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Eosinophils and CCR3 Regulate Interleukin-13 Transgene-Induced Pulmonary Remodeling

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Interleukin (IL)-13 transgene overexpression in the lung induces features of chronic inflammatory lung disorders, including an eosinophil-rich inflammatory cell infiltration, airway hyper-reactivity, and remodeling of the airway (eg, subepithelial fibrosis, goblet cell metaplasia, and smooth muscle hypertrophy and hyperplasia). Here, we aimed to define the role of eosinophils and eosinophil signaling molecules [eg, eotaxins and CC chemokine receptor (CCR) 3] in IL-13-mediated airway disease. To accomplish this, we mated IL-13-inducible lung transgenic mice with mice deficient in eosinophil chemoattractant molecules (eotaxin-1, eotaxin-2, and their receptor CCR3) and with mice genetically deficient in eosinophils (ΔdblGATA). We report that in the absence of eotaxin-2 or CCR3, there was a profound reduction in IL-13-induced eosinophil recruitment into the lung lumen. In contrast, in the absence of eotaxin-1, there was a fourfold increase in IL-13-mediated eosinophil recruitment into the airway. IL-13 transgenic mice deficient in CCR3 had a 98% reduction in lung eosinophils. Furthermore, the reduction in pulmonary eosinophils correlated with attenuation in IL-13-induced mucus cell metaplasia and collagen deposition. Mechanistic analysis identified alterations in pulmonary protease and transforming growth factor-β_1 expression in eosinophil-deficient mice. Taken together, these data definitively identify a functional contribution by eosinophils on the effects of chronic IL-13 expression in the lung. ***(Am J Pathol 2006, 169:2117–2126; DOI: 10.2353/ajpath.2006.060617)***

Asthma, one of the most common serious chronic diseases of childhood, is an inflammatory lung disease characterized by airway wall remodeling and reduced respiratory function. The chronic inflammatory process, with an airway infiltrate composed primarily of $CD4^+$ lymphocytes and eosinophils, contributes to airway remodeling, defined as altered lung structural changes.[1] These structural changes in the airway include mucus cell metaplasia and increased deposition of collagen, proteoglycans, and other matrix proteins in the subepithelial layer.[2] Importantly, these structural changes are implicated, at least partially, in the clinical manifestations of asthma.[1,3,4]

Animal studies have defined a central effector role for interleukin (IL)-13 in many pathological features of experimental asthma.[5–12] Pulmonary overexpression of IL-13 results in inflammation, airway fibrosis, mucus metaplasia, airway hyper-responsiveness, and enhanced lung volumes and compliance.[6,13] IL-13 receptors are expressed by a number of structural cells in the lung, including epithelial and smooth muscle cells, suggesting that direct signaling in these cells may be largely responsible for lung remodeling.[14] However, IL-13 also induces a profound inflammatory infiltrate composed of numerous cell types, especially eosinophils; it has been proposed that IL-13-induced chemokine and protease expression and activity are at least partially responsible for cellular recruitment.[15,16] IL-13 is known to be a potent stimulator of an array of chemokines, including the eotaxins (CCL11, CCL24, and CCL26), eosinophil-specific chemoattractants.[15,17] The eotaxins act locally in tissues to promote eosinophil accumulation by enhancing and orchestrating migration to the site of inflammation by stimulating chemotaxis.[18,19] Whereas chemokines are notorious for stimulating several receptors, the eotaxins are unusual in that they signal through a single chemokine receptor CC chemokine receptor (CCR) 3, a receptor abundantly expressed on eosinophils.[20,21] In the mouse system, CCR3 expression is almost exclusively detected on eosinophils.[22,23] Importantly, recent studies have demonstrated that CCR3 disruption impaired eosin-

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ophil recruitment in acute models of experimental asthma.[24–26] In addition to regulating eosinophil trafficking into mucosal tissues, the eotaxins activate the respiratory burst apparatus, induce degranulation, and up-regulate adhesion molecule expression.[27–29] Activated eosinophils have been shown to be a source of several molecules implicated in tissue remodeling processes, including IL-13-induced transforming growth factor (TGF)-β_1.[30,31] Prior studies have shown that IL-13-induced eosinophil recruitment in the lung airway and lung tissue in an acute model are regulated by eotaxin-2 and eotaxin-1, respectively,[32] but the role of these molecules in regulating other aspects of IL-13-induced pathology has not been addressed.

In this study, our aim was to elucidate the role of eosinophils in chronic IL-13-mediated lung pathology. In contrast to recent studies using genetically engineered eosinophil-deficient mouse strains that have primarily focused on examining the role of eosinophils in allergen-induced allergic lung disease,[33,34] we aimed to test the role of eosinophils under conditions of a strong chronic model of experimental asthma elicited by overexpression of a IL-13 transgene in the lung. Although transgenic systems produce relatively high levels of cytokines compared with physiological concentrations, they may more closely model the human condition, which typically involves ongoing exposure to triggers (such as viral infections or perennial allergens). As such, we examined several independent genetically altered mouse lines with defects in eosinophil development or recruitment aiming to identify pathways downstream from eosinophils. Specifically, we used multiple independent genetic approaches by generating an inducible transgenic system that targets expression of IL-13 to the lung in wild-type mice and mice deficient in eosinophils (ΔdblGATA), eotaxin-1, eotaxin-2, and CCR3. Herein, we report the consequences of these genetic engineering events on the outcome of chronic IL-13-mediated pulmonary disease. The data to be presented support a central regulatory role for eosinophils and the eotaxin/CCR3 axis in the development of multiple aspects of lung pathology resulting from chronic IL-13 exposure, including a marked effect on the development of airway remodeling. As such, these results draw attention to the therapeutic potential of anti-eosinophil-directed therapeutics.

