# RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS MEDIATES ALLERGIC AIRWAY INFLAMMATION VIA GROUP 2 INNATE LYMPHOID CELLS

by

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**RECEPTOR FOR ADVANCED GLYCATION ENDPRODUCTS MEDIATES ALLERGIC AIRWAY INFLAMMATION VIA GROUP 2 INNATE LYMPHOID CELLS**

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Half of all asthmatics suffer from allergic airway inflammation driven by type 2 immune responses in the lung, yet the molecular mechanisms that control early, initiating events in allergic asthma are poorly understood. Recently, scientists have discovered a new subset of innate cells that can trigger rapid type 2 allergic inflammation, even in the absence of an adaptive immune system. These cells, termed group 2 innate lymphoid cells (ILC2s), are activated by the epithelial alarmin, interleukin (IL)-33, and secrete copious amounts of the type 2 cytokines IL-5 and IL-13 to drive tissue eosinophilia, mucus hypersecretion, and airway hyperresponsiveness (AHR). It is unknown how these cells are recruited to the lung to promote allergic airway inflammation (AAI).

The receptor for advanced glycation endproducts (RAGE) is a proinflammatory receptor abundantly expressed in the lung. Previous studies have found that, in the absence of RAGE, IL-5 and IL-13 cytokine responses are impaired, and mouse lungs are completely protected from development of AAI and AHR. It was therefore hypothesized that RAGE was necessary for recruitment of type-2-cytokine-producing ILC2s to the lung during allergen challenge to initiate AAI.

The data presented here demonstrate that RAGE is, in fact, necessary for ILC2 accumulation in the lung after allergen challenge. Furthermore, this mechanism appears to be

lung-specific and independent of RAGE expression on ILC2s themselves: tissues that do not normally express RAGE (i.e. gastrointestinal tract) mount normal ILC2-driven allergic responses even in RAGE KO mice. The ILC2-activating cytokine, IL-33, was also dependent on RAGE signaling, both upstream to trigger its release from epithelial cells and also downstream to mediate its inflammatory effects.

This is the first study examining a parenchymal factor in the recruitment of ILC2s to a specific organ. The exact mechanism by which this is occurring is still under investigation, but it may lead to the discovery of important new therapeutic targets early in the development of AAI.

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# PREFACE

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

| | |
|---|---|
| AAI | allergic airway inflammation |
| Ab | antibody |
| AGE | advanced glycation endproduct |
| AHR | airway hyperresponsiveness |
| ANOVA | analysis of variance |
| AT1 | alveolar type 1 |
| AT2 | alveolar type 2 |
| BALF | bronchoalveolar lavage fluid |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| DAMP | damage-associated molecular pattern |
| DC | dendritic cell |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| ECL | enhanced chemiluminescence |
| ELISA | enzyme-linked immunosorbent assay |
| ERK | extracellular signal-regulated kinase |
| Fab | fragment antigen-binding |
| FACS | fluorescence-activated cell sorting |
| FBS | fetal bovine serum |
| Fc | fragment crystallizable |
| FEV1 | forced expiratory volume in one minute |
| FVC | forced vital capacity |
| G | tissue damping |
| GI | gastrointestinal |
| GWAS | genome wide association study |
| H | tissue elastance |
| H&E | hematoxylin and eosin |
| HDM | house dust mite |
| HLMVEC | human lung microvascular endothelial cell |
| HMGB1 | high mobility group box 1 protein |
| HRP | horseradish peroxidase |
| HUVEC | human umbilical vein endothelial cell |
| IACUC | Institutional Animal Care and Use Committee |
| ICAM-1 | intercellular adhesion molecule 1 |
| ICOS | inducible T cell costimulator |
| Ig | immunoglobulin |
| iILC2 | inflammatory group 2 innate lymphoid cell |
| IL1RAcP | interleukin-1 receptor accessory protein |
| IL1RL1 | interleukin-1 receptor-like 1 |
| ILC | innate lymphoid cell |
| ILC1 | group 1 innate lymphoid cell |
| ILC2 | group 2 innate lymphoid cell |
| ILC3 | group 3 innate lymphoid cell |
| i.n. | intranasal |
| IL-*n* | interleukin-*n* |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| kDa | kilodalton |
| KO | knockout |
| $LXA_4$ | lipoxin $A_4$ |
| Mac-1 | macrophage antigen 1 |
| MadCAM-1 | mucosal vascular addressin cell adhesion molecule 1 |
| MAPK | mitogen-activated protein kinase |
| MBP-1 | major basic protein 1 |
| MHC II | major histocompatibility complex, class II |
| mRAGE | membrane receptor for advanced glycation endproducts |
| mRNA | messenger ribonucleic acid |
| MSA | mouse serum albumin |
| NF-κB | nuclear factor kappa B |
| nILC2 | natural group 2 innate lymphoid cell |
| OVA | ovalbumin |
| PAGE | polyacrylamide gel electrophoresis |
| PAS | periodic acid Schiff |

| | |
|---|---|
| PBB | PBS + 0.5% BSA buffer |
| PBS(T) | phosphate-buffered saline (Tween-20) |
| p-IκB | phosphorylated IκB |
| PMA | phorbol 12-myristate 13-acetate |
| PRR | pattern recognition receptor |
| PVDF | polyvinylidene difluoride |
| qRT-PCR | quantitative real-time polymerase chain reaction |
| *Rag* | recombination activation gene |
| RAGE | receptor for advanced glycation endproducts |
| rIL-33 | recombinant interleukin-33 |
| Rn | resistance |
| RORα | retinoid-related orphan receptor alpha |
| rpm | rotations per minute |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulfate |
| SEM | standard error of the mean |
| sRAGE | soluble receptor for advanced glycation endproducts |
| Th*n* | T helper *n* (i.e. 1, 2, or 17) |
| TNF-α | tumor necrosis factor alpha |
| Treg | T regulatory cell |
| TSLP | thymic stromal lymphopoietin |
| VCAM-1 | vascular cell adhesion molecule-1 |
| WT | wild-type |

# 1.0 INTRODUCTION

Asthma is a heterogeneous disease that afflicts millions of people worldwide and accounts for huge health care expenditures annually. The mechanisms that control asthma pathogenesis are complex and are still not fully understood. The receptor for advanced glycation endproducts (RAGE) has been studied for over twenty years, yet despite its high expression in the lung, only recent studies have begun to examine RAGE in inflammatory responses in the lung. A role for RAGE in promoting asthma pathogenesis was characterized within the last five years. During this same time, a new field of immunology was born with the discovery of innate lymphoid cells, which promote inflammation in a variety of organs without the use of specific antigen receptors. Group 2 innate lymphoids cells (ILC2s), upon stimulation with the proinflammatory epithelial-derived cytokine, IL-33, promote type 2 responses in the lung during allergic airway inflammation (AAI) and asthma. This work sought to study possible interactions among these innate immune components to determine if they are modulating a novel pathway in the development of AAI and asthma.

## 1.1 ASTHMA

### 1.1.1 Epidemiology

Approximately 300 million people worldwide suffer from asthma.[1] In western nations, about 10% of adults and 30% of children are asthmatics, and the prevalence continues to slowly increase each year.[2] In 2009, 8.2% of the American population suffered from asthma; these rates were highest among females, children, non-Hispanic blacks and Puerto Ricans, those living near the poverty level, and residents of the Northeast and Midwestern United States.[3] Asthma exacerbations, which are usually triggered by allergens (e.g., house dust mite (HDM)), infections (e.g., RSV), or exercise, resulted in 1.75 million emergency department visits, 456,000 hospital admissions, and over 3,000 deaths in 2007.[2, 3] Asthma increases an individual's direct medical costs by over $3,000 every year, and in 2007, it was estimated that the total cost of asthma in the United States was $56 billion when direct medical costs and loss of productivity from asthma-related morbidity and mortality was taken into account.[4]

Asthma has an unknown etiology, but is thought to have both genetic and environmental influences. Several genome wide association studies (GWAS) have been carried out, the largest of which examined over 10,000 patients with asthma.[5] Thirteen susceptibility loci have consistently been linked to asthma, and these genes were involved in coordination of the innate and adaptive immune responses (especially the allergic type 2 response) and in epithelial barrier function.[5, 6] Among environmental triggers of asthma (including smoke,[7, 8] strong odors,[9] viruses/infections,[10] weather,[11, 12] medications,[13] exercise,[14] and hormones[15, 16]), indoor and outdoor allergens are the most common and are some of the most potent exacerbating factors for asthmatics.[17] *Alternaria alternata* is a fungal allergen composed of at least sixteen known

allergic molecules.[18] It has been well-studied as a trigger of asthma exacerbations, especially in children with severe asthma.[19-21] House dust mites are also highly prevalent in the environment and have been linked to increased asthma severity and airway inflammation.[22, 23] In one study, >99% of observed homes had detectable levels of dog, cat, or *Alternaria alternata* allergen and 85% of households had detectable levels of HDM allergens.[24] In this same study, it also was found that homes with greater numbers of asthmatics had greater numbers of allergens present, and that allergen burden correlated with the presence of asthma symptoms in these patients.[24]

Asthma is now recognized as a heterogeneous disease consisting of many different subtypes (or "endotypes").[25-27] While asthma is classically thought of as being driven by allergic type 2 immune responses, recent studies have shown that only about 50% of asthmatics have the classical T helper 2 (Th2)-high asthma subtype (more information on Th2 responses can be found in Section 1.1.3 below).[28] The studies presented here will focus on allergen-driven, Th2-high asthmatic phenotypes.

### 1.1.2 Pathophysiology

Allergic asthma is characterized by a persistent, mostly eosinophilic inflammation in the airways, which results in airway hyperresponsiveness (AHR). During acute exacerbations, airway smooth muscle cells constrict, causing a reversible obstruction of airflow through the respiratory tract. Patients often experience wheezing, coughing, and shortness of breath as a result. Airway edema, increased mucus production, leaky vasculature, and the sloughing of epithelial cells further exacerbate bronchoconstriction.[1] As a result of this increased airway resistance, patients cannot sufficiently expire, thus air becomes trapped in the lungs, causing an increase in the air left in the lungs after expiration (residual volume) and a decrease in the amount of air that can be forcibly

exhaled in one second (forced expiratory volume, FEV1). Obstructive lung diseases such as asthma are characterized by a decrease in the FEV1 to forced vital capacity (FVC) ratio (FEV1/FVC ratio), such that it falls below the normal value of 80%.[1] Patients with suspected asthma are diagnosed with pulmonary function testing following challenge with methacholine or histamine, both potent bronchoconstrictors. Asthmatics have hyperreactive airways and thus will show signs of bronchospasm and obstruction (FEV1 decreased by 20%) with lower doses of methacholine. Physicians assess the reversibility of the disease by administering a bronchodilator (e.g., a β2 agonist) after the methacholine challenge. The obstruction is considered "reversible" if FEV1 increases by 12% or 200 mL within fifteen minutes of inhalation of the β2 agonist.[1]

### 1.1.3 Cellular and molecular pathology

The majority of asthmatics have a genetic predisposition for strong type I hypersensitivity (allergic) reactions. Allergens, as well as parasitic infections, trigger a type 2 immune response (described below, as summarized from Abbas and Lichtman,[29] Barnes,[1] Oliphant *et al.*,[30] and Ray and Cohn[31])(Figure 1). The initial sensitization to an allergen occurs when dendritic cells (DCs) in the lung phagocytose an allergen. The particle or organism is processed, and the antigen is displayed on the DC major histocompatibility complex II (MHC II). The DC presents the antigen to a naïve CD4+ T cell in the lymph node, triggering the naïve T cell to differentiate into a Th2 cell. Th2 cells begin to produce the classic "type 2 cytokines" IL-4, IL-5, and IL-13. IL-4 and IL-13 activate B cells to undergo class switching to produce large amounts of IgE. IgE binds to and crosslinks the Fc receptor on mast cells to trigger degranulation and the release of histamine, prostaglandins, and cysteinyl-leukotrienes. These molecules are potent smooth muscle cell constrictors and contribute to the bronchospasms that are a hallmark of asthma. They can

also promote vascular permeability and the recruitment of more inflammatory cells. Once mast cells are sensitized, the IgE remains bound to and can easily reactivate the mast cell upon repeated exposure to the allergen. IL-4, in addition to contributing to IgE production, also promotes the differentiation and recruitment of more Th2 cells in the lung by increasing expression of the Th2-promoting transcription factor GATA-3 in naïve T cells.[32] IL-5 promotes recruitment of eosinophils to the lung. IL-13, which aids IL-4 in stimulating IgE production, promotes AHR and activates goblet cells in the airway to secrete copious amounts of mucus. In addition to Th2 cells, newly discovered ILC2s also secrete large amounts of the type 2 cytokines IL-5 and IL-13 and are crucial early players in AAI.[33-35] Additionally, ILC2s are important stimulators of the adaptive Th2 cell response.[36] (More information on ILC2s in AAI will be discussed in Section 1.4.)

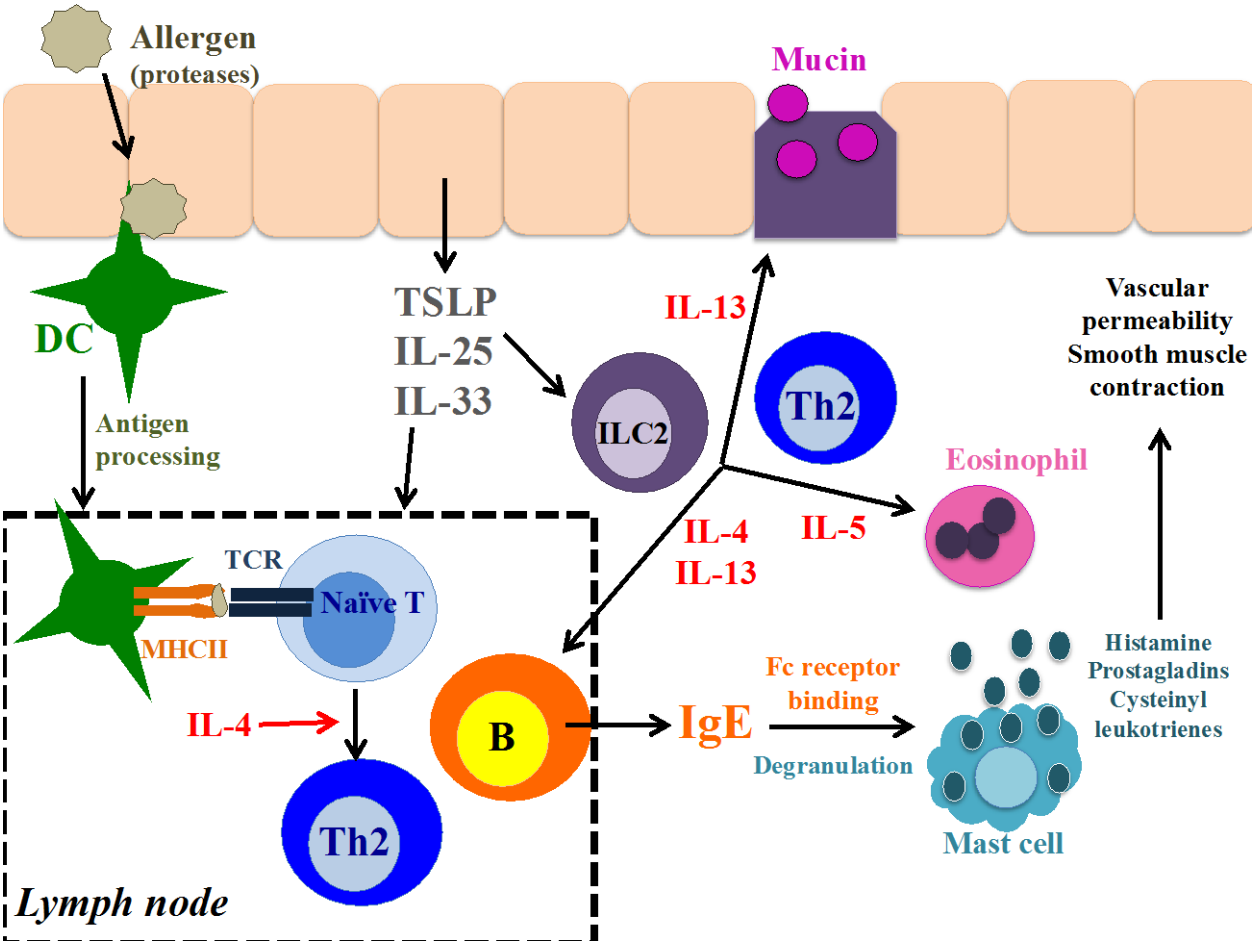


**Figure 1. Summary schematic of the type 2 immune response in allergic asthma.**

Allergens are taken up and processed by dendritic cells (DCs) in the lung epithelial tissue. DCs migrate to the lymph node where they present allergen antigen on MHC II to a naïve T cell via T cell receptor (TCR). T cells differentiate into T helper 2 (Th2) cells and migrate to the lung to secrete IL-4, IL-5, and IL-13. Allergen-damaged lung epithelium also releases TSLP, IL-25, and IL-33, which can activate T cells and group 2 innate lymphoid cells (ILC2s). ILC2s produce additional IL-5 and IL-13. IL-4 and IL-13 activate B cells and induce immunoglobulin (Ig) class switching to IgE. IgE binds mast cells via Fc receptors and causes degranulation and release of histamine, prostaglandins, and leukotrienes from mast cells. These molecules contribute to vascular permeability and smooth muscle cell contraction. IL-5 from Th2 cells and ILC2s recruits eosinophils to the lung. IL-13 causes goblet cell hyperplasia and mucus hypersecretion.

Recent studies have identified three asthma "initiator" cytokines upstream of Th2 cell activation that are released from damaged or stressed epithelial cells to promote type 2 immune responses. It is thought that allergen proteases as well as chronic inflammatory cellular infiltrates contribute to epithelial damage in AAI, thus causing release of these initiator cytokines: thymic stromal lymphopoietin (TSLP), IL-25, and IL-33.[23, 37, 38] Involvement of epithelium in the pathogenesis of asthma is supported by the previously-mentioned GWAS studies, which showed that SNPs in several genes that are crucial to airway epithelial cell function (i.e. *Tslp*, *Il33*, and *Il1rl1* (IL-33 receptor)) are associated with asthma.[6, 39] TSLP, IL-25, and IL-33 have all been shown to promote AAI. TSLP, when overexpressed in the lung, promotes AAI and AHR, and thus has been identified as an initiator of type 2 immune responses.[40] TSLP also promotes secretion of the type 2 cytokines, IL-4, IL-5, and IL-13, while at the same time inhibiting the action of type 1 immune response cytokines, likely through inhibition of IL-12.[30, 37] In animals lacking a receptor for TSLP, IL-17A-driven neutrophilic inflammation predominates, suggesting that TSLP also acts to inhibit IL-17A signaling.[41] In humans, epithelial dendritic cells express a great deal of TSLP receptor on their surface, suggesting that they are potent responders to TSLP when it is released from the epithelium.[38] A recent clinical trial has shown some promise for anti-TSLP therapy in asthmatics. Thirty-one patients with mild allergic asthma were studied in a double-blind, placebo-controlled study, and it was found that use of the anti-TSLP antibody reduced both AAI and AHR in allergen-induced asthma exacerbations.[42] IL-25, a member of the IL-17 family, is elevated in the lungs of mice after HDM challenge[43] and is released from epithelial cells, Th2 cells, or activated mast cells.[44] IL-25 contributes extensively to airway remodeling in asthma,[43, 45] and when administered to mice intranasally, IL-25 is sufficient to induce AHR and a Th2 response.[34, 46, 47] IL-33, a member of the IL-1 family, is located in the

nucleus and is constitutively expressed in the lungs.[48, 49] IL-33 is suspected to be the crucial cytokine for initiating type 2 responses to allergens in the lungs.[50] (A more detailed review of IL-33 in AAI can be found in Section 1.3.)

Prolonged type 2 inflammatory responses driven by IL-4, IL-5, and IL-13 result in the classic pathological findings of lung and airway tissue in asthma and allergic airway disease.[1] Abundant numbers of eosinophils, T-lymphocytes, and mast cells reside in the airway mucosa and are visualized easily by histology. This persistent inflammation can damage the epithelium, resulting in sloughing off of dead and injured cells. Goblet cell hyperplasia from IL-13-induced mucus production is another characteristic pathological feature of asthmatic airways. Additionally, eosinophils release fibrotic factors that can lead to collagen deposits underneath the epithelium and resultant basement membrane thickening.[1] New blood vessels are also formed in asthmatic lungs, and many of the existing vessels become dilated and leaky.[51]

In summary, at the molecular level, allergens trigger epithelial damage and innate and adaptive immune responses leading to activation of ILC2s and Th2 cells. These cells then secrete high levels of the type 2 cytokines IL-4, IL-5, and IL-13, which promote pulmonary eosinophilia, chronic inflammation, mucus hypersecretion, increased vascular permeability, and AHR.

### 1.1.4 Treatment

There is no cure for asthma. Bronchodilators and inhaled corticosteroids are the mainstays of therapy for asthmatics. A “stepwise” treatment plan is often initiated for asthma sufferers.[1] The first level of therapy is bronchodilators, which, when inhaled, rapidly reverse bronchoconstriction and inhibit mast cells during acute asthma attacks.[52] Because asthma is often associated with chronic inflammation in the airways, many patients supplement bronchodilator

use with daily adminstration of inhaled corticosteroids to best control their disease. Corticosteroids are anti-inflammatory and act by recruiting histone deacetylases to modify transcription of inflammatory cytokines and signaling molecules such as NF-κB.

When patients' symptoms fail to improve with steroids and bronchodilators, more expensive, molecular-based therapies are initiated. Montelukast and zafirlukast block leukotriene receptors, thus preventing bronchoconstriction and some eosinophilic inflammation. These were some of the first new asthma drugs in decades when they were introduced to the market in the mid-1990s, but it has since been discovered that only ~30-40% of asthma sufferers show any clinical benefit from these drugs.[53] Nevertheless, the leukotriene antagonists are used in addition to steroids in some patients that see a reduction in symptoms with their use. Monoclonal antibodies have also been developed against numerous type 2 immune response elements, including IgE, IL-4, IL-4R, IL-5, IL-5R, IL-13, and IL-13R (extensively reviewed in Walford and Doherty,[54] Fahy,[26] and Fajt and Wenzel[55]). Biologics targeting TSLP, IL-25, and IL-33 pathways are also in development and have shown efficacy in mouse models.[56-58] Many asthma patients treated with biologics did not see a significant decrease in their symptoms. However, it was found that certain subsets of patients responded very well to these treatments, suggesting that defining biomarkers in patients to identify responders and non-responders is crucial for treatment success.[26, 55] For example, only patients with high eosinophilia responded to anti-IL-5 treatments.[59-61] Serum periostin, a biomarker for eosinophilia,[62] was also a good indicator of response to IL-13 monoclonal antibodies.[63] This suggests that these therapies only work in Th2-high asthma populations, which, as mentioned previously, only account for about half of all asthma sufferers.[28] As a result of phenotypic heterogeneity among asthmatics and because of the

high cost of prescribing these drugs, biologic therapies will likely not be widely used until better ways of identifying responders and refining these therapies are discovered.

Less than 10% of patients experience asthma symptoms that are refractory to all of the above treatments, but this population accounts for the majority of asthma-related health care spending.[54, 64] Thus, new therapies are needed to control inflammation and curb health care spending, especially in these hard-to-treat patients. The mechanisms explored here (involving RAGE and ILC2s) could be attractive new therapeutic targets as they may not only be involved in the chronic inflammatory response, but also in the early allergic response that triggers acute asthma attacks and exacerbations.

## 1.2 RAGE BIOLOGY

### 1.2.1 Structure

RAGE is a 35 kDa protein from the immunoglobulin superfamily.[65] RAGE exists in the body in two main forms: membrane-bound RAGE (mRAGE) and soluble RAGE (sRAGE).[66] Membrane-bound RAGE has three domains: an extracellular domain that recognizes and binds RAGE ligands, a hydrophobic transmembrane domain, and a charged cytoplasmic domain that functions in intracellular signaling. Soluble RAGE does not contain the transmembrane or cytosolic portions of the protein. It is a product of either alternative splicing events[66, 67] or the cleavage of mRAGE by ADAM10 or matrix metalloproteinase 9.[68, 69] The extracellular domain of RAGE is composed of two “constant” regions and one “variable” region.[65] The variable region contains two N-glycosylation sites where most of RAGE’s many ligands bind,[65] though recent studies

have also identified a positively charged binding pocket in the variable domain (K52, R98, K110) that promotes tight binding of hydroimidozolone-containing AGEs or negatively charged nucleic acids.[70-73] RAGE is considered a pattern recognition receptor (PRR), as it identifies ligands based on their three-dimensional structure rather than a specific amino acid sequence.[70, 74] As such, RAGE can be activated by numerous ligands (discussed in Section 1.2.3). While sRAGE is usually monomeric, ligand binding to mRAGE mediates its oligomerization.[70, 75-77] Additionally, twenty splice forms of RAGE have been identified in the human body, and seventeen exist in mice; tissue type dictates what splice form is expressed.[78-80]

### 1.2.2 Expression

RAGE is highly expressed in many tissues of the developing embryo, but this expression decreases as the organism enters adult life.[81] In adult tissues at baseline, RAGE is constitutively highly expressed in the lung, while other tissues show little to no RAGE expression at all (Figure 17).[82-85] Subsequent studies have localized RAGE expression to the basal membrane of type 1 alveolar epithelial (AT1) cells, and RAGE has been defined as a specific marker of AT1 cells.[86-89] Even so, some reports have shown RAGE mRNA in type 2 alveolar epithelial (AT2) cells as well, though these findings are less well supported in the literature.[83] In addition to expression in the lung epithelium, RAGE expression has also been noted in vascular smooth muscle cells,[82] airway smooth muscle cells,[90] endothelial cells,[82] neurons,[82, 91] and immune cells such as macrophages,[82] dendritic cells,[92] eosinophils,[93] T cells,[94-97] and B cells.[94] Many of these cells and tissues induce RAGE expression only when they are activated to do so or are surrounded by RAGE ligands.[81] Notably, RAGE expression is upregulated on various cell types in pathological inflammatory states such as diabetes mellitus, vascular disease, cancer, and neurodegenerative

diseases.[98] However, decreased RAGE expression in the lung has been associated with lung cancer and pulmonary fibrosis.[84, 99] sRAGE normally circulates in the blood at low levels, but its physiological relevance has not yet been identified. Interestingly, levels of sRAGE increase in patients with inflammatory diseases, highlighting a potential role for sRAGE as a biomarker.[100]

### 1.2.3 Ligands

Although RAGE was first identified as a receptor for advanced glycation end products (AGEs),[65] a plethora of studies have shown that it can bind a large variety of endogenous ligands, leading to its classification as a PRR.[74, 101] Understanding the complexities of RAGE-ligand binding are complicated not only by the fact that RAGE can bind many ligands, but also that many RAGE ligands bind to other receptors as well.

Common RAGE ligands include AGEs, S100/calgranulin proteins, and high mobility group box 1 protein (HMGB1 or amphoterin). AGEs are the result of a non-enzymatic Maillard reaction between the carbonyl group on an aldose sugar (most commonly glucose) and the amino group on a protein.[102-104] AGEs are found at increased levels in patients with diabetes due to these patients' high blood glucose levels. Age and oxidative stress can also elevate AGE levels.

S100 proteins are small calcium-binding proteins that are often found at high levels in inflammatory states and were first found to interact with RAGE on endothelium.[105] They are usually localized to the site of inflammation as they are released by activated inflammatory cells. There are numerous S100 proteins; some do not activate RAGE at all, and others activate RAGE to varying degrees.[106] For example, S100A4 proteins activate RAGE in pulmonary artery smooth muscle cells induce a pulmonary arterial hypertension-like phenotype in the cells.[107] In inflamed airway epithelial cells, S100A12 from neutrophils has been shown to increase expression of a

mucin protein in a RAGE-dependent manner, resulting in increased mucus production in the airway.[108] Lastly, in atopic dermatitis, S100A9 was shown to amplify allergic inflammatory responses by signaling through RAGE on keratinocytes, causing release of the inflammatory initiator cytokine IL-33.[109] These studies are just a glimpse into the diverse pathogenic phenotypes resulting from S100 protein activation of RAGE signaling in different tissues.

HMGB1, also known as amphoterin, was discovered as a novel RAGE binding partner that played a role in neurite outgrowth in developing embryos.[91] Since then, HMGB1 has been studied as a nuclear protein that is important in chromatin remodeling. However, it can also be passively released from damaged cells as a proinflammatory alarmin.[110] Additionally, there is evidence that macrophages, natural killer cells, and dendritic cells can actively secrete HMGB1.[111] In addition to binding RAGE, HMGB1 also binds to and activates toll-like receptors,[112] demonstrating the promiscuity of this molecule and highlighting the potential for HMGB1 to activate non-specific targets when administered to cells or animals in an experimental model.

Other recently discovered RAGE binding partners include collagen I, collagen IV, and laminin in the extracellular matrix.[113] RAGE's ability to bind extracellular matrix components such as collagen have been shown to be important for its role in the spreading of AT1 cells in the lung.[114] In Alzheimer's disease, RAGE has the ability to bind soluble amyloid-β, resulting in oxidative stress and release of inflammatory cytokines in the neuronal compartment.[115] RAGE also can transport amyloid-β across endothelial cells, contributing to plaque formation in the central nervous system.[116] Through electrostatic interactions between a positive cavity on RAGE and the negative charges on the backbone of nucleic acid, RAGE has now also been shown to bind DNA and RNA in inflammatory reactions.[73, 77] RAGE binds nucleic acids and facilitates

their uptake into the cell via endosomes. The RAGE-nucleic acid complex is then thought to associate with TLR9 inside the cell to promote inflammation.[77] Finally, RAGE has also been shown to bind to Mac-1 (αMβ2, CD11b/CD18) integrin on leukocytes to facilitate their recruitment to inflamed tissue (detailed in Section 1.2.4).[117]

As RAGE discoverer Ann Marie Schmidt noted, "the ligand appears to dictate the biology of the receptor..."[98] Each RAGE ligand interacts with the receptor in a slightly different way. For example, AGEs and amphoterin seem to bind only the variable domain of RAGE,[70, 118, 119] while S100 proteins and nucleic acids bind to both the variable domain and one of the constant domains.[71, 75, 77] The downstream signaling pathway that is stimulated by RAGE-ligand binding depends on the identity of the ligand, the tissue type where the inflammation is occurring, and the ligand oligomerization state. Generally, oligomerized ligands activate RAGE more strongly than monomers do.[120] The presence of RAGE ligands in the extracellular environment has been shown in several cases to cause upregulation of RAGE expression, which leads to further amplification of inflammatory signaling cascades (detailed in Section 1.2.4).[121-123] Importantly, RAGE ligands are not degraded or altered to prevent further signaling when they bind and signal through RAGE. Therefore, as ligands accumulate, they continuously amplify the inflammatory response by pooling in the inflamed region.

