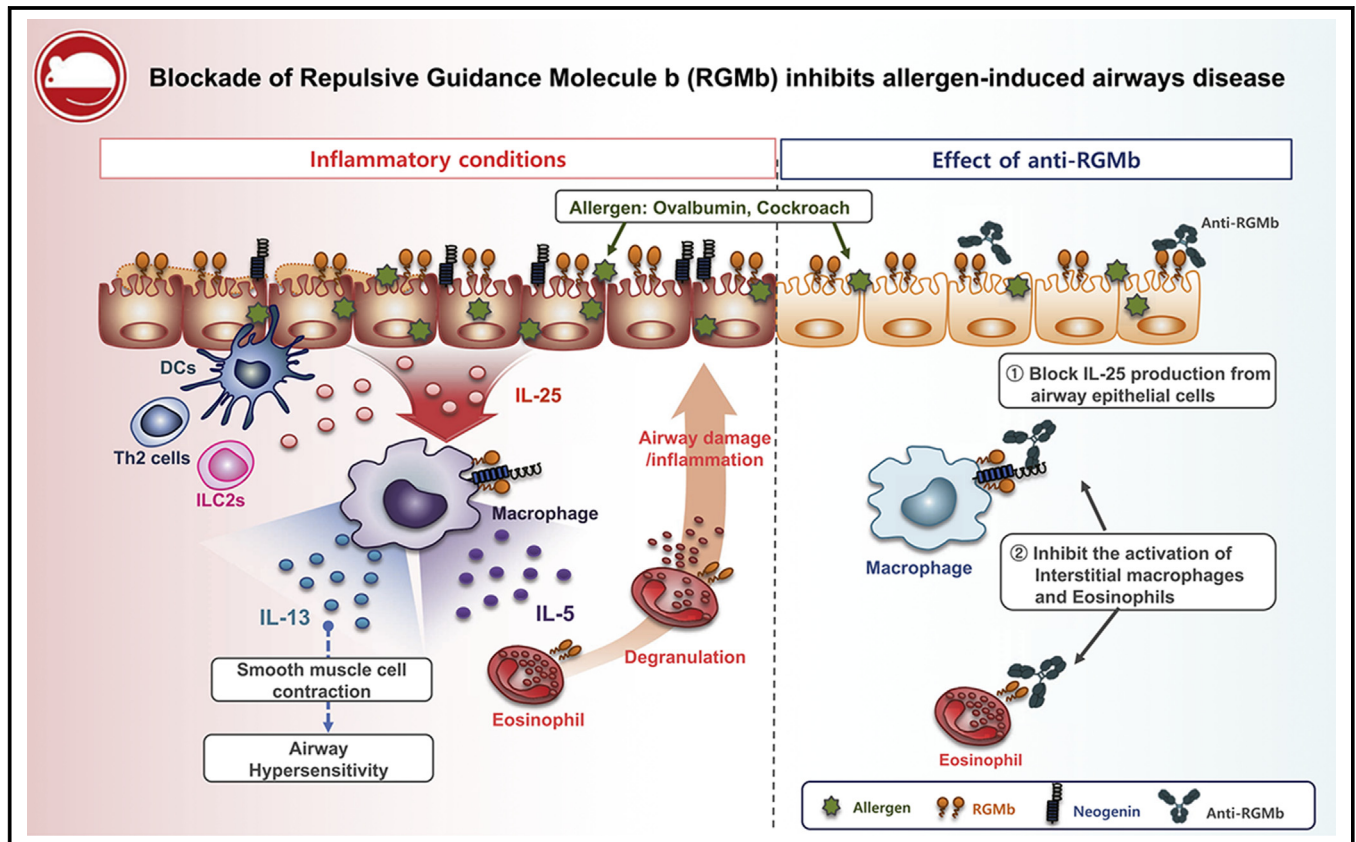


Blockade of RGMb inhibits allergen-induced airways disease



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GRAPHICAL ABSTRACT



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Disclosure of potential conflict of interest: S. Yu, Y. Xiao, G. J. Freeman, and R. H. DeKruyff have patents pending on the use of RGMb reagents in asthmatic patients and

patients with cancer. G. J. Freeman has patents/pending royalties on the PD-1 pathway from Bristol-Myers-Squibb, Roche, Merck, EMD-Serono, Boehringer-Ingelheim, AstraZeneca, and Novartis and has served on advisory boards for CoStim, Novartis, Roche, Eli Lilly, Bristol-Myers-Squibb, Seattle Genetics, and Bethyl Laboratories. The rest of the authors declare that they have no relevant conflicts of interest.

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Background: Allergic asthma causes morbidity in many subjects, and novel precision-directed treatments would be valuable.

Objective: We sought to examine the role of a novel innate molecule, repulsive guidance molecule b (RGMb), in murine models of allergic asthma.

Methods: In models of allergic asthma using ovalbumin or cockroach allergen, mice were treated with anti-RGMb or control mAb and examined for airway inflammation and airway hyperreactivity (AHR), a cardinal feature of asthma. The mechanisms by which RGMb causes airways disease were also examined.

Results: We found that blockade of RGMb by treatment with anti-RGMb mAb effectively blocked the development of airway inflammation and AHR. Importantly, blockade of RGMb completely blocked the development of airway inflammation and AHR, even if treatment occurred only during the challenge (effector) phase. IL-25 played an important role in these models of asthma because IL-25 receptor-deficient mice did not develop disease after sensitization and challenge with allergen. RGMb was expressed primarily by innate cells in the lungs, including bronchial epithelial cells (known producers of IL-25), activated eosinophils, and interstitial macrophages, which in the inflamed lung expressed the IL-25 receptor and produced IL-5 and IL-13. We also found that neogenin, the canonical receptor for RGMb, was expressed by interstitial macrophages and bronchial epithelial cells in the inflamed lung, suggesting that an innate RGMb-neogenin axis might modulate allergic asthma.

Conclusions: These results demonstrate an important role for a novel innate pathway in regulating type 2 inflammation in patients with allergic asthma involving RGMb and RGMb-expressing cells, such as interstitial macrophages and bronchial epithelial cells. Moreover, targeting this previously unappreciated innate pathway might provide an important treatment option for allergic asthma. (J Allergy Clin Immunol 2019;144:94-108.)

Key words: Asthma, airway hyperreactivity, macrophages, repulsive guidance molecule b, neogenin, IL-17RB, allergy

Asthma, which has increased dramatically in prevalence over the past 3 decades, is a major public health concern. Allergic asthma, the most common form of asthma, is mediated by type 2 inflammatory cells, including T_H2 cells acting in concert with group 2 innate lymphoid cells (ILC2s) and eosinophils, resulting in airway hyperreactivity (AHR), a cardinal feature of asthma. Airway epithelial cells, which are among the first to encounter inhaled allergens, initiate type 2 responses through production of innate cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin. In particular, IL-25 plays a key role in type 2 inflammation in asthmatic patients¹⁻³ by binding to its receptor, IL-17RB, expressed on T_H2 cells, ILC2s, and type 2 myeloid (T2M) cells⁴ and promoting production of type 2 cytokines, thus orchestrating many of the classical features of asthma. However, little is known about innate pathways and molecules that affect and regulate IL-25-driven airway inflammation and AHR.

We examined a novel innate protein, repulsive guidance molecule b (RGMb), which appears to play a key role in IL-25-driven airway pathology. RGMb, a member of the repulsive guidance molecule (RGM) family, was discovered as a repulsive

Abbreviation used

AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
BMP:	Bone morphogenetic protein
CRA:	Cockroach allergen
FACS:	Fluorescence-activated cell sorting
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
H&E:	Hematoxylin and eosin
IHC:	Immunohistochemistry
ILC2:	Group 2 innate lymphoid cell
NEO1:	Neogenin
OVA:	Ovalbumin
PD-L2:	Programmed death ligand 2
RGM:	Repulsive guidance molecule
RGMb:	Repulsive guidance molecule b
RGMb ⁺ macrophage:	F4/80 ⁺ CD11b ⁺ CD11c ⁻ macrophage
T2M:	Type 2 myeloid

axon guidance cue, which directs embryonic brain development by linking the cell surface receptor neogenin (NEO1)⁵⁻⁹ and bone morphogenetic protein (BMP) 2, BMP-4, or BMP-6,¹⁰ providing a hub for multiple signaling pathways^{7,11} that play critical roles in bone^{10,12,13} and lung¹⁴⁻¹⁶ morphogenesis.

Several previous reports have suggested important postnatal roles for RGMb, NEO1, BMP-2, BMP-4, and BMP-6 in the lung. First, lung injury results in induction of NEO1 expression¹⁷ and also activates the BMP pathway in bronchial epithelial cells,¹⁵ potentially promoting lung inflammation. Furthermore, in an allergic airway model, bronchial epithelial cells upregulated expression of BMP-2, BMP-4, and BMP-6 and upregulated BMP/SMAD signaling,¹⁴ further indicating a postnatal role of BMPs in lung inflammation. Much less is known about the role of RGMb postnatally in the lung. However, the first report of a role for RGMb in the postnatal lung demonstrated that RGMb mRNA was highly expressed by lung interstitial macrophages, that RGMb bound to programmed death ligand 2 (PD-L2), and that this RGMb-PD-L2 pathway played a key role in development of respiratory tolerance.¹⁸

In the current report, using experimental mouse models of allergic asthma with ovalbumin (OVA) or cockroach allergen (CRA), we demonstrated a critical role for RGMb in coordinating type 2 lung inflammation. Thus blockade of RGMb by means of treatment with an anti-RGMb mAb profoundly blocked the development of allergic asthma. Surprisingly, both airway epithelial cells and interstitial macrophages in naive and inflamed lung tissue expressed RGMb, as did activated eosinophils, which accumulated in the lungs of mice sensitized and challenged with OVA or German CRA extract. Moreover, a large fraction of RGMb⁺ interstitial macrophages in the inflamed lung expressed IL-17RB, the receptor for IL-25, which accounted for approximately 95% of the IL-17RB⁺ cells in the inflamed lung. This is important because in this model IL-25, but not IL-33, was required for AHR development. Furthermore, F4/80⁺CD11b⁺CD11c⁻ macrophages (RGMb⁺ macrophages) in the inflamed lung produced IL-4, IL-5, and IL-13 and coexpressed NEO1, suggesting the importance of a type 2 innate RGMb-NEO1 signaling hub in these cells. Because treatment with anti-RGMb potently reduced airway inflammation and AHR, even when given late after sensitization, these findings

suggest that anti-RGMb treatment might be effective as a treatment for ongoing established asthma in human subjects.

METHODS

Animals

BALB/cBy and C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Me). PD-L2^{-/-} mice on a BALB/c background have been described previously.¹⁸ Mice were maintained and used according to institutional and National Institutes of Health guidelines in a pathogen-free facility. IL-17RB^{-/-} mice were provided by Amgen (Thousand Oaks, Calif).² ST2^{-/-} mice were provided by Andrew McKenzie.¹⁹ Animal protocols were approved by the Animal Care and Use Committees at Boston Children's Hospital and the Dana-Farber Cancer Institute.

Antibodies

Monoclonal antibodies specific for IL-17RB and NEO1 were generated by means of subcutaneous immunization of female Lewis strain rats (Harlan Sprague-Dawley, Indianapolis, Ind) with mouse IL17-RB-Ig (Arg18-Gly286) or mouse NEO1-Ig (Ala42-Ile 1033 and Asp442-Leu461 del; R&D Systems, Minneapolis, Minn). Hybridoma supernatants were screened and selected for use based on cell-surface staining of mouse IL-17RB-transfected 300.19 cells or NEO1-transfected 300.19 cells, respectively, and for lack of reactivity with untransfected cells (see Fig E1 in this article's Online Repository at www.jacionline.org). Anti-IL-17RB mAb 9B10, which was used in these studies, blocked binding of IL-17RB-huIgG Fc to ELISA plates coated with rIL-25 (see Fig E1), further demonstrating its specificity for the IL-25 receptor. Monoclonal antibodies were purified by using protein G-agarose (Pierce Biotechnology, Rockford, Ill) and conjugated to fluorochromes by BioLegend (San Diego, Calif) or biotin conjugated by using standard techniques.

Generation of RGMb mAbs was previously described.¹⁸ Clone 9D1 was used for *in vivo* studies. RGMb mAb clone 9D1 blocks interaction of RGMb with BMP-2/4 and PD-L2¹⁸ and partially blocks the interaction of RGMb with NEO1 (about 60%, data not shown). Because RGMb binds NEO1 at 2 distinct sites on the RGM molecule, forming a complex of 2 RGMs with 2 NEO1 molecules,⁷ clone 9D1 might block interaction at only one of these sites but not both.

RGMb mAb clone 9D3 was used for fluorescence-activated cell sorting (FACS), immunofluorescence, and immunohistochemistry (IHC) studies because its epitope is not covered by interactions with PD-L2, NEO1, or BMPs. PD-L2 mAb 2C9 blocks the interaction of PD-L2 with RGMb but does not block the interaction of PD-L2 with PD-1.¹⁸

AHR measurement

To induce AHR, mice were sensitized with 100 μ g of OVA (ICN Biomedicals, Costa Mesa, Calif) adsorbed in 2 mg of alum administered intraperitoneally on day 0 and were given intranasal OVA (100 μ g) or saline (50 μ L) on days 7, 8, and 9. In some experiments mice were treated with RGMb mAb 9D1, PD-L2 mAb 2C9, or control mAb intraperitoneally (500 μ g per treatment), as indicated.

