A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation

Patricia C. Fulkerson*, Christine A. Fischetti†, Melissa L. McBride†, Lynn M. Hassman†, Simon P. Hogan†, and Marc E. Rothenberg†‡

*Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45257-0524; and †Division of Allergy and Immunology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, OH 45229-3039

Communicated by K. Frank Austen, Harvard Medical School, Boston, MA, September 11, 2006 (received for review August 1, 2006)

To clarify the role and regulation of eosinophils, we subjected several key eosinophil-related genetically engineered mice to a chronic model of allergic airway inflammation aiming to identify results that were independent of the genetic targeting strategy. In particular, mice with defects in eosinophil development (Δdbl-GATA) and eosinophil recruitment [mice deficient in CCR3 (CCR3 knockout) and mice deficient in both eotaxin-1 and eotaxin-2 (eotaxin-1/2 double knockout)] were subjected to *Aspergillus fumigatus*-induced allergic airway inflammation. Allergen-induced eosinophil recruitment into the airway was abolished by 98%, 94%, and 99% in eotaxin-1/2 double knockout, CCR3 knockout, and Δdbl-GATA mice, respectively. Importantly, allergen-induced type II T helper lymphocyte cytokine production was impaired in the lungs of eosinophil- and CCR3-deficient mice. The absence of eosinophils correlated with reduction in allergen-induced mucus production. Notably, by using global transcript expression profile analysis, a large subset (29%) of allergen-induced genes was eosinophil- and CCR3-dependent; pathways downstream from eosinophils were identified, including *in situ* activation of coagulation in the lung. In summary, we present multiple lines of independent evidence that eosinophils via CCR3 have a central role in chronic allergic airway disease.

allergy | asthma | chemokine | cytokine

Asthma is a chronic inflammatory disease involving an eosinophil-rich infiltrate that has been linked with lung remodeling (1, 2). Although not completely specific for inhibiting murine eosinophils, blockade or deletion of IL-5 improves multiple markers of lung remodeling associated with reduced levels of eosinophil-derived TGF-β_1 in experimental asthma (3, 4). Furthermore, studies of two distinct strains of mice specifically deficient in eosinophils have suggested that eosinophils are an integral part of experimental asthma, yet they reported different results regarding the contribution of eosinophils (5, 6). Collectively, these results draw attention to the need to identify critical pathways involved in the recruitment and activation of eosinophils in the asthmatic lung, as well as the mechanism by which eosinophils contribute to disease. Early results with two different humanized antibodies against human IL-5 have revealed that this strategy does not effectively deplete tissue eosinophils in the asthmatic lung (7–9). As such, there is a pressing need to develop new approaches to block eosinophils in the asthmatic lung.

Although chemokines are notorious for stimulating several receptors, the eotaxins are unusual in that they signal through a single chemokine receptor CCR3 (now designated CD193), a receptor abundantly expressed on eosinophils (10, 11). Recent studies have demonstrated that CCR3 gene deletion impairs eosinophil recruitment in acute models of experimental asthma (12–14). It remains unknown whether CCR3 is a dominant pathway in chronic models of allergic airway inflammation. The contribution of CCR3 toward other important asthma parameters, including mucus production, has not yet been addressed.

In this study we elucidate the role of eosinophils in experimental allergic airway inflammation in a chronic model induced by repeated intranasal doses of the natural aeroallergen *Aspergillus fumigatus* rather than ovalbumin (OVA). We examined several independent genetically altered mouse lines with defects in eosinophil development or recruitment aiming to identify pathways downstream from eosinophils in multiple systems (5, 12, 14). Our results definitively identify a central role for eosinophils in regulating multiple aspects associated with allergic lung inflammation, draw attention to the therapeutic potential of anti-eosinophil-directed therapeutics, and identify numerous eosinophil-associated pathways that deserve further attention.

Results

CCR3 and Its Ligands Regulate Pulmonary Tissue and Airway Eosinophilia. Allergen-induced bronchoalveolar lavage fluid (BALF) eosinophilia was profoundly inhibited (>94%, $P < 0.0001$) in mice deficient in CCR3 or eotaxin-1/2 and in the Δdbl-GATA mice compared with WT mice (Fig. 1*A*). Allergen challenge resulted in a marked increase in peribronchial and perivascular eosinophils in WT mice (Fig. 1 *B–D*). There was a dramatic reduction in eosinophil accumulation in peribronchial (90 ± 9% and 84 ± 14%; mean ± SD) and perivascular (95 ± 2% and 83 ± 8%) lung tissue in both eotaxin-1/2 double knockout and CCR3 knockout (KO) mice, respectively, compared with allergen-challenged WT mice (Fig. 1 *B–D*). Examination of Δdbl-GATA mice revealed lung tissue essentially devoid of eosinophils, as measured by the absence of major basic protein-positive cells (data not shown). These results demonstrate a critical role for CCR3 and its ligands in allergen-induced pulmonary eosinophilia despite the induction of other chemoattractant mediators. Indeed, cysteinyl leukotrienes were detectable in lung homogenates from both WT and CCR3 KO allergen-challenged mice (175 ± 2.3 and 174 ± 2.1 pg/ml, respectively; $n = 8$ mice per group).

Allergen-Induced Leukocyte Recruitment in Genetically Engineered Mice. Total BALF cells recovered from the airways of allergen-challenged WT mice were significantly increased (≈20-fold) compared with saline controls ($101 \pm 28.8 \times 10^4$ vs. $5.8 \pm 3.6 \times 10^4$, respectively, representative of three experiments). Cell recruitment into the airway in response to allergen challenge was dramatically

Author contributions: P.C.F., S.P.H., and M.E.R. designed research; P.C.F., C.A.F., M.L.M., L.M.H., and S.P.H. performed research; P.C.F., C.A.F., L.M.H., S.P.H., and M.E.R. analyzed data; and P.C.F. and M.E.R. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: BALF, bronchoalveolar lavage fluid; PAS, periodic acid/Schiff; Th2, T helper lymphocytes, type II; KO, knockout; OVA, ovalbumin.