Materials and Methods

Inducible IL-13 Lung Transgenic Mice

Bitransgenic mice (CC10-iIL-13) were generated in which IL-13 was expressed in a lung-specific manner that allowed for external regulation of transgene expression, as previously described.[35] CC10-iIL-13 mice deficient in eosinophils, CCR3, eotaxin-1, and eotaxin-2 were generated by breeding the CC10-iIL-13 (FVB/n) with the ΔdblGATA (BALB/c), CCR3 (BALB/c), eotaxin-1 (SVEV), and eotaxin-2 (SVEV) gene-targeted mice for three generations. CCR3 gene-targeted (CCR3KO) and ΔdblGATA (dbl-Gata) mice were generously provided by Drs. Alison Humbles and Craig Gerard (Children's Hospital, Boston, MA). For all experiments, wild-type mice with the appropriate mixed backgrounds were used as controls. Transgene expression was induced by feeding bitransgenic mice doxycycline-impregnated (dox) food (625 mg/kg; Purina Mills, Richmond, IN). Animals were housed under pathogen-free conditions in accordance with institutional guidelines.

Bronchoalveolar Lavage Fluid Collection and Analysis

Mice were sacrificed by an intraperitoneal injection of Ketaject (ketamine hydrochloride; 0.2 mg/kg; Phoenix Pharmaceuticals, St. Joseph, MO). A midline neck incision was made, and the trachea was cannulated. The lungs were lavaged two times with 1.0 ml of lavage buffer [phosphate-buffered saline (PBS) containing 1% fetal calf serum]. The recovered bronchoalveolar lavage fluid (BALF) was centrifuged at 400 $\times$ *g* for 5 minutes at 4°C and resuspended in 200 μl of lavage buffer. Lysis of red blood cells was performed using RBC lysis buffer (Sigma, St. Louis, MO) according to the manufacturer's recommendations. Total cell numbers were counted with a hemacytometer. Cytospin preparations of 1×10^5 cells were stained with the Hema 3 Staining System (Fisher Diagnostics, Middletown, VA), and differential cell counts were determined.

Northern Blot Analysis

RNA was electrophoresed in an agarose-formaldehyde gel, transferred to Gene Screen transfer membranes (NEN, Boston, MA) in 10$\times$ sodium chloride and sodium citrate, and cross-linked by UV radiation. The cDNA probes, generated by polymerase chain reaction (PCR) or from commercially available vectors [I.M.A.G.E. Consortium obtained from American Tissue Culture Collection (Rockville, MD) or Incyte Genomics (Palo Alto, CA)], were sequence confirmed, radiolabeled with ^{32}P, and hybridized using standard conditions.

Eosinophil Quantitation

Lung tissue eosinophils were identified by anti-major basic protein (MBP) staining. The lungs were inflation-fixed in 10% neutral buffered formalin at 25 cm H_2O, embedded in paraffin, cut into 5-μm sections, and fixed to positively charged slides. Endogenous peroxidase in the tissues was quenched with 0.5% hydrogen peroxide in methanol. Lung sections were digested (10 minutes, 37°C) with pepsin (Zymed, San Francisco, CA) and blocked by incubation at room temperature in 3% normal goat serum in PBS for 2 hours. The blocked sections were treated with rabbit anti-mouse MBP at 1:10,000 dilution (a kind gift of James and Nancy Lee, Mayo Clinic, Scottsdale, AZ) in 3% normal goat serum/PBS overnight at 4°C. The slides were subsequently washed free of primary antibody with several changes of PBS, followed by

Table 1. Primer Sequences and LightCycler Conditions for Pulmonary Proteases

Protease	Forward primer	Reverse primer	Product size (bp)	Annealing temperature (°C)
Cathepsin B	5′-GGCTGGACGCAACTTCTACA-3′	5′-CAGAAGCTCCATGCTCCAGA-3′	360	60
Cathepsin K	5′-TCTGCTGCTACCCATGGTGA-3′	5′-TGGCCCACATATGGGTAAGC-3′	600	60
Cathepsin S	5′-CAATGGAGCAACTGCAGAGA-3′	5′-CCAACAGGCACCACAAGAAC-3′	380	58
MMP-12	5′-CAGCAGTTCTTTGGGCTAGA-3′	5′-TGTGCTGGGGTTAAGGTATC-3′	540	58
MMP-13	5′-CTTCTGGCACACGCTTTTCCTC-3′	5′-CGCAGCGCTCAGTCTCTTCACC-3′	605	66
MMP-19	5′-TCTTACTTCCCATGACAGTC-3′	5′-CCATCAAAGGTATTGGAGCA-3′	490	56
Adam-8	5′-ACCTCCTTTGCCCCATGTGA-3′	5′-GGTGCTAATACTGGCAGCTG-3′	310	60

incubation with biotinylated goat anti-rabbit IgG (1:250 dilution) and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 2 hours and 45 minutes, respectively. These slides were developed with nickel diaminobenzidine-cobalt chloride solution to form a black precipitate and counterstained with nuclear fast red. Replacing the primary antibody with normal rabbit serum ablated the immunostaining. Quantification of immunoreactive cells was performed by counting the positively stained cells under low-power magnification of longitudinal sections, and eosinophil levels are expressed as the number of eosinophils per square millimeter.

Enzyme-Linked Immunosorbent Assay Measurements

Cytokine levels were measured in the BALF using enzyme-linked immunosorbent assay kits specific for murine IL-13, eotaxin-1, and eotaxin-2 (R&D Systems, Minneapolis, MN) and TGF-β_1 (Promega Corporation, Madison, WI). Detection limits for the enzyme-linked immunosorbent assays were 8, 10, 12, and 15 pg/ml for IL-13, eotaxin-1, eotaxin-2, and TGF-β_1, respectively.