On day 10, mice were anesthetized with 50 mg/kg body weight pentobarbital, tracheostomized, and ventilated. Direct measurement of airway resistance and dynamic compliance was performed by using invasive plethysmography (BUXCO Systems, Wilmington, NC).²⁰ Lung resistance and compliance was measured in response to aerosolized saline (0.9% NaCl), followed by increasing doses of aerosolized acetyl- β -methylcholine chloride (methacholine; 0.125–40 mg/mL; Sigma-Aldrich, St Louis, Mo).

CRA-induced allergic airway model

CRA purchased from Greer laboratories (XBP46D3A4; Lenoir, NC) was suspended in PBS to a protein concentration of 2 mg/mL. For the short stimulation protocol, mice were sensitized with 10 μ g of CRA in alum on day 0 and then challenged with 10 μ g of CRA in PBS intranasally on days 7, 8, and

9. Lung histology, FACS analysis of total lung cells, and bronchoalveolar lavage (BAL) were done at day 10. For the chronic model, mice were immunized with 10 μ g of CRA emulsified in incomplete Freund adjuvant on day 0, followed by challenge with 5 μ g of CRA intranasally on days 14, 18, 22, and 26. Anti-RGMb mAb was administered on days 13, 17, 21, and 25. Lung histology, FACS analysis of total lung cells, and BAL fluid analysis were performed on day 27.

BAL, histopathology, and FACS staining of lung cells

After death, lungs were lavaged 3 times with 1 mL of PBS containing 2% FBS, and fluid was pooled. The relative number of different types of leukocytes was determined from slide preparations of BAL fluid stained with hematoxylin and eosin (H&E). For histopathology, lungs were flushed with PBS to remove blood, infused with 10% (vol/vol) formalin, and embedded in paraffin. Lung sections 5 μ m in thickness were cut and stained with H&E. H&E sections on slides were scored based on degree of inflammation as follows: 0, no significant inflammation; 1, small clusters (10–30 cells) of inflammatory cells adjacent to vessels and airways; 2, moderately sized clusters (30–100 cells) of inflammatory cells adjacent to vessels and airways; and 3, large aggregates (>100 cells) of inflammatory cells adjacent to vessels and airways.

FACS antibodies and analysis

For FACS analysis of lung cells, the lung was perfused with PBS; cut into small pieces; digested in RPMI 1640 with 5% FBS, 1.6 mg/mL collagenase IV (Worthington Biochemical, Lakewood, NJ), and 200 U/mL DNase I (Roche, Mannheim, Germany); and then treated with red blood cell lysing buffer (Sigma). Antibodies used for staining can be found in Table E1 in this article's Online Repository at www.jacionline.org. For FACS staining of RGMb expression, cells were first stained for surface marker expression, fixed, and permeabilized with eBioscience Intracellular Fixation and Permeabilization Buffer (Thermo Fisher Scientific, Waltham, Mass) and then stained with Alexa Fluor 647-conjugated RGMb mAb 9D3. For detection of intracellular cytokines, total lung cells were cultured in the presence of Golgi Stop for 6 hours before staining for surface marker expression. Cells were then fixed, permeabilized, stained with anti-cytokine or control mAb, and analyzed by means of FACS.

Immunofluorescence microscopy staining

Frozen sections (7 μ m) of lung tissue were prepared on day 10 from mice sensitized and challenged with OVA. Slides were then stained with antibodies in 2% rat serum and 0.5% BSA. See Table E1 for a list of antibodies used.

Fluorescent images were visualized with a Zeiss AxioImager upright microscope and Plan-NEOFLUAR 40 $\times$ /1.30 lens (Carl Zeiss, Oberkochen, Germany). Pictures were acquired with the AxioCam Hrc and analyzed on AxioVision software (release 4.8). Z-stack images were acquired with a Carl Zeiss LSM 700 Laser Scanning Microscope with ZEN 2009 imaging software (Carl Zeiss). Image analysis was performed with ImageJ software (National Institutes of Health, Bethesda, Md). Resultant stacks were rendered in 3 dimensions by using Bitplane Imaris 7.1 software (Bitplane, Concord, Mass).

IHC

IHC staining for RGMb or NEO1 was performed on sections from tissue frozen in OCT. Tissue was blocked with BLOXALL and stained with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, Calif). Biotin-conjugated RGMb mAb 9D3, biotin-conjugated NEO1 mAb 3C9, or control antibody was used for detection of RGMb or NEO1. After development with peroxidase substrate (DAB Chromogen DAKO K3468; DAKO, Glostrup, Denmark), slides were counterstained with hematoxylin.

Cell isolation and stimulation

Splenic cells were isolated by means of mechanical tissue disruption, depleted of B cells, and restimulated *in vitro* with OVA. Culture supernatants were collected after 4 days for cytokine analysis by means of ELISA.

Quantitative real-time RT-PCR

Total RNA was isolated from lung tissue with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized with the iScript cDNA Synthesis Kit (Invitrogen, Carlsbad, Calif). Quantitative real-time RT-PCR with TaqMan Universal Master Mix (Applied Biosystems, Foster City, Calif) and a gene-specific TaqMan probe was carried out in a 7500 Sequence Detection System (Applied Biosystems). Levels of target gene expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression by using the $2^{-\Delta\Delta C_t}$ method. The following primers and probes were purchased from Applied Biosystems: mouse *Gapdh* (4352339E), *Il4* (Mm00445258_g1), *Il5* (Mm00439645_m1), *Il13* (Mm00434204_m1), *Il25* (Mm00499822_m1), and *Il17rb* (Mm00444709_m1).

Microarray

Total RNA was extracted with the RNeasy Kit (Qiagen). RNA samples were sent to the Microarray Core Facility at the Dana-Farber Cancer Institute (Boston, Mass) for amplification and hybridization on the Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, Calif) to obtain genome-wide expression profiles. Raw data for sample populations were preprocessed and normalized by using the RMA algorithm in the ExpressionFileCreator module in GenePattern.²¹ The resulting data were subsequently analyzed in the Multiplot Suite in GenePattern by first filtering out genes with low expression values (<100 in all subsets) and noisy genes (covariance > 0.8) and then selecting for genes upregulated or downregulated (fold change ≤ 0.6 or fold change ≥ 1.6 , respectively; $P < .05$, 1-way ANOVA Tukey honest significant difference) in inflamed lung tissue (isotype) relative to lung tissue from the saline-treated group. These genes of interest were subsequently divided into further subgroups based on the degree of upregulation or downregulation resulting from treatment with RGMb mAb. Heat maps were generated with the GENE-E module (<http://www.broadinstitute.org/cancer/software/GENE-E/>) in GenePattern (and compiled for publication using Adobe Illustrator; Gene Expression Omnibus submission no. GSE79156).²¹

RESULTS

Treatment with anti-RGMb mAb inhibits AHR development

In an experimental mouse model of allergic asthma, treatment of OVA-sensitized and challenged mice with anti-RGMb mAb at the time of sensitization and in the effector phase (days -1, 4, and 8; Fig 1, A) blocked the development of AHR (Fig 1, B) and cytokine production (Fig 1, C), as assessed on day 10. The reduction in airway inflammation was confirmed by gene expression studies, showing that expression of *Il4*, *Il13*, and *Il33*, as well as *Ctla4*, *Cxcl5*, and *Icos*, was increased in the inflamed airway but significantly reduced by treatment with anti-RGMb (see Fig E2, A, in this article's Online Repository at www.jacionline.org). Expression of *Ifng*, *Tslp*, *Il17a*, *Il22*, and *Il23* was unchanged across the saline-treated, RGMb mAb-treated, and isotype control mAb-treated groups (see Fig E2, B). Furthermore, treatment with anti-RGMb mAb after sensitization had occurred (ie, administration of anti-RGMb only on days 6 and 8) also greatly reduced AHR (Fig 1, D) and airway inflammation (Fig 1, E and F), indicating that the anti-RGMb mAb can function at the effector stage to inhibit AHR and inflammation. Importantly, treatment with anti-RGMb mAb before and during sensitization (days -1 and 4) did not block sensitization because splenic T cells from

OVA-sensitized and anti-RGMb mAb-treated mice proliferated vigorously and produced IL-4 and IFN- γ upon *in vitro* restimulation with OVA (Fig 1, G). Therefore blockade of AHR and inflammation by anti-RGMb was not mediated through effects on T-cell sensitization, which was required in our model, because T-cell sensitization to OVA was required for expression of IL-25 and IL-13 and for OVA-induced airway inflammation (Fig 1, H and I).

RGMb mAb inhibits established airways disease

Anti-RGMb treatment was also effective in a model of established airway inflammation in which mice were exposed to several rounds of OVA administered intranasally and treated with anti-RGMb on day 15 (Fig 2, A). After the first round of challenge with intranasal OVA (days 7-8), airway inflammation was shown to be present on day 9, with the presence of eosinophils, interstitial macrophages (F4/80⁺CD11b⁺CD11c⁻), and CD4⁺ lymphocytes (Fig 2, B) and peribronchiolar cellular infiltration (Fig 2, C). Importantly, just one dose of anti-RGMb on day 15 blocked airway inflammation measured on day 18 after a second round of intranasal OVA, demonstrating that anti-RGMb inhibits established airways disease (Fig 2, B and D). Lung inflammation is quantitated in Table E2 in this article's Online Repository at www.jacionline.org.

RGMb mAb inhibits CRA-induced airway inflammation

We also examined the effects of anti-RGMb in a model of CRA-induced allergic asthma (Fig 2, E) and showed that treatment with anti-RGMb significantly reduced numbers of eosinophils, neutrophils, interstitial macrophages, and lymphocytes in lung tissue and BAL fluid (Fig 2, F and G). Examination of lung sections stained with H&E showed that exposure to CRA induced peribronchiolar and perivascular infiltrates consisting of eosinophils, lymphocytes, and interstitial macrophages and significant bronchiolar mucus production (Fig 2, H, middle panels). Anti-RGMb-treated mice showed significantly reduced peribronchial inflammation and normal bronchiolar epithelium (Fig 2, H, right panels; inflammation is quantitated in Table E3 in this article's Online Repository at www.jacionline.org). These results indicate that treatment with anti-RGMb effectively reduces lung inflammation in CRA-induced and OVA-induced allergen models and in a model of established airway inflammation.

RGMb is expressed by innate rather than adaptive cells in the lung

Using IHC, we found that RGMb was highly expressed by bronchial epithelial cells (Fig 3, A) and interstitial macrophages (Fig 3, B) in both OVA-sensitized and challenged mice and in saline-treated control mice. Additionally, in areas of inflammation in the lungs, other myeloid but not lymphoid cells expressed RGMb. This was confirmed by using immunofluorescence microscopy, showing that CD11b⁺ and F4/80⁺ cells in the lung (markers of interstitial macrophages) highly express RGMb (Fig 3, C, white arrows), whereas CD11c⁺ cells (dendritic cells) and CD4⁺ T cells did not (see Fig E3 in this article's Online Repository at www.jacionline.org). In

