Granulocyte-Macrophage Colony-Stimulating Factor Is Required for Bronchial Eosinophilia in a Murine Model of Allergic Airway Inflammation[1]

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GM-CSF plays an important role in inflammation by promoting the production, activation, and survival of granulocytes and macrophages. In this study, GM-CSF knockout (GM-CSF$^{-/-}$) mice were used to investigate the role of GM-CSF in a model of allergic airway inflammation. In allergic GM-CSF$^{-/-}$ mice, eosinophil recruitment to the airways showed a striking pattern, with eosinophils present in perivascular areas, but almost completely absent in peribronchial areas, whereas in wild-type mice, eosinophil infiltration appeared in both areas. In the GM-CSF$^{-/-}$ mice, mucus production in the airways was also reduced, and eosinophil numbers were markedly reduced in the bronchoalveolar lavage (BAL)[3] fluid. IL-5 production was reduced in the lung tissue and BAL fluid of GM-CSF$^{-/-}$ mice, but IL-4 and IL-13 production, airway hyperresponsiveness, and serum IgE levels were not affected. The presence of eosinophils in perivascular but not peribronchial regions was suggestive of a cell migration defect in the airways of GM-CSF$^{-/-}$ mice. The CCR3 agonists CCL5 (RANTES) and CCL11 (eotaxin-1) were expressed at similar levels in GM-CSF$^{-/-}$ and wild-type mice. However, IFN-γ mRNA and protein were increased in the lung tissue and BAL fluid in GM-CSF$^{-/-}$ mice, as were mRNA levels of the IFN-γ-inducible chemokines CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-Tac). Interestingly, these IFN-γ-inducible chemokines are natural antagonists of CCR3, suggesting that their overproduction in GM-CSF$^{-/-}$ mice contributes to the lack of airway eosinophils. These findings demonstrate distinctive abnormalities to a model of allergic asthma in the absence of GM-CSF. *The Journal of Immunology,* **2008, 180: 2600–2607.**

Granulocyte-macrophage CSF plays a role in the growth, development, and maturation of granulocytes, macrophages, and dendritic cells (1). Although GM-CSF was originally considered as a hemopoietic cytokine, more recent studies indicate it also has a major role in a variety of inflammatory responses (2). GM-CSF has important effects on eosinophils, which are prominent in allergic inflammation. GM-CSF not only accelerates the growth and maturation of eosinophils, but also primes them for activation, and enhances their survival (3, 4). GM-CSF, IL-5, and IL-3 have similar effects on eosinophils, and each cytokine binds to a receptor consisting of a specific α-chain and a common β-chain (5).

GM-CSF has been implicated in allergic inflammation in human and animal studies. For example, a higher proportion of cells expressed GM-CSF mRNA in bronchoalveolar lavage (BAL) fluid from asthma patients compared with healthy controls (6). When asthma patients were challenged with allergen, GM-CSF levels increased in the BAL fluid, and the levels correlated with the number and percentage of BAL eosinophils (7). In mice exposed to allergen by aerosol inhalation, infection intranasally with a viral construct expressing GM-CSF was followed by marked airway inflammation with eosinophil infiltration and increased levels of IL-4 and IL-5 (8). Administration of a GM-CSF-neutralizing mAb attenuated allergic airway inflammation in a murine model of asthma, significantly reducing airway hyperresponsiveness (AHR), airway eosinophilia, and pulmonary inflammation (9). In a study on inhalation of diesel exhaust particles in mice, AHR and Clara cell hyperplasia were attenuated by anti-GM-CSF Ab (10). In a report on intranasal exposure to house dust mite, administration of a GM-CSF-neutralizing mAb markedly reduced inflammatory responses in the lung and spleen and resulted in reduced Th2 effector cells and airway eosinophilia (11).

Leukocyte tissue recruitment in inflammation is orchestrated by the coordinated induction of chemokines. In the case of eosinophils, ligands of the chemokine receptor CCR3 are critical for migration (12, 13). By contrast, the chemokines CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (I-Tac), which are induced by IFN-γ, selectively signal through CXCR3, a chemokine receptor expressed preferentially on Th1 cells (14, 15). CXCL9, CXCL10, and CXCL11 have been shown to function as CCR3 antagonists in vitro (16, 17). Furthermore, in vivo, CXCL9 inhibited eosinophil recruitment into airway tissue in allergic inflammation (18).

In the present study, GM-CSF$^{-/-}$ mice were used to investigate the role of GM-CSF in a murine model of allergic asthma. We show that GM-CSF deficiency impairs the inflammatory response in this model. Several key features of allergic inflammation were reduced in GM-CSF$^{-/-}$ mice, including airway eosinophilia, and IL-5 levels in the

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[3] Abbreviations used in this paper: BAL, bronchoalveolar lavage; AHR, airway hyperresponsiveness; WT, wild type; PAS, periodic acid-Schiff; PTLN, peritracheal lymph node; PAP, pulmonary alveolar proteinosis.