### 1.2.4 Functions and signaling pathways

RAGE has been shown to have a myriad of functions, but its most well-studied role is in the amplification of cellular inflammatory responses. RAGE is a PRR, meaning that it can recognize many different endogenous ligands (detailed in Section 1.2.3) that then promote innate immune responses. Activation of RAGE causes a sustained NF-κB response by maintaining a steady pool

of newly synthesized NF-κBp65 mRNA and protein.[124] A large byproduct of RAGE signaling is the formation reactive oxygen species (ROS), which can also activate NF-κB and promote other inflammatory mechanisms such as increased vascular cell adhesion molecule 1 (VCAM-1) expression or cellular apoptosis.[125-127] Further perpetuation of the inflammatory response comes from the fact that NF-κB can directly bind to the gene encoding RAGE to promote RAGE expression.[121] This positive feedback loop between RAGE and NF-κB contributes to chronic, pathological inflammation in many diseases including atherosclerosis, diabetes, Alzheimer's disease, cancer, and asthma.

The specific signaling pathway that becomes activated by RAGE and ultimately leads to NF-κB activation is dependent on the identity of the RAGE ligand and the tissue type where the receptor is expressed. Studies have identified several of these intermediary players in the RAGE signaling cascade and they include ERK 1/2 MAP kinases,[128] p38 MAP kinase,[129] Rho GTPases,[130, 131] phosphatidylinositol-3 kinase,[127] and JAK/STAT,[132] among others. Signaling through RAGE is dependent on expression of full-length RAGE containing the cytoplasmic tail.[130, 133] Interestingly, the C-terminus of RAGE has no tyrosine kinase activity, suggesting that RAGE must interact with other molecules in the cytoplasm to transduce extracellular signals.[131] Indeed, studies have now shown that the cytoplasmic domain of RAGE contains binding sites for ERK 1/2[133] and diaphanous-1 (a Rho effector protein).[131] Activation of either of these proteins leads to activation of Rac/Cdc42 small GTPases that then control cellular migration.

In addition to promoting inflammation, RAGE has also been shown to play a role in numerous other cellular functions. In developing embryos, amphoterin binds to N-glycans on RAGE, leading to activation of Rac/Cdc42, stimulation of cell motility, and subsequent neurite outgrowth.[119, 130] In the adult lung, RAGE promotes the adherence of AT1 cells to collagen in the

basement membrane and facilitates cell spreading.[114] Because AT1 cells are the primary cells through which gas exchange occurs, the cells need to be stretched over the basement membrane as long, thin sheets to work most efficiently. When RAGE is absent in mice, the animals have an increased risk of developing idiopathic pulmonary fibrosis, likely due to loss of AT1 cell adhesion and subsequent exposure of the basement membrane to harmful free radicals and other damaging agents.[84] RAGE has also been implicated in the differentiation of AT1 cells from AT2 cells.[88] Thus, damaged lung cells may not be easily replaced in RAGE knockout (KO) mice due decreased differentiation of AT2 cells to AT1 cells, which may also contribute to fibrosis.

Interestingly, RAGE is only expressed in mammals and its protein composition is closely related to that of cellular adhesion molecules (CAMs), suggesting that it evolved to play a role in cell-to-cell adhesion.[134] Indeed, multiple studies have shown that RAGE plays both a direct and indirect role in leukocyte adhesion and recruitment to inflamed tissues. In an animal model of acute peritonitis and subsequent *in vitro* studies, RAGE was shown to directly bind and recruit leukocytes via interactions with Mac-1 (αMβ2, CD11b/CD18) on the white blood cell surface.[117] In a follow-up study using an acute traumatic injury model in mouse cremaster muscle venules, endothelial RAGE was found to bind Mac-1 in conjunction with intercellular adhesion molecule 1 (ICAM-1) to recruit leukocytes into the tissue.[135, 136] RAGE was also crucial for leukocyte adhesion in studies using blood cells from preterm infants, suggesting a role for RAGE in young, developing animals that have very high receptor expression.[137] RAGE signaling also indirectly promotes adhesion and recruitment of inflammatory cells by inducing the expression of VCAM-1 on vascular endothelial cells[138] and peritoneal mesothelial cells.[139] Unlike RAGE binding with β2 integrin, VCAM-1 interacts with β7 integrins on leukocytes to recruit them to sites of inflammation.[140]

In 2004, a study was published stating that RAGE was important for the initiation and maintenance of innate immune responses, but that it played no role in adaptive immune repsonses.[141] The following year, however, a study showed that dendritic cell maturation and Th1 cell polarization of CD4+ cells was dependent on RAGE signaling, suggesting that RAGE could have effects on the adaptive immune system as well.[92] Another group showed that alloimmune T cell proliferation following heart transplant was reliant on RAGE,[142] and later, the same group demonstrated that RAGE on T cells was required for T cell priming.[96] In these experiments, RAGE KO mice had decreased T cell proliferation and a preferential Th2 polarization. Further experiments demonstrated a role for RAGE in Th1 polarization of T cells [95] and in dendritic cell homing to lymph nodes to facilitate this T cell priming and polarization.[143] Interestingly, human T cells were found to express RAGE not on the extracellular surface as in mouse cells, but intracellularly in endosomes.[97] The reasons for this altered cellular location are unknown and are still being investigated. As a whole, these investigations illustrate that RAGE can play a role in both the innate and adaptive immune systems to facilitate inflammatory responses.

### 1.2.5 Soluble RAGE and other inhibitors

As previously discussed, RAGE can exist in a full-length, membrane-bound form (mRAGE) or in a cleaved, soluble form (sRAGE). sRAGE can still bind ligands with the same affinity as mRAGE, but it cannot elicit downstream signaling due to its lack of a cytoplasmic tail. As such, sRAGE is considered an anti-inflammatory decoy receptor: it scavenges RAGE ligands, preventing them from signaling through mRAGE.

Administration of sRAGE as a therapeutic has shown promising results in studies of asthma,[144] autoimmune myocarditis,[145] and chronic hypoxia.[146] Another study blocked RAGE signaling in diabetic mice with an anti-RAGE $F(ab')_2$ antibody to improve neovascularization of ischemic tissue.[147] These studies have sparked interest in developing small molecule inhibitors of RAGE that could potentially be used to treat human disease, especially Alzheimer's disease. One group has identified pyrazole-5-carboxamides molecules as potent RAGE inhibitors and has been able to increase the molecules' inhibitory effects on RAGE through fluoride substitutions.[148] These agents have shown promise in a preliminary study examining inhibition of RAGE binding to amyloid-β. Another study inhibited RAGE-T cell effects both *in vivo* and *in vitro* through the use of the small molecule inhibitor of RAGE, TTP488 (azeliragon, Trans-Tech Pharma, LLC).[95] This drug can be given to patients orally and is currently in a Phase 3 clinical trial for Alzheimer's disease (ClinicalTrials.gov Identifier: NCT02080364).[149, 150] Another small molecule inhibitor of RAGE that has been used in animal studies is FPS-ZM1, which interferes with the variable domain of RAGE to prevent ligand binding.[151] These studies demonstrate that administration of sRAGE, a RAGE blocking antibody, or a small molecule inhibitor to interfere with RAGE signaling may be a novel therapeutic approach for many diseases that involve RAGE-mediated inflammation.

### 1.2.6 RAGE and asthma

Even though RAGE is highly expressed in the lung, little is known about RAGE's role in inflammatory diseases of the lung such as asthma. Two genome-wide association studies suggest that RAGE is important in asthma pathogenesis in humans. In patients with decreased FEV1 (a common finding on pulmonary function testing in patients with asthma), an association with a

single nucleotide polymorphism (SNP) (rs2070600) in the RAGE ligand-binding domain was found.[152, 153] This mutation results in a glycine to serine substitution at amino acid 82 (G82S), which increases RAGE's affinity for its ligands, leading to amplified inflammatory responses when compared to wild-type controls.[154, 155] Despite these studies, currently very little is known about the molecular mechanisms by which RAGE promotes asthma pathogenesis in humans.

In mice, laboratory studies have found that RAGE plays a key role in the pathogenesis of asthma in several models of AAI.[144, 156, 157] In one study, wild-type and RAGE KO mice were treated with HDM extract for seven weeks, after which wild-type mice presented with airway eosinophilia, goblet cell hyperplasia, and impaired pulmonary function after methacholine challenge.[144] In the absence of RAGE, however, the mice were protected against this asthma-like phenotype, as the airway was normal both physiologically and histologically. IL-4, which is important for activation of T cells, was increased normally in both wild-type and RAGE KO mice treated with HDM. IL-5 and IL-13, which direct eosinophil recruitment and mucus secretion, respectively, were increased in wild-type mice, but remained at baseline levels in RAGE KO mice, suggesting that RAGE is important for the production of these two type 2 cytokines. Eotaxins also were not elevated in RAGE KO mice, but were increased in wild-type mice. All of these studies were recapitulated using an ovalbumin (OVA) model with similar results.[144] Another study showed that HMGB1 release during HDM- or cockroach-induced AAI was dependent on TLR4 signaling early and then dependent on RAGE signaling later in the reaction.[156] RAGE also appears to play a role in T cell activation in OVA-induced allergic airway sensitization.[157]

Additional studies have shown associations between RAGE ligands and asthma. One report showed that HMGB1 promotes the recruitment of eosinophils to lungs in asthma, and that

levels of HMGB1 are positively correlated with the expression of TNF-α, IL-5, and IL-13.[158] This same study demonstrated that HMGB1 levels are elevated in the sputum of asthmatics and are positively correlated with the severity of the disease and the number of inflammatory cells in the lungs. S100A8/A9, a heterodimer complex that binds to RAGE, has also been implicated in airway remodeling and inflammation in asthma.[159] Additionally, S100A12 from eosinophils signals through RAGE to promote mast cell degranulation and IgE-mediated responses in the lung.[160] Notably, asthmatic patient sputum samples contained elevated levels of S100A12, and asthmatic patient lung tissue had greater numbers of S100A12+ eosinophils when compared to non-asthmatic controls.[160]

Overall, there is strong evidence to support a role for RAGE in asthma/AAI, yet the complexities of the receptor itself make determining the mechanism by which this is occurring a somewhat difficult (but not impossible) problem to address.

## 1.3 INTERLEUKIN-33 BIOLOGY

While TSLP, IL-25, and IL-33 have all been shown to be important type 2 immune response initiating cytokines, the studies here will focus on IL-33. IL-33 has been shown to be the crucial cytokine mediating allergic airway inflammatory responses.[50, 161] Furthermore, studies from our lab have shown that in the RAGE KO asthma model, TSLP and IL-25 expression are not dependent on RAGE.[162] These investigationss found that there is no significant difference in TSLP expression in whole-lung homogenates after seven weeks of HDM treatment in either wild-type or RAGE KO mice. In contrast, IL-25 secretion in the bronchoalveolar lavage fluid (BALF) after seven weeks of HDM exposure is upregulated in response to allergen in both wild-

type and RAGE KO mice. This expression of IL-25, however, is inadequate to precipitate a type 2 immune response in RAGE KO in this disease model.[144, 162] Therefore, IL-33 has become the focus of the studies presented here because its functions are dependent on RAGE (as will be explored in upcoming chapters).

### 1.3.1 Discovery and functions

The protein now known as IL-33 was first discovered as a nuclear factor possessing a novel helix-turn-helix DNA binding domain and was highly expressed in endothelial cells.[163] Two years later, IL-33 was re-discovered as a cytokine when it was found to be a ligand for the orphan receptor ST2.[49] Upon ST2 binding, IL-33 activates MAP kinases and NF-κB to induce type 2 immune responses. When administered to mice intraperitoneally, IL-33 causes splenomegaly, eosinophilic inflammation in the gastrointestinal (GI) tract, IgE/IgA production, vascular changes in the lung, and mucus hypersecretion in both the lungs and the gut.[49] Both lung and gut tissue also had increased type 2 cytokine mRNA after IL-33 treatment.

As a cytokine, IL-33 binds to ST2 and signals only in the presence of an accessory protein, IL-1 receptor accessory protein (IL-1RAcP).[164, 165] IL-33 must first bind to ST2 and then this complex is recognized by IL-1RAcP to initiate downstream signaling events.[166] ST2 also exists in a soluble form and functions as a decoy receptor.[49, 167] As a nuclear binding factor, IL-33 binds to heterochromatin via its N-terminal homeodomain-like helix-turn-helix motif and has transcriptional repressor activities.[168] Specifically, the N-terminus of IL-33 binds to NF-κB p65 to sequester it in the nucleus and repress its proinflammatory transcriptional activity.[169]

IL-33's dual roles as a transcriptional repressor when localized to the nucleus and as a proinflammatory cytokine when released from the nucleus are strikingly similar to those of the

RAGE ligand and prototypical alarmin, HMGB1.[170, 171] Supporting its classification as an alarmin, IL-33 is normally localized to the nucleus of cells but is released as a proinflammatory mediator when the cell becomes damaged or stressed. The localization of IL-33 is important for inflammatory homeostasis in the body. When the nuclear domain of one IL-33 allele was deleted in mice, IL-33 was released into the circulation, and the mice developed fatal, multi-organ, eosinophil-dominant inflammation within several months of birth.[172] Importantly, IL-33 mediates innate inflammatory signals but is not crucial for acquired tissue injury inflammatory responses.[173] It plays an especially important role in mucosal innate immunity in the gut and lung, especially in AAI, colitis, and endotoxin shock. Mouse models of contact hypersensitivity, experimental autoimmune encephalitis, diabetes, and hepatitis, all of which rely on acquired immune responses mediated by T cells, were found to be independent of IL-33 function.[173]

Cellular injury and necrosis are well-known causes of passive release of full-length IL-33, yet other reasons for IL-33 release from the nucleus have been studied as well. As will be discussed further in Section 1.3.3, apoptosis induces cleavage of IL-33 by caspase-3 and -7, resulting in release of inactive forms of IL-33 so that unwanted inflammatory responses do not occur.[170, 174] While the majority of IL-33 release is passive when cells become necrotic, it has been hypothesized that some cells can actively secrete IL-33 in the face of biomechanical or cellular stress. One study showed a role for IL-33 as a mechano-sensing paracrine factor that was secreted from cells after cyclic strain or cellular stretch.[175] Another showed that full-length IL-33 was actively secreted in an ATP-dependent manner from bronchial epithelial cells without accompanying cellular injury after exposure to the allergen *Alternaria alternata*.[176] A more recent study has shown that endogenous uric acid, a damage-associated molecular pattern (DAMP), is released from cells after treatment with allergen-associated cysteine proteases; the

increase in uric acid then stimulated IL-33 release from cells without any observable cell death.[177] Together, these studies show that IL-33 can be passively released when cells undergo necrosis, or it can be actively secreted in response to mechanical stress, ATP, uric acid, or other signals that are commonly found in cells that are under stress.

### 1.3.2 Expression

Early studies examining cell types that express IL-33 demonstrated a strong IL-33 presence in the nuclei of vascular endothelial cells.[48, 163, 178] The first studies examining IL-33 expression also focused on endothelial cells and showed expression of IL-33 in the nuclei of vascular endothelial cells from human skin, small intestine, umbilical vein, lung, colon, mammary gland, and kidney.[178] This study also revealed that endothelial IL-33 expression is increased in non-proliferating cells and is downregulated upon cellular migration and proliferation. Another study published at the same time found that IL-33 was found to colocalize with chromatin and was constitutively expressed in a widespread array of human tissues and cell types. Importantly, IL-33 was identified not just in endothelial cells, but also in epithelial cells that were exposed to the environment (i.e. skin and mucosal surfaces).[48] Examination of mouse tissue IL-33 expression revealed differences from that of humans. Notably, mouse endothelium does not express IL-33.[179] Instead, IL-33 was found in lymph nodes, spleen, nervous tissue (brain and eye), embryonic tissues, and abundantly in adult epithelial cells at barrier surfaces (skin, lung, salivary glands, stomach, and vagina).[179] This study also showed that IL-33 expression could be induced in many tissue types upon onset of inflammation.

The discovery of IL-33 in epithelial cells at barrier surfaces combined with its role in type 2 inflammatory responses (detailed in Section 1.3.4) sparked many investigations into IL-33

expression in the airways. Endobronchial biopsy specimens from human asthmatics showed increased IL-33 mRNA expression when compared to non-asthmatic control samples.[180] Further experiments localized IL-33 expression to airway epithelial, endothelial, and smooth muscle cells and demonstrated that TNF-α stimulated IL-33 expression in airway smooth muscle cells.[180] A follow-up study by the same group showed increased IL-33 expression in bronchial epithelium of severe asthmatics both in endobronchial biopsy samples and in primary airway epithelial cell cultures.[181] These results were recapitulated in a study by another group examining levels of IL-33 in airway epithelial cells from asthmatic and non-asthmatic control biopsy specimens.[182] Patients with moderate asthma also possessed higher levels of IL-33 in the BALF than those with mild asthma or non-asthmatic controls.[181] Later studies explored IL-33 expression in distal alveolar epithelial cells and found very prominent expression of the protein in AT2 epithelial cells.[183-186] AT2 cells seem to be the biggest source of IL-33 in AAI.

Finally, some studies have suggested that IL-33 is expressed and secreted from circulating immune cells and that this is a potential source of IL-33 during allergic type 2 reactions. Peritoneal macrophages,[187] alveolar macrophages,[188, 189] mast cells,[187] and dendritic cells[189, 190] have all been shown to upregulate IL-33 expression when stimulated. One group hypothesized that all cells expressing FcRγ-associated receptors, including dendritic cells, monocytes, macrophages, mast cells, natural killer cells, and basophils, were sources of IL-33 in allergic type 2 immune responses.[190] A study using an IL-33 citrine reporter mouse to examine IL-33 expression after exposure to allergens, however, put these expression profiles into context.[185] In these experiments, mice treated with OVA showed a 15-fold increase in overall IL-33 expression in lung tissues. Cells positive for the hematopoietic marker, CD45, and citrine increased 28-fold in response to OVA. These cells were mostly eosinophils, macrophages, and B

cells. Importantly, however, the authors also pointed out that 80-90% of the citrine positive cells after OVA exposure were negative for CD45 and were instead positive for the epithelial marker, EpCam. This study demonstrated that while circulating immune cells can express IL-33, the most prominent IL-33 producers, at least in allergic reactions, are AT2 epithelial cells. Another study further supported these results by utilizing bone marrow chimeric mice to illustrate that IL-33 from hematopoietic immune cells is not essential for development of HDM-induced AAI.[191]

### 1.3.3 Cleavage products

There has been much debate over the years about the biological activity of the various cleaved forms of IL-33. When the protein was first studied as a cytokine, scientists believed that caspase-1 cleaved the full-length 30 kDa IL-33 to an 18 kDa mature, active form.[49] This was proved to be untrue by Cayrol and Girard when they showed that full-length IL-33 is, in fact, biologically active and that it is inactivated by cleavage by caspase-3 and caspase-7.[170] Because caspase-3 and caspase-7 are involved in cellular apoptosis, it was hypothesized that IL-33 is inactivated during programmed cell death so that an inflammatory response is not triggered as it would be if the cell released IL-33 due to damage or stress.[174]

Questions about full-length IL-33 having biological activity also emerged because it appeared that cleaved forms of the protein had much greater activity. Several studies confirmed that full-length IL-33 is active, but that cleavage of the protein greatly increases its biological activity.[176, 192] Using both mouse and human IL-33, studies demonstrated that the inflammatory cell proteases neutrophil elastase and cathepsin G cleaved full-length IL-33 to mature forms that had approximately 10-fold higher biological activity than the full-length form.[192] In humans, cleavage of full-length 31 kDa IL-$33_{1\text{-}270}$ by neutrophil elastase results in a 20 kDa IL-$33_{99\text{-}270}$

product, and cleavage by cathepsin G yields two products, 21 kDa IL-$33_{95\text{-}270}$ and 18 kDa IL-$33_{109\text{-}270}$. In mice, the full-length form of IL-$33_{1\text{-}266}$ (~35 kDa) is cleaved by cathepsin G to generate a 20 kDa cleavage product, IL-$33_{102\text{-}266}$. Cleavage by neutrophil elastase results in a major cleavage product at 20 kDa, IL-$33_{102\text{-}266}$, and a minor cleavage product at ~19-20 kDa, IL-$33_{109\text{-}266}$. When further studies emerged highlighting the role of IL-33 in type 2 responses (which normally do not involve a large influx of neutrophils), researchers focused on the ability of mast cell proteases chymase and tryptase to cleave IL-33. The mature forms of IL-33 after mast cell serine protease cleavage had ~30-fold higher biological activity than full-length human IL-33.[193] Human mast cell chymase resulted in cleavage of IL-$33_{1\text{-}270}$ to 18 kDa IL-$33_{95\text{-}270}$ and 21 kDa IL-$33_{109\text{-}270}$ products. Human tryptase cleaves IL-$33_{1\text{-}270}$ into a major product at 18 kDa (IL-$33_{107\text{-}270}$), and two minor products at 25 kDa (IL-$33_{79\text{-}270}$) and 26 kDa (IL-$33_{72\text{-}270}$). Murine IL-33 was also cleaved by mast cell tryptase.[193]

Researchers have now defined a central activation region of IL-33 corresponding to amino acids 66-111 in the human form.[193] This is the region where proteases cleave full-length IL-33 to promote its biological activity during inflammation. Amino acids 1-65 compose the nuclear localization domain of the protein while the IL-1-like cytokine domain is formed by amino acids 112-270.

### 1.3.4 IL-33 in allergic diseases/asthma

IL-33 has emerged as a very important player in type 2 immune responses, especially asthma/AAI. An important role for IL-33 in asthma has been supported by numerous GWAS that show a strong association between SNPs in genes encoding IL-33 or its receptor, ST2 (IL1RL1), and the development of asthma.[5, 194-197] While it had been well-established that IL-33 was

released from pulmonary epithelial cells as an alarmin to trigger type 2 immune responses, an exciting new role for IL-33 was uncovered in 2010 with the discovery of innate lymphoid cells (See Section 1.4). ILC2s express ST2, and IL-33 was found to potently activate these cells to produce large amounts of the type 2 cytokines IL-5 and IL-13.[198-201]

IL-33 was soon found to be a crucial mediator of AHR and AAI after viral infection[188, 202-204] or allergen exposure.[33-36, 205] Importantly, IL-33 is not just an acute-acting cytokine; it is also necessary for chronic allergen reactions and is driven by adaptive immune responses as well.[206] IL-33 was also characterized as a major player in food allergies,[207] nasal polyps and chronic rhinosinusitis,[208, 209] atopic dermatitis,[210] parasitic infections,[183] and other ILC2-driven type 2 immune responses. During eosinophilic inflammation, IL-33 can activate eosinophils directly via ST2 on eosinophils or indirectly via activation of ILC2s (which then drive eosinophilic responses).[211] In asthmatic inflammation and hyperresponsiveness, both IL-33 and IL-25 are released from epithelial cells and both can activate ILC2s. Studies have shown, however, that IL-33 is the more important cytokine in allergic airway disease. Both *in vivo* and *in vitro* studies showed that IL-33 was more important than IL-25 for development of AHR and it more potently activated ILC2s.[50] This same finding was reiterated in a later study examining the differences between IL-33 and IL-25 structural and functional effects in AAI.[161]

The kinetics of initial IL-33 release during AAI are very rapid. Several studies have shown that IL-33 is found in the BALF (or nasal lavage fluid[209]) within one hour of allergen exposure, but is no longer detectable in the BALF three to six hours after exposure.[176, 186, 206, 209] One thought is that IL-33 is rapidly released and cleaved by allergen-associated proteases. *Alternaria alternata* serine proteases were shown to cause rapid release of IL-33 to drive type 2 immune responses.[186] Another study administered common allergen-associated cysteine

proteases, papain and bromelain, directly to naïve mice, which induced IL-33 expression within three hours.[177] This group also administered *Alternaria, Aspergillus*, or HDM allergen extracts to mice to show a rapid increase in IL-33 expression dependent on allergen protease activity. Interestingly, proteases were also found to induce expression of the cellular DAMP, uric acid, which was important for IL-33 release after direct administration of proteases or after administration of protease-containing allergen extract.[177] Similarly, chitin, while not a protease, is a polysaccharide found in many allergens and has also been shown to be a potent allergen-associated stimulant of IL-33 release in AAI.[212]

IL-33 has been shown to upregulate ICAM-1 and VCAM-1 expression in endothelial cells.[213, 214] This function is dependent on NF-κB signaling and promotes recruitment of immune cells from the blood to sites of inflammation. It was hypothesized that this upregulation is a result of IL-33's nuclear transcriptional regulatory function and not its function as a cytokine since upregulation of adhesion molecules occurred even in the absence of ST2.[214] This directly contrasts to another study that showed this process to be dependent upon ST2.[213] Together, these studies may indicate that IL-33 can upregulate adhesion molecules both intracellularly as a nuclear factor and extracellularly as a cytokine, depending on the cellular environment and inflammatory stimulus. Another way that IL-33 helps to recruit cells to sites of inflammation is by working with TSLP to induce hematopoietic progenitor cell homing to the lung via an IL-13-dependent pathway.[215]

IL-33, as a major player early on in development of type 2 innate immune responses, has been identified as an attractive target for novel therapies in allergic diseases.[216] Early studies in animal models appear promising,[217, 218] but anti-IL-33 therapies have yet to move forward to clinical trials. In fact, the only clinical trials involving IL-33 at this time are ones evaluating the

cytokine as a biomarker for disease pathogenesis (searched "IL-33" in clinicaltrials.gov). Very recent studies have shown that IL-33 driving ILC2 responses in adipose tissue stimulates beiging of white fat and is beneficial for metabolic homeostasis (see Section 1.4.3.4).[219-221] Therefore, until a more complete understanding of IL-33's many functions in the body can be determined, a tissue-specific targeted IL-33 therapy may be needed since systemic blockade may cause unwanted, pathological side effects in clinical trials.[222]

## 1.4 GROUP 2 INNATE LYMPHOID CELLS

### 1.4.1 Discovery

Two papers published in the early 2000s first identified a new cell population that could trigger type 2 immune responses following administration of IL-25 both in the presence (wild-type mice) and absence (*Rag2-/-* mice) of an adaptive immune system.[46, 47] In 2006, Richard Locksley's group demonstrated that IL-13 expression from a hematopoietic, non-T-cell was necessary for parasitic worm expulsion and Th2 cell recruitment in the lung.[223] However, it was not until 2010 that the first papers on innate lymphoid cells were published, showing that these cells lacked T cell lineage markers (CD3, CD4, and CD8) and specific antigen receptors, yet were highly active at mucosal surfaces and in fat-associated molecular clusters (FALCs) to promote type 2 immune responses in mice. [198-201] These studies showed that, even in the absence of an adaptive immune system, innate lymphoid cells were stimulated by IL-25 and/or IL-33 to produce large amounts of IL-5 and IL-13, but not IL-4, to aid in parasitic worm expulsion in the GI tract. Neill and colleagues called these cells nuocytes, named for their ability to produce large

amounts of IL-13 ("nu" is the thirteenth letter of the Greek alphabet) in response to a parasitic infection.[199] Other groups referred to the cells as natural helper cells,[198] innate helper 2 cells,[200] and type 2 multipotent progenitor cells.[201] The variability in naming early on led to a petition for uniform nomenclature and classification of these cells as "group 2 innate lymphoid cells."[224]

### 1.4.2 ILC classifications and surface markers

Innate lymphoid cells (ILCs) act as mediators between the innate and adaptive immune systems in humans and mice and have been classified into three distinct groups, mostly based on their transcriptional regulators and cytokine production. This classification scheme has been extensively reviewed in the literature.[225-229] The cells of the three ILC groups tend to functionally mirror Th1, Th2, and Th17 adaptive immune cells, respectively. All ILCs possess a distinctly lymphoid morphology, but do not express markers of any other known cellular lineage (and thus are defined as being lineage negative). Importantly, they also have no antigen receptors and completely lack antigen specificity, making them innate-acting cells. All ILCs require the bZIP transcription factor, Nfil3, for proper development and function[230, 231] and they differentiate from a common precursor defined by expression of the transcription factor PLZF.[232]

Group 1 ILCs (ILC1s) include natural killer (NK) cells and are activated by IL-12 and IL-15 to produce IFN-γ in response to viruses and tumors.[228, 233] ILC1s largely function in the intestine, spleen, liver, and peritoneal cavity and express CD127, NK1.1, and the T-box transcription factor, T-bet.

Group 3 ILCs (ILC3s), which include lymphoid tissue inducer (LTi) cells, are a very complex population of ILCs and are beyond the focus of this dissertation, so this summary will just briefly describe their main characteristics. ILC3s reside mostly in the intestine and produce

IL-22 and IL-17 when stimulated by IL-23 and IL-1β. Their development is dependent on expression of the nuclear hormone receptor, retinoic acid receptor related orphan receptor γt (RORγt). ILC3s are involved in gut bacterial defense,[234] lymph node development[235] and repair,[236] protection of intestinal stem cells from graft-versus-host disease,[237] and intestinal inflammation (i.e. inflammatory bowel disease, Crohn's disease, etc.).[234] A recent study has also shown that ILC3s producing IL-17 in the lung mediate obesity-associated asthma pathogenesis.[238] Further adding to ILC3 complexity, it was shown that a subpopulation of ILC3s can transition into IFN-γ-producing ILC1-like cells.[239] This demonstrates that ILCs retain some plasticity and are influenced by environmental cues.

