Negative regulation of eosinophil recruitment to the lung by the chemokine monokine induced by IFN-γ (Mig, CXCL9)

Patricia C. Fulkerson*, Nives Zimmermann[†], Eric B. Brandt[†], Emily E. Muntel[†], Matthew P. Doepker[†], Jessica L. Kavanaugh[†], Anil Mishra[†], David P. Witte[‡], Hongwei Zhang[§], Joshua M. Farber[§], Ming Yang[¶], Paul S. Foster[¶], and Marc E. Rothenberg[†‖]

*Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45257-0524; [†]Division of Allergy and Immunology, [‡]Division of Pathology, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039; [§]Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Room 11N-228, Building 10, Bethesda, MD 20892-1888; and [¶]Division of Molecular Bioscience, John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra 0200, Australia

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Experimental analysis of allergic airway inflammation (AAI) in animals and humans is associated with coordinate gene induction. Using DNA microarray analysis, we have identified a large panel of AAI signature genes. Unexpectedly, the allergen-challenged lung (a T helper 2 microenvironment) was found to be associated with the expression of T helper 1-associated CXCR3 ligands, monokine induced by IFN-γ (Mig), and IFN-γ-inducible protein of 10 kDa (IP-10). Here we report that Mig functions as a negative regulator of murine eosinophils. Whereas Mig was not able to induce chemotaxis of eosinophils, pretreatment with Mig induced a dose-dependent inhibition of chemoattractant-induced eosinophil transmigration *in vitro*. Moreover, i.v. administration of low doses of Mig ($\approx$10–30 μg/kg) induced strong and specific dose-dependent inhibition of chemokine-, IL-13-, and allergen-induced eosinophil recruitment and, conversely, neutralization of Mig before allergen challenge increased airway eosinophilia. Importantly, Mig also inhibited a CCR3-mediated functional response in eosinophils. These results indicate that the ultimate distribution and function of inflammatory cells within the allergic lung is dictated by a balance between positively and negatively regulatory chemokines. The identification of a naturally occurring eosinophil inhibitory chemokine pathway *in vivo* provides a strategic basis for future therapeutic consideration.

allergy | asthma | cytokine | eotaxin

Eosinophil accumulation in the blood and tissues is a hallmark feature of several important medical diseases, including atopic disorders, parasitic infections, and numerous systemic diseases (e.g., Churg–Strauss syndrome, eosinophilic gastroenteritis, and the idiopathic hypereosinophilic syndrome) (1, 2). The finding that eosinophils normally account for only a small percentage of circulating or tissue-dwelling cells and that their numbers markedly and selectively increase under specific disease states indicates the existence of molecular mechanisms that tightly regulate the selective generation and accumulation of these leukocytes. Numerous mediators have been identified as eosinophil chemoattractants, including diverse molecules such as lipid mediators (platelet activating factor, leukotrienes) and recently chemokines such as the eotaxin subfamily of chemokines (3, 4). However, to date, a role for naturally occurring inhibitory cytokines of eosinophil chemoattraction *in vivo* has not been identified.

During induction of eosinophil-associated allergic airway inflammation (AAI), leukocyte tissue recruitment is orchestrated by the coordinated induction of chemokines (3, 5). Focusing on eosinophils, a paradigm has emerged implicating the T helper (Th)2 cytokines, IL-4 and IL-13, in the induction of eosinophil-active chemokines that signal through CCR3, a chemokine receptor selectively expressed on eosinophils. In contrast, Th1 cytokines (e.g., IFN-γ) induce a different set of chemokines [e.g., IFN-γ-inducible protein of 10 kDa (IP-10, CXCL10), monokine induced by IFN-γ (Mig, CXCL9), and IFN-inducible T cell chemoattractant (I-TAC, CXCL11)] (3, 6). These chemokines are unique in that they selectively signal through CXCR3, a receptor expressed on activated T cells (preferentially of the Th1 phenotype). This Th1 and Th2 chemokine dichotomy may be even more complex in view of recent publications suggesting that these Th1- and Th2-associated chemokines may inhibit CCR3 and CXCR3, respectively. For example, human CXCR3 ligands have been demonstrated to be CCR3 antagonists, inhibiting the action of CCR3 ligands on human eosinophils and CCR3^{+} cells *in vitro* (7, 8). In addition, eotaxin has been reported to be an antagonist for CXCR3 (9). These results suggest a feedback loop by which Th1- and Th2-associated chemokines coordinately regulate eosinophil responses, but this has not been proven *in vivo*. Recently, we have taken an empiric approach to define a broad spectrum of genes associated with induction of AAI (experimental asthma) in mice (10). In the 291 "AAI signature genes" identified, we found overexpression of expected Th2-associated cytokines [IL-4, eotaxin-1, monocyte chemoattractant protein (MCP)-1, MCP-2]; however, several Th1-associated chemokines were also upregulated. In this manuscript, we focus attention on the paradoxical induction of CXCR3 ligands (Th1 regulated) during experimental AAI in mice, and we report an inhibitory role for the CXCR3 ligand Mig in regulating eosinophil chemoattraction. Mig potently inhibits eosinophil migration *in vitro* and markedly attenuates eosinophil lung recruitment to diverse stimuli, including chemokines, IL-13, and allergen *in vivo*. In addition, Mig effectively inhibited a CCR3-mediated functional response. As such, these results demonstrate the existence of naturally occurring eosinophil-inhibitory cytokines, such as Mig, identifying a pathway with potential therapeutic significance.