Lung Histopathological Changes

Mice were sacrificed by an intraperitoneal injection of Ketaject (ketamine hydrochloride; 0.2 mg/kg; Phoenix Pharmaceuticals). Lungs were inflation fixed with 10% neutral-buffered formalin at 25 cm H_2O and immersed in the same fixative. The inflated lungs were embedded in paraffin and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or Masson's trichrome stain. PAS-stained airway goblet cells were enumerated by light microscopy examination (magnification, ×400). To quantitate the level of mucus expression in the airway, the number of PAS^+ and total epithelial cells in individual bronchioles was counted. At least three medium-sized bronchioles (defined by having approximately 90 to 150 luminal airway epithelial cells) were evaluated in each slide. Results are expressed as the percentage of PAS^+ cells per bronchiole, which is calculated from the number of PAS^+ epithelial cells per bronchiole divided by the total number of epithelial cells in each bronchiole. Quantification of collagen was performed by morphometric analysis using the Metamorph Imaging System (Universal Imaging Corporation, West Chester, PA). The lung sections were taken from the same position in each set of mice, and at least three to four random sections/mouse were analyzed. Using digital image capture, collagen-stained areas surrounding large airways were quantified (square micrometers) and normalized to length of basement membrane (micrometers).

Protease Expression by Real-Time PCR Analysis

RNA samples were subject to reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Genes of interest were quantitated by real-time PCR using the LightCycler instrument in conjunction with the ready-to-use LightCycler FastStart DNA Master SYBR Green I reaction mix (Roche Diagnostics, Indianapolis, IN). Primer pairs and annealing temperatures for each gene of interest can be found in Table 1. Results (picograms of cDNA) were normalized to glyceraldehyde-3-phosphate dehydrogenase (picograms of cDNA) amplified from the same cDNA mix.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical significance comparing different sets of mice was determined by Student's *t*-test.

Results

Effect of IL-13 on Eotaxin and CCR3 Expression

We initially assessed the levels of the eosinophil-specific chemokines, eotaxin-1 and eotaxin-2, and CCR3 in an inducible transgenic system that targets expression of IL-13 to the lung. At 6 weeks of age, CC10-iIL-13 mice were fed normal food pellets or food pellets impregnated with doxycycline (dox). After 4 weeks, markedly increased levels of eotaxin-1, eotaxin-2, and CCR3 mRNA were detectable by Northern blot analysis in the lungs of CC10-iIL-13 mice fed dox food compared with control double transgenic mice fed normal food (Figure 1A). Eotaxin-1 and eotaxin-2 protein levels were also strikingly elevated (16- and 277-fold, respectively) in the BALF from dox-fed CC10-iIL-13 mice ($P \leq 0.001$; Figure 1B).

To identify the role of eosinophils and CCR3 in IL-13-induced inflammation, we generated CC10-iIL-13 mice

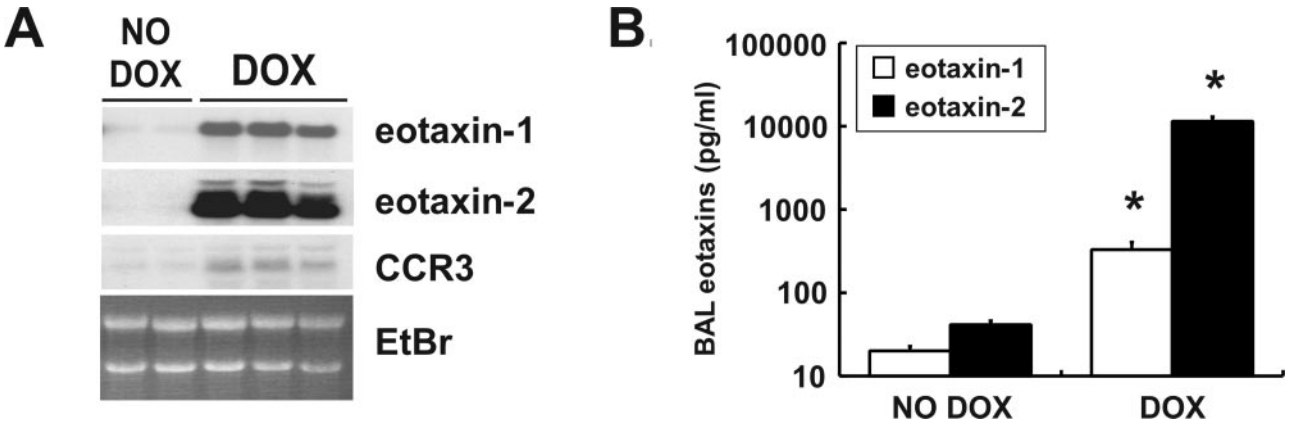

Figure 1. Expression of CCR3 and the eotaxin chemokines is induced by lung IL-13 transgenic overexpression. **A:** Northern blot analysis of total lung RNA from CC10-iIL13 mice that were fed normal (NO DOX) or dox-impregnated (DOX) food for 4 weeks reveals IL-13-induced expression of eotaxin-1, eotaxin-2, and CCR3. Each lane represents a separate mouse. Ethidium bromide (EtBr)-stained gel is also shown. **B:** The levels of eotaxin-1 and eotaxin-2 protein were assessed in the BALF of CC10-iIL13 mice that were fed normal (NO DOX) or dox-impregnated (DOX) food for 4 weeks. Protein levels represent the mean ± SEM of six to seven mice per group (derived from two experiments). $^*P < 0.001$.