‡To whom correspondence should be addressed. E-mail: rothenberg@cchmc.org.

www.pnas.org/cgi/doi/10.1073/pnas.0607863103

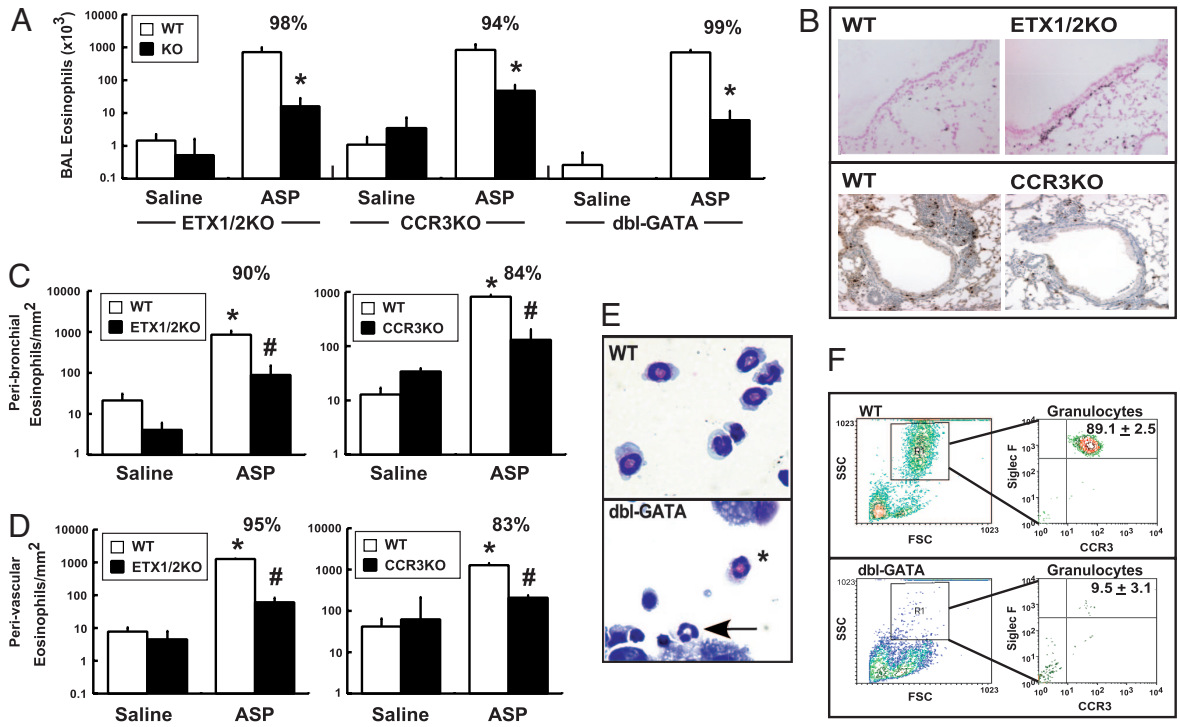

Fig. 1. Airway and tissue eosinophilia in genetically engineered mice. (*A*) Eosinophils in the BALF from WT (white bars) and gene-targeted (black bars) mice after induction of experimental asthma are shown (mean ± SD): eotaxin-1/2 KO (ETX1/2KO), CCR3 KO ($n = 3$ experiments with three to seven mice per group per experiment), and ΔdbI-GATA (dbl-GATA, $n = 2$ experiments with three to five mice per group per experiment). *, $P < 0.0001$ when compared with WT control mice. Percent reduction in cell recruitment when compared with WT control mice is shown. (*B*) Pulmonary eosinophil infiltration, as detected by anti-major basic protein immunohistochemistry, is shown in WT, eotaxin-1/2 KO (ETX1/2KO), and CCR3 KO mice. (Magnification: ×100.) Quantitation of eosinophil infiltration around bronchioles (peribronchial) (*C*) and blood vessels (perivascular) (*D*) in WT (white bars) and gene-targeted (black bars) mice is presented as mean ± SEM ($n = 3$ experiments with two to five mice per group per experiment). *, $P \leq 0.05$ when compared with saline-challenged WT mice; #, $P \leq 0.05$ when compared with allergen-challenged WT mice. (*E*) BALF cytospins from allergen-challenged WT and ΔdbI-GATA (dbl-GATA) mice. The asterisk indicates an eosinophil, and the arrow indicates neutrophilic granulocytes with nuclei more typical of eosinophils. (*F*) A CCR3- and Siglec-F-expressing population in BALF of allergen-challenged WT and ΔdbI-GATA (dbl-GATA) mice. A representative histogram of anti-CCR3 and anti-Siglec-F is shown ($n = 2$ experiments).

impaired in the absence of eotaxin-1/2 or CCR3 expression (68 ± 22 and 77 ± 4%, respectively; $n = 3$ experiments each). Cell accumulation into the airways of ΔdbI-GATA mice was also reduced (59 ± 20%, $P < 0.0001$; $n = 2$ experiments). We next examined the effect of dbl-GATA, eotaxin-1/2, and CCR3 deficiency on allergen-induced chemoattraction of other cell types. Lymphocyte and macrophage accumulation into the airway in response to allergen challenge was unchanged in the eotaxin-1/2 double knockout mice, compared with the WT control mice (data not shown). Allergen-induced mononuclear cell recruitment was significantly reduced (53%) in the CCR3 KO mice; specifically, macrophages ($20.5 \pm 5 \times 10^4$ vs. $9.6 \pm 3 \times 10^4$, $P < 0.0001$) and lymphocytes ($4.5 \pm 2 \times 10^4$ vs. $1.0 \pm 0.8 \times 10^4$, $P < 0.0001$) were reduced (77%) compared with WT control mice. Notably, no CCR3 was detected on the surface of $CD3^+$ or $CD19^+$ lymphocytes from the spleen or lungs of allergen-challenged mice, suggesting that the decrease in lymphocyte accumulation in the airway is an indirect result of CCR3 deficiency on eosinophils (data not shown).

