

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

RAGE-dependent VCAM-1 expression in the lung endothelium mediates IL-33-induced allergic airway inflammation

T. N. Perkins^{1,2}  | E. A. Oczypok¹ | P. S. Milutinovic^{3,4} | R. E. Dutz¹ | T. D. Oury¹

¹Department of Pathology, University of Pittsburgh, School of Medicine, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

²Department of Pediatrics, Division of Pulmonary, Allergy, and Clinical Immunology, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, PA, USA

³Department of Pediatrics, Duke University Medical Center, Durham, NC, USA

⁴Department of Medicine, Duke University Medical Center, Durham, NC, USA

Correspondence

Tim D. Oury, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA.
Email: tdoury@pitt.edu

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Abstract

Background: The receptor for advanced glycation endproducts (RAGE) has been implicated as a critical molecule in the pathogenesis of experimental asthma/allergic airway inflammation (AAI). It has been previously shown that RAGE acts both upstream of interleukin-33 (IL-33) release and downstream of IL-33 release via RAGE-dependent IL-33-induced accumulation of type 2 innate lymphoid cells (ILC2s) in the lungs, which perpetuate type 2 inflammation and mucus metaplasia. However, the mechanism by which RAGE mediates downstream IL-33-induced type 2 inflammatory responses is unknown.

Objective: This study tested the hypothesis that ILC2s are recruited to the lungs via RAGE-dependent vascular cell adhesion molecule 1 (VCAM-1) expression on lung endothelial cells.

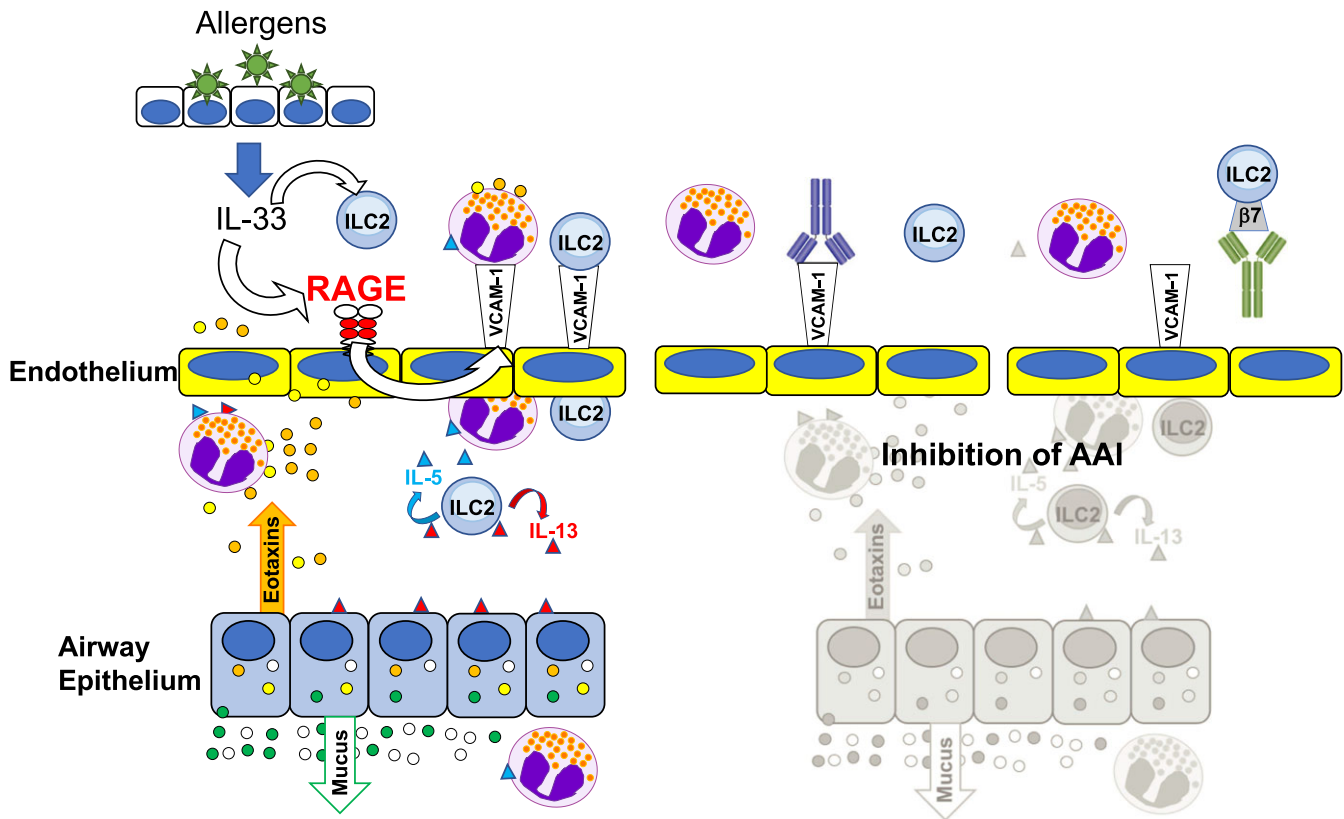
Methods: House dust mite extract, *Alternaria alternata* extract, or rIL-33 was used to induce AAI/VCAM-1 expression in wild-type (WT) and RAGE-knockout (RAGE-KO) mice. Intravenous (i.v.) anti-VCAM-1 or intraperitoneal (i.p.) $\beta 7$ blocking antibody administration was used to determine the role of VCAM-1 in IL-33-induced AAI.

Results: Enhanced VCAM-1 expression in the lungs by HDM, AA, or rIL-33 exposure was found to be RAGE-dependent. In addition, stimulation of primary mouse lung endothelial cells with IL-33 induced VCAM-1 expression in WT, but not RAGE-KO cells. Administration of VCAM-1 and $\beta 7$ -integrin blocking antibodies reduced IL-33-induced eosinophilic inflammation, mucus metaplasia, and type 2 inflammatory responses.

Conclusion: This study demonstrates that allergen- and cytokine-induced VCAM-1 expression is RAGE-dependent and contributes to lung ILC2 accumulation and downstream eosinophilic inflammation, mucus metaplasia, and type 2 inflammatory responses.

KEYWORDS

asthma, endothelium, IL-33, RAGE, VCAM-1



GRAPHICAL ABSTRACT

Exposure to allergens or recombinant interleukin-(IL)-33 induces expression of vascular cell adhesion molecule-1 (VCAM-1) in the lung endothelium in a RAGE-dependent manner and activates type 2 innate lymphoid cells (ILC2). IL-33 recruits ILC2s to the lungs, which produce IL-5 and IL-13, which induces eotaxin production, eosinophil recruitment, and mucus secretion. Blocking VCAM-1 or $\beta 7$ integrins with neutralizing antibodies inhibits IL-33 induced eosinophilic inflammation and mucus production in the lungs. IL-33 mediates ILC2 and eosinophil recruitment to the lungs through RAGE-dependent VCAM-1 expression and interaction with $\beta 7$ integrins.

1 | INTRODUCTION

Asthma affects nearly 300 million people worldwide, resulting in about 2 million emergency room visits and nearly 3000 deaths per year.¹ IL-33 is an epithelial-derived cytokine and endogenous danger signal that plays a central role in allergic asthma^{2,3} by initiating type 2 immune responses.^{4,5} The primary mode of action of IL-33 in allergic asthma/airway inflammation (AAI) is by stimulating type 2 innate lymphoid cells (ILC2s).^{6–10} ILC2s have been shown to be critical players in the pathogenesis of asthma^{10–13} and are the major producers of IL-5, IL-9, and IL-13 in asthma.¹³

The mechanisms leading to ILC2 accumulation in the lung in models of AAI are not well understood. However, a recent study found that in conjunction with local proliferation,¹⁴ ILC2s are produced in the bone marrow and recruited from the peripheral blood to the lungs.¹⁵ The exact mechanisms of ILC2 recruitment and trafficking into the lung tissue in models of AAI remain unclear and may vary depending on the model used, as different allergens and cytokines have divergent effects on ILC2 activation.

The receptor for advanced glycation endproducts (RAGE) is a member of the immunoglobulin family of proteins¹⁶ with various

ligands including amyloid peptides, S100/calgranulins, and high-mobility group box-1 (HMGB1).¹⁷ Notably, RAGE expression is highest in the lung,^{18,19} and recent studies suggest it plays an important role in asthma pathogenesis.^{20,21} Two genomewide association studies were conducted to find loci associated with altered forced expiratory volume in 1 second (FEV₁),^{22,23} a key pulmonary function parameter impaired in asthma. Both studies found a strong association between decreased FEV₁ and rs2070600, a SNP that leads to an amino acid substitution (G82S) in the ligand-binding domain of RAGE. In addition, recent studies found that the RAGE ligands S100A8/A9 and S100A12 contribute to airway inflammation and remodeling in asthma.²⁴ Furthermore, soluble RAGE, which acts as a decoy receptor to dampen persistent RAGE signaling, is decreased in the blood and sputum of patients with severe asthma.^{25,26} These findings suggest that RAGE contributes to the pathogenesis of AAI, although the RAGE-expressing cells that initiate RAGE-related AAI remain undefined.