Materials and Methods

Mice. Eight- to 12-week-old male and female CD2-IL-5 transgenic, WT mice (National Cancer Institute, Frederick, MD) and signal transduction and activator of transcription (STAT)6-deficient mice (The Jackson Laboratory) of the BALB/c background were maintained as described (11), according to institutional guidelines. The CD2–IL-5 transgenic mice (BALB/c) were used as a source of eosinophils, as reported (12).

Abbreviations: AAI, allergic airway inflammation; Th, T helper; IP-10, IFN-γ-inducible protein of 10 kDa; Mig, monokine induced by IFN-γ; STAT, signal transduction and activator of transcription; OVA, ovalbumin; i.n., intranasal; i.t., intratracheal; BALF, bronchoalveolar lavage fluid; NBT, nitroblue tetrazolium.

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

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Experimental AAI Induction and Cytokine Challenge Models. The ovalbumin (OVA)-induced AAI model was generated as described (13). *Aspergillus fumigatus* antigen-induced AAI was stimulated by 3 weeks of mucosal sensitization with repeated intranasal (i.n.) administration, as described (13). Eotaxin-induced eosinophilia was generated by administration of 3 μg of recombinant eotaxin (a kind gift of PeproTech, Rocky Hill, NJ) by i.n. delivery, according to a previous publication (14). For intravenous (i.v.) chemokine delivery, 200 μl (1 μg) of the recombinant chemokine (PeproTech) or saline was injected into the lateral tail vein 30 min before intratracheal (i.t.)/i.n. cytokine or allergen delivery. Some mice were treated with 500 μg of neutralizing rabbit polyclonal anti-murine Mig (prepared by J. M. Farber) or rabbit IgG control 24 h before allergen challenge. Subsequently, the bronchoalveolar lavage fluid (BALF) and/or lung tissue was harvested 18–24 h after challenge. For i.t. delivery of IL-13, mice were anesthetized with ketamine (5 mg/100 μl) and hung upright, and 20 μl of recombinant cytokine or saline was delivered into the trachea with a Pipetman (Gilson, Middleton, WI). Mice were treated i.t. with recombinant IL-13 (a kind gift of Debra Donaldson, Wyeth Laboratory, Cambridge, MA) on days 0 (4 μg) and 2 (10 μg), before BALF and lung tissue harvest 36 h later.

Microarray Data Analysis. Microarray hybridization was performed by the Affymetrix Gene Chip Core facility at Cincinnati Children's Hospital Medical Center as described (10). The analysis was performed with one mouse per chip ($n \geq 3$ for each allergen challenge condition and $n \geq 2$ for each saline challenge condition).

Northern Blot Analysis. Lung RNA (10–20 μg) was subjected to Northern blot analysis as described (10). Mig and IP-10 cDNA probes were kind gifts of A. D. Luster (Massachusetts General Hospital, Boston).

Cytokine Quantitation. Cytokine protein concentration in the BALF of allergen- and saline-challenged mice was quantified by using a DuoSet ELISA Development kit specific for Mig/CXCL9 (R & D Systems); the detection limit was 0.9 pg/ml.

Eosinophil Quantitation. BALF differential cell counts and lung tissue eosinophils identified by anti-major basic protein (MBP) staining were performed as reported (13).

***In Situ* Hybridization.** *In situ* hybridization was performed as described (10). In brief, murine Mig cDNA in pBluescript (Stratagene) was linearized by *Eco*RI or *Not*I digestion, and antisense and sense RNA probes, respectively, were generated by T7 and SP6 RNA polymerase (Riboprobe Gemini Core Systems II transcription kit, Promega). The radiolabeled (α-[^{35}S]thio-UTP) probes were hybridized and washed under high-stringency conditions.

Chemotaxis Assay. All chemotactic responses were determined by transmigration through respiratory epithelial cells as described (15). Leukocytes (1.5×10^6) were placed in the upper chamber and the chemoattractant (eotaxin-2 at 1 ng/ml or eotaxin-1 at 10 ng/ml) was placed in the lower chamber. Eosinophils were obtained by immunomagnetic negative selection of splenocytes from IL-5 transgenic mice (12). Pretreated cells were incubated with chemokine (Mig, JE, or eotaxin-2) for 15 min at 37°C and then washed twice to remove chemokine from the medium. Transmigration was allowed to proceed for 1.5–3 h.

Eosinophil Mobilization. BALB/c mice were administered i.v. eotaxin (1 μg) alone, eotaxin (1 μg) and Mig (1 μg) together, or saline in 200 μl. After 1 h, mice were bled from the tail vein. Blood was diluted (1:10) in Discombe's solution prepared as described (16).

Flow Cytometry. Cells (5×10^5) were washed with fluorescence-activated cell sorting buffer (2% BSA/0.1% NaN_3 in PBS) and incubated with 150 ng (1.5 μg/ml) of phycoerythrin-conjugated anti-murine CCR3 antibody (R & D Systems), 300 ng (3 μg/ml) of anti-murine CXCR3 (a generous gift of Jerry Di Salvo, Merck Research Laboratories, Rahway, NJ), 1 μg (10 μg/ml) of FITC-conjugated anti-murine CD4 (BD Biosciences Pharmingen) or the isotype-matched control IgG for 30 min at 4°C. After two washes in fluorescence-activated cell sorting buffer, cells probed for CXCR3 were incubated with FITC-conjugated isotype-specific secondary antibody (Pharmingen) for 30 min at 4°C in the dark. After two washes, labeled cells were subjected to flow cytometry on a FACScan flow cytometer (Becton Dickinson) and analyzed by using CELLQUEST software (Becton Dickinson). Internalization of surface CCR3 was assayed as reported (15).

