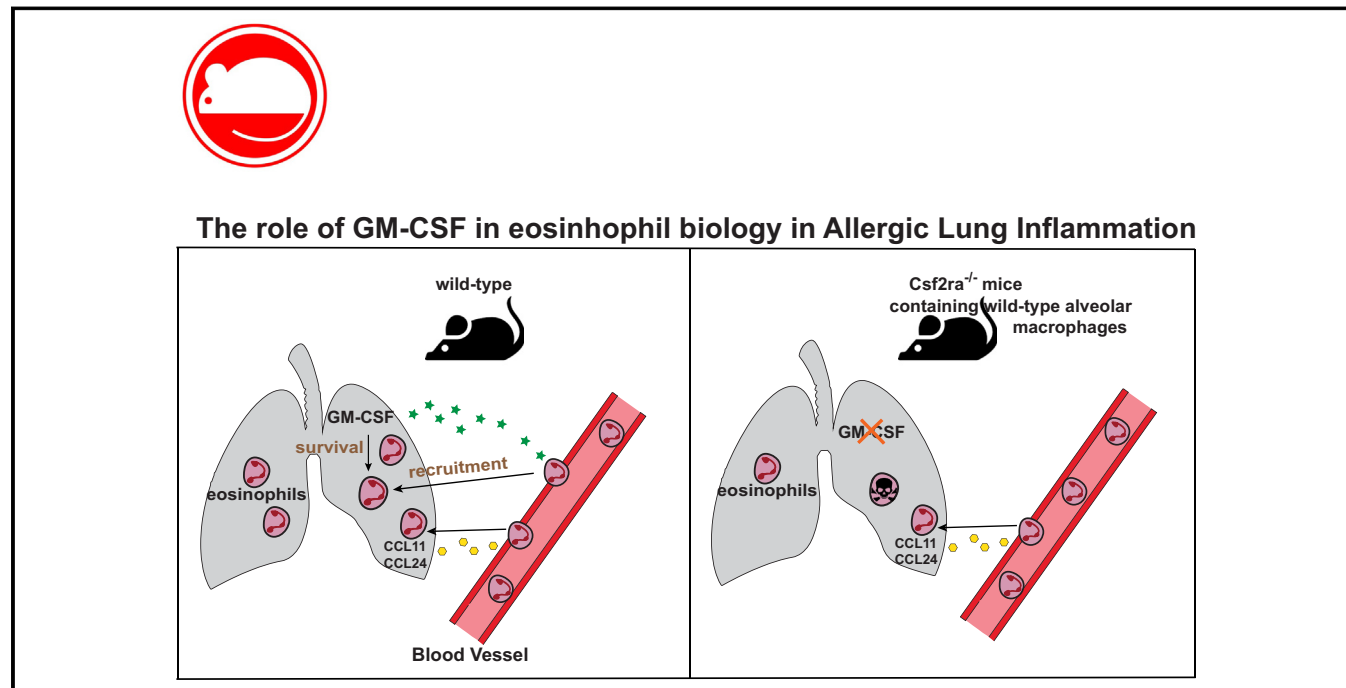


GM-CSF intrinsically controls eosinophil accumulation in the setting of allergic airway inflammation



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GRAPHICAL ABSTRACT



Background: Eosinophils are a therapeutic target in asthmatic patients, and GM-CSF has been suggested to control various aspects of eosinophil biology, including development, function, and survival. However, to date, the role of GM-CSF signaling in eosinophils *in vivo* is largely unclear.

Objective: We sought to elucidate the role of GM-CSF signaling in asthmatic inflammation.

Methods: Wild-type and GM-CSF receptor α (*Csf2ra*)-deficient mice reconstituted with *Csf2ra*-proficient alveolar macrophages were subjected to different models of airway inflammation to

evaluate the effect of GM-CSF signaling deficiency on asthmatic inflammation in general and on eosinophils in particular.

Results: We demonstrate that GM-CSF signaling, although being largely dispensable for eosinophil development at steady state, intrinsically promotes accumulation of eosinophils in the lung during allergic airway inflammation. In contrast, chitin-induced eosinophil accumulation in the peritoneal cavity occurs independent of GM-CSF, indicating organ specificity. We show that GM-CSF induces chemokinesis and promotes eosinophil survival *in vitro*, which likely contribute to eosinophil accumulation in the airways *in vivo*.

Conclusion: GM-CSF intrinsically promotes eosinophil accumulation in the setting of pulmonary allergic inflammation. (J Allergy Clin Immunol 2019;143:1513-24.)

Key words: Eosinophils, asthma, allergic airway inflammation, GM-CSF, chemokinesis

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Eosinophils are a subtype of granulocytes and are most classically associated with type 2 inflammation characteristic of helminth infections. They are recognized as one of the hallmarks of asthma and have recently received much attention as a therapeutic target in patients with this airway disease.¹ They have been suggested to modulate multiple aspects of

Abbreviations used

AM:	Alveolar macrophage
BAL:	Bronchoalveolar lavage
BM:	Bone marrow
DC:	Dendritic cell
EdU:	5-Ethynyl-2'-deoxyuridine
FACS:	Fluorescence-activated cell sorting
GM-CSFR:	GM-CSF receptor
HDM:	House dust mite
OVA:	Ovalbumin
PAP:	Pulmonary alveolar proteinosis
WT:	Wild-type

asthmatic inflammation, including mucus production, smooth muscle cell hyperplasia, angiogenesis, and fibrosis,² and thereby contribute to the asthma exacerbation symptoms. Indeed, specifically targeting eosinophils was shown to be a successful strategy in a subset of patients with steroid-resistant asthma, who often present with the most serious symptoms.³ More recently, additional important roles of eosinophils in other contexts have been unraveled, including regulation of glucose metabolism in adipose tissue,⁴ mammary gland development,⁵ and regulation of intestinal health.⁶ Thus understanding eosinophil biology is also highly relevant for disease states other than airway inflammation.

Eosinophilia in peripheral tissues during inflammation and to some degree also eosinophil development is dependent on IL-5,⁷ and IL-5 signaling requires the common β -chain, which is shared with the cytokines IL-3 and GM-CSF. Indeed, many aspects of eosinophil biology have been suggested to be controlled also by GM-CSF, including eosinophil development at steady state and their survival,⁸ activation,⁶ and migration⁹ in inflammation. However, the lack of a receptor knockout specific for GM-CSF has hampered efforts to understand the intrinsic role of GM-CSF signaling in eosinophils thus far. In the lung in particular, the role of GM-CSF in patients with airway inflammation has not been comprehensively addressed to date because of the essential role of GM-CSF in development of alveolar macrophages (AMs).¹⁰ Mice lacking GM-CSF¹⁰ or GM-CSF receptor (GM-CSFR)¹¹ develop strong pulmonary alveolar proteinosis (PAP), which has a profound effect on the lung environment. This has been a strong confounding factor in previous studies looking at the role of GM-CSF in allergic inflammation in either ligand-deficient¹² or common β -chain-deficient animals.¹³

In this study we now report the combined use of a recently described *Csf2ra*-deficient mouse line¹¹ with reconstitution of a wild-type (WT) AM compartment to study the role of GM-CSF signaling in eosinophils in homeostasis and inflammation. We find that although being largely dispensable for the development of eosinophils at steady state, GM-CSF controls eosinophil accumulation in the inflamed lung by inducing chemokinesis and promoting eosinophil survival *in vitro*. This activity seems to be organ specific because it has been observed in the inflamed lung but not the peritoneal cavity. Thus we uncover a novel organ-specific regulation of eosinophil biology through GM-CSF signaling.

METHODS**Mice**

C57BL/6J mice were bred in house or purchased from Charles River (Wilmington, Mass). *Csf2ra*^{-/-} animals were recently described.¹¹ *Csf2rb*^{-/-} mice were provided by B. Becher. All animals were housed in individually ventilated cages under specific pathogen-free conditions at the ETH Phenomics Facility (Zurich, Switzerland) and used for experiments at between 6 and 14 weeks of age, unless stated otherwise. All animal experiments were approved by the local animal ethics committee (Kantonales Veterinärwesen Zurich) and were performed according to local rules (Swiss Animal Protection Ordinance [TschV] Zurich) and Swiss animal protection law (TschG).

Cell suspension preparations

Mice were killed with an overdose of sodium pentobarbital by means of intraperitoneal injection. Organs were removed and then processed according to the following procedure: lungs were digested with 1 mg/mL hyaluronidase (Sigma, St Louis, Mo), 25 μ g/mL Collagenase XI (Sigma), 50 μ g/mL Liberase TM (Roche, Mannheim, Germany), and 0.02 mg/mL DNaseI (Sigma) at 37°C for 45 minutes and subsequently passed through a 70- μ m cell strainer. Small intestines and colons were treated as described by Bogunovic et al.¹⁴ Bone marrow (BM) cells were flushed from femurs and tibia and then used directly for staining. ACK buffer was used for erythrocyte lysis for all organs.

Chitin administration

Chitin flakes were washed 3 times with PBS and then sonicated for 15 minutes at 25 Hz. Chitin was then filtered through a 70- μ m cell strainer, and mice received 800 ng intraperitoneally or 5 μ g intratracheally in PBS.