RAGE-null mice are markedly protected against the development of AAI in multiple models.^{20,21,27} In response to allergens, RAGE-KO mice develop a normal humoral response; however, they elicit no ILC2 accumulation or production of IL-5, IL-9, or IL-13.^{20,27} Interestingly, not only is RAGE required for allergen-induced accumulation

of lung IL-33, but RAGE is also required to induce IL-33-mediated responses when IL-33 is administered exogenously.²⁷

It has been shown that ILC2 recruitment in the gut depends on $\alpha 4\beta 7$ expression on ILC2s interacting with MAdCAM-1 on gut endothelial cells.^{28,29} Notably, VCAM-1 and not MAdCAM-1 has been shown to promote recruitment of $\alpha 4\beta 7$ -expressing allergic inflammatory precursor cells into the lung.³⁰ RAGE signaling has been shown to promote VCAM-1 expression on endothelial cells,³¹ and IL-33 is a potent inducer of VCAM-1 expression on endothelial cells.³² IL-33-induced accumulation of ILC2s is RAGE-dependent in the lungs, but not in the gut.²⁷ This study investigates the hypothesis that differential expression of adhesion molecules and RAGE in the lung compared to the intestine contributes to the lung-specific effects of RAGE in ILC2 accumulation. The results of this study suggest that RAGE promotes ILC2 accumulation in the lung in response to allergens and IL-33 by promoting lung endothelial VCAM-1 expression.

2 | MATERIALS AND METHODS

*A detailed description of methods can be found in the Supporting Information.

2.1 | Animals and reagents

Eight-week-old wild-type C57BL/6 mice were purchased from Taconic (Hudson, NY, USA). RAGE-knockout (KO) mice congenic with the C57BL/6 strain were provided by Dr. A. Bierhaus (University of Heidelberg), and a breeding colony was established.^{33,34} Animal studies were carried out under the National Research Council's guidelines in the "Guide for the care and use of laboratory animals," with oversight by the University of Pittsburgh's Institutional Animal Care and Use Committee. HDM and *Alternaria alternata* extracts were purchased from Greer Laboratories. Recombinant IL-33 was purchased from BioLegend.

2.2 | Models of AAI/asthma

For more information on models of asthma/AAI, see Figure S1 (adapted from a previously published figure²⁷). A chronic model of AAI was induced by treating wild-type or RAGE-KO mice intranasally 4 times per week for 7 weeks with 40 μ g of HDM extract in 25 μ L of saline. Mice were killed 48 hours after the last treatment.²⁰ In an acute model of AAI,³⁵ mice were treated intranasally with 25 μ g of *Alternaria alternata* extract in 25 μ L of saline on days 0, 3, 6, and 9 and killed on day 10. For the IL-33 intranasal protocol, mice were treated with 1 μ g of IL-33 in 25 μ L of saline daily for 4 days and then killed after 24 hours. In all models, control mice were treated with saline vehicle alone.

2.3 | Fluorescence microscopy

In brief, mouse lung sections were stained with anti-mouse VCAM-1 (R&D AF643) followed by straining with nuclear Hoechst stain. Sections were coverslipped with Gelvatol and visualized. Images were taken at 20 $\times$ magnification.

2.4 | Isolation and culture of primary mouse lung endothelial cells

In brief, lungs were excised and digested, and a single-cell suspension was prepared and subjected to magnetic bead separation. Negative selection was performed using anti-mouse CD45 beads and positive selection was performed using anti-mouse CD31 beads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA). Cells were cultured and maintained in growth medium, grown to near confluence, and then stimulated with saline or rIL-33 (100 ng/mL).

2.5 | VCAM-1 blocking and $\beta 7$ blocking

Detailed methods are given in Supporting Information.

2.6 | Bronchoalveolar lavage and histological analysis

Bronchoalveolar lavage, cytospin analysis, and lung fixation and staining were carried out as previously described;²⁰ see Supporting Information for a detailed description.

2.7 | Flow cytometry

Whole-lung single-cell suspensions were prepared, and cells were stained with the following antibody cocktail: anti-mouse lineage cocktail (BioLegend, San Diego, CA, USA), anti-mouse CD11c (eBioscience, San Diego, CA, USA), anti-mouse Fc ϵ RI (BioLegend), anti-mouse NK1.1 (eBioscience), anti-mouse CD90.2 (Thy1.2) (BD), and anti-mouse ST2 (eBioscience). ILC2s were counted as lineage-negative, CD90.2-positive, and ST2-positive.² Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Gating strategy for mouse lung ILC2s was previously published;²⁷ see Supporting Information for a detailed description.

2.8 | Immunoblotting, ELISA, and qRT-PCR

Methods were based on those previously published. Full details can be found in the Supporting Information.

2.9 | Statistical analysis

Statistics were performed with GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). Results are expressed as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's posttest or the unpaired Student's *t* test. *P* values of <.05 were considered significant.

3 | RESULTS

3.1 | RAGE is required for enhanced VCAM-1 expression in the lung endothelium in experimental AAI

In addition to various cell types in the lungs, lung endothelial cells express RAGE (Figure S1). Expression levels of VCAM-1 were

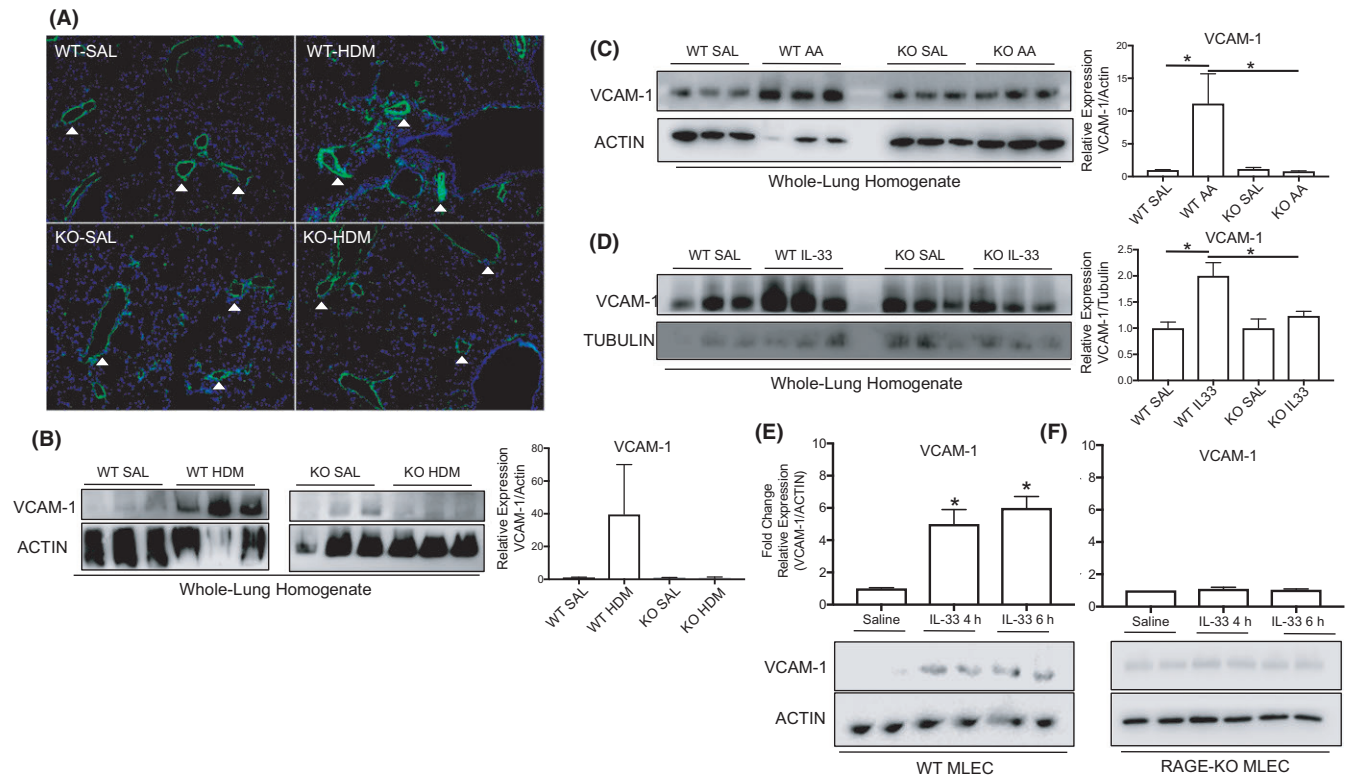


FIGURE 1 RAGE is required for enhanced VCAM-1 expression in the lung endothelium in experimental AAI. VCAM-1 expression is enhanced in the lungs of WT but not RAGE-KO mice in multiple models of experimental AAI. (A) Immunofluorescently stained frozen tissue sections of lungs from WT or RAGE-KO mice chronically exposed intranasally to HDM extract or saline (vehicle) control. VCAM-1 in green and nuclear counterstain (DAPI) in blue. Western blot and densitometry quantification of whole-lung homogenates from WT and RAGE-KO mice exposed intranasally to (B) HDM extract or saline control, (C) AA extract or saline control, and (D) rIL-33 or saline control. Blots were probed for VCAM-1 (100 kDa) or β -actin (42 kDa) or β -tubulin (50 kDa). Western blot and densitometry quantification of (E) WT and (F) RAGE-KO mouse lung endothelial cells (MLEC) treated with saline or rIL-33 (100 ng/ml) for 4 or 6 h; blots were probed for VCAM-1 (100 kDa) or β -actin (42 kDa). * $P < .05$. $N = 2-3$ per group, per experiment. Data are representative of at least 2 independent experiments

examined in the lungs of WT and RAGE-KO mice subjected to both a chronic allergen exposure model (house dust mite (HDM)) and an acute allergen exposure model (*Alternaria alternata* (AA)), as well as an acute cytokine-induced model (rIL-33) of AAI (Figure S2A-C).^{20,27} Figure 1 illustrates that VCAM-1 expression was enhanced in WT mice exposed to HDM (A-B), AA (C), and rIL-33 (D) compared to saline-exposed control mice. RAGE-KO mice subjected to the same exposures exhibited no change in whole-lung VCAM-1 expression levels compared to control mice. Enhanced VCAM-1 levels in WT mice were most notable in the endothelium of large bronchial vessels as determined by immunofluorescence (Figure 1A).