Analysis of the ΔdbI-GATA mice revealed a reduction (50%) in lymphocyte recruitment (2.5 ± 1.4 vs. $5.0 \pm 2.3 \times 10^4$, $P < 0.02$, $n = 3$ experiments) and an increase (≈3-fold) in airway neutrophilia compared with WT controls (19.2 ± 11.5 vs. $6.7 \pm 6.5 \times 10^4$, $P < 0.02$, $n = 3$ experiments). Some of the neutrophilic granulocytes contained nuclei more typical of eosinophils (Fig. 1*E*). These neutrophil/eosinophil-like cells were increased in the airways of allergen-challenged ΔdbI-GATA mice compared with WT mice (2.2 ± 0.9% vs. 7.6 ± 1.5% of BALF cells from WT and ΔdbI-GATA mice, respectively, $P = 0.04$, $n = 7$–10 mice per group combined from two independent experiments). FACS analysis on BALF cells from allergen-challenged WT and ΔdbI-GATA mice identified a population (≈10%) of granulocytes in the ΔdbI-GATA mice that expressed high levels of CCR3 and Siglec-F (Fig. 1*F*). In WT mice, 90% of the BALF granulocyte cells were CCR3- and Siglec-F-positive.

Eosinophils and CCR3 Regulate Allergen-Induced Cytokine Production. To investigate whether the decrease in eosinophils in the airway of CCR3-deficient and ΔdbI-GATA mice was associated with an impaired T helper lymphocyte type II (Th2) response, we first examined pulmonary levels of Th2 cytokines, including IL-4, IL-5, and IL-13. CCR3-deficiency resulted in impaired IL-4 (84%, 50.2 ± 19 vs. 8 ± 2.3 pg/ml, $P = 0.002$, representative of two experiments) and IL-13 (33%, 148.4 ± 41.9 vs. 98.9 ± 17.7 pg/ml, $P = 0.008$, representative of two experiments) production after chronic allergen exposure compared with allergen-challenged WT control mice. Dbl-GATA deficiency also resulted in decreased pulmonary IL-4 (69%, 59.9 ± 17.2 vs. 15.7 ± 6 pg/ml, $P < 0.001$, representative of two experiments) and IL-13 (30%, 196.8 ± 16.4 vs. 138.4 ± 22.1 pg/ml, $P = 0.009$, representative of two experiments). In contrast, pulmonary IL-5 production was not consistently reduced in CCR3 KO or ΔdbI-GATA mice after chronic allergen exposure compared with WT control mice (data not shown). Next we examined the impact of dbl-GATA and CCR3 deficiency on plasma levels of antigen-specific IgG_1. We found no decrease in *A. fumigatus*-specific IgG_1 plasma levels in allergen-challenged ΔdbI-GATA or CCR3 KO mice compared with controls (data not shown). We also examined the consequences of dbl-GATA deficiency on total IgE production and found no decrease in plasma IgE levels from

IMMUNOLOGY

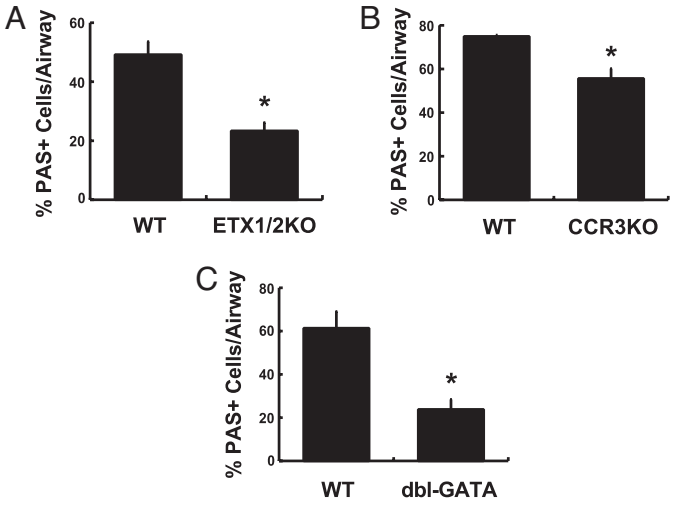

Fig. 2. Allergen-induced mucus production in genetically engineered mice. Quantitation of PAS^+ cells (mean ± SD) in airways of allergen-challenged WT and gene-targeted mice 24 h after the last allergen challenge. Data are from eotaxin-1/2 KO (ETX1/2KO) (*A*), CCR3 KO ($n = 3$ experiments with three to seven mice per group per experiment) (*B*), and ΔdblGATA (dbl-GATA) (*C*) mice ($n = 7$–10 mice per group combined from two experiments). *, $P \leq 0.001$ when compared with WT control mice.

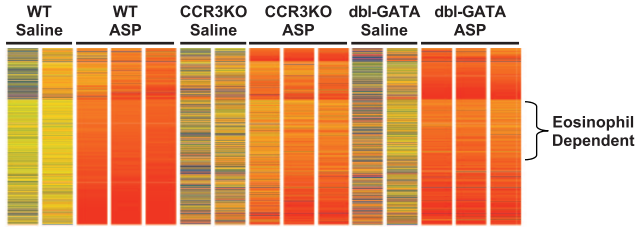

Fig. 3. Allergen-induced transcripts in genetically engineered mice. The 685 genes differentially expressed ($P < 0.05$) in the allergen-challenged (ASP) lungs of WT, CCR3-deficient (CCR3 KO), and ΔdblGATA (dbl-GATA) mice compared with saline-challenged controls is shown; up-regulated genes are represented in red, and down-regulated genes are represented in blue. The magnitude of the gene changes is proportional to the darkness of the color. Each column represents an individual mouse, and each line represents a gene.

allergen-challenged ΔdblGATA mice compared with controls (data not shown).