Table I. *Sequences of primers and TaqMan probes*

Transcript	Primer	Sequence (5′–3′)	cDNA Product (bp)	Ref.
CCR3	Forward	TGGCCTTGTACAGCGAGATCT	105	42
	Reverse	CAAAAGTCACAGTTCGGGCTC		
	TaqMan probe[a]	CTGGCTATCGTCCATGCTGTGTTTGC		
CCL11 (eotaxin-1)	Forward	CCCAACACACTACTGAAGAGCTACAA	87	42
	Reverse	TTTGCCCAACCTGGTCTTG		
	TaqMan probe	CAACAACAGATGCACCCTGAAAGCCA		
CXCL10 (IP-10)	Forward	GCCGTCATTTTCTGCCTCAT	125	43
	Reverse	GCTTCCCTATGGCCCTCATT		
	TaqMan probe	TCTCGCAAGGACGGTCCGCTG		
IFN-γ	Forward	CAGCAACAGCAAGGCGAAA	141	44
	Reverse	CGCTTCCTGAGGCTGGATT		
	TaqMan probe	AGGATGCATTCATGAGTATTGCCAAGTTTGA		
CCL5 (RANTES)	Forward	GCAAGTGCTCCAATCTTGCA	71	44
	Reverse	CTTCTCTGGGTTGGCACACA		
	TaqMan probe	TCGTGTTTGTCACTCGAAGGAACCGC		
CXCR3	Forward	TGCTGTGCTACTGAGTCAGCG	67	45
	Reverse	TACAGCCAGGTGGAGCAGG		
	TaqMan probe	CTGCCCTGAGCAGCACGGACAC		
CXCL9 (Mig)	Forward	CTTTTCCTTTTGGGCATCATCT	72	45
	Reverse	TCGTGCATTCCTTATCACTAGGG		
	TaqMan probe	TCCTCGAACTCCACACTGCTCCAGG		
CXCL11 (I-Tac)	Forward	GCCCTGGCTGCGATCAT	65	45
	Reverse	CAGCGCCCCTGTTTGAAC		
	TaqMan probe	TGGGCCACAGCTGCTCAAGGC		

[a] TaqMan probes were labeled with FAM at the 5′ end and TAMRA at the 3′ end.

lung, BAL fluid, and peritracheal lymph nodes. These changes were associated with overproduction of the CCR3 antagonists CXCL9, CXCL10, and CXCL11.

Materials and Methods

Animals

GM-CSF gene knockout mice (GM-CSF$^{-/-}$) mice, derived on a C57BL/6 background, were provided by the Ludwig Institute for Medical Research (Melbourne, Australia) (19). C57BL/6 wild-type (WT) mice were purchased from the Animal Resource Centre (Perth, Western Australia, Australia). Mice were housed in the pathogen-free animal facility at the Garvan Institute of Medical Research. All procedures were approved by the Garvan Institute of Medical Research/St. Vincent's Hospital Animal Ethics Committee and the University of Newcastle Animal Care and Ethics Committee and were conducted according to the Animal Welfare Guidelines of the National Health and Medical Research Council of Australia.

Allergic asthma model, BAL, and histology

Experiments were performed as previously described (20). Briefly, 6- to 8-wk-old female WT and GM-CSF$^{-/-}$ mice were immunized by i.p. injection with 100 μg of OVA (grade V; Sigma-Aldrich) absorbed onto 0.1 ml of $Al(OH)_3$ gel (alum; Sigma-Aldrich) at days 0 and 14, and unimmunized mice were injected with alum in PBS. All groups were challenged with aerosolized OVA (1% w/v in PBS) on days 21, 23, 25, and 27 for 20 min each day and euthanized on day 28. The right lung was lavaged twice with 0.5 ml of normal saline and cells in BAL fluid were counted and subjected to cytocentrifugation. Slides were fixed in methanol and stained with modified Giemsa.

A lobe of the nonlavaged lung was fixed in 10% formalin (Sigma-Aldrich), embedded in paraffin, and 3-μm sections were stained with H&E or periodic acid-Schiff (PAS). All slides were read by an observer who was blinded to the treatment groups, and severity of inflammation was quantitated on a 0–3 scale. The appearances in untreated control mice were defined as 0 and maximum changes were given a score of 3 as previously described (20).

Cell isolation from lymphoid organs and in vitro stimulation

Single-cell suspensions were prepared from peritracheal lymph nodes (PTLN) and spleens. Cells were filtered through cell strainers to remove debris. Cells were washed twice with PBS and counted in a hemocytometer. Single-cell suspensions were cultured at 10^6 cells/ml in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% (v/v) FCS (HyClone), 2 mM L-glutamine (Invitrogen Life Technologies), 20 mM HEPES (Invitrogen Life Technologies), and 10 U/ml and 10 μg/ml penicillin/streptomycin (Invitrogen Life Technologies). Cells were cultured for 48 h at 37°C in a moisturized incubator with 5% CO_2 in air, in conditions of no stimulus, 10 μg/ml OVA or 5 μg/ml Con A (Sigma-Aldrich). Supernatants were harvested and stored at −80°C pending analysis.

Serum IgE and cytokine assays

Total serum IgE was detected with the Mouse IgE ELISA Set (BD Biosciences) according to the manufacturer's instructions. Absolute concentrations were determined with reference to the manufacturer's IgE standard. OVA-specific IgE detection was performed by coating 384-well ELISA plates with 20 μl of 20 μg/ml OVA overnight at 4°C and then incubating with diluted sera. All subsequent steps were the same as for total serum IgE. Sera were diluted with PBS: 1/50 for total serum IgE and 1/5 for OVA-specific IgE.

Cytokine concentrations were measured using mouse IL-4, IL-5, and IFN-γ ELISA sets (BD Biosciences) according to the manufacturer's instructions. IL-13 detection was performed with a murine IL-13 ELISA development kit (PeproTech) according to the manufacturer's instructions. The minimum detection limits for individual cytokines were: IL-4, 7.8 pg/ml; IL-5, 15.6 pg/ml; and IFN-γ, 31.3 pg/ml. BAL fluids used for cytokine detection were taken from the first lavage.