Primary mouse lung endothelial cells (MLEC) were isolated from WT and RAGE-KO mouse lungs to determine the effects of in vitro stimulation with rIL-33. MLEC were stimulated with IL-33 (100 ng/mL) or saline (control) for 4 and 6 hours, and VCAM-1 levels were assessed by Western blot of whole-cell lysates. IL-33 induced VCAM-1 expression in WT (Figure 1E), but not RAGE-KO MLEC (Figure 1F). There was no difference in baseline levels of VCAM-1 in WT vs RAGE-KO MLEC (Figure S3). Figure 1 indicates that RAGE is required for enhanced expression of VCAM-1 in mouse lung endothelium and in isolated mouse lung endothelial cells in response to allergens or signaling molecules (ie, IL-33).

ST2 is the primary receptor for IL-33, and we see similar expression of ST2 in endothelial cells from both WT and RAGE-KO cells. The finding that IL-33 induces VCAM-1 in WT but not RAGE-KO cells suggests that RAGE is directly contributing to IL-33-induced expression of VCAM-1 in endothelial cells. IL-33 has never been shown to be a ligand for RAGE, but these data suggest that RAGE is required for IL-33-induced VCAM-1 expression in lung endothelial cells.

3.2 | Blockade of VCAM-1 inhibits IL-33-induced lung eosinophilia, mucus metaplasia, and type 2 inflammatory responses

Upon allergen exposure, ILC2s accumulate in the lungs where they secrete copious amounts of IL-5 and IL-13.¹³ Local release of IL-5 and IL-13 induces eotaxin and eotaxin-2 production which promotes recruitment and proliferation of eosinophils in the lungs.³⁶ To determine the role of RAGE-dependent VCAM-1 in trafficking of effector cell types in IL-33-induced AAI, intravenous anti-VCAM-1 blocking antibody (AVA) or isotype control antibody (ICA) was administered 1 hour prior to intranasal challenge with rIL-33 or saline control (Figure S1D) and lung eosinophilic inflammation was assessed. Cytospin analysis and differential cell counts revealed significant BALF

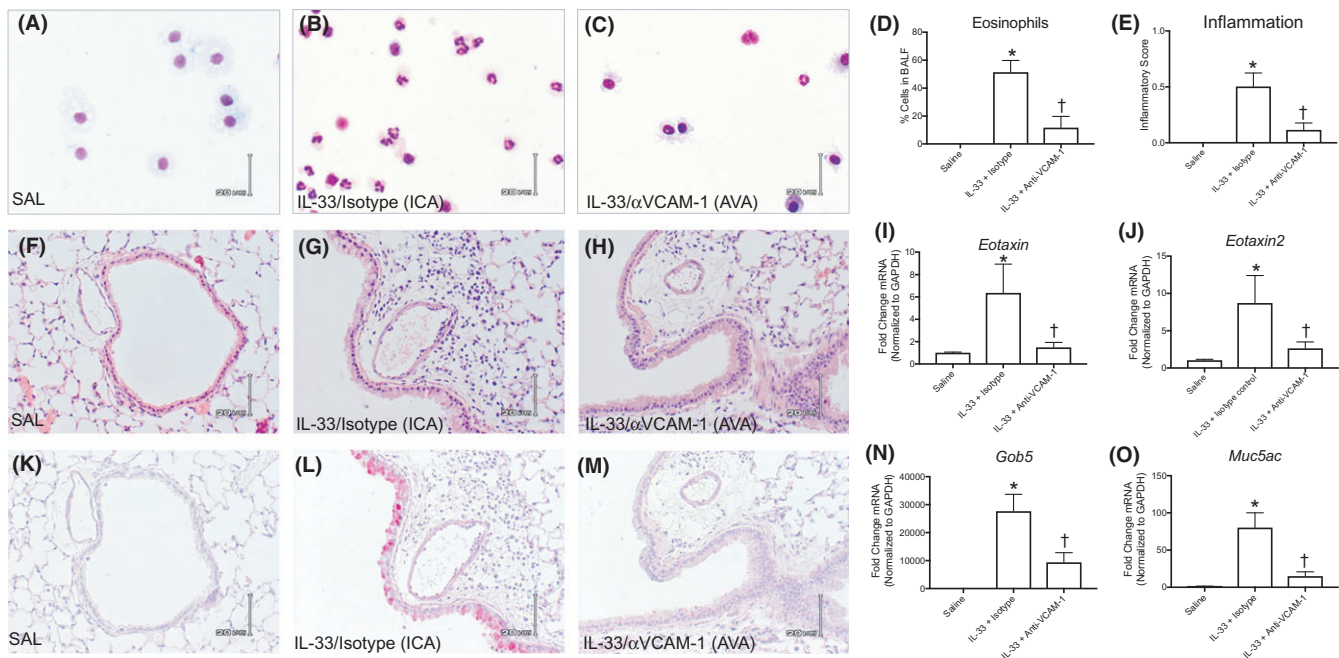


FIGURE 2 Blockade of VCAM-1 inhibits IL-33-induced lung eosinophilic inflammation and mucus metaplasia. Cytospin analysis was performed on BALF from mice treated with saline, IL-33 + isotype control antibody (ICA), and anti-VCAM-1 blocking antibody (AVA). Representative photomicrographs of BALF cytospin preps from (A) saline control mice, (B) IL-33 + ICA mice, and (C) IL-33 + AVA mice. (D) Differential cell counts of cytospin preps represented as the percentage of eosinophils in the BALF (see Figure S2 for cell numbers). Lung histology inflammatory scores (E) and representative photomicrographs of H&E-stained lung sections from (F) saline control mice, (G) IL-33 + ICA mice, and (H) IL-33 + AVA mice. qRT-PCR analysis of mRNA levels of eosinophil chemoattractants (I) *Eotaxin/Ccl11* and (J) *Eotaxin2/Ccl24* from whole-lung homogenates. Representative photomicrographs of periodic acid-Schiff (PAS)-stained lung sections (mucus hypersecretion, bright pink stain) of mice treated with (K) saline, (L) IL-33 + ICA, and (M) IL-33 + AVA. qRT-PCR analysis of whole-lung mRNA levels of mucus markers (N) *Gob5* and (O) *Muc5ac*. qRT-PCR data are represented as the mean fold change relative to saline controls and normalized to the housekeeping gene *Gapdh*. N = 4–6 animals per group, per experiment; data are representative of 2–3 repeat experiments. Data are presented as the mean $\pm$ SEM. * $P < .05$ vs saline controls † $P < .05$ vs IL-33 + ICA

eosinophilia in mice treated with IL-33 + ICA compared to saline controls. However, administration of AVA significantly reduced IL-33-induced BALF eosinophilia (Figure 2A–D, Figure S4A).

Histological examination of the lungs of WT mice exposed to IL-33 + ICA showed marked peribronchiolar eosinophilic inflammation compared to saline-exposed control mice which was diminished in mice treated with IL-33 + AVA (Figure 2E–H). qRT-PCR analysis of whole-lung RNA from mice showed increased mRNA levels of the *Eotaxin/Ccl11* (Figure 2I) and *Eotaxin2/Ccl24* (Figure 2J) in mice treated with IL-33 + ICA, which was significantly reduced in mice treated with AVA. These data indicate that blockade of VCAM-1 signaling prevents IL-33-driven eosinophilic inflammation in the lungs.

Periodic acid-Schiff (PAS)-stained lung sections indicate excessive mucus production in the airways of mice treated with IL-33 + ICA compared to saline-treated control mice, which was almost completely reversed to control levels by treatment with AVA (Figure 2K–M). Expression of markers of mucus metaplasia, *Gob5* (Figure 2N) and *Muc5ac* (Figure 2O), was assessed by qRT-PCR, and mRNA levels were increased in the lungs of mice treated with IL-33 + ICA compared to saline control mice. Treatment with the AVA significantly reduced IL-33-induced *Gob5* and *Muc5ac* levels.

The levels of IL-5, IL-13, and ILC2 accumulation were assessed in mice treated with IL-33 + ICA or AVA. Analysis of whole-lung mRNA

showed a marked increase in mRNA levels of both *Il13* and *Il5* (Figure 3A,B) as well as whole-lung (Figure 3C,D) and BALF (Figure 3E–F) protein levels of IL-5 and IL-13 in mice treated with IL-33 + ICA. In contrast, there was no significant increase in mRNA levels of *Il5* or *Il13* (Figure A–B), or whole-lung or BALF protein levels of IL-5 or IL-13 (Figure 3C–F) in mice treated with IL-33 + AVA. Lung ILC2s (Lin[−], CD90.2⁺, ST2⁺; see Figure S5 for gating strategy) were increased in mice treated with IL-33 + ICA, which was reduced by AVA (Figure 3G). The levels of the Th2 cytokine IL-4 were not measured as it has been demonstrated that IL-33 does not induce significant production of IL-4 from ILC2s or Th2 cells,^{37,38} which we confirmed in our rIL-33 model of AAI (Figure S6). These results support the hypothesis that VCAM-1 is contributing to IL-33-induced ILC2 responses in the lungs of mice.