Eosinophils and CCR3 Regulate Allergen-Induced Mucus Production. Examination of periodic acid/Schiff (PAS)-positive cells in the bronchial epithelium of eotaxin-1/2 double knockout mice revealed a significant decrease (53 ± 4%, $n = 3$ experiments, $P < 0.001$) in mucus production in response to allergen challenge compared with WT control mice (Fig. 2*A*). In the CCR3-deficient mice, PAS-positive epithelium was significantly reduced (26 ± 6%, $n = 3$ experiments, $P < 0.001$) after allergen challenge (Fig. 2*B*). Notably, a reduction in PAS-positive epithelium was also observed in the ΔdblGATA mice (60 ± 12%, $n = 2$ experiments with three to five mice per group per experiment) (Fig. 2*C*).

Eosinophils and CCR3 Regulate Gene Induction in Allergic Inflammation. We next examined the impact of CCR3 and dbl-GATA deficiency on the induction of the global asthma transcriptome (15). In our chronic model of experimental asthma, allergen challenge resulted in a 2-fold or more induction of ≈1.5% of represented genes (685 of 45,101) on the DNA chip. Clustering of the signal intensities of the individual lung samples in each group revealed a high similarity of transcript expression pattern between allergen-challenged CCR3 KO and ΔdblGATA mice (Fig. 3). Analysis of the allergen-induced transcripts revealed a subset of 197, or 29% of the chronic asthma transcriptome, that were eosinophil- and CCR3-dependent, because they were induced in the lung after chronic allergen exposure in WT mice, but not in CCR3 KO and/or ΔdblGATA mice (Table 1).

Gene ontology analysis of the allergen-induced genes dependent on eosinophils and CCR3 expression revealed that the overexpressed genes were involved in macromolecule metabolism (22%), protein metabolism (19%), response to biotic stimulus (10%), defense response (9%), and response to wounding (5%). Thirty-six percent of the gene products were extracellular molecules, supporting a role for eosinophils in regulating the local microenvironment.

A number of genes not previously associated with eosinophils *in vivo* were identified, including transcription factors (e.g., Spi-C and Oct-6), channel proteins (e.g., Kcnn4, Aqp9, and Slc15a4), proteases (Ctse), and cytokines (e.g., IL-21 and Pdgfc). Interestingly, three of the eosinophil- and CCR3-dependent genes, ST2, Egr2, and Pirb, have been shown to be involved in down-regulating or inhibiting an immune response (16–18).

Eosinophil Deficiency Results in Impaired Induction of Genes Involved in the Coagulation Cascade. Eosinophil-associated genes included an impressive number of genes involved in the coagulation cascade (PAI-2, F5, F10, and P2X1). As such, we measured a marker of clotting activation, thrombin-anti-thrombin III (TAT) complexes, in the BALF of allergen-challenged WT, CCR3 KO, and ΔdblGATA mice. In response to allergen challenge, BALF TAT complexes significantly increased (18-fold) in WT mice (2.93 ± 1.4 vs. 51.3 ± 7.6 pM, $n = 2$ experiments with four to seven mice per group per experiment, $P \leq 0.0001$). CCR3 and dbl-GATA deficiency resulted in a 39% (31.2 ± 7.8 pM, $P \leq 0.001$) and 46% (27.5 ± 7.2 pM, $P \leq 0.0001$) reduction in allergen-induced TAT complexes, respectively, compared with WT control mice.

Dbl-GATA Deficiency Results in Increased Expression of Mast Cell-Related Genes. Dbl-GATA deficiency also resulted in marked gene induction not seen in the WT mice. These dysregulated genes included the mast cell protease carboxypeptidase A3 (Cpa3). Examination of other mast cell-related allergen-induced genes revealed a greater fold increase in ΔdblGATA asthmatic lungs compared with WT for MMCP-1 (16-fold vs. 3-fold) and MMCP-2 (48-fold vs. 17-fold). The increase in MMCP-1 expression was associated with a 4-fold increase and a 2-fold increase in plasma MMCP-1 levels in the allergen-challenged CCR3-deficient and ΔdblGATA mice, respectively, compared with WT controls (120 ± 55 vs. 29 ± 11 pg/ml, $n = 7$ mice per group for CCR3 KO, $P \leq 0.001$; 106.5 ± 28 vs. 50.6 ± 17 pg/ml, $n = 7$–8 mice per group for ΔdblGATA, $P \leq 0.001$).

Discussion

Recent elegant studies using two distinct lines of eosinophil-deficient mice highlight an important role for eosinophils with certain hallmark features of asthma, but the results of the two studies were divergent and did not identify the key contributing eosinophil effector functions (5, 6). In this study we aimed to define the importance of eosinophils and CCR3 and its ligands in several hallmark features of chronic experimental asthma. First, we demonstrate that eosinophil recruitment is primarily dependent on CCR3 and the eotaxins in chronic experimental asthma, despite the expression of numerous other eosinophil-active chemoattractants. Second, we demonstrate a significant regulatory role for eosinophils in Th2 cytokine production with reduced levels of IL-4 and IL-13 measured in allergen-challenged lungs. Third, we demonstrate that reduction in lung