Group 2 ILCs (ILC2s), which are the focus of the studies presented here, have been discussed above and are primarily responsible for mounting type 2 immune responses in parasitic infections and allergic reactions by secreting large amounts of IL-5 and IL-13. ILC2s are activated by IL-25, IL-33, and TSLP. ILC2s express IL-7 receptor (CD127), IL-33 receptor (ST2), IL-2 receptor (CD25), inducible T cell costimulator (ICOS), thymocyte marker (CD90), hematopoietic progenitor marker c-kit (CD117), stem cells antigen 1 (Sca-1; Ly6A/E), and a hematopoietic marker (CD45).[198, 199, 240] The receptors for IL-2, IL-7, IL-25, and IL-33 are all important for ILC2 development and activation. ICOS and ICOS-ligand are both expressed on ILC2s and interactions between these molecules on ILC2s has recently been shown to be important for ILC2 survival and cytokine release.[241] ILC2s express CD45, suggesting a bone marrow origin, and require IL-7 signaling for their differentiation, suggesting a lymphoid origin.[242] A series of elegant experiments by Wong *et al.* demonstrated that ILC2s are, indeed, derived from a common lymphoid progenitor cell.[243] Id2, γ-chain, IL-2R, IL-7R, GATA-3, RORα, and Notch signaling are all required for the development of ILC2s from common

lymphoid progenitor cells.[240, 243-246] Variability in ILC2 expression of the IL-33 receptor (ST2) and the IL-25 receptor (IL-17RB) in the earliest reports of ILC2s led to the hypothesis that mixed ILC2 populations were present in these samples. Recently a new study showed that ILC2s can follow two pathways: a natural ILC2 (nILC2) pathway responding to IL-33 and an inflammatory ILC2 (iILC2) pathway responding to IL-25.[247] The authors found that iILC2s are inflammatory-stimulated progenitor cells that can develop into nILC2s. Since nILC2s proliferate rather slowly in response to infection, iILC2s comprise a significant pool of progenitor cells from which nILC2s can be generated quickly in response to infection.[247] iILC2s can be identified by their high expression of the surface marker, KLRG1, and a lack of ST2 expression.

A human equivalent of the mouse ILC2 exists, is defined as being lineage negative and CRTH2+CD161+IL-7Rα+CD25+CD45+, and has been found to play a role in several inflammatory diseases.[248-250] These cells were found at increased levels in human cases of chronic rhinosinusitis[208, 251] and have been isolated from the BALF of patients undergoing lung transplants.[202] They have also been identified in high numbers in eosinophilic nasal polyps and are decreased with steroid treatment.[252, 253]

### 1.4.3 Functional roles of ILC2s in disease and repair

ILC2s function in a variety of tissues to promote type 2 inflammatory responses. They are involved in both harmful, pathological inflammatory processes and in beneficial tissue repair, homeostasis, and defense against parasitic infections.

#### 1.4.3.1 Cytokine production

When stimulated by IL-25, IL-33, or TSLP, ILC2s release large amounts of IL-5 and IL-13. IL-5 recruits mast cells, basophils, IgE-producing B cells, and especially eosinophils. IL-13 triggers goblet cell hyperplasia and tissue remodeling. Another cytokine produced by ILC2s is IL-9, which has been found to promote ILC2 survival and cytokine secretion via autocrine signaling.[254, 255] ILC2s also release amphiregulin, an epidermal growth factor that has been shown to be important for pulmonary tissue repair after viral infections[202, 256] and IL-6, which mediates B cell antibody production.[198, 199]

#### 1.4.3.2 Tissue localization

ILC2s can be found in many tissues in the body, but have been most widely studied in the GI tract, lungs and nasal cavity, skin, spleen, lymph tissue, adipose tissue, and in human peripheral blood samples.[257] At baseline, these are very rare cell populations, but efforts have been made to identify their location within various tissues, especially in the airway where they play a major role in allergic inflammation. In the naïve mouse airway, one report identified IL-5-expressing ILC2s in collagen-rich regions near mid-sized blood vessels and airways, but notably not in alveoli.[258] A more recent study by the same group visualized IL-5-expressing ILC2s around medium-to-large blood vessels that expressed VCAM-1 in the endothelium and around collagen-containing airways after chitin exposure.[212] Another report using *Il13*-reporter mice demonstrated an IL-33-induced increase in ILC2s in the submucosa surrounding airway α-smooth muscle actin.[50] In healthy human lung tissue samples, ILC2s were found surrounding medium and small airways and were accompanied by mast cells.[259]

#### 1.4.3.3 ILC2s in the gastrointestinal tract

The first studies on ILC2s focused on GI tract ILC2s and demonstrated that these cells were crucial mediators of helminth expulsion via induction of type 2 immune responses.[199-201] Interestingly, ILC2 populations also expand in the GI tract in response to vitamin A deficiency.[260] The authors of the study hypothesized that this change occurs to enhance innate type 2 barrier immunity in the gut in the face of malnutrition-induced repression of the adaptive immune system and to help expel parasites from the GI tract that compete with the host for nutrition. ILC2s in the GI tract, which constitutively express IL-5 and IL-13, also appear to respond to food intake by upregulating IL-13 secretion.[258] This is mediated in part by vasoactive intestinal peptide (VIP) signaling through VIP receptor type 2 (VPAC2) on ILC2s, suggesting that ILC2s play an important role in metabolic pathways and circadian rhythm.[258] ILC2s also produce large amounts of amphiregulin, which has been shown to be reparative in colitis models by attenuating the immune response via upregulation of T regulatory cells (Tregs).[261]

The role of ILC2s in chronic, pathological inflammation in the GI tract has also been studied, though ILC3s seem to be more prominent players in gut inflammation.[262] ILC2s increased in number and contributed to inflammation in response to IL-25 in a mouse oxazolone colitis model.[263] It has not yet been studied what role, if any, ILC2s play in human inflammatory bowel disease, but it is likely they contribute somewhat since ulcerative colitis is mediated by type 2 cytokines such as IL-13.[264]

#### 1.4.3.4 ILC2s in adipose tissue and skin

As briefly mentioned in Section 1.3.4, a new beneficial role for ILC2s in adipose tissue metabolic homeostasis has recently been established. Healthy animal visceral adipose tissue (VAT) contains eosinophils and alternatively activated macrophages (AAMs) that control

metabolic homeostasis.[265] In the absence of eosinophils, adipose tissue expands, AAM numbers decrease, and insulin resistance develops.[266] Resident ILC2s in the VAT were found to control eosinophil populations via IL-5 and IL-13 secretion to aid in the maintenance of a healthy metabolism, and stimulation of type 2 immune responses elsewhere in the body promoted ILC2-mediated effects in the VAT.[219] ILC2s were later shown to be important in the beiging of white adipose tissue in both human and mouse tissues.[220, 221] Beige adipocytes, commonly activated by cold temperatures, are highly thermogenic cells that reside within white adipose tissue to regulate energy expenditure and metabolic homeostasis.[267] High levels of beige adipocytes are found in lean individuals and restrict development of obesity. Therefore, it was not surprising that decreased expression of ILC2s in adipose tissue is associated with obesity.[221]

Skin is another barrier organ where ILC2s reside and function. Patients with atopic dermatitis were found to have increased levels of ILC2s in their skin when compared to healthy controls.[210, 268] ILC2s in the skin have both pro- and anti-inflammatory functions, and resident ILC2s in the skin promote homeostasis via constitutive production of IL-13.[269] Notably, ILC2s are inhibited when bound to E-cadherin, and E-cadherin is downregulated in atopic dermatitis, suggesting a possible mechanism by which ILC2s become activated in atopic dermatitis.[210] Murine basophils also participate in ILC2 responses in atopic dermatitis. Early secretion of IL-4 from basophils was crucial for maximum accumulation of ILC2s in skin inflammatory clusters.[270]

#### 1.4.3.5 ILC2s in the airway

Pulmonary ILC2s play a major role in promoting type 2 responses in asthma and AAI. OVA-induced ILC2 accumulation in mice was noted in the lung, and these cells were found to make up 77% of the IL-13-producing, non-CD4+ cells after twelve days of allergen exposure.[34] Direct

intranasal administration of the ILC2-stimulating cytokines IL-25 or IL-33 was able to induce AAI and increases in ILC2 numbers in the BALF of mice. ILC2s were also shown to be able to stimulate AHR in mouse models of asthma.[34] Additional studies demonstrated that ILC2s functioned independently of the adaptive immune system to promote AAI in mice after IL-33, *Alternaria alternata*, or papain protease exposure.[33, 35] ILC2s were even present in the lungs of naïve mice,[33, 35, 271] and resident ILC2s were also identified in the lungs of healthy human lungs.[202, 208, 259] ILC2s are major producers of IL-5 and IL-13 in the lung in response to cytokines or allergens, and in some cases are the principal IL-5- and IL-13-producing cells.[271, 272] In fact, 50% and 80% of the cells producing these cytokines were ILC2s after intranasal IL-25 or IL-33 administration, respectively, and in OVA-induced AAI, ILC2s and Th2 cells produced equal amounts of IL-5 and IL-13.[271] ILC2s, and not T cells, were also found to be necessary for the persistence of chronic asthma by stimulating an IL-33/IL-13-mediated positive feedback loop between epithelial cells and immune cells.[273] These early studies opened up an exciting new field of ILC2 research as they identified pulmonary ILC2s that functioned independent of adaptive immune responses to promote type 2 inflammatory reactions in the lung in multiple models of asthma and AAI.

Allergens are potent stimulators of ILC2s, usually via an IL-33-mediated pathway. Allergen-associated serine proteases,[186] cysteine proteases,[177] or chitin[212] stimulate IL-33 release from epithelial cells to promote rapid ILC2 accumulation and function in the lung. *Alternaria* was able to induce ILC2 recruitment to the lung within one to three days, resulting in AAI and AHR.[186, 256] Chitin caused upregulation of IL-13-expressing ILC2 numbers in the lung within forty-eight hours.[212] Treatment of cells with the HDM-associated protease, papain, caused an increase in endogenous uric acid, IL-33 release, and ILC2 stimulation.[177] As the authors point

out, this study suggests that airway cells sense allergen protease activity and not necessarily the protease molecule itself to induce ILC2 activation and AAI.

In addition to allergen exposure, viral infections were also found to promote ILC2 responses in the lung. Viral infections are responsible for a large percentage of asthma exacerbations every year, especially in children. ILC2s accumulated in mouse lungs after administration of the PR8 mouse influenza A virus in the presence or absence of an adaptive immune system.[202] Furthermore, ILC2s caused IL-13-dependent AHR following infection with an H3N1 influenza virus; the virus triggered IL-33 release from macrophages, which in turn activated ILC2s in the lung, independent of the adaptive immune response.[188] Virus infection as a child can disturb the immune system and induce onset of asthma-like symptoms in a previously healthy individual. Neonatal rhinovirus infection in young (6-day-old) mice was found to induce expression of IL-25 and subsequent activation of type-2-cytokine-producing ILC2s, resulting in chronic AHR and mucus hypersecretion.[274] This same response was not found in adult mice (8-week-old), suggesting that the ILC2 response to virus infection changes as animals mature. Finally, although studies have shown associations between viral infection and a pathological role of ILC2s, ILC2s are also necessary for proper lung tissue repair following viral infections.[202] When ILC2s were absent from lung tissue, long-term lung function and tissue repair were severely impaired. ILC2 secretion of amphiregulin was found to restore lung integrity and function after viral infections.[202]

Though much of the work on ILC2s and asthma has been done in mouse models, ILC2s are likely to play a role in human asthma/AAI as well. A large-scale GWAS showed an association between physician-diagnosed asthma and SNPs in genes that encode IL-13 (rs1295686) and RORα (rs11071559), crucial proteins for ILC2 function and development,

respectively.[5] Additionally, human BALF samples from asthmatic patients possessed elevated levels of IL-33 and ILC2s (Lin-FcεRI-CD127+IL-33R+ cells) compared to those of non-asthmatic control subjects.[273] Another study examined human peripheral blood ILC2s in asthmatics and non-asthmatic patients. Not only did the asthmatic patients have more peripheral blood ILC2s, but the ILC2s from these patients also had enhanced type 2 cytokine release when stimulated.[275] Peripheral blood samples from allergic individuals contained ILC2s expressing GATA-3 and E26 transformation-specific sequence-1 (ETS-1) transcription factors, which were shown to be important for generating STAT6-dependent ILC2 proliferation in an *Alternaria* mouse model of asthma.[256] This suggests that human ILC2s, since they possess similar transcriptional regulators, function similarly to mouse ILC2s in response to allergens. Besides asthma, chronic rhinosinusitis is a common atopic pathology in the upper respiratory tract/sinuses that is associated with the development of nasal polyps. Many studies have also linked ILC2s with chronic rhinosinusitis in human patients: ILC2s are abundantly present in many nasal polyps and are associated with greater eosinophilia.[252, 253, 276-278]

Many diverse mechanisms exist to regulate ILC2 function in the airway. Specialized pro-resolving mediators (SPMs), including lipoxins, resolvins, protectins and maresins, are derived from omega-3 fatty acids and recently have been described as anti-inflammatory molecules.[279] Bruce Levy's group isolated peripheral blood human ILC2s that express both the prostaglandin $D_2$ ($PGD_2$) receptor, CRTH2, and the lipoxin receptor, ALX/FPR2.[259] They showed that ILC2s are stimulated to produce IL-13 via $PGD_2$ signaling on CRTH2, and that IL-13 secretion is greatly decreased upon lipoxin $A_4$ ($LXA_4$) signaling via ALX/FPR2. This was the first study demonstrating that ILC2s could be negatively regulated by a lipid mediator. A follow-up study by the same group showed a role for another SPM, maresin-1, in decreasing ILC2 production of

IL-5 and IL-13 and increasing the cell's production of the repair cytokine, amphiregulin.[280] Importantly, the dampening of the ILC2 response was mediated via ILC2 interactions with Tregs that were stimulated by maresin-1. Conversely, lipid mediators derived from arachidonic acid, known as cysteinyl leukotrienes, have been shown to promote type 2 immune responses by signaling through cysteinyl leukotriene receptors on ILC2s.[281] Lipids have also been shown to be important for ILC2 accumulation in the lung during inflammation. Human and mouse lung ILC2s express lower levels of the CRTH2 receptor than peripheral blood ILC2s do, and, as a result, lung ILC2s migrate towards the CRTH2 ligand, $PGD_2$, less robustly than spleen-derived ILC2s *in vitro*.[282] It was thought that CRTH2 is downregulated upon $PDG_2$ binding. Thus, lung-derived ILC2s would be expected to express lower levels of the receptor since they had already bound $PGD_2$ and accumulated in the lung. *In vivo* studies also demonstrated a need for CRTH2 on ILC2s for $PGD_2$-mediate ILC2 accumulation in the lung in response to helminth infection.[282] There is evidence in the literature that basophils also act to recruit and stimulate ILC2s via production of IL-4. Papain protease stimulates basophils to produce massive amounts of IL-4, which, in conjunction with IL-2 and IL-33, stimulates proliferation of ILC2s.[283] Another study has shown that basophil-derived IL-4 is necessary for optimal accumulation of ILC2s in the skin in models of atopic dermatitis.[270]

ILC2s and their functions in AAI have only recently been discovered, but ideas for new targets for asthma therapies already are emerging from this research. Much work is left to do to uncover and understand the many functions and complexities of these new immune cells.

### 1.4.4 ILC2 and Th2 cell interactions

Though ILC2s can function in the absence of an adaptive immune system, they are in no way completely separated from interacting with adaptive immune system components. In fact, ILC2s bridge innate and adaptive immunity and interact with T cells in numerous ways. The crosstalk between ILC2s and Th2 cells is complex and is an active area of investigation.

Many allergic diseases are caused by an initial sensitization to an allergen followed by repeated exposures and inflammatory exacerbations. These recall responses are mediated by memory CD4+ Th2 cells. In a series of elegant experiments, Liu and colleagues demonstrated that antigen-specific CD4+ Th2 cells were the primary mediators of recall responses to OVA allergen and that increased numbers of these cells promoted ILC2 secretion of IL-13 without an expansion of ILC2 numbers.[284] Without Th2 cells, ILC2s were not sufficient to mount a recall response on their own. Unlike acute models where ILC2s can function independently of the adaptive immune system to promote AAI, both ILC2s and CD4+ T cells are required for maintenance of chronic allergic reactions to airborne allergens.[206] Both cell types contributed to airway IL-5 and IL-13 levels, though Th2 cells were more numerous in these chronic models. Another group proposed that T cells increase the severity of chronic allergic asthma, but are not necessary for the persistence of AHR and lung remodeling.[273] Instead, persistence of chronic asthma-like phenotypes was mediated by ILC2s. These ILC2s were driven by epithelial-derived IL-33, which was controlled by two positive feedback loops: IL-13 driven IL-33 release and IL-33 autoinduction.[273] More studies will be needed to fully understand the role of ILC2s and Th2 cells in asthma exacerbations as a result of chronic, repeated exposure to allergens.

Allergen proteases are often necessary for stimulation of ILC2s, while Th2 cells rely on ILC2 activation for their induction. This has been demonstrated in an *Alternaria*-induced model

of AAI, where ILC2s are reliant on serine protease activity to induce their function, but Th2 cells are not directly reliant on this protease activity.[186] In papain-induced AAI, on day two of treatments, ILC2s comprised the majority of IL-5+ and IL-13+ cells. On day twenty-one, the majority of IL-5+ and IL-13+ cells were CD4+ Th2 cells, though ILC2s still comprised 30% of the IL-5+ IL-13+ population.[36] Thus, ILC2s stimulated by papain-induced IL-33 release were crucial early mediators responsible for mounting robust Th2 responses. They did so by secreting IL-13, which was found to stimulate dendritic cell migration to the lymph nodes to prime T cells to differentiate to Th2 cells.[36]

ILC2s have also recently been shown to express major histocompatibility complex class II (MHCII), meaning that ILC2s have the potential to process antigens.[284-286] MHCII expression was found on 50-70% of ILC2s from naïve mice, and the level of expression varied depending on the tissue type in which the ILC2s were expressed.[286] Without MHCII, ILC2s could not mediate successful helminth expulsion. ILC2s were found to be able to process and present antigen, though less efficiently than professional antigen-presenting cells.[285, 286] ILC2s interacted with antigen-specific T cells via MHCII to promote T cell function and differentiation to the Th2 cell phenotype.[285, 286] T cells, in return, produced large amounts of IL-2 to stimulate ILC2 proliferation and secretion of IL-5 and IL-13.[285, 286]

Other ligand-receptor pairs are also thought to mediate ILC2-T cell interactions. Tumor necrosis factor receptor, OX40 (CD134), was shown to be expressed on T cells and its ligand OX40L was expressed on ILC2s.[287] CD4+ T cell proliferation and cytokine production in culture could be enhanced through the addition of ILC2s: CD4+ T cells triggered enhanced IL-4 production from ILC2s, while ILC2s greatly promoted CD4+ T cell production of IL-5.[287] ICOS is another receptor that is highly expressed on both ILC2s and T cells and is important for

cellular activation and function.[199, 288] ILC2s also express ICOSL, the ligand for ICOS.[241] Therefore, not only do ILC2s interact with each other to promote ILC2 survival and function, but they may also be able to directly activate T cells through ICOS interactions (though this has not yet been shown in the literature).

# 2.0 RATIONALE AND HYPOTHESIS

For over twenty years, RAGE has been studied in a variety of inflammatory diseases and tissues. Though RAGE is most highly expressed in the lung, surprisingly very little is known about its role in this organ. Recently, however, an important role for pulmonary RAGE in asthma pathogenesis was uncovered.[144] In these studies, when RAGE was absent, mice were completely protected from development of AAI and AHR in response to chronic exposure to HDM allergen extract. Surprisingly, IL-4, IgG, and IgE responses were normal in RAGE knockout (KO) mice exposed to HDM, demonstrating that some humoral and T cell immune responses are intact in these mice. However, RAGE KO mice fail to upregulate IL-5 or IL-13 in response to allergen. Because IL-5 and IL-13 are the major drivers of asthma pathogenesis (eosinophilia, mucus hypersecretion, AHR), lack of these cytokines results in a complete absence of an asthmatic phenotype in RAGE KO mice.

In the past five years, an entirely new branch of immunology has emerged with the discovery of innate lymphoid cells. Group 2 innate lymphoid cells (ILC2s) are potent inducers of type 2 immune responses and have been extensively implicated as early players in the pathogenesis of asthma/AAI.[33-35] ILC2s secrete profuse amounts of the type 2 cytokines IL-5 and IL-13, but little to no IL-4.

Both RAGE and ILC2s are important early initiators in AAI. RAGE has been characterized as a PRR, meaning it can bind to a variety of damage-associated molecular patterns

(DAMP) to stimulate the innate immune system. Allergic airway responses are initiated by allergen-triggered release of DAMPs such as uric acid or IL-33, resulting in recruitment and stimulation of ILC2s to the lung. The exact mechanisms by which allergens induce DAMP release and ILC2 recruitment/stimulation in the lung are unknown.

The complete lack of an IL-5 and IL-13 response in RAGE KO mice after allergen challenge suggests that RAGE KO mice have a defect in the ILC2 response. These findings, therefore, led to the central hypothesis of this work that ***RAGE mediates allergic airway inflammation by promoting the amount and/or function of group 2 innate lymphoid cells in the lung***.

# 3.0 MATERIALS AND METHODS

## 3.1 ANIMAL STUDIES

### 3.1.1 Mice

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 Institutional Animal Care and Use Committee (IACUC). All animal protocols were approved by the IACUC. Wild-type male and female C57BL/6 mice were obtained from Taconic. RAGE KO mice in a C57BL/6 background were a gift from Dr. A. Bierhaus (University of Heidelberg, Germany) and were bred in-house to establish a colony. These mice contain a global deletion of exons 2 to 7 in the RAGE locus on chromosome 17.[289] Additionally, they express an EGFP reporter protein wherever RAGE is knocked out, thus causing the animals' cells to fluoresce green. Eight to twelve week old RAGE KO mice were age- and sex-matched to wild-type mice for each experiment.

### 3.1.2 Induction of allergic airway inflammation

Three agents were used for induction of AAI: house dust mite (HDM) extract (Greer), *Alternaria alternata* extract (Greer), or recombinant IL-33 (rIL-33) (Biolegend). The reagents were diluted

with sterile saline to the proper dosage and administered to the mice via intranasal (i.n.) instillation. To do this, mice were anesthetized with isoflurane and then given 12.5 µL of cytokine or allergen drop-wise in each nostril for a total of 25 µL. Control mice were given 25 µL of saline vehicle alone.

Models of AAI are illustrated in Figure 2. In the chronic HDM model, male mice were given 40 µg of HDM extract four days a week for seven weeks. In the acute *Alternaria* model, female mice were given 25 µg of extract on days 0, 3, 6, and 9. In the IL-33 model, male mice were given 1 µg of cytokine every day for four consecutive days. All mice were sacrificed 24 hours after the last treatment. In certain cases, mice were given 50 µg of *Alternaria* in a single 50 µL dose (25 µL per nostril) and sacrificed one hour after i.n. treatment.

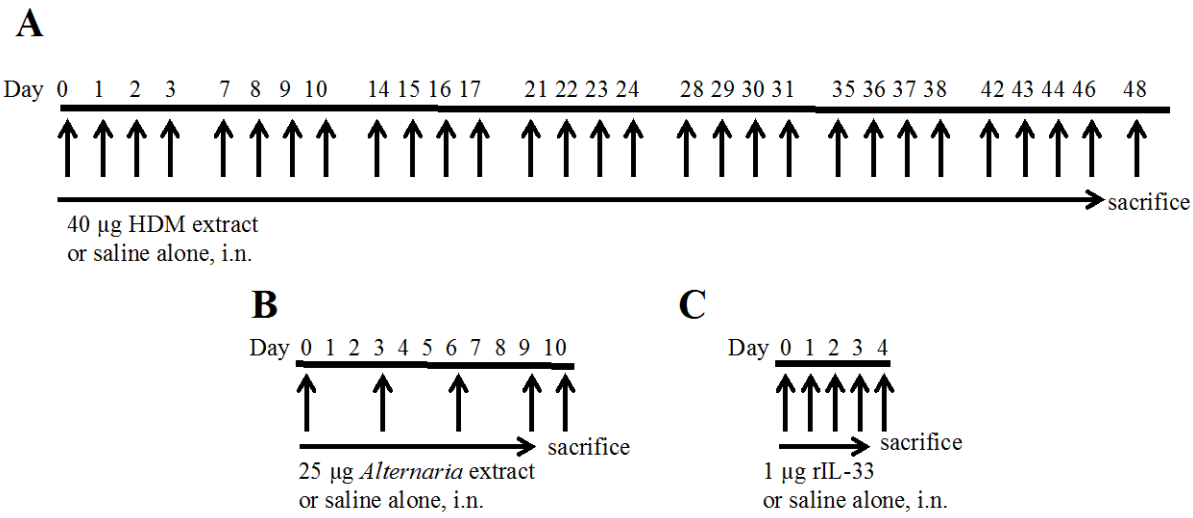


**Figure 2. Mouse models of allergic airway inflammation**

The volume of each dose of allergen, cytokine, or saline alone was 25 µL. All doses were given intranasally, and mice were sacrificed 24 hours after the last treatment. (A) Male mice were given 40 µg of HDM extract in saline or saline alone (control) four days a week for seven weeks. (B) Female mice were given 25 µg of *Alternaria* extract in saline or saline alone on days 0, 3, 6, and 9. (C) Male mice were given 1 µg of recombinant mouse IL-33 (rIL-33) or saline alone for four consecutive days.

### 3.1.3 sRAGE studies

Mouse soluble RAGE was purified in-house and was administered alongside allergen in an attempt to reduce the inflammatory response in both the IL-33 and *Alternaria alternata* models of AAI (described above). C57BL/6 wild-type mice were each given 25 μg of mouse sRAGE simultaneously with allergen (either 1 μg of mouse rIL-33 or 25 μg of *Alternaria alternata* extract) in 40 μL of saline intranasally. Twelve-week-old male mice received these treatments for four consecutive days and were sacrificed 24 hours after the last treatment. Eight-week-old female mice received these treatments on days 0, 3, 6, and 9 and were sacrificed on day 10. Proper single-reagent solution controls were run in parallel, as well as a control solution containing both the allergen and 25 μg of mouse serum albumin (MSA) to test for the effects of nonspecific protein interactions.

### 3.1.4 VCAM-1 blocking antibody studies

This experiment was carried out using both the IL-33 AAI model (male mice, treatment with 1 μg of rIL-33 (Biolgend) for four consecutive days) and the *Alternaria* model (female mice, treatment with 25 μg of *Alternaria alternata* extract (Greer) on days 0, 3, 6, and 9). A rat anti-mouse VCAM-1 blocking antibody (clone M/K-2, Millipore) or isotype control (rat $IgG_1$ κ, Biolegend) was diluted in sterile saline such that a dose of 1.5 mg/kg animal weight could be administered in 100 μL volumes to each mouse.[290] The VCAM-1 antibody was given via intravenous (i.v.) tail vein injection one hour prior to intranasal treatment with allergen or cytokine. Three control groups were also utilized: (1) one that received i.n. saline alone with no i.v. antibody injection, (2) one that received i.n. allergen or cytokine alone with no i.v. antibody

injection, and (3) one that received an i.v. injection of the isotype control one hour prior to treatment with allergen or cytokine. All mice were sacrificed 24 hours after the final treatment.

### 3.1.5 Processing of animal tissues after treatments

In experiments where no pulmonary function testing was performed, mice were sacrificed with a single 150 μL intraperitoneal (i.p.) injection of a 1:8 dilution of Euthasol (0.5 mg/mL pentobarbital sodium and 62.5 μg/mL phenytoin sodium in each administered dose; Henry Schein Animal Health). To collect bronchoalveolar lavage fluid (BALF), a small hole was cut into the trachea and a 24G cannula was inserted and sutured into place. Eight hundred microliters (0.8 mL) of sterile 0.9% saline was instilled into the lungs via the cannula and withdrawn. A small amount of BALF was removed for cytospin (described in Section 3.4.1) and the remainder was centrifuged at 10,000 rpm for 10 minutes at 4°C. The BALF supernatant was separated from the cell pellet and both were stored at -80°C for later use. The left lung was tied off at the bronchus, removed from the animal, and either flash-frozen in liquid nitrogen or immediately processed for flow cytometry. The right lung was inflation fixed *in situ* via the trachea with 0.6 mL of 10% formalin for eight minutes, removed from the animal, and then submerged in 10% formalin for four hours to continue fixation. Fixed tissue was then placed in 70% ethanol and given to the University of Pittsburgh's Research Histology Services for further processing. In cases were histology samples were not needed, the right lung was simply removed from the animal without fixation and flash-frozen in liquid nitrogen for future use.

### 3.1.6 Pulmonary function testing

Pulmonary function testing was performed with the help of Michelle Manni, Ph.D. at the Alcorn Lab in the Department of Pediatrics at the Children's Hospital of Pittsburgh. A flexiVent machine (SCIREQ) was used as previously described.[291] Mice were anesthetized with an i.p. injection of a 1:10 dilution of sodium pentobarbital (~90 mg/kg), tracheostomized, cannulated with an 18G IV adapter, and attached to the clean, calibrated flexiVent machine. The animals were ventilated at a rate of about 200 breaths/minute with a tidal volume of 0.25 mL and a positive-end expiratory pressure (PEEP) of approximately 3 cm $H_2O$. Pressure and volume measurements were taken and multiple linear regression was used to fit the pressure-volume curve of each mouse to a mathematical model of lung motion (coefficients of determination <0.85 were discarded). A methacholine challenge was then initiated. A nebulizer was used to administer increasing doses of methacholine (Sigma) (0, 0.75, 3.125, 12.5, and 50 mg/mL) to the mice. For each aerosolized dose, the tidal volume was increased to 1.0 mL and the respiratory rate was slowed to allow for maximum exposure to the methacholine over a period of two seconds. This perturbation was applied every ten seconds over the course of three minutes to measure the response. Data for Newtonian resistance (Rn), elastance (H), and tissue damping (G) were collected at each time point and were used to generate dose-response curves. BALF and lung tissue were then harvested from mice as described above.