3.3 | Blocking $\beta 7$ -integrin inhibits IL-33-induced lung eosinophilia, mucus metaplasia, and type 2 inflammatory responses

To determine the role of $\beta 7$ -integrin in IL-33-induced trafficking of ILC2s and eosinophils to the lungs, mice were treated intraperitoneally with anti- $\beta 7$ -integrin antibody (ABA) or ICA 1 hour prior to intranasal challenge with rIL-33 or saline control (Figure S1E). BALF

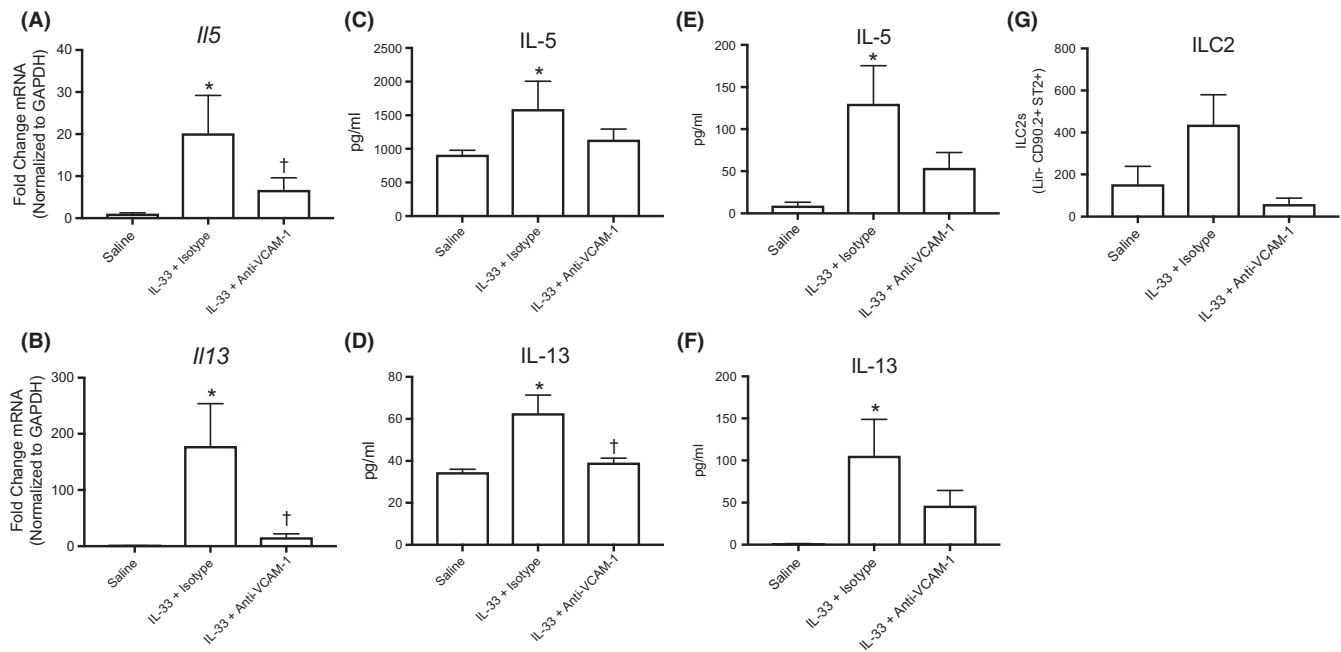


FIGURE 3 Blocking VCAM-1 reduces IL-33-induced type 2 inflammatory responses in the lungs of WT mice. Markers of type 2 inflammatory response, IL-5 and IL-13, were assessed by qRT-PCR analysis of RNA isolated from whole-lung homogenates, as well as protein levels from whole-lung homogenates and BALF from mice treated with saline, IL-33 + isotype control antibody (ICA), or anti-VCAM-1 antibody (AVA). Relative mRNA levels of (A) *Il5* and (B) *Il13*; data are represented as the fold change relative to saline controls and normalized to the housekeeping gene *Gapdh*. Protein levels from whole-lung homogenates of (C) IL-5 and (D) IL-13 and protein levels from BALF of (E) IL-5 and (F) IL-13 as determined by ELISA. Percentage of lung ILC2s (lineage-, CD90.2+, ST2+) as determined by flow cytometry (G). N = 4-6 animals per group, per experiment; data are representative of 2-3 repeat experiments. BALF sample data are representative of pooled samples from 3 independent experiments (N = 12-18). Data are presented as the mean $\pm$ SEM. * $P < .05$ vs saline controls † $P < .05$ vs IL-33 + ICA

cytospin analysis revealed significant BALF eosinophilia in mice treated with IL-33 + ICA compared to saline controls, which was significantly reduced by treatment with ABA (Figure 4A-D, Figure S4B).

Histological examination of the lungs of WT mice exposed to IL-33 + ICA showed marked peribronchiolar eosinophilic inflammation compared to saline-exposed control mice, which was significantly diminished in mice treated with ABA (Figure 4E-H). qRT-PCR analysis of whole-lung mRNA showed increased levels of *Eotaxin/Ccl11* (Figure 4I) and *Eotaxin2/Ccl24* (Figure 4J) in mice treated with IL-33 + ICA, which was significantly reduced in mice treated with ABA. These data suggest that blockade of $\beta 7$ -integrin prevents IL-33-driven eosinophilic inflammation in the lungs.

PAS staining revealed minimal positive staining, only in the IL-33 + ICA group (Figure 4K-M). However, similar to findings of VCAM-1 blocking experiments, mRNA levels of *Gob5* (Figure 4N) and *Muc5ac* (Figure 4O) were increased in the lungs of mice treated with IL-33 + ICA compared to saline control mice, and treatment with ABA significantly reduced this effect.

To determine the potential role of $\beta 7$ -integrin in trafficking of ILC2s to the lung in response to IL-33, the levels of IL-5, IL-13, and ILC2 accumulation were assessed. Analysis of whole-lung homogenates from mice showed a marked increase in mRNA levels of both *Il5* and *Il13* in mice treated with IL-33 + ICA, which was significantly abolished by treatment with ABA (Figure 5A,B). Although treatment with ABA did not completely abrogate IL-33-induced IL-5 and IL-13

in the BALF, there was no significant increase compared to saline controls, as there was a significant increase only in mice treated with IL-33 + ICA (Figure 5C,D). Lung ILC2s were significantly increased in mice treated with IL-33 + ICA, and this effect was reduced with ABA antibody (Figure 5E). These data suggest that $\beta 7$ -integrins contribute to IL-33-induced ILC2 responses in the lungs of mice.

4 | DISCUSSION

Local proliferation of inflammatory cells, including ILC2s, contributes to propagation of allergic inflammation.^{14,39} However, it is well known that mature eosinophils⁴⁰⁻⁴² and eosinophil progenitors⁴³⁻⁴⁵ are trafficked from the bone marrow to sites of allergic inflammation. In addition, a recent study indicates that in addition to local proliferation,¹⁴ ILC2s of hematopoietic origin are trafficked to the lungs in response to challenge with *Alternaria alternata* extract.¹⁵ Moreover, ILC2s are elevated in the blood of subjects with allergic asthma compared to nonasthmatic controls,⁴⁶ and ILC2s are increased in the sputum of subjects with severe asthma.³⁹ In line with those findings, this report is the first to indicate ILC2 and eosinophil trafficking via RAGE-dependent activation of the lung endothelium in an IL-33-induced model of AAI.

Activation and altered expression of surface molecules on eosinophils as well as the lung endothelium in asthma have been well

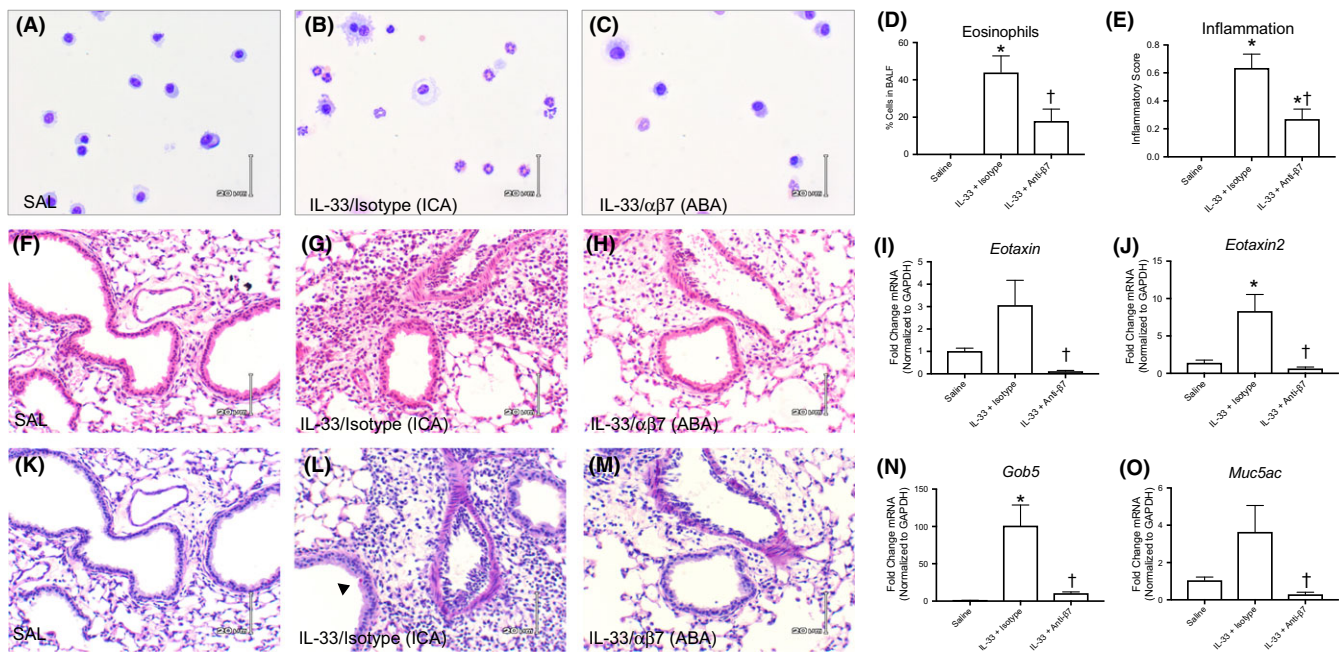


FIGURE 4 Blockade of $\beta 7$ -integrin inhibits IL-33-induced lung eosinophilic inflammation and mucus metaplasia. Cytospin analysis was performed on BALF from mice treated with saline, IL-33 + isotype control antibody (ICA), and anti- $\beta 7$ -integrin blocking antibody (ABA). Representative photomicrographs of cytospin preps from (A) saline control mice, (B) IL-33 + ICA mice, and (C) IL-33 + ABA mice. (D) Differential cell counts of cytospin preps represented as the percentage of eosinophils in the BALF (see Figure S2B for cell numbers). Lung histology inflammatory scores (E) and representative photomicrographs of H&E-stained lung sections from (F) saline control mice, (G) IL-33 + ICA mice, and (H) IL-33 + ABA mice. qRT-PCR analysis of mRNA levels of eosinophil chemoattractants (I) *Eotaxin/Ccl11* and (J) *Eotaxin2/Ccl24* from whole-lung homogenates. Representative photomicrographs of periodic acid-Schiff (PAS)-stained lung sections (mucus hypersecretion, bright pink stain) of mice treated with (K) saline, (L) IL-33 + ICA (black arrow indicates positive PAS), and (M) IL-33 + ABA. qRT-PCR analysis of whole lung mRNA levels of mucus markers (N) *Gob5* and (O) *Muc5ac*. Data are represented as the mean fold change relative to saline controls and normalized to the housekeeping gene *Gapdh*. N = 4–6 animals per group, per experiment; data are representative of 2–3 repeat experiments. Data are represented as the mean $\pm$ SEM. * $P < .05$ vs saline controls $^{\dagger}P < .05$ vs IL-33 + ICA (N = 5–6)