Table 1. Eosinophil- and CCR3-dependent allergen-induced genes

Gene	Description	GenBank accession no.	Fold induction WT	CCR KO	dbl-GATA
Oit3	Oncoprotein-induced transcript 3	NM_010959	35	—	—
Spi-C	Spi-C transcription factor	NM_011461	21	—	—
Adora3	Adenosine A3 receptor	NM_009631	17	—	—
Pou3f1	POU domain, class 3, transcription factor	NM_011141	16	—	—
Serpinb2/PAI-2	Plasminogen activator inhibitor 2	NM_011111	16	1.0	0.8
Prg2/MBP	Proteoglycan 2, bone marrow	NM_008920	13	1.8	—
CCR3	Chemokine receptor 3	NM_009914	10	—	—
Matk	Megakaryocyte-associated tyrosine kinase	NM_010768	10	—	—
Ltb4r1	Leukotriene B4 receptor 1	NM_008519	9	—	—
Sytl1	Synaptotagmin-like 1	NM_031393	8	—	—
F5	Coagulation factor V	NM_007976	8	1.6	0.6
Epx	Eosinophil peroxidase	NM_007946	5	—	—
Galnt6	*N*-acetylgalactosaminyltransferase 6	NM_172451	5	—	—
Man2b1	Mannosidase 2, αB1	NM_010764	4	1.9	1.5
F10	Coagulation factor X	NM_007972	4	1.5	—
Kcnn4	Potassium calcium-activated channel	NM_008433	4	1.3	1.6
IL4	IL-4	NM_021283	3	1.9	1.6
P2rx1	Purinergic receptor P2X	NM_008771	3	—	—
Aqp9	Aquaporin 9	NM_022026	3	0.9	0.8
Ncf1	Neutrophil cytosolic factor 1	NM_010876	3	1.2	1.4
Egr2	Early growth response 2	NM_010118	3	1.3	1.2
Csf2rb2	CSF 2 receptor, β2, low-affinity	NM_007781	3	1.3	1.6
Pirb	Paired Ig-like receptor B	NM_011095	3	1.0	1.7
Il1rl1/ST2	IL-1 receptor-like 1	NM_010743	3	1.2	1.3
Bmp1	Bone morphogenetic protein 1	NM_009755	3	1.0	1.6
Ctsl	Cathepsin L	NM_009984	2.5	1.0	1.9
Hgfac	Hepatocyte growth factor activator	NM_019447	2.5	1.8	1.8
Ctse	Cathepsin E	NM_007799	2.5	1.2	1.0
Msr1	Macrophage scavenger receptor 1	NM_031195	2.5	1.5	1.9
IL21	IL-21	NM_021782	2.4	—	1.0
Csf2rb1	CSF 2 receptor, β1, low-affinity	NM_007780	2.4	1.3	1.3
Cklfsf7	Chemokine-like factor superfamily 7	NM_133978	2.3	1.3	1.7
Ncf4	Neutrophil cytosolic factor 4	NM_008677	2.3	1.0	1.3
Slc13a4	Solute carrier family 13, member 4	NM_172892	2.3	—	1.6
Pdgfc	Platelet-derived growth factor, C polypeptide	NM_019971	2.3	0.8	1.6
Fcer2a	Fc receptor, IgE, low-affinity II, α	NM_013517	2.3	1.4	1.8
Siglecg	Sialic acid binding Ig-like lectin G	NM_172900	2.2	—	1.5
Grn	Granulin	NM_008175	2.1	1.3	1.8
Itgb2	Integrin β2	NM_008404	2.1	1.6	1.6
Col6a1	Procollagen type VI, α1	NM_009933	2.1	1.5	1.8
Slc35c2	Solute carrier family 35, member C2	NM_144893	2.1	1.7	1.8
Hck	Hematopoietic cell kinase	CK330863	2.1	0.7	0.7
Ninj1	Ninjurin 1	NM_013610	2.0	1.0	1.6
Slc15a4	Solute carrier family 15, member 4	NM_133895	2.0	1.3	1.8
C3	Complement component 3	NM_009778	2.0	1.7	1.8

Data show fold change in transcript levels in allergen-challenged lungs compared with saline-challenged controls in WT, CCR3 KO, and dbl-Gata mice. —, Transcript levels of the gene are below the level of detection.

eosinophilia and Th2 cytokine production is associated with diminished airway mononuclear cell accumulation and a reduction in PAS staining evident in asthmatic lungs. And, finally, global transcript expression profiling was used to identify eosinophil-associated effector pathways.

In acute OVA models of experimental asthma where sensitized animals are exposed to the antigen OVA over the course of a few days the effects of CCR3 on cell trafficking have been shown to be specific to the eosinophil because no differences in allergen-induced mononuclear or neutrophil accumulation in the lung lumen were observed (12, 13). In our chronic model of allergic airway disease, we observed impaired allergen-induced recruitment of eosinophils, macrophages, and lymphocytes. Our data showing that murine CCR3 is predominantly expressed by eosinophils are consistent with previous studies (19, 20). These observations suggest that leukocyte recruitment in chronic allergic inflammation is orchestrated, at least in part, via CCR3 signaling in eosinophils, although we cannot exclude signal transduction events in other cells such as basophils and epithelial cells, because they have been shown to express low levels of CCR3 at least in the human system (21, 22). Interestingly, only eosinophil trafficking is impaired in the eotaxin-1/2-deficient mice, suggesting that other CCR3 ligands provide sufficient stimulation to induce the infiltration by other cell types. Although eosinophil recruitment into the lung is profoundly reduced (95%) in the eotaxin-1/2-deficient mice (and CCR3-deficient mice), it is important to note that the residual perivascular and peribronchial eosinophils may still contribute to disease.

