-eotaxin Tg/IL-13^{-/-} and IFABPp-eotaxin Tg mice, and (C) vehicle- or allergen-sensitized and oral-challenged mice treated with IL-13R α 2Fc or IgG control. Data are expressed as the mean enhanced pause (Penh; percentage over baseline) $\pm$ SEM from 6–10 mice per group. Methacholine challenge was measured by barometric plethysmography, and the data are represented as the percentage increase in Penh over baseline reactivity. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired *t* test. Significant differences ($*P < 0.05$) between groups are shown. (C) Significant differences ($*P < 0.05$) compared with OVA + IL-13R α 2Fc.

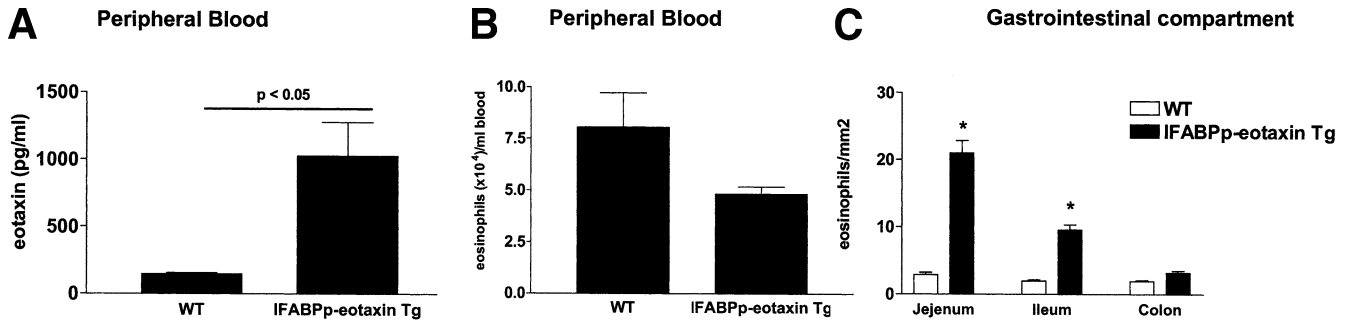


Figure 6. Peripheral blood, pulmonary, and gastrointestinal eosinophil levels in IFABPp-eotaxin Tg and WT mice. (A) Eotaxin levels in serum of IFABPp-eotaxin Tg mice and wild-type (WT) mice. Eosinophil levels in (B) peripheral blood and (C) the gastrointestinal compartment of IFABPp-eotaxin Tg mice and WT mice. (A) Eotaxin and IL-5 levels were determined by ELISA as described in Methods. Data represent the mean $\pm$ SEM of 4–5 mice per group from duplicate experiments. (B) The total number of eosinophils in peripheral blood. Data are expressed as the mean number of eosinophils ($\times 10^4$ /mL) $\pm$ SEM and are representative of 3–4 mice per group from triplicate experiments. (C) Eosinophil numbers per square millimeter in jejunum, ileum, and colon of WT and IFABPp-eotaxin Tg mice are shown. Eosinophils were identified by immunohistochemistry staining by using anti-MBP mAb as described in Materials and Methods. Eosinophils were quantitated by counting 20 similar high-power fields (HPF; magnification 32 $\times$) for each group. Data represent the mean $\pm$ SEM of 4–5 random sections per mouse for 4–5 mice per group from duplicate experiments. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired *t* test.