documented [reviewed in 47,48]. However, the mechanisms by which the changes in adhesion molecule expression contribute to trafficking and sequestration of eosinophils and other key inflammatory cells (eg, ILC2s) are poorly understood. Previous studies have indicated increased endothelial expression of cell adhesion molecules (i.e., ICAM-1, E-selectin, and VCAM-1) in human asthma biopsies.⁴⁹ Interestingly, VCAM-1 had the largest increase in expression in bronchial explants in response to *ex vivo* allergen challenge.⁵⁰ RAGE signaling has been linked to endothelial activation and dysfunction in a number of studies,^{51–54} and RAGE ligands are known to induce VCAM-1 expression on endothelial cells.^{51,52,54}

The current study demonstrates that VCAM-1 expression is enhanced in the lungs of mice challenged with HDM, AA, and rIL-33 *in vivo* and in endothelial cell cultures *in vitro*, in a RAGE-dependent manner (Figure 1). These findings are the first report that IL-33 signals either directly or indirectly through RAGE to induce VCAM-1 expression. It is important to note that although IL-33 is known to signal through its receptor ST2, ST2 levels were similar in both WT and RAGE-KO whole lung and isolated MLEC (not illustrated). Moreover, RAGE-KO mice are nonresponsive to direct intranasal rIL-33 administration, suggesting further that RAGE is required for IL-33 signaling.²⁷ IL-33 is extremely similar to other “dual-function

cytokines,” including the RAGE ligand, HMGB1, and IL-1 α . These dual-function cytokines act as transcriptional regulators when localized to the nucleus and have proinflammatory functions when released by both passive and active processes.^{55–57} RAGE is promiscuous in its ability to bind various ligands. RAGE ligands such as HMGB1 also bind multiple receptors, including Toll-like receptors (TLRs).^{58–60} It is possible that IL-33 also binds to RAGE, or RAGE may act as a coreceptor, similar to RAGE-TLR4 interactions demonstrated with HMGB1 binding.⁶¹

Blocking VCAM-1 *in vivo* reduced airway eosinophilic inflammation, type 2 inflammation, and mucus metaplasia induced by IL-33. These findings are in agreement with a previous study which found blocking VCAM-1 reduced ovalbumin-induced AAI, as well as airway hyperresponsiveness.⁶² Data in the current study indicate that blocking VCAM-1 reduced IL-5 and IL-13 levels in the lungs, as well as lung ILC2 levels. Although it remains unclear as to the definitive role of VCAM-1 blocking on ILC2 accumulation, VCAM-1 blocking did inhibit airway eosinophilic inflammation and mucus metaplasia, which are downstream pathological endpoints mediated by ILC2-driven IL-5 and IL-13 signaling. This was evidenced by abrogated mRNA levels of *Il5*, *Il13*, *Eotaxin*, *Eotaxin2*, *Gob5*, and *Muc5ac*, as well as lung eosinophilia and mucus metaplasia (Figures 2 and 3).

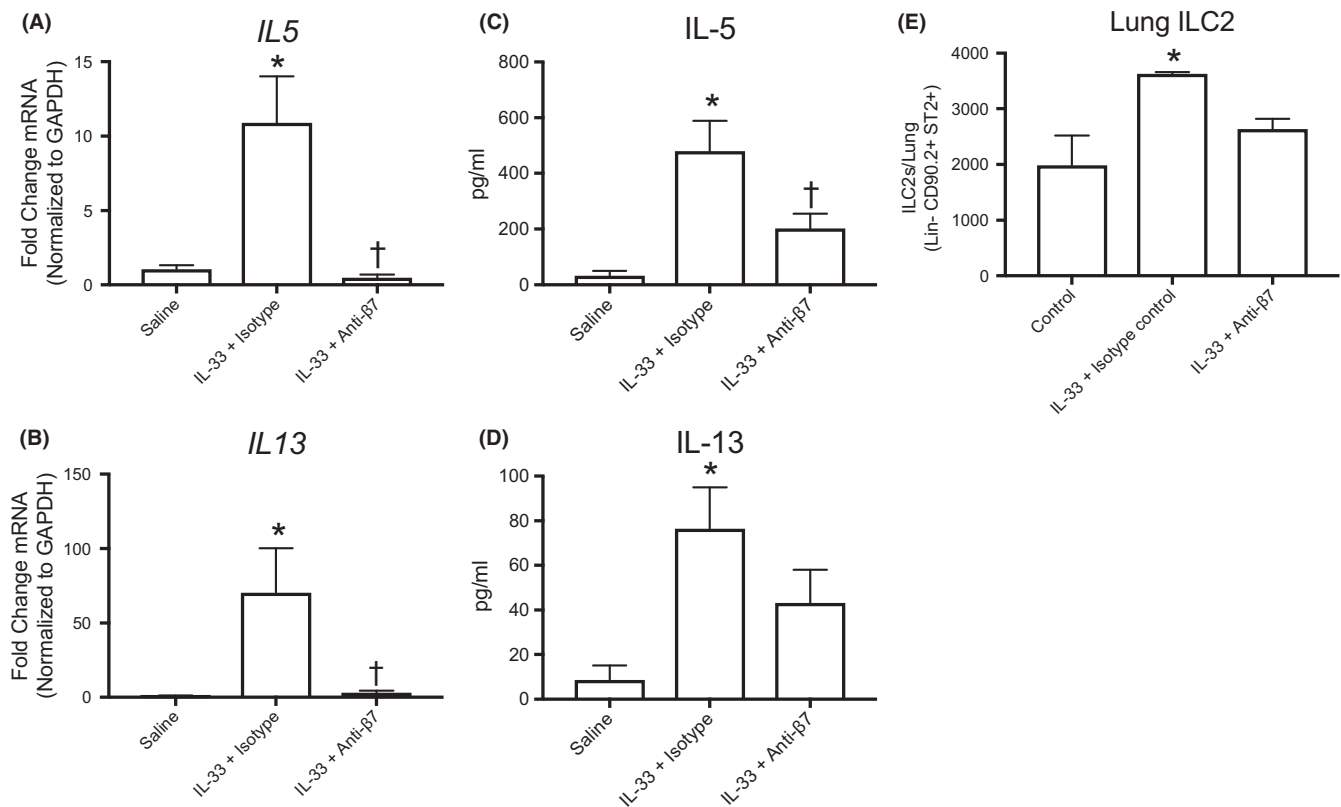


FIGURE 5 Blocking $\beta 7$ -integrin reduces of IL-33-induced type 2 inflammatory responses in the lungs of WT mice. Markers of type 2 inflammatory response, IL-5 and IL-13, were assessed by qRT-PCR analysis of RNA isolated from whole-lung homogenates, as well as protein levels in the BALF from mice treated with saline, IL-33 + ICA, or ABA. Relative mRNA levels of (A) *Il5* and (B) *Il13*; data are represented as the fold change relative to saline controls and normalized to the housekeeping gene *Gapdh*. Protein levels from BALF of (C) IL-5 and (D) IL-13 as determined by ELISA. Number of lung ILC2s (lineage-, CD90.2+, ST2+) as determined by flow cytometry (F). N = 4-6 animals per group, per experiment; data are representative of 2-3 repeat experiments. BALF sample data are representative of pooled samples from 3 independent experiments (N = 14-18). Data are represented as the mean $\pm$ SEM. * $P < .05$ vs saline controls † $P < .05$ vs IL-33 + ICA

It is also important to point out the potential direct or indirect role of IL-33/RAGE/VCAM-1 signaling in regulating Th2-mediated responses. Functionality of both Th2 cells and ILC2s is highly dependent on each other, and these two cell types work synergistically to promote allergic inflammation.⁶³⁻⁶⁵ Th2 responses are absent in mice lacking ILC2s,⁶³ whereby ILC2s skew the differentiation of naïve T cells to Th2 cells, and Th2-derived cytokines induce production of type 2 cytokines from ILC2s.⁶⁶ Similar to ILC2s, IL-33 induces the production of IL-5 and IL-13, but not IL-4 in Th2 cells,^{37,38} which is consistent with our findings regarding IL-5, IL-13, and IL-4. Moreover, our previous study also found that RAGE was required for recruitment of not only ILC2s, but also Th2 lymphocytes in response to *Alternaria*.²⁷ It is likely that lack of ILC2 recruitment to the lungs may prevent Th2 differentiation and ILC2-Th2 cross talk, leading to dampened type 2 inflammatory responses.