In all three strains of genetically engineered eosinophil impaired mice, we observed a significant decrease in the percentage of PAS^+ airway epithelial cells in response to chronic allergen exposure. A role for adenosine signaling in regulating mucus production and eosinophil recruitment has been shown, as antagonizing the A_3 adenosine receptor (A3R) prevented airway eosinophilia and mucus production in ADA-deficient mice (23). Importantly, we iden-

tified A3R as an eosinophil-dependent allergen-induced gene (Table 1), supporting an important role for eosinophils and adenosine in allergen-induced PAS$^+$ staining of lung epithelial cells. We also found impaired local cytokine accumulation, including IL-13 and IL-4, in lung homogenates of allergen-challenged CCR3-deficient and Δdbl-GATA mice. It is important to note that in our model the reduction in global lung IL-4, a cytokine strongly associated with goblet cell hyperplasia, was greater than IL-13. Also, preliminary *ex vivo* studies in our laboratory demonstrate a marked deficit in antigen-induced IL-4, IL-5, and IL-13 production by splenocytes isolated from allergen-challenged Δdbl-GATA mice compared with WT control mice, suggesting a defect in lymphocyte activation (data not shown). This is in agreement with studies demonstrating that a combined deficiency in IL-5 and eotaxin-1 results in a defect in IL-13 production by Th2 lymphocytes (24). Our *in vivo* studies demonstrate that release of cytokines and other mediators by eosinophils within the local microenvironment may modulate pulmonary pathology and immune responses where eosinophils have accumulated (e.g., peribronchial regions). Using microarray analysis we identified multiple potential molecules through which eosinophils and CCR3 may regulate local immune responses. For instance, we found a sharp decrease in transcript levels of leukotriene B_4 receptor 1 (BLT1) in allergen-challenged CCR3- and dbl-GATA deficient mice. BLT1 has been shown to be important in early recruitment of effector T lymphocytes, and BLT1 deficiency results in decreased allergen-induced eosinophil recruitment and decreased hyperplasia of goblet cells (25, 26). In addition, IL-4, Egr-2, and ST2, known to be important regulators of T cell activation (17, 18), and now identified to be eosinophil-associated, may regulate the Th2 lung response. We also identify the involvement of PAI-2, factor 5, factor 10, and P2X1 receptor in eosinophil-associated allergic inflammation, implicating a role for eosinophils in the regulation of fibrin deposition in the allergic lung. Indeed, PAI-2 and factor 5 have been shown to be expressed by eosinophils *in vitro* (27, 28), but their association with eosinophilic lung disease has not been reported. The potential relationship between eosinophils and the coagulation system was further supported by the reduction in an allergen-induced clotting activation marker (TAT) in the BALF from CCR3 KO and Δdbl-GATA mice. Our data are in agreement with a previous study demonstrating a significant correlation between sputum concentrations of eosinophilic cationic protein and sputum TAT levels in human asthmatics (29). This relationship warrants further investigation because disordered coagulation and fibrinolysis, including the accumulation of fibrin and thrombin in the airway, have been associated with AHR (30).

In our efforts to identify pathways downstream of eosinophils, we also observed an increase in mast cell associated transcripts in the lungs of dbl-GATA deficient mice, but not CCR3 KO mice. Mechanistically, we cannot exclude the influence of alterations in the promoter of GATA-1 on mast cell gene transcription in the Δdbl-GATA mice, because GATA-1 has been shown to be important in mast cell differentiation, although mast cell differentiation in the Δdbl-GATA mice has been reported to be normal (31, 32). In our model, the increase in MMCP-1 was confirmed by a 2-fold increase and a 4-fold increase in plasma MMCP-1 levels in the allergen-challenged eosinophil- and CCR3-deficient mice, respectively. Our data are in agreement with a previous report of increased allergen-induced intraepithelial mast cells in the trachea of CCR3 KO mice (12). Interestingly, we also observed significantly increased plasma MMCP-1 in saline-challenged CCR3 KO and Δdbl-GATA mice, compared with WT control mice (data not shown). Our data suggest a role for eosinophils in regulating allergen-induced and baseline mast cell homing and/or activation.

To define a causative relationship between the recruitment of eosinophils and the onset or progression of pulmonary pathologies associated with allergic asthma, multiple experimental approaches have been used to deplete animals of eosinophils, including manipulation of cytokine (e.g., IL-5 and eotaxins) levels via gene targeting and neutralizing antibodies and depletion of eosinophils using an anti-CCR3 antibody (19, 20, 33–35). More recently, Lee *et al.* (6) targeted the depletion of eosinophils (these mice are designated as PHIL) using an eosinophil-specific promoter to drive expression of a cytocidal protein, diphtheria toxin A. In comparison, Yu *et al.* (31) developed mice harboring a deletion of a high affinity double palindromic GATA binding site in the GATA-1 promoter (Δdbl-GATA), which led to the specific ablation of the eosinophil lineage. Interestingly, RT-PCR analysis of gene expression in the bone marrow of the Δdbl-GATA mice revealed no expression of eosinophil peroxidase, but expression of major basic protein was reduced and CCR3 expression remained unchanged. In our study we found mature eosinophils (≈1% of that found in WT) in the BALF from Δdbl-GATA mice subjected to our experimental chronic asthma protocol. We also found cells, presumably eosinophils, expressing CCR3 and Siglec-F in the BALF from these same mice. We cannot exclude the possibility that the CCR3$^+$ cells identified in the BALF of Δdbl-GATA mice may include basophils. The low level of eosinophils in our chronic model of experimental asthma may be due to IL-5-independent events, because these mice have been shown to be devoid of classic eosinophils by IL-5 overexpression (31). Interestingly, we found increased BAL neutrophils in the Δdbl-GATA mice in our chronic model of experimental asthma. Some of these cells had the nuclear morphology of murine eosinophils but did not stain for eosinophilic granules. It is tempting to speculate that these "neutrophils" are granule-less eosinophils, but further studies are needed to define this cell population. Regardless of whether these cells may be related to eosinophils in part, our results are consistent with the presence of a profound deficiency of eosinophils in the Δdbl-GATA mice. Perhaps residual eosinophil-like cells identified in the Δdbl-GATA mice may be partially responsible for the different phenotype compared with PHIL mice (Table 2). In our model we identified a defect in Th2 cytokine production locally within the lung that was associated with profound reductions in pulmonary eosinophil recruitment. This defect was also associated with reduced mucus production in the lung and diminished recruitment of mononuclear cells into the airway in response to chronic allergen challenge. Taken together, the emerging role for the eosinophil as a prominent effector cell in multiple parameters of allergic asthma, including airway remodeling, suggests effective depletion of eosinophils from the airways could have useful therapeutic benefit.