### 3.1.7 Gastrointestinal studies

Nine-week-old male C57BL/6 wild-type and RAGE KO mice were treated with intraperitoneal injections of 0.5 μg of mouse rIL-33 (Biolegend) in 150 μL of saline for four consecutive days.

Twenty-four hours after the last treatment, mice were sacrificed with Euthasol as above. Peritoneal lavage was then carried out by injecting 10 mL of 2% fetal bovine serum (FBS) in saline into the peritoneal cavity with a 25 gauge needle and withdrawing the fluid with a 21 gauge needle. An aliquot of peritoneal lavage fluid was saved for cytospin and DiffQuik staining (see Section 3.4.1). The rest was centrifuged at 10,000 rpm for 10 minutes at 4°C to pellet the cells and the supernatant was saved for ELISAs (see Section 3.3.4). Spleens were removed and placed in 2% FBS in saline on ice and then processed for ILC2 cell counting by flow cytometry (see Section 3.1.8). BALF samples were taken, left lungs were flash frozen, and right lungs were fixed in 10% formalin as described above (Section 3.1.5). Small intestines were removed from the duodenum to the cecum, fecal material was rinsed out with saline, and the specimen was stored in 10% formalin until dissection. The intestines were cut longitudinally and then separated into three equal lengths and rolled onto a toothpick using the Swiss Roll method.[292] Samples were fixed for 6 hours in 10% formalin and then submitted to Research Histology Services for paraffin embedding and periodic acid Schiff staining.

### 3.1.8 Flow cytometry

#### 3.1.8.1 *In vitro* generation of Th2 cells and ST2 antibody titration

In order to determine the optimal concentration of the anti-mouse ST2-Percpefluor710 antibody (eBiosciences) to use for identifying ILC2s, T cells were isolated from a wild-type mouse spleen and differentiated *in vitro* into Th2 cells. These cells were then used to titrate the new ST2 antibody. An eight-week old male C57BL/6 mouse was sacrificed and the spleen was harvested. The spleen was mashed through a 70 μm cell strainer into a solution of phosphate-buffered saline (PBS) and 2% FBS. The spleen was passed through a second filter and then red blood cells were

lysed with ACK Lysing Buffer (Gibco). Cells were pelleted and then resuspended in PBS and 2% FBS and plated at a density of 0.5 million cells/mL. To stimulate T cell differentiation into Th2 cells, the following reagents were added to the cells in culture and allowed to incubate for 5 days: 1 μg/mL hamster anti-mouse CD3 (BD Biosciences), 5 μg/mL rat anti-mouse IFN-γ (BD Biosciences), 20 U/mL recombinant mouse IL-2 (Biolegend), and 100 ng/mL recombinant IL-4 mouse (Biolegend). After differentiation, ~3 x $10^6$ Th2 cells were lysed with cell lysis buffer (25 mM HEPES, 150 mM sodium chloride (NaCl), 5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1% Triton-X, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and a 1:100 dilution of a protease inhibitor cocktail (Sigma P8340, containing the following inhibitors: AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstain A), pH 7.5), and then 25 μg of cell protein was run on a Western blot (see details in Section 3.3.3) to assess RAGE expression.

The Th2 cells that were not frozen down were used for titration of the ST2 antibody. Aliquots containing 1 x $10^6$ cells were stimulated for 3-4 hours at 37°C with 50 ng/mL PMA (Sigma) and 750 ng/mL ionomycin (Sigma). One hour after stimulation was initiated, cells were treated with GolgiStop (BD Biosciences) at 1:1000 (1 μL per 1 mL) to halt cytokine secretion for the remainder of the stimulation incubation. Following stimulation, cells were pelleted and resuspended in 100 μL of PBS + 0.5% bovine serum albumin (BSA) + 2mM EDTA containing 2 μL of Fc receptor block (CD16/CD32) for ten minutes at room temperature. Extracellular staining was then carried out for 30 minutes at 4°C in the dark with five cocktails containing a 1:100 dilution of PE-conjugated anti-mouse CD4 antibody (eBiosciences) and then varying concentrations of the anti-mouse ST2-Percpefluor710 antibody (eBiosciences) (concentrations tested were 1:1000, 1:200, 1:80, 1:50, and 1:25). The cells were then washed and resuspended in fixation/permeabilization solution (eBiosciences) for 30 minutes in the dark at 4°C. Intracellular

cytokine staining was then carried out using an APC-conjugated anti-mouse IL-5 antibody (IL-5) diluted 1:50 in permeabilization buffer at 4°C for 30 minutes in the dark. Flow cytometry to identify CD4+ST2+IL-5+ Th2 cells was carried out with the help of Dr. Anupriya Khare in the Ray Lab at the University of Pittsburgh School of Medicine. Cell brightness and labeling efficiency was analyzed. It was decided that the best concentration for future ST2 antibody staining in lung ILC2s would be a 1:33 dilution (3 μL in 100 μL of staining buffer).

#### 3.1.8.2 ILC2 counts in the lung

For whole lung single cell suspension preparation, left lungs were harvested from mice into 1.0 mL of PBS. Lungs were diced into small pieces and digested in 12 mL of a 1 mg/mL collagenase media (50 mL Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) + 50 mg collagenase (Sigma)) for 30 minutes at 37°C. The digested tissue was then passed through a 70 μm filter. Red blood cells were lysed with ACK Lysing Buffer (Gibco), and the solution was passed through another 70 μm filter. Single cell suspensions were then treated with Fc receptor block CD16/CD32 (eBioscience) for 10 minutes at room temperature to prevent nonspecific antibody binding. Cells were stained for 30 minutes in the dark at 4°C with the following antibody cocktail: phycoerythrin (PE)-conjugated anti-mouse lineage cocktail (containing antibodies against CD3ε (clone 145-2C11) , Ly-6G/Ly-6C (clone RB6-8C5), CD11b (clone M1/70), CD45R/B220 (clone RA3-6B2), TER-119 (clone Ter-119), Biolegend), 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 (APC)-conjugated CD90.2 (Thy1.2) (clone 53-2.1, BD Bioscience), and peridinin chlorophyll protein (PerCP)-eFluor710-conjugated ST2 (IL-33R) (clone RMST2-2, eBioscience). ILC2s were counted as cells that were lineage negative, CD90.2 positive, and ST2

positive (PE-, APC+, PerCP-eFluor710+). Unstained controls, isotype controls and bead color controls were also prepared. All staining was carried out in PBS + 0.5% bovine serum albumin (BSA) + 2mM EDTA. Cells were counted on an LSRII machine (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.).

#### 3.1.8.3 ILC2 counts in the spleen

For splenocyte preparation, whole spleens were removed from mice after i.p. IL-33 treatment and mashed through a 70 μm filter into a solution of PBS and 2% FBS. The filter was rinsed and the solution was passed through a second 70 μm. RBC cell lysis and CD16/CD32 Fc-receptor blocking and cell staining was carried out as above for lung cells.

#### 3.1.8.4 Th2 cell counts in the lung

Left lungs were removed from animals after allergen treatment and placed in 1.0 mL of PBS on ice. Collagenase digestion and processing of a single cell lung suspension was carried out as above for lung ILC2s. Following RBC lysis, cells were washed with PBS, passed through a 70 μm filter, and pelleted. The cells were resuspended in 1.0 mL of T cell media (DMEM + 10% FBS + 1% penicillin/streptomycin) and stimulated for 3-4 hours at 37°C with 50 ng/mL PMA (Sigma) and 750 ng/mL ionomycin (Sigma). One hour after stimulation was initiated, cells were treated with GolgiStop (BD Biosciences) at 1:1000 (1μL per 1 mL) to halt cytokine secretion for the remainder of the stimulation incubation. Following stimulation, cells were pelleted and resuspended in fixation/permeabilization solution (eBiosciences) to allow for intracellular staining. The cells were incubated at 4°C for 30 minutes in the dark. Cells were then washed with permeabilization buffer (eBioscience), pelleted, and treated with Fc receptor block (CD16/CD32) for ten minutes at room temperature. Cells were then washed, pelleted, and

stained for 30 minutes in the dark at 4°C with the following antibody cocktail: PE-conjugated anti-mouse CD4 (clone GK1.5, eBioscience), PE-Cy7-conjugated anti-mouse IL-4 (clone 11B11, BD Biosciences), APC-conjugated anti-mouse IL-5 (clone TRFK5, BD Biosciences), and eFluor450-conjugated anti-mouse IL-13 (clone eBio13A, eBioscience). Unstained controls, isotype controls and bead color controls were also prepared. Stained cells were washed, pelleted, and resuspended in PBS + 0.5% BSA + 2mM EDTA and counted on an LSRII machine (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.). CD4+ cells were examined for expression of each of the type 2 cytokines: IL-4, IL-5, and IL-13.

#### 3.1.8.5 Flow-activated cell sorting from the lung

Eight wild-type male C57BL/6 mice were treated with IL-33 intranasally as described above to stimulate ILC2 expression. Sixteen lungs were digested in collagenase media and processed into a single cell suspension as above. After CD16/CD32 block, cells were stained only with the PE-conjugated lineage antibodies for 30 minutes in the dark at 4°C. The suspensions were pooled and then treated with anti-PE magnetic microbeads (Miltenyi Biotec) and passed over a MACS LS Separation Column (Miltenyi) to deplete lineage positive cells. The flow-through was incubated with the CD90.2 and ST2 antibodies for 30 minutes in the dark at 4°C. A FACSAria III Cell Sorter (BD Biosciences) was used to isolate lineage negative, CD90.2 positive, ST2 positive ILC2s.

#### 3.1.8.6 Flow-activated cell sorting from the spleen

Wild-type and RAGE KO mice were treated as above for gut studies with three consecutive days of intraperitoneal injections of 1 μg mouse rIL-33 in saline. Twenty-four hours after the last

treatment, the mice were sacrificed with Euthasol and spleens were harvested into a solution of HBSS + 2% FBS.

A single cell suspension was created by mashing the spleens through a 70 μm cell strainer, and then passing the solution through a second 70 μm strainer. Red blood cells were lysed with ACK lysing buffer (Gibco), and the reaction was quenched with 30 mL of cold PBS + 2% FBS then filtered through a third 70 μm cell strainer. Lineage cell depletion was carried out on the cells using a lineage cell depletion kit (Miltenyi #130-090-858). Briefly, the cells were incubated with 10 μL of a biotin-conjugated lineage antibody cocktail (containing monoclonal antibodies against CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly6G/C), 7-4, and Ter-119) per every $10^7$ cells in magnetic bead staining buffer (PBS + 0.5% BSA + 2mM EDTA) for 10 minutes at 4°C. Next, 20 μL of anti-biotin microbeads were added in magnetic bead staining buffer per $10^7$ cells. This mixture was incubated at 4°C for 15 minutes and then the reaction was quenched with 1.5 mL magnetic bead staining buffer per $10^7$ cells. Cells were pelleted and resuspended in 0.5 mL of magnetic bead staining buffer. Lineage positive cells were removed using an AutoMACS machine (Miltenyi; located in the Thomas E. Starzl Transplantation Institute (STI) Flow Cytometry Core Facility).

The resulting cells were treated with a 1:50 dilution of anti-CD16/CD32 antibody to block Fc receptors and then were stained for extracellular markers as above (same cocktail that was used to identify ILC2 numbers in lung) for 30 minutes at 4°C in the dark. Lineage negative, CD90.2+, ST2+ ILC2s were sorted into FBS at the Children's Hospital of Pittsburgh Rangos Research Center on a FACSAria III Cell Sorter (BD Biosciences). Unfortunately, the machine broke down and no samples could be sorted from the RAGE KO mice.

## 3.2 CELL CULTURE

### 3.2.1 ILC2s

ILC2s were flow-sorted from wild-type mouse spleens after intraperitoneal IL-33 treatment as described (see Section 3.1.8). Cells were cultured for 6 days at 37°C with 5% $CO_2$ in ILC2 growth media (RPMI-1640 media + 10% FBS + 1% penicillin/streptomycin + 2 mM L-glutamine + 50 mM β-mercaptoethanol + 150 ng/mL mouse rIL-33 (Biolegend) + 50 ng/mL recombinant mouse IL-2 (Biolegend) + 50 ng/mL recombinant mouse IL-7 (Biolegend)). Media was changed daily and was saved to monitor cytokine production by ELISA (see below). Cells were counted daily on a hemocytometer to observe proliferation *in vitro*.

### 3.2.2 Generation of advanced glycation endproducts for use in cell culture

To generate AGEs, 25 mg/mL of BSA and 0.5 M glucose was dissolved in 100 mL of PBS, sterile filtered, and stored in a 37°C incubator for 28 weeks (≥ 4 weeks is sufficient time for AGEs to form).[130, 138, 293] A control solution of BSA without glucose was also prepared. After incubation, formation of BSA-AGEs was confirmed by measuring protein concentration (Bradford Assay) and fluorescence (excitation/emission at 370 nm/440 nm). A >10-fold increase in the fluorescence ratio between glucose-treated BSA and control is sufficient to confirm the presence of AGEs; the fluorescence ratio here was 63. Finally, the solutions were run over a Detoxi-Gel Resin column (Thermo Scientific) to remove endotoxin contamination before use on cells. A limulus amebocyte lysate (LAL) endotoxin gel-clotting test (Pyrotell #GS003) was

performed according to the manufacturer's instructions to confirm that the endotoxin level was <0.03 EU/mL.

### 3.2.3 Human umbilical vein endothelial cells (HUVECs)

#### 3.2.3.1 Culture conditions

Cryopreserved HUVECS (ATCC CRL-2480, passage 2) were thawed and cultured on 0.1% gelatin-coated plates (Sigma G1393) in HUVEC media (F12K media + 10% FBS + 0.03 mg/mL endothelial growth supplement (Sigma E2759) + 0.1 mg/mL heparin (Sigma H3149) + 1% L-glutamine) at 37°C. Media was changed every other day and the cells were passaged at least once after thawing before being used in an experiment.

#### 3.2.3.2 Collection of cellular lysate

One 75 $cm^2$ flask of HUVECs (~5 x $10^6$ cells) was lysed with 1 mL of RIPA Buffer (Thermo Scientific) containing protease inhibitors (1:100 dilution of Halt Protease/Phosphatase Inhibitor Cocktail and 1:100 dilution of EDTA) (Thermo Scientific)) for five minutes. The cells were scraped and the lysate was collected in a centrifuge tube and spun down at 14,000 x g for 15 minutes at 4°C to pellet debris. Supernatant was saved for Western blot analysis (see Section 3.3.3).

#### 3.2.3.3 Cellular stimulation assays

HUVECs (passage 5) were plated at a density of ~2 x $10^5$ cells per well in two 6-well tissue culture plates coated with 0.1% gelatin. Cells were given two days to adhere before treatments. To stimulate the cells, they were cultured for 3 hours at 37°C with 100 μg/mL[138] BSA-AGEs

(see "Generation of AGEs" above), 100 μg/mL[138] BSA negative control, 10 ng/mL[49] human rIL-33 (Biolegend), or 0.1 μg/mL[294] HMGB1 (gift to lab). A second rIL-33 well was also set-up and incubated for only one hour. Following incubation, cell media was suctioned off of the cells and saved for IL-33 ELISA studies (see Section 3.3.4). Cells were lysed with 100 μL of RIPA Buffer as described above and the supernatant was stored at -80°C.

A second stimulation assay was run using a time course to assess when stimulation occurs. Cryopreserved HUVECs (passage 3) were thawed and grown to passage 5 in HUVEC media. Cells were plated on four 6-well plates coated with 0.1% gelatin at a density of ~2 x $10^5$ cells per well. Cells were given two days to adhere to the plates and then stimulation was initiated. Cells were treated with BSA-AGEs, IL-33, and HMGB1 (same concentrations as described in the three-hour assay above). This time, however, a positive control treatment was added: 10 ng/mL of TNF-α (Sigma). Cell lysate for each of the four conditions was collected as above with RIPA Buffer at 0, 5, 15, 30, 60, and 120 minutes after the start of the stimulation.

### 3.2.4 Human lung microvascular endothelial cells (HLMVECs)

#### 3.2.4.1 Culture conditions

HLMVECs were originally purchased from Lonza (CC-2527), but a cryopreserved sample (passage 4) was generously provided by Dr. Claudette St. Croix in the Center for Biological Imaging at the University of Pittsburgh. Cells were grown in Endothelial Growth Media (EGM-2MV Bullet Kit, Lonza CC-3202) containing human endothelial growth factor (hEGF), hydrocortisone, gentamicin, amphotericin-B, FBS, vascular endothelial growth factor (VEGF), human fibroblast growth factor B (hFGF-B), recombinant human long $R^3$ insulin-like growth factor-1 ($R^3$-IGF-1), ascorbic acid, and heparin. Media was changed every other day. One

confluent 10 $cm^2$ plate of cells (passage 5) was lysed with 733 μL RIPA Buffer as described above and supernatants were saved for Western blot.

#### 3.2.4.2 Cellular stimulation assay

HLMVECs (passage 5) were plated at a density of ~1 x $10^4$ cells per well on four 6-well culture dishes. Cells were given four days to adhere and proliferate before treatment. Cells were treated with 10 ng/mL TNF-α (Sigma), 0.1 μg/mL human HMG-1 (Sigma), or 10 ng/mL human rIL-33 (Biolegend) in endothelial growth media. Reactions were stopped by removing the media from the cells at 0, 5, 15, 30, 60, 120, and 180 minutes after stimulation was begun. Cells were trypsinized with 500 μL 0.25% trypsin/EDTA (Life Technologies), quenched with endothelial growth media, and pelleted. Cell pellets were stored at -80°C until RNA isolation and qRT-PCR processing (see below).

## 3.3 BIOCHEMICAL ANALYSES

### 3.3.1 Tissue homogenization

Frozen lungs were homogenized rapidly in ~1.0 mL of cold CHAPS buffer (50 mM Tris-HCL, 150 mM NaCl, 10 mM CHAPS in distilled water, pH 7.4) with protease inhibitors (100 μM 3,4-dichloroisocoumarin (DCI), 10 μM, *trans*-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), and 1 mM 1,10 phenanthroline). The homogenate was incubated at 4°C on a rotary mixer for at least two hours. Debris was sedimented by spinning the homogenate at 20,000 x g for 20 minutes at 4°C. The supernatant was frozen at -80°C until use.

### 3.3.2 Bradford assay

Protein concentrations of lung homogenate, cell lysates, and BALF were calculated using a Bradford assay. Lung homogenates and cell lysates were diluted 1:10 in PBS before being read, while BALF was not diluted. Ten microliters of each sample were plated on a 96-well plate along with bovine serum albumin standards diluted in the appropriate buffer (0-1.0 mg/mL). Two hundred and ninety microliters of Coomassie Plus Assay Reagent (Pierce) was added to each well and incubated at room temperature for five minutes. The plate was then read on a plate reader at a wavelength of 595 nm using SoftMax Pro software (Molecular Devices, LCC).

### 3.3.3 SDS-PAGE and immunoblotting

#### 3.3.3.1 SDS-PAGE

Proteins were separated in an ammediol (2-amino-2-methyl-1,3-propanediol)-buffered polyacrylamide gel electrophoresis system. Five- to fifteen-percent polyacrylamide gradient gels were made and used within one month. Twenty-five to thirty micrograms of each protein sample was mixed with sodium dodecyl sulfate (SDS) buffer (1% SDS in final sample concentration) and 50 mM dithiothreitol (DTT), boiled for ten minutes, and then loaded into each well of a stacking gel. For non-reducing gels, DTT was not added. Two buffers were used to run the proteins through the gel. In the upper compartment of the gel apparatus, the buffer contained 200 mM glycine, 200 mM ammediol, and 0.5% SDS. The lower reservoir buffer contained 310 mM ammediol and 2% concentrated hydrochloric acid (HCl). Gels were run for two hours at constant milliamperes (25 mA for one gel and 50 mA for two gels), or until the dye front ran off of the gel.

#### 3.3.3.2 Immunoblotting

Polyvinylidene difluoride (PVDF) membranes were activated for five minutes in methanol before use. For transfer of protein from PAGE gel to membrane, gels were equilibrated in CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) gel blotting buffer (10 mM CAPS, pH 11 and 10% methanol) for fifteen minutes. Gels and membranes were sandwiched between sponges and blotting paper, then submerged in blotting buffer in a chamber with constant cooling. Protein was allowed to transfer over a 50-minute period at 500 mA. Ponceau S was used to confirm successful transfer of proteins to the membrane. To do this, blots were incubated in a solution containing 0.2% Ponceau S and 3% trichlorocacetic acid for twenty minutes and then destained for two five-minute washes in destain solution (0.04% methanol and 0.0075% glacial acetic acid). Membranes were rinsed with water, dried, and scanned.

The membrane was then reactivated in methanol for five minutes and washed in PBS + 3% Tween-20 (PBST) for three ten-minute washes. Blots were then blocked in 5% milk in PBST overnight at 4°C. The blots were washed three times, ten minutes per wash, with PBST and then incubated with primary antibody (details below) at room temperature for one hour. In some cases blots were incubated in primary antibody overnight at 4°C; in these instances, the blocking condition was also changed to one hour at room temperature. After further washing, the blot was then incubated for one hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (details below). After a final set of three ten-minute washes, the blots were developed using enhanced chemiluminescence (ECL) HRP substrate (SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) or ECL Plus Substrate (Pierce)). Blots were imaged on a Kodak Gel Logic 2200 Imaging System. When appropriate, blots were stripped with stripping buffer (25 mM glycine, 1% SDS, pH 2.0), blocked with 5%

milk, and reprobed with a monoclonal mouse anti-β-actin primary antibody (1:5000 in PBST, one hour at room temperature; Sigma A5316) for normalization. Quantification was carried out using NIH ImageJ software.

In addition to the β-actin antibody listed above, the following primary antibodies were used at the following concentrations for one hour at room temperature unless otherwise specified: 1:1000 rabbit anti-HMGB1 (Abcam #18256) in PBST; 1:1000 monoclonal mouse anti-phospho-IκBα (Ser32/26) (Cell Signaling #9246) overnight at 4°C in 5% milk in PBST; 1:1000 monoclonal mouse anti-IκBα (Cell Signaling #4814) overnight at 4°C in 5% milk in PBST; 1:2000 polyclonal goat anti-mouse ICAM-1 (CD54) (R&D Systems AF796) in PBST; 1:500 goat anti-mouse IL-33 (R&D Systems AF3626) in PBST; 1:5000 rabbit anti-mouse RAGE (GenScript 35131-9B) in PBST; 1:100 rabbit anti-human RAGE (H300; Santa Cruz sc-5563) in PBST; 1:1000 goat anti-human ST2 (R&D AF523) in PBST; 1:1000 rat anti-mouse VCAM-1 (R&D Systems MAB6434) in PBST; 1:2000 goat anti-mouse VCAM-1 (R&D Systems AF643) in PBST; and 1:500 monoclonal mouse anti-human VCAM-1 (R&D BBA5) overnight at 4°C in 5% milk in PBST in non-reducing conditions (no DTT).

The following HRP-conjugated secondary antibodies were used at the following concentrations for one hour at room temperature: 1:5000 goat anti-rabbit IgG (Bio-Rad 170-6515) in 1% milk; 1:5000 rabbit anti-mouse IgG (Jackson 315-035-003) in 1% milk or 1:10,000 anti-mouse IgG in PBST; 1:5000 donkey anti-goat IgG (Jackson 705-035-147) in PBST or in 1% milk in PBST; and 1:5000 rabbit anti-rat IgG (Jackson 312-035-003) in PBST.

### 3.3.4 Enzyme-linked immunosorbent assay (ELISA)

Kits were purchased from BD Biosciences (mouse IL-4 and IL-5), eBioscience (mouse IL-13), or R&D Systems (mouse IL-2, mouse IL-7, mouse IL-33, and human IL-33). Samples were plated on 96-well polystyrene high-binding plates (R&D Systems). ELISAs were carried out on undiluted BALF, undiluted peritoneal lavage fluid, or 1:1000 dilutions of cell culture media according the manufacturer's instructions with two minor changes: (1) For all ELISAs, samples were incubated on the plates overnight at 4°C instead of at room temperature for two hours and (2) for the BD Biosciences and R&D Systems ELISA kits, the signal was detected with 150 μL SigmaFast OPD reagent (peroxidase substrate; Sigma P9187) for 30 minutes in the dark instead of adding substrate solution and stop solution separately. Each plate was read on a plate reader at an absorbance of 450 nm and analyzed using SoftMax Pro software (Molecular Devices, LCC). Cytokine concentrations in the BALF, peritoneal lavage fluid, or cell media were calculated by comparing the absorbance of each sample to a standard curve generated from known cytokine standard concentrations.

### 3.3.5 RNA isolation and qRT-PCR

RNA was isolated using the Qiagen RNeasy Mini Kit. Cell pellets were lysed in 350 μL Buffer RLT and homogenized in QIAshredder spin columns (Qiagen). RNA was isolated as per manufacturer's instructions and eluted into 30 μL of nuclease-free water. A 1:20 dilution of the isolated nucleic acid was used to obtain the absorbance of the sample at 260 and 280 nm. The purity of the isolated sample (i.e. how much protein contamination is in the sample) was calculated by evaluating the ratio of absorbance at 260 nm to absorbance at 280 nm for each

sample. RNA concentration for each sample was calculated with the following formula: absorbance at 260 nm x dilution factor x 44 ug/mL (because one absorbance unit at 260 nm is equal to 44 μg/mL of RNA according to the Qiagen Mini Kit handbook). In experiments where RNA yields were small (i.e. from ILC2s), RNA concentration was measured on a nanodrop machine.

An equal amount of RNA (<1 μg) from each sample was taken for reverse transcription. The RNA was combined with a master mix containing 10X reverse transcriptase buffer, 25 mM magnesium chloride, 10 mM dNTPs, 20 U/μL RNase inhibitor, 50 U/μL Multiscribe reverse transcriptase enzyme, and 50 μM random hexamers (Applied Biosystems/Life Technologies). Reverse transcription was carried out in a Techne thermocycler using the following program: 42°C for 40 minutes, 95°C for 5 minutes, 5°C for five minutes. When RNA yields were low (i.e. from ILC2s), reverse transcription was carried out using SuperScript VILO Master Mix (Invitrogen/Life Technologies) because of its increased sensitivity for very small amounts of RNA. The VILO reverse transcription reaction was carried out in the Techne thermocycler using the following program: 25°C for 10 minutes, 42°C for 60 minutes, 85°C for five minutes.

Quantitative real-time PCR was carried out on 1-100 ng of cDNA (samples were diluted as needed with nuclease-free water, and equal amounts were taken for each sample). cDNA was mixed with a master mix containing 2X Taqman Universal PCR Master Mix (Life Technologies), Taqman 20X single tube gene expression assay reagent (Life Technologies), and nuclease-free water. Each Taqman gene expression assay reagent contained probes/primers for a specific gene of interest. The following primers were used: human VCAM1 (Hs01003372_m1); human AGER (RAGE) (Hs00542584_g1); human IL1RL1 (ST2) (Hs00545033_m1); human GAPDH (Hs02758991_g1); mouse AGER (RAGE) (Mm00545815_m1); mouse IL-4

(Mm00445259_m1); mouse IL-5 (Mm00439646_m1); mouse IL-13 Mm00434204_m1); and mouse GAPDH (Mm99999915_g1). Samples were plated in triplicate and GAPDH-probed controls were plated in duplicate on a MicroAmp Optical 96-well Reaction Plate (Life Technologies). The plate was sealed with an adhesive film, gently vortexed, and centrifuged for five minutes at 2000 rpm. Plates were run on a 7300 Real-time PCR System (Applied Biosystems) with the following program: 50°C for 2 minutes; 95°C for 10 minutes; then 40 cycles of 95°C for 15 seconds and 60°C for one minute. Data was analyzed using the ΔΔCt method, where a sample probed with the gene of interest was compared to the same sample probed with GAPDH. Baseline conditions (i.e. wild-type saline-treated animals or untreated cells) were arbitrarily set to a fold-change of 1.0.

### 3.3.6 Binding studies

#### 3.3.6.1 sRAGE hybrid ELISA

A hybrid ELISA was set up to assess sRAGE binding to various cytokines of interest based on previously published methods.[114, 295, 296] A high-binding polystyrene 96-well plate (R&D Systems) was coated with 300 nM of the following molecules diluted in PBS (50 μL per well): mouse rIL-33 (Biolegend), recombinant mouse IL-25 (R&D Systems), recombinant mouse TSLP (eBiosciences), mouse HMGB1 (gift to the lab), and bovine serum albumin (BSA) (Pierce). The molecules were plated along the length of a column in duplicate or triplicate. The plate was incubated at 4°C overnight. The following day, the plate was washed three times with PBST (300 μL per well) and then blocked in PBS/10% BSA (200 μL per well) for one hour at 37°C with rocking. The plate was again washed three times with PBST. Various concentrations of purified mouse sRAGE (0 nM, 1 nM, 10 nM, 30 nM, 60 nM, 100 nM, 300 nM, 600 nM) in

PBS/10% BSA were then added to the plate (each concentration extending along the length of a row), and the plate was incubated at 37°C with rocking for 90 minutes. The plate was washed three times with 300 µL per well of PBST. A 1:2500 dilution of a goat anti-mouse RAGE antibody (#3) in PBS/0.2% BSA was added at a volume of 60 µL per well. The plate was incubated at 37°C with rocking for one hour and then washed with PBST three times. A 1:20,000 dilution of an HRP-conjugated donkey anti-goat IgG antibody (Jackson 705-035-147) in PBS/0.2% BSA was added to the plate, and the samples were incubated at 37°C with rocking for one hour. The wells were washed three times with PBST and then incubated with 100 µL per well of SigmaFast OPD peroxidase substrate (Sigma P9187) for 30 minutes at room temperature in the dark. The plate was read on a plate reader at an absorbance of 450 nm and analyzed using SoftMax Pro software (Molecular Devices, LCC).