It should be noted that VCAM-1, as well as other adhesion molecules, contributes to eosinophil trafficking. It has been well demonstrated that adhesion and transendothelial migration of eosinophils from the circulation are dependent on VCAM-1 interaction with very late antigen-4 (VLA-4),⁶⁷⁻⁷¹ although adhesion, rolling, and transendothelial migration are also mediated in part by other

adhesion molecules including ICAM-1 and E-selectin. However, in regard to eosinophil recruitment, the effects on expression of eotaxins would be upstream of VCAM-1-eosinophil interactions, suggesting that blocking VCAM-1 prevents eosinophil recruitment in this model by preventing eotaxin synthesis (induced by IL-13).

ILC2 recruitment in the gut depends on $\alpha 4\beta 7$ -MAdCAM-1 interactions.^{28,29} However, VCAM-1 has been shown to promote recruitment of $\alpha 4\beta 7$ -expressing allergic inflammatory precursor cells into the lung.³⁰ IL-33-induced accumulation of ILC2s is RAGE-dependent in the lungs, but not in the gut.²⁷ Blocking $\beta 7$ -integrins had remarkably similar results compared to treatment with ABA. Pretreatment with ABA diminished lung eosinophilic inflammation and mucus metaplasia marker expression (Figure 4). Interestingly, only the IL-33 + ICA group had significant numbers of lung ILC2s, suggesting $\beta 7$ -integrin contributes to IL-33-induced accumulation of ILC2s in the lungs. A recent study, which strongly demonstrates the recruitment of ILC2s to lungs in experimental AAI, suggests that $\beta 2$ -integrins are the primary mediator of ILC2 recruitment to the lungs in response to *Alternaria* extract challenge. In this study, the authors also found that blocking $\alpha 4$ -integrin had no effect in this model.¹⁵ It is possible that blocking $\alpha 4$ -integrin directly does not inhibit the

ability of $\alpha 4\beta 7$ -integrin to bind its receptor molecule (ie, VCAM-1), as the cytoplasmic domain of $\beta 7$ contributes to $\alpha 4\beta 7$ binding to VCAM-1.^{72,73} In addition, differences between this study and that of Karta et al¹⁵ may also be due to the agent used to induce AAI (IL-33 vs *Alternaria* extract), as ILC2s behave differently depending on the type of stimulation (eg, cytokine vs allergen). Nonetheless, both studies suggest ILC2 recruitment from circulation contributes to the development of experimental AAI.

It is also interesting to note the role of differential integrin expression in maturation, trafficking, and sequestration of eosinophils during allergic responses. For instance, integrin expression levels change sequentially as eosinophils mature in circulation.^{74–76} As noted above, $\beta 7$ -MAdCAM-1 interactions are crucial to eosinophil recruitment in the small intestine;^{77,78} however, in the large intestine, this is primarily mediated by $\beta 2$ -ICAM-1 interactions.⁷⁹ Conversely, in the lungs, eosinophil attachment and transendothelial migration are mediated by $\beta 1$ - and $\beta 2$ -integrins, and not $\beta 7$ -integrin.^{48,78} This suggests that although blocking VCAM-1 can potentially contribute to blocking eosinophil recruitment to the lungs, $\beta 7$ -integrin blocking should not directly influence eosinophil trafficking, and this is rather due to inhibition of upstream mechanisms (ie, ILC2 recruitment/activation, which then promotes eotaxin production).

Overall, this study suggests that RAGE-dependent activation of the lung endothelium contributes to the development of AAI. RAGE is required for allergens and IL-33 to induce VCAM-1 on lung endothelial cells. Blocking VCAM-1 on the endothelium, and $\beta 7$ -integrins systemically, decreases type 2 inflammatory responses mediated by ILC2s and abrogates eosinophilic inflammation and mucus metaplasia. Future studies investigating the effects of endothelial-specific RAGE signaling are needed to further delineate the contribution of endothelial RAGE signaling, and downstream pathogenic mechanisms in experimental models of AAI.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

TNP, EAO, and TDO designed the study. TNP, EAO, PSM, and RED performed and analyzed experiments. TNP and TDO wrote the manuscript. TNP, EAO, PSM, RED, and TDO have all read and edited the manuscript prior to submission.

ORCID

T. N. Perkins  <http://orcid.org/0000-0002-4719-4856>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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SUPPLEMENTAL MATERIALS

Materials and Methods

Animals and reagents

Eight-week-old wild-type C57BL/6 mice were purchased from Taconic (Hudson, NY). RAGE knockout (KO) mice congenic with the C57BL/6 strain were provided by Dr A. Bierhaus (University of Heidelberg), and a breeding colony was established.^{1, 2} Animal studies were carried out under the National Research Council's guidelines in the "Guide for the care and use of laboratory animals," with oversight by the University of Pittsburgh's Institutional Animal Care and Use Committee. HDM and *Alternaria alternata* extracts were purchased from Greer Laboratories (Lenoir, NC). Recombinant mouse IL-33 was purchased from BioLegend (San Diego, Calif).

Models of AAI/asthma

Figure E1 represents models of AAI and treatment regimens. A chronic model of AAI was induced by treating wild-type or RAGE KO mice intranasally 4 times per week for 7 weeks with 40 µg of HDM extract (Greer) in 25 µL of saline. Mice were sacrificed 48 hours after the last treatment.³ In an acute model of AAI⁴, mice were treated intranasally with 25 µg of *Alternaria alternata* extract (Greer) in 25 µL of saline on days 0, 3, 6, and 9 and sacrificed on day 10. For the rIL-33 intranasal protocol, mice were treated with 1 µg of recombinant mouse IL-33 in 40 µL of saline daily for four days and then sacrificed after 24 hours. In all models control mice were treated with saline vehicle alone.

Fluorescence microscopy

Mouse lung sections were hydrated through a series of ethanol solutions, followed by antigen retrieval by heat treatment in citrate solution (Dako) for 30min. sections were washed three times in PBS, and blocked in 2% BSA-PBST for 1 hour. Sections were then stained with Goat

Anti-mouse VCAM-1 (R&D AF643) diluted 1:100, RAGE (Santa Cruz H-300) diluted 1:100, or Anti-mouse CD31 (Abcam ab28364) in 0.5% BSA-PBST overnight at 4°C. Sections were washed three times for five minutes in PBST. Sections were then incubated with secondary antibody (Donkey anti-goat-AlexaFluor 488 or Anti-Rabbit-AlexaFluor 555) for 1 hour at RT (in dark). Sections were washed two times in PBST and three times in PBS, followed by 30 seconds in Hoechst and then washed three times for one min in PBS. Sections were coverslipped with Gelvatol and visualized. Images were taken at 200X magnification.

Isolation and culture of primary mouse lung endothelial cells

Male wild-type and C57BL/6 (Taconic) and in-house RAGE-KO mice aged 6-8 weeks were used for isolation of mouse lung endothelial cells (MLEC). Mice were euthanized by i.p. injection with a lethal dose of pentobarbital. Lungs were then perfused with 10ml of Hank's buffered saline solution (HBSS) (Gibco), with 10U/ml heparin (Sigma). Lungs were instilled through the trachea with 1 ml digestion buffer [2mg/ml Collagenase (Sigma), 50U/ml Dispase (Gibco), in Calcium/Magnesium-free HBSS], and trachea was tied off with suture silk. Lungs are excised and placed in 5ml digestion buffer and placed on ice. Lungs are then decanted into a 6-well plate, and 1000U DNase I (Invitrogen) is added to each set of lungs and lungs are cut into 1-2mm pieces, and then transferred to a 50ml conical tube and incubated at 37°C shaking for 45min. Digested lungs are then filtered through a series of filters (100µm, 70µm, and 40µm). Red blood cells were lysed using ACK lysis buffer (Gibco) and the single cell suspension was subjected to Magnetic bead separation using (Insert MB system). Negative selection was performed using anti-mouse-CD45 beads and positive selection was performed using anti-mouse-CD31 beads according to the manufacturer's protocol (Miltenyi Biotec). Flasks and plates were pre-coated with 0.1%Gelatin (Sigma), and 0.1mg/ml Bovine Collagen Type I (Gibco). MLEC were cultured and maintained in Opti-MEM (Gibco) supplemented with 10% FBS (Corning), 1X Penicillin/Streptomycin (Gibco), 2mM L-glutamine, Non-essential Amino acids

(1X) (Gibco), 2-mercaptoethanol, Bovine pituitary extract (Lonza). Cells were grown to near confluence and then stimulated with recombinant mouse IL-33 (Biolegend), at 100ng/ml.

Human umbilical cord endothelial (HUVEC) cell culture.

HUVEC cells were purchased from ATCC and cultured according to ATCC recommendation. In brief, cells were seeded on flasks and plates coated with 0.1% Gelatin and 0.1mg/ml Collagen type I. Cells were grown and maintained in F12K media supplemented with 10% FBS, 1X Penicillin/Streptomycin, 25mM HEPES (Gibco), 0.03mg/ml Endothelial Growth Supplement (Sigma), and Heparin (10U/ml).

Human lung microvascular endothelial cells (HLMVEC).

HLMVEC cells were purchased from Lonza, and cultured according to manufacturer's recommendation in EGM2-MV.

VCAM-1 Blocking

Mice were given intravenous injections (200µl) (1.5mg/kg) of Anti-VCAM-1(Clone M/K) antibody (Millipore, CBL1300) or isotype control antibody (R&D, RTK2071) in saline, 1 hour prior to intranasal administration of 1µg IL-33 daily for 4 days and mice were sacrificed 24 hours after the last treatment.

7 Blocking

Mice were given intravenous injections (200µl) (65µg) of Rat anti-mouse β 7 integrin (Clone FIB27) antibody (BD) or isotype control antibody (BioLegend, RTK2071) in saline 1 hour prior to intranasal administration of 1µg IL-33 daily for 3 days and mice were sacrificed 24 hours after the last treatment.