Transwell assays

Eosinophils or neutrophils were seeded into 5- μ m Costar polycarbonate membrane transwell plates (Corning, Corning, NY), and medium containing either 200 ng/mL eotaxin-2 (BioLegend, San Diego, Calif), 200 ng/mL CCL3 (BioLegend), 100 ng/mL GM-CSF (BioLegend), or a mixture of these chemokines was added to the top or bottom of the well, as indicated in each experiment. Plates were incubated for 3 hours at 37°C, and subsequently, migrated cells were harvested and analyzed by using flow cytometry. The number of migrated cells was normalized to the medium-only control.

Generation of BM-derived eosinophils. BM-derived eosinophils were generated, as previously described.¹⁵

Survival assays. For *ex vivo* BM eosinophils, BM cells were directly cultured *in vitro* with 100 ng/mL GM-CSF and IL-5 for the indicated time periods in complete RPMI-medium. For *in vitro*-derived BM eosinophils, cells were stimulated after 14 days of culture with the indicated cytokines.

5-Ethynyl-2'-deoxyuridine incorporation assay

Mice were injected with 0.5 mg of 5-ethynyl-2'-deoxyuridine (EdU; Life Technologies, Grand Island, NY), and mice were killed after overnight incorporation. Tissues were harvested and then treated according to the manufacturer's instructions by using the Click-iT Alexa Fluor 488 Imaging Kit (Life Technologies).

Flow cytometry

Multiparameter analysis was performed on a FACSCanto II or LSR Fortessa (BD, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Ashland, Ore). Fluorochrome-conjugated or biotinylated mAbs specific to mouse CD11c (N418), CD11b (M1/70), Siglec-F (E5-2440; BD Biosciences, San Jose, Calif), CD45 (30-F11), CD45.2 (104), CD4 (GK1.5), CD8 α (53-6.7), CD3e (145-2C11), CD64 (X54-5/7.1), IL-4 (11B11), IL-5 (TRFK5), IFN- γ (XMG1.2), Ly-6G (1A8-Ly6g), XCR1 (ZET), and GM-CSFR α (R&D Systems, Minneapolis, Minn) were purchased from BioLegend, unless otherwise stated. Dead cells were excluded by using the live/dead marker eFluor780 (eBioscience, San Diego, Calif). Before all staining,

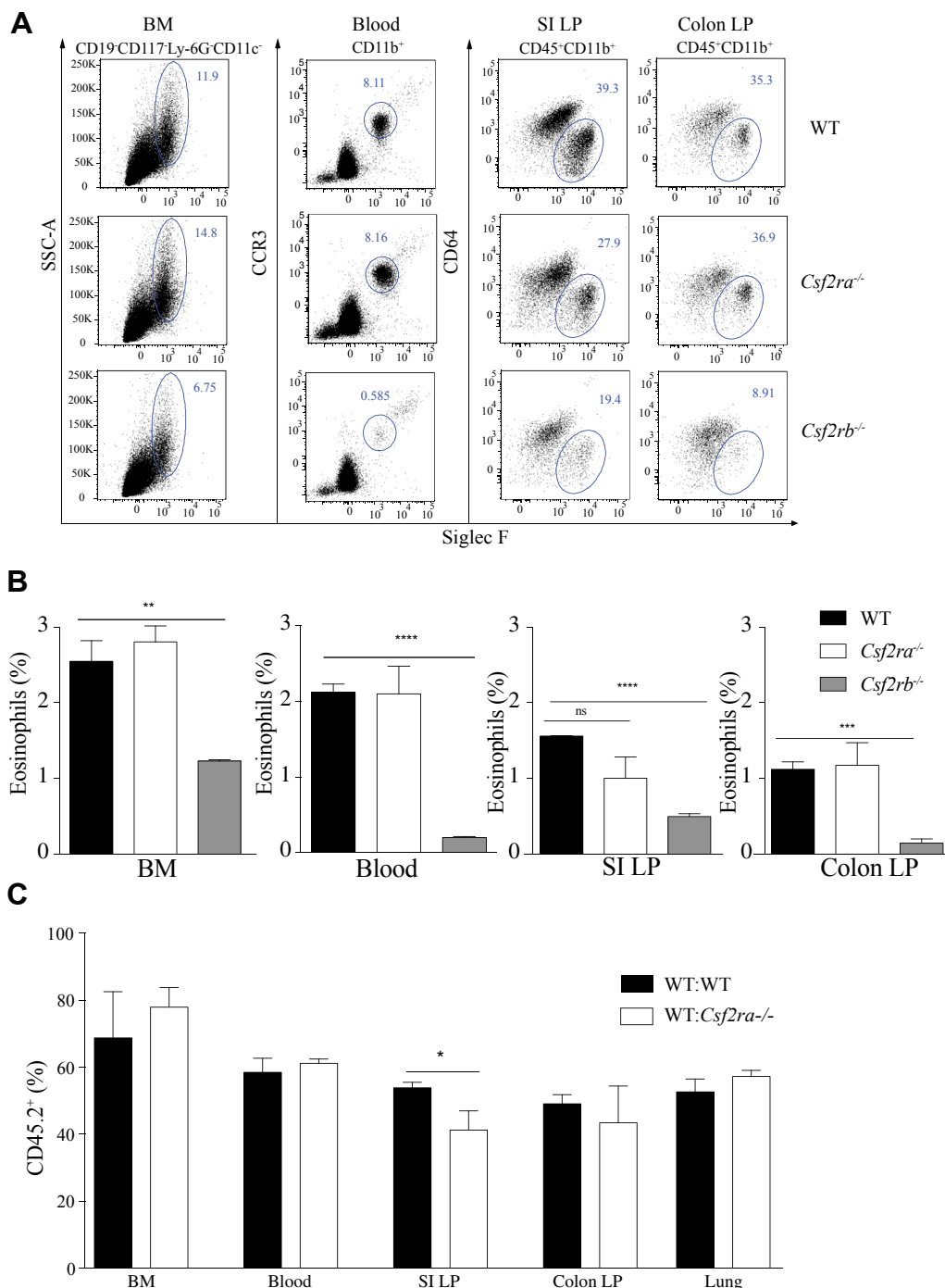


FIG 1. GM-CSF signaling is largely dispensable for eosinophil development. **A** and **B**, Organs and tissues of naive *Csf2ra*^{-/-} and *Csf2rb*^{-/-} animals were analyzed for their eosinophil populations at steady state. Shown are representative dot plots (Fig 1, A) and a summary of FACS data (Fig 1, B; mean ± SEM) for the indicated organs (n = 3-4 per group). Eosinophils were identified as indicated in the figure. **C**, WT (CD45.1⁺CD45.2⁺) mice were lethally irradiated and reconstituted with a mixture of either WT (CD45.1) and WT (CD45.2) or WT (CD45.1) and *Csf2ra*^{-/-} (CD45.2) BM at a 1:1 ratio. After 10 weeks of reconstitution, organs of naive recipient mice were analyzed for eosinophils. Shown is a summary of FACS data (mean ± SEM) in which the frequency of CD45.2⁺ cells is shown for the indicated organs (n = 3-4 per group). Data are representative of 2 independent experiments. **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001, ANOVA. LP, Lamina propria; SI, small intestine; SSC, side scatter.