with a severe defect in eosinophil development (ΔdblGATA) and mice that were gene-targeted for CCR3, eotaxin-1, or eotaxin-2. We first compared protein levels of IL-13 and the eotaxin chemokines in lungs from wild-type double-transgenic mice and double-transgenic mice that are deficient in eosinophils, eotaxin-1, eotaxin-2, or CCR3. After 4 weeks on dox food, BALF IL-13 protein levels increased dramatically in wild-type CC10-iIL13 mice (from 0.18 ± 0.02 to 112 ± 21 ng/ml, $P \leq 0.001$, $n = 5$ to 8 mice/group). No significant differences in IL-13 protein levels were detected in the gene-altered CC10-iIL-13 mice compared with their wild-type control mice (data not shown). Thus, expression of IL-13 was not attributed to the eotaxins and/or eosinophils, consistent with the transgene being expressed in respiratory Clara cells.[33,34] Lung protein levels of the eotaxin chemokines were also examined. Similar levels of IL-13-induced eotaxin-1 and eotaxin-2 proteins were detected in the CCR3 and the eotaxin gene-targeted mice with no difference in either eotaxin chemokine accumulation between the gene-targeted mice and their wild-type control mice, demonstrating that there is no compensatory overexpression of the remaining eotaxin chemokine in either of the gene-targeted mice (data not shown).

Effect of CCR3 and Its Ligands on IL-13-Induced Airway Eosinophilia

IL-13-induced BALF eosinophilia was profoundly inhibited (84 ± 1.7%, $n = 2$ experiments, with 3 to 4 mice/group/experiment, $P = 0.03$) in the absence of eotaxin-2 (Figure 2A). However, eotaxin-1 deficiency resulted in an increase (fourfold, $P = 0.002$) in IL-13-induced airway eosinophilia when compared with wild-type controls (23.7 ± 4.3 versus $7 \pm 1.7 \times 10^4$ eosinophils, respectively; Figure 2B). Notably, IL-13-induced airway eosinophilia was markedly reduced in CCR3-deficient mice (95 ± 3%, $n = 3$ experiments, with 4 to 5 mice/group/experiment, $P \leq 0.007$) and absent in the Δdbl-GATA mice when compared with wild-type controls (Figure 2C; data not shown).

Effect of CCR3 and Its Ligands on IL-13-Induced Lung Tissue Eosinophilia

Pulmonary tissue accumulation of eosinophils is a prominent feature of IL-13-induced inflammation; we therefore examined the effect of eotaxin-1, eotaxin-2, and CCR3 deficiencies on eosinophil infiltration into the lung tissue. IL-13 expression resulted in a marked increase in peribronchial eosinophils in wild-type mice (Figure 2, D–F). However, there was a dramatic reduction (98%) in eosinophil accumulation in peribronchial lung tissue in CCR3-deficient mice when compared with wild-type mice [7.1 ± 3.4 versus 338 ± 170 eosinophils/mm^2 (mean ± SEM), $n = 7$ to 8 mice/group, $P = 0.05$; Figure 2D]. There was a modest (42%) but significant reduction in IL-13-induced eosinophil accumulation surrounding the airway of eotaxin-1-deficient mice [275 ± 62 versus 474 ± 64 eosinophils/mm^2 (mean ± SEM), $n = 7$ to 10 mice/group, $P = 0.05$; Figure 2E]. Notably, eotaxin-2 deficiency had no effect on IL-13-induced peribronchial eosinophil recruitment when compared with wild-type mice (474 ± 64 versus 473 ± 177 eosinophils/mm^2; Figure 2E). Examination of Δdbl-GATA mice revealed lung tissue devoid of eosinophils, as measured by the absence of MBP$^+$ cells (Figure 2F; data not shown).

Effect of Eosinophils and CCR3 on IL-13-Induced Airway Inflammation

Total BALF cells recovered from the airways of CC10-iIL13 wild-type mice after 4 weeks of transgene expression were significantly increased (ninefold) compared with controls (Figure 3). Cell recruitment into the airway in response to IL-13 transgene expression was impaired in the absence of CCR3 expression or eosinophils (Figure 3). The reduction in total inflammatory cell accumulation suggested a role, either directly or indirectly, for eosinophils and CCR3 signal transduction in chemoattraction of leukocytes other than eosinophils in IL-13-induced airway disease. As such, we next examined the effect of eosin-