79

80

81 *Bronchoalveolar Lavage*

82 For bronchoalveolar lavage, 0.8 mL of saline was instilled in the lungs through the trachea and
83 withdrawn. Cell counts and differentials were obtained, as previously described.³

84

85 *Histological analysis*

86 Lung fixation and staining (Standard Hematoxylin and Eosin, and Periodic Acid-Schiff were
87 carried out, as previously described.³ For inflammatory scoring, Standard hematoxylin and
88 eosin staining was performed on 5- μ m-thick lung sections and was scored by a pathologist
89 (Oury) who was blinded to the sample groups as previously described^{2, 5, 6}. In brief, every field
90 (20-25 fields) in the entire lung was observed with a light microscope (X200 magnification).
91 Scoring was based on the percentage of lung tissue according to the following scale: 0 = no
92 inflammation, 1 = up to 25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%. The pathological
93 index score was then reported as a ratio of the sum of all the scores divided by the total number
94 of fields counted for each sample.

95

96 *Flow cytometry*

97 For whole-lung single-cell suspension preparation, lungs were harvested from mice, digested in
98 12 mL of a 1 mg/mL collagenase media for 30 minutes at 37°C, and then passed through a 70-
99 mm filter. Red blood cells were lysed with ACK Lysing Buffer (Gibco, Carlsbad, Calif), and the
100 solution was passed through another 70-mm filter. Single-cell suspensions were incubated with
101 anti-CD16/CD32 antibody (eBioscience, San Diego, Calif) for 10 minutes at room temperature.
102 Cells were stained for 30 minutes in the dark at 48C with the following antibody cocktail:
103 phycoerythrin (PE)–conjugated anti-mouse lineage cocktail (BioLegend; containing antibodies
104 against CD3 ϵ [clone 145-2C11], Ly-6G/Ly-6C [clone RB6-8C5], CD11b [clone M1/70],

CD45R/B220 [clone RA3-6B2], and TER-119 [clone Ter-119]), PE-conjugated anti-mouse CD11c (clone N418, eBioscience), PE-conjugated anti-mouse FCεRI (clone MAR-1, BioLegend), PE-conjugated anti-mouse NK1.1 (clone PK136, eBioscience), allophycocyanin-conjugated CD90.2 (Thy1.2) (clone 53-2.1, BD Bioscience), and peridinin chlorophyll protein-eFluor710–conjugated ST2 (IL-33 receptor; clone RMST2-2, eBioscience). ILC2s were counted as lineage-negative, CD90.2-positive, and ST2-positive ⁷ (PE2, allophycocyanin positive, peridinin chlorophyll protein-eFluor710) cells on an LSRII machine (BD Biosciences, San Jose, Calif) and analyzed with FlowJo software (Tree Star, Ashland, Ore). Gating strategy for mouse lung ILC2s was previously published. ⁸ See figure E3 for details.

Immunoblotting

Frozen lungs were homogenized in T-PER buffer (Sigma), with 1% protease and phosphatase inhibitor cocktail (Sigma), and 25 to 30 mg of protein was separated by means of SDS-PAGE and immunoblotted, as previously described. ³ Membranes were probed with 1:1000 goat anti-mouse VCAM-1 polyclonal antibody (AF643; R&D Systems, Minneapolis, Minn. Even sample loading was assessed with a mouse anti-β-actin mAb (1:5000; Sigma, St Louis, Mo) or Mouse anti-β-tubulin mAb (1:5000; Sigma, St. Louis, Mo). Human cell cultures were probed with Anti-RAGE (H-300) Rabbit pAb (1:500; Santa Cruz Biotechnology).

Enzyme-linked immunosorbent assays

ELISAs for IL-5 (BD Biosciences), and IL-13 (eBioscience) were performed per the manufacturer's instructions on bronchoalveolar or neat whole lung homogenates (homogenates were spun for 10 minutes at 10,000 rpm to remove debris).

Quantitative RT-PCR

RNA extraction, reverse transcription, and quantitative RT-PCR were carried out, as previously described.³ Primers are listed in Supplemental table 1. (Applied Biosystems, Foster City, Calif).

Supplemental Table 1

Taqman Primers

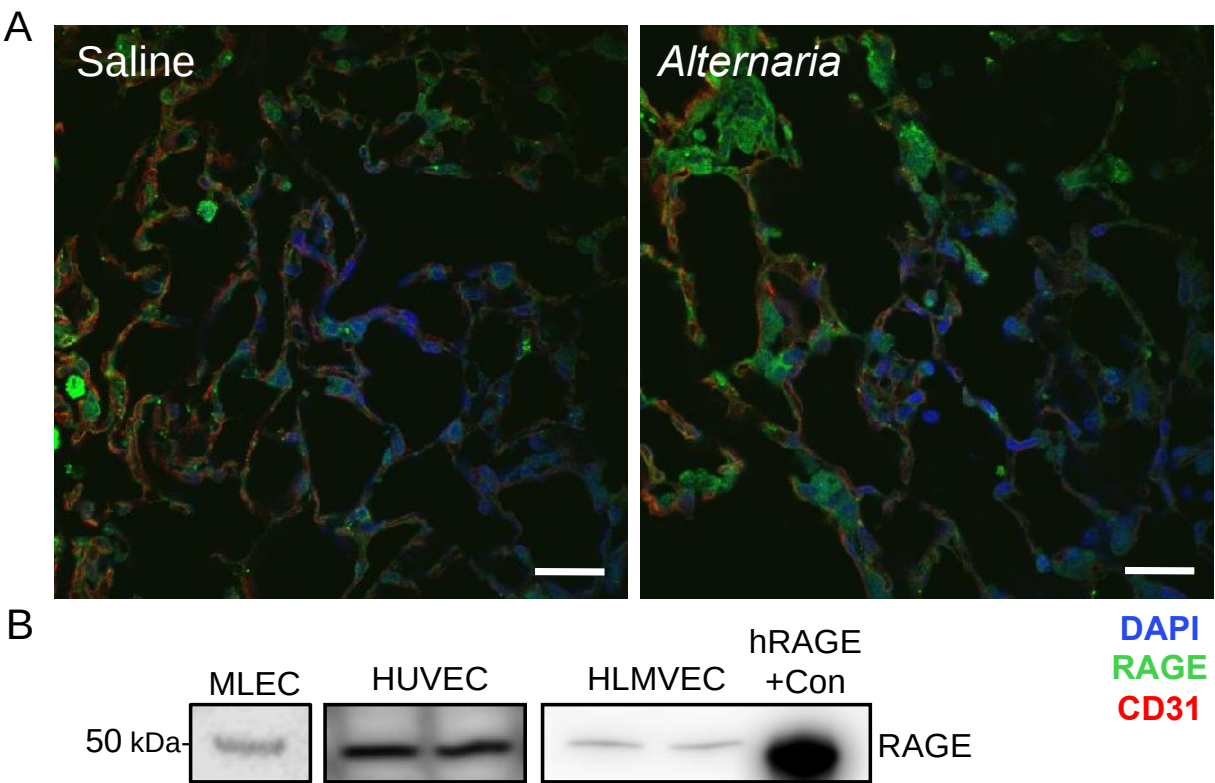
Gene	Probe ID
<i>Eotaxin/Ccl11</i>	Mm00441238_m1
<i>Eotaxin-2/Ccl24</i>	Mm00444701_m1
<i>Gapdh</i>	Mm99999915_g1
<i>Gob5/Clca1</i>	Mm01320697_m1
<i>Il5</i>	Mm00439646_m1
<i>Il13</i>	Mm00505403_m1
<i>Muc5ac</i>	Mm01276718_m1

Statistical analysis

Statistics were performed with GraphPad Prism 7 software (GraphPad Software, La Jolla, Calif). Results are expressed as the mean $\pm$ SEM. Statistical significance was determined by using One-way ANOVA with a Tukey post-test or the unpaired Student's t test. P values of less than 0.05 were considered significant.

153

154 **Supplemental Figures**



155

156 **Figure S1**

157 (A) Representative immunofluorescent images of inflated WT mouse lungs exposed to saline or
158 *A. alternata*. RAGE (green), endothelial cell marker CD31 (red) and nuclear DAPI counterstain.
159 Scale bar = 10µm. Yellow color indicates co-localization of RAGE and CD31 in endothelial cells.
160 (B) From left to right, western blots of primary mouse lung endothelial cells (MLEC), human
161 umbilical vein endothelial cells (HUVEC) and primary human lung microvascular endothelial
162 cells (HLMVEC), and RAGE positive control (50kDa).