involves T cell–dependent signals (CD40/CD40L ligation and T cell–derived cytokines).^{36,37} We therefore examined the T-cell cytokine profiles of splenocytes from WT and IFABPp-eotaxin Tg mice after polyclonal activation by α CD3/CD28. Polyclonal stimulation of WT and IFABPp-eotaxin Tg splenocytes promoted the production of IL-4, IL-5, IL-13, and IFN- γ (Figure 8A–D). The levels of Th₂ cytokines (IL-4, IL-5, and IL-13), but not Th₁ cytokines (IFN- γ), from IFABPp-eotaxin Tg mice were significantly increased when compared with those from WT mice. To show that the exaggerated production of Th₂ cytokines by cells derived from IFABPp-eotaxin Tg mice was not due to differences in the percentages of CD4⁺ T cells in the splenocyte population, CD4⁺ T-cell numbers in splenic populations were determined by fluorescence-activated cell sorting analysis. No significant difference in the number of CD4⁺ T cells in splenocyte populations was observed between WT and IFABPp-eotaxin Tg mice (results not shown). Furthermore, no significant difference in MTT incorporation in splenocyte populations was observed between WT and IFABPp-eotaxin Tg mice after polyclonal activation (Figure 8E). This indicates that the increased levels of Th₂ cytokines were not due to differences in the ability of T cells to proliferate. Collectively, these findings show that ectopic expression of eotaxin in the GI tract selectively promotes a biased Th₂ immunity. Furthermore, our studies show that differences in the intrinsic production of Th₂ cytokines are not caused by altered CD4⁺ T-cell numbers or proliferative responses between WT and IFABPp-eotaxin Tg splenocytes.

The demonstration that CD4⁺ T cells from IFABPp-eotaxin Tg mice produced Th₂ cytokines after stimulation with the polyclonal activators α CD3/ α CD28 led us

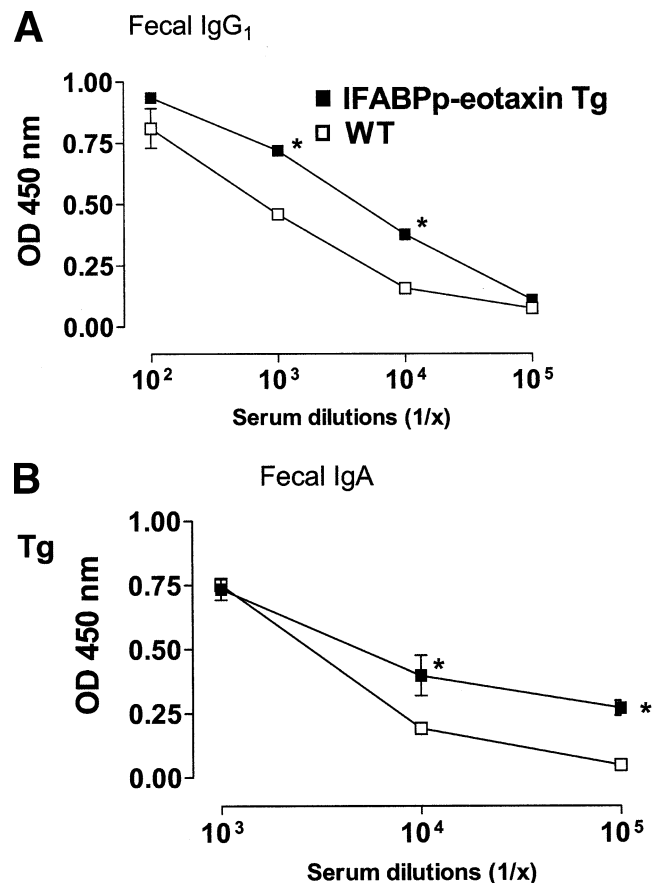


Figure 7. Ectopic expression of eotaxin in the gastrointestinal tract promotes an altered immunoglobulin profile. Fecal IgG₁ (A) and IgA (B) levels in IFABPp-eotaxin Tg and WT mice are shown. Serum and fecal material was collected, and antibody titers were determined as described in Materials and Methods. Data represent the mean optical density of the serum dilution (1/x) $\pm$ SEM from 4–6 mice per group from triplicate experiments. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired *t* test. Significant differences (* $P < 0.05$) between groups are shown.