#### 3.3.6.2 Biacore

Biacore binding studies were carried out with Dr. Eric Goetzman at Children's Hospital of Pittsburgh on a Biacore 3000 platform (GE Healthcare). CM5 sensor chips (GE Healthcare) were warmed, coated with running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20; GE Healthcare), and primed twice. The surface of the chip was activated with a 1:1 mixture of EDC amine coupling reagent (0.4 M 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride in water) and NHS amine coupling reagent (0.1 M N-hydroxysuccinimide in water) for 7 minutes at a rate of 10 µL/minute. Mouse sRAGE (purified in-house) was diluted to 50 µg/mL in 10 mM acetate, pH 5.5, and then injected onto the chip for seven minutes at a rate of 10 µL/minute. Excess reactive groups on the chip were deactivated with an injection of 1M ethanolamine-HCl, pH 8.5 for 7 minutes at a rate of 10 µL/minute. The chip was washed for 1 minute with 1M NaCl and then 5 µM of mouse rIL-33 (Biolegend) was

injected onto the chip for 3 minutes at 20 μL/minute and binding was assessed. The reciprocal of this procedure was also attempted to see if IL-33 could be attached to the chip to assess for RAGE binding in the final step, but IL-33 could not bind to the activated chip. Finally, with sRAGE bound to the chip, 5 μM of mouse HMGB1 (gift to our lab) was injected as the ligand in the final step for 3 minutes at a rate of 20 μL/minute as a positive control.

#### 3.3.6.3 ForteBIO Octet

ForteBIO studies were carried out in the Lotze Lab at the University of Pittsburgh Hillman Cancer Center to analyze possible IL-33/sRAGE binding kinetics based on a previously described method.[113] Amine-reactive tips (ForteBIO #18-5029) were hydrated for 5 minutes in PBS in a black, polypropylene 96-well plate (Greiner Bio-One #655209). A second plate was loaded into the machine with 200 μL per well of the proteins of interest and wash buffers: columns 1, 4, and 6 contained PBS for washing, baseline readings, and dissociation steps; column 2 contained the protein to be loaded onto the tip (1 μM mouse sRAGE, purified in-house); column 3 contained 100 nM BSA in PBS for quenching; and column 5 contained the protein to be ligated to sRAGE (1 μM mouse rIL-33 (Biolegend) or 1 μM mouse HMGB1 (gift to lab)). 100 nM of BSA was also used as a negative control for both receptor and ligand. The experiment was run at 30°C with the following program times: 60s for an initial baseline reading, 300s for sRAGE loading onto the tip, 100s of quenching the reaction in BSA, 60s of washing with PBS, 60s of a second baseline reading, 1800s for the association of the ligand, and 1800s for dissociation of the ligand into PBS. A kinetic analysis was carried out using the ForteBIO software and a graph was generated to express binding capacity on the needle over time. This experiment was repeated again using a higher concentration of rIL-33 (5 μM) to see if more ligand was required in order to observe sRAGE binding. It was also repeated a third time in

a reciprocal fashion in which either rIL-33 or HMGB1 was loaded onto the amine reactive tips and then dipped into sRAGE during the association step.

## 3.4 HISTOLOGICAL ANALYSES

### 3.4.1 Lavage cytospins and cell differential

Immediately after lavage fluid was removed from the animal, a small aliquot was taken for cytospins. The sample was diluted 1:2 in most cases, and 1:5 or 1:10 in cases where large cellular inflammatory responses were expected. One hundred microliters of diluted BALF or peritoneal lavage fluid was centrifuged onto glass slides at 750 rpm for 5 minutes in a Shandon Cytospin 4 Cytocentrifuge (Thermo Scientific). The slides dried overnight and then were stained using the Diff-Quik Stain Set (Siemens #B4132-1A). To do this, slides were submerged in the fixative solution (1.8 mg/mL triarylmethane dye in methanol) for 1 minute, “Solution 1” (1g/L xanthene dye with 0.01% sodium azide) for 30s, and “Solution 2” (1.25 g/L thiazine dye mixture (0.625g/L azure A and 0.625 g/L methylene blue)) for 10s. The slides were dried overnight and then were coverslipped with Permount mounting media (Fisher).

To differentiate cell types in the lavage fluid, stained cytospin slides were observed on a light microscope using the 40X objective. The number of monocytes, neutrophils, lymphocytes, and eosinophils were counted in the field of view and recorded. This was repeated in nine more fields of view for a total of ten fields per sample. The percentage of each cell type in the sample was calculated by summing the total number of each cell type across all ten fields for that sample

and then dividing the sum of each cell type by the total number of all cells counted in that sample and finally multiplying by 100 (Example: Percentage of monocytes in sample 1 = (Σ monocytes in 10 fields from sample 1 / Σ all cells in 10 fields from sample 1) x100). To calculate the concentration of total cells in the BALF or peritoneal lavage fluid, it was first determined that 144 fields of view cover the entire area of cells in the cytospin sample. Therefore, the cellular concentration of the lavage fluid was determined by the following formula: (Total cells counted/number of fields counted) x total fields x dilution factor x (1/volume of lavage fluid used for cytospin). For example, if 200 total cells were counted in ten fields in a standard 1:2 dilution of BALF in a 100 μL cytospin sample, the cellular concentration of the BALF would be: (200 cells/10 fields) x 144 fields x 2 x (1/0.1 mL) = 57,600 cells/mL.

### 3.4.2 Hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) stains

All tissue processing and staining was carried out by the University of Pittsburgh's Research Histology Services. Briefly, formalin-fixed mouse lung or intestinal tissue was dehydrated with ethanol, cleared with xylenes, and then embedded in paraffin wax for sectioning. Sections were cut and mounted on glass slides before being deparaffinized with xylenes, rehydrated with ethanol and distilled water, and then stained with H&E or PAS.

### 3.4.3 Immunohistochemistry

#### 3.4.3.1 Lung fixation and freezing

Right lungs were inflation fixed *in situ* with 2% paraformaldehyde in PBS for 15 minutes. The lungs were then cut from the mouse and submerged in 2% paraformaldehyde for another 2 hours

at room temperature. Lung tissue was then placed in a 30% sucrose solution at 4°C overnight. The solution was changed two times during this period. The following day, the fixed lungs were placed on small squares of filter paper and flash frozen for 30 seconds in 2-methylbutane cooled with liquid nitrogen. The lungs were then further cooled in liquid nitrogen directly for 10 seconds. Samples were stored in small sample boxes at -80°C until sectioned.

#### 3.4.3.2 Cryosectioning

Fixed and frozen lung tissue was embedded in OCT, and 7 μm sections were cut using a cryostat microtome. Sections were placed onto labeled Superfrost Plus glass slides and stored at -20°C until used for immunofluorescence studies (below).

#### 3.4.3.3 Immunofluorescent labeling

Tissue sections were rehydrated with two washes of PBS and then permeabilized for ten minutes with 0.1% Triton X-100 in PBS. Tissue was blocked in 2% BSA for 45 minutes at room temperature and washed five times with PBS + 0.5% BSA buffer (PBB). Tissues were then incubated in primary antibody diluted in PBB overnight at 4°C. Goat anti-mouse VCAM-1 antibody (R&D AF643) was diluted 1:200, goat anti-mouse ICAM-1 (R&D AF796) was diluted either 1:25 or 1:50, rabbit anti-mouse RAGE (GenScript 35131-9B) was diluted 1:500 or 1:250, rat monoclonal anti-mouse CD31 (Abcam or Pitt's Center for Biological Imaging (CBI)) was diluted 1:500 or 1:100, and rat anti-major basic protein (MBP) monoclonal antibody (provided by Dr. J. Lee, Mayo Clinic) was diluted 1:500. After five washes with PBB, sections were then incubated for one hour at room temperature in the dark with the appropriate secondary antibody. The secondary antibodies included donkey anti-goat IgG conjugated to Cy3, donkey anti-rabbit IgG conjugated to Cy5, donkey anti-rat IgG conjugated to DyLight488, donkey anti-goat IgG

conjugated to Cy5, goat anti-rat IgG conjugated to Cy3, and donkey anti-rabbit IgG conjugated to AlexaFluor488. All secondary antibodies were from Jackson ImmunoResearch, Molecular Probes, or the CBI and were diluted 1:500 in PBB except for the donkey anti-goat Cy5 antibody, which was diluted 1:1000 in PBB. Tissue sections were washed five times with PBB and then five times with PBS to remove residual BSA protein prior to Hoechst staining. Tissue sections were stained with Hoechst (10 μg/mL) for 30 seconds and then washed with PBS four times. Gelvatol was used to adhere a coverslip to each slide. Slides were stored at 4°C in the dark until imaging.

#### 3.4.3.4 Imaging

Imaging was carried out on a Nikon Eclipse 90i advanced automated research microscope, an Olympus Fluoview 1000 confocal micrscope, or an Olympus IX71 inverted fluorescent microscope. All 90i imaging was done using a 20X objective; 10x10 fields were collected for all samples, except control samples, which were imaged as 4x4 fields. Confocal imaging was carried out using a 60X objective.

#### 3.4.3.5 Quantification

All quantification was done using NIS Elements software (Nikon).

For the RAGE-CD31 co-localization studies, a threshold level was set for the red channel (CD31) and another threshold was set for the green channel (RAGE). Each confocal image was analyzed using the same red and green thresholds. Binary area data was collected for each threshold (red and green) as well as for the intersection of the two thresholds (where RAGE and CD31 are both expressed).

For the VCAM-1 quantification, a region of interest (ROI) was drawn around each lung section such that large areas of empty space were excluded from the analysis. The threshold level for the TRITC (VCAM-1) channel was set and used for each image being analyzed. Each object (i.e. vessel) in the image was counted and the mean intensity was recorded. The ROI area was also recorded for each sample. Mean intensities were divided by ROI area to normalize. The data was further normalized such that the wild-type saline-treated group was set to 1.0 for easier comparison of values.

## 3.5 STATISTICS

Statistical analysis was performed using GraphPad Prism 5 and quantitative results are expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using 2-way ANOVA and, where appropriate, unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

# 4.0 RESULTS

## 4.1 RAGE KNOCKOUT MICE DO NOT DEVELOP ALLERGIC AIRWAY INFLAMMATION IN RESPONSE TO *ALTERNARIA ALTERNATA*

Previous studies have shown that RAGE KO mice do not develop AAI or AHR in a HDM model.[144] In the HDM model, mice were treated chronically: four times a week for seven weeks. In order to better investigate RAGE's role in the initiation of AAI, an acute model of AAI using *Alternaria alternata* was employed. *Alternaria* is a fungal allergen that is well-known to cause asthma exacerbations[19-21] and has been well established as a rapid trigger of AAI in mouse models.[33, 176, 186, 256]

Wild-type and RAGE KO mice were treated intranasally with four 25 μg doses of *Alternaria* or saline vehicle control over a ten-day period (Figure 2). Following treatment, eosinophilic inflammation, mucus hypersecretion, airway hyperreactivity, and type 2 cytokine levels were examined. As expected, BALF cellularity was increased in wild-type animals that were given *Alternaria*, and the majority (~70%) of these cells were eosinophils (Figure 3). H&E staining of lung tissue also revealed a large eosinophilic infiltrate around airways and vessels in the lung parenchyma (Figure 4). Immunohistochemical staining of lung tissue using an antibody against eosinophil major basic protein 1 (MBP-1) confirmed that these cells were indeed eosinophils (Figure 5). In contrast, RAGE KO mice treated with *Alternaria* were

indistinguishable from saline-treated controls: BALF cellularity was not increased from baseline and there were little to no eosinophils present in the lung tissue.

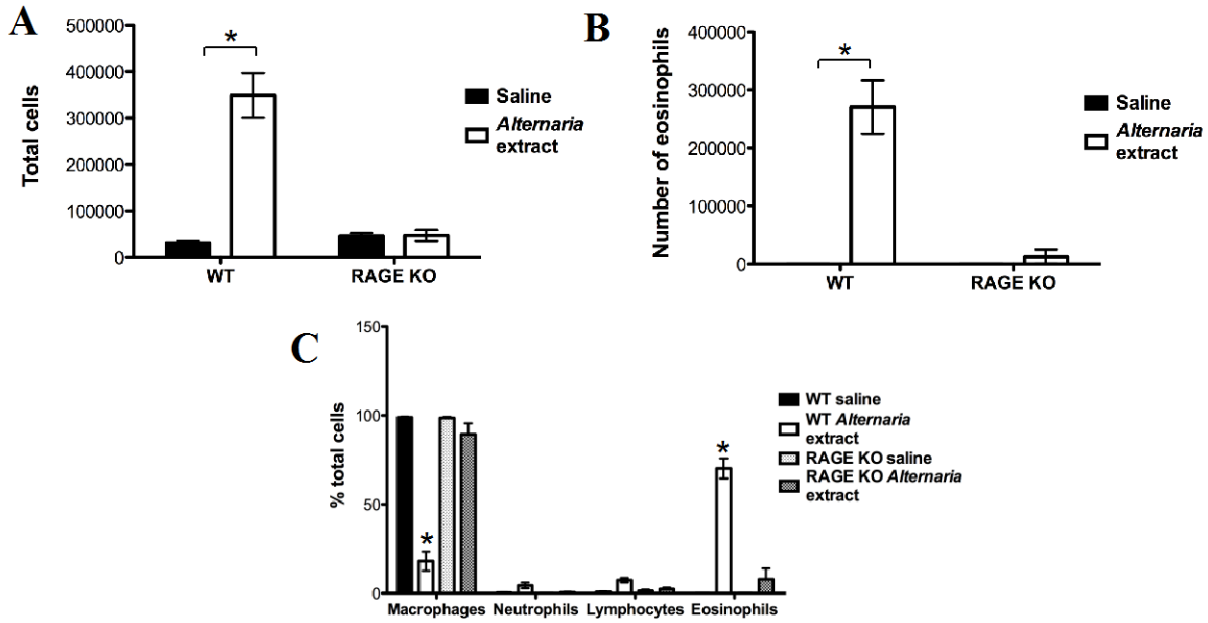


**Figure 3. Eosinophilic inflammation is present in the BALF of wild-type mice after *Alternaria* challenge.**

(A) Total cell counts and (B) total eosinophil counts in the BALF of wild-type (WT) and RAGE KO mice at Day 10 after intranasal challenge with saline or *Alternaria* extract. (C) Cell differential from the BALF illustrating that the majority of cells in the BALF are eosinophils only in wild-type, *Alternaria*-treated mice. In all cases results are expressed as means ± SEMs. $n$ = 7-14 mice per strain per treatment group. *$p < 0.05$ versus comparison. Experiment was replicated at least three separate times.

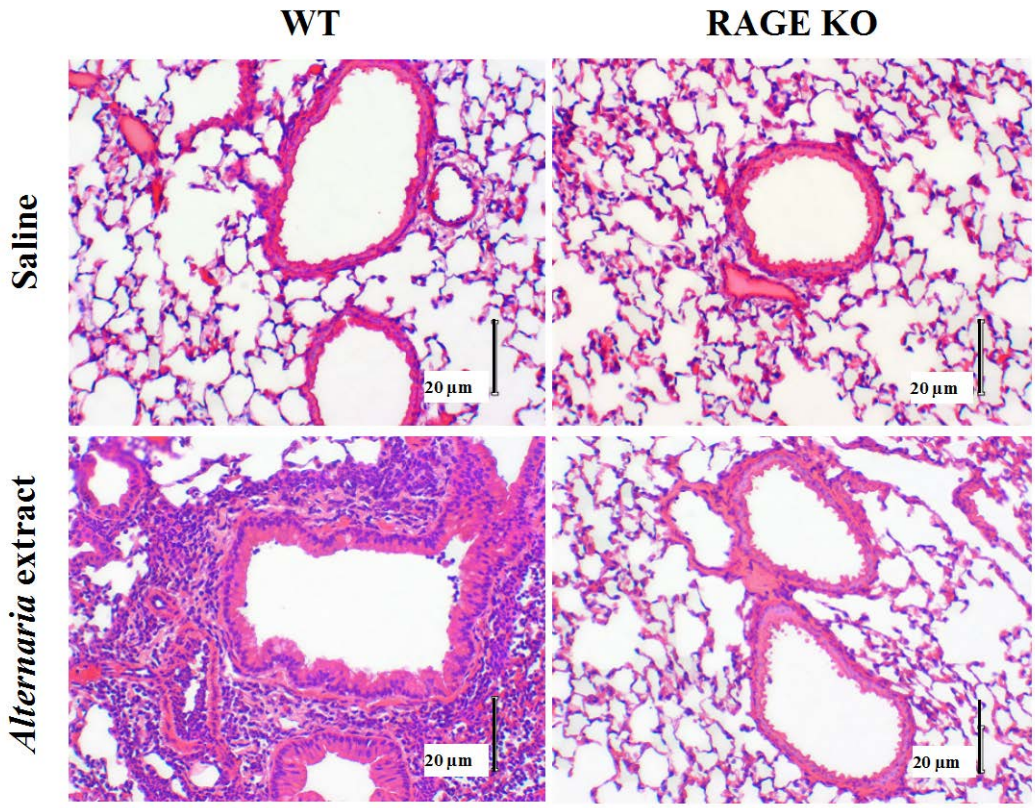


**Figure 4. Eosinophils surround the airways and vessels of wild-type mouse lungs after *Alternaria* challenge.**

Representative H&E stains of wild-type (WT) and RAGE KO mouse lung tissue after mice were subjected to the 10-day *Alternaria* model. Images were taken at 200X magnification. Scale bars = 20 µm. *n* = 7-14 mice per strain per treatment group. Experiment was replicated at least three separate times.

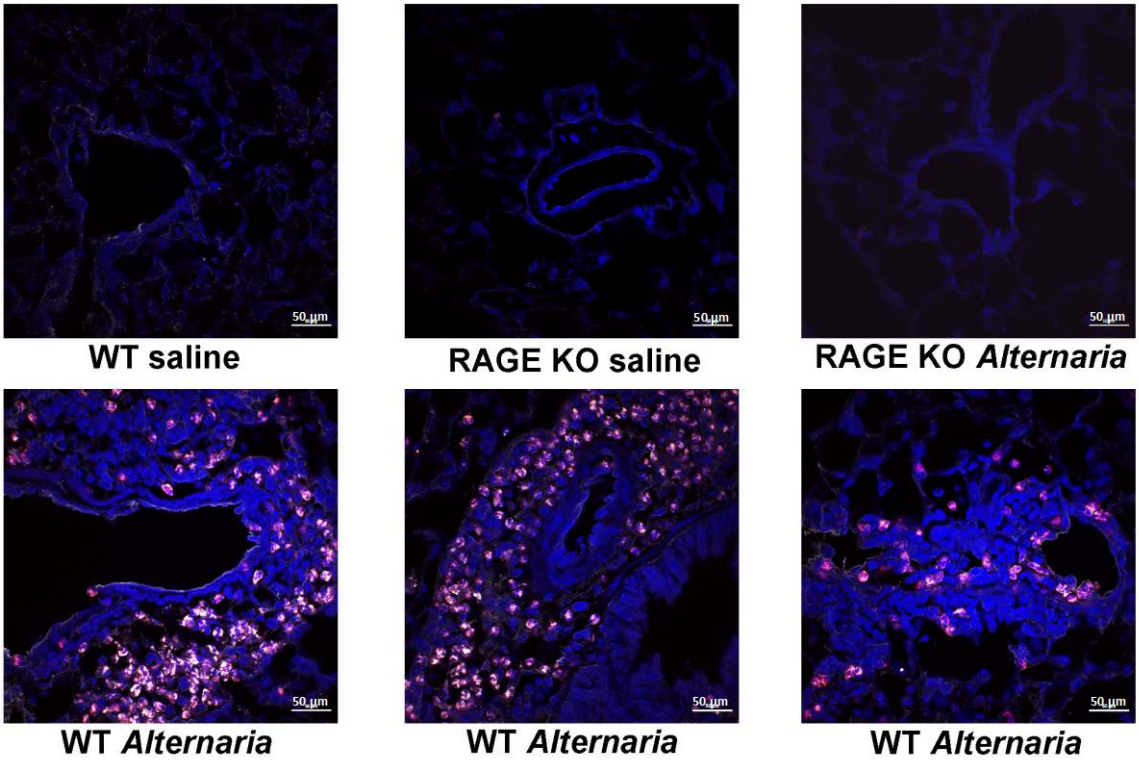


**Figure 5. MBP-labeled eosinophils are found in the lungs of wild-type, but not RAGE KO, mice after *Alternaria* challenge.**

*Pink: Major basic protein (MBP; eosinophils), blue: nuclei*. Representative confocal images of wild-type (WT) and RAGE KO mouse lung tissue after mice were subjected to the 10-day *Alternaria* model. *Top*: No eosinophils are seen in WT saline, RAGE KO saline, or RAGE KO *Alternaria* treatment groups. *Bottom*: Three representative confocal images of eosinophilic infiltrates in the WT lung tissue after allergen treatment. $n = 1$ representative animal per strain/treatment group. Scale bars = 50 μm in all images.

PAS staining revealed bright pink mucus deposits within many of the large airways in wild-type *Alternaria*-treated mice (Figure 6). Mucus hypersecretion was absent in RAGE KO *Alternaria*-treated mice.

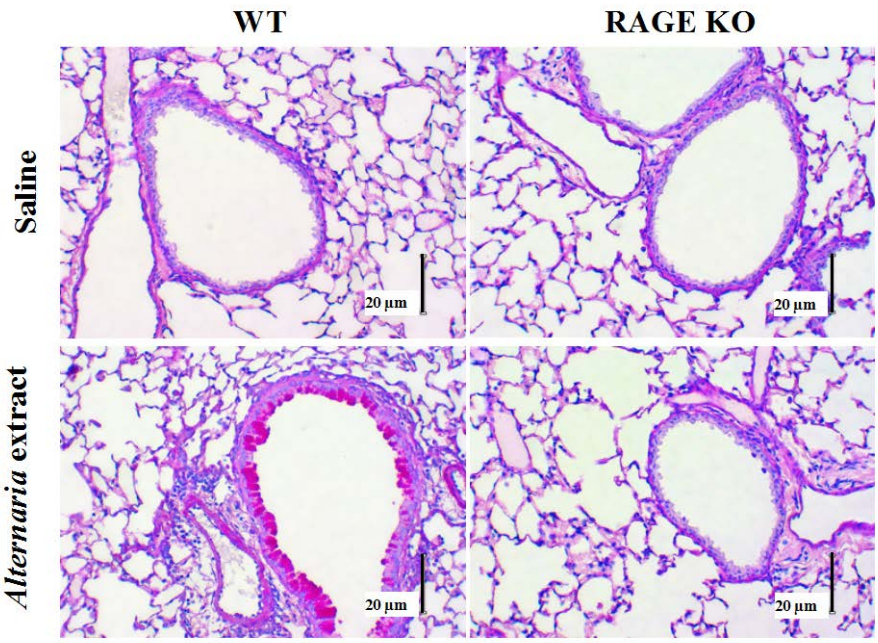


**Figure 6. Mucus hypersecretion is evident in wild-type, but not RAGE KO, mouse lungs after *Alternaria* challenge.**

Representative PAS stains of wild-type (WT) and RAGE KO mouse lung tissue after mice were subjected to the 10-day *Alternaria* model. Mucin stains bright pink. Images were taken at 200X magnification. Scale bars = 20 µm. *n* = 7-14 mice per strain per treatment group. Experiment was replicated at least three separate times.

Airway hyperreactivity was assessed in *Alternaria*-treated mice using a Flexivent apparatus and a methacholine challenge test. Changes in airway resistance (Rn), tissue elastance (H), and tissue damping (G) parameters were recorded with each increasing dose of methacholine from 0-50 mg/mL. Resistance was significantly increased in wild-type mice treated with *Alternaria* at the two highest doses of methacholine (12.5 and 50 mg/mL)(Figure 7A). When RAGE was absent, however, tissue resistance remained equal to that of saline-treated controls throughout the entire methacholine challenge. Likewise, tissue elastance was increased in wild-type, but not RAGE KO, mice treated with *Alternaria* (Figure 7B). Tissue damping was

the same in both wild-type and RAGE KO mice treated with *Alternaria* and was not significantly different from that of saline-treated mice (Figure 7C). Interestingly, lung compliance measurements revealed a significantly lower level of lung compliance in RAGE KO mice at baseline when compared to wild-type controls (Figure 7D). RAGE KO compliance did not change in response to *Alternaria*, while wild-type compliance decreased with allergen challenge to levels similar to those of RAGE KO mice. In summary, *Alternaria* does not induce AHR in mice that lack RAGE.

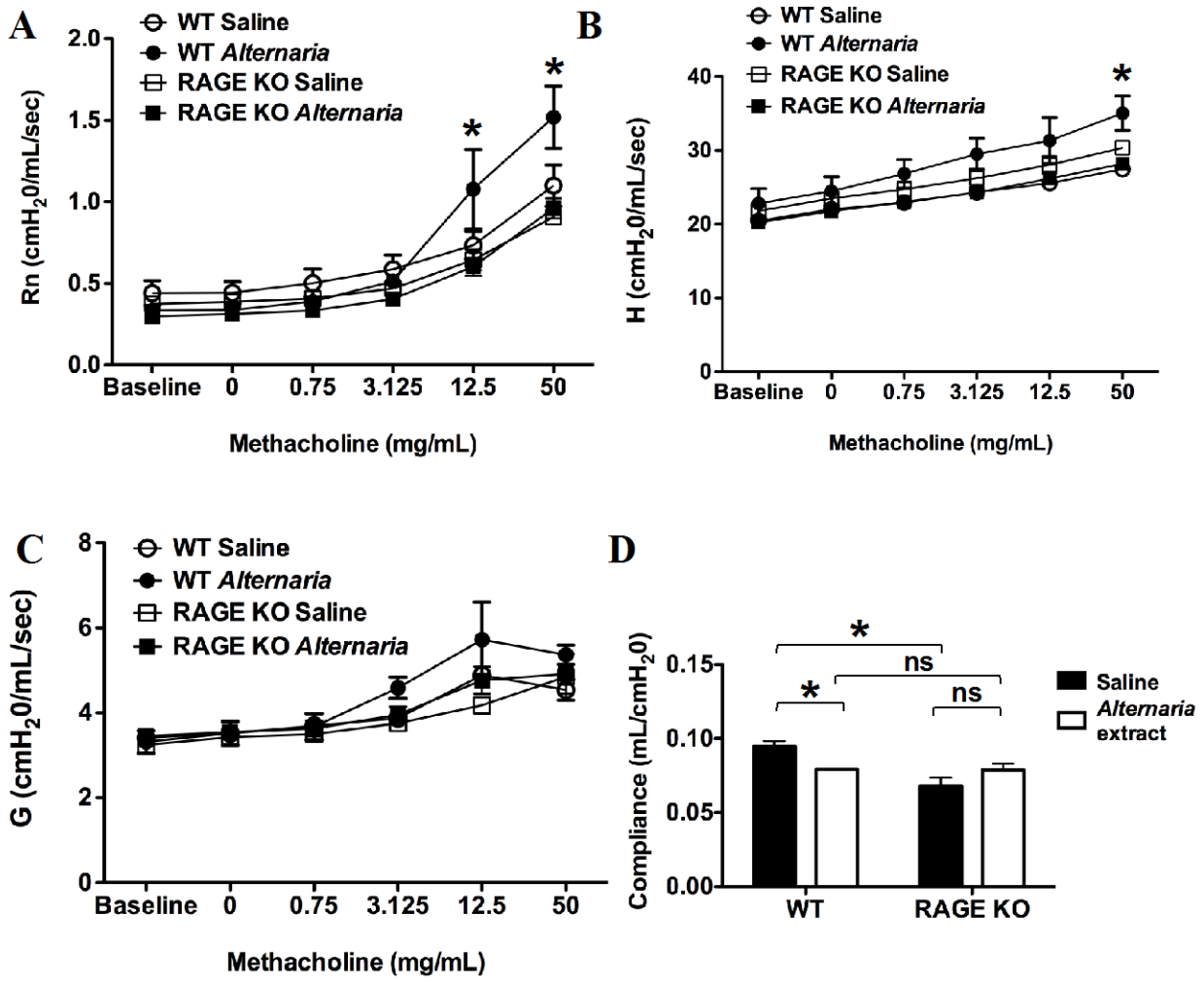


**Figure 7. *Alternaria* exposure causes abnormal pulmonary functioning in wild-type mice.**

Pulmonary function testing demonstrating changes in (A) airway resistance (Rn) and (B) elastance (H) in wild-type (WT), *Alternaria*-treated mice during methacholine challenge. (C) Tissue damping (G) was not significantly altered in response to *Alternaria* challenge. (D) Lung compliance measurements were noticeable lower at baseline in RAGE KO animals when compared to WT controls. All functional analyses were collected on Day 10 of the *Alternaria* model. Results are expressed as means ± SEMs. *n* = 3-4 mice per strain per treatment group. *p < 0.05 versus comparison. *ns* = not significant.

Because type 2 cytokines mediate the development of AHR, tissue inflammation, and mucus hypersecretion in response to allergens, levels of IL-4, IL-5, and IL-13 were assessed in the BALF of wild-type and RAGE KO mice after *Alternaria* treatment. Levels of all three cytokines were significantly increased in response to allergen in wild-type mice (Figure 8). In RAGE KO mice, however, IL-4, IL-5, and IL-13 levels remained at baseline. In the HDM model, RAGE KO mice also had blunted IL-5 and IL-13 responses, yet IL-4 levels increased normally.[144] Levels of IL-2 and IL-7, which are important for ILC2 and T cell development and differentiation, were also analyzed by ELISA in the BALF of wild-type and RAGE KO mice in the *Alternaria* model. The levels of these cytokines were undetectable in the BALF (data not shown) and may be better analyzed using whole lung homogenate for ELISA or qRT-PCR.