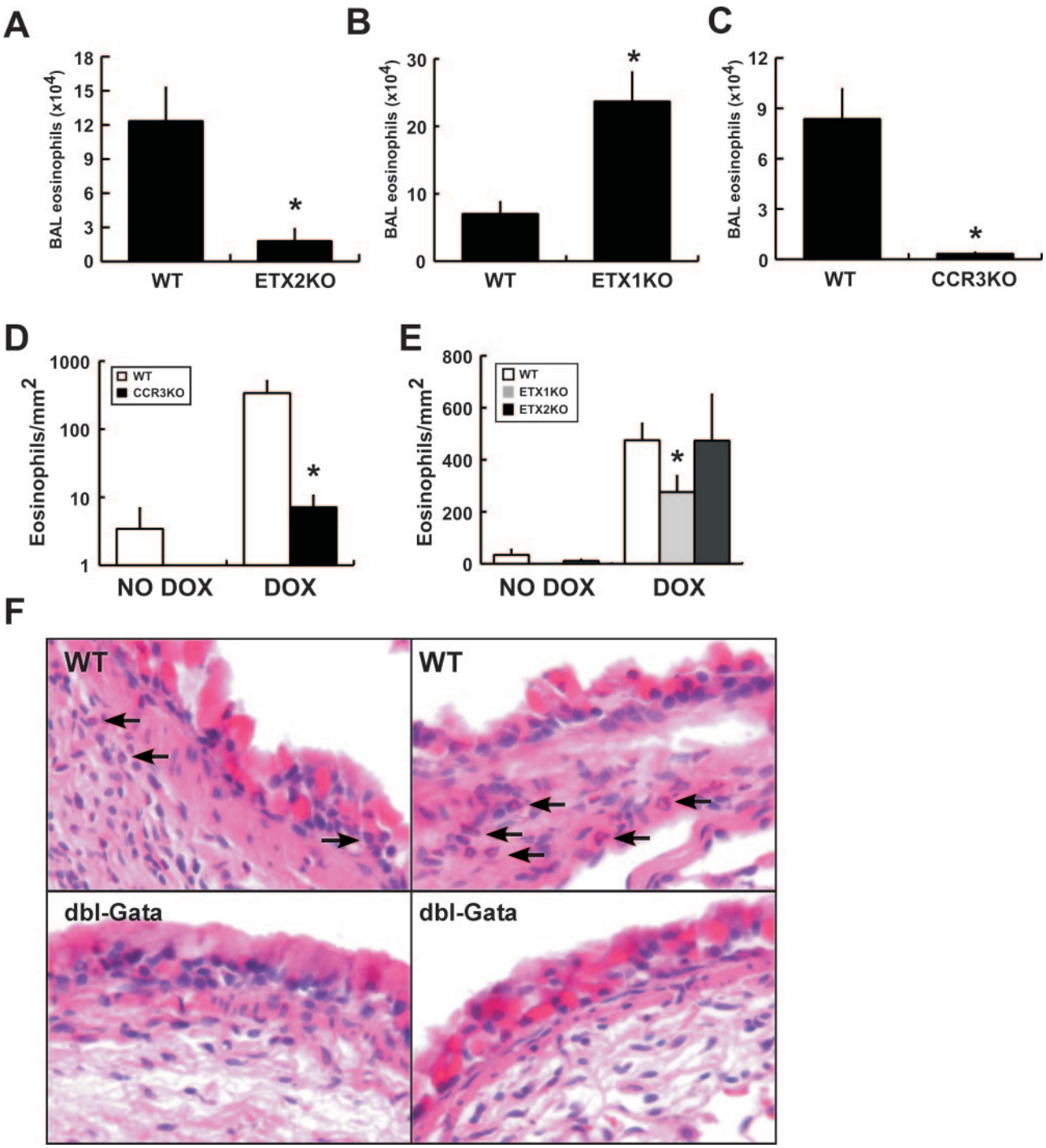

Figure 2. IL-13 expression results in airway eosinophilia that is eotaxin-2 and CCR3-dependent and tissue eosinophilia that is CCR3 and eotaxin-1-dependent. Eosinophils in BALF from wild-type (WT) and eotaxin-2-deficient (ETX2KO; **A**), eotaxin-1-deficient (ETX1KO; **B**), or CCR3-deficient (CCR3KO; **C**) CC10-iIL13 mice fed dox food for 4 weeks. A representative experiment is shown (mean ± SEM, $n = 3$ with three to six mice per group per experiment). $^*P = 0.03$. Quantitation of eosinophil infiltration, as detected by anti-MBP immunohistochemistry, in the lungs in wild-type (WT, **white bars**) and CCR3- (CCR3KO, **black bars; D**), eotaxin-1- (ETX1KO, **gray bars E**), and eotaxin-2-deficient (ETX2KO, **black bars**) mice is shown before (NO DOX) and after (DOX) 4 weeks on dox food. Data are presented as mean ± SEM ($n = 4$ to 10 mice per group). $^*P \leq 0.05$. **F:** H&E-stained lungs from wild-type (WT) and ΔdblGATA (dbl-Gata) mice fed dox food for 4 weeks are shown. **Arrow**, eosinophils.

ophil and CCR3 deficiency on IL-13-induced chemoattraction of other cell types. Neutrophil and lymphocyte accumulation in the airway in response to IL-13 transgene expression was unchanged in the CCR3KO or ΔdblGATA mice when compared with wild-type control mice (data not shown). However, airway macrophage accumulation after 4 weeks of transgene expression was significantly reduced in both the CCR3KO and the ΔdblGATA mice when compared with the wild-type control mice ($3.3 \pm 0.6 \times 10^5$ wild-type versus $1.8 \pm 0.3 \times 10^5$ CCR3KO [$P = 0.02$] and $2.1 \pm 0.4 \times 10^5$ ΔdblGATA [$P = 0.05$] BAL macrophages, $n = 3$ experiments).

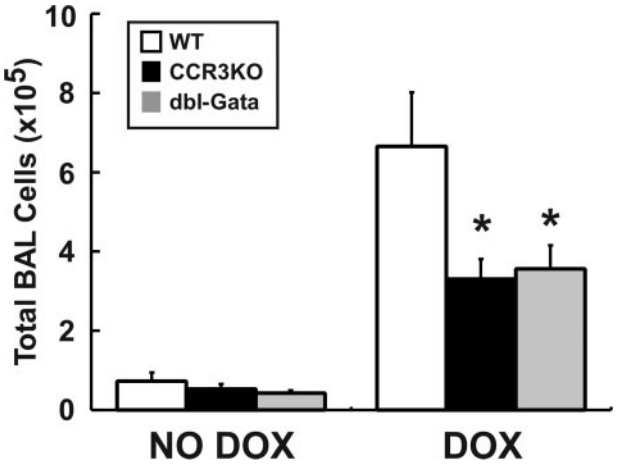

Figure 3. IL-13 transgene expression results in leukocyte recruitment that is partially regulated by eosinophils and CCR3. IL-13 transgene-induced total leukocyte accumulation (mean ± SEM) in the airways of wild-type (WT, **white bars**), CCR3KO (**black bars**), and ΔdbI-GATA (dbl-Gata, **gray bars**) mice; $n = 3$ experiments. $^*P \leq 0.05$ when compared with wild-type control mice.