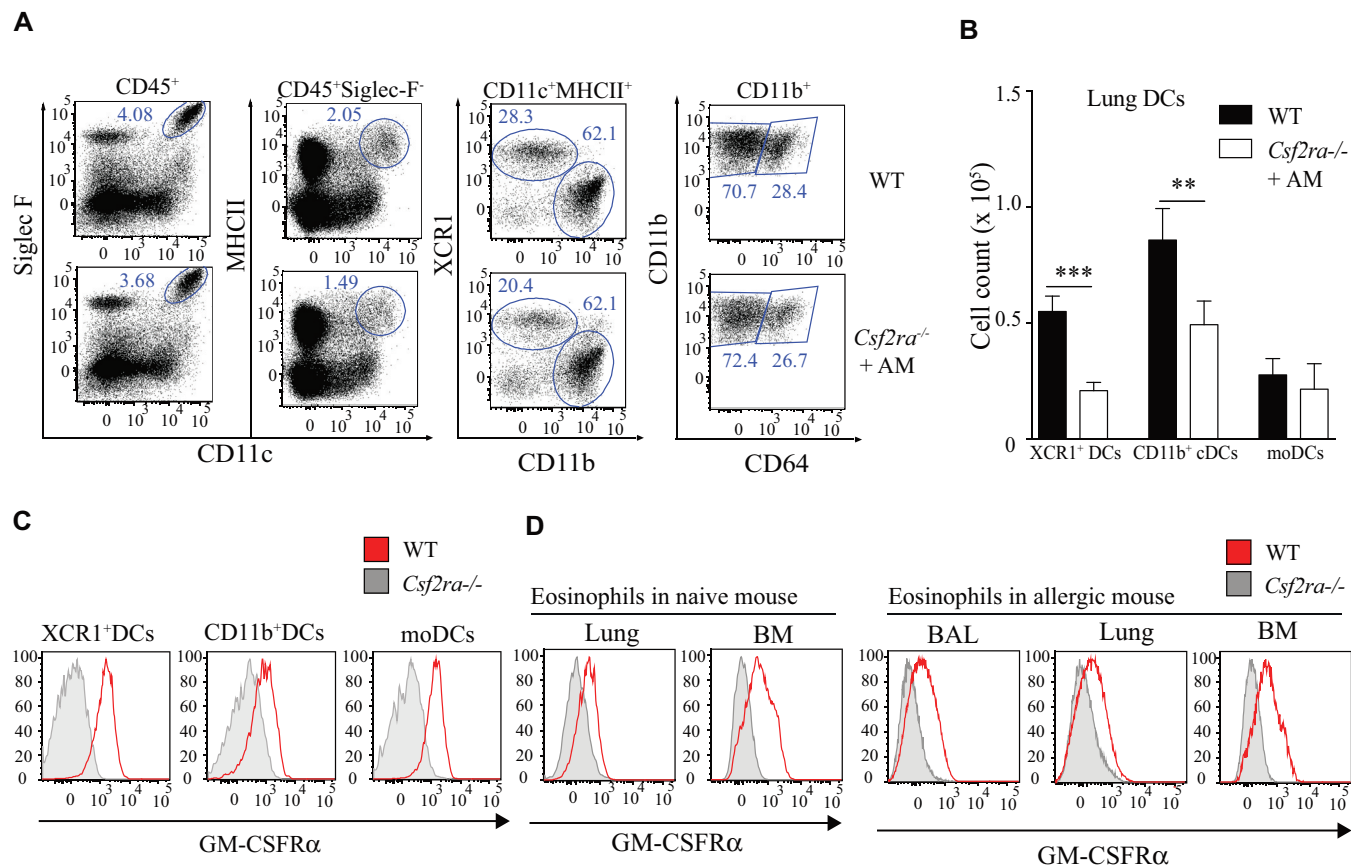


FIG 2. GM-CSFR is expressed on eosinophils and lung-resident DCs at steady state and in inflammation. **A**, Fetal (E17.5) lung AM precursors from WT CD45.1⁺ C57BL/6 embryos were transferred intranasally to neonatal (day 3 after birth) *Csf2ra*^{-/-} mice. Shown are representative dot plots of naive lungs of AM-reconstituted *Csf2ra*^{-/-} mice and WT control mice. AMs were identified as CD45⁺CD11c⁺Siglec-F⁺ cells. Lung DCs were gated as CD11c⁺MHCII⁺Siglec-F⁺CD45⁺, and XCR1⁺ DCs were identified as XCR1⁺CD11b⁺, CD11b⁺ DCs were identified as XCR1⁻CD11b⁺CD64⁺, and monocyte-derived DCs were identified as XCR1⁻CD11b⁺CD64⁺ cells, respectively. **B**, Summary of FACS data (mean $\pm$ SEM; n = 4 per group). **C** and **D**, GM-CSFR α surface expression on lung DC subsets, as well as eosinophils, was measured by using flow cytometry. Shown are representative histograms of the indicated cell types. Data are representative of 2 independent experiments. ***P* < .01 and ****P* < .001, Student *t* test (unpaired).

FcγIII/II receptors were blocked by means of incubation with homemade anti-CD16/CD32 (2.4G2). Annexin V on apoptotic cells were stained with the Annexin V detection kit (eBioscience), according to the manufacturer's instructions.

Murine models of airway inflammation

For the ovalbumin (OVA) model, animals were injected intraperitoneally with 100 μg of OVA (Invitrogen, Carlsbad, Calif) resuspended in aluminum hydroxide solution (Serva, Heidelberg, Germany). At the indicated times, animals were challenged with intratracheal injection of 20 μg of OVA in PBS. For the house dust mite (HDM) extract model, animals received either 10 μg of HDM extract (Greer Laboratories, Lenoir, NC) intratracheally or PBS for sensitization and were then challenged at the indicated times with 10 μg of HDM extract.

BM chimeras

For BM chimeras, C57BL/6 CD45.1⁺CD45.2⁺ mice were lethally irradiated (9.5 Gy by using a caesium source) and reconstituted with 5 to 10 × 10⁶ BM cells of the genetic background and with the ratio indicated for each experiment. Mice were analyzed 10 weeks after reconstitution.

Transfer of AM precursors into neonates

Lungs from E17.5 embryos of CD45.1⁺ WT mice were harvested, minced, and digested with collagenase IV, as described above. CD45⁺ cells were then purified with MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and cells were then transferred intranasally into neonates 1 to 7 days after birth to allow reconstitution of the WT AM compartment.

Statistical analysis

Mean values, SDs, SEMs, Student *t* test (unpaired) results, and ANOVA (1-way) results were calculated with Prism software (GraphPad Software, La Jolla, Calif).

RESULTS

GM-CSFR signaling is largely dispensable for eosinophil development

To address the specific role of GM-CSF signaling in eosinophil development, we analyzed the eosinophil compartment of naive *Csf2ra*^{-/-} and *Csf2rb*^{-/-} mice. *Csf2ra*^{-/-} animals are deficient only in GM-CSF signaling, whereas *Csf2rb*^{-/-} mice in addition