Eosinophil Oxidase Activity Assay. Reduction of nitroblue tetrazolium (NBT; Sigma) was used as a measure of superoxide production. Cells were seeded onto chamber slides in RPMI medium 1640 for 1 h at 37°C to allow cells to adhere, and then stimulated with eotaxin-1 (10 nM), Mig (100 nM), or buffer alone in saturated NBT solution for 20 min at 37°C. PBS-washed cells were fixed with methanol and counterstained with safranin O (Sigma). At least 200 cells were examined to determine the percentage of NBT^+ cells.

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

Results

Induction of CXCR3 Ligands in Experimental AAI. We were first interested in identifying genes that were differentially expressed in a well established model of eosinophilic AAI. Three or 18 h after allergen challenge, lung RNA was subjected to microarray analysis using the Affymetrix chip U74Av2, which contains oligonucleotide probe sets representing 12,422 genetic elements (10). Of the allergen-induced genes, it was notable that chemokines represented a large subset; 14 of the 27 chemokines represented on the chip were induced compared with saline-challenged control mice. In particular, there was strong induction of the Th1-associated chemokines Mig (Fig. 1*a*), and IP-10 (Fig. 1*b*). To verify that the microarray data reflected gene induction, we examined Mig and IP-10 expression by using Northern blot analysis. Indeed, Mig and IP-10 mRNA were strongly induced after allergen challenge, and the kinetic pattern mimicked the microarray data (Fig. 1*c*). Analysis of Mig protein expression in the lungs of OVA-challenged mice revealed an increase in Mig expression (175 ± 81 pg/ml; mean ± SD, $n = 4$ mice per group) in comparison with saline-challenged mice (<0.9 pg/ml). In an attempt to further define the cellular sources of Mig, we performed *in situ* hybridization for Mig mRNA. Antisense staining of asthmatic lung revealed high levels of Mig in the perivascular and peribronchial inflammatory regions (Fig. 1*d* and data not shown). In contrast, the antisense probe did not detect significant staining in the saline-challenged lung (data not shown). Additionally, no specific staining with the sense probe in OVA-challenged mice was seen (data not shown).

We were next interested in determining whether the induction of Mig and IP-10 was limited to the OVA model of eosinophilic AAI. We therefore induced experimental AAI in naive mice with repeated i.n. doses of *A. fumigatus* antigens. Compared with mice challenged with saline, *A. fumigatus* antigen-challenged mice had marked Mig and IP-10 expression (Fig. 2*a*). The level of Mig protein expression in the BALF increased from 1.3 ± 1.2 to 12.8 ± 7.5 pg/ml (mean ± SD, $n = 3$ or 4 mice per group). Thus, the induction of Mig and IP-10 by allergen challenge was not specific to the antigen used, confirming that these chemokines were indeed AAI signature genes.

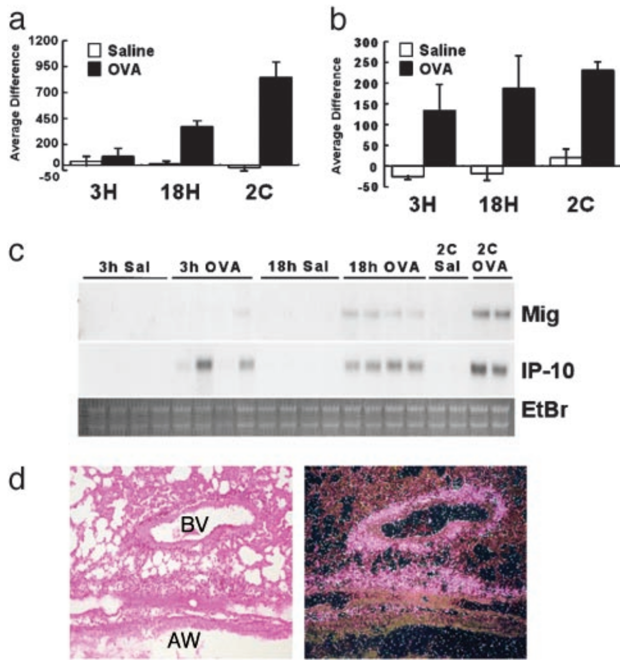

Fig. 1. Mig and IP-10 mRNA expression in OVA-induced AAI. (*a* and *b*) The average difference (mean ± SD, $n = 2$ or 3 mice per group) for the microarray hybridization signal of Mig (*a*) and IP-10 (*b*) in saline- and OVA-treated mice. (*c*) Northern blot analysis of Mig and IP-10 mRNA expression in saline- (Sal) and OVA-challenged mice. Ethidium bromide (EtBr) staining of the RNA gels is also shown. Each lane represents RNA from a single mouse. Time points: 3H, 3 h after one challenge; 18H, 18 h after one challenge; and 2C, 18 h after two challenges. (*d*) *In situ* hybridization of Mig mRNA in the lung of OVA-induced allergic mice. The staining pattern of Mig antisense riboprobe in corresponding bright-field (*Left*) and dark-field (*Right*) images of the OVA-challenged lungs are shown. BV, blood vessel; AW, airway.