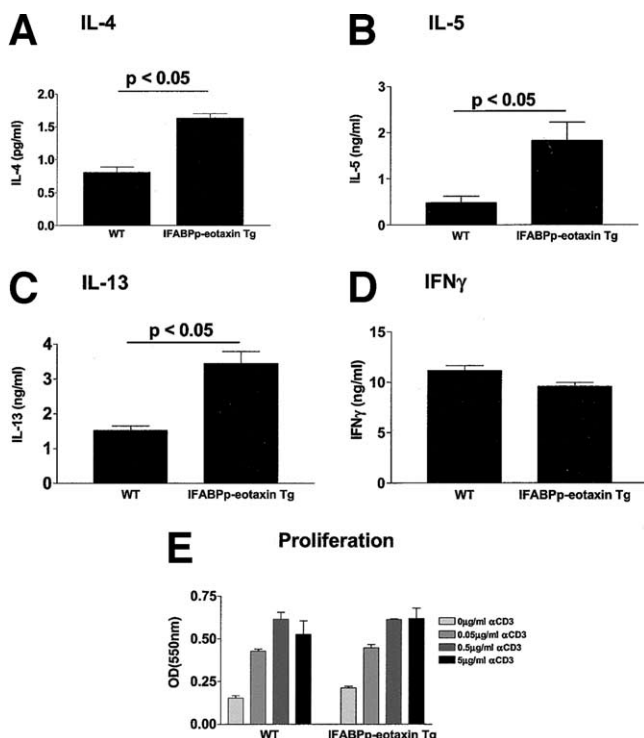


Figure 8. Ectopic expression of eotaxin in the gastrointestinal tract predisposed to systemic CD4⁺ Th₂-type immunity. IL-4 (A), IL-5 (B), IL-13 (C), and IFN- γ (D) levels in supernatants from α CD3/ α CD28-stimulated splenocytes from WT and IFABPp-eotaxin Tg mice are shown. (E) Proliferative dose response of α CD3/ α CD28-stimulated splenocytes from WT and IFABPp-eotaxin transgenic mice. Data are expressed as the mean $\pm$ SEM level of cytokines from 4–5 mice per group from duplicate experiments. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired t test.

to examine the effect of eotaxin on CD4⁺ T cell–derived cytokine production. Eotaxin has been shown to exclusively signal through the G protein–coupled CCR3, so we first examined the expression of CCR3 on splenocytes from WT and IFABPp-eotaxin Tg mice (Figure 9A). Two-color fluorescence-activated cell sorting analysis showed that $16.67\% \pm 0.61\%$ of splenocytes were positive for CCR3. Of the CCR3-positive cells, $26.80\% \pm 1.40\%$ were CD4⁺ cells. By comparing mean fluorescence intensities, we observed no significant difference in the expression of CCR3 or CCR3/CD4 on splenocytes between WT and IFABPp-eotaxin Tg mice (results not shown). After the demonstration that a subpopulation of splenic CD4⁺ T cells expressed the eotaxin receptor CCR3, we next examined α CD3/ α CD28-stimulated cytokine production by CD4⁺ T-cell splenocytes from WT mice after incubation with increasing doses of eotaxin. Twenty-four-hour preincubation of splenocytes with eotaxin (0.01 or 0.1 ng/mL) induced a significant increase in IL-13 production by CD4⁺ T cells after polyclonal activation. IL-13 release by CD4⁺ T cells after 0.01 and 0.1 ng/mL eotaxin exposure increased by ~ 4 - and 9-fold, respectively. These studies suggest that eotaxin can enhance IL-13 production by CD4⁺ T cells.