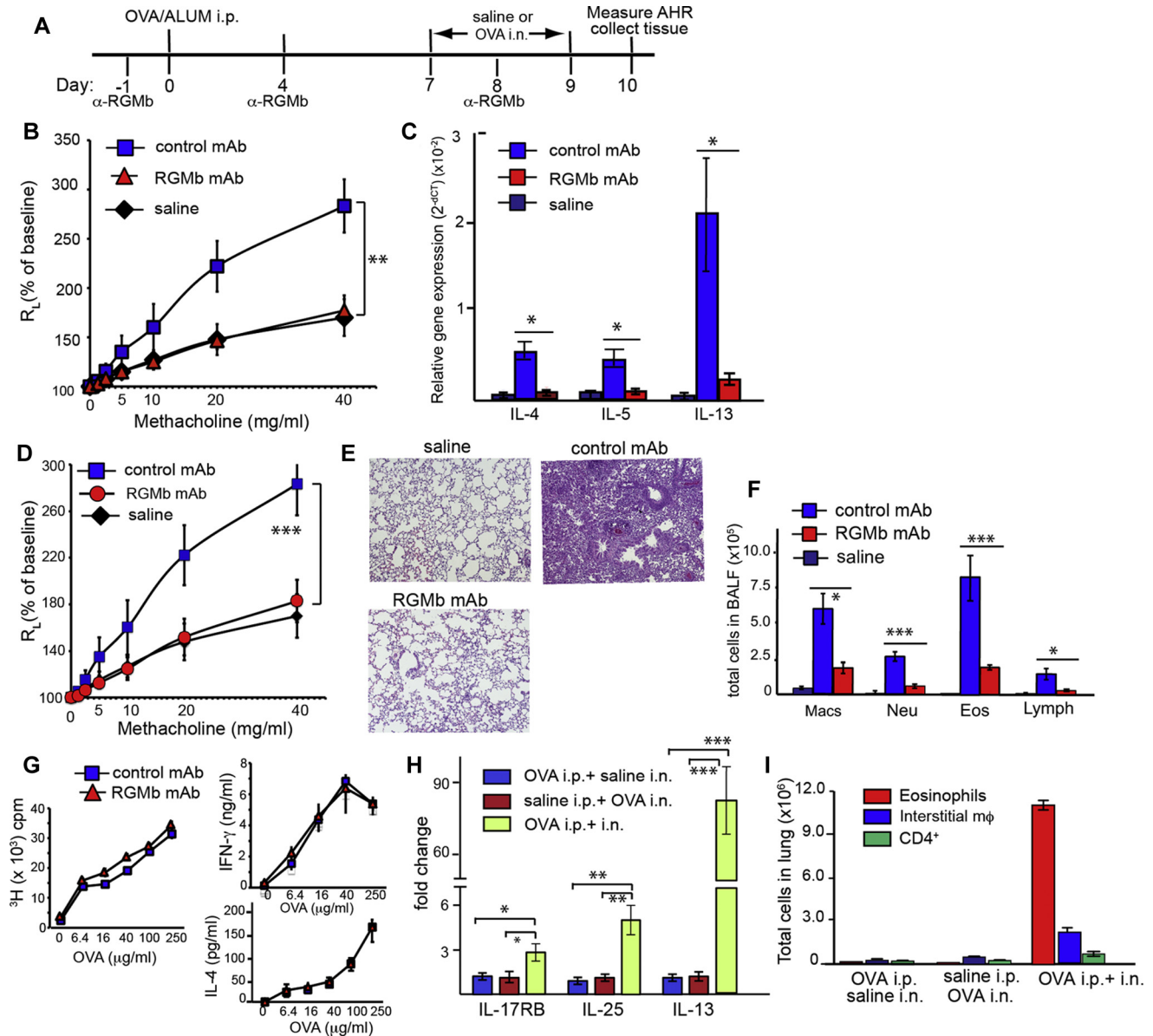


FIG 1. Anti-RGMb mAb treatment blocks development of AHR and allergic airway inflammation. **A**, Protocol for OVA sensitization and challenge. **B**, OVA-sensitized and challenged mice were treated with RGMb mAb or control mAb on days -1, 4, and 8. Results show changes in lung resistance (R_L). **C**, Level of target gene expression in mRNA from lung tissue was expressed relative to *Gapdh*. **D**, OVA-sensitized and challenged mice were treated with RGMb or control mAb on days 6 and 8. Results show changes in lung resistance (R_L). **E**, Lung sections from mice in Fig 1, **D**, stained with H&E. **F**, Inflammatory cells in BAL fluid (cells/lung) of mice from Fig 1, **D**. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Macs*, macrophages; *Neu*, neutrophils. Fig 1, **B** and **D**-**F**, $n = 4$. **G**, OVA-sensitized mice were treated with RGMb mAb or control mAb on days -1 and 4 and splenic T-cell responses assayed on day 7. **H**, *Il17rb*, *Il25*, and *Il13* gene expression in lung tissue of mice sensitized and challenged with OVA, saline, or both. **I**, Total number of eosinophils (CD11b⁺Siglec-F⁺), interstitial macrophages (CD11b⁺F4/80⁺CD11c⁻Siglec-F⁻), and CD4⁺ T cells in lung tissue from mice in Fig 1, **H**. Data are means $\pm$ SEMs and representative of 3 experiments. $*P < .05$, $**P < .01$, and $***P < .005$. Fig 1, **B** and **D**, Control mAb compared with the RGMb mAb group (2-way ANOVA with Bonferroni *post hoc* test). Fig 1, **C**, **F**, and **H**, Control mAb compared with RGMb mAb group (unpaired *t* test). *i.n.*, Intranasal; *i.p.*, intraperitoneal.

F4/80⁺CD11b⁺ cells, most but not all of the RGMb expression was intracellular (Fig 3, C, center panel), which was confirmed by confocal microscopy of CD11b⁺RGMb⁺ cells (see Video E1 in this article's Online Repository at www.jacionline.org). These findings are consistent with our IHC studies (Fig 3, B)

and with other studies identifying interstitial macrophages as F4/80⁺CD11c⁻ cells in naive mice²² and also with our previous studies showing RGMb mRNA expression in naive lung tissue, with the greatest RGMb mRNA expression in F4/80⁺CD11c⁻ cells.¹⁸

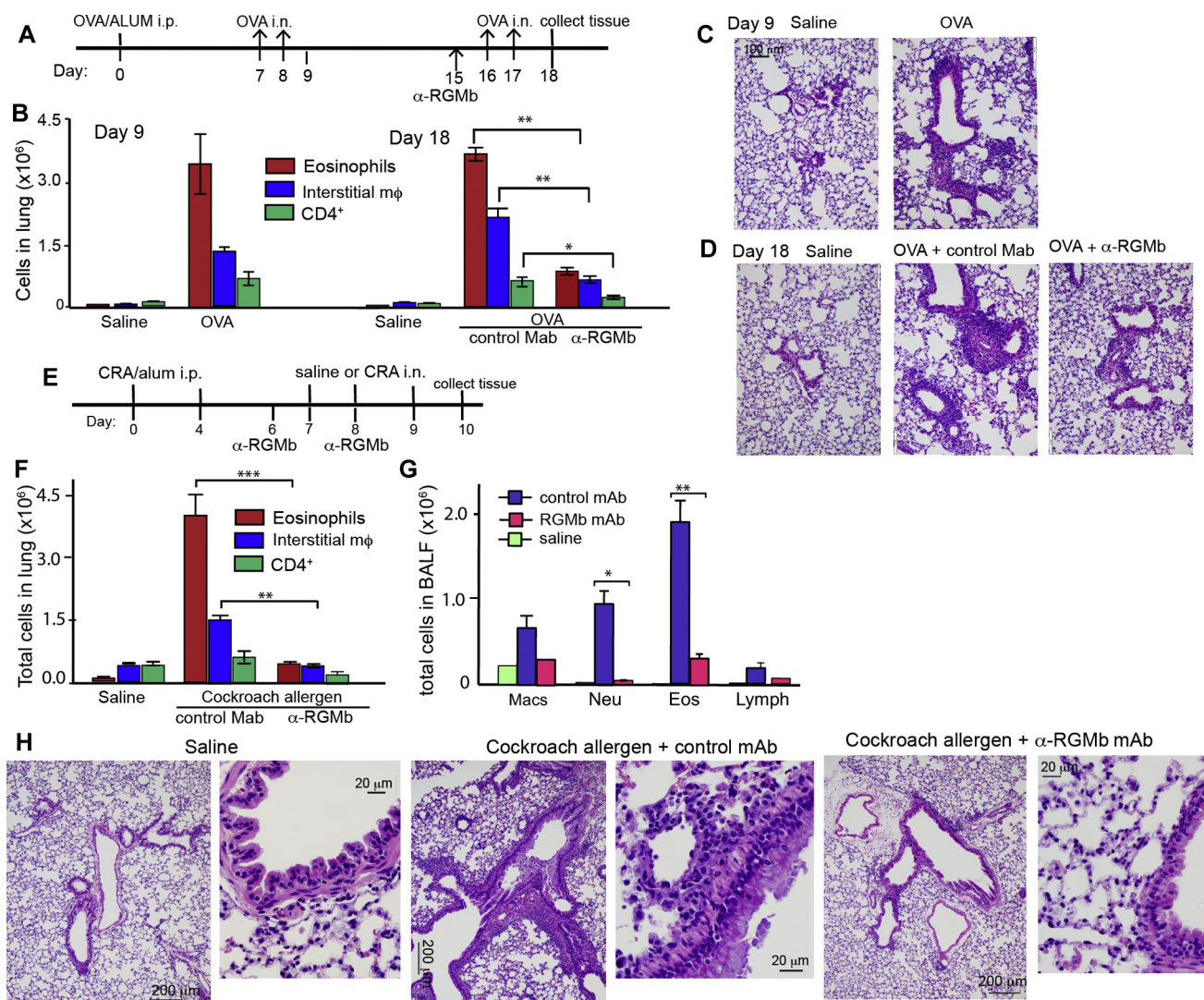


FIG 2. RGMB mAb inhibits established airways disease and CRA-induced inflammation. **A**, Protocol for experiment shown in Fig 2, **B-D**. Mice were treated with RGMB mAb or control mAb on day 15. **B**, Lungs were collagenase digested and stained. Total numbers of eosinophils, interstitial macrophages, and CD4⁺ T cells in lungs on days 9 and 18 are shown. **C** and **D**, Lung sections from mice on day 9 (Fig 2, **C**) and day 18 (Fig 2, **D**) stained with H&E ($\times 200$ magnification). **E**, Protocol for experiment shown in Fig 2, **F-H**. Mice sensitized and challenged with CRA were treated with RGMB or control mAb on days 6 and 8. **F**, Lungs were collagenase digested and stained as in Fig 2, **B**. **G**, Differential cell counts in BAL fluid (cells/lung) of mice shown in Fig 2, **F**. **Eos**, Eosinophils; **Lymph**, lymphocytes; **Macs**, macrophages; **Neu**, neutrophils. **H**, Lung sections from mice in Fig 2, **F** and **G**, stained with H&E. **Left panels**, Lung tissue from negative control mice that received intranasal saline but no CRA shows no significant inflammation. High-power magnification of alveolar and bronchiolar epithelium is shown. **Center panels**, Lung tissue from positive control mice that received CRA. Dense perivascular and peribronchiolar inflammation is noted. High-power magnification shows the inflammatory infiltrate consists of eosinophils, lymphocytes, and interstitial macrophages. Bronchiolar epithelium shows abundant cytoplasmic mucin. **Right panels**, Lung tissue from mice that received CRA and anti-RGMB treatment. Mild peribronchiolar inflammation is noted. High-power magnification shows minimal peribronchiolar inflammation and normal bronchiolar epithelium. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

Eosinophils in the inflamed lung also expressed RGMB because CD11b⁺Siglec-F⁺ cells (eosinophils) expressed RGMB (Fig 3, **D**, white arrows in the overlay). Moreover, flow cytometry showed that RGMB expression by eosinophils was highly upregulated in OVA-sensitized and challenged mice (>99% of activated eosinophils expressed RGMB compared with only 13% to 18% of the few eosinophils present in saline-challenged mice; Fig 3, **E** and **F**).

RGMB⁺ interstitial macrophages express IL-17RB and produce IL-5 and IL-13

Because the RGMB⁺ interstitial macrophages appeared to be similar to recently described T2M cells (F4/80⁺CD11b⁺Gr-1^{mid}, IL-17RB⁺ cells that produce IL-13 and IL-4),⁴ we examined the RGMB⁺ cells for expression of IL-17RB and type 2 cytokines. Flow cytometry showed that most (80% to 90%) of the

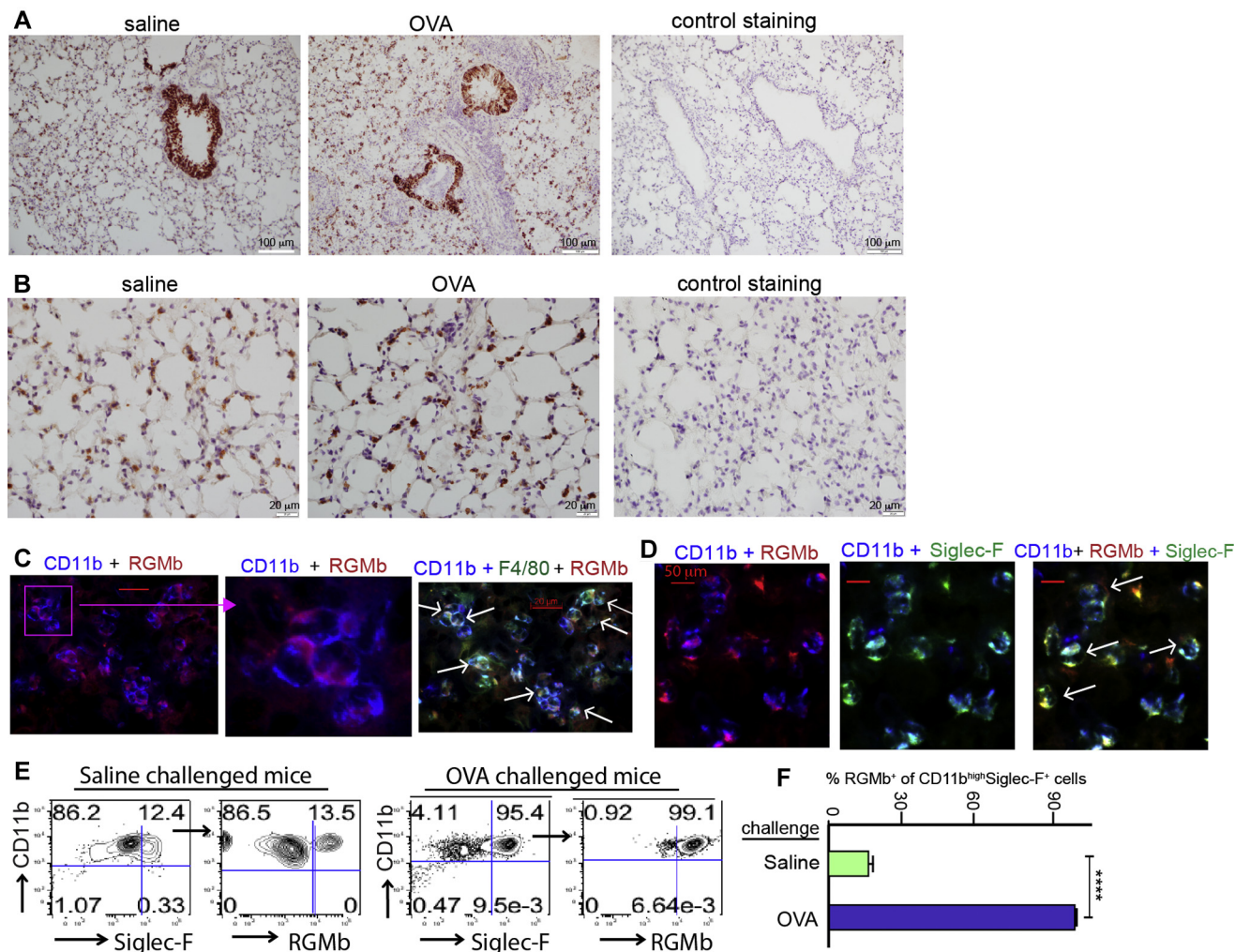


FIG 3. RGMb is expressed by innate rather than adaptive cells in the lung. **A** and **B**, IHC staining for RGMb on lung tissue from mice sensitized and challenged with OVA or saline shows strong staining in bronchiolar epithelium and interstitial macrophages. The *far right panels* of Fig 3, **A** and **B**, show staining with control mAb. **C** and **D**, Immunofluorescent staining for RGMb of lung sections from mice sensitized and challenged with OVA. Fig 3, **C**: The *left panel* shows RGMb (red) and CD11b (blue), the *center panel* shows higher magnification of the left panel; and in the *right panel* white arrows indicate colocalization of RGMb (red), CD11b (blue), and F4/80 (green). Colocalization appears as white. Fig 3, **D**, Coexpression of RGMb (red) by cells that express Siglec-F (green), a marker of eosinophils, and CD11b (blue) is indicated with *white arrows* (right panel). Images are representative of at least 5 animals and 3 experiments. **E**, Flow cytometric analysis of RGMb expression by eosinophils from lung digests of mice challenged with saline (*left panel*) or OVA (*right panel*). Analyses of 1 representative mouse per group are shown. Numbers on plots reflect the percentage of cells in the plot present within the gated area. Data are representative of 3 experiments. **F**, Bar graph summary of data from the experiment shown in Fig 3, **E** (3 mice per group). **** $P < .001$.