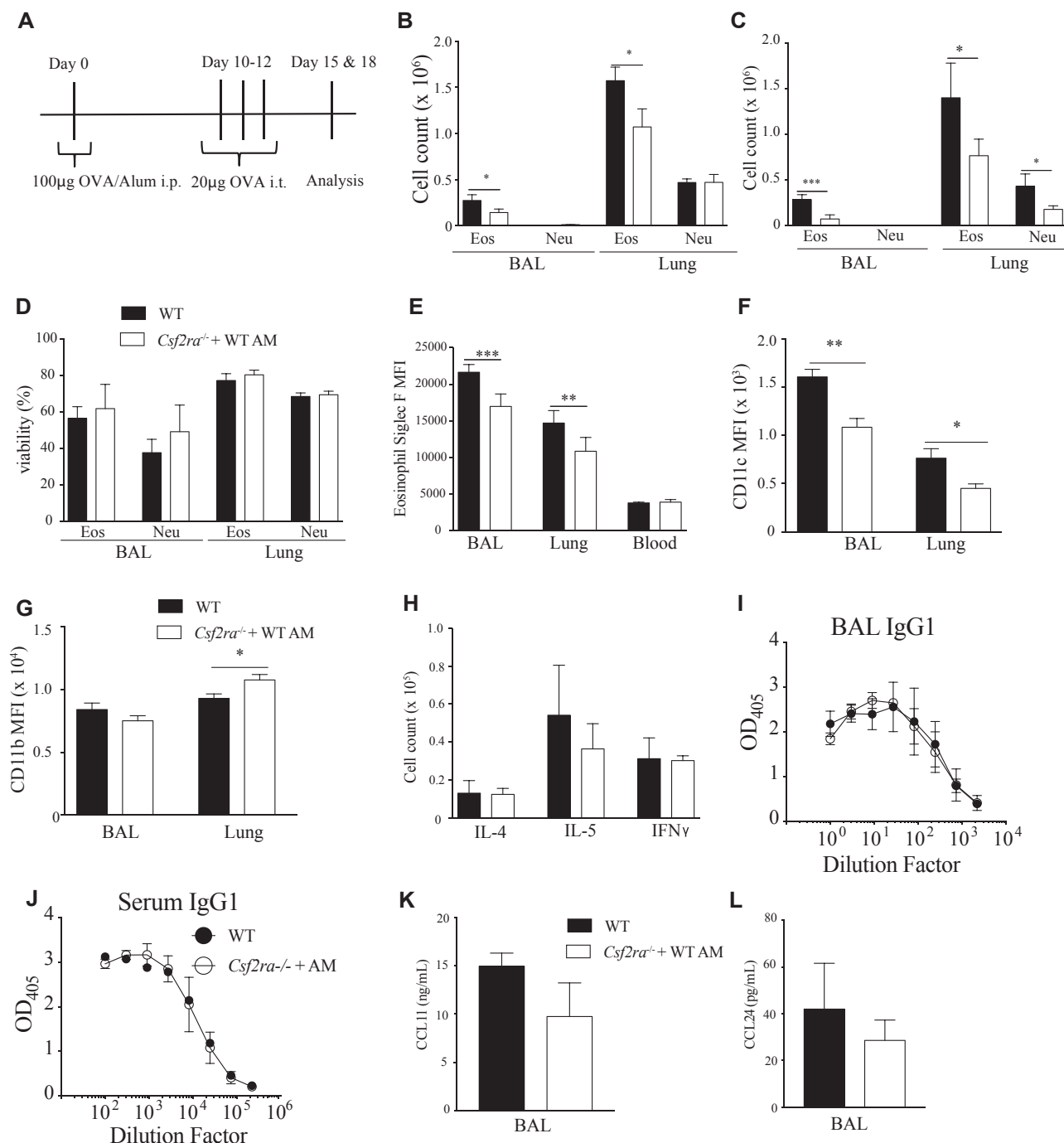


FIG 3. GM-CSF signaling promotes pulmonary eosinophilia in allergic inflammation. **A–J**, AM-reconstituted *Csf2ra*^{-/-} mice were sensitized and challenged with OVA, as shown in the scheme (Fig 3, A), and analyzed 3 and 6 days after the last challenge. Fig 3, B and C, Summary of FACS data (mean $\pm$ SEM) of BAL fluid and lung tissue for eosinophils and neutrophils for the indicated time points (n = 5 per group). Eosinophils were identified as CD45⁺CD11c⁺CD11b⁺Siglec-F⁺SSC-A^{high} and neutrophils as CD45⁺CD11c⁺CD11b⁺Ly-6G⁺ cells, respectively. Fig 3, D, Viability of eosinophils and neutrophils in BAL fluid and lung tissue 6 days after the last challenge. Viability was defined as the frequency of Annexin-V⁻ cells. Shown is a summary of FACS data (mean $\pm$ SEM; n = 5 per group). Fig 3, E–G, Mean fluorescent intensity (MFI) of Siglec-F, CD11c, and CD11b of eosinophils in indicated organs in allergic mice. Shown is a summary of FACS data (mean $\pm$ SEM; n = 5 per group). Fig 3, H, Lung cells were restimulated for 4 hours with phorbol 12-myristate 13-acetate/ionomycin. Shown is the total number of lung IL-4⁺, IL-5⁺, and IFN- γ -producing CD4⁺ T cells as a summary of FACS data (mean $\pm$ SEM; n = 5 per group). Fig 3, I and J, OVA-specific antibody titers were measured in immunized mice 6 days after the last challenge. Shown are levels of IgG₁ in BAL fluid (Fig 3, I) and serum (mean $\pm$ SEM; n = 5 per group; Fig 3, J). **K** and **L**, CCL11 and CCL24 levels were quantified in BAL fluid of immunized mice 6 days after the last challenge, (mean $\pm$ SEM; n = 5 per group). Data are representative of 2 independent experiments. Eos, Eosinophils; i.p., intraperitoneal; Neu, neutrophils. *P < .05, **P < .01, and ***P < .001, Student t test (unpaired).

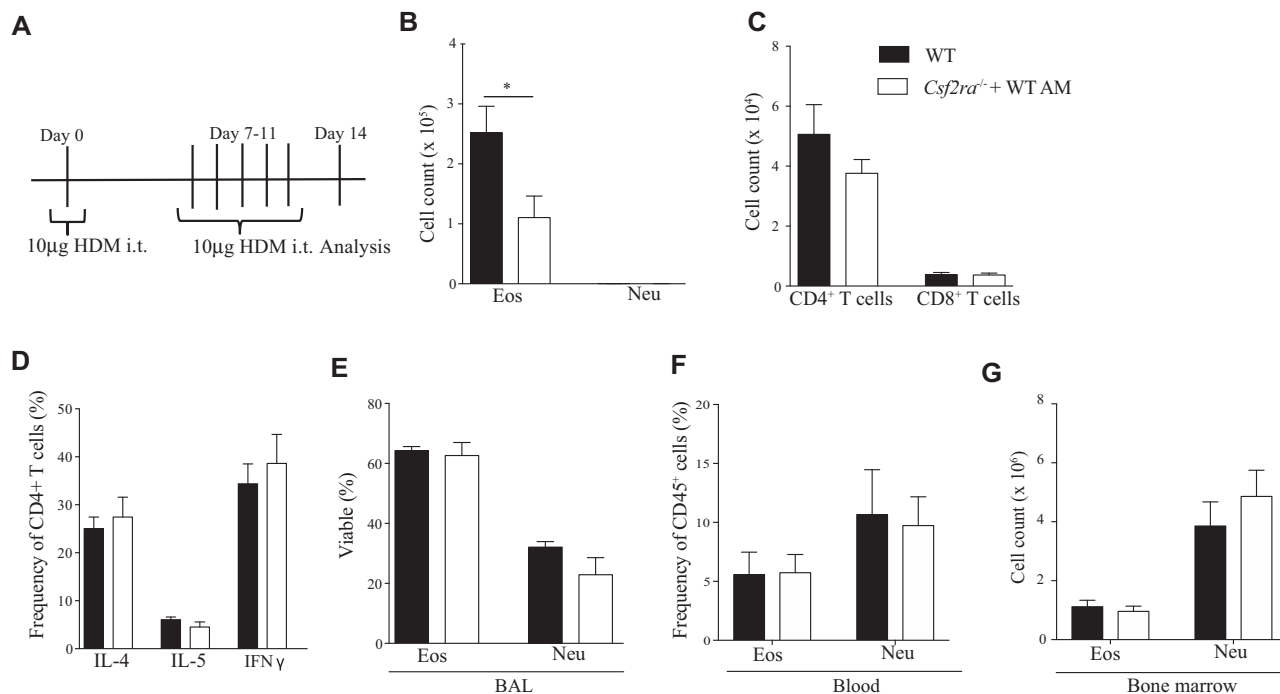


FIG 4. GM-CSFR signaling controls pulmonary eosinophilia in HDM-induced pulmonary allergic inflammation. **A**, AM-reconstituted *Csf2ra*^{-/-} mice were sensitized and challenged with HDM, as indicated in the scheme. *i.t.*, Intratracheal. **B-D**, Numbers of BAL fluid eosinophils and neutrophils (Fig 4, **B**), CD4⁺ and CD8⁺ T cells in BAL fluid (Fig 4, **C**), and frequencies of IL-4-, IL-5- and IFN- γ -producing CD4⁺ T cells (Fig 4, **D**) as a summary of FACS data (mean $\pm$ SEM). **E**, Viability of eosinophils and neutrophils in BAL fluid. Viability was defined as the frequency of Annexin-V⁻ cells (n = 5-6 per group). **F** and **G**, Frequencies of eosinophils and neutrophils in blood (Fig 4, **F**) and total numbers in BM (Fig 4, **G**; n = 4-8 per group). Data are representative of 2 independent experiments. *Eos*, Eosinophils; *Neu*, neutrophils. **P* < .05, Student *t* test (unpaired).

to GM-CSF can also not respond to IL-3 and IL-5. As expected, *Csf2rb*^{-/-} mice show a reduction in mature eosinophils in the BM and peripheral organs analyzed, including blood, the small intestine, and colon lamina propria. In contrast, no significant differences were found in *Csf2ra*^{-/-} mice (Fig 1, **A** and **B**).

To address a possible intrinsic role of GM-CSF signaling in a competitive setting, we generated mixed BM chimeras with WT and *Csf2ra*^{-/-} BM cells. Analyzing again the eosinophil population of these chimeric mice at steady state, we did not observe any significant impairments for CD45.2⁺ *Csf2ra*^{-/-} BM-derived cells in the BM, blood, colon, and lung (Fig 1, **C**). However, a minor developmental disadvantage could be observed for eosinophils in the small intestine lamina propria (Fig 1, **C**). WT BM-derived CD45.1⁺ cells made up the remainder of the eosinophil compartment in all organs, indicating complete replacement of CD45.1⁺CD45.2⁺ host-derived eosinophils (see Fig E1, **A**, in this article's Online Repository at www.jacionline.org).

Overall, these results confirmed a major requirement for common β -chain signaling for eosinophil development while indicating that intrinsic sensing of GM-CSF is largely dispensable for eosinophil generation at steady state.

GM-CSFR signaling controls pulmonary eosinophilia in the setting of experimental asthma

Having established that GM-CSFR signaling does not play a major role in eosinophil development, we wanted to investigate the regulation of eosinophils during inflammation, focusing on

experimental asthma as a classical disease model for these cells. *Csf2ra*^{-/-} mice have PAP caused by the absence of AMs,¹¹ similar to *Csf2rb*^{-/-} and *Csf2*^{-/-} mice, which is a confounding factor in understanding lung immune responses in these knockout mice. However, we have recently established a stem cell therapy precluding PAP in mice lacking GM-CSFR chains.^{10,16} For this purpose, we transferred CD45.1⁺ AM precursors harvested from E17.5 lungs of WT embryos into neonatal *Csf2ra*^{-/-} CD45.2⁺ pups. Indeed, AM precursors fully reconstituted *Csf2ra*^{-/-} mice with a population of mature WT AMs (Fig 2, **A**), which prevented development of PAP, as recently described.¹⁷ Further analysis of naive AM-reconstituted *Csf2ra*^{-/-} mice revealed that they had a reduced number of lung conventional but not monocyte-derived dendritic cells (DCs; Fig 2, **B**), confirming earlier reports about a role of GM-CSF signaling in lung DC development.¹⁸ Analyzing the surface expression of GM-CSFR α on lung DCs (Fig 2, **C**), it was evident that all lung DC subsets significantly expressed the receptor. Similarly, eosinophils from both naive and immunized animals in different tissues significantly expressed GM-CSFR α on the cell surface (Fig 2, **D**).