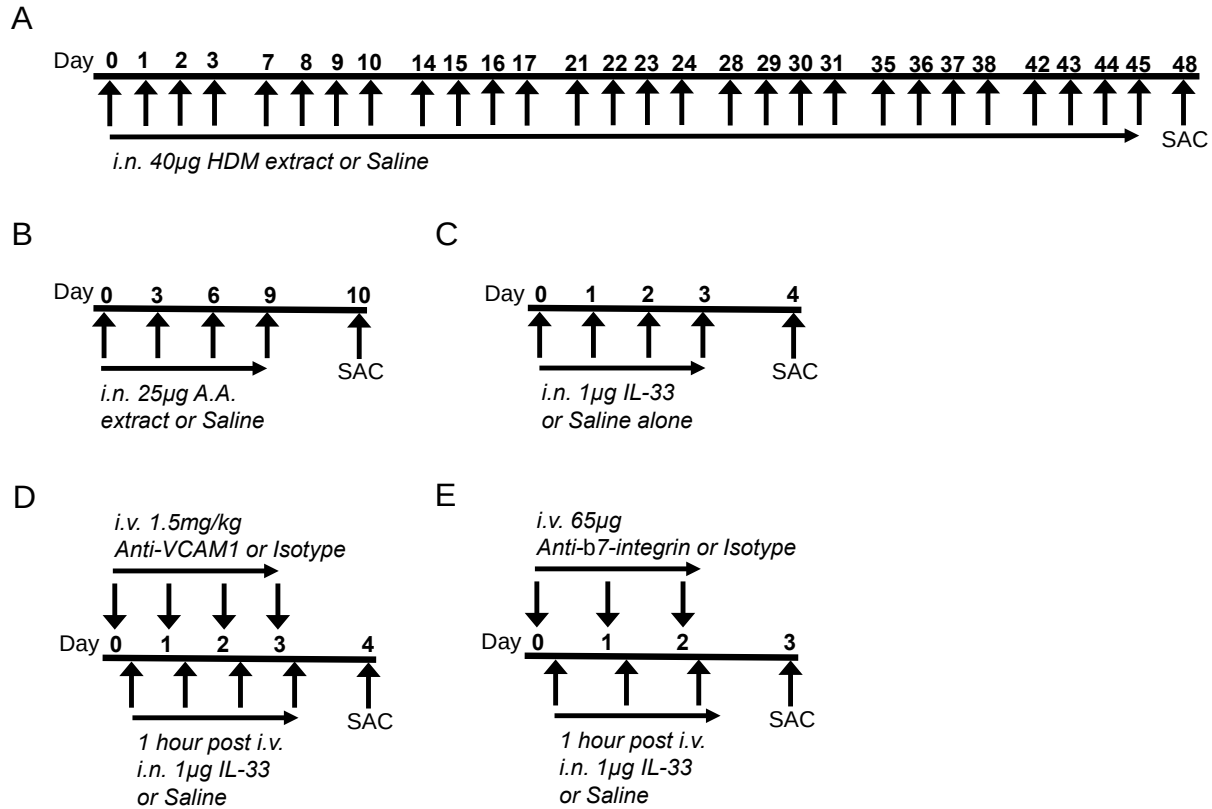


Figure S2

Schematic representations of experimental models of AAI used throughout this study. (A) HDM: Mice were intranasally administered 40µg house dust mite extract (Greer) in saline or saline alone, 4 times a week for 7 weeks Days 0-3, 7-10, 14-17, 21-24, 28-31, 35-38, 42-45, and then sacrificed on day 48. (B) AA: Mice were intranasally administered 25µg *Alternaria alternata* extract in saline or saline alone every 3 days (Day 0, 3, 6, and 9), 4 doses total and then sacrificed 24 hours after last treatment. (C) IL-33: Mice were intranasally administered 1µg of recombinant mouse IL-33 (BioLegend) in saline or saline alone daily for 4 days and sacrificed 24h after last treatment. (D) VCAM-1 blocking: Following the same protocol as IL-33, mice were intravenously pretreated with (1mg/kg) anti-VCAM-1 antibody (Millipore) or isotype control antibody (IgG1; R&D Systems) 1h prior to intranasal treatment. (E) β7 integrin blocking: Following the same protocol as IL-33 (except 3 days treatment instead of 4), mice were

intraperitoneally pretreated with 65µg Anti-β7 (BD) integrin antibody or isotype control (IgG2a; BioLegend). For all intranasal administrations, mice were given 40µl (20µl each nostril). This figure was adapted from a previous publication (Oczypok et al J Allergy and Clin Immunol. 2015 Sep;136(3):747-756.e4)

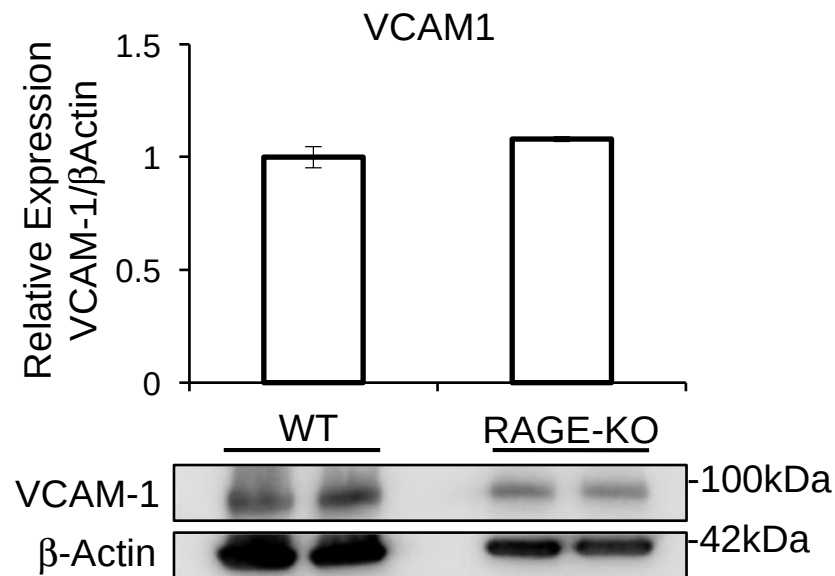


Figure S3

Comparison of baseline expression levels of VCAM-1 in whole cell lysates of primary mouse lung endothelia cells from WT and RAGE-KO mice. Western blots probed for VCAM-1 (100kDa) and b-Actin (42kDa), with relative densitometry expression values above.

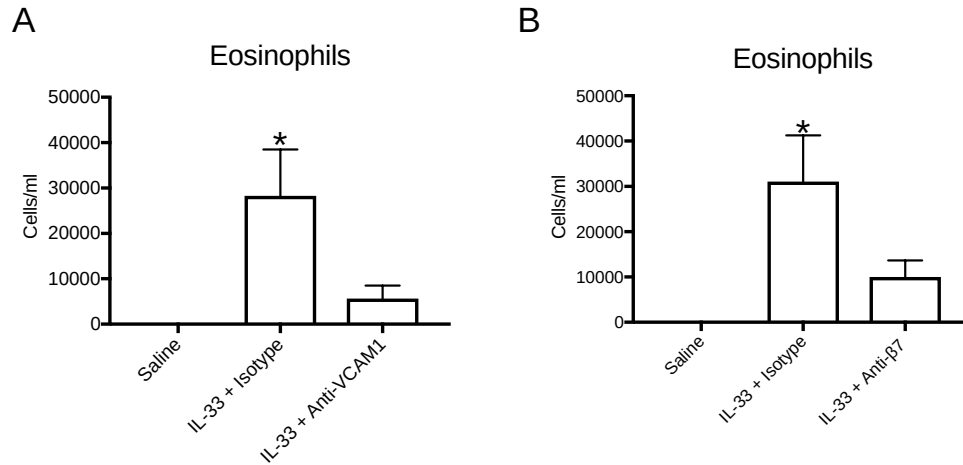


Figure S4

(A) BALF cytospin data corresponding to Figure 3D, data are represented as total Eosinophils/ml BALF. (B) BALF cytospin data corresponding to Figure 6D, data are represented as total Eosinophils/ml BALF. N=4-7 in each experiment and data are representative of 2-3 repeat experiments. Data are represented at the mean $\pm$ SEM. *P<0.05 vs. Saline Controls.

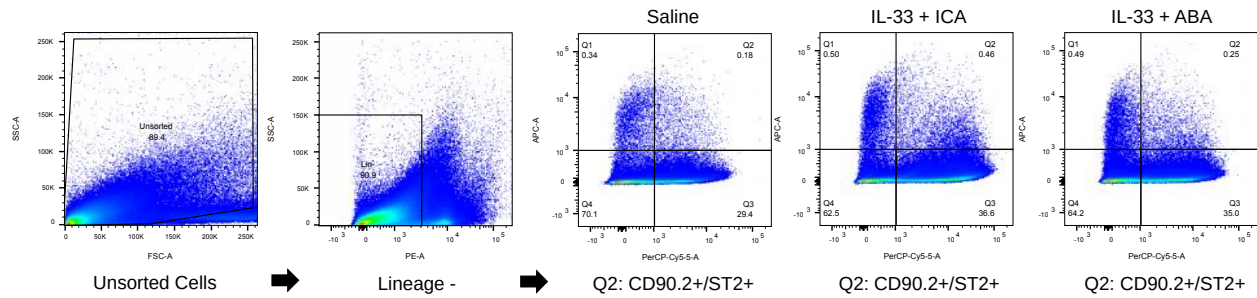


Figure S5

Schematic representation of gating strategy for lung ILC2s based on previously published findings (Oczypok et al J Allergy and Clin Immunol. 2015 Sep;136(3):747-756.e4). Unsorted cells were then gated for PE-negative cells (Lineage negative). These cells were then gated for the population positive for APC (CD90.2), and PerCP-Cy5-5-A (ST-2), which are located in Quadrant 2 as denoted in examples.

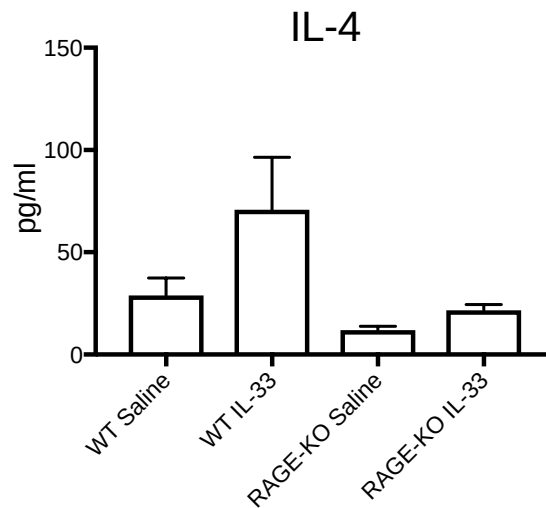


Figure S6

Levels of IL-4 in mouse BALF, measured by ELISA. Although there was a trend toward increased IL-4 in WT mice, there were no significant changes in IL-4 levels in WT or RAGE-KO mice administered rIL-33 (Fig. E2C). N=4 in each experiment and data are representative of 2 repeat experiments. Data are represented at the mean $\pm$ SEM.

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