CD11b⁺F4/80⁺ macrophages expressed RGMb, as expected (Fig 4, **A** and **C**), and surprisingly, many of these CD11b⁺RGMb⁺ cells from OVA-challenged mice also expressed IL-17RB (Fig 4, **D**). They did not express ST2, the receptor for IL-33 (data not shown). Histologically, the interstitial macrophages purified by using FACS from lungs of OVA-sensitized and challenged mice contained numerous cytoplasmic vacuoles and often displayed indented, kidney-shaped nuclei (Fig 4, **F**, left panel) similar to those described for T2M cells⁴ and lung interstitial macrophages,²² which were also shown to express F4/80 and CD11b. In contrast, eosinophils displayed the typical eosinophil morphology with red cytoplasmic granules (Fig 4, **F**, right panel).

We also found that the F4/80⁺CD11b⁺ macrophages from sensitized and challenged mice that expressed IL-17RB (40% to 60% in various experiments) produced IL-5, IL-13, and some IL-4 (Fig 4, **D**), which was shown as a bar graph summary in Fig 4, **G**. Moreover, although many FACS-sorted interstitial macrophages expressed IL-17RB (approximately 67%) on culture (48 hours) with recombinant IL-25, they responded by increasing their expression of IL-17RB (>98%, see Fig E4 in this article's Online Repository at www.jacionline.org). Together, these findings suggest that in our model RGMb⁺ macrophages express IL-17RB⁺ and can respond to IL-25, which likely leads to production of IL-5 and IL-13 and a role as effector cells.

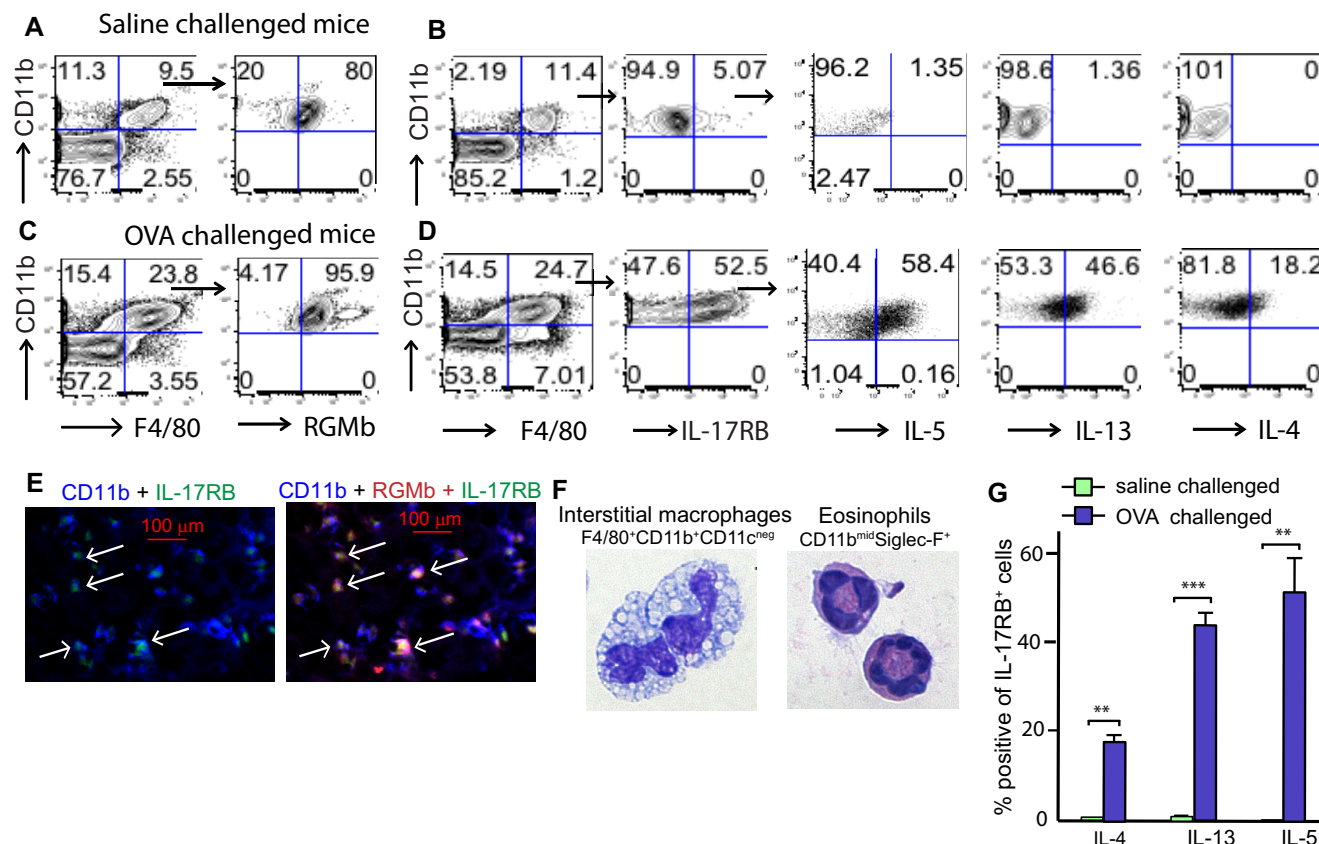


FIG 4. Interstitial macrophages from OVA-sensitized and challenged mice express RGMb and produce type 2 cytokines. **A–D**, Flow cytometric analysis of cells from lung digests of mice challenged with saline (Fig 4, A and B) or OVA (Fig 4, C and D). Interstitial macrophages (F4/80⁺CD11b⁺CD11c^{neg}Siglec-F^{neg} cells) were stained for IL-17RB, intracellular and surface RGMb, and intracellular cytokines. RGMb expression (Fig 4, A and C) and IL-17RB expression (Fig 4, B and D) and intracellular cytokine expression by interstitial macrophages are shown. Numbers on plots reflect percentages of cells in the plot that are present within the gated area. Analyses of 1 representative mouse per group are shown. Data are representative of 3 experiments. **E**, Coexpression of IL-17RB (green) with CD11b (blue; left panel, white arrows) and RGMb (red; right panel, white arrows). **F**, Morphology of eosinophils and interstitial macrophages FACS purified from the lungs of OVA-sensitized and challenged mice. Cells were sorted as CD45⁺Siglec-F⁺CD11b^{mid} (eosinophils) and CD45⁺Siglec-F^{neg}CD11b⁺F4/80⁺CD11c^{neg} (interstitial macrophages) and stained with HEMA-3. **G**, Bar graph representation of the experiment shown in Fig 4, B and D (summary of 3 mice per group). ***P* < .01 and ****P* < .005.

Most IL-17RB⁺ cells in inflamed lungs are RGMb⁺ interstitial macrophages

The number of inflammatory cells in the lung increased after intranasal challenge of sensitized mice with OVA (Fig 5, A). Twenty-four hours after the first intranasal challenge, numbers of CD4⁺ T cells in the lung were significantly increased compared with those in control mice. Numbers of eosinophils and interstitial macrophages increased more slowly but were markedly different from control mice on days 9 and 10. Most of the inflammatory cells in the lung on day 10 were eosinophils, followed by RGMb⁺ interstitial macrophages and CD4⁺ T cells (ILC2s and invariant natural killer T cells were present in the lungs in much lower numbers; Fig 5, B, and see Table E4 in this article's Online Repository at www.jacionline.org). Importantly, most (about 95%) of the IL-17RB⁺ cells present in the inflamed lung on day 10 were interstitial macrophages (that expressed RGMb; Fig 5, C), suggesting that interstitial macrophages were the primary cells responding to IL-25. Moreover,

IL-17RB⁺ interstitial macrophages were present at much greater frequencies in the lungs of OVA-challenged mice than in the lungs of saline-challenged mice (Fig 5, C and E, and see Table E4). Treatment with anti-RGMb mAb, even with only 1 dose during the effector phase of the protocol, greatly reduced the number of IL-17RB⁺ cells in the lungs (Fig 5, E), as well as total numbers of inflammatory cells (Fig 5, D), suggesting an important role for IL-17RB⁺RGMb⁺ interstitial macrophages.

Exposure of mice to CRA in a chronic exposure model over 27 days (Fig 5, F) resulted in greater lung inflammation and significant accumulations of eosinophils, interstitial macrophages, and CD4⁺ T cells in the lung (Fig 5, G–J [middle panel]). Histologic inflammation is quantitated in Table E5 in this article's Online Repository at www.jacionline.org. As with OVA, the most numerous IL-17RB⁺ cells were interstitial macrophages (Fig 5, G). Treatment with anti-RGMb mAb on days 13, 17, 21, and 25 significantly reduced the number of IL-17RB⁺ cells, as well as the total number of inflammatory cells in the lung (Fig 5, G and H)