AM-reconstituted *Csf2ra*^{-/-} mice were then sensitized and challenged with OVA, as indicated in the scheme (Fig 3, **A**), to determine the importance of GM-CSFR signaling for pulmonary eosinophilia in experimental asthma. Quantification of the number of eosinophils and neutrophils in the bronchoalveolar lavage (BAL) fluid and lung tissue of immunized mice at days 3 and 6 after the last challenge revealed that the number of eosinophils was significantly reduced in the BAL fluid at both time points,

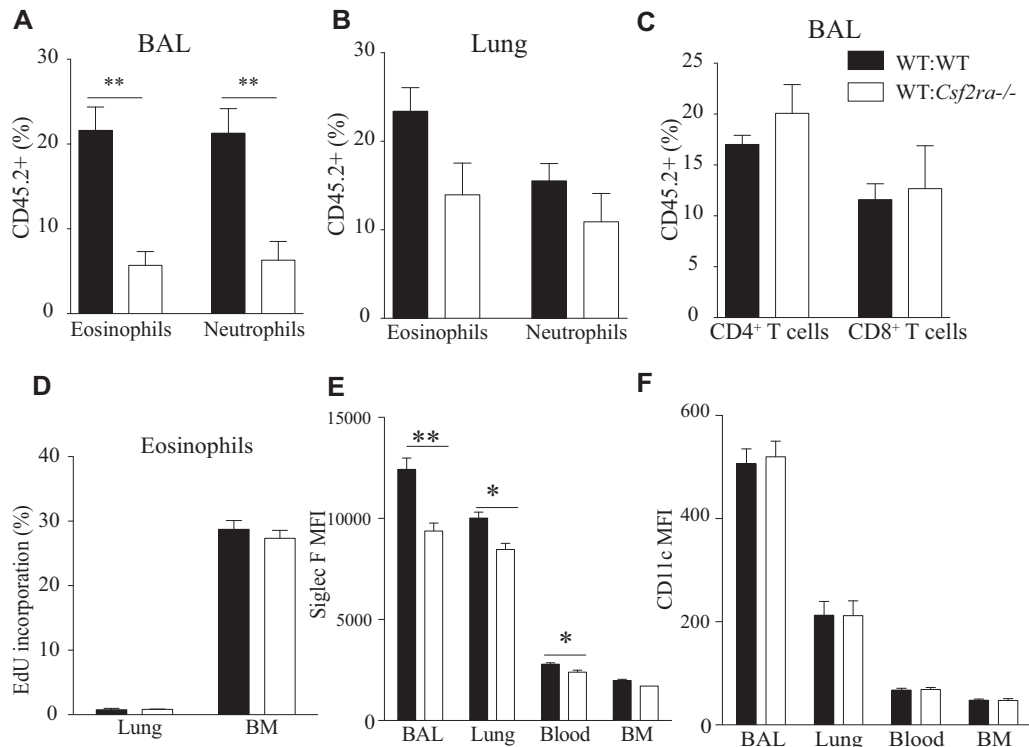


FIG 5. GM-CSF signaling intrinsically controls eosinophilia in the setting of allergic inflammation. **A-D**, WT mice were lethally irradiated and reconstituted with a mixture of either WT (CD45.1) and WT (CD45.2) or WT (CD45.1) and *Csf2ra*^{-/-} (CD45.2) BM at a 4:1 ratio. After 10 weeks of reconstitution, animals were sensitized and challenged with OVA, as described in Fig 2. **A-C**, Summary of FACS data (mean $\pm$ SEM; $n = 6-7$ per group) in which the frequency of CD45.2⁺ cells of granulocytes is shown for BAL fluid (Fig 5, A) and lung tissue (Fig 5, B), as well as BAL fluid CD4⁺ and CD8⁺ T cells (Fig 5, C). **D**, One milligram of EdU was injected 1 day before analysis, and shown are the frequencies of EdU⁺ cells of CD45.2⁺ eosinophils in indicated organs. Shown is a summary of FACS data (mean $\pm$ SEM; $n = 6-7$ per group). **E** and **F**, Mean fluorescent intensity (MFI) of Siglec-F and CD11c on eosinophils in indicated organs. Shown is a summary of FACS data (mean $\pm$ SEM; $n = 6-7$ per group). Data are representative of 2 independent experiments. * $P < .05$ and ** $P < .01$, Student t test (unpaired).

although the difference was more pronounced at the later time point (Fig 3, B and C). Moreover, neutrophil counts were reduced in the lung but not the BAL fluid 6 days after challenge (Fig 3, D and E), when inflammation was declining.

To evaluate whether the difference in pulmonary eosinophilia was observable already at an early time point, we analyzed eosinophils and neutrophils in BAL fluid and lung tissue 24 hours after the last challenge. Although we observed a trend toward fewer eosinophils in BAL fluid and lungs of *Csf2ra*^{-/-} mice, these were not found to be statistically significant (see Fig E1, B).

Because we observed a more pronounced deficiency in pulmonary eosinophilia after the peak of inflammation and there had been some reports that GM-CSF regulates eosinophil survival *in vitro*,⁸ we hypothesized that eosinophil survival might be impaired in the absence of functional GM-CSF signaling. Therefore we quantified the frequency of viable granulocytes using Annexin V and viability dye staining in the BAL fluid and lung tissue of asthmatic animals. However, no differences could be observed between eosinophils and neutrophils of WT and *Csf2ra*^{-/-} animals in any compartment (Fig 3, D). Furthermore, reports in a colitis model suggested that GM-CSF controls activation of eosinophils by directly regulating

Siglec-F expression,⁶ a key eosinophil marker.¹⁹ Indeed, we found that the increase in the mean fluorescent intensity of Siglec-F on eosinophils in *Csf2ra*^{-/-} mice in both the BAL fluid and lung tissue compared with blood was significantly lower than that of WT control mice (Fig 3, E), whereas this difference could not be observed in eosinophils from naive mice (see Fig E1, C). In addition, eosinophils from AM-reconstituted *Csf2ra*^{-/-} mice displayed lower surface levels of the integrin CD11c (Fig 3, F), but not CD11b (Fig 3, G), which again was not visible in naive mice (see Fig E1, D).

Because of the pronounced reduction in pulmonary eosinophilia in mice lacking GM-CSF signaling, the importance of a functional T_H2 immune response for recruitment of eosinophils into the lung, and the observed deficiency in lung DCs, we quantified the number of CD4⁺ T cells producing the T_H2-type cytokines IL-4 and IL-5, which are essential drivers of eosinophilia in this type of asthma model.²⁰ No differences were observed in the number of cells producing T_H2 cytokines, and also the number of IFN- γ ⁺ cells was the same between WT and *Csf2ra*^{-/-} mice (Fig 3, H). In addition, no difference was observed in the amount of OVA-specific IgG₁ antibody titers in the BAL fluid and serum of immunized mice (Fig 3, I and J). Furthermore, levels of the eosinophil-attracting chemokines CCL11