Discussion

Patients with eosinophilic inflammation of the GI tract and nonasthmatic patients with various manifestations of food allergy have abnormal respiratory function, including increased frequency of asymptomatic BHR.^{38–40} We have developed model systems

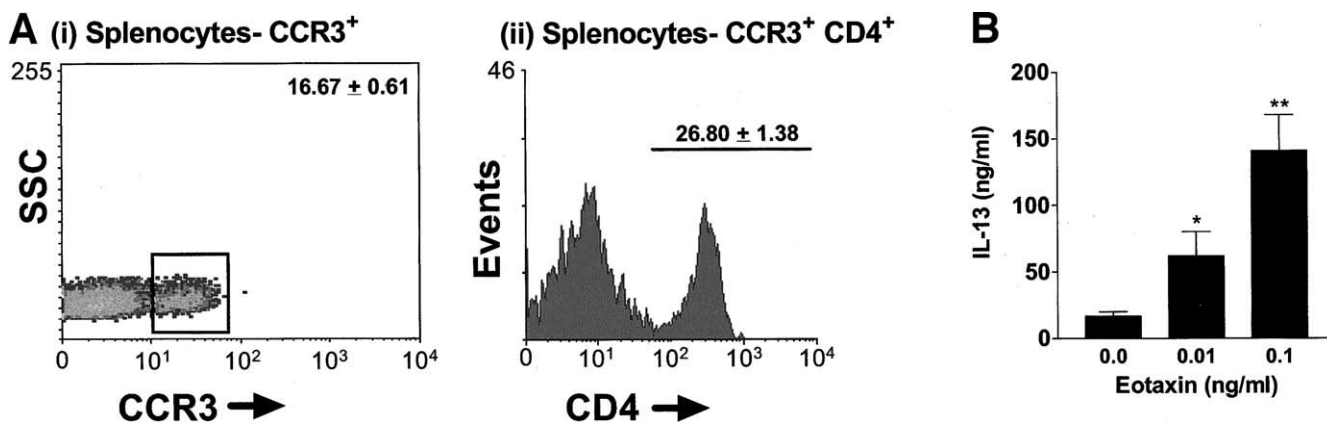


Figure 9. CCR3 Expression on CD4⁺ T cells and the effects of eotaxin exposure on polyclonal T-cell activation. (A) Representative contour plot of (i) CCR3 expression and (ii) CD4 expression on CCR3⁺ splenic cells. (B) IL-13 levels in supernatants from α CD3/ α CD28-stimulated splenocytes treated with eotaxin 0.01 and 0.1 ng/mL. Splenocytes from WT mice were stained with APC-conjugated anti-CD4 and Alexa-488-conjugated anti-CCR3. (ii) Cells were gated on CCR3⁺ expression, and CD4 expression was examined. The percentage of (i) CCR3⁺ splenocytes and (ii) CD4⁺ CCR3⁺ splenocytes represents the mean percentage of positive cells per total cells $\pm$ SEM (where $n = 6$ per group). (B) Data are expressed as the mean $\pm$ SEM level of IL-13 from triplicate cultures from duplicate experiments. The statistical significance of differences ($P < 0.05$) was determined with the Student unpaired t test. Significant differences (* $P < 0.05$; ** $P < 0.01$) are shown as compared with eotaxin 0.0 ng/mL.

to identify the cellular and molecular mechanisms involved in EGID-associated BHR. We showed that oral antigen-induced EGID, characterized by eosinophilic inflammation, leads to the development of BHR. This extraintestinal manifestation of disease is dependent on the increased expression of eotaxin and the recruitment of eosinophils to the GI tract. In Tg mice, we showed that ectopic expression of eotaxin in the GI tract is sufficient to promote GI eosinophilia and induce BHR. BHR in these mice was associated with increased production of Th₂-type cytokines (IL-4, IL-5, and IL-13) and increased production of Th₂-type antibodies (IgG1). BHR was further shown to be dependent on the presence of CD4⁺ T cells and the production of IL-13. In vitro stimulation of WT CD4⁺ T cells in the presence of eotaxin promotes increased IL-13 production. These data suggest that increased levels of eotaxin and eosinophils in the GI tract can predispose to a systemic Th₂-type immunity and alter bronchial reactivity. It is important to note that these studies suggest that allergic inflammation of the GI tract may alter bronchial reactivity in an eotaxin/eosinophil-dependent manner.