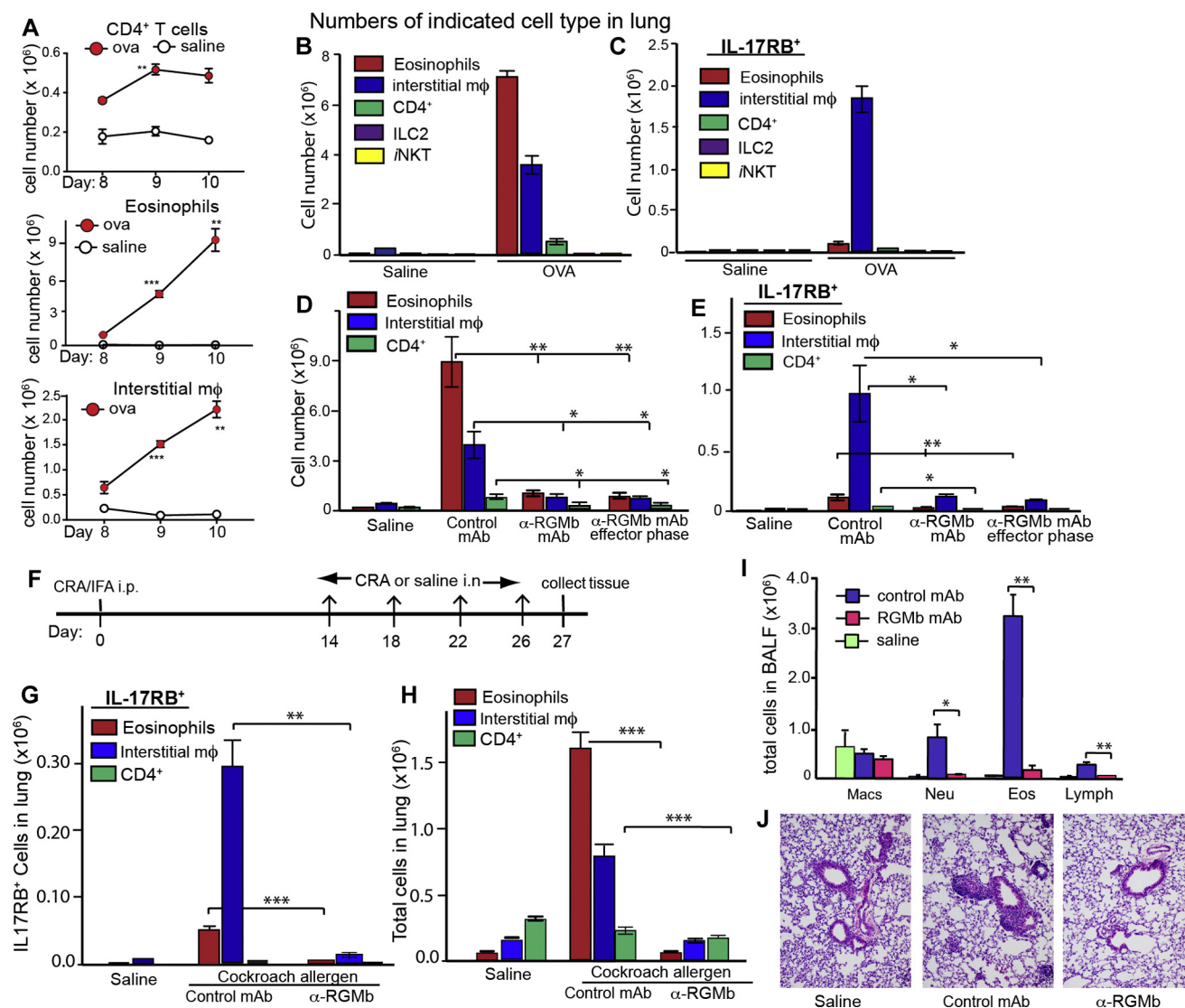


FIG 5. Numbers of interstitial macrophages and eosinophils in the lung increase after allergen exposure, which is inhibited by treatment with RGMb mAb. **A–C**, Lungs from mice sensitized and challenged with OVA or saline as in Fig 1, A, were harvested on the indicated day, collagenase digested, and analyzed by using flow cytometry. Fig 5, A, Total numbers of eosinophils, interstitial macrophages, and CD4⁺ T cells in the lungs on days 8, 9, and 10 are shown. Fig 5, B, Lungs from mice sensitized and challenged with OVA or saline were collagenase digested and analyzed by using flow cytometry. Fig 5, C, Total numbers of eosinophils, interstitial macrophages, CD4⁺ T cells, ILC2s, and invariant natural killer T (iNKT) cells and numbers of IL-17RB⁺ cells of these cell types in the lung on day 10 are shown. **D** and **E**, Mice sensitized and challenged with OVA were treated with RGMb mAb or isotype control on days 0, 4, and 8 (α-RGMb) or on days 6 and 8 (α-RGMb effector phase). Total numbers of eosinophils, interstitial macrophages, and CD4⁺ T cells in the lung on day 10 (Fig 5, D) and IL-17RB⁺ numbers of these cell types (Fig 5, E) are shown (n = 4). Data are means ± SEMs. *P < .05, **P < .01, and ***P < .005, unpaired t test. **F**, Protocol for experiment shown in Fig 5, G–J. Mice sensitized and challenged with CRA were treated with RGMb mAb or isotype control on days 13, 17, 21, and 25. **G** and **H**, Numbers of IL-17RB⁺ eosinophils, interstitial macrophages, and CD4⁺ T cells (Fig 5, G) and total numbers of cells of these cell types in the lung (Fig 5, H) on day 27 are shown. **I**, Differential cell counts in BAL fluid (cells/lung) of mice shown in Fig 5, G and H. Eos, Eosinophils; Lymph, lymphocytes; Macs, macrophages; Neu, neutrophils. **J**, Lung sections from mice in Fig 5, G and H, stained with H&E (x200 magnification). *P < .05, **P < .01, and ***P < .005.

and BAL fluid (Fig 5, I). Moreover, IL-17RB expression by interstitial macrophages present in the lungs after anti-RGMb treatment was substantially reduced, nearly to the level of that observed in saline-treated control mice (see Fig E5 in this article's Online Repository at www.jacionline.org). Examination of lung sections stained with H&E showed that exposure to CRA

induced peribronchiolar and perivascular infiltrates consisting of eosinophils, lymphocytes, and interstitial macrophages (Fig 5, J, middle panel), whereas anti-RGMb-treated mice showed minimal peribronchial inflammation and normal bronchiolar epithelium (Fig 5, J, right panel, inflammation is quantitated in Table E5). This indicates that treatment with anti-RGMb blocks

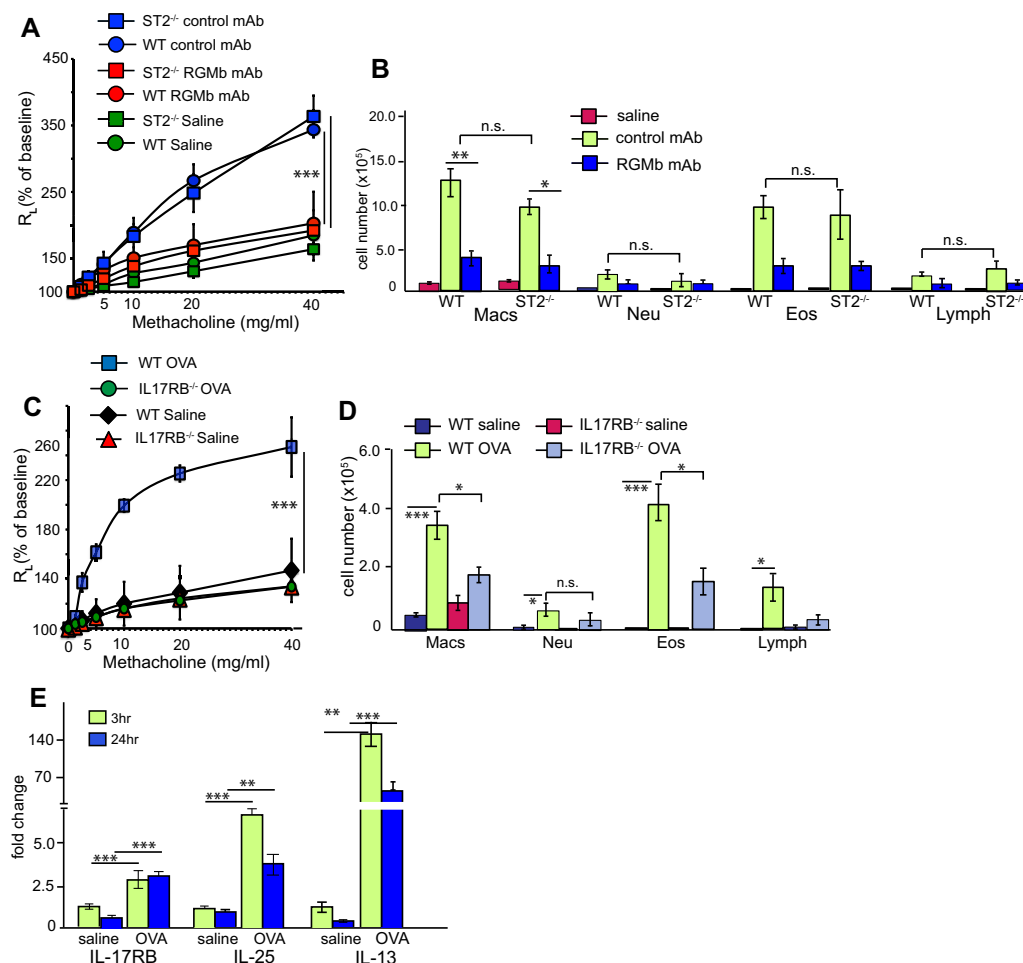


FIG 6. IL-25 but not IL-33 signaling is required for development of OVA-induced AHR. **A–D**, ST2^{−/−} (Fig 6, A and B), IL17RB^{−/−} (Fig 6, C and D), or wild-type (WT) mice sensitized and challenged with OVA or saline as in Fig 1, A, were assessed for AHR on day 10. Mice in Fig 6, A, were treated with control mAb or RGMb mAb on days −1, 4, and 8, as indicated. Fig 6, B and D, Inflammatory cells in BAL fluid (cells/lung) of the mice in Fig 6, A and C. **E**, Expression of *Il17rb*, *Il25*, and *Il13* mRNA in lungs of WT mice at 3 and 24 hours after the last allergen challenge measured by using quantitative real-time RT-PCR. *Gapdh* was used as an internal control. Values are normalized to saline-treated mice at 3 hours. Fig 6, A and C, $n = 4$. Data are shown as means $\pm$ SDs. * $P < .05$, ** $P < .01$, and *** $P < .005$, 2-way ANOVA with the Bonferroni *post hoc* test. Fig 6, B, D, and E, Unpaired *t* test. *n.s.*, Not significant; R_L , lung resistance.

accumulation/expansion of inflammatory cells, particularly of RGMb⁺ interstitial macrophages, which occurs in the lungs in response to allergen.

IL-17RB, but not ST2, is required for allergen-induced AHR

IL-25^{23,24} but not IL-33^{25,26} played a critical role in our model of asthma because IL-33 receptor-deficient ST2^{−/−} mice sensitized and challenged with OVA had similar levels of AHR and inflammation, as did wild-type mice (Fig 6, A and B). Furthermore, treatment with anti-RGMb mAb blocked the development of AHR and lung inflammation in both ST2^{−/−} and wild-type mice. In contrast, IL-17RB^{−/−} mice did not have AHR (Fig 6, C) and airway inflammation (Fig 6, D) after OVA sensitization and challenge. Moreover, mRNA expression for IL-17RB, IL-25, and IL-13 was increased in the lungs of OVA-sensitized mice at 3 and 24 hours after challenge with OVA in the lungs (Fig 6, E). Together, these studies

indicate that IL-25 and IL-17RB were critical for the development of allergen-induced AHR.

The RGMb pathway in allergic asthma is independent of PD-L2

Because we recently demonstrated that RGMb can promote respiratory tolerance by interacting with one of its ligands, PD-L2,¹⁸ we asked whether this pathway had a role in AHR development. Treatment with an anti-PD-L2 mAb (2C9) that blocks the interaction of PD-L2 with RGMb but does not inhibit the interaction of RGMb with NEO1 or BMP-2, BMP-4, or BMP-6,¹⁸ had no effect on AHR or airway inflammation (Fig 7, A and B), whereas treatment with anti-RGMb abolished AHR (Fig 7, C and D). These findings were confirmed in PD-L2^{−/−} mice, which had robust AHR and inflammation when sensitized and challenged with OVA²⁷ that was abolished by treatment with anti-RGMb mAb (Fig 7, C–E). These results demonstrate that interaction of RGMb with PD-L2 did not affect AHR development.

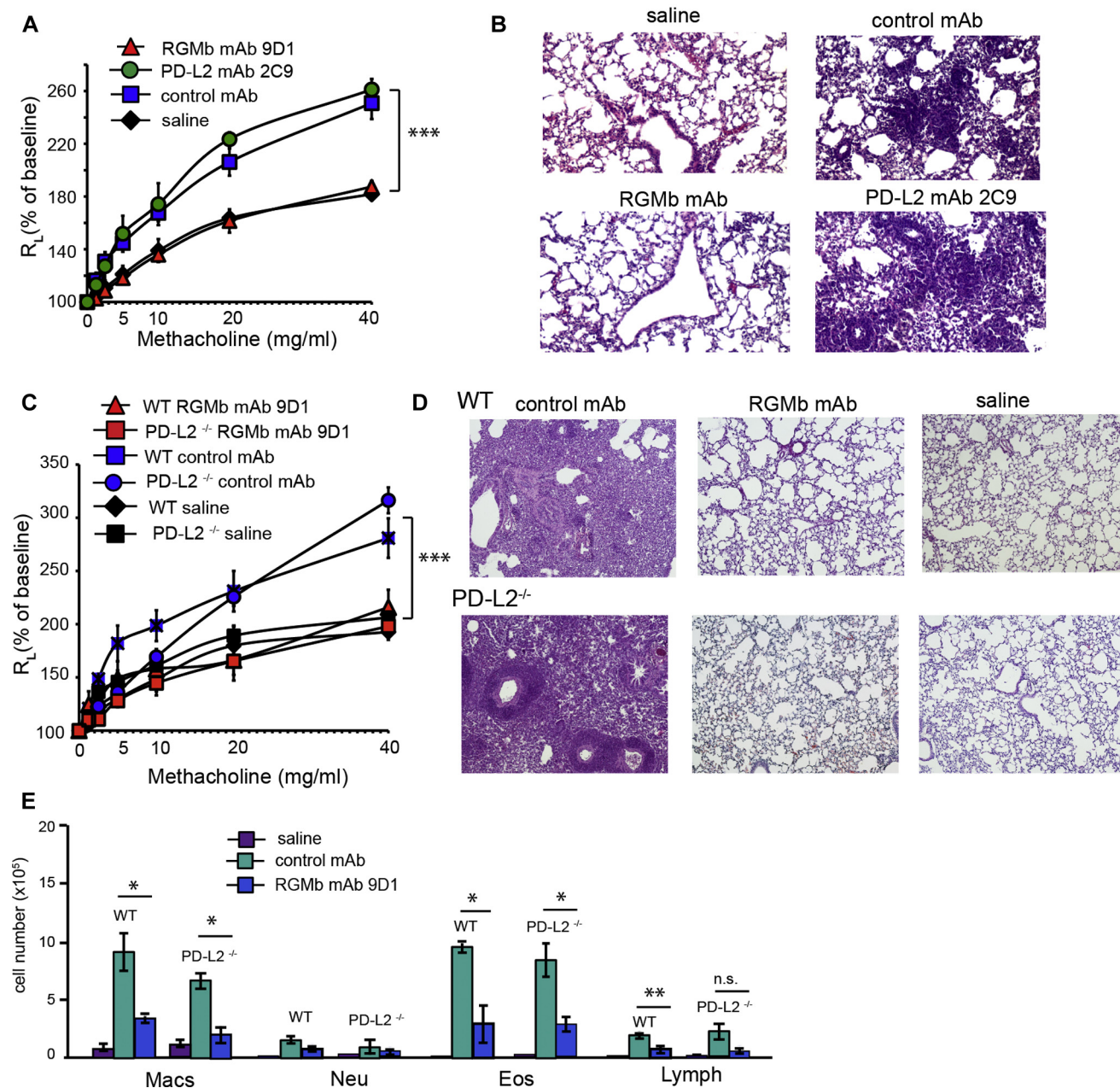


FIG 7. RGMb blockade of AHR and lung inflammation does not involve RGMb:PD-L2 interaction. **A**, OVA-sensitized and challenged mice were treated with RGMb mAb 9D1, PD-L2 mAb 2C9, or isotype control on days -1, 4, and 8. **B**, Lung tissue from the mice in Fig 7, A, was stained with H&E. **C**, PD-L2-deficient or wild-type (WT) mice sensitized and challenged with OVA were treated with RGMb mAb or isotype control (500 μ g) on days -1, 4, and 8. WT saline and PD-L2^{-/-} saline denote WT and PD-L2^{-/-} mice sensitized with OVA and challenged with saline as negative controls. **D**, Lung sections from mice in Fig 7, C, were stained with H&E. **E**, Inflammatory cells in BAL fluid (cells/lung) of the mice in Fig 7, C. Fig 7, A-D, $n = 4$. Data are means $\pm$ SEMs and representative of 3 experiments. * $P < .05$, ** $P < .01$, and *** $P < .005$, control mAb group compared with RGMb mAb group (Fig 7, A and C), 2-way ANOVA with Bonferroni post-hoc test; Fig 7, E, unpaired t test. Eos, Eosinophils; Lymph, lymphocytes; Macs, macrophages; Neu, neutrophils; n.s., not significant; R_L , lung resistance.