RT-PCR

RNA was extracted from lung tissues with TRIzol reagent (Invitrogen Life Technologies). RNA samples were then purified using RNase-free DNaseI (Qiagen), according to the manufacturer's instructions, and passed through RNeasy mini kits (Qiagen). RNA preparation, reverse transcription, and quantitative real-time PCR analysis with internal TaqMan probes were performed as described elsewhere (20). Primer sequences are shown in Table I or in Ref. 20.

Immunohistochemistry

One lobe of unlavaged lung was embedded in Tissue-Tek OCT compound (Proscitech), snap frozen, and sections (5 μm) were cut on a cryostat (Leica CM1900). Sections were fixed with methanol/acetone, air-dried, rinsed with PBS for 5 min, and incubated with 3% H_2O_2 for 1 min. The sections were incubated with blocking buffers, provided by Histomouse Max (Broad Spectrum DAB) kit (Zymed Laboratories), and stained with anti-mouse IFN-γ or CXCL10 mAb (R&D Systems) according to the manufacturer's instructions.

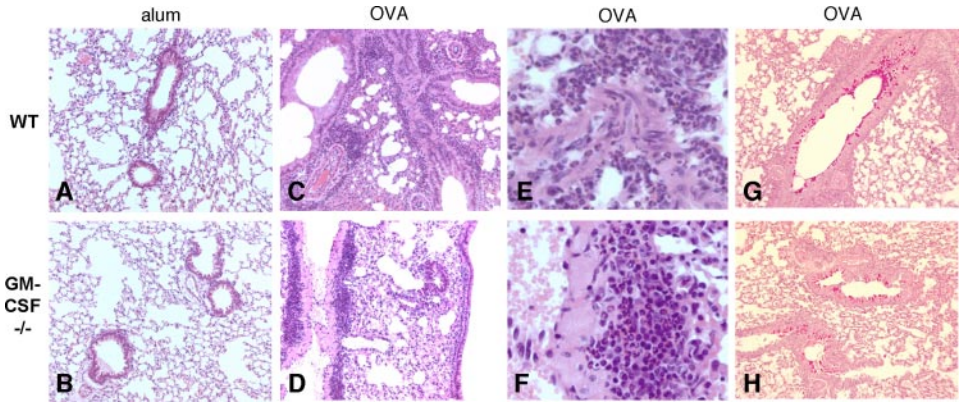

FIGURE 1. Peribronchial eosinophilia was reduced in GM-CSF$^{-/-}$ mice. The photomicrographs are representative of lungs from WT/alum mice (*A*), GM-CSF$^{-/-}$/alum mice (*B*), WT/OVA mice (*C*, *E*, and *G*), and GM-CSF$^{-/-}$/OVA mice (*D*, *F*, and *H*). Sections were stained with H&E (*A–F*) or PAS (*G* and *H*). *A–D* and *G* and *H* were photographed at ×100 magnification and *E* and *F* at ×400. The data shown are representative of two separate experiments.

AHR measurement

Mice were immunized i.p. with a single injection of 100 μg of OVA plus 0.1 ml of alum at day 0 and then were challenged, according to the standard protocol, four times at days 21, 23, 25, and 27 with 1% OVA aerosol. A single immunization with OVA/alum instead of two immunizations was considered more satisfactory for measuring AHR in C57BL/6 mice. Twenty hours after the last OVA challenge, mice were transported to the University of Newcastle and AHR was measured. Mice were anesthetized with i.p. ketamine-xylazine (80–100 mg/kg ketamine; 10 mg/kg xylazine, 100 μl/10 g body weight). Dynamic compliance and pulmonary resistance in response to increasing concentrations of inhaled β-methacholine were determined as previously described (21).

Statistical analysis

Data are presented as mean ± SEM. The two-tailed unpaired *t* test was used to determine statistical significance. Data were analyzed by Statview statistical software (version 5.0; Abacus Concepts). Values of $p < 0.05$ were considered statistically significant.

Results

Absence of eosinophils in peribronchial areas and BAL fluid in GM-CSF$^{-/-}$ mice

Lung histology was assessed 24 h after the last OVA challenge. Under low-power examination, control unimmunized WT mice given i.p. alum alone (WT/alum mice) had no cellular infiltration in the perivascular and peribronchial areas, and air spaces were intact (Fig. 1*A*). Similar results were found in unimmunized GM-CSF$^{-/-}$ mice (Fig. 1*B*). In OVA-immunized WT mice (WT/OVA mice), there was prominent cellular infiltrate in both perivascular and peribronchial areas. Tissue consolidation and loss of air space were also noted (Fig. 1*C*). By contrast, lung histology of OVA-immunized GM-CSF$^{-/-}$ mice showed marked cellular infiltrate in the perivascular areas (Fig. 1*D*, *left* of field), but not in the peribronchial areas (Fig. 1*D*, *right* of field). Under high power, the inflammatory cell infiltrate in the perivascular and peribronchial areas in the lungs of WT/OVA mice was shown to be ~60% eosinophils (Fig. 1*E*), as was the infiltrate in the perivascular areas in immunized GM-CSF$^{-/-}$ mice (Fig. 1*F*).

In PAS-stained sections, there was a substantial amount of mucus in the airway epithelium and the airway lumen of WT/OVA mice (Fig. 1*G*). Immunized GM-CSF$^{-/-}$ mice showed less mucus in the airway epithelium than the WT/OVA mice and little mucus occlusion in the airways (Fig. 1*H*). All sections were examined by an observer who was blinded to the treatment groups, and were scored for inflammation on a scale of 0–3. In immunized mice, eosinophils around airways and airway mucus production were significantly reduced in the GM-CSF$^{-/-}$ mice (Fig. 2). In the control groups given alum or no treatment, all scores were low. The data revealed allergic airway inflammatory features in both GM-CSF$^{-/-}$ and WT mice, but GM-CSF$^{-/-}$ mice show remarkably reduced cellular infiltrate in the peribronchial areas and reduced mucus production. These data suggest that GM-CSF is a key cytokine in mediating inflammation, especially in the recruitment of eosinophils to the airways.

Eosinophils were completely absent in the BAL fluid of immunized GM-CSF$^{-/-}$ mice, whereas there were abundant eosinophils in the BAL fluid in WT mice (Fig. 3). There were more lymphocytes and neutrophils in the GM-CSF$^{-/-}$ mice than in their WT counterparts (Fig. 3). In GM-CSF$^{-/-}$ mice, the absence of eosinophils in the BAL fluid (Fig. 3) was consistent with markedly reduced eosinophilic inflammation in the peribronchial lung tissue (Fig. 1). Blood eosinophil counts were determined at the time of tissue harvest. The levels in immunized GM-CSF$^{-/-}$ and WT

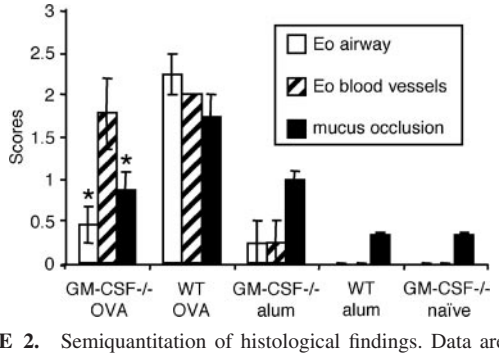

FIGURE 2. Semiquantitation of histological findings. Data are shown as mean ± SEM, $n = 6$. In columns without error bars, the SEM equals 0. *, $p < 0.05$ compared with WT/OVA mice. The data shown are representative of two separate experiments.

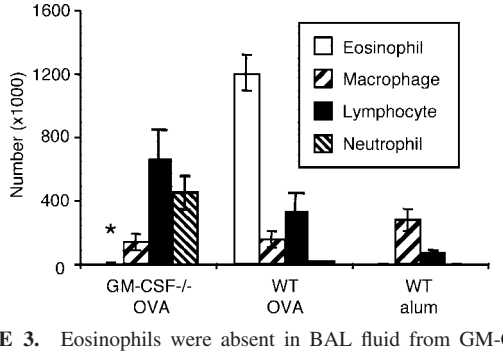

FIGURE 3. Eosinophils were absent in BAL fluid from GM-CSF$^{-/-}$ mice. Data are shown as mean ± SEM, $n = 6$. *, $p < 0.05$ compared with WT/OVA mice. The lymphocyte values were not significantly different between GM-CSF$^{-/-}$ and WT-immunized mice. The experiment shown is representative of two separate experiments.

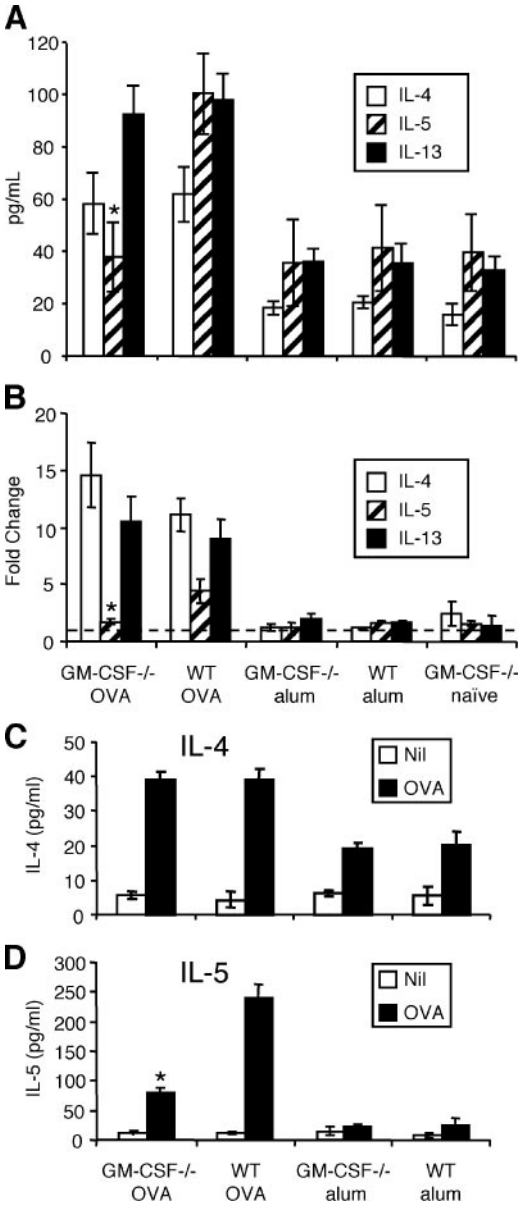

FIGURE 4. GM-CSF$^{-/-}$ mice had reduced IL-5 but normal IL-4 and IL-13. *A*, Cytokines were measured in BAL fluid by ELISA. *B*, Cytokine mRNA levels were determined by quantitative RT-PCR using the TaqMan probe technique. Data were normalized with β-actin. Data are expressed as fold change, with values from control untreated WT mice defined as 1, as indicated by the dashed line. *C* and *D*, Cytokines were measured after culture of PTLN cells with no stimulus or 10 μg/ml OVA for 48 h. Data are shown as mean ± SEM, $n = 6$. *, $p < 0.05$ compared with WT/OVA mice. The experiment shown is representative of two separate experiments.

mice were similar, 0.9 ± 0.1 and 0.6 ± 0.3, respectively (data are $\times 10^9$/L, mean ± SEM, $n = 6$). Therefore, the reduction in eosinophils in the peribronchial regions and BAL of GM-CSF$^{-/-}$ mice could not be attributed to any reduction in blood eosinophil levels.

Reduced IL-5 production in GM-CSF$^{-/-}$ mice

In BAL fluid, IL-5 was significantly reduced in immunized GM-CSF$^{-/-}$ mice compared with their WT counterparts (Fig. 4*A*). By contrast, BAL fluid IL-4 and IL-13 was similar in the immunized GM-CSF$^{-/-}$ and WT mice. In the unimmunized control groups, the levels of these cytokines were low. The IL-5 in the immunized GM-CSF$^{-/-}$ mice was not significantly different from that in the unimmunized groups.

Similar results were obtained for mRNA in lung tissue. IL-5 mRNA was significantly lower in immunized GM-CSF$^{-/-}$ mice compared with their WT counterparts. (Fig. 4*B*). In the immunized GM-CSF$^{-/-}$ mice, IL-5 mRNA was similar to that in the unimmunized groups. By contrast, IL-4 and IL-13 mRNA were increased to a similar extent in both immunized groups. In the unimmunized groups, IL-4, IL-5, and IL-13 mRNA levels were very low, similar to the basal levels of these cytokines in completely untreated WT mice.

When PTLN cells were cultured with OVA in vitro, IL-4 and IL-5 secretion was increased in immunized WT mice compared with unimmunized mice. In cultures from immunized GM-CSF$^{-/-}$ mice, IL-5 was markedly lower than in WT mice and almost as low as in the unimmunized groups. By contrast, IL-4 production was similar in immunized GM-CSF$^{-/-}$ and WT mice (Fig. 4, *C* and *D*). The cytokine data from BAL, lung tissue, and PTLN of immunized animals, taken together, demonstrate a consistent pattern of much lower IL-5 in GM-CSF$^{-/-}$ compared with WT mice, whereas levels of IL-4 and IL-13 were not affected by the absence of functional GM-CSF genes.

No effect on IgE and AHR in GM-CSF$^{-/-}$ mice

Total and Ag-specific serum IgE levels were significantly higher in immunized mice. GM-CSF deficiency did not alter either total IgE or OVA-specific IgE production (Fig. 5). In the AHR study, immunized WT mice showed significantly reduced dynamic compliance (Fig. 6*A*) and increased pulmonary resistance (Fig. 6*B*) compared with unimmunized WT mice, indicating the successful induction of AHR in immunized mice. The AHR data in immunized GM-CSF$^{-/-}$ mice were very similar to their WT counterparts, with no statistically significant difference between the two groups. These data indicate that GM-CSF deficiency did not have a detectable effect on serum IgE or AHR.

Reduced lung CCR3 mRNA in GM-CSF$^{-/-}$ mice

CCR3 is an important chemokine receptor on eosinophils, as mice lacking functional CCR3 genes show impaired eosinophil recruitment to the lungs in a model of allergic airway inflammation (22). Therefore, changes to CCR3 or its ligands could explain the lack of infiltration of eosinophils in the airways of GM-CSF$^{-/-}$ mice. Lung mRNA levels of two CCR3 ligands, CCL11 and CCL5, were increased in immunized mice and were similar in the GM-CSF$^{-/-}$ and WT groups (Fig. 7). mRNA levels in the unimmunized alum-treated groups were lower and close to those in completely untreated mice. The lack of a significant difference in CCL11 or CCL5 between immunized WT and GM-CSF$^{-/-}$ mice indicates that the reduction in peribronchial eosinophils in GM-CSF$^{-/-}$ mice is not associated with reduced chemokine mRNA expression. Lung CCR3 mRNA levels were lower in immunized GM-CSF$^{-/-}$ mice compared with the immunized WT group (Fig. 7). CCR3 mRNA in the alum groups was close to the basal levels of untreated mice. Therefore, although there are fewer eosinophils in the lungs of GM-CSF$^{-/-}$ mice, there is no evidence that they lack CCR3 expression.

Increased IFN-γ and IFN-γ-inducible chemokines in GM-CSF$^{-/-}$ mice

Levels of the key Th1 cytokine IFN-γ were assessed, as we hypothesized that reduced airway eosinophils may be related to induction by IFN-γ of CCR3 antagonist chemokines. IFN-γ protein was increased in the BAL fluid of immunized GM-CSF$^{-/-}$ mice (Fig. 8) compared with WT mice. IFN-γ was increased in the BAL

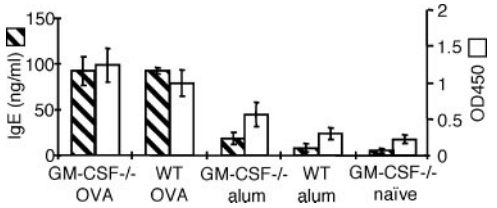

FIGURE 5. Serum IgE was not affected in GM-CSF$^{-/-}$ mice. Total serum IgE (▨, ng/ml) and OVA-specific IgE (□, OD units) are shown. Data are shown as mean ± SEM, $n = 6$. The experiment shown is representative of two separate experiments.

fluid of unimmunized GM-CSF$^{-/-}$ mice, both in the alum-treated and untreated groups, although the IFN-γ levels in these groups were not as high as in immunized GM-CSF$^{-/-}$ mice. Similarly, IFN-γ mRNA levels in the lung tissue of GM-CSF$^{-/-}$ mice were increased (Fig. 8). These data suggest that GM-CSF is important in regulating IFN-γ expression. In the lungs of GM-CSF$^{-/-}$ mice, regardless of OVA immunization, there were peribronchial lymphoid aggregates in some areas, as previously described (19). IFN-γ was detected in these lymphoid aggregates of GM-CSF$^{-/-}$ mice by immunohistochemical staining, both in OVA-immunized and in unimmunized mice (Fig. 9).

IFN-γ induces the production of the chemokines CXCL9, CXCL10, and CXCL11 that are ligands for CXCR3 (14, 15) and antagonists of CCR3 (16, 17). mRNAs of all of these chemokines were significantly increased in immunized GM-CSF$^{-/-}$ mice compared with immunized WT mice (Fig. 10). Levels in the immunized WT group were similar to those of the control unimmunized groups. In immunohistochemistry, CXCL10 staining was associated with pulmonary lymphoid aggregates in GM-CSF$^{-/-}$ mice in both OVA-immunized and alum control groups (Fig. 9). These data suggest that deficiency of GM-CSF induces increased amounts of IFN-γ, which in turn induces higher levels of the IFN-γ-inducible chemokines CXCL9, CXCL10, and CXCL11. CXCR3 mRNA was also significantly higher in immunized GM-CSF$^{-/-}$ mice than in their WT counterparts (Fig. 10). Thus, increased production of IFN-γ-inducible chemokines in the immunized GM-CSF$^{-/-}$ mice may have attracted increased numbers of CXCR3-expressing cells into the lung tissue.

Discussion

This study reveals a striking requirement for GM-CSF in a murine model of allergic asthma. After allergen aerosol challenge, lung

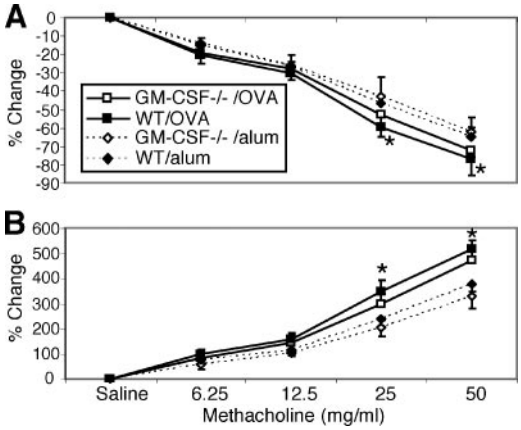

FIGURE 6. AHR was not affected in GM-CSF$^{-/-}$ mice. *A*, Dynamic compliance; *B*, lung resistance. Data are shown as mean ± SEM. *, $p < 0.05$ indicates WT/OVA mice compared with WT/alum mice.

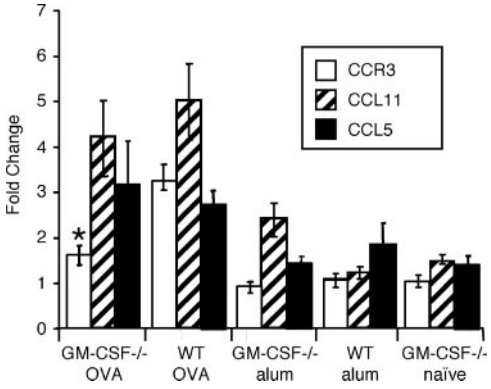

FIGURE 7. CCR3 was reduced, but CCL11 and CCL5 were not affected in GM-CSF$^{-/-}$ mice. CCR3, CCL11, and CCL5 lung mRNA levels were determined by quantitative RT-PCR as in Fig. 4. Data are expressed as fold change, with values from control untreated WT mice defined as 1, as indicated by the dashed line. *, $p < 0.05$ compared with WT/OVA mice, $n = 6$. In the GM-CSF$^{-/-}$ groups, although CCR3 was higher in OVA-treated than in alum-treated mice, the difference did not reach statistical significance ($p = 0.083$). The data shown are representative of two separate experiments.

histology demonstrated very few inflammatory cells in the peribronchial areas in GM-CSF$^{-/-}$ mice, although they were abundant in the perivascular regions (Fig. 1*D*). By contrast, in WT mice, inflammatory cells, especially eosinophils, were plentiful in both peribronchial and perivascular areas. These results were consistent with findings in the cellular composition of BAL fluid, in which eosinophils were absent in GM-CSF$^{-/-}$ mice (Fig. 3). Another striking finding is the markedly reduced production of IL-5 in the GM-CSF-deficient mice (Fig. 4).

Activated eosinophils are a source of both GM-CSF and IL-5 (23, 24). Both cytokines maintain eosinophil survival (25, 26) and can act on eosinophils in an autocrine fashion (27, 28). The reduction in pulmonary eosinophils in the GM-CSF$^{-/-}$ mice may be due in part to the loss of GM-CSF survival signals on eosinophils. The reduction in eosinophil numbers could account for the reduction in IL-5 levels in the lungs and BAL fluid of GM-CSF$^{-/-}$ mice (Fig. 4). Alternatively, reduced production by T cells could account for lower IL-5 levels in the GM-CSF$^{-/-}$ mice. The reduced IL-5 production in draining lymph nodes of GM-CSF$^{-/-}$ mice (Fig. 4*D*) most likely reflects an effect on T cell production of IL-5. Regardless of the source, reduced IL-5 in the lungs of the GM-CSF$^{-/-}$ mice could affect the survival of eosinophils and could contribute to reduced eosinophil numbers in the peribronchial tissue. Alternatively, IL-5 has been reported to stimulate eosinophil chemokinesis (29). Reduced IL-5 in the lungs might be associated

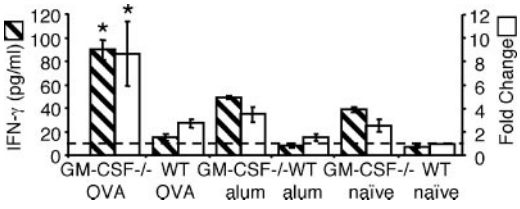

FIGURE 8. Lung IFN-γ was increased in GM-CSF$^{-/-}$ mice. IFN-γ in BAL fluid was measured by cytokine ELISA (▨, pg/ml) and lung IFN-γ mRNA was determined by quantitative RT-PCR (□, fold change) as in Fig. 4. The dashed line indicates mRNA values in control untreated WT mice, defined as 1. Data are shown as mean ± SEM, $n = 6$. *, $p < 0.05$ compared with WT/OVA mice. The experiment shown is representative of two separate experiments.

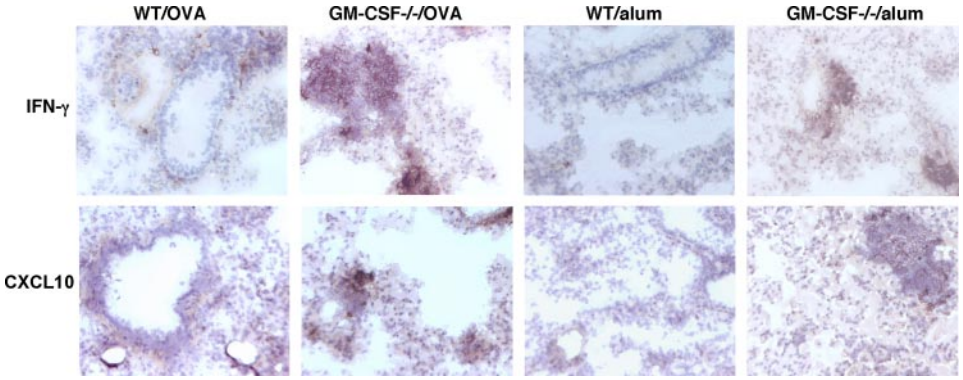

FIGURE 9. IFN-γ and CXCL10 staining was detected in lymphoid aggregates in GM-CSF$^{-/-}$ mice. Immunohistochemical staining was performed and representative lung sections are shown. The genotype and treatment are indicated above each panel. Sections in *upper row* are stained for IFN-γ and in the *lower row* for CXCL10.

with reduced eosinophil chemokinesis and therefore reduced numbers of these cells in the peribronchial tissue. The blood levels of eosinophils were similar in WT and GM-CSF$^{-/-}$ mice. These data suggest that in the GM-CSF$^{-/-}$ mice, eosinophils are produced normally, but their migration or survival in the lungs is deficient.

IL-4 levels were not affected in GM-CSF$^{-/-}$ mice (Fig. 4), nor was serum IgE (Fig. 5). Because IL-4 is essential for IgE production in mice (30), the lack of effect of GM-CSF deficiency on IL-4 is consistent with the IgE data. Although IL-4 and IL-5 are commonly coproduced, dissociation of these two cytokines has been reported (31). There was no effect on AHR, an important parameter of allergic asthma, in GM-CSF$^{-/-}$ mice (Fig. 6). The data indicate that GM-CSF does not play an important role in mediating AHR in this model. IL-13 is the principal cytokine required for AHR (32). IL-13 levels were not affected by GM-CSF deficiency (Fig. 4). Therefore, the lack of effect of GM-CSF deficiency on IL-13 is consistent with the AHR results. Airway mucus was reduced in GM-CSF$^{-/-}$ mice (Fig. 1). This may be a direct consequence of loss of effects of GM-CSF on the airway epithelium, because GM-CSF receptors have been reported on airway epithelial cells in the rat (33).

In models of allergic diseases, Ab neutralization of GM-CSF attenuated eosinophil inflammation and AHR (9, 11). The phenotype in GM-CSF$^{-/-}$ mice in the present study is similar, but not identical, to these reports. The principal differences are the selective absence of eosinophils in the peribronchial areas in GM-CSF$^{-/-}$ mice, which was not described in the Ab studies, and the normal AHR in the GM-CSF$^{-/-}$ mice compared with reduced AHR in the Ab reports. These differences may have arisen because of differences in mouse strains and sensitization and challenge protocols. Furthermore, complex compensatory changes in GM-CSF knockout mice may produce different findings to studies using Ab neutralization.

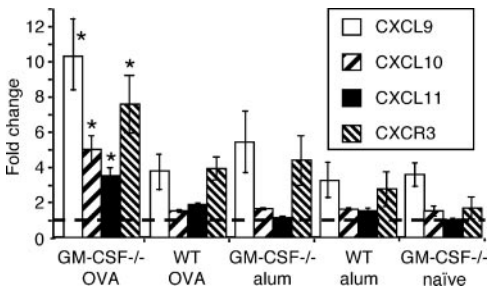

FIGURE 10. mRNA for IFN-γ-inducible chemokines and CXCR3 were increased in GM-CSF$^{-/-}$ mice. mRNA levels were determined by quantitative RT-PCR as in Fig. 4. Data are shown as mean ± SEM, $n = 6$. *, $p < 0.05$ compared with WT/OVA mice. The experiment shown is representative of two separate experiments.

In allergic GM-CSF$^{-/-}$ mice, CCR3 mRNA levels in the lung were significantly reduced, but mRNA levels of the CCR3 ligands CCL11 and CCL5 were not affected (Fig. 7). The absence of eosinophils in peribronchial areas in allergic GM-CSF$^{-/-}$ mice (Fig. 1) is consistent with the reduced levels of CCR3 in total lung mRNA, because CCR3 is principally expressed on eosinophils (34) and eosinophils are abundant in WT animals in this model. In CCR3$^{-/-}$ mice, eosinophil accumulation in airways and AHR were reduced in allergic airway inflammation (22, 35). Similarly, a recent report indicates that orally administered small molecule CCR3 antagonists selectively reduced pulmonary eosinophilic inflammation (36). In CCR3$^{-/-}$ mice, eosinophils were limited to the subendothelial space, indicating that they were incapable of crossing the elastic tissue into the lung parenchyma (22). The reduction in pulmonary eosinophils in these reports is reminiscent of our study on GM-CSF$^{-/-}$ mice. However, eosinophils in the GM-CSF$^{-/-}$ mice did reach the lung parenchyma adjacent to the vessels, but were not found near the airways (Fig. 1). In the CCR3$^{-/-}$ mice, there was reduced AHR (35), whereas in the GM-CSF$^{-/-}$ mice, AHR was not affected (Fig. 6). These data indicate that CCR3$^{-/-}$ mice have a similar but not identical defect to GM-CSF$^{-/-}$ mice in allergic asthma models.

High levels of the Th1 cytokine IFN-γ mRNA and protein were found in the lungs of GM-CSF$^{-/-}$ mice (Fig. 8). Although allergic asthma has been considered a Th2-driven disease, increased IFN-γ production has been reported in human studies (37) and in mouse models (38). Although the Th1 cytokine IFN-γ was overproduced in GM-CSF$^{-/-}$ mice, the Th2 cytokines IL-4 and IL-13 were not reduced (Fig. 4), indicating that deficiency of GM-CSF is not associated with a simple switch from a Th2 to a Th1 response. In the present study, it is not surprising that high levels of mRNA of the IFN-γ-inducible chemokines CXCL9, CXCL10, and CXCL11 and their receptor CXCR3 were found in GM-CSF$^{-/-}$ mice (Fig. 10). These chemokines competitively inhibit the binding of CCR3 ligands to their receptor (16, 17). We conclude that high expression of CXCL9, CXCL10, and CXCL11 in the lung may contribute to the absence of eosinophils in the peribronchial regions.

GM-CSF$^{-/-}$ mice have abnormal lungs, with surfactant accumulation in the alveoli, when the mice are older than 12 wk, and the presence of focal lymphoid aggregates from 3 wk of age (19). This disease is similar to human pulmonary alveolar proteinosis (PAP), which is usually caused by Abs to GM-CSF that impair surfactant clearance by pulmonary macrophages (39). GM-CSF$^{-/-}$ mice and patients with PAP are at increased risk of pulmonary infections (19, 39). Our findings of defective allergic airway inflammation in GM-CSF$^{-/-}$ mice provide further characterization of the extent of the immunodeficiency associated with the lack of GM-CSF. In the present study, the immunization protocol was started in 6- to 8-wk-old GM-CSF$^{-/-}$ mice to minimize

the impact of PAP. Nevertheless, PAP and the lymphoid aggregates may have contributed to the phenotype of the GM-CSF$^{-/-}$ mice. IFN-γ and CXCL10 were readily detected in these lymphoid aggregates (Fig. 9). Thus, IFN-γ inducible chemokines, derived from the pulmonary aggregates, may contribute to the lack of eosinophils in the airways of the GM-CSF$^{-/-}$ mice. Furthermore, it is possible that pulmonary infection in the GM-CSF$^{-/-}$ mice could contribute to this phenotype. In this context, it is notable that IFN-γ was significantly elevated in the BAL of untreated GM-CSF$^{-/-}$ mice (Fig. 8), indicating a potential for skewing to a Th1 response before experimental intervention. This raises the possibility that the reduced eosinophilic inflammation in the GM-CSF$^{-/-}$ mice may be caused by the pulmonary abnormalities of these animals, rather than by a defect in allergic inflammatory reactions. However, a study on inflammation in another site, the peritoneal cavity, reported fewer eosinophils in the peritoneal lavage of GM-CSF$^{-/-}$ mice compared with that of WT animals (40). These findings indicate that defects in tissue eosinophilia are not limited to the lung, but are a general feature of GM-CSF$^{-/-}$ mice.

In conclusion, we propose possible mechanisms to explain the absence of eosinophils in the airways of GM-CSF$^{-/-}$ mice. Eosinophil viability may be directly impaired by the absence of GM-CSF and further affected by reduced IL-5 production. Our findings are relevant to the interpretation of clinical trials in asthma on the effect of neutralizing Abs to IL-5, which showed little benefit. In treated patients, substantial numbers of eosinophils were detected in bronchial biopsies, and it was suggested that GM-CSF may have contributed to eosinophil survival in the tissues (41). A second mechanism in GM-CSF$^{-/-}$ mice involves excessive IFN-γ production in the airways, eliciting the generation of IFN-γ-inducible chemokines, which may antagonize CCR3 ligands and impair eosinophil migration to the airways. Both survival of eosinophils and effects on their migration to peribronchial areas most likely contribute to the phenotype in our studies with GM-CSF mice. Regardless, our findings provide further evidence for the important role of GM-CSF in allergic airway inflammation.

Disclosures

The authors have no financial conflict of interest.

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