Mig Is Negatively Regulated by STAT6 in Experimental AAI. Having identified Mig as a gene associated with allergic airway responses, we were next interested in identifying factors involved in regulating Mig expression. We examined the role of STAT6 because this transcription factor has been identified as a critical regulator of Th2 responses, including allergen-induced chemokine expression (17–19). For example, as a positive control, allergen-induced eotaxin-2 expression was completely STAT6 dependent (Fig. 2*b*). However, allergen-induced Mig expression was enhanced in the lungs of mice that were STAT6 deficient, when compared with mice that were WT (Fig. 2*b*). These results indicate that the molecular signals that regulate allergen-induced Mig are different from those that regulate allergen-induced eotaxins.

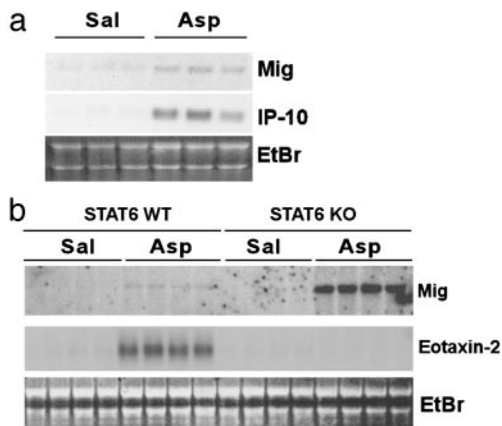

Fig. 2. Regulation of Mig expression. (*a*) Northern blot analysis of Mig and IP-10 mRNA expression after repeated doses of i.n. saline (Sal) or *A. fumigatus* antigen (Asp) treatment. (*b*) Northern blot analysis of Mig and eotaxin-2 mRNA expression in WT and STAT6-deficient mice after saline or *A. fumigatus* antigen treatment. Each lane represents a separate mouse. The ethidium bromide (EtBr) staining of the RNA gel is shown as a control for RNA loading.

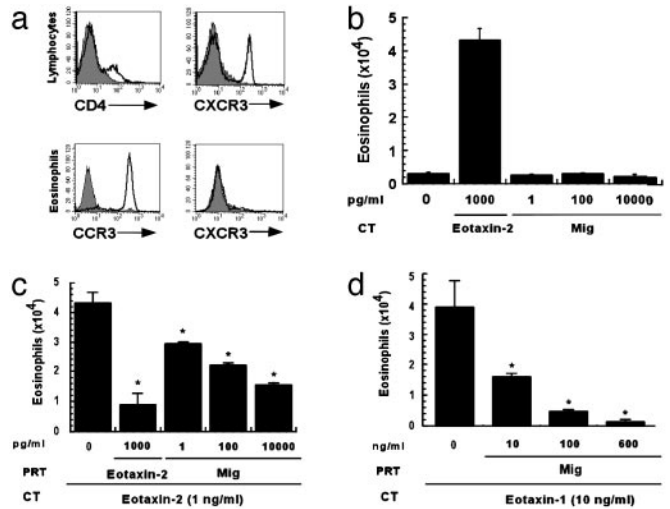

Fig. 3. Murine eosinophils lack CXCR3 on their surface and fail to migrate toward Mig. (*a*) Lymphocytes express CXCR3, but eosinophils have no detectable CXCR3 on their surface (all cells were derived from the spleen of IL-5 transgenic mice). Filled histogram, isotype-matched control; solid line, CCR3, CXCR3, or CD4. These results are representative of three experiments. (*b*) Transmigration of eosinophils in response to indicated doses of Mig. Cells (1.5×10^6) were allowed to transmigrate in response to the chemoattractants (CT) Mig and eotaxin-2. Data represent mean ± SD of eosinophils that migrated through a layer of respiratory epithelial cells. The results are representative of three experiments. (*c* and *d*) Mig inhibits eosinophil movement toward various concentrations of eotaxin-2 and eotaxin-1 *in vitro*. Cells were allowed to transmigrate after pretreatment (PRT) with buffer, Mig, or eotaxin-2. Data represent mean ± SD of eosinophils that migrated toward eotaxin-2 or eotaxin-1. *, $P < 0.05$. The results are representative of three experiments.

Murine Eosinophils Do Not Migrate in Response to Mig. A subset of human eosinophils, especially after cytokine treatment, has been reported to express the Mig receptor CXCR3 (20). To determine whether allergen-induced expression of Mig could be responsible, at least in part, for eosinophil lung recruitment, we examined CXCR3 expression on murine eosinophils. Although significant CXCR3 expression was demonstrated on lymphocytes, no CXCR3 was detected on the surface of murine eosinophils (Fig. 3*a*). Similar results were observed with murine eosinophils isolated from distinct sources (e.g., from the lung of IL-4/IL-5 bitransgenic mice and the lungs of allergen-challenged asthmatic mice; data not shown). Consistent with the absence of CXCR3 expression, murine eosinophils did not respond to a full concentration range of Mig in a transmigration assay (Fig. 3*b*). As a control, replicate eosinophils strongly responded to eotaxin-2 (Fig. 3*b*). Taken together, these data suggest that Mig does not directly promote murine eosinophil chemotaxis.