NEO1 is expressed by F4/80⁺CD11b⁺ cells after OVA sensitization and challenge

Because the canonical pathway for RGMb signaling is through binding the receptor NEO1,^{7,9} providing a hub for interactions with BMPs,^{11,28} we examined NEO1 expression in lung tissue. Using a novel anti-NEO1 mAb, we found that NEO1 colocalized

with expression of RGMb in the lungs of OVA-sensitized and challenged mice (Fig 8, A, left panel). Numerous cells were observed that coexpressed NEO1, F4/80, and RGMb (Fig 8, A, right panel, white arrows), although not all RGMb⁺ cells expressed NEO1 and a few F4/80⁺ cells expressed neither NEO1 nor RGMb (red arrows). Confocal microscopy showed that

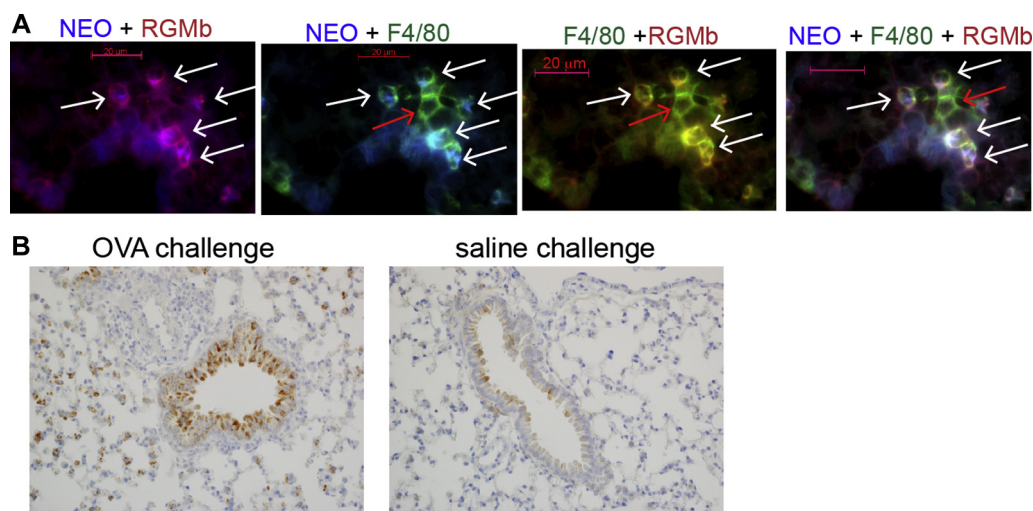


FIG 8. NEO1 is expressed by RGMb⁺ interstitial macrophages and bronchiolar epithelium in lung tissue from OVA-sensitized and challenged mice. **A**, *Left panel*, Colocalization of NEO1 (blue) and RGMb (red) appears magenta (*white arrows*). *Second panel*, Colocalization of F4/80 (green) and NEO1 (blue; *white arrows*). *Third panel*, Colocalization of F4/80 (green) and RGMb (red) appears yellow (*white arrows*). *Right panel*, Triple colocalization of NEO1, RGMb, and F4/80 appears white (*white arrows*). Images are representative of at least 4 animals. **B**, IHC staining of lung tissue for NEO1. Bronchiolar epithelium from the saline-challenged group shows patchy weak cytoplasmic staining. In the OVA-challenged group the bronchiolar epithelium shows diffuse strong cytoplasmic staining. NEO1-positive interstitial macrophages are also present in the OVA-challenged lungs compared with the saline-treated mice.

NEO1 was predominantly expressed intracellularly, although some surface expression was detectable (see Fig E6 and Video E2 in this article's Online Repository at www.jacionline.org). Using IHC to detect NEO1, we found that bronchial epithelium from OVA-challenged mice showed diffuse strong cytoplasmic staining for NEO1. NEO1 was also expressed by interstitial macrophages in lungs of the OVA-challenged mice. In contrast, bronchial epithelium from saline-challenged mice showed patchy weak cytoplasmic staining for NEO1, and NEO1 expression was not detected on interstitial macrophages (Fig 8, B).

DISCUSSION

Using several murine models of asthma, we discovered that RGMb contributes greatly to the development of allergen-induced AHR and type 2 lung inflammation through novel innate mechanisms. The importance of RGMb in the lung is supported by our findings that RGMb was expressed in the lung by (1) bronchial epithelial cells, which are known to respond to environmental stimuli by secreting IL-25, IL-33, thymic stromal lymphopoietin, and GM-CSF, thus initiating type 2 responses,^{3,29,30} and (2) interstitial macrophages, which expressed the IL-25 receptor and responded to IL-25 by secreting IL-5 and IL-13, a critical cytokine causing AHR.^{31,32} After exposure to either OVA or CRA, RGMb⁺ interstitial macrophages greatly increased in number in the lung. Moreover, treatment with anti-RGMb mAb blocked expansion of RGMb⁺ interstitial macrophages and eosinophils in the lung and inhibited development of AHR, even when given late after sensitization. Thus our studies identify RGMb as a novel innate molecular entity critically involved in AHR development and suggest that targeting RGMb could be an effective therapy for established asthma.

The critical role of RGMb-expressing interstitial macrophages in asthmatic patients was suggested by a previous study in a chronic CRA model describing macrophages, called T2M cells, that produced IL-13.⁴ T2M cells expressing the surface markers IL-17RB⁺, F4/80⁺, CD11b⁺ and Gr-1^{mid} accumulated in the lungs of mice after repeated allergen exposure and were critical in asthma pathogenesis.⁴ In another setting F4/80⁺CD11b⁺ macrophages were also shown to play an essential role in IL-13-dependent lung inflammation in which depletion of these cells at the time of airway challenge prevented recruitment of eosinophils to the airways.³³ We greatly extended these studies and demonstrated that lung interstitial macrophages that produce IL-5 and IL-13 expressed RGMb and that treatment with anti-RGMb inhibited airway inflammation and AHR. A recent report showing that targeting RGMb decreased lung inflammation is consistent with our findings.³⁴

Moreover, we showed that the RGMb⁺ interstitial macrophages also played an important role because they expressed IL-17RB, the receptor for IL-25, which was required in our mouse model of allergen-driven AHR. An important role for IL-25 in human asthma has been demonstrated in clinical studies^{35,36} and confirmed by human genetic studies.³⁷ IL-25 is produced by inflamed airway epithelial cells,^{3,35} eosinophils,³⁸ tuft cells in the intestinal and respiratory tracts,³⁹⁻⁴¹ and solitary chemosensory cells in the airways.⁴² IL-25, in combination with neuropeptides, such as neuromedin U, which is released from sensory neurons in the lungs, and calcitonin gene-related peptide, which is released from pulmonary neuroendocrine cells, can promote type 2 immunity in allergen-driven AHR^{1,2,43-45} by enhancing production of IL-4, IL-5, and IL-13 from ILC2s, T_H2 cells, and RGMb⁺ interstitial macrophages.^{2,23,44-46} Although RGMb⁺ interstitial macrophages in saline-treated control mice express only low levels of IL-17RB, we found that after either OVA or CRA sensitization

and challenge, RGMb⁺ interstitial macrophages were the most numerous IL-17RB-expressing cells, accounting for about 98% of IL-17RB⁺ cells in the lung. Levels of both IL-25 and IL-17RB mRNA and protein increase in the lungs after infection with respiratory syncytial virus or rhinovirus^{35,47} or after allergen exposure, as shown in the present study and in a previous study.⁴ IL-25 was shown to induce IL-17RB surface expression by human CD4⁺ T cells,⁴⁸ and IL-17RB expression by human monocytic cells was induced and amplified by IL-4 and IL-25.⁴⁹ We found that although about 50% of the RGMb⁺ interstitial macrophages taken directly from inflamed lungs expressed IL-17RB, on incubation with rIL-25, expression increased to greater than 98%.

In OVA- or CRA-sensitized and challenged mice treated with anti-RGMb mAb, IL-17RB expression by interstitial macrophages was reduced almost to level seen in saline-treated control mice. These findings suggest that production of IL-25 by, for example, bronchial epithelial cells in the inflamed lung results in enhanced expression of IL-17RB on interstitial macrophages and subsequent production of type 2 inflammatory cytokines and suggests that treatment with anti-RGMb mAb can inhibit production of IL-25.

The important role of RGMb-expressing cells (epithelial cells, interstitial macrophages, and eosinophils) was shown by treating mice with anti-RGMb, which blocked accumulation of eosinophils and RGMb⁺ interstitial macrophages. Blocking IL-25 in the challenge phase with anti-IL-25 mAb was shown to prevent AHR but did not reduce cell infiltration or eosinophil numbers in BAL fluid,¹ indicating that RGMb plays a more dominant role than IL-25 at this time point. In contrast, IL-33 was not required in our model because development of AHR occurred normally in mice deficient in ST2, the receptor for IL-33. IL-33 has been shown to be required in other models of asthma, such as that induced by influenza infection, in which infected alveolar macrophages produce IL-33, which then activates ILC2s.^{50,51}

Numbers of RGMb⁺ macrophages in the lung increased rapidly after allergen challenge. This increase might be due to local proliferation of lung-resident macrophages driven by the cytokine IL-4^{52,53} or recruitment from the blood or bone marrow.³⁸ The number of eosinophils recruited into the airways by local production of IL-5 and by eotaxin produced by IL-13-exposed epithelial cells⁵⁴ also increased after allergen challenge.

Allergen-primed T cells, particularly pathogenic effector T_H2 cells,^{55,56} are required in our model, and we believe they are involved in initiating the anamnestic allergic inflammatory response in the lung, perhaps in conjunction with invariant natural killer T cells and ILC2s.⁵⁷ Although ILC2s have been shown to have critical roles in many models of asthma, particularly with protease-containing allergens that damage airway epithelium⁵⁸ or in the response to viral infection,^{47,50,59} ILC2s might not be required in all conditions leading to asthma. For example, ILC2s were found to be dispensable in inducing type 2 lung inflammation when T cells were first sensitized with allergen and adjuvant.⁶⁰ Moreover, in our models ILC2s were present at low numbers in the lung and did not increase after allergen challenge.

RGMb signals through linking the NEO1 and BMP pathways (ie, by binding BMP-2, BMP-4, or BMP-6),^{11,28,61} which are key signaling molecules, and NEO1,⁵⁻⁸ thus forming a BMP-RGM-NEO1 superreceptor complex. We speculate that RGMb can upregulate airway inflammation and AHR by forming a superreceptor complex with NEO1 and BMP-2, BMP-4, or BMP-6,

which are shown to be upregulated in bronchial epithelial cells in acute allergic airway inflammation,^{14,15} and interstitial macrophages in the inflamed lung, thus activating BMP signaling pathways. This is consistent with the observation that when both NEO1 and RGM are expressed on the same cell, as we observed for interstitial macrophages and bronchial epithelial cells in inflamed lung tissue, cis (same-cell) interactions occur in a BMP-dependent manner.¹¹ BMPs mediate clustering of RGM-NEO1 at the cell surface,^{7,11} and engagement of BMPs triggers intracellular phosphorylation,⁶² leading to downstream signaling by the SMAD cascade or alternative pathways.⁶³ The RGMb mAb used in our *in vivo* studies blocks the interaction of RGMb with BMP-2 and BMP-4¹⁸ and partially blocks the interaction of RGMb with NEO1 (data not shown), likely disrupting formation of the signaling hub.

Although both RGMb and NEO1 proteins can be cell-surface molecules, they are primarily localized intracellularly (Fig 3 and see Videos E1 and E2), which is in agreement with recent data from crystal structure analysis suggesting that the RGM-NEO1 complex transports BMP dimers from the cell surface to endosomes that are rich in BMP type I receptors.¹¹ Although the site of action of BMP receptors is under debate, endocytosis has been shown to be important for BMP signaling.⁶⁴

Anti-RGMb mAb treatment can inhibit airway inflammation at several stages of the allergic response by blocking expansion of the RGMb-expressing macrophages, which, through production of IL-13, contribute to lung smooth muscle contraction and eosinophil recruitment.⁵⁴ RGMb mAb treatment might also inhibit production of type 2 mediators by bronchial epithelial cells, which we have shown express RGMb. Bronchial epithelial cells have been termed “gatekeepers of allergic sensitization”²⁹ because on detection of inhaled allergens through pattern recognition receptors they release cytokines that initiate type 2 responses. Further studies will be required to fully elucidate these mechanisms.