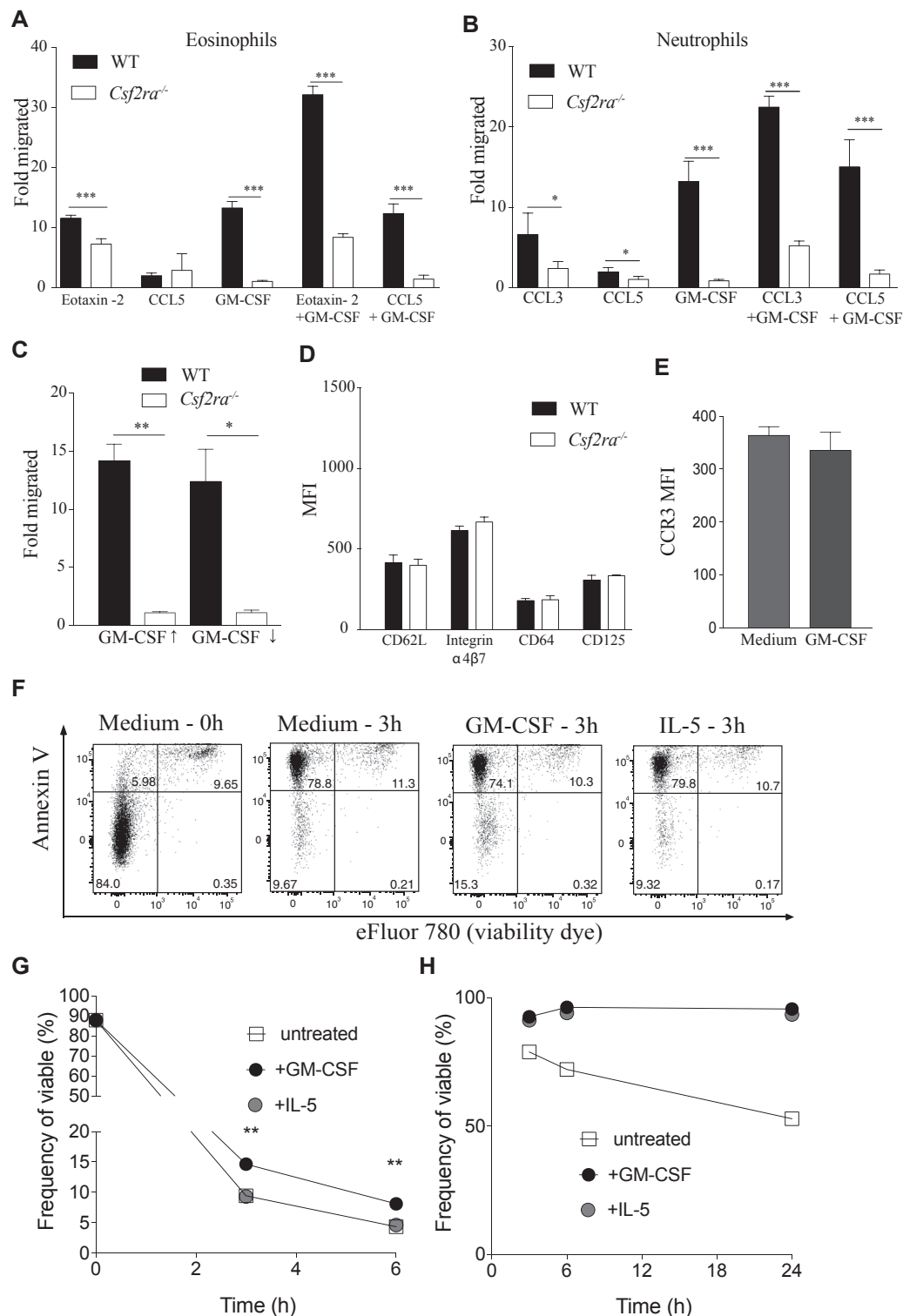


FIG 6. GM-CSF signaling intrinsically controls eosinophil migration *in vitro* by inducing chemokinesis. **A** and **B**, Eosinophils and neutrophils were sorted (>99%) from the BM of WT or *Csf2ra*^{-/-} mice, and a 3-hour transwell assay was conducted. Fig 6, **A**, Eosinophils migrated toward eotaxin-2 (200 ng/mL), CCL5 (200 ng/mL), GM-CSF (100 ng/mL), or a combination of these (n = 6 per condition). Fig 6, **B**, Neutrophils migrated toward CCL3 (200 ng/mL), CCL5 (200 ng/mL), GM-CSF (100 ng/mL), or a combination of those (n = 6 per condition). Shown is fold migration normalized to the medium control (mean ± SEM). **C**, GM-CSF (100 ng/mL) was added to the upper or lower transwell, and migration was quantified after 3 hours. Shown is fold migration normalized to the medium control (mean ± SEM). **D**, Indicated cell-surface markers were quantified *ex vivo* on untreated BM eosinophils from WT and *Csf2ra*^{-/-} animals. Cells were identified as described in Fig 1. Shown is mean fluorescent intensity (MFI; mean ± SEM; n = 3 per group). **E**, Eosinophils

and CCL24 were comparable between AM-reconstituted *Csf2ra*^{-/-} and WT mice (Fig 3, K and L).

Overall, these results indicate that GM-CSF signaling generally promotes pulmonary eosinophilia during allergic inflammation and is not required for an intact T_H2 immune response and normal levels of eosinophil-attracting chemokines.

GM-CSFR signaling controls pulmonary eosinophilia in the setting of HDM-induced pulmonary allergic inflammation

After having established an essential requirement of GM-CSF signaling for pulmonary eosinophilia in a classical model of asthmatic inflammation using OVA and alum, we wanted to confirm these results by using the more physiologic allergen HDM extract. Again, AM-reconstituted *Csf2ra*^{-/-} mice were sensitized and challenged with HDM, as indicated in the scheme (Fig 4, A). Similar to the results observed in the OVA model, the number of infiltrating eosinophils in BAL fluid was significantly reduced in *Csf2ra*^{-/-} mice (Fig 4, B), and almost no recruitment was observed in PBS-sensitized control mice (see Fig E1, E). No differences could be observed in numbers of infiltrating CD4⁺ and CD8⁺ T cells (Fig 4, C) or frequencies of T_H2 cytokine-producing CD4⁺ T cells (Fig 4, D). Similarly, no differences could be observed in frequencies of viable cells among the granulocytes (Fig 4, E).

To address the possibility that the pool of eosinophils is affected differentially in the absence of GM-CSFR signaling during allergic inflammation, we quantified granulocytes in blood and BM during HDM-induced inflammation. The frequency of eosinophils in the blood of allergic *Csf2ra*^{-/-} mice was no different from that in WT control mice (Fig 4, F). Similarly, no differences could be observed in granulocyte numbers in the BM (Fig 4, G).

Overall, these results corroborated the observations made in the OVA/alum model. In addition, they indicated that accumulation of eosinophils in the lungs is specifically impaired, whereas the pool of eosinophils in blood and bone marrow is unaffected in the absence of GM-CSFR signaling.

Eosinophils intrinsically require GM-CSFR signaling for lung and alveolar recruitment during asthmatic inflammation

After having established an important role for GM-CSF signaling for pulmonary eosinophilia in allergic inflammation, we wanted to determine whether GM-CSF signaling plays an intrinsic role in eosinophils in this setting. To address this question, we generated mixed BM chimeras by reconstituting lethally irradiated C57BL/6 recipients with a mixture of BM cells

derived from *Csf2ra*^{-/-} (CD45.2) and WT (CD45.1) mice and tested these animals in an OVA/alum experimental asthma model. In BAL fluid the frequency of both eosinophils and neutrophils lacking *Csf2ra* was significantly reduced compared with the WT control cells (Fig 5, A). In the lung a similar trend could be observed, but this was not quite statistically significant (Fig 5, B). The reduction was indeed specific to *Csf2ra*-deficient granulocytes because other cell types in BAL fluid, such as CD4⁺ and CD8⁺ T cells, showed no obvious impairment (Fig 5, C).

To determine whether the reduction in *Csf2ra*-deficient granulocyte numbers was due to impaired proliferation, we measured their turnover based on incorporation of the DNA analogue EdU. The frequency of EdU⁺ eosinophils was high (up to 30%) in the BM and negligible (approximately 1%) in the lungs of WT mice, indicating expansion of eosinophils in the BM and recruitment to the lung rather than local expansion in the lung on airway allergen exposure. No difference was observed in the absence of GM-CSFR (Fig 5, D).

Finally, to establish whether impaired upregulation of Siglec-F and CD11c expression on eosinophils (Fig 3, E and F) was indeed due to an intrinsic requirement of the GM-CSFR, we compared the mean fluorescent intensities of eosinophils in the different compartments. Reduced expression of Siglec-F could be observed in *Csf2ra*^{-/-} eosinophils from lung tissue and BAL fluid in particular (Fig 5, E). However, CD11c surface expression was comparable in WT and *Csf2ra*^{-/-} eosinophils (Fig 5, F). Surprisingly, CCR3 surface expression levels were also greater on *Csf2ra*-deficient eosinophils compared with WT control cells in the BAL fluid and lung tissue but not in the blood of asthmatic animals (see Fig E1, F).

Overall, these results indicate that GM-CSF signaling in granulocytes and in particular eosinophils intrinsically controls accumulation and activation during pulmonary allergic inflammation.

GM-CSF intrinsically controls eosinophil migration *in vitro* by inducing chemokinesis

After having established an important role for GM-CSF signaling in granulocytes in the setting of asthmatic inflammation, we wanted to identify the underlying mechanistic basis for this phenomenon. We hypothesized that GM-CSF could be directly involved in recruitment of granulocytes to the lung.

To first address a potential role of GM-CSF in regulating eosinophil migration, we initially conducted simple *in vitro* transwell assays with *ex vivo* fluorescence-activated cell sorting (FACS)-sorted eosinophils and neutrophils from the BM of WT and *Csf2ra*^{-/-} mice. Interestingly, GM-CSF by itself also triggered potent GM-CSFR-dependent migration of both eosinophils

harvested from BM of WT and *Csf2ra*^{-/-} mice were treated with GM-CSF for 3 hours. Shown is CCR3 MFI normalized to the untreated control (n = 3 per group; mean ± SEM). F and G, BM eosinophils from WT and *Csf2ra*^{-/-} mice were treated with GM-CSF or IL-5 for indicated times. Fig 6, F, FACS dot plots show staining with Annexin-V and eFluor 780. Fig 6, G, Values indicate the frequency (mean ± SEM) of viable cells defined as Annexin-V⁻eFluor780⁺ of BM eosinophils (n = 3 per group). H, Eosinophils were differentiated from BM stem cells. At day 14, cells were washed and grown overnight in the presence of medium and FCS before addition of IL-5, GM-CSF, or neither for indicated times and analyzed by using FACS. Values show the frequency (mean ± SEM) of viable cells defined as Annexin-V⁻eFluor780⁺ of BM-derived eosinophils (n = 3 per group). Data are representative of 3 independent experiments. *P < .05, **P < .01, and ***P < .001, Student t test (unpaired).