Mig Is an Inhibitor of Eosinophils *in Vitro*. We next hypothesized that Mig was an inhibitor for CCR3 ligand-induced eosinophil chemoattraction. To address this hypothesis, we pretreated eosinophils with Mig and examined their subsequent chemotactic response to potent CCR3 ligands. Mig pretreatment strongly inhibited eosinophil transmigration in response to eotaxin-2 in a dose-dependent manner (Fig. 3*c*). As a positive control, pretreatment of eosinophils with eotaxin-2 inhibited transmigration. As a negative control, pretreat-

ment of eosinophils with 1 ng/ml monocyte chemoattractant protein 1 (JE, CCL2) did not inhibit eosinophil transmigration (data not shown). Mig also inhibited eosinophil responses to eotaxin-1 in a dose-dependent fashion (Fig. 3*d*); analysis of the concentration dependence suggested that Mig was more effective at inhibiting eotaxin-1. In addition, pretreatment of eosinophils with another CXCR3 ligand, IP-10 (CXCL10), also markedly inhibited eosinophil transmigration to eotaxin-2 [from $7.6 \pm 0.25 \times 10^4$ to $2.9 \pm 0.09 \times 10^4$ cells (mean $\pm$ SD, $n = 2$)]. We wanted to rule out the possibility that the inhibitory effect of Mig on eosinophils was due to toxicity. Accordingly, we determined that Mig was not toxic to eosinophils, as determined by exclusion of a viability dye (trypan blue) and by the ability of IL-5 to promote eosinophil survival (21) even in the presence of Mig (data not shown).

Mig Inhibits Eotaxin-Induced Eosinophil Recruitment to the Lung. We were next interested in determining whether Mig could serve as an inhibitor of eosinophil migration *in vivo*. To test this possibility, we examined the ability of Mig to inhibit eotaxin-2-induced eosinophil recruitment into the lung. First, i.n. administration of eotaxin-2 (3 μg) to IL-5 transgenic mice was shown to induce marked eosinophil lung recruitment. For example, 3 h after eotaxin-2 treatment, eosinophil levels in the BALF increased from $7.2 \pm 2.7 \times 10^3$ to $19.6 \pm 4.5 \times 10^5$. To test the inhibitory role of Mig, mice were intravenously injected with Mig 30 min before i.n. eotaxin-2 delivery. After i.n. treatment with 0.1, 0.5, and 1.0 μg of Mig, there was a dose-dependent inhibition of eosinophil recruitment to the lung (21%, 51%, and 88%, respectively) (Fig. 4*a*). Blood eosinophilia was unaffected with any dose of Mig (data not shown). The mean decrease in eotaxin-2-induced BALF eosinophilia after Mig (1 μg) treatment was $82 \pm 0.5\%$ (Fig. 4*b*). For comparison, mice were treated with (i.v.) eotaxin-1 (1 μg) before i.n. eotaxin-2 delivery. Mig and eotaxin-1 had similar inhibitory activities (Fig. 4*c*). As a negative control, mice were also treated with the chemokine JE before eotaxin-2 i.n. administration. Intravenous treatment with JE (1 μg) had no effect on eotaxin-2 induced eosinophil recruitment to the airway (data not shown). The ability of Mig to inhibit eosinophil chemokine responses *in vivo* was not limited to eotaxin-2; Mig also inhibited the effects of eotaxin-1. For example, pretreatment with 1 μg of Mig reduced eotaxin-1-induced BALF eosinophilia from $2.6 \pm 0.42 \times 10^6$ to $4.0 \pm 1.5 \times 10^5$ cells (mean $\pm$ SD, $n = 3$ mice per group). Chemokine-induced eosinophil recruitment into the lung was also inhibited by IP-10. With IP-10 treatment before eotaxin-1 challenge, eosinophil recruitment into the airways was reduced from $2.6 \pm 0.42 \times 10^6$ to $4.0 \pm 1.6 \times 10^5$ cells (mean $\pm$ SD, $n = 3$ mice per group). We were also interested in determining whether Mig had an effect on lung tissue eosinophilia. Histological examination revealed that eosinophil migration into the lung was dramatically inhibited after i.v. Mig treatment before eotaxin-2 i.n. delivery (Fig. 4*d*). When Mig was administered i.n. (1 μg) before eotaxin-2 administration, eosinophil recruitment was not significantly inhibited (data not shown), suggesting that Mig's inhibitory activity depends on systemic (i.v.), rather than local (lung), administration.

Mig Inhibits Eotaxin-Induced Eosinophil Mobilization to the Blood. Because eotaxin has been shown to induce a pronounced blood eosinophilia in WT mice when administered systemically (22), we were interested in determining whether Mig could inhibit eotaxin-induced eosinophil mobilization. To test this possibility, we treated mice with i.v. eotaxin-1 (1 μg) alone or in combination with Mig (1 μg) and examined the effect on blood eosinophilia. After 1 h, eotaxin-1 induced a rapid increase in circulating eosinophil levels (Fig. 4*e*). With i.v. Mig treatment, there was a significant reduction in eotaxin-induced eosinophil mobilization (Fig. 4*e*).