Table 2. Effects of eosinophil depletion or impaired eosinophil recruitment on experimental asthma parameters

	OVA model		*Aspergillus* allergen-challenged model		
Asthma parameter	PHIL	Δdbl-GATA	CCR3 KO	ETX1/2 DKO	Δdbl-GATA
BALF eosinophils	+	+	+	+	+
Lung tissue eosinophils	+	+	+	+	+
BALF mononuclear cells	NE	NE	+	NE	+
AHR	+	NE	ND	ND	ND
Mucus production	+	NE	+	+	+
Collagen deposition	ND	+	ND	ND	ND
Th2 antibody production	ND	NE	NE	NE	NE
Th2 cytokines	ND	NE	+	NE	+

+, the genetic manipulation of the mouse resulted in protection from or reduction in severity of the asthma parameter. NE, no effect: no change in the asthma parameter between the genetically modified mouse and WT control mice. ND, not determined: the asthma parameter was not measured.

Materials and Methods

Mice. BALB/c and 129SvEv WT mice were obtained from Taconic (Germantown, NY). CCR3 gene-targeted and Δdbl-GATA mice

(BALB/c background) were generously provided by Alison Humbles and Craig Gerard (Children's Hospital, Boston, MA) (5, 12). Eotaxin-1 and eotaxin-2 double deficient mice (SvEv background) were described previously (14).

Experimental Asthma Induction. Asthma-like lung disease was induced with intranasal administration of saline or *A. fumigatus* allergenic extract (Hollister-Stier Laboratories, Spokane, WA) three times per week for 3 weeks (15). Subsequently, the BALF, plasma, and/or lung tissue was harvested 24 h after the final challenge. RNA for microarray analysis was harvested from unlavaged lungs 24 h after the final challenge.

BALF Collection and Analysis. BAL differential cell counts were performed as previously described (36).

Preparation of RNA and Microarray Hybridization. Microarray hybridization was performed, as previously described (15), with one mouse per chip ($n \geq 3$ for each allergen challenge condition, and $n = 2$ for each saline challenge condition). The genome-wide mouse MOE430 2.0 GeneChip (Affymetrix, Santa Clara, CA) was used.

Microarray Data Analysis. From data image files, gene transcript levels were determined by using algorithms in the Microarray Analysis Suite (Affymetrix) and GeneSpring software as previously reported (15). The list of allergen-induced genes dependent on eosinophils and CCR3 was compiled by identifying genes that were induced ≥2-fold in allergen-challenged WT lungs compared with WT saline control lungs, but not induced ≥2-fold in allergen-challenged gene-targeted mice. Ontology analysis of the differentially expressed transcripts was done by using DAVID (database for annotation, visualization, and integrated discovery; http://david.abcc.ncifcrf.gov) (37).

Flow Cytometry. To identify Siglec-F$^+$/CCR3$^+$ cells, 1×10^6 BALF cells were incubated with FITC-conjugated anti-CCR3 (rat IgG_{2a}, clone 83101; R & D Systems, Minneapolis, MN) (1 μg per 10^6 cells), phycoerythrin-conjugated Siglec-F (Rat IgG_{2a}, clone E50-2440; BD Biosciences Pharmingen, San Jose, CA) (1 μg per 10^6 cells), or FITC-, phycoerythrin-isotype-matched control Ig (rat IgG_{2a}). To identify CCR3$^+$ cells, lungs were perfused with PBS, removed from the pleural cavity, minced into small pieces, and incubated in a Liberase Blendzyme 3 (0.1 units/ml; Roche, Indianapolis, IN) and DNase I (0.5 mg/ml; Sigma, St. Louis, MO). Lung and spleen cell suspensions were incubated with fluorescein-conjugated anti-mouse CCR3 (250 ng/ml; R & D Systems), phycoerythrin-conjugated anti-mouse CD19 (1D3, 1 μg/ml; Pharmingen), anti-mouse CD3ε (145-2C11, 1 μg/ml; Pharmingen), or the isotype-matched control IgG. Flow cytometric data acquisition was performed by using a two-laser, four-color FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data analysis was done by using CELLQUEST version 3.3 (BD Biosciences Pharmingen) software.

Lung Histopathological Changes. Quantitation of lung tissue eosinophils, PAS-stained airway goblet cells, and cytokine levels in lung homogenates was performed by a blinded observer as previously described (36).

ELISA Measurements. MMCP-1, IgE, and cysteinyl leukotriene levels were measured by ELISA according to the manufacturer's instructions (respectively, Moredun Scientific, Midlothian, U.K.; BD Pharmingen; and Cayman Chemical, Ann Arbor, MI). Thrombin–anti-thrombin III (TAT) complexes were measured in BALF by ELISA according to the manufacturer's instructions (Enzyme Research Laboratories, South Bend, IN). Cytokine levels were measured in lung homogenates by using DuoSet ELISA Development kits specific for IL-13 (R & D Systems), IL-4, and IL-5 (BD Pharmingen); the detection limits for IL-4, IL-5, and IL-13 were 4, 6, and 19 pg/ml, respectively. Plasma *Aspergillus*-specific IgG_1 was measured as previously reported (38).

We thank Dr. Fred Finkelman for helpful discussions and review of the manuscript; Drs. Sam Pope (Cincinnati Children's Medical Center, Cincinnati, OH), Jamie Lee (Mayo Clinic, Scottsdale, AZ), Nancy Lee (Mayo Clinic, Scottsdale, AZ), Allison Humbles, and Craig Gerard for critical reagents; and Andrea Lippelman for assistance with preparation of the manuscript. This work was supported in part by National Institutes of Health Grants R01 AI42242, R01 AI45898, and P01 HL-076383-01 (all to M.E.R.).