Effect of Eosinophils and CCR3 on IL-13-Induced Mucus Production

We next examined the role of eosinophils and CCR3 on IL-13-induced mucus production. Examination of PAS^+ cells in the bronchial epithelium of CCR3-deficient mice revealed a significant decrease (40 ± 5%, $n = 3$ experiments, $P < 0.001$) in mucus production after 4 weeks of IL-13 transgene expression compared with wild-type control mice (Figure 4, A and B). Notably, a marked reduction in PAS^+ epithelium was also observed in the ΔdbI-GATA mice (37 ± 9%, $n = 3$ experiments; Figure 4, A and B).

Effect of Eosinophils and CCR3 on IL-13-Induced Collagen Deposition

To examine the effect of eosinophil and CCR3 deficiency on IL-13-induced lung fibrosis, we quantitated the area of collagen deposition surrounding large airways in the lungs of CC10-iIL13 wild-type, CCR3KO, and ΔdbI-GATA mice. Four weeks of IL-13 transgene expression resulted in a threefold increase in area of collagen deposition surrounding large airways in the lungs of CC10-iIL13 wild-type mice (from 26.2 ± 8.5 to 77.9 ± 13 $\mu m^2/\mu m$ basement membrane). The absence of CCR3 or eosinophils resulted in a 47 to 50% reduction in IL-13-induced collagen deposited around large airways (Figure 5, A and B). The fibrogenic effects of IL-13 have been proposed to be mediated by TGF-β_1; eosinophils, along with macrophages and epithelial cells, are sources of TGF-β_1.[31] As such, we next determined whether protein levels of active BAL TGF-β_1 were altered in the absence of CCR3 or eosinophils. CCR3 deficiency had no effect on the protein levels of IL-13-induced active BAL TGF-β_1 compared with wild-type controls (Figure 5C). In contrast, eosinophil deficiency resulted in a significantly reduced accumulation of active TGF-β_1 in the BALF of ΔdbI-GATA mice (Figure 5C).

Effect of Eosinophils and CCR3 on IL-13-Induced Protease Expression

Previous studies have shown that IL-13 induces the expression of a number of proteases that contribute to IL-13-induced inflammation and lung remodeling.[6,16] We were next interested in the contribution of eosinophils to IL-13-induced protease expression. The levels of mRNA encoding cathepsin B, cathepsin K, cathepsin S, matrix metalloproteinase (MMP)-12, MMP-13, MMP-19, and Adam-8 were increased after 4 weeks of IL-13 expression (Table 2). Eosinophil deficiency resulted in significantly decreased levels of mRNA encoding for cathepsin B, cathepsin S, and MMP-13 (Table 2).

Discussion

We generated an inducible transgenic system that targets IL-13 expression to the lungs of wild-type mice and mice deficient in eosinophils, CCR3, eotaxin-1, or eotaxin-2. First, we demonstrated that airway eosinophilia induced by IL-13 is CCR3- and eotaxin-2-dependent. Second, we established a role for eotaxin-1 in negatively regulating airway eosinophil accumulation, as demonstrated by an increase in eosinophils in the lung lumen in the absence of eotaxin-1. Third, we established that IL-13-induced lung tissue eosinophilia is CCR3-dependent

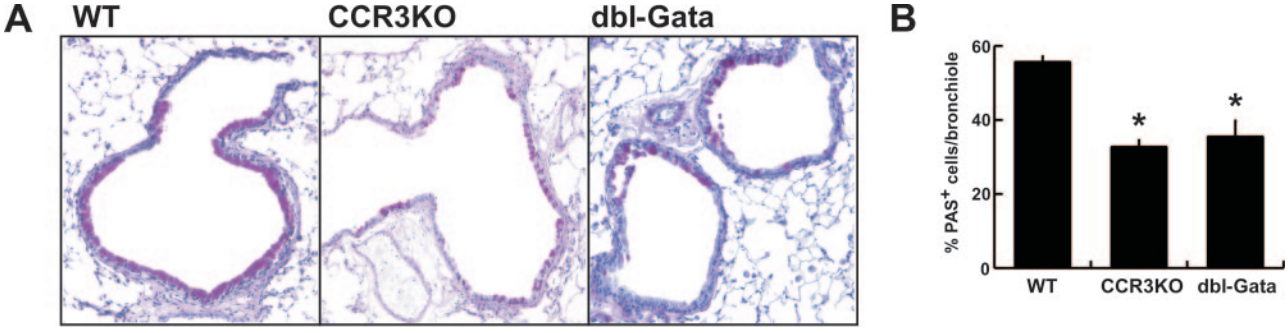

Figure 4. IL-13 transgene expression results in mucus cell metaplasia that is partially eosinophil and CCR3-dependent. PAS staining for mucus (**A**) and quantitation of PAS^+ cells (mean ± SEM; **B**) in airways of CC10-iIL13 wild-type (WT), CCR3-deficient (CCR3KO), and ΔdbI-GATA (dbl-Gata) mice after 4 weeks on dox food; $n = 3$ experiments with four to six mice per group per experiment. $^*P \leq 0.001$ when compared with wild-type control mice.