Mig Inhibits OVA-Induced Eosinophil Recruitment to the Lung. We were next interested in determining whether pharmacological

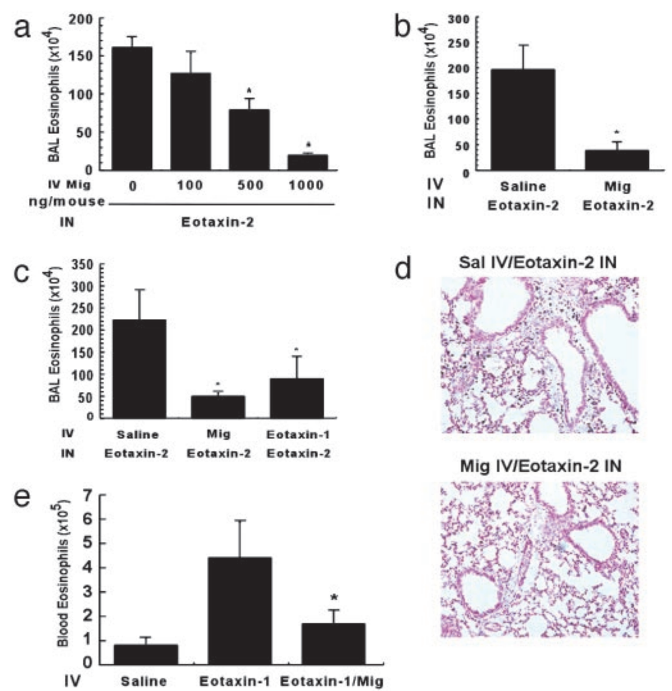

Fig. 4. Mig inhibits chemokine-induced eosinophil recruitment. (*a*) Mean $\pm$ SD of eosinophils that migrated into the airway toward eotaxin-2. IL-5 transgenic mice were treated intravenously (IV) with saline or Mig 30 min before i.n. (IN) delivery of eotaxin-2. (*b*) Mice treated with saline or Mig before eotaxin-2 i.n. delivery. Data represent mean $\pm$ SD of airway eosinophils with nine mice in each group. *, $P = 0.001$. (*c*) Eosinophils that migrated into airway in response to i.n. (IN) eotaxin-2 delivered after i.v. treatment with saline, Mig, or eotaxin-1. Data represent mean ($\pm$SD) of lung or airway eosinophils. Representative experiment ($n = 2$) with four mice in each group per experiment. *, $P \leq 0.04$. (*d*) Eosinophils, detected by anti-major basic protein immunohistochemistry, are shown in lung tissue after i.v. (IV) saline (*Upper*) or Mig (*Lower*) pretreatment before eotaxin-2 delivery (IN). (For higher-resolution image, see Fig. 7, which is published as supporting information on the PNAS web site.) (*e*) Mig inhibits eotaxin-induced eosinophil mobilization to the blood. Data represent mean $\pm$ SD of blood eosinophils after i.v. (IV) cytokine administration from three experiments with 12 mice in each group. *, $P < 0.0001$.

administration of Mig down-regulated eosinophil recruitment to the lung in OVA-induced experimental eosinophilic AAI. To test this hypothesis, we subjected sensitized mice to a single challenge with i.n. OVA or saline. We examined the ability of i.v. Mig, given 30 min before allergen challenge, to inhibit leukocyte recruitment into the lung. Notably, when mice were treated with i.v. Mig, there was a marked reduction of BALF eosinophils (Fig. 5*a*). The mean decrease in OVA-induced BALF eosinophilia was $69 \pm 1.4\%$. In contrast, there was no reduction in allergen-induced BALF neutrophils or lymphocytes (data not shown). As a control, mice were treated intravenously with JE (1 μg) before antigen challenge, but there was no change in BALF eosinophils compared with saline-treated mice (data not shown).

Mig Inhibits IL-13-Induced Eosinophil Recruitment. Because IL-13 has been shown to induce the expression of multiple CCR3 ligands (23), we wanted to determine whether Mig could also inhibit IL-13-induced eosinophil recruitment *in vivo*. To test this possibility, we examined the effect of i.v. Mig on i.t. IL-13-induced eosinophil recruitment to the lung. Mice were treated with Mig 30 min before a second dose of i.t. IL-13 or saline and we examined the effect on leukocyte recruitment. Recombinant IL-13 induced marked recruitment of leukocytes into the airway (Fig. 5*b*). In contrast to mice treated with i.v. saline, mice treated with i.v. Mig demonstrated a significant reduction in airway eosinophils (Fig. 5*b*). The mean

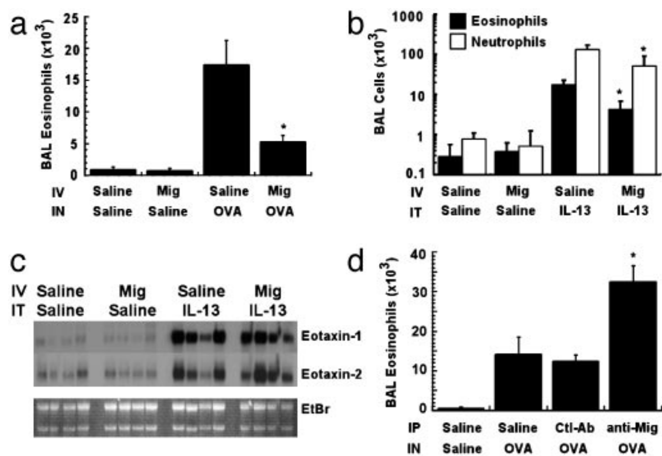

Fig. 5. Mig inhibits allergen- and IL-13-induced eosinophil recruitment to the lung and functions as an eosinophil inhibitor *in vivo*. (*a*) OVA-sensitized mice were treated with i.v. (IV) saline or Mig 30 min before i.n. (IN) saline or OVA challenge. A representative experiment ($n = 3$) with four mice in each group is shown. *, $P = 0.0009$. (*b*) Mice were treated with i.v. (IV) saline or Mig 30 min before i.t. (IT) IL-13 delivery. A representative experiment ($n = 2$) with four mice in each group is shown. *, $P = 0.003$. (*c*) Northern blot analysis of eotaxin-1 and -2 mRNA expression in i.v. saline- and Mig-treated mice after IL-13 delivery. Ethidium bromide (EtBr) staining of the RNA gels is also shown. Each lane represents RNA from a single mouse. (*d*) Mig neutralization increases antigen-induced eosinophil recruitment to the lung. OVA-sensitized mice were treated with i.p. (IP) injection of anti-Mig antibody or control IgG (Ctl-Ab) antibody. A representative experiment ($n = 2$) with four mice in each group is shown. Data in *a*, *b*, and *d* represent mean ± SD of airway eosinophils.