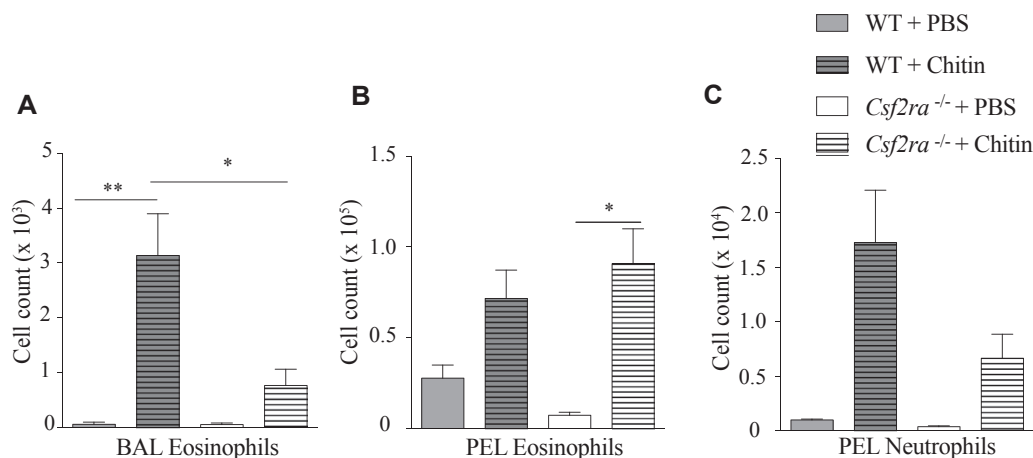


FIG 7. GM-CSF signaling controls lung-specific eosinophil migration *in vivo*. **A**, WT and *Csf2ra*^{-/-} animals received 5 μ g of chitin intratracheally, and eosinophil infiltration was quantified after 48 hours. Shown are pooled data from 2 independent experiments (mean $\pm$ SEM; n = 6-14 per group). **B** and **C**, WT and *Csf2ra*^{-/-} animals received 800 ng of chitin intraperitoneally, and granulocyte infiltration was quantified after 48 hours. Shown is a summary of FACS data (mean $\pm$ SEM; n = 3-7 per group) of eosinophils and neutrophils. Data are representative of 2 independent experiments. **P* < .05 and ***P* < .01, Student *t* test (unpaired). PEL, Peritoneal lavage.

and neutrophils in these assays (Fig 6, A). Both eotaxin-2, a well-known eosinophil-attracting chemokine, and, to a lesser extent, CCL5 also induced eosinophil migration *in vitro*, which was slightly affected in the absence of GM-CSFR α (Fig 6, A). Notably, an additive function of eotaxin-2 and GM-CSF could be observed, which was not the case for CCL5 and GM-CSF (Fig 6, A). Similar to eosinophils, GM-CSF also induced potent migration of neutrophils *in vitro*, which was again strictly dependent on the presence of GM-CSFR α (Fig 6, B). Also, for neutrophils, the combination of CCL3 and GM-CSF induced more potent migration, although this was again not the case for CCL5 and GM-CSF (Fig 6, B).

Having observed potent migration of eosinophils in the presence of GM-CSF, we wanted to further characterize the underlying mechanism. To evaluate whether GM-CSF induces directional chemotaxis or just nondirectional chemokinesis, we conducted transwell experiments in which GM-CSF was added to the upper or lower well. These experiments revealed that GM-CSF displayed equal capacity to induce migration in both conditions, indicating that GM-CSF is a potent inducer of chemokinesis rather than chemotaxis (Fig 6, C). No differences were observed in cell-surface expression of a variety of activation markers on BM-localized eosinophils from WT and *Csf2ra*^{-/-} mice *ex vivo*, ruling out potential developmental differences before GM-CSF stimulation *in vitro* (Fig 6, D). Furthermore, GM-CSF stimulation did not alter CCR3 expression on viable WT eosinophils, indicating that it does not promote chemokinesis by inducing eotaxin-CCR3 pathway (Fig 6, E).

After elucidating a role for GM-CSF in regulating eosinophil migration, we sought to also investigate its capacity to influence eosinophil survival. Notably, the majority (ie, 80%) of *ex vivo*-isolated BM eosinophils upregulated Annexin V, a marker of apoptosis, already after 3 hours of culture. GM-CSF, but not IL-5, stimulation moderately reduced apoptosis (Fig 6, F and G). Moreover, 98% of eosinophils were dead according to staining with a viability dye (ie, eFluor780) after 24 hours of culture in

the absence or presence of GM-CSF or IL-5 (data not shown). Next, using a well-established protocol for *in vitro* differentiation of eosinophils from BM stem cells,¹⁵ we found that both IL-5 and GM-CSF potently inhibited eosinophil cell death for at least 24 hours (Fig 6, G).

Overall, these results indicate that GM-CSF potently induces eosinophil chemokinesis and eosinophil survival *in vitro*.

GM-CSF controls lung-specific eosinophil migration *in vivo*

To address whether GM-CSF directly promotes eosinophil migration *in vivo*, chitin, as a potent inducer of innately driven eosinophilia,²¹ was injected intratracheally. Similar to results observed in *in vitro* assays, the number of eosinophils in the BAL fluid was significantly lower in *Csf2ra*^{-/-} compared with WT control mice 48 hours post treatment (Fig 7, A).

To investigate whether the *in vivo* requirement for GM-CSF signaling for eosinophil migration was a general phenomenon or lung-specific, we injected chitin intraperitoneally and again evaluated granulocyte recruitment to the peritoneal cavity. To our surprise, eosinophil recruitment was completely intact in *Csf2ra*-deficient mice (Fig 7, C). Although not statistically significant, a minor decrease in neutrophil recruitment was observed in the peritoneal cavity (Fig 7, C). Overall, these results suggest that defective GM-CSFR signaling in eosinophils results in impaired migration to the lung but not the peritoneal cavity.

DISCUSSION

GM-CSF signaling has been reported to affect many aspects of eosinophil biology, but to date, no study has addressed the function of this key cytokine in eosinophils *in vivo* in an intrinsic and comprehensive manner. In this article we demonstrate that GM-CSF signaling intrinsically promotes eosinophils accumulation in inflamed lungs in patients with allergic inflammation. We

found that although appearing to be largely dispensable for development and expansion *in vivo*, GM-CSF directly controls the trafficking of granulocytes to allergic lungs and potentially their survival *in situ*. Indeed, we show that the requirement of GM-CSF appears to be lung specific because eosinophil accumulation was not affected in the peritoneal cavity of *Csf2ra*^{-/-} mice. Mechanistically, GM-CSF appears to directly induce chemokinesis of eosinophils to the inflamed lung, acting by itself, as well as in synergistic fashion with other chemokines, such as the eotaxin family.

Interestingly, eosinophils in BAL fluid and lung tissue of asthmatic *Csf2ra*^{-/-} mice expressed significantly higher levels of CCR3 than WT control animals, indicating that CCR3 receptor internalization is potentially impaired in the absence of functional GM-CSF signaling. CCR3 receptor internalization has been shown to be an important step in regulating multiple aspects of eosinophil chemotaxis.²² However, GM-CSF treatment by itself appears to have no effect on CCR3 expression, suggesting a more complex mechanism *in vivo* than can be modeled *in vitro*. Therefore GM-CSF also appears to promote the ability of eosinophils to respond to chemokines, but the underlying mechanism remains to be unraveled in future studies.

In addition to promoting eosinophil recruitment, we found that GM-CSF can also directly regulate eosinophil survival *in vitro*, as has already been suggested in the literature.⁸ Indeed, the deficiency in pulmonary eosinophilia is more pronounced at later time points, supporting the idea that survival might also play a role *in vivo* in this context. Although we did not find clear evidence for this in our *in vivo* models, we cannot exclude that survival contributes to this phenomenon because dying cells are rapidly removed by macrophages and not readily detected.²³

In addition, to the intrinsic role of GM-CSF signaling in eosinophils, we also found that GM-CSF potentially promotes eosinophil accumulation indirectly. Although no differences were observed in levels of eosinophil-attracting chemokines, eosinophils from *Csf2ra*^{-/-} mice exhibited significantly reduced surface expression of the integrin CD11c. Recent preliminary evidence suggests that CD11c is required for transepithelial eosinophil migration.²⁴ Interestingly, although eosinophil accumulation in BAL fluid was strongly defective in the absence of functional GM-CSF signaling, the reduction was less pronounced for lung tissue. It is likely that this represents eosinophils at different stages of migration going through the lung parenchyma to the bronchoalveolar space.²⁴ This notion would suggest that the strong reduction in eosinophil counts in the BAL fluid of *Csf2ra*^{-/-} animals is due to a specifically impaired migration to the bronchoalveolar space in the absence of functional GM-CSFR signaling while leaving extravasation and migration of eosinophils to the lung parenchyma largely intact. This would indicate that the last transepithelial migration step is strongly defective without functional GM-CSF signaling.

In addition, GM-CSF appears to directly control eosinophil activation in the lung, as shown by promoting expression of Siglec-F. The question remains of why GM-CSF signaling appears to play a much more important role in mediating eosinophil migration to the bronchoalveolar space than other peripheral sites, such as the peritoneum. It is known that GM-CSF levels are particularly high in the lung, and indeed, it is strongly induced in allergic inflammation.²⁵ It is likely that at other sites different tissue-derived cytokines promote eosinophil migration and function. This observation also raises the question about the

actual source of GM-CSF in the lung in this context. It is known that in the lung GM-CSF is mainly produced by nonhematopoietic cells,¹⁰ but whether bronchoalveolar epithelial cells are in fact the main producers of this cytokine in allergic inflammation remains to be elucidated in future studies. In the context of asthma, these findings are important because recent evidence points to a potentially detrimental role of eosinophils in a subset of patients with steroid-resistant asthma, which is particularly prevalent in obese subjects.²⁶ Thus understanding which factors contribute to the recruitment of eosinophils to the inflamed lung and their activation *in situ* in this context is of paramount importance.

In addition, the results presented in this article further underline the importance of GM-CSF for lung immunity in general after previous studies have already established key functions of GM-CSF for AM^{17,27} and lung DC¹⁸ development. Importantly, *Csf2ra*-deficiency did not prevent induction of a potent T_H2 response in the evaluated experimental asthma models despite the significant reduction in numbers of lung conventional DCs at steady state. This suggests that the remaining DCs are sufficient for induction of type 2 immunity or that recruitment of monocyte-derived DCs can overcome the deficiency in lung-resident DCs. Furthermore, it raises questions about previous studies²⁸ that used eosinophils just as a readout for a T_H2 response, excluding possible direct effects of GM-CSF on eosinophils.