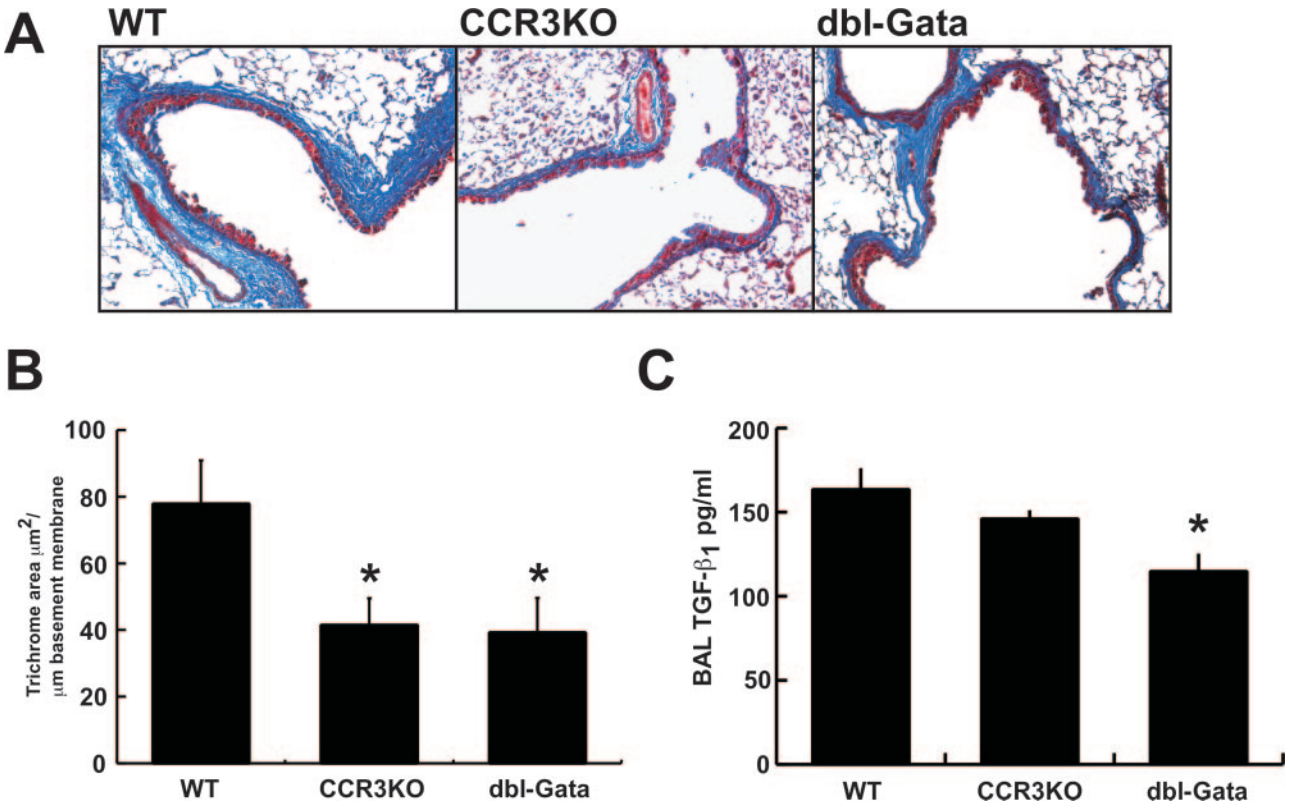

Figure 5. IL-13 transgene expression results in collagen deposition that is partially eosinophil and CCR3-dependent. **A:** Collagen deposition as shown in Masson's trichrome-stained lungs from wild-type (WT), CCR3-deficient (CCR3KO), and ΔdblGATA (dbl-Gata) CC10-iIL13 mice after being fed dox food for 4 weeks. Each panel is an individual mouse. Magnification, ×100. **B:** Quantitation (mean ± SEM) of area of collagen staining surrounding airways of CC10-iIL13 wild-type (WT), CCR3-deficient (CCR3KO), and ΔdblGATA (dbl-Gata) mice after 4 weeks on dox food; $n = 2$ to 3 experiments. $^*P \leq 0.04$ when compared with wild-type control mice. **C:** TGF-β_1 protein levels (mean ± SEM) were measured in the BALF of CC10-iIL13 wild-type (WT), CCR3-deficient (CCR3KO), and ΔdblGATA (dbl-Gata) mice after 4 weeks on dox food; $n = 8$ to 14 mice/group (derived from two to three experiments). $^*P \leq 0.02$ when compared with wild-type control mice.

and partially eotaxin-1-dependent. Fourth, we reveal a role for eosinophils and CCR3 in amplifying IL-13-induced mucus and collagen production. Finally, we identified a contributory role for eosinophils in regulating IL-13-induced TGF-β_1 and protease expression.

IL-13-induced inflammation has been shown to be regulated by a number of different mediators, including chemokines. Signal transduction through CCR1 and CCR2 is important in IL-13 effector pathways because deficiency in these cytokine receptors resulted in decreased IL-13-induced pulmonary inflammation associated with macrophages.[15,36] Mechanistic analysis revealed attenuated IL-13-induced chemokine expression in the absence of CCR1 expression.[36] Although IL-13 has been shown to be a potent inducer of the eosinophil chemoattractants, CCL11/eotaxin-1 and CCL24/eotaxin-2, and lung eosinophilia, the contribution of these chemokines, their receptor CCR3, and eosinophils in IL-13-mediated lung inflammation has remained largely unexplored.[15] Our studies addressed this deficiency by demonstrating that chronic IL-13 exposure induces production of CCR3 and its ligands, resulting in eosinophil recruitment, and that these events have a central role in IL-13-mediated lung remodeling *in vivo*. In addition, because eosinophils have been shown to produce IL-13,[37,38] these observations allow for speculation that an IL-13- and CCR3-feedback

Table 2. Quantitative Analysis of Protease Expression (pg × 10^{-3}) in Lung (Normalized to Glyceraldehyde-3-Phosphate Dehydrogenase)

Protease	WT		CCR3KO	dbl-GATA
	No Dox	Dox	Dox	Dox
Cathepsin B	315 ± 121	1470 ± 147	1420 ± 210	688 ± 137†
Cathepsin K	89.3 ± 83.3	1104 ± 148	1542 ± 1076	1006 ± 213
Cathepsin S	50.5 ± 35.8	440 ± 89.6	454 ± 32.9	181 ± 27.1†
MMP-12	0.4 ± 0.5	507 ± 56.5	751 ± 69.6	364 ± 119
MMP-13	60 ± 38	487 ± 54	512 ± 141	163 ± 90†
MMP-19	274 ± 157	4120 ± 1995	9988 ± 9769	2910 ± 429
Adam-8	0.02 ± 0.009	0.3 ± 0.2	0.49 ± 0.15	0.15 ± 0.07