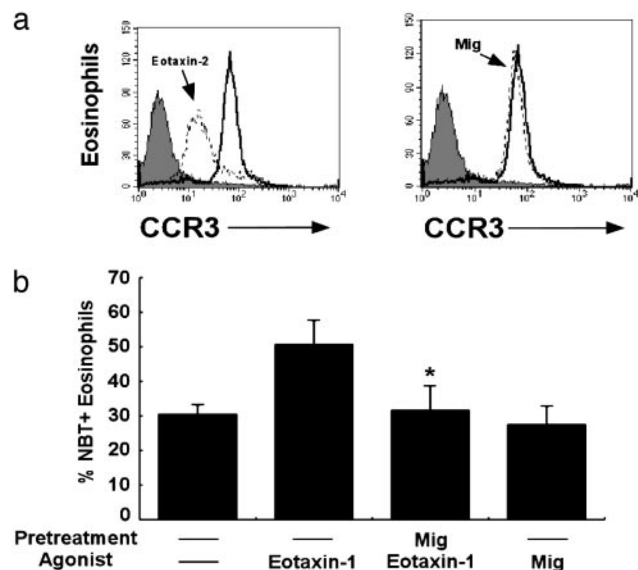

Fig. 6. Mig does not induce CCR3 internalization but is a functional inhibitor of eosinophils. (*a*) Analysis of surface CCR3 on eosinophils after incubation with buffer (solid line), eotaxin-2 (*Left*, dashed line), or Mig (*Right*, dashed line). The filled histogram is the isotype-matched control antibody. (*b*). Mig inhibits superoxide production. Eosinophils were pretreated with buffer or Mig (100 nM) and analyzed for superoxide production by reduction of NBT in response to eotaxin-1 (10 nM), Mig (100 nM), or buffer. The results represent the percentage of positive cells. Error bars show the mean ± SD; $n = 3$. *, $P < 0.03$.

decrease in IL-13-induced BALF eosinophilia was 71 ± 7%. Interestingly, Mig pretreatment also decreased BALF neutrophils (Fig. 5*b*), but it had no effect on BALF lymphocyte levels (data not shown). We have not yet determined whether the inhibitory effect on IL-13-induced neutrophilia is directly mediated by Mig or indirectly through inhibition of eosinophils. These data, together with the previous *in vivo* studies, indicate that i.v. Mig potently inhibits eosinophil recruitment into the lung in response to diverse stimuli.

A potential mechanism for inhibition of leukocyte recruitment is a reduction in expression of chemoattractant molecules. We were interested in determining whether Mig treatment before IL-13 administration altered expression of eosinophil-specific chemokines. In control treated mice, IL-13 induced marked expression of eotaxin-1 and eotaxin-2 (Fig. 5*c*). However, Mig treatment had no effect on the expression of the eosinophil-specific chemokines (Fig. 5*c*), suggesting that the inhibitory effect does not depend on blockade of chemokine production.

Mig Neutralization Increases Airway Eosinophil Recruitment. Although we have demonstrated that pharmacological administration of Mig inhibited eosinophil recruitment in three model systems, it was important to determine whether endogenous allergen-induced Mig was a functional inhibitor of eosinophil migration. To test this possibility, we treated OVA-sensitized mice with anti-murine Mig IgG. Twenty-four hours after antibody treatment, mice were i.n. challenged with either OVA or saline and then examined for eosinophil recruitment into the airways. After neutralizing anti-Mig treatment, BALF eosinophils increased ≈3-fold over control IgG-treated mice (Fig. 5*d*). As an additional control, the ability of the antibody to neutralize Mig was confirmed by the reduction of Mig protein levels in the BALF 24 h after one i.n. allergen challenge. Mig levels in the BALF of OVA-challenged mice were 3.7 ± 3.9 pg/ml and 28.6 ± 14.4 pg/ml (mean ± SD, $n = 4$ mice per group) after anti-Mig or control IgG treatment, respectively. These data suggest that the antigen-induced expression of the chemokine Mig functions as an inhibitor of eosinophil recruitment into the airways.

Mig Does Not Induce CCR3 Internalization. Human CXCR3 ligands have been shown to bind to eosinophils and CCR3-transfected cells (7, 8). As such, we were interested in ruling out the possibility that Mig's inhibitory action was mediated (at least in part) by induction of CCR3 internalization. Mig pretreatment had no effect on the level of CCR3 on the surface of eosinophils (Fig. 6*a*). As a control, eotaxin-2 pretreatment induced marked CCR3 internalization. This effect was not seen when the preincubation was conducted at 4°C, verifying that we were indeed assaying receptor internalization rather than epitope blockade by eotaxin-2, consistent with previous reports (15).