Lastly, for the first time, this report comprehensively addressed the role of GM-CSF signaling in eosinophil development. The results demonstrate that GM-CSF has no major role in generation of eosinophils in the BM and blood. GM-CSF signaling was found not to be required for eosinophil development in peripheral tissues, such as the colon, at steady state, although we observed a minor deficiency in eosinophils in the small intestine in a competitive setting. Interestingly, intestinal eosinophils were shown to express high levels of Siglec-F and CD11c,²⁹ which were similar to those in infiltrating cells in our asthma models. This would suggest a possible role of GM-CSF also in this context but whether this is the case remains to be elucidated in future studies.

Overall, our results reveal a lung-specific requirement of GM-CSF for accumulation of eosinophils during allergic inflammation, underlining the role of GM-CSF as tissue-specific regulator of immunity.

We acknowledge the use of the Immgen database as an informative tool for the design of this study.

Key messages

- GM-CSF signaling intrinsically promotes eosinophil accumulation in the lung during airway inflammation.
- GM-CSF induces eosinophil chemokinesis and promotes survival *in vitro*.
- GM-CSF signaling has no major role in eosinophil development at steady state or in inflammation.

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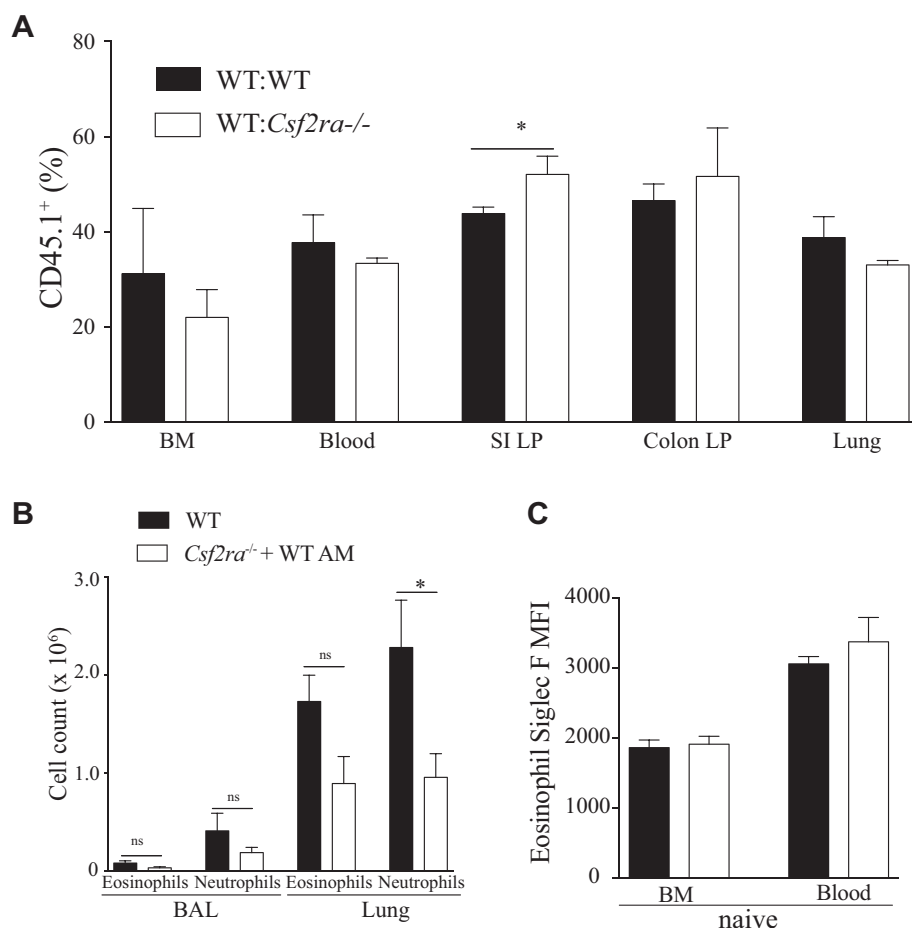


FIG E1. GM-CSF signaling promotes pulmonary eosinophilia in the setting of allergic inflammation. **A**, WT (CD45.1⁺CD45.2⁺) mice were lethally irradiated and reconstituted with a mixture of either WT (CD45.1) and WT (CD45.2) or WT (CD45.1) and *Csf2ra*^{-/-} (CD45.2) BM at a 1:1 ratio. After 10 weeks of reconstitution, organs of naive recipient mice were analyzed for eosinophils. Shown is a summary of FACS data (mean $\pm$ SEM) in which the frequency of CD45.1⁺ cells is shown for the indicated organs (n = 3-4 per group). **B**, Fetal (E17.5) lung AM precursors from WT CD45.1⁺ C57BL/6 embryos were transferred intranasally to neonatal (day 3 after birth) *Csf2ra*^{-/-} mice. After 8 weeks, animals were sensitized and challenged with OVA, as shown in the scheme and analyzed 1 day after the last challenge. Shown is a summary of FACS data (mean $\pm$ SEM) of BAL fluid and lung tissue for eosinophils and neutrophils (n = 5 per group). **C** and **D**, Shown is the mean fluorescent intensity (MFI) of Siglec-F and CD11c of eosinophils in indicated organs in naive mice. Shown is a summary of FACS data (mean $\pm$ SEM; n = 5 per group). **E**, AM-reconstituted *Csf2ra*^{-/-} mice were sensitized and challenged with HDM, as indicated in Fig 4. Shown is a summary of FACS data (mean $\pm$ SEM) of BAL fluid and lung tissue for eosinophils and neutrophils (n = 4-8 per group). Eosinophils were identified as CD45⁺CD11c⁻CD11b⁺Siglec-F⁺SSC-A^{high} and neutrophils as CD45⁺CD11c⁻CD11b⁺Ly-6G⁺ cells, respectively. **F**, WT mice were lethally irradiated and reconstituted with a mixture of either WT (CD45.1) and WT (CD45.2) or WT (CD45.1) and *Csf2ra*^{-/-} (CD45.2) BM at a 1:1 ratio. After 10 weeks of reconstitution, animals were sensitized and challenged with OVA, as described in Fig 2. Shown is the MFI of CCR3 on eosinophils in indicated organs. Shown is a summary of FACS data (mean $\pm$ SEM; n = 6-7 per group). Data are representative of 2 independent experiments. *P < .05 and **P < .01, Student t test (unpaired). LP, Lamina propria; ns, not significant; SI, small intestine.

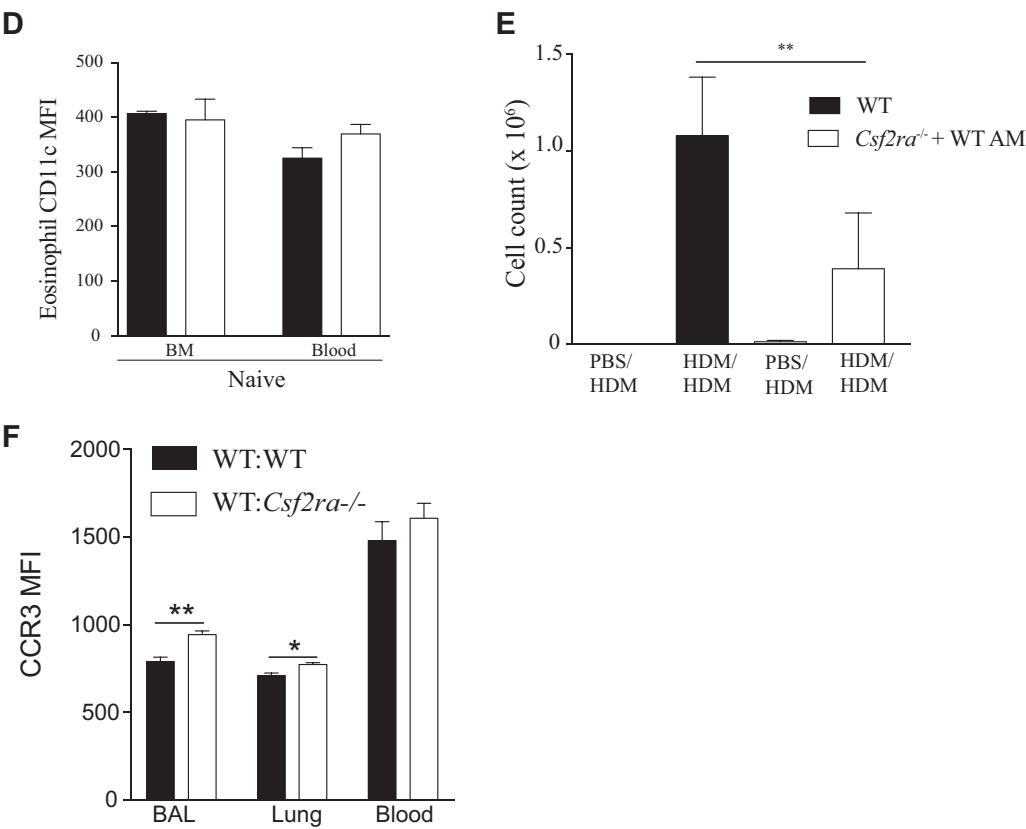


FIG E1. (Continued).