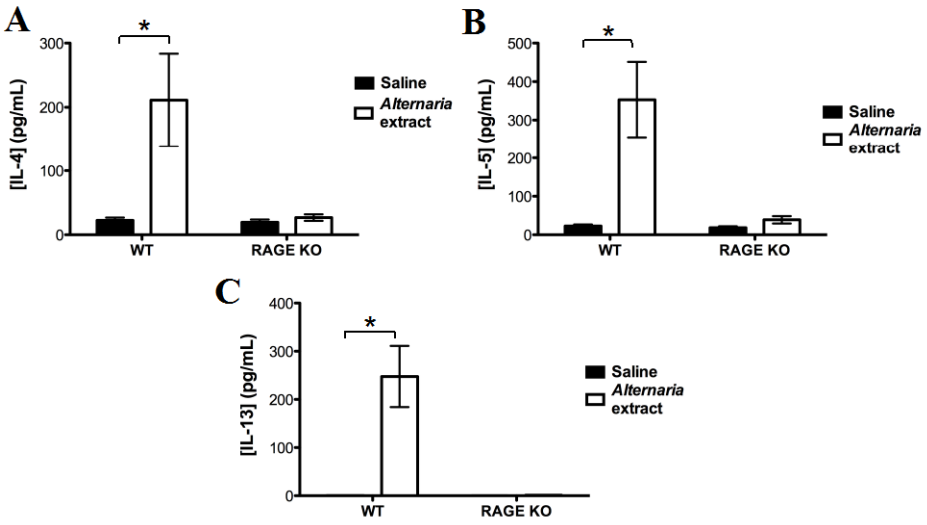


**Figure 8. IL-4, IL-5, and IL-13 cytokine levels do not increase in the BALF of RAGE KO mice treated with *Alternaria*.**

ELISA analyses of undiluted BALF samples on Day 10 of the *Alternaria* model reveal increases in (A) IL-4, (B) IL-5, and (C) IL-13 cytokine levels in wild-type (WT), but not RAGE KO, mouse airways. Results are expressed as means ± SEMs. *$p < 0.05$ versus comparison. $n$ = 7-14 mice per strain per treatment group. Experiment was replicated at least three separate times.

Therefore, in an acute allergen model, lack of RAGE protects mice from developing pathological changes in the lung associated with AAI and AHR by preventing increases in type 2 cytokines, just as in the chronic HDM model. While subtle differences do exist between the two models, they show that RAGE is crucial for both early and late stages of AAI.

## 4.2 GROUP TWO INNATE LYMPHOID CELLS DO NOT ACCUMULATE IN RAGE KNOCKOUT MOUSE LUNGS IN RESPONSE TO ALLERGEN

RAGE KO mice do not upregulate IL-5 or IL-13 in response to allergen in either the chronic HDM model[144] or the acute *Alternaria* model (Figure 8). In type 2 immune responses, IL-5 and IL-13 are produced by both CD4+ Th2 cells and ILC2s, suggesting that one or more of these cell types may be defective or not present in RAGE KO mice during an allergic airway response.

Studies have focused on the importance of ILC2s for type 2 responses in both HDM and *Alternaria* models. Evidence in the literature demonstrates that ILC2s can be major producers of IL-5 and IL-13 in the lung in models of AAI and thus are important initiators of eosinophilic inflammation.[271] Notably, mice lacking an adaptive immune system (*Rag 1 -/-* or *Rag 2-/-* mice) can mount type 2 responses in response to allergen via ILC2 activation alone.[33, 35] Additionally, rapid induction of AAI in the *Alternaria* model (within twelve hours of allergen exposure)[256] leaves little time for a full Th2 response to be mounted. Therefore, it seemed likely that the lack of an IL-5 and IL-13 response in RAGE KO mice was due to loss of ILC2s.

To test this hypothesis, wild-type and RAGE KO mice were treated with *Alternaria* for ten days and one lung from each animal was harvested for flow cytometry. ILC2s were identified as lineage negative cells that were positive for expression of the extracellular markers CD90.2

and ST2 (IL-33R) (Lin-CD90.2+ST2+)(Figure 9). Wild-type mice had approximately 1,500 ILC2s in the lungs at baseline (Figure 10). This number increased to over 7,500 ILC2s after allergen treatment. RAGE KO mice, in contrast, had approximately 1,000 ILC2s at baseline and this level remained unchanged after allergen challenge. These data indicate that RAGE is required for ILC2 accumulation in the lungs after *Alternaria* challenge.

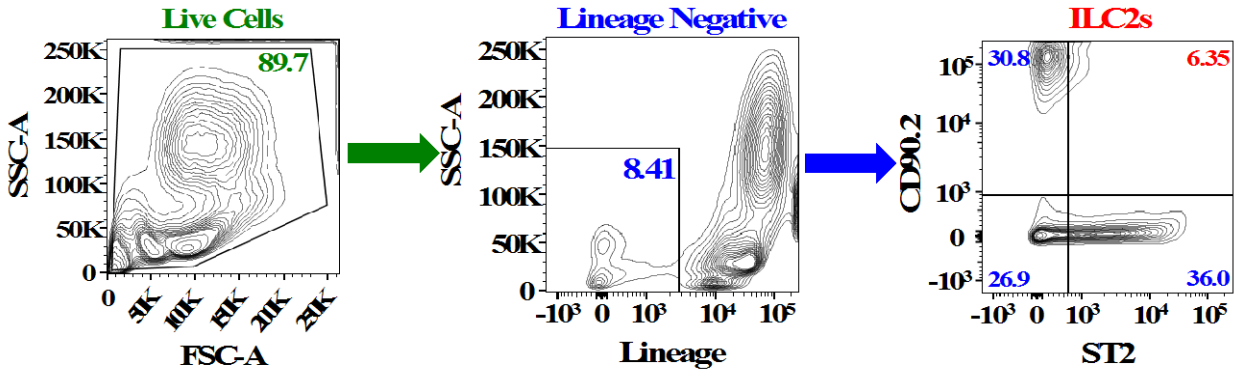


**Figure 9. Flow gating strategy for lung ILC2s**

ILC2s were identified in lung single cell suspensions by gating for live cells that were negative for lineage markers and positive for both CD90.2 and ST2 (upper right quadrant, in red). Numbers within the gates are percentages of the parent population.

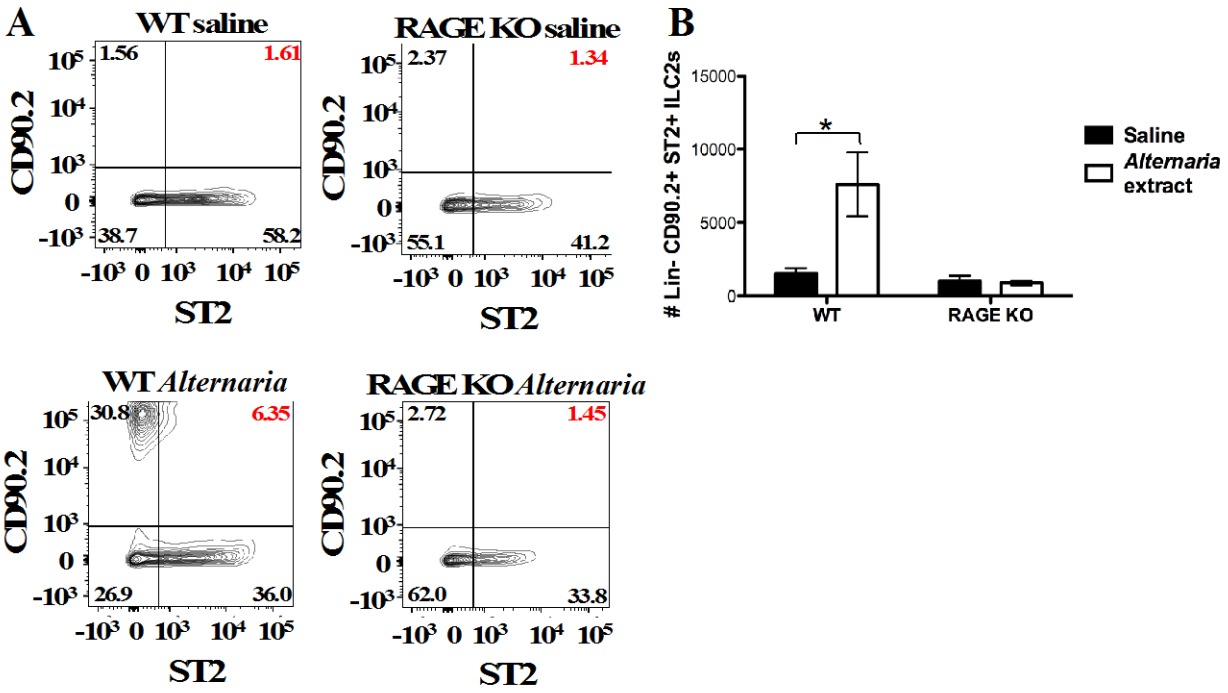


**Figure 10. ILC2s increase in the lungs of wild-type mice, but not RAGE KO mice, in response to *Alternaria*.**

(A) Representative flow plots of the live, lineage-negative cell populations gated for expression of CD90.2 and ST2. Numbers in each quadrant are percentages of the parent population. ILC2s were identified as lineage-negative cells that coexpress CD90.2 and ST2. (B) Graphic summary of ILC2s enumerated by flow cytometry in one lung after 10 days of treatment with *Alternaria*. Results are expressed as mean ± SEM. *n* = 3-11 mice per strain per treatment group. *p < 0.05 versus comparison. Results were replicated in at least three separate experiments.

To confirm that Lin-CD90.2+ST2+ cells were indeed ILC2s, the cell population was isolated from IL-33-stimulated wild-type lungs and RNA was extracted for qRT-PCR. RAGE KO mice did not have enough ILC2s in the lungs for isolation. Lin-CD90.2+ cells that were both positive and negative for ST2 were isolated in separate populations, as ST2 has shown to be variably expressed on ILC2s in some cases.[297] Approximately 40,000 Lin-CD90.2+ST2+ ILC2s

and about 100,000 Lin-CD90.2+ST2- cells were isolated from a combination of lungs from eight wild-type mice. Both Lin-CD90.2+ST2+ and Lin-CD90.2+ST2- cells expressed large amounts of IL-5 and IL-13 and little IL-4, confirming their identity as ILC2s (Figure 11). The ST2- population expressed slightly less IL-5 and IL-13 than the ST2+ population did. Therefore, ST2- cells may represent a precursor ILC2 population or a less-stimulated ILC2 subset.

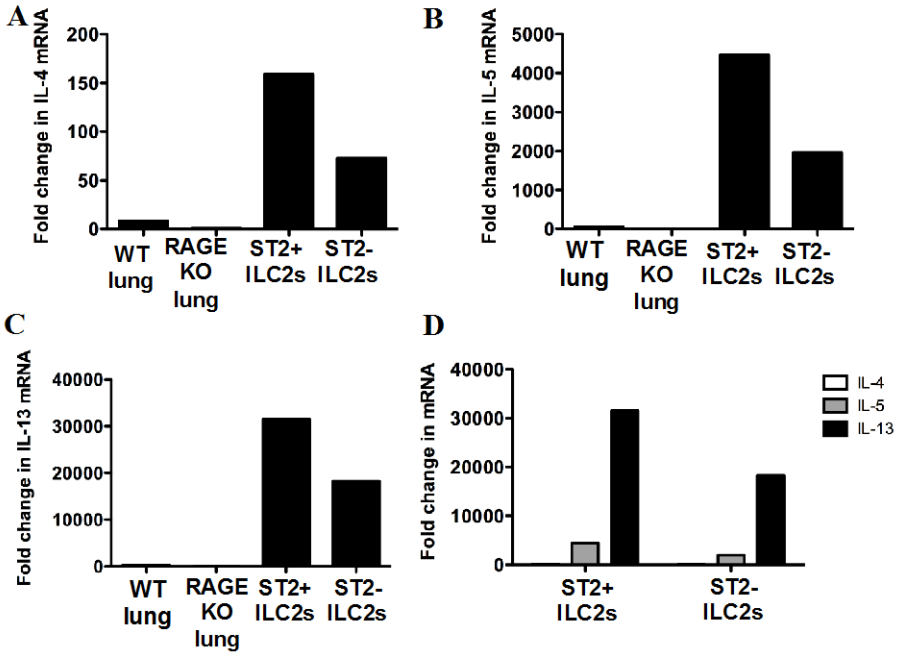


**Figure 11. mRNA for IL-5 and IL-13 is highly expressed in isolated lung ILC2s.**

qRT-PCR analysis of RNA from isolated lung ILC2 populations. Both Lin-CD90.2+ST2+ and Lin-CD90.2+ST2- cells were isolated from IL-33-treated WT mouse lungs for analysis. Though not ideal, HDM-treated WT mouse lung homogenate served as a positive control for the type 2 cytokines (set to fold-change value of 1.0). HDM-treated RAGE KO mouse lung homogenate served as a negative control since no type 2 immune response developed in these animals. ST2+ and ST2- ILC2 populations expressed large amounts of (A) IL-4 (B) IL-5, and (C) IL-13 mRNA when compared to WT lung homogenate. (D) IL-4 mRNA was much less abundant than IL-5 and IL-13 mRNA in these cells. $n$ = 1 sample per group, run on the plate in triplicate.

For completeness, CD4+IL-5+ and CD4+IL-13+ Th2 cells were also counted in the lung following *Alternaria* challenge in both wild-type and RAGE KO mice. IL-5- and IL-13-producing CD4+ cells increased in wild-type, but not RAGE KO, mice treated with allergen (Figure 12).

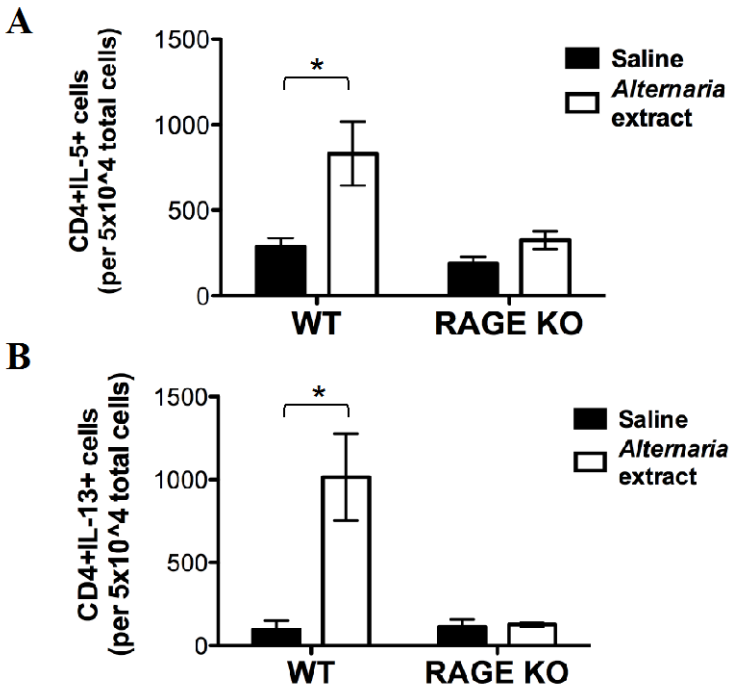


**Figure 12. CD4+ IL-5- and IL-13-producing Th2 cell responses are attenuated in RAGE KO mice after *Alternaria* challenge.**

(A) Numbers of CD4+IL-5+ cells and (B) CD4+IL-13+ cells are increased in wild-type (WT) mice after 10-day *Alternaria* treatment, but are not increased in RAGE KO mice. Results are expressed as means ± SEMs. *n* = 3-5 mice per strain per treatment group. *$p < 0.05$ versus comparison.

## 4.3 RAGE FUNCTIONS BOTH UPSTREAM AND DOWNSTREAM OF IL-33

IL-33, moreso than IL-25, is a potent activator of ILC2s and is a strong inducer of AAI and AHR.[50, 161] Because ILC2s do not accumulate in the lungs of RAGE KO mice in response to allergens that are known to act through IL-33-dependent pathways, it was hypothesized that RAGE was needed for IL-33 release. Levels of IL-33 in total lung homogenate from wild-type and RAGE KO mice treated with *Alternaria* were analyzed by Western blot (Figure 13). These studies revealed that after allergen treatment, RAGE KO mice have an attenuated IL-33 response when compared to wild-type mice. A similar result was seen using the HDM model.[162] This suggests that RAGE is necessary for IL-33 production or release in AAI.

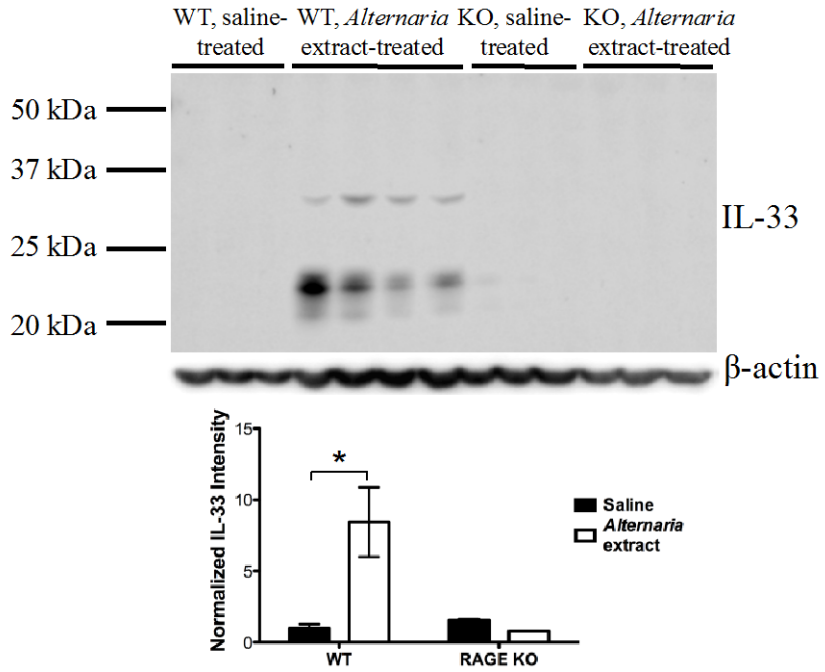


**Figure 13. RAGE is necessary for upregulation of IL-33 in response to *Alternaria*.**

Immunoblot probing for IL-33 in whole lung homogenate after 10-day *Alternaria* treatment and summary of normalized IL-33/β-actin signal intensity ratios (saline controls set arbitrarily to 1.0). Results are expressed as mean ± SEM. *n* = 3-4 mice per strain/treatment group. *p < 0.05 versus comparison.

Several studies have shown that IL-33 is released into the BALF within one hour of acute allergen exposure.[176, 206, 209, 298] To determine if loss of RAGE affected IL-33 release into the extracellular space, wild-type and RAGE KO mice were given a high dose of intranasal *Alternaria* (50 μg) and were sacrificed exactly one hour after treatment. ELISA analysis of the BALF revealed a large increase in IL-33 levels in the lavage fluid of wild-type allergen-treated animals, but not RAGE KO mice (Figure 14). The results were consistent with previously reported levels of the cytokine in the BALF of C57BL/6 mice,[176] however, there was a high amount of variability among the mice within this trial and among different attempts to repeat the experiment. Therefore, no statistically significant difference between strain or treatment group was officially noted.

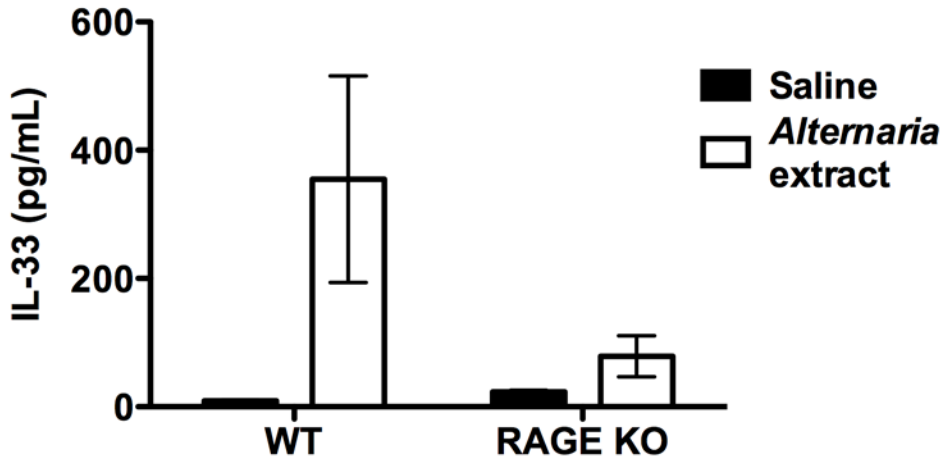


**Figure 14. IL-33 levels seem to increase in the BALF of wild-type, but not RAGE KO, mice one hour after *Alternaria* challenge.**

ELISA analysis of undiluted BALF from wild-type (WT) and RAGE KO mice treated intranasally with saline or high-dose (50 μg) *Alternaria*. BALF samples were collected one hour after allergen challenge. Results are presented as mean ± SEM. *n* = 3-5 mice per strain per treatment group. This is one representative experiment from five separate replicates. No statistically significant differences were found between any of the groups.

The results presented thus far have shown that RAGE acts upstream of IL-33 to promote expression of the cytokine in the lung after allergen challenge. Therefore, it was hypothesized that bypassing the need for RAGE by administering exogenous IL-33 directly to RAGE KO mice would be able to induce AAI in these animals. Intranasal administration of mouse rIL-33 over a four-day period (Figure 2) induced a type 2 inflammatory response in wild-type mice (Figure 15). Eosinophils surrounded airways and vasculature and were highly abundant in the BALF (Figure 15A&C). Mucus secretion was significantly increased in the airways (Figure 15B). IL-5 and IL-13 levels in the BALF were highly elevated (~1600 pg/mL and ~450 pg/mL, respectively) (Figure 15D). Surprisingly, however, RAGE KO mice treated with IL-33 displayed no eosinophilia, no mucus hypersecretion, and no increase in IL-5 or IL-13 levels over baseline. Bypassing the necessity for RAGE and giving IL-33 directly was not able to induce AAI. This suggested that RAGE was needed not only upstream of IL-33 release, but also downstream of IL-33 to aid in activation of the type 2 immune response.

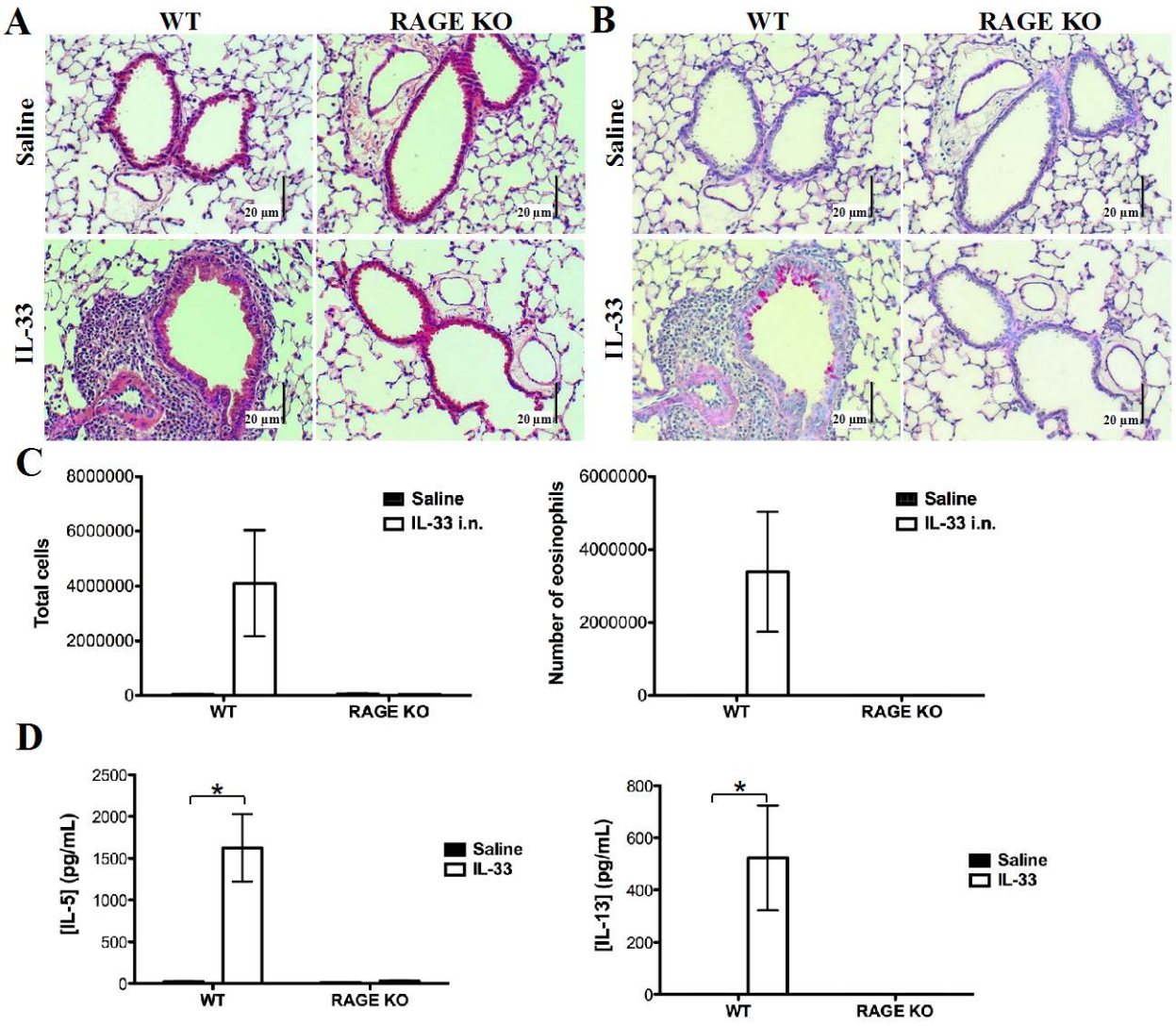


**Figure 15. Intranasal administration of recombinant IL-33 induces type 2 immune responses in wild-type, but not RAGE KO, mice.**

(A) Representative H&E and (B) PAS stains of lung sections (200x magnification; scale bars = 20 µm) from wild-type (WT) and RAGE KO mice after exogenous intranasal IL-33 administration. (C) BALF total cell and eosinophil counts normalized to 1.0 mL. (D) ELISA analyses of IL-5 and IL-13 levels in undiluted BALF after treatment with IL-33. All samples were collected after 4 days of IL-33 treatment. In all cases results are expressed as means ± SEMs. *n* = 3-11 mice per strain per treatment group. *$p < 0.05$ versus comparison. Results were replicated in at least three experiments.

Because one of the main downstream functions of IL-33 is to promote ILC2 expansion and recruitment to the lung during AAI,[34, 199] ILC2 numbers in the lung following IL-33 challenge were assessed by flow cytometry. Wild-type mice had an increase in ILC2s from a baseline level of about one hundred after saline treatment to several thousand ILC2s (~4,300) in one lung after IL-33 administration (Figure 16). RAGE KO mice, meanwhile, showed no increase in lung ILC2 numbers over saline controls after IL-33 treatment. These data demonstrated that RAGE is required for IL-33-induced ILC2 accumulation in the lung in AAI.

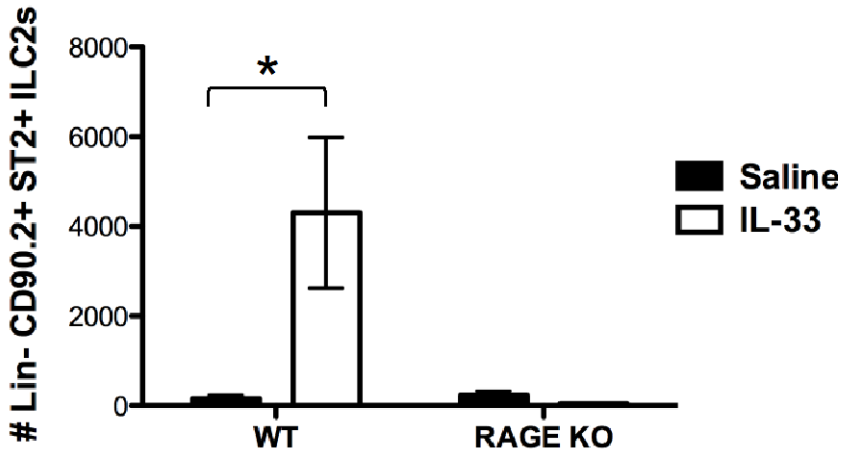


**Figure 16. Recombinant IL-33 induces ILC2 accumulation in wild-type, but not RAGE KO, mouse lungs.**

Graphic summary of ILC2s (Lin-CD90.2+ST2+ cells) enumerated by flow cytometry in one lung after 4 days of intranasal IL-33 treatments. Results are expressed as mean ± SEM. *n* = 3-9 mice per strain per treatment group. *p < 0.05 versus comparison. Results were replicated among at least three separate experiments.

## 4.4 ILC2S FUNCTION NORMALLY IN THE GASTROINTESTINAL TRACT OF RAGE KO MICE

In untreated wild-type mice, RAGE is highly expressed in the lungs and is not expressed in the spleen, small intestine, or large intestine (Figure 17).[84, 85] This suggests that RAGE's role in IL-33-mediated inflammation may be specific to the lung and led to the hypothesis that ILC2-driven type 2 inflammatory responses in RAGE KO mice would occur normally in tissues that do not express high levels of RAGE.

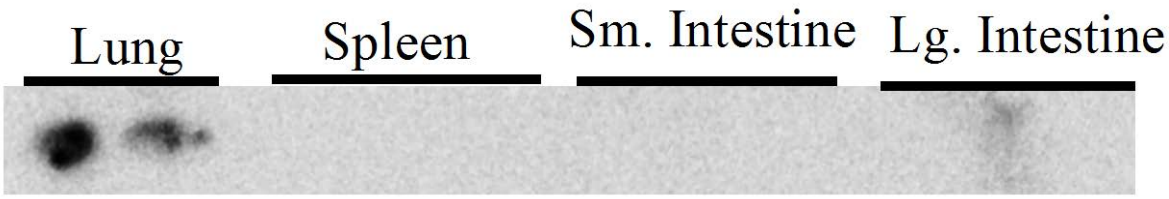


**Figure 17. RAGE is highly expressed in the lung, but not in other mouse tissues.**

Immunoblot analysis of wild-type mouse tissue homogenate probed for expression of RAGE. *n* = 2 mice for each organ. This experiment was replicated on at least one other separate occasion with similar results. *Sm* = small; *Lg* = large.

In addition to their role in AAI, ILC2s have been widely studied in the GI tract since parasites elicit type 2 immune responses in this organ system.[199-201] Studies have shown that i.p. IL-33 injections are sufficient to induce intestinal eosinophilia, mucus hypersecretion, and splenomegaly in wild-type mice.[49] To test if ILC2s and their downstream effects are functional in RAGE KO mice intestinal tracts, wild-type and RAGE KO mice were given i.p. IL-33

injections for three days, and GI and splenic tissue was harvested for analysis. PAS stains of both wild-type and RAGE KO mouse ileum sections showed increased mucus production after IL-33 treatment (Figure 18). Additionally, IL-5 and IL-13 levels were elevated in the peritoneal lavage fluid of both mouse strains (Figure 19). Importantly, the numbers of ILC2s in the spleen increased equally in both wild-type and RAGE KO mice after the IL-33 injections (Figure 20). Together, these data demonstrate that even in global RAGE KO mice, ILC2s function normally in tissues that do not normally express RAGE.

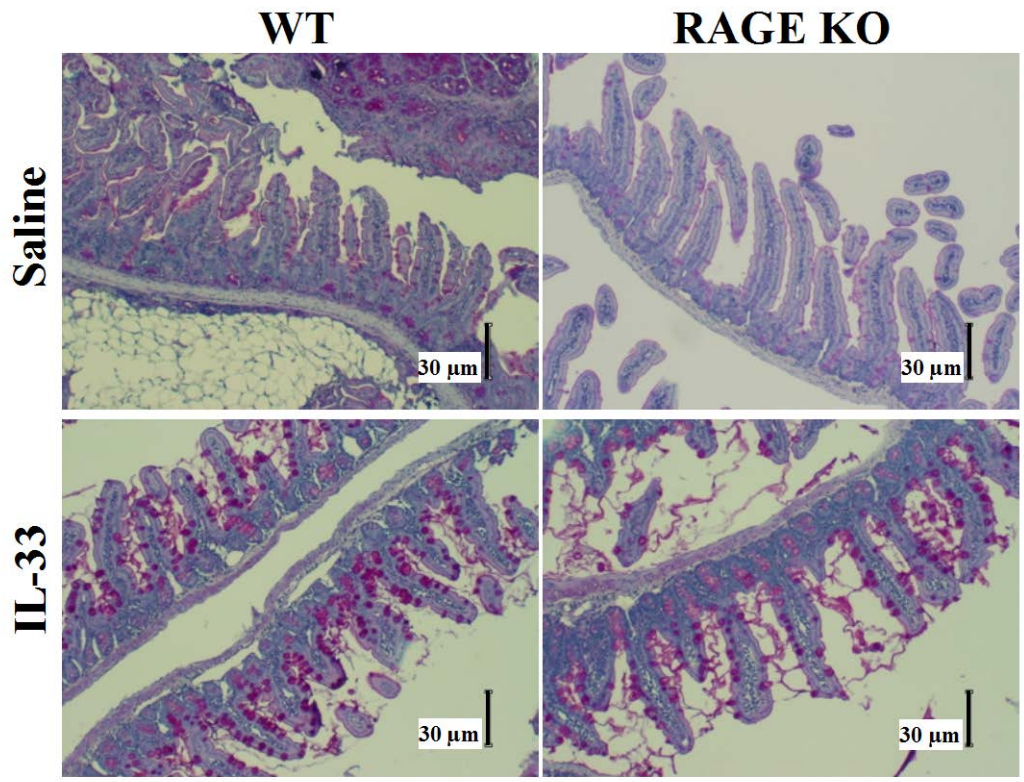


**Figure 18. Both wild-type and RAGE KO mice develop mucus hypersecretion in the small intestine in response to intraperitoneal IL-33 administration.**

Representative PAS stains of wild-type (WT) and RAGE KO mouse ileum tissue after mice were subjected to three days of intraperitoneal IL-33 injections. Mucin stains bright pink. Images were taken at 100X magnification. Scale bars = 30 µm. *n* = 3-5 mice per strain per treatment group.

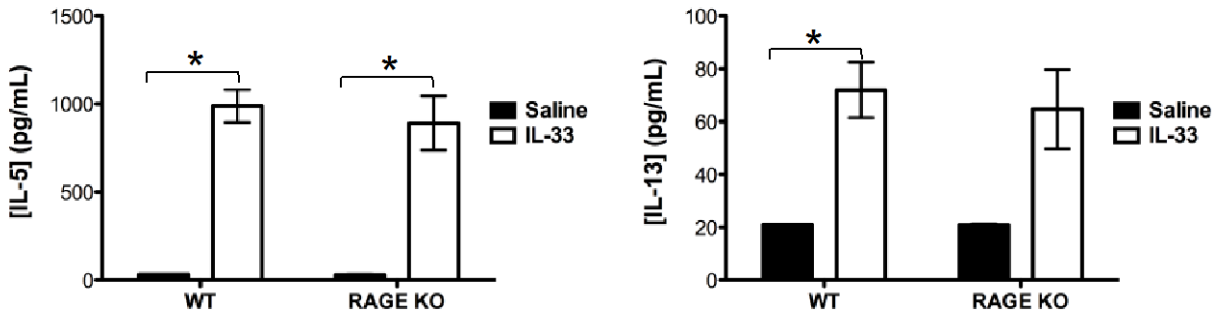


**Figure 19. IL-5 and IL-13 cytokine levels increase in the peritoneal lavage fluid of both wild-type and RAGE KO mice after intraperitoneal IL-33 administration.**

ELISA analyses of undiluted peritoneal lavage fluid after three days of intraperitonal IL-33 reveal increases in IL-5 and IL-13 cytokine levels in wild-type (WT), but not RAGE KO, mice. Results are expressed as means ± SEMs. *$p < 0.05$ versus comparison. $n$ = 3-5 mice per strain per treatment group.

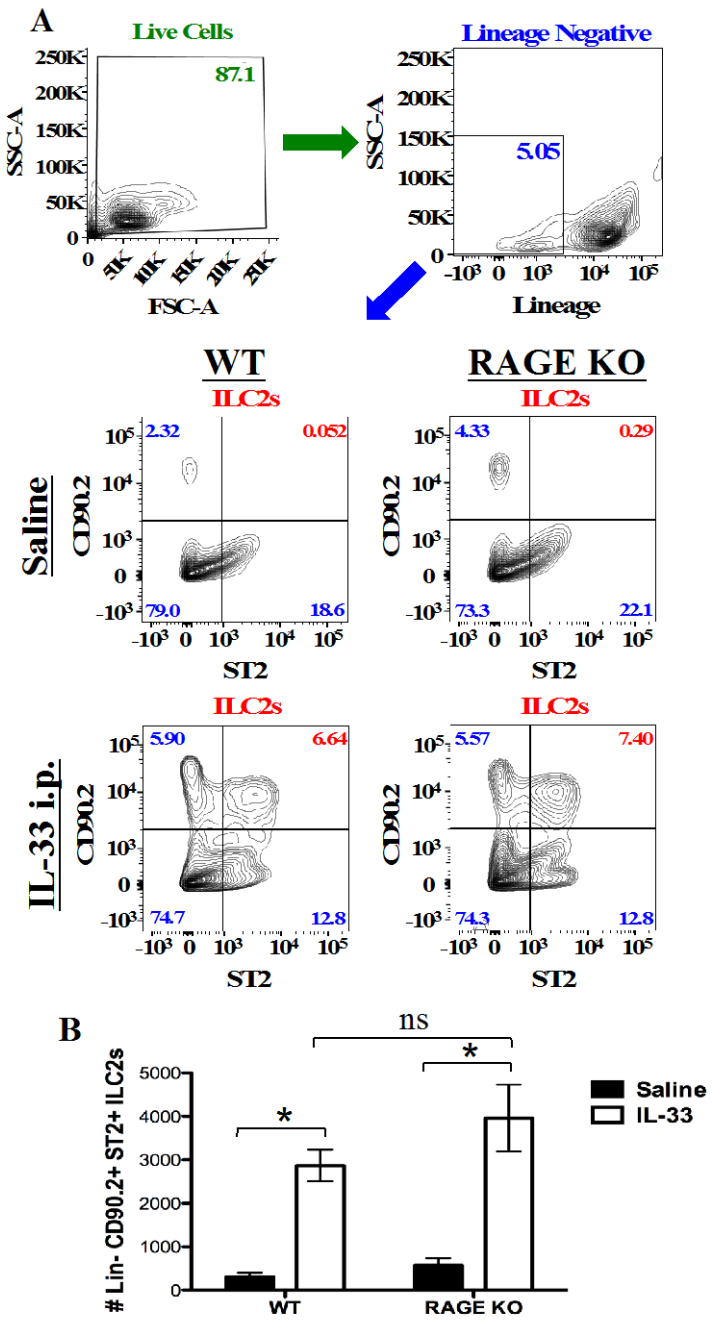


**Figure 20. Splenic ILC2s increase in response to IL-33 in both wild-type and RAGE KO mice.**

(A) Gating strategy to identify ILC2s in the spleen. Numbers within each gate represent the percentage of cells from the parent population that fall within the gate. ILC2s were identified as live, lineage-negative, CD90.2-positive, ST2-positive cells. (B) The number of ILC2s in each spleen was enumerated by flow cytometry. Results are expressed as mean ± SEM. *p < 0.05 versus comparison. *n* = 3-5 mice per strain per treatment group. *ns* =not significant.

Lin-CD90.2+ST2+ cells isolated from the spleens of wild-type animals treated with IL-33 i.p. were cultured *in vitro* to confirm their identity as ILC2s. After six days in culture with IL-2, IL-7, and IL-33 stimulation, the isolated cells secreted copious amounts of IL-5 and IL-13, but very little IL-4 (Figure 21). The cells were also able to proliferate in culture, suggesting that these populations can be expanded *in vitro* for further experimentation (Figure 22).

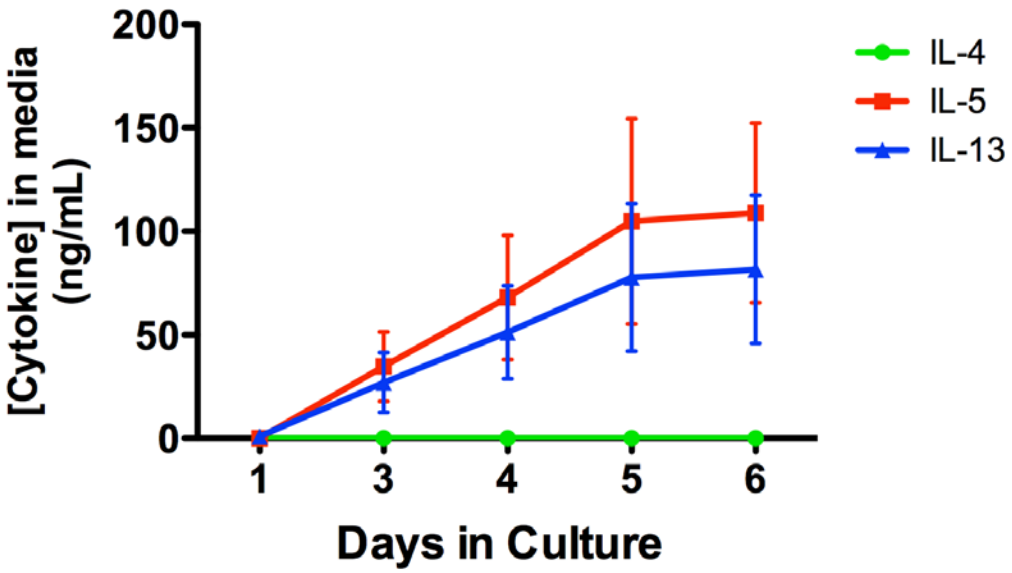


**Figure 21. Isolated spleen ILC2s produce large amounts of IL-5 and IL-13 in culture.**

Wild-type splenic ILC2s were cultured for six days with IL-2, IL-7, and IL-33 stimulation. Media was collected each day (except day 2), then a 1:1000 dilution was analyzed by ELISA to detect IL-4, IL-5, and IL-13 cytokine concentrations. Results are expressed as mean ± SEM. $n$ = 5 per time point (one population of cells was isolated from each of five wild-type mice and plated in separate wells for culturing).

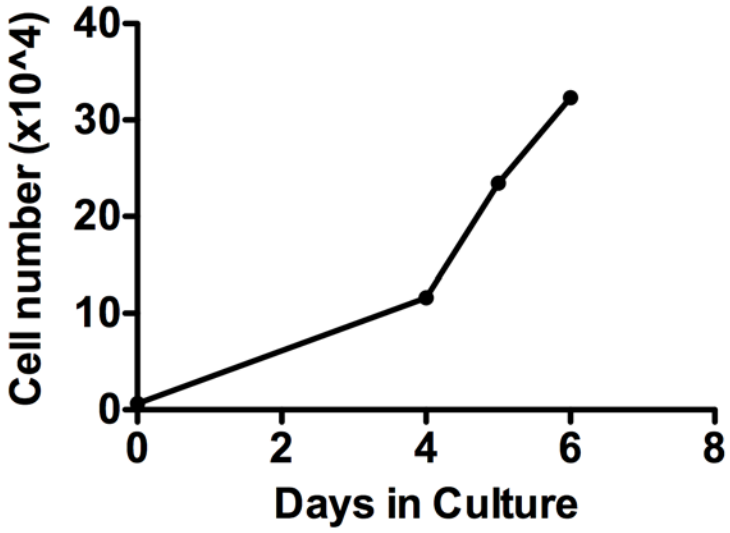


**Figure 22. Isolated wild-type splenic ILC2s proliferate well in culture.**

Wild-type splenic ILC2s were cultured for six days with IL-2, IL-7, and IL-33 stimulation. Cells were periodically counted over the six-day culture period. The mean number of cells was graphed at each time point. *n* = 5 per time point (one population of cells was isolated from each of five wild-type mice and plated in separate wells for culturing).

## 4.5 RAGE ON ILC2S IS NOT AS IMPORTANT AS PULMONARY RAGE FOR DEVELOPMENT OF AAI

While RAGE is known to be highly expressed on AT1 epithelial cells in the lung,[86, 87, 114] it has also been shown to be expressed on a variety of blood immune cells including macrophages,[82, 299] eosinophils,[93] and T cells.[97, 157] Previous studies from our lab using wild-type and RAGE KO bone marrow chimeric mice illustrated that mice expressing RAGE in their stromal tissue developed AAI in response to HDM treatment, regardless of whether they received wild-type or RAGE KO bone marrow.[162] In contrast, mice that lacked RAGE in the stromal tissue did not

develop AAI in response to allergen, even when donor bone marrow contained wild-type, RAGE-expressing hematopoietic cells. In the IL-33 i.p. experiments described above, ILC2s from RAGE KO mice were shown to function normally in the GI tract and spleen, suggesting that RAGE is not needed directly on ILC2s in order for them to perform their role in allergic inflammatory responses.

Preliminary studies show that RAGE is likely not expressed on Th2 cells, but may be expressed on ILC2s. T cells were isolated from wild-type mouse spleens and differentiated *in vitro* into Th2 cells. Western blot analysis revealed no RAGE in the cell lysate of these Th2 cells (Figure 23). ILC2s (ST2+) were isolated from wild-type lungs, and qRT-PCR showed a threshold-crossing fluorescent signal for RAGE mRNA at a cycle threshold (Ct) of 31.8 out of 40 total cycles (Figure 24). It is difficult to determine from this value if RAGE is actually present or not on these cells, especially when a good negative control was not available at the time. RAGE KO mouse lung homogenate was used because RAGE KO lungs do not possess enough ILC2s for sufficient cellular isolation. One of the advantages of isolating ILC2s from the spleen is that both wild-type and RAGE KO mice greatly increase the number of ILC2s in response to IL-33. Therefore, large numbers of both wild-type and RAGE KO mice can be isolated from stimulated spleens in future experiments in order to have good RAGE KO ILC2 negative controls for qRT-PCR analysis of RAGE expression in wild-type ILC2s.

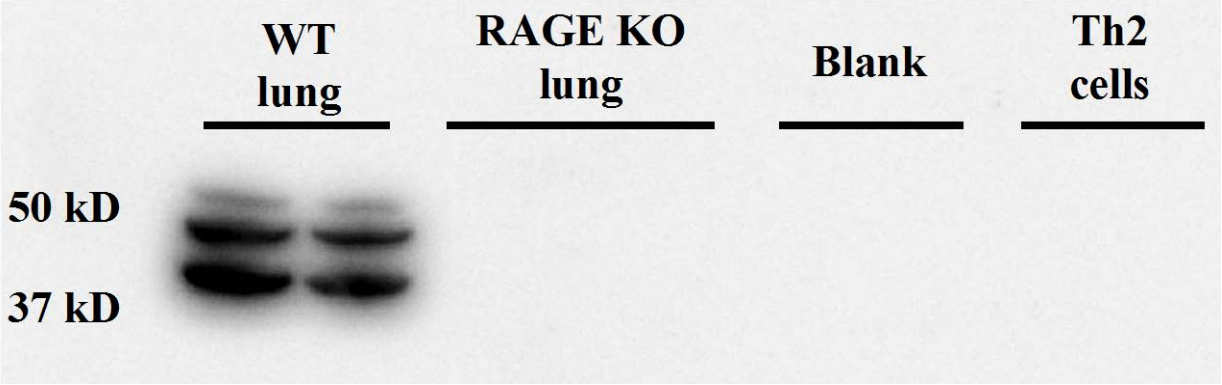


**Figure 23. RAGE is not expressed in mouse Th2 cells.**

Immunoblot probing for RAGE in *in-vitro* differentiated Th2 cells from the spleen. RAGE appears as a doublet (mRAGE and sRAGE) in the positive control (wild-type mouse lung homogenate). RAGE KO mouse lung homogenate served as a negative control. *n* = 2 samples per group.

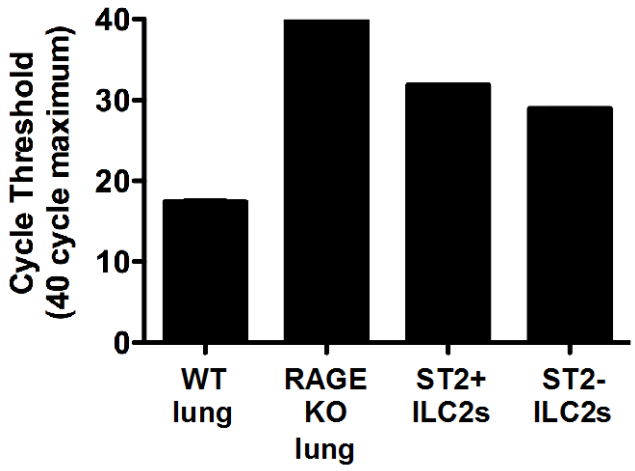


**Figure 24. Isolated ILC2s from the lung may express small amounts of RAGE.**

Cycle threshold (Ct) values from qRT-PCR analysis of RNA from wild-type (WT) (positive control) or RAGE KO (negative control) mouse whole lung homogenate and lung ST2+ and ST2- ILC2 populations after IL-33 stimulation *in vivo*. Lower Ct values indicate greater presence of RAGE mRNA. RAGE KO mice never reach the threshold before the maximum number of PCR cycles is completed (Ct = 40). ST2+ and ST2- ILC2 populations crossed the cycle detection threshold before the maximum number of cycles was reached, suggesting that small amounts of RAGE mRNA may be present in these cells. *n* = 1 sample per group, run on the plate in triplicate.

## 4.6 IL-33 MAY BE A NOVEL RAGE LIGAND

Many comparisons have been drawn between IL-33 and HMGB1, a known RAGE ligand.[170, 171] Both molecules reside in the nucleus of cells and are released upon cellular damage as "alarmins" to activate the immune system. Since RAGE is a promiscuous receptor with many binding partners, it seemed reasonable to explore if IL-33 was a novel RAGE ligand.

A hybrid ELISA has been used in the past to assess sRAGE binding to various ligands and extracellular matrix proteins.[114, 295, 296] A 96-well high-binding polystyrene plate was coated overnight with IL-33, IL-25, TSLP, HMGB1 (positive control), and BSA (negative control), and sRAGE's ability to bind these molecules was assessed (Figure 25). sRAGE bound well to HMGB1 as expected. It did not bind to BSA or TSLP. It only slightly bound to IL-25. sRAGE bound to IL-33 at a level only slightly less than that of HMGB1 binding, suggesting that IL-33 is a unique RAGE binding partner.

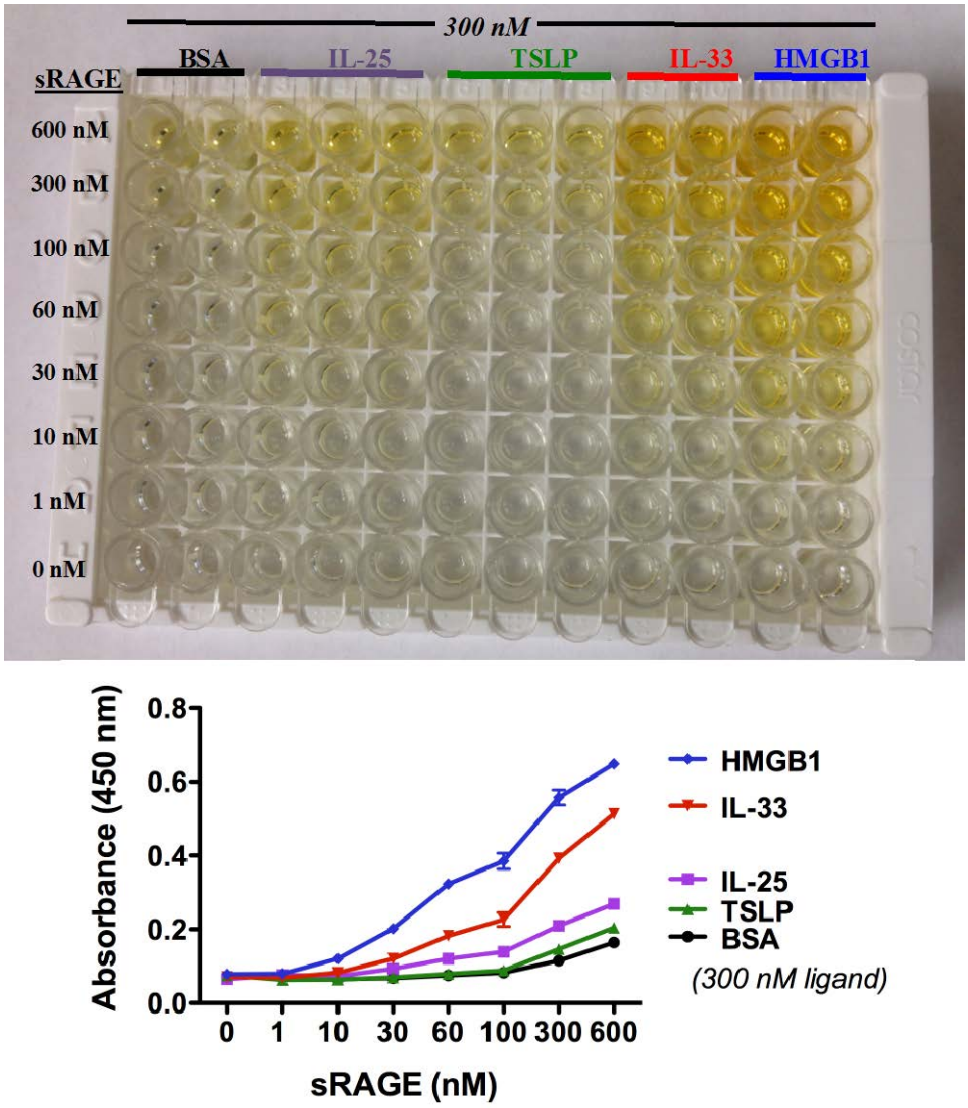


**Figure 25. A hybrid ELISA demonstrates that IL-33 can bind to sRAGE.**

*Top*: Each well was coated with 300 nM of BSA (negative control), IL-25, TSLP, IL-33, or HMGB1 (positive control) and then varying concentrations (0-600 nM) of sRAGE was added. *Bottom*: Antibodies were used to detect bound sRAGE in each well and absorbance measurements were collected and graphed against the concentration of sRAGE in that well. Plotted values are means ± SEM. IL-25 and TSLP samples were run in triplicate. BSA, IL-33, and HMGB1 samples were run in duplicate and the analysis was replicated in a separate experiment.

To better characterize the possible binding interaction between RAGE and IL-33, binding kinetic studies were pursued using the Biacore 3000 system. RAGE-ligand binding has been assessed successfully in this system previously.[300] In the Biacore 3000 system, sRAGE is bound to a sensor chip via its amine groups. The chip is then coated with an IL-33-containing solution and the amount of IL-33 bound and the kinetics of the binding are measured through the chip. Unfortunately, IL-33 did not appear to bind to RAGE (data not illustrated). However, the HMGB1 positive control that was used also did not show any binding. It is possible that the way sRAGE is bound to the chip prevents it from interacting with its ligands. IL-33 would not bind to the chip itself, so the reverse set-up would not work either. This system, therefore, did not seem ideal for studying RAGE-IL-33 binding interactions.

Another common platform for measuring protein-protein binding kinetics is the ForteBIO Octet system, and this machine has been used successfully by our lab in the past to measure sRAGE binding kinetics.[113] Amine-reactive tips were used to bind sRAGE, then sRAGE binding to IL-33 or HMGB1 was assessed. HMGB1 bound to sRAGE during the association phase of the study, while IL-33 did not bind and its binding kinetics resembled that of the BSA negative control (Figure 26A). This suggested that IL-33 does not bind sRAGE. An increased concentration of IL-33 ligand was also used, yet this made no difference in its sRAGE binding kinetics (Figure 26B). The reverse set-up was also attempted, but IL-33 did not bind to the amine-reactive tip (Figure 26C).

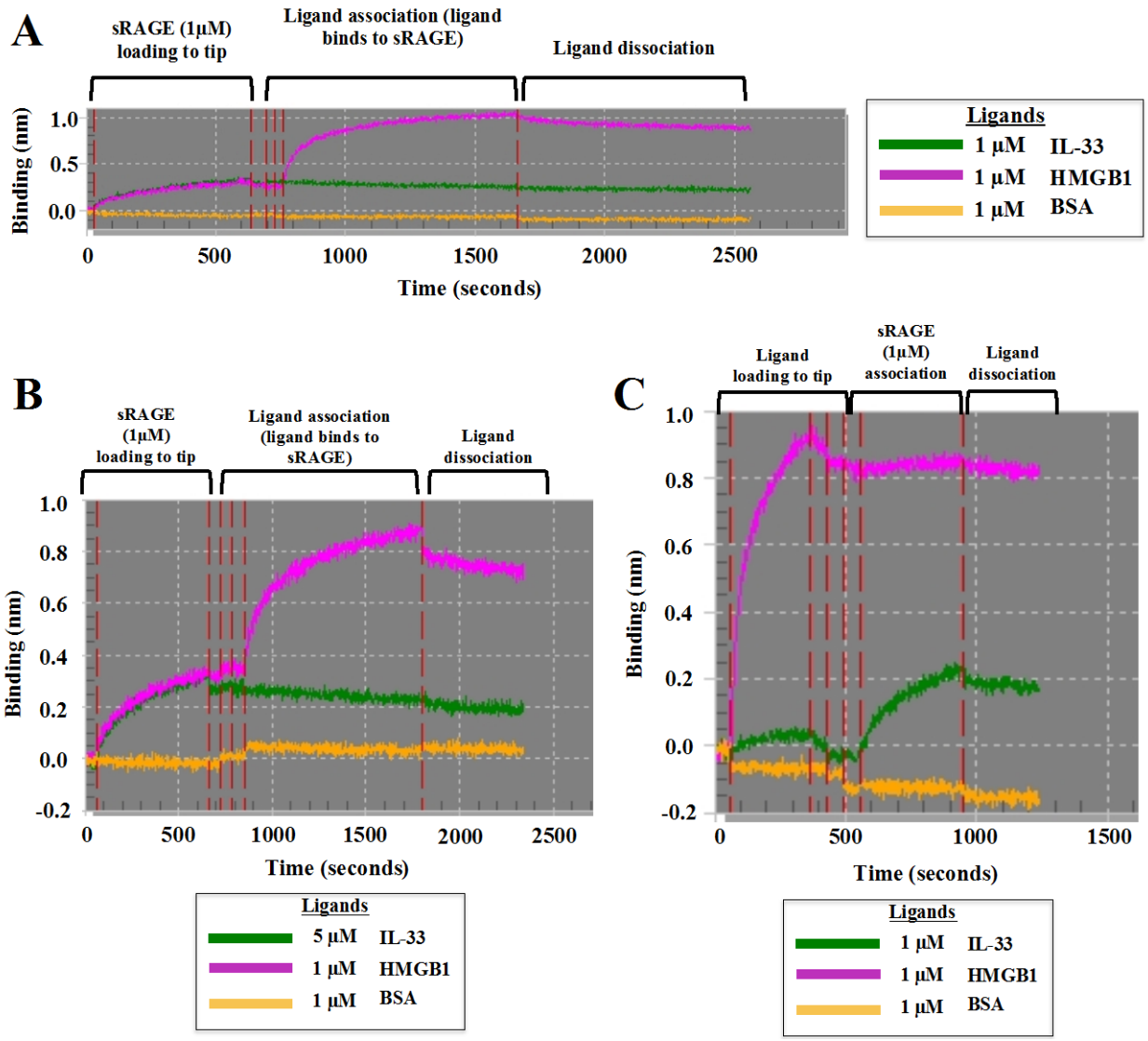


**Figure 26. ForteBIO Octet studies show that IL-33 does not bind sRAGE.**

(A) ForteBIO Octet association/dissociation binding curve first shows sRAGE loading onto the amine-reactive tip. Next, HMGB1 (pink) binds to sRAGE while IL-33 (green) and BSA (yellow) do not. Finally, HMGB1 dissociates from the tip. (B) In another trial, the same experiment as (A) was repeated using a higher concentration of IL-33 (5 µM) to promote binding to sRAGE. IL-33 (green) still did not bind to sRAGE. (C) In the final experiment, a reverse set-up was employed. IL-33 (green) and BSA (yellow) could not be loaded onto the amine-reactive tip. HMGB1 (pink) loaded well. The slight increase in the IL-33 signal (green) in the sRAGE association phase is sRAGE binding to the amine-reactive tip since IL-33 did not bind to it. *n* = 1 per group.

At this time, biochemical analyses show conflicting results about possible RAGE-IL-33 binding interactions. *In vitro* cell culture signaling studies are an alternative method by which RAGE-IL-33 binding and signaling may be assessed in the future.

## 4.7 HMGB1 PROTEIN IS ALTERED IN WILD-TYPE MOUSE LUNGS DURING AAI, BUT REMAINS UNCHANGED IN RAGE KO MOUSE LUNGS

The ligand(s) involved in activation of RAGE in AAI is unknown. In addition to exploration of a possible new RAGE ligand (IL-33), HMGB1 was also analyzed because it is a well-known RAGE ligand known to be upregulated in asthma pathogenesis. First, HMGB1 expression was analyzed by Western blot in the BALF of wild-type and RAGE KO animals after treatment with *Alternaria*. The 25 kDa band for HMGB1 was present in the BALF of almost all of the mice, but with no apparent pattern to its expression; HMGB1 levels varied within and among the strain and treatment groups (Figure 27).

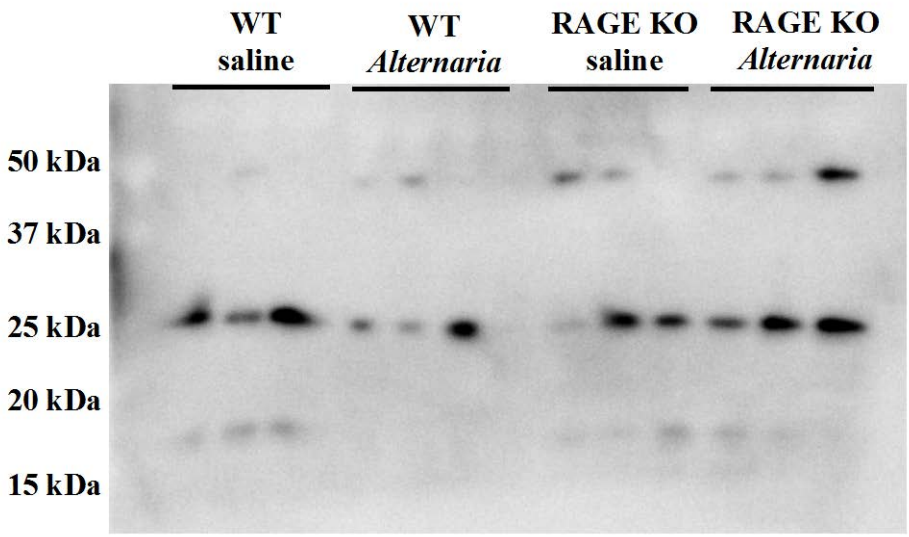


**Figure 27. HMGB1 is present in the BALF of wild-type and RAGE KO mice after *Alternaria* exposure.**

HMGB1 immunoblot of undiluted BALF from wild-type (WT) and RAGE KO mice after the ten-day *Alternaria* challenge. HMGB1 appears as a band at 25 kDa. *n* = 3-4 mice per strain per treatment group.

HMGB1 was also analyzed in the total lung homogenate of the same mice (Figure 28). The levels of HMGB1 appeared to be increased in wild-type treated mice when compared to RAGE KO treated mice, mostly due to the added expression of an additional lower molecular weight band: HMGB1 ran as a doublet in wild-type *Alternaria*-treated mice. A 25 kDa HMGB1 band was seen in all mice, while a ~22 kDa second band appeared only in wild-type *Alternaria*-treated mice (these were also the only mice to mount an allergic inflammatory reaction in the experiment (see Section 4.1)). The lower molecular weight HMGB1 band may be a cleavage product,[301] or it may be HMGB1 in a different redox state. Recent studies have shown that the redox state of three cysteine residues on HMGB1 change both how it migrates in a gel and its chemoattractant/cytokine activity.[302, 303] All-thiol HMGB1 (containing fully reduced cysteines)

can be found both intracellularly and extracellularly and possesses chemoattractant capabilities. When two cysteines form a disulfide bond, HMGB1 gains cytokine-inducing activity. This type of HMGB1 is usually found extracellularly. When HMGB1 is fully oxidized by reactive oxygen species, it loses both its chemoattractant and cytokine-inducing abilities. This led to the hypothesis that the 22 kDa band on the HMGB1 gel was perhaps a cytokine-inducing disulfide form of HMGB1.

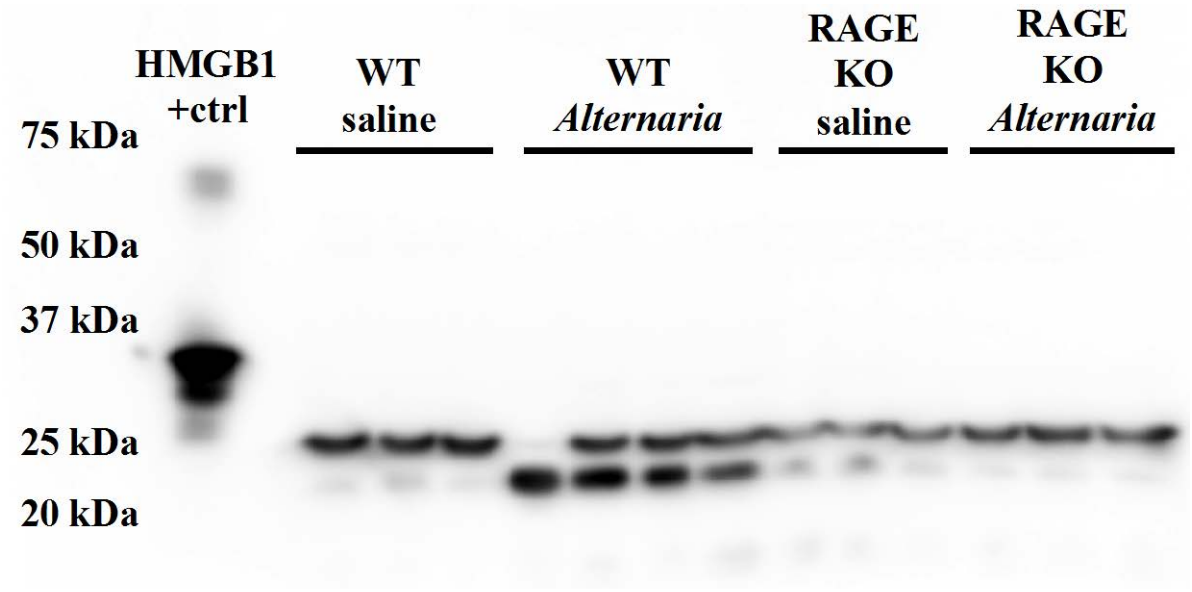


**Figure 28. HMGB1 banding patterns are different in wild-type and RAGE KO mouse lung homogenate after *Alternaria* challenge.**

HMGB1 immunoblot of whole lung homogenate from wild-type (WT) and RAGE KO mice after the ten-day *Alternaria* challenge. HMGB1 appears as a band at 25 kDa in most samples and an additional ~22 kDa band is present in WT, *Alternaria*-treated mice. $n$ = 3-4 mice per strain per treatment group.

To further test this hypothesis, the same samples were run on a new gel using non-reducing conditions. When DTT was eliminated from the sample preparation, the lower band

was still present in the samples (Figure 29). In the non-reducing gel, the lower band is much fainter in the last two samples, but the 25 kDa band is also lighter in these same samples suggesting that this faintness is a gel developing/imaging issue and not due to a lower amount of protein. Because the banding pattern seemed unaffected by changing the redox conditions of the gel, the lower band is likely a cleavage product of HMGB1 and not disulfide HMGB1.

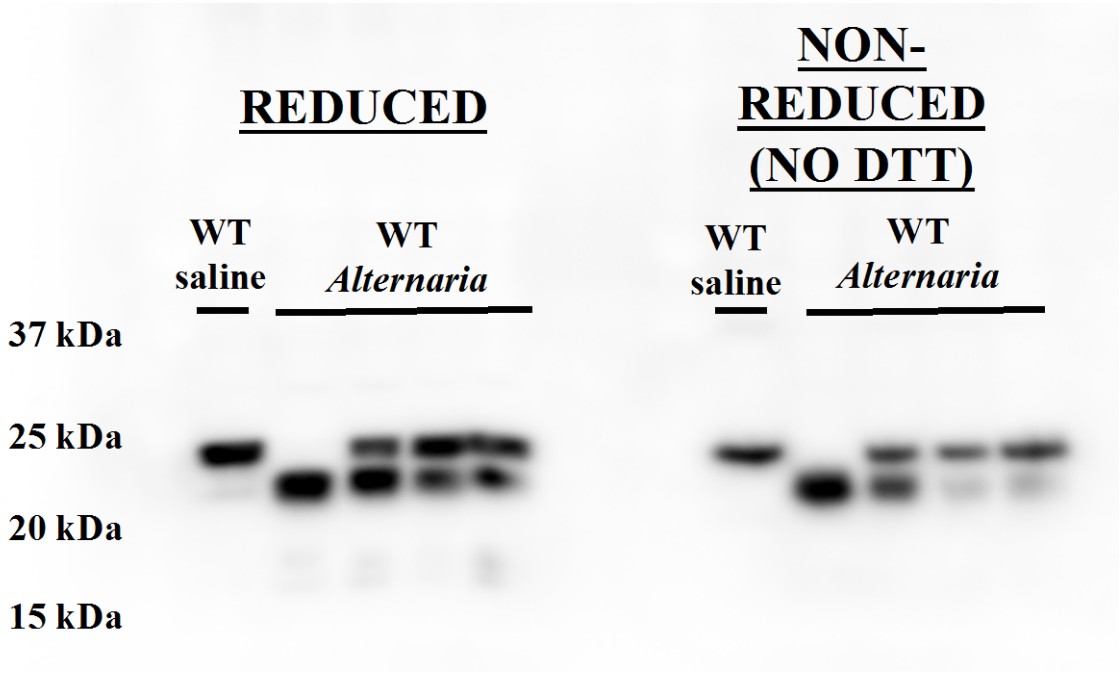


**Figure 29. HMGB1 banding pattern does not significantly change in non-reducing conditions.**

HMGB1 immunoblot of whole lung homogenate under reducing and non-reducing conditions from wild-type (WT) mice after the ten-day *Alternaria* challenge. HMGB1 appears as a band at 25 kDa in the saline-treated sample and as both 25 kDa and ~22 kDa bands in *Alternaria*-treated mice. The banding pattern is not significantly altered in non-reducing conditions when compared to reducing conditions.

HMGB1 levels were also examined in lung homogenates from wild type and RAGE KO mice after chronic HDM treatment (Figure 30). It was difficult to assess if there were appreciable

changes in HMGB1 protein levels over time, yet it was clear that no low molecular weight band was present in any of the HDM-treated samples. Once again, as with the analysis of IL-33 (Figure 13), there seems to be a difference in the banding pattern of HMGB1 between *Alternaria*-treated mice and HDM-treated mice.

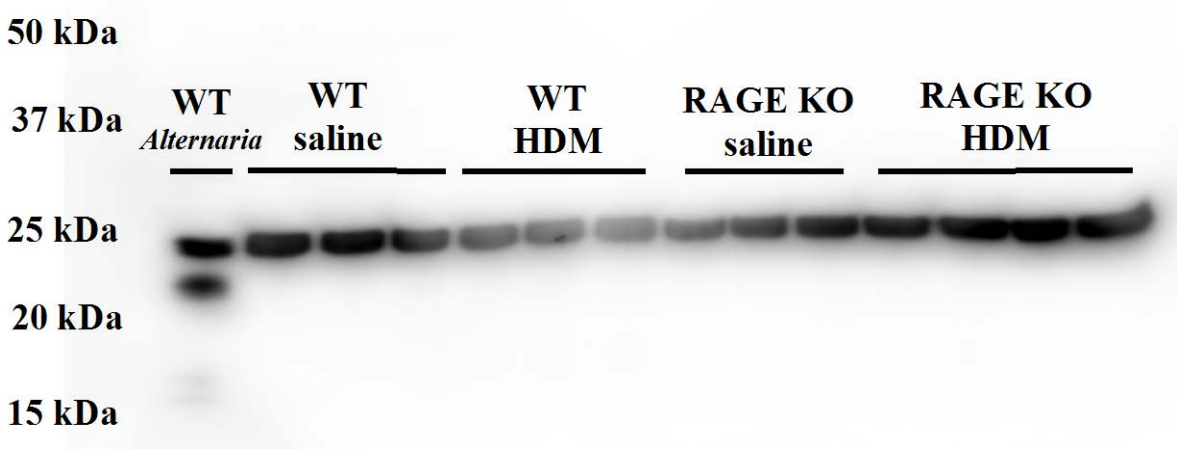


**Figure 30. HMGB1 levels are constant and appear in a single-band pattern on Western blot after chronic HDM challenge.**

HMGB1 immunoblot of whole lung homogenate from wild-type (WT) and RAGE KO mice after seven weeks of HDM exposure. HMGB1 appears as a band at 25 kDa in all samples (as compared to it running as a doublet with a band at 25 kDa and ~22 kDa in WT, *Alternaria*-treated mice, far left). The lightening of the bands in the middle of the blot was likely due to an exposure issue; re-runs of this blot show these bands to be of equal intensity to bands in other lanes (not illustrated). The Ponceau staining showed even loading and transfer (not illustrated), also suggesting an ECL development issue. $n$ = 3-4 mice per strain per treatment group.

## 4.8 SOLUBLE RAGE DOES NOT INHIBIT AAI IN WILD-TYPE MICE AFTER INTRANASAL IL-33 OR *ALTERNARIA* EXPOSURE

Soluble RAGE (sRAGE) is a decoy receptor that can sequester RAGE ligands and decrease overall RAGE signaling. sRAGE has been shown to decrease airway inflammation in wild-type animals in the chronic HDM model,[144] highlighting RAGE signaling as a therapeutic target in AAI. To see if sRAGE also could block AAI in the acute *Alternaria* model, sRAGE was administered simultaneously with *Alternaria* to wild-type mice. Mouse serum albumin (MSA) was given as a nonspecific protein control. In the ten-day *Alternaria* model, sRAGE had no effect on eosinophilia (Figure 31A&B) or mucus hypersecretion (Figure 31C); any mouse that received *Alternaria* developed AAI.

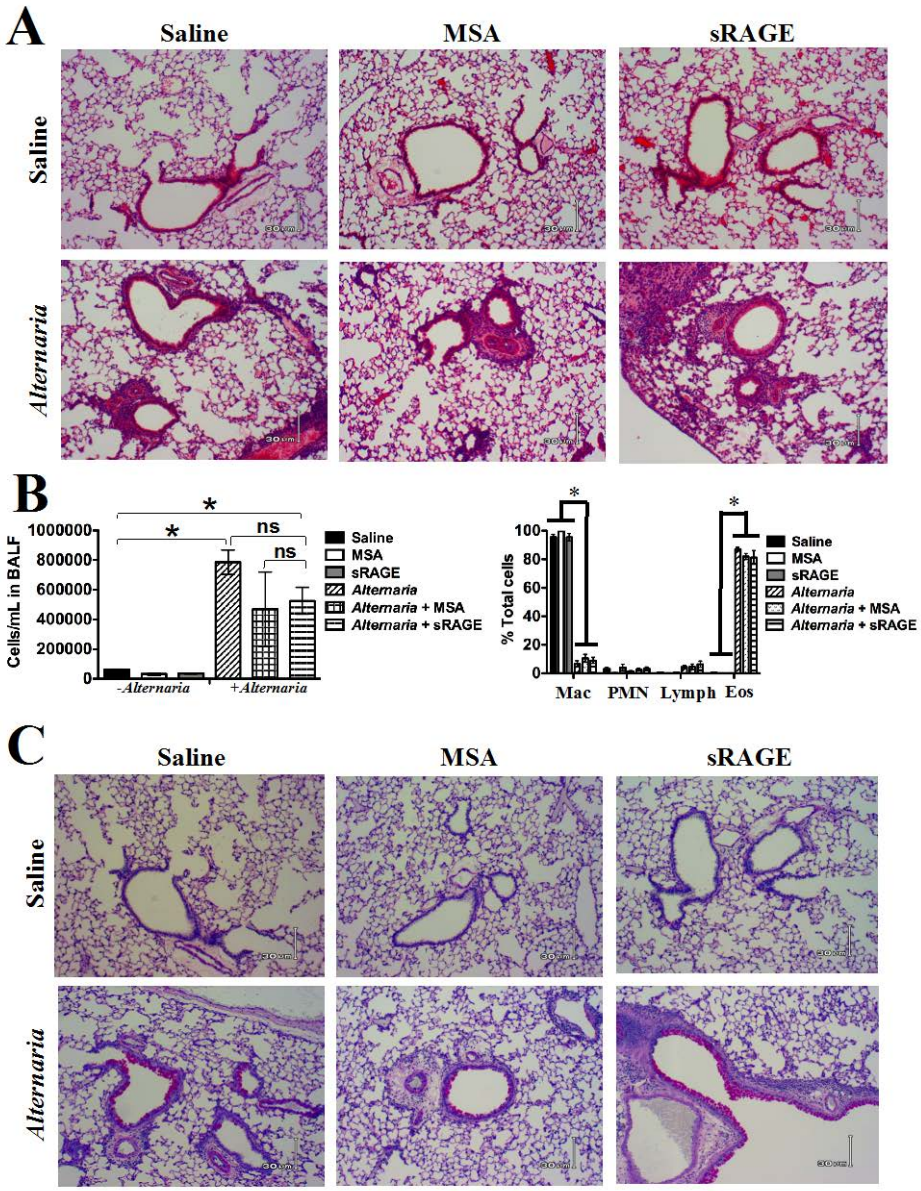


**Figure 31. sRAGE does not block *Alternaria*-induced AAI.**

(A) Representative H&E stains of wild-type (WT) mouse lung tissue after the 10-day *Alternaria* model and simultaneous sRAGE or mouse serum albumin (MSA, control) treatments. (B) BALF cell concentrations and differential illustrating influx of eosinophils after *Alternaria* treatments. (C) Representative PAS images showing bright pink mucin in *Alternaria*-treated mice. All data was collected on Day 10 of the model. Images were taken at 100X magnification. Scale bars = 30 μm. Results are expressed as mean ± SEM. *p < 0.05 versus comparison. *n* = 3-4 mice per strain per treatment group. *ns* = not significant, *Mac* = macrophages, *PMN* = polymorphonuclear leukocytes (neutrophils), *Lymph* = lymphocytes, *Eos* = eosinophils.

This experiment was also repeated using IL-33 instead of *Alternaria*. After four consecutive days of treatments, eosinophilic inflammation (Figure 32A&B) and mucus hypersecretion (Figure 32C) developed in any mouse that had received IL-33, regardless if it had also received sRAGE. Therefore, it appears that sRAGE only blocks AAI in a chronic HDM model and has little to no effect in short-term *Alternaria* and IL-33 models.

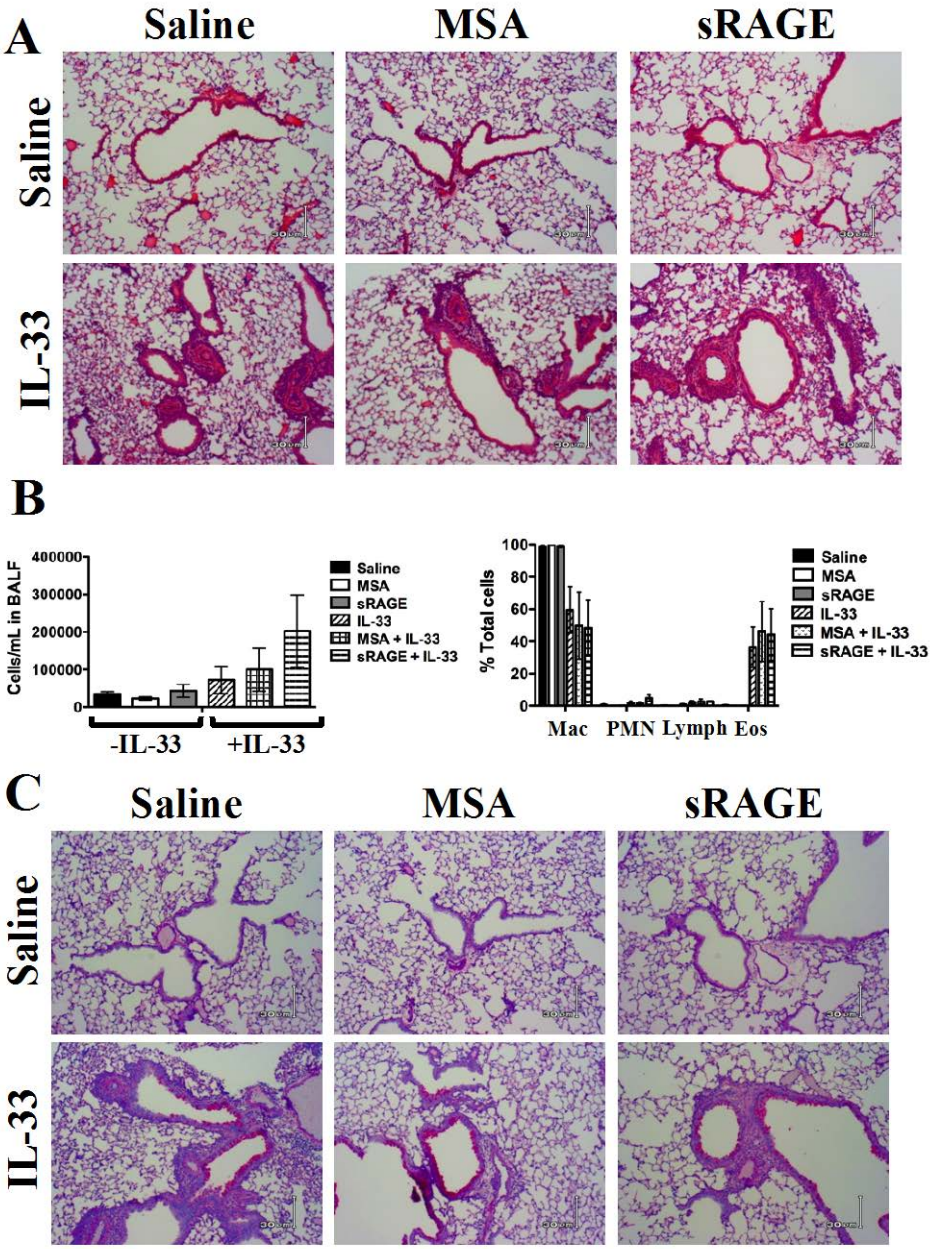


**Figure 32. sRAGE does not prevent IL-33-induced AAI.**

(A) Representative H&E stains of wild-type (WT) mouse lung tissue on Day 4 of the IL-33 model and simultaneous sRAGE or mouse serum albumin (MSA, control) treatments. (B) BALF cell concentrations and differential illustrating influx of eosinophils after IL-33 treatments. (C) Representative PAS images showing bright pink mucin in IL-33-treated mice. Images were taken at 100X magnification. Scale bars = 30 μm. Results are expressed as mean ± SEM. *p < 0.05 versus comparison. *n* = 3-4 mice per strain per treatment group. *Mac* = macrophages, *PMN* = polymorphonuclear leukocytes (neutrophils), *Lymph* = lymphocytes, *Eos* = eosinophils

In a follow-up experiment, IL-33 levels were measured in lung homogenate from wild-type and RAGE KO mice that had been treated with HDM and sRAGE to see if sRAGE had any effect on IL-33 expression. IL-33 levels overall were increased with HDM treatment when compared to saline controls, but the levels were highly variable among mice (Figure 33). For example, treatment with sRAGE resulted in two mice with high levels of IL-33 and two mice with low levels of IL-33 in their lungs. It is difficult to draw any conclusions from this data.

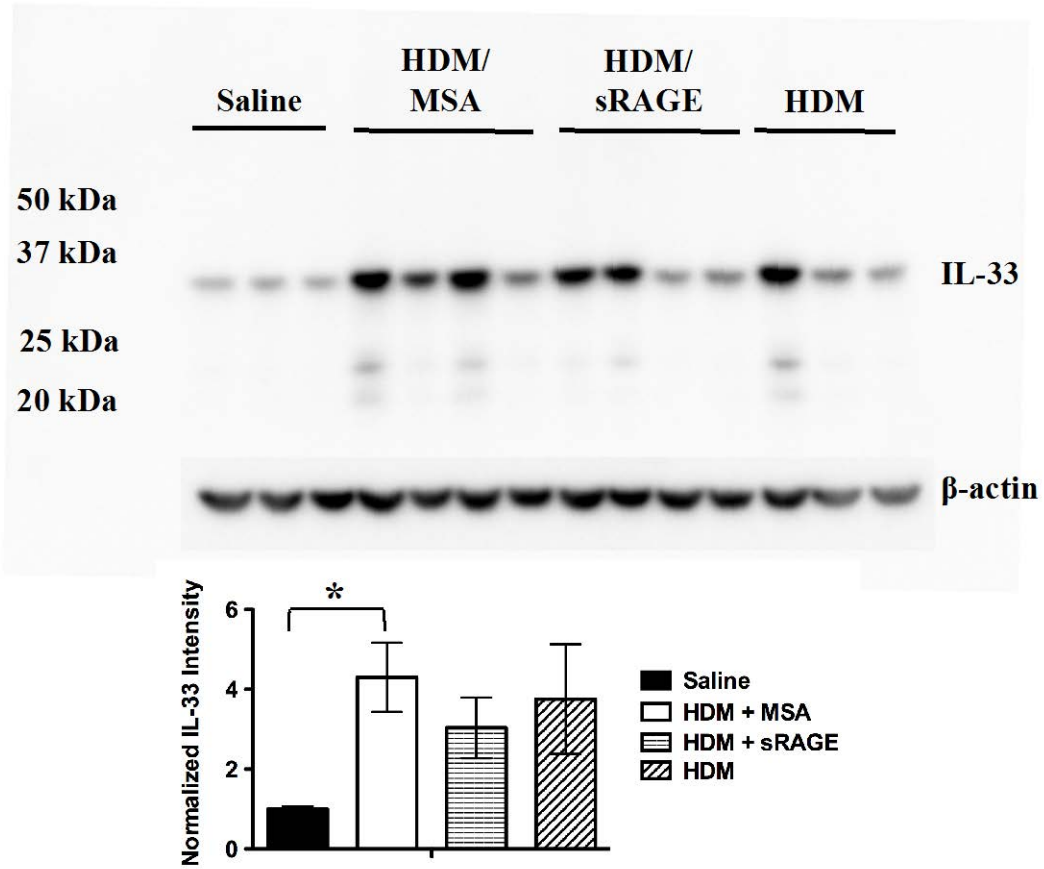


**Figure 33. sRAGE does not seem to greatly affect IL-33 levels in HDM-induced AAI.**

Immunoblot probing for IL-33 in whole lung homogenate after seven weeks of HDM, HDM and mouse serum albumin (MSA, control), or HDM and sRAGE treatments. Below, graphical summary of normalized IL-33/β-actin signal intensity ratios (saline controls set arbitrarily to 1.0). Results are expressed as mean ± SEM. *n* = 3-4 mice per strain/treatment group. *$p < 0.05$ versus comparison.

## 4.9 RAGE IS EXPRESSED ON MOUSE AND HUMAN LUNG ENDOTHELIAL CELLS

As previously discussed, pulmonary parenchymal, and not hematopoietic, RAGE seems to be important for development of AAI. While RAGE is known to be highly expressed on AT1 epithelial cells in the lung, it was first studied in diabetes on endothelial cells in blood vessels. It is unknown, therefore, whether pulmonary epithelial or endothelial RAGE (or both) is involved in development of AAI and ILC2 accumulation in the lung.

To pursue this question, RAGE expression on endothelial cells was confirmed using several methods. First, immunohistochemistry was carried out on frozen wild-type mouse lung sections using antibodies against RAGE and an endothelial marker, CD31. Confocal microscopy was used to visualize the expression patterns and co-localization of these two proteins in the lung. Although the signal was dim in the images, quantification of RAGE-CD31 overlap revealed that RAGE does co-localize with CD31 (Figure 34). When examining fluorescent signal area in the images (binary areas), the ratio of RAGE signal to CD31 signal was 2.7. By analyzing the intersection of RAGE-positive areas with CD31-positive areas, it was found that roughly 28% of the total RAGE signal was expressed in areas also positive for CD31, meaning that 28% of total pulmonary RAGE is expressed on endothelial cells. Conversely, 76.5% of the endothelial cell area expressed RAGE.