BHR is a hallmark pathophysiological manifestation of allergic asthma, and clinical and experimental investigations have provided corroborative evidence suggesting that CD4⁺ T cell-derived IL-4, IL-5, and IL-13 regulate this process.⁴¹ In particular, CD4⁺ T cell-derived IL-13 has been shown to directly induce BHR.^{42,43} Administration of recombinant IL-13 to the airways induces BHR, and adoptive transfer of CD4⁺ IL-13-secreting T cells into IL-13-deficient mice can also reconstitute antigen-induced BHR.^{32,33,42} It is currently believed that IL-13-induced BHR is mediated by direct interaction of IL-13 on airway smooth muscle, because IL-13 receptors have been identified on bronchial smooth muscle cells.⁴⁴ In this study, by using an in vivo model of EGID and Tg mice (IFABPp-eotaxin Tg and IFABPp-eotaxin Tg IL-13-deficient mice), we showed that CD4⁺ T cells and IL-13 critically regulate EGID-associated BHR.

Although we showed an important role for CD4⁺ T cells and IL-13 in EGID-associated BHR, the temporal and spatial localization of CD4⁺ T cells that mediate this process requires further investigation. It is possible that CD4⁺ T cell-mediated BHR is primarily caused by localization of IL-13-producing CD4⁺ T cells throughout the pulmonary compartment during immune surveillance. Increased levels of T cell-derived IL-13 in the airways could thus lead to the development of BHR. However, this is unlikely because we did not observe any

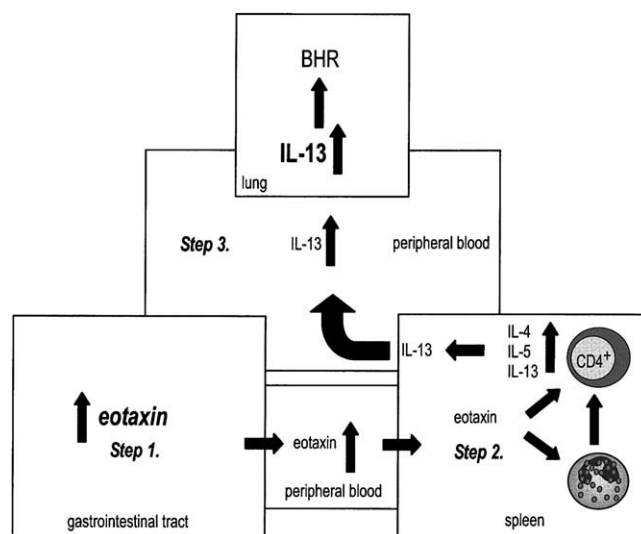


Figure 10. Schematic overview of the proposed mechanism of EGID-associated BHR. (1) Increased levels of eotaxin in the gastrointestinal compartment lead to increased systemic eotaxin. (2) Eotaxin enhances IL-13 synthesis by splenic CD4⁺ T cells, possibly via eosinophils. (3) Increased levels of CD4⁺ T cell-derived IL-13 in the periphery and pulmonary compartment predispose to BHR.

cellular inflammation (lymphocytic infiltrate) in the airways of these animals and, importantly, because BHR occurred independently of classic features of CD4⁺ Th₂ activation in the pulmonary compartment, such as mucus hypersecretion and eosinophilic infiltrate. Alternatively, increased production of IL-13 by CD4⁺ T cells localized within distant immune organs (spleen) could lead to an aberrant systemic increase in IL-13, which predisposes to BHR (Figure 10). In support of the latter concept, we showed that CD4⁺ T cells from the spleen of IFABPp-eotaxin Tg mice produced increased levels of IL-13 after polyclonal activation and that adoptive transfer of splenocytes from IFABPp-eotaxin Tg mice was sufficient to promote BHR. Furthermore, we showed that the increased production of IL-13 was associated with increased levels of this cytokine in the periphery and pulmonary compartment of naive IFABPp-eotaxin Tg mice. Collectively, these findings suggest that BHR in EGID is not a result of CD4⁺ T cell-mediated inflammation in the airways but is rather a consequence of the extrapulmonary Th₂ inflammatory response (Figure 10). It is interesting to note that the development of BHR in patients with EGID can also occur in the absence of symptoms of respiratory disease or pulmonary inflammation.^{39,45,46}