Mig Inhibits Functional Response of Eosinophils. Eosinophils have been shown to produce abundant reactive superoxide anion and related reactive oxygen species (24). We examined the ability of Mig to inhibit agonist-induced superoxide anion formation in eosinophils. To test this ability, we treated eosinophils with eotaxin-1 after Mig pretreatment and measured oxidase activity. Eotaxin activation of eosinophils resulted in an increase in NBT^+ cells (Fig. 6*b*). Mig pretreatment inhibited the formation of eotaxin-induced NBT^+ eosinophils by a remarkable 94% (Fig. 6*b*). The high background NBT staining in the untreated eosinophils is likely caused by their endogenous exposure to IL-5 because they are derived from IL-5 transgenic mice.

Discussion

Inappropriate expression of chemokines can result in excessive leukocyte recruitment and activation, resulting in extensive tissue inflammation and injury (25). As such, influencing pathological processes with chemokine receptor blockade is an active area of investigation (26). In this study, we have shown that Mig is a potent inhibitor of eosinophil recruitment both *in vitro* and *in vivo*. Migration of eosinophils in response to CCR3 ligands *in vitro* was markedly inhibited by pretreatment of eosinophils with Mig. Like-

wise, accumulation of eosinophils in the lungs in response to eotaxin-1 and eotaxin-2 was greatly inhibited after treatment with i.v. Mig. Furthermore, Mig inhibited IL-13-induced eosinophil recruitment and allergen-induced lung eosinophilia. Notably, when Mig was neutralized during induction of experimental asthma, airway eosinophil migration increased with no change in other leukocyte chemoattraction. In addition, Mig inhibited eosinophil oxidase activity induced by eotaxin-1, providing supportive evidence that Mig blocks both eosinophil recruitment and the effector function of this leukocyte. Collectively, these data demonstrate that allergen-induced Mig acts as a specific and natural inhibitor of eosinophil recruitment *in vivo*.

Mig's inhibitory actions were elicited when it was administered intravenously (rather than directly to the lung), supporting a mechanism involving interference with eosinophil recruitment into the lung. Although one study has shown that human eosinophils express CXCR3 and transmigrate toward both IP-10 and Mig *in vitro* (20), we did not detect CXCR3 expression on murine eosinophils from multiple distinct tissue locations. Consistent with the absence of CXCR3, murine eosinophils did not respond to a range of Mig concentrations, suggesting that Mig exerts its inhibitory effects through a different mechanism. In our study, the magnitude of Mig-induced reduction of eosinophil trafficking is fairly profound, comparable if not greater than that seen in eotaxin-1 or CCR3 gene targeted mice (27–29), suggesting a mechanism beyond CCR3 antagonism alone. There are conflicting reports regarding the *in vitro* interaction of human CXCR3 ligands and CCR3. One study suggests that CXCR3 ligands can competitively inhibit the binding of the eotaxin chemokines (7), yet in a recent report the CXCR3 ligand CXCL11 did not compete with CCL11 for binding to CCR3 (8). We extend these *in vitro* results by demonstrating that Mig is a potent naturally occurring eosinophil-inhibitory chemokine in multiple models of AAI.

The identification of Mig as an AAI signature gene suggests the codevelopment of both Th1 and Th2 responses during allergic airway inflammation because Mig is primarily induced by IFN-γ (30). Indeed, although asthma is a Th2-associated disease, numerous studies have shown coinvolvement of Th1 and Th2 cells in the pathogenesis and/or effector phase of human asthma and experimental asthma in rodents (31–35). For example, the Th1-associated chemokine IP-10 has been shown to be upregulated in human asthma (induction of Mig has not been examined) (36). Notably, chronic overexpression of IP-10 in the lungs (because of adenovirus infection or transgenesis in mice) influences several features of experimental asthma, primarily by affecting levels of Th1 and Th2 cells and their cytokines (37–39). In addition, adoptive transfer experiments in rodent AAI models have elegantly demonstrated cooperative roles for Th1 and Th2 cells in both suppressing and augmenting disease (33). Defining the role of Th1 responses in the development of AAI is not just an academic question because numerous therapeutic strategies (including conventional allergen immunotherapy) are designed to promote Th1 responses in attempt to inhibit Th2 responses. Our experiments, strictly focused on the development of lung eosinophilia, support an inhibitory role for Th1-associated responses during the development of experimental asthma, at least with regard to the development of lung eosinophilia. Our finding that Mig consistently induced an inhibitory action on eosinophil recruitment may be a result of Mig delivery via the i.v. route. Notably, when we administered Mig directly to the respiratory tract, we did not observe inhibition of eosinophil migration.

The inhibition of eosinophil recruitment by Mig may be an endogenous mechanism of limiting the immune response and lung injury. Recently, several naturally occurring ligands (including the eotaxins) have been shown to be effective chemokine receptor antagonists (7, 40, 41), but these studies have not been verified *in vivo*. In our study, we demonstrate that pretreatment with Mig induced a dose-dependent inhibition of chemoattractant-induced eosinophil transmigration *in vitro*. In addition, we have translated these *in vitro* observations to demonstrate that Mig's inhibitory activity may be exploited to control eosinophil infiltration in a variety of inflammatory lung models. We also demonstrate that Mig inhibits a CCR3-mediated functional response of eosinophils induced by eotaxin-1. Taken together, our results provide evidence for a feedback loop by which Th1- and Th2-associated chemokines (e.g., eotaxin and Mig, respectively) coordinately regulate eosinophil responses *in vivo*. As such, the identified pathway may serve as a prototype for the development of novel and selective therapies for eosinophil-associated disorders.

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