It should be noted that RGMb appears to have 2 distinct functions in the lung involving 2 distinct immunologic pathways. We previously described a pathway in which RGMb, through interaction with PD-L2, promotes respiratory tolerance in the lung.¹⁸ Respiratory tolerance induced by exposure to OVA in the respiratory tract without prior T-cell priming was inhibited by treatment with an anti-PD-L2 antibody, which blocked the interaction of PD-L2 with RGMb but not the interaction of RGMb with NEO1 or BMPs.¹⁸ In the inflammatory pathway described herein, which involves allergen sensitization before allergen challenge in the respiratory tract, administration of the anti-PD-L2 mAb, which blocks the RGMb:PD-L2 interaction, had no effect on AHR. In contrast, in lung inflammation a RGMb:NEO1:BMPs interaction can be involved (without PD-L2) based on our finding that (1) NEO1 is expressed in the inflamed lung on bronchial epithelial cells and interstitial macrophages; (2) the anti-RGMb mAb we used inhibits interaction of RGMb with BMP-2, BMP-4, and NEO1; (3) previous work demonstrated an increase in BMP-2, BMP-4, and NEO1 in inflamed lung tissue^{14,17}; and (4) crystal structure analyses demonstrated that RGMb forms a ternary complex with NEO1 and BMP-2, BMP-4, or BMP-6, creating a potent signaling hub.¹¹ Upstream events, such as activation of epithelial cells by allergen or sensitized T cells, likely promotes the RGMb:NEO1:BMP pathway. Alternatively, it is possible that an RGMb interaction with BMP-2, BMP-4, or BMP-6 or with an unidentified ligand is involved. In any case,

in the development of AHR, the RGMb pathway is distinct from that seen in respiratory tolerance.

In summary, we identified a novel innate immune pathway in the lungs in which blockade of RGMb inhibited the development of AHR and lung inflammation. We identified lung cells that expressed RGMb and NEO1 and that modulated the development of AHR. RGMb mAb treatment was effective when given during the challenge phase of the allergic lung response and even after several rounds of allergen exposure. RGMb treatment might be effective in reducing IL-25-driven disease, including allergen-induced AHR, respiratory syncytial virus-induced pulmonary disease,⁴⁷ and rhinovirus-induced allergic airway inflammation,³⁵ which have both been shown to be driven by IL-25. Agents that target RGMb could provide an effective therapy for asthma.

We thank Amgen for providing the IL-17RB-deficient mice and Andrew N. J. McKenzie, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, for providing the ST2-deficient mice.

Key messages

- RGMb regulates type 2 inflammation in allergic asthma.
- RGMb is expressed primarily by innate cells in the lungs, including activated eosinophils and interstitial macrophages, and by bronchial epithelial cells.
- Treatment with anti-RGMb mAb after allergen sensitization inhibits allergen-induced asthma (AHR) and lung inflammation.

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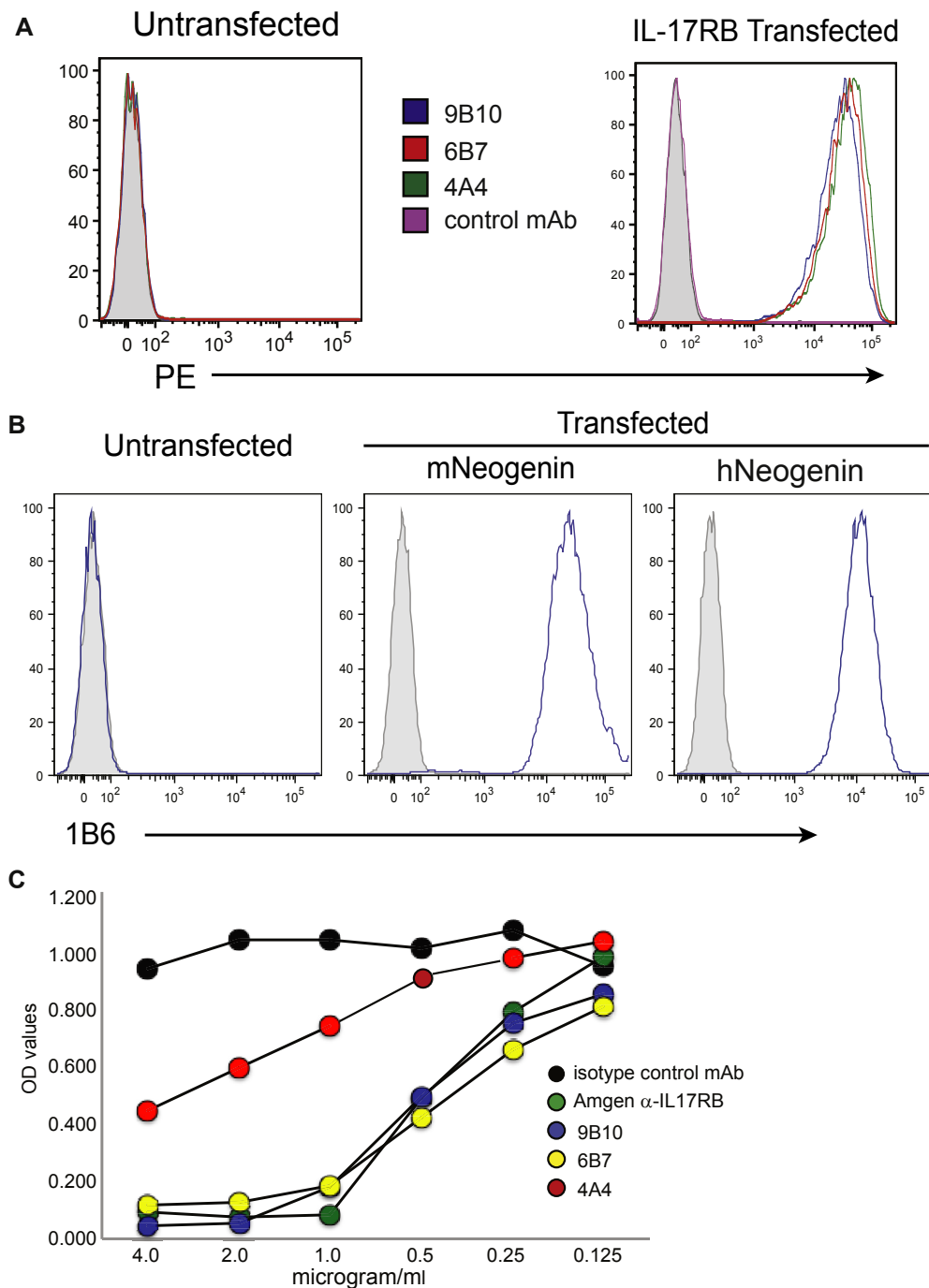


FIG E1. Specificity of IL-17RB and NEO1 mAbs. **A**, Untransfected 300.19 cells (300.19) or mouse IL-17RB-transfected 300.19 cells were stained with IL-17RB mAb 9B10, 6B7, or 4A4 or control mAb (10 μ g/mL). The filled histogram shows unstained cells. PE, Phycoerythrin. **B**, Untransfected 300.19 cells or 300.19 cells stably transfected with mouse (*m*) NEO1 or human (*h*) NEO1 were stained with NEO1 mAb 1B6 (solid line) or isotype control (filled histogram). **C**, Anti-IL-17RB mAbs were tested in a solid-phase assay for the ability to block binding of IL-17RB-huIgG Fc to ELISA plates coated with recombinant murine IL-25 (0.25 μ g/mL). mAb 9B10 was an effective blocking antibody.

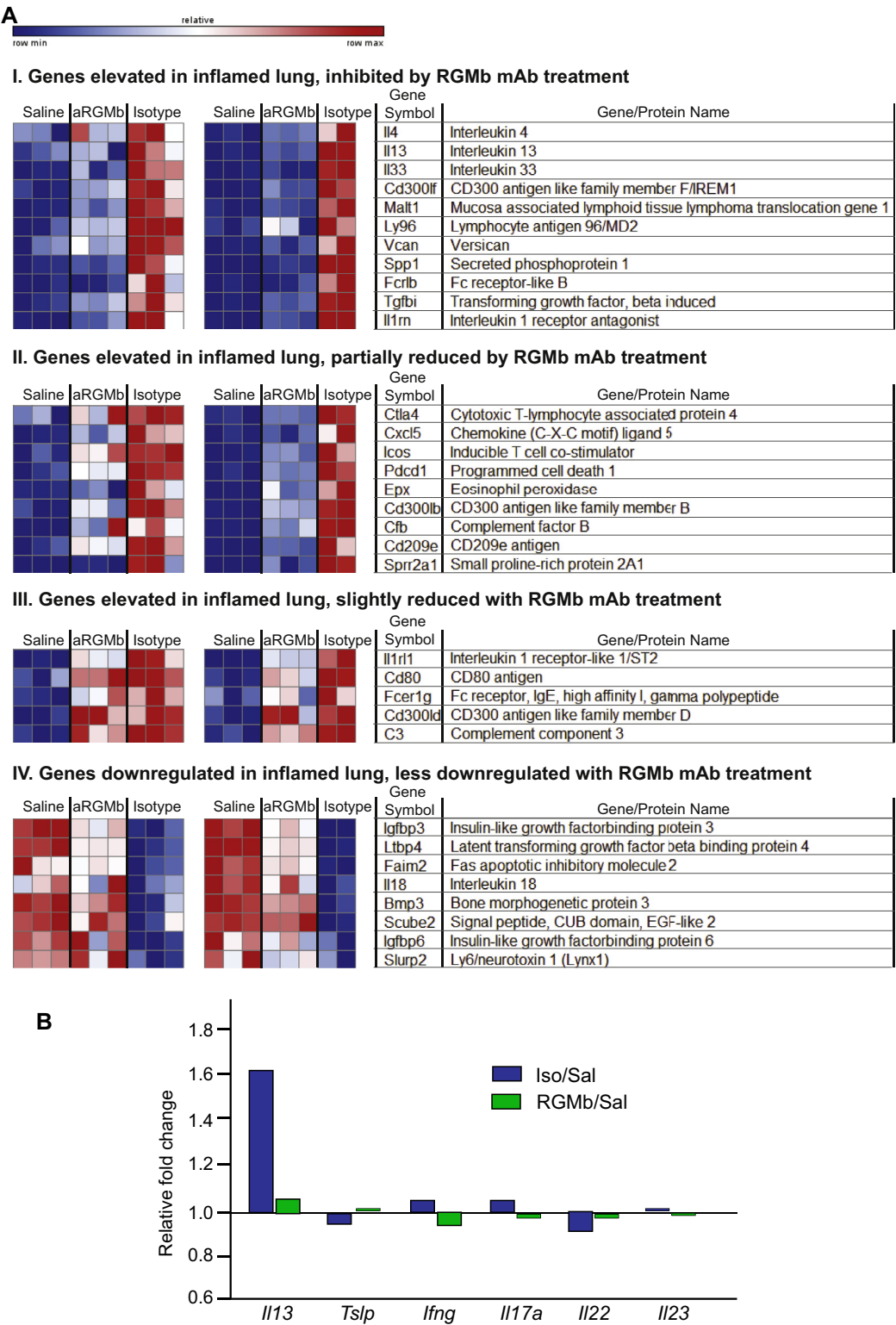


FIG E2. RGMb mAb treatment can reverse changes in inflammatory gene expression in the inflamed lung. **A**, Wild-type mice sensitized and challenged with OVA were treated with RGMb mAb or isotype control as in Fig 1, A. On day 10, lung tissues were harvested and used for microarray analysis. Genes upregulated in the isotype (*Iso*) group (inflamed lung) relative to the saline (*Sal*) group were reduced to the level of the saline group ($Iso/Sal > 1.6$, $RGMb/Iso < 0.6$, and $0.6 < RGMb/Sal < 1.6$; *group I*), partially reduced ($Iso/Sal > 1.6$, $RGMb/Iso < 0.6$, and $RGMb/Sal > 1.6$; *group II*), and unchanged by RGMb mAb treatment ($Iso/Sal > 1.6$, $RGMb/Iso > 0.6$, and $RGMb/Sal > 1.6$; *group III*). In *group IV* Genes downregulated in inflamed lung tissue relative to the saline group were less downregulated with RGMb mAb treatment ($Iso/Sal < 0.6$, $RGMb/Iso > 1.6$, and $0.6 < RGMb/Sal < 1.6$). Two microarray experiments were performed on 2 to 3 individual mice per treatment group. **B**, Bar graph of expression levels of cytokine genes that were unchanged across the 3 groups, with *Il13* (reduced by RGMb treatment, *group I*, Fig E2, A) included for reference. The figure presents isotype and RGMb values relative to saline, where a ratio of 1 shows a lack of change.

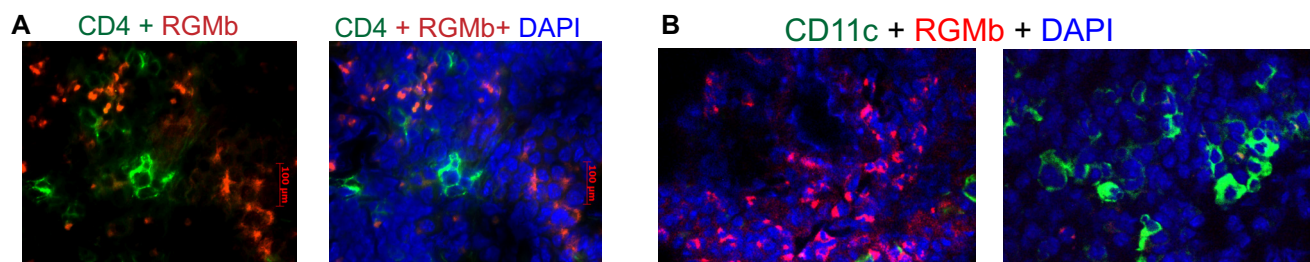


FIG E3. CD4⁺ T cells and CD11c⁺ cells in the lungs do not express RGMb protein. **A**, CD4⁺ T cells in the lungs do not express RGMb. *Left panel*, Lack of colocalization of CD4 (green) and RGMb (red). *Right panel*, Nuclei were visualized with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). **B**, CD11c⁺ cells do not express RGMb protein. The image on the left shows an optic field containing large numbers of RGMb⁺ cells (red) and very few CD11c⁺ cells (green). DAPI staining is shown in blue. The *right panel* shows a different field of the same section containing many CD11c⁺ cells but very few RGMb⁺ cells.