GI disease-associated BHR in IFABPp-eotaxin Tg mice was associated with increased levels of eotaxin/eosinophils and heightened Th₂ immunity, suggesting that eotaxin/eosinophils in the GI tract may be important in the development of Th₂ immune responses. In-

deed, we showed in vitro that polyclonal activation of splenocytes in the presence of eotaxin promotes enhanced IL-13 production. Recent studies support an important role for eotaxin in the regulation of Th₂ immunity and the intrinsic modulation of IL-13 production by CD4⁺ T cells.⁴⁷ In vitro antigen stimulation of CD4⁺ T cells from eotaxin-deficient mice results in impaired production of IL-13.⁴⁷ How eotaxin alters Th₂ development is not yet fully understood. We showed that a small percentage of CD4⁺ cells express the eotaxin receptor, CCR3, and this suggests that eotaxin may directly stimulate IL-13 production in T cells. Previous investigations have shown CCR3 expression on human CD4⁺ T cells and eotaxin-stimulated CD4⁺ T-cell activation.⁴⁸ Alternatively, eotaxin-mediated effects may be indirectly mediated through splenic CCR3⁺ non-T cells. CCR3 is expressed on eosinophils, basophils, mast cells, and CD4⁺ T cells,^{48–50} and we showed that >90% of CCR3⁺ cells were CD4⁺ cells. There is emerging evidence that eosinophils are an important source of IL-4, which is a critical regulator of the development of Th₂ immune responses.^{51–54} Furthermore, eotaxin/CCR3 signaling has been shown to induce IL-4 release from eosinophils and basophils.^{50,54} Eosinophils store IL-4 as a preformed mediator in eosinophil granules and, on stimulation with eotaxin, rapidly release IL-4 through a vesicular transport-dependent mechanism.^{52,53} Thus, increased levels of eotaxin and increased numbers of eosinophils in defined tissue compartments (such as mucosal surfaces where Th₂ cells dwell), as seen in IFABP-eotaxin Tg mice or mice with oral antigen-induced EGID, may promote Th₂ immunity through increased production of IL-4. We are further investigating whether eotaxin-mediated eosinophil activation may stimulate IL-4 production and promote CD4⁺ Th₂-type cell polarization in the GI tract.

Although our findings suggest that increased levels of eotaxin in the GI tract can predispose to altered Th₂ immunity and BHR, we cannot unequivocally exclude the possibility that chronic overexpression of eotaxin in any tissue can promote a similar phenotype. However, previous studies have shown that overexpression of eotaxin in other mucosal surfaces does not predispose to tissue eosinophilia, Th₂ immunity, and BHR.^{55,56} Chronic expression of eotaxin in the lung by using the human surfactant protein C promoter did not alter baseline levels of eosinophils in the pulmonary compartment.⁵⁵ Furthermore, overexpression of eotaxin in the lung by gene transfer did not induce BHR.⁵⁶ Collectively these data suggest that localized overexpression of eotaxin in the airways alone does not predispose to BHR and altered T-cell responses.

In summary, we showed that EGID predisposes to BHR in an eotaxin-dependent manner. Further, we showed by using a Tg system that ectopic expression of eotaxin in the GI tract induces GI eosinophilia, CD4⁺ T cell-derived Th₂ cytokine production, and enhanced intrinsic BHR. The GI disease-associated BHR was regulated by CD4⁺ T cells and IL-13. Finally, we showed in vitro that treatment of CD4⁺ T cells with eotaxin enhances IL-13 production after polyclonal activation. These observations suggest that increased levels of eotaxin, eosinophils, or both in the GI tract can modulate T-cell immunity and airway function. Furthermore, these findings provide a possible explanation for the extraintestinal manifestations (BHR) observed in some patients with an underlying EGID.

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