1. Fahy JV, Corry DB, Boushey HA (2000) *Curr Opin Pulm Med* 6:15–20.
2. Bousquet J, Chanez P, Lacoste JY, White R, Vic P, Godard P, Michel FB (1992) *Allergy* 47:3–11.
3. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, McElwain K, McElwain S, Friedman S, Broide DH (2004) *J Clin Invest* 113:551–560.
4. Flood-Page P, Menzies-Gow A, Phipps S, Ying S, Wangoo A, Ludwig MS, Barnes N, Robinson D, Kay AB (2003) *J Clin Invest* 112:1029–1036.
5. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, *et al.* (2004) *Science* 305:1776–1779.
6. Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, Protheroe C, Pero R, Nguyen T, Cormier SA, *et al.* (2004) *Science* 305:1773–1776.
7. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, Mathur AK, Cowley HC, Chung KF, Djukanovic R, *et al.* (2000) *Lancet* 356:2144–2148.
8. Kips JC, O'Connor BJ, Langley SJ, Woodcock A, Kerstjens HA, Postma DS, Danzig M, Cuss F, Pauwels RA (2003) *Am J Respir Crit Care Med* 167:1655–1659.
9. Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS (2003) *Am J Respir Crit Care Med* 167:199–204.
10. Gao JL, Sen AI, Kitaura M, Yoshie O, Rothenberg ME, Murphy PM, Luster AD (1996) *Biochem Biophys Res Commun* 223:679–684.
11. Daugherty BL, Siciliano SJ, DeMartino JA, Malkowitz L, Sirotina A, Springer MS (1996) *J Exp Med* 183:2349–2354.
12. Humbles AA, Lu B, Friend DS, Okinaga S, Lora J, Al Garawi A, Martin TR, Gerard NP, Gerard C (2002) *Proc Natl Acad Sci USA* 99:1479–1484.
13. Ma W, Bryce PJ, Humbles AA, Laouini D, Yalcindag A, Alenius H, Friend DS, Oettgen HC, Gerard C, Geha RS (2002) *J Clin Invest* 109:621–628.
14. Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME (2005) *J Immunol* 175:5341–5350.
15. Zimmermann N, Mishra A, King NE, Fulkerson PC, Doepker MP, Nikolaidis NM, Kindinger LE, Moulton EA, Aronow BJ, Rothenberg ME (2004) *J Immunol* 172:1815–1824.
16. Ujike A, Takeda K, Nakamura A, Ebihara S, Akiyama K, Takai T (2002) *Nat Immunol* 3:542–548.
17. Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, Liew FY (2004) *Nat Immunol* 5:373–379.
18. Safford M, Collins S, Lutz MA, Allen A, Huang CT, Kowalski J, Blackford A, Horton MR, Drake C, Schwartz RH, *et al.* (2005) *Nat Immunol* 6:472–480.
19. Justice JP, Borchers MT, Crosby JR, Hines EM, Shen HH, Ochkur SI, McGarry MP, Lee NA, Lee JJ (2003) *Am J Physiol* 284:L169–L178.
20. Grimaldi JC, Yu NX, Grunig G, Seymour BW, Cottrez F, Robinson DS, Hosken N, Ferlin WG, Wu X, Soto H, *et al.* (1999) *J Leukocyte Biol* 65:846–853.
21. Khodoun MV, Orekhova T, Potter C, Morris S, Finkelman FD (2004) *J Exp Med* 200:857–870.
22. Stellato C, Brummet ME, Plitt JR, Shahabuddin S, Baroody FM, Liu MC, Ponath PD, Beck LA (2001) *J Immunol* 166:1457–1461.
23. Young HW, Molina JG, Dimina D, Zhong H, Jacobson M, Chan LN, Chan TS, Lee JJ, Blackburn MR (2004) *J Immunol* 173:1380–1389.
24. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, Hogan SP, Koskinen A, McKenzie AN, Dent LA, *et al.* (2002) *J Exp Med* 195:1433–1444.
25. Tager AM, Bromley SK, Medoff BD, Islam SA, Bercury SD, Friedrich EB, Carafone AD, Gerszten RE, Luster AD (2003) *Nat Immunol* 4:982–990.
26. Terawaki K, Yokomizo T, Nagase T, Toda A, Taniguchi M, Hashizume K, Yagi T, Shimizu T (2005) *J Immunol* 175:4217–4225.
27. Swartz JM, Bystrom J, Dyer KD, Nitto T, Wynn TA, Rosenberg HF (2004) *J Leukocyte Biol* 76:812–819.
28. Voehringer D, Shinkai K, Locksley RM (2004) *Immunity* 20:267–277.
29. Gabazza EC, Taguchi O, Tamaki S, Takeya H, Kobayashi H, Yasui H, Kobayashi T, Hataji O, Urano H, Zhou H, *et al.* (1999) *Lung* 177:253–262.
30. Wagers SS, Norton RJ, Rinaldi LM, Bates JH, Sobel BE, Irvin CG (2004) *J Clin Invest* 114:104–111.
31. Yu C, Cantor AB, Yang H, Browne C, Wells RA, Fujiwara Y, Orkin SH (2002) *J Exp Med* 195:1387–1395.
32. Migliaccio AR, Rana RA, Sanchez M, Lorenzini R, Centurione L, Bianchi L, Vannucchi AM, Migliaccio G, Orkin SH (2003) *J Exp Med* 197:281–296.
33. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG (1996) *J Exp Med* 183:195–201.
34. Rothenberg ME, Maclean JA, Pearlman E, Luster AD, Leder P (1997) *J Exp Med* 185:785–790.
35. Yang Y, Loy J, Ryseck RP, Carrasco D, Bravo R (1998) *Blood* 92:3912–3923.
36. Fulkerson PC, Fischetti CA, Hassman LM, Nikolaidis NM, Rothenberg ME (2006) *Am J Respir Cell Mol Biol* 35:337–346.
37. Dennis G, Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) *Genome Biol* 4(5):P3.
38. Akei HS, Mishra A, Blanchard C, Rothenberg ME (2005) *Gastroenterology* 129:985–994.