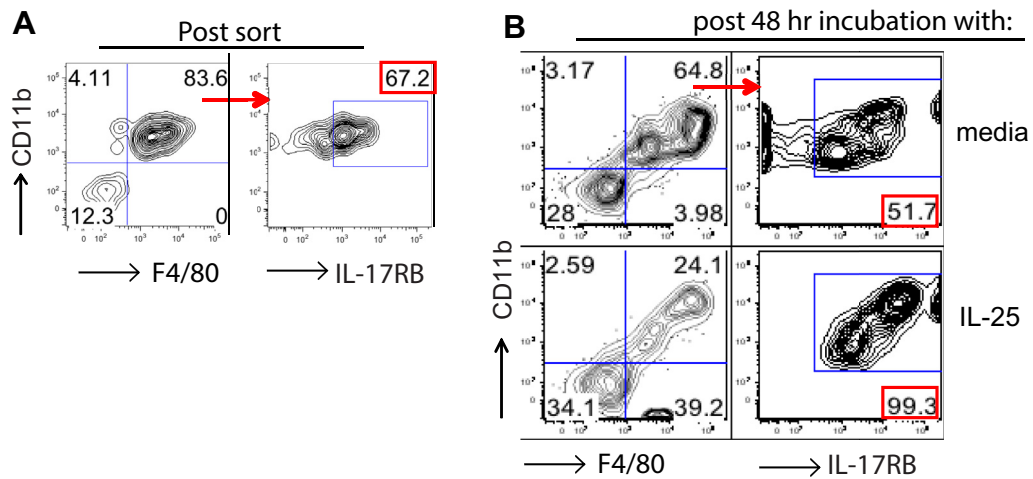


FIG E4. Expression of IL-17RB by interstitial macrophages is enhanced by incubation with rIL-25. Lungs from mice sensitized and challenged with CRA or saline were collagenase digested and analyzed by using flow cytometry. Cells were sorted as CD45⁺ Siglec-F⁻ CD11b⁺ F4/80⁺ CD11c⁻ (interstitial macrophages). **A**, Cells were stained for IL-17RB expression immediately after sorting. **B**, Cells were stained for IL-17RB expression after 48 hours of culture with media or rIL-25.

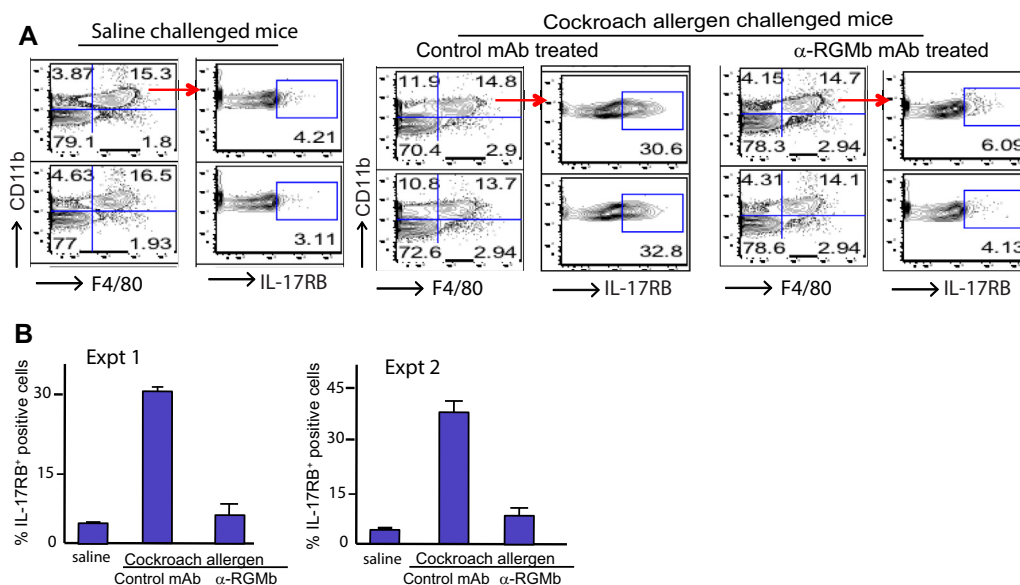


FIG E5. Expression of IL-17RB by interstitial macrophages of CRA-sensitized and challenged mice is reduced by treatment with anti-RGMb mAb. Mice sensitized and challenged with CRA were treated with RGMb mAb or isotype control on days 13, 17, 21, and 25. Lungs from mice sensitized and challenged with CRA or saline were collagenase digested and analyzed by using flow cytometry. **A**, IL-17RB expression by interstitial macrophages. Numbers on plots reflect the percentage of cells in the plot that are present within the gated area. Analyses of 2 representative mice per group are shown. **B**, Bar graph representation of experiment shown in Fig E5, A (experiment 1), and a duplicate experiment (experiment 2): summary of 3 mice per group.

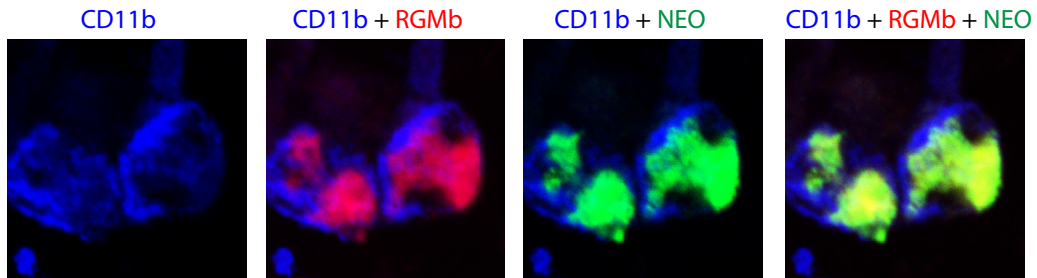


FIG E6. RGMb and NEO1 proteins are expressed on the cell surface but primarily expressed intracellularly. Confocal images showing a single Z-stack section from [Video E2](#). Image shows 2 CD11b⁺ cells (blue) with intracellular and surface expression of NEO (green) and RGMb (red). The *last panel* is an overlay of all fluorescent channels. Overlap of NEO (green) with RGMb (red) appears as yellow.

TABLE E1. Antibodies used for immunofluorescence microscopy or IHC and flow cytometry

Antibody	Clone	Conjugate	Company
Immunofluorescence microscopy or IHC			
CD11b	M1/70	Pacific Blue	BioLegend, San Diego, Calif
F4/80	BM8	Alexa Fluor 488	BioLegend
Siglec-F (CD170)	1RNM44N	Unconjugated	eBioscience, San Diego, Calif
CD11c	N418	Alexa Fluor 488	BioLegend
CD4	GK1.5	Alexa Fluor 488 or Alexa Fluor 594	BioLegend
Goat anti-rat		Alexa Fluor 488	Molecular Probes, Eugene, Ore
RGMb	9D3	Alexa Fluor 594	
NEO1	1B6	Brilliant Violet 421	
IL-17RB	9B10	Alexa Fluor 488	
RGMb	9D3	Biotin	
NEO1	3C9	Biotin	
Flow cytometry			
CD11c	BU15	APC-eFluor 780	eBioscience
CD11b	M1/70	PerCP/Cy5.5	BioLegend
F4/80	BM8	Alexa Fluor 700, Brilliant Violet 421 or APC-Cy7	BioLegend
CD45	104	Brilliant Violet 605	BD Biosciences, San Jose, Calif
Gr-1	RB6-8C5	PE-Cy7	BioLegend
TCR β	H57-597	Alexa Fluor 488	eBioscience
Siglec-F	E50-2440	PE	BD Biosciences
c-Kit	2B8	Brilliant Violet 421	BD Biosciences
Scal-1	D7	APC-Cy7	BioLegend
CD4	RM4-5	APC-eFluor780	eBioscience
ST2	RMST2-2	Biotin	eBioscience
Streptavidin		PE	eBioscience
CD1d-tetramer		PE	NIH Tetramer Facility
CD3	145-2C11	FITC	BioLegend
CD19	6D5	FITC	BioLegend
CD11C	N418	FITC	BioLegend
F4/80	BM8	FITC	BioLegend
Fc ϵ RI α	1-Mar	FITC	BioLegend
NK1.1	PK136	FITC	BioLegend
CD14	Sa14-2	FITC	BioLegend
CD11b	M1/70	FITC	BD Biosciences
IL-4	11B11	PE	BioLegend
IL-13	eBio13A	PE	eBioscience
IL-5	TRFK5	PE	BioLegend
IL-17RB	9B10	Alexa Fluor 647	
RGMb	9D3	Alexa Fluor 647	
NEO1	1B6	Brilliant Violet 421	
NEO1	1B6	Alexa Fluor 488	

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.

TABLE E2. Treatment with anti-RGMb mAb reduces lung inflammation: Mice sensitized and challenged with OVA or saline

Experiment 1			Experiment 2		
Group	Mouse	Inflammation score	Group	Mouse	Inflammation score
Saline	8	0	Saline	8	0
	9	0		9	0
	10	0		10	0
Control mAb	11	2	Control mAb	11	2
	12	2		12	3
	13	2		13	2
Anti-RGMb	14	1	Anti-RGMb	14	1
	15	1		15	1
	16	1		16	1
	17	1		17	1

OVA-treated mice were given anti-RGMb or control mAb on day 15 (protocol: Fig 2, A). Lung sections were stained with H&E (Fig 2, D) and scored for inflammation as follows: 0, no significant inflammation; 1, small clusters (10-30 cells) of inflammatory cells adjacent to vessels and airways; 2, moderately sized clusters (30-100 cells) of inflammatory cells adjacent to vessels and airways; and 3, large aggregates (>100 cells) of inflammatory cells adjacent to vessels and airways. $P < .0001$, anti-RGMb versus control mAb treated (when the 2 experiments were combined).

TABLE E3. Treatment with anti-RGMb mAb reduces lung inflammation: Mice sensitized and challenged with CRA or saline

Experiment 1			Experiment 2		
Group	Mouse	Inflammation score	Group	Mouse	Inflammation score
Saline	1	0	Saline	1	0
	2	0		2	0
	3	0		3	0
Control mAb	4	2	Control mAb	4	1
	5	2		5	2
	6	1		6	1
Anti-RGMb	7	0	Anti-RGMb	7	0
	8	1		8	1
	9	0		9	1
	10	1			

CRA-treated mice were given anti-RGMb mAb or control mAb. Lung sections were stained with H&E (see Fig 2, *H*) and scored for inflammation as above (see Table E2). $P = .045$, anti-RGMb treated versus isotype control (when the 2 experiments were combined).

TABLE E4. Numbers of indicated cell types in lung tissue after allergen exposure

	Eosinophils		Interstitial macrophages	
	Total	IL-17RB ⁺	Total	IL-17RB ⁺
Saline	$0.06 \pm 0.007 \times 10^6$	$0.02 \pm 0.006 \times 10^5$	$0.30 \pm 0.02 \times 10^6$	$0.02 \pm 0.002 \times 10^6$
OVA	$7.07 \pm 0.27 \times 10^6$	$1.11 \pm 0.10 \times 10^5$	$3.60 \pm 0.31 \times 10^6$	$1.89 \pm 0.12 \times 10^6$
CD4 ⁺ T cells				
Total	IL-17RB ⁺			
$0.27 \pm 0.02 \times 10^5$	$0.38 \pm 0.10 \times 10^3$			
$4.60 \pm 0.50 \times 10^5$	$6.86 \pm 0.54 \times 10^3$			
	iNKT cells		ILC2s	
	Total	IL-17RB ⁺	Total	IL-17RB ⁺
Saline	$0.14 \pm 0.02 \times 10^4$	$0.00 \pm 0.40 \times 10^2$	$0.10 \pm 0.02 \times 10^4$	$0.00 \pm 0.10 \times 10^2$
OVA	$2.13 \pm 0.20 \times 10^4$	$5.30 \pm 1.50 \times 10^2$	$2.92 \pm 0.30 \times 10^4$	$1.04 \pm 0.06 \times 10^3$

Mice that were sensitized with OVA/alum intraperitoneally on day 0 were challenged with OVA or saline on days 7, 8, and 9. Lungs were harvested on day 10, collagenase digested, and analyzed by using flow cytometry.

iNKT, Invariant natural killer T.

TABLE E5. Treatment with anti-RGMb mAb reduces lung inflammation: Mice sensitized and challenged with CRA or saline in a chronic exposure model over 27 days

Experiment 1			Experiment 2		
Group	Mouse	Inflammation score	Group	Mouse	Inflammation score
Saline	1	0	Saline	101	0
	2	0		102	0
	3	0		103	0
Control mAb	4	3	Control mAb	104	2
	5	3		105	2
	6	3		106	2
Anti-RGMb	7	0	Anti-RGMb	107	0
	8	0		108	1
	9	0		109	1

CRA-treated mice were given anti-RGMb mAb or control mAb. Lung sections were stained with H&E (see [Fig 5, J](#)) and scored for inflammation (see [Table E2](#)). $P < .0001$, anti-RGMb versus control mAb treated (when the 2 experiments were combined).