Quantitation of protease mRNA in the lung as determined by LightCycler. Data are presented as means ± SD ($n = 3$ mice per group).
$^\dagger P \leq 0.02$ when compared with wild-type Dox.

loop exists in IL-13-mediated responses. In these responses, IL-13 would induce CCR3 ligand elaboration by resident lung cells and macrophages; this would, in turn, induce tissue eosinophilia and production of eosinophil-derived IL-13. These amplification events could contribute to the intensity and persistence of the inflammatory response in chronic lung diseases such as asthma and chronic obstructive pulmonary disease.

Our current study establishes that IL-13-induced lung eosinophilia is differentially regulated by eotaxin-1 compared with eotaxin-2. In particular, we demonstrate that eotaxin-2 is crucial for IL-13-induced airway eosinophilia. We also demonstrate that eotaxin-1 is more important for lung tissue eosinophilia because peribronchial eosinophil accumulation was reduced in eotaxin-1-deficient mice. Interestingly, there was a significant increase in airway eosinophil accumulation in the absence of eotaxin-1, suggesting that eotaxin-1 may be critical for retention of eosinophils in the lung tissue. This finding is compatible with a prior study from our laboratory in which acute IL-13-induced lung eosinophilia, but not airway eosinophilia, was shown to be dependent on eotaxin-1. It is interesting to note that in that model, the relative level of eosinophils in the lung of eotaxin-1-deficient mice was increased compared with wild-type mice.[32] Our current study describes a mechanism by which airway eosinophilia is regulated by localized expression of the eotaxin chemokines. Notably, CCR3 was required for both airway and peribronchial eosinophilia in our transgenic mice, demonstrating a cooperative mechanism for CCR3 and its ligands in directing the recruitment and localization of pulmonary eosinophils. Further studies are needed to identify the downstream mechanism, such as alteration in adhesion molecules on resident lung cells by the eotaxin chemokines, in the cooperative orchestration of eosinophil localization in the asthmatic lung.[39,40] Because lung tissue eosinophilia was only partially eotaxin-1-dependent and was independent of eotaxin-2 expression, other CCR3 ligands must contribute to the peribronchial eosinophil accumulation. Taken together, these results support an essential role for CCR3 signaling in orchestrating IL-13-mediated leukocyte recruitment and spatial localization into the lung.

Our data suggest that eosinophils and CCR3 have a fundamental role in amplifying IL-13-mediated lung remodeling in a chronic model of cytokine exposure. To identify the causative relationship between eosinophils and the onset of IL-13-induced lung pathologies, we used a mouse line harboring a deletion of a high-affinity double palindromic GATA binding site in the GATA-1 promoter (ΔdblGATA), which led to the specific ablation of the eosinophil lineage.[41] After chronic IL-13 exposure, we did not find MBP^+ cells in the lungs of the ΔdblGATA mice, suggesting that the lungs were devoid of eosinophils, because the granule protein MBP has been shown to be the core of eosinophil granules[42,43]; however, we cannot exclude the possibility of a granule-less eosinophil in these genetically altered mice. In this study, we focused on the role of eosinophils in lung remodeling; future studies are warranted to examine airway hyperreactivity induced in our chronic model. We observed a significant decrease in the percentage of PAS^+ airway epithelial cells in response to chronic IL-13 expression in the CCR3-deficient and the ΔdblGATA mice. Although our data are consistent with prior studies, a recent study using the ΔdblGATA mice in an allergen-induced experimental asthma model showed no reduction in mucus staining with eosinophil deficiency.[33,34,44,45] The differences in experimental protocol (chronic and continuous cytokine exposure versus allergen challenges) may explain the divergent results. Neutrophils have been implicated in IL-13-induced mucin production through activation of epidermal growth factor receptor signaling.[46,47] Our studies offer a parallel example of how myeloid cells regulate mucus production in the lung. We also demonstrate a significant reduction in IL-13-mediated collagen deposition surrounding the large airways in CCR3-deficient and ΔdblGATA mice. TGF-β_1 has been shown to be a critical mediator of the fibrotic effects of IL-13.[31] Although we found significantly reduced levels of active TGF-β_1 in the BAL of ΔdblGATA mice, there were no significant differences between wild-type and CCR3-deficient mice, suggesting that multiple mechanisms, including those that do not use TGB-β_1-induced fibrosis, are operational in IL-13-mediated remodeling the lung. Expression of MMP-13, cathepsin S, and cathepsin B was significantly attenuated in the absence of eosinophils. Apart from digesting components of the ECM, metalloproteinases modulate the activity of other proteases and cytokines, including chemokines and TGF-β_1, which are important in lung diseases.[48] In addition, cathepsin S and MMP-13 have collagenase activity, suggesting an important role for eosinophils in regulating fibrotic tissue responses.[49,50]

Taken together, we provide multiple lines of evidence that eosinophils are prominent effector cells involved with multiple parameters of IL-13-associated diseases, including goblet cell metaplasia and collagen deposition. Our chronic model uses an inducible transgenic system that results in expression of high levels of cytokine in the lung compared with levels that are likely operational in patients; such analyses have been shown to be useful for establishing pathophysiological paradigms and uncovering molecular insight into possible disease pathogenesis, as documented by a variety of similar transgenic systems.[6,31,51–53] As such, our results highlight the need to develop effective clinical reagents (such as CCR3 antagonists substantiated by our present findings) that block or deplete lung eosinophils to test the compelling eosinophil hypothesis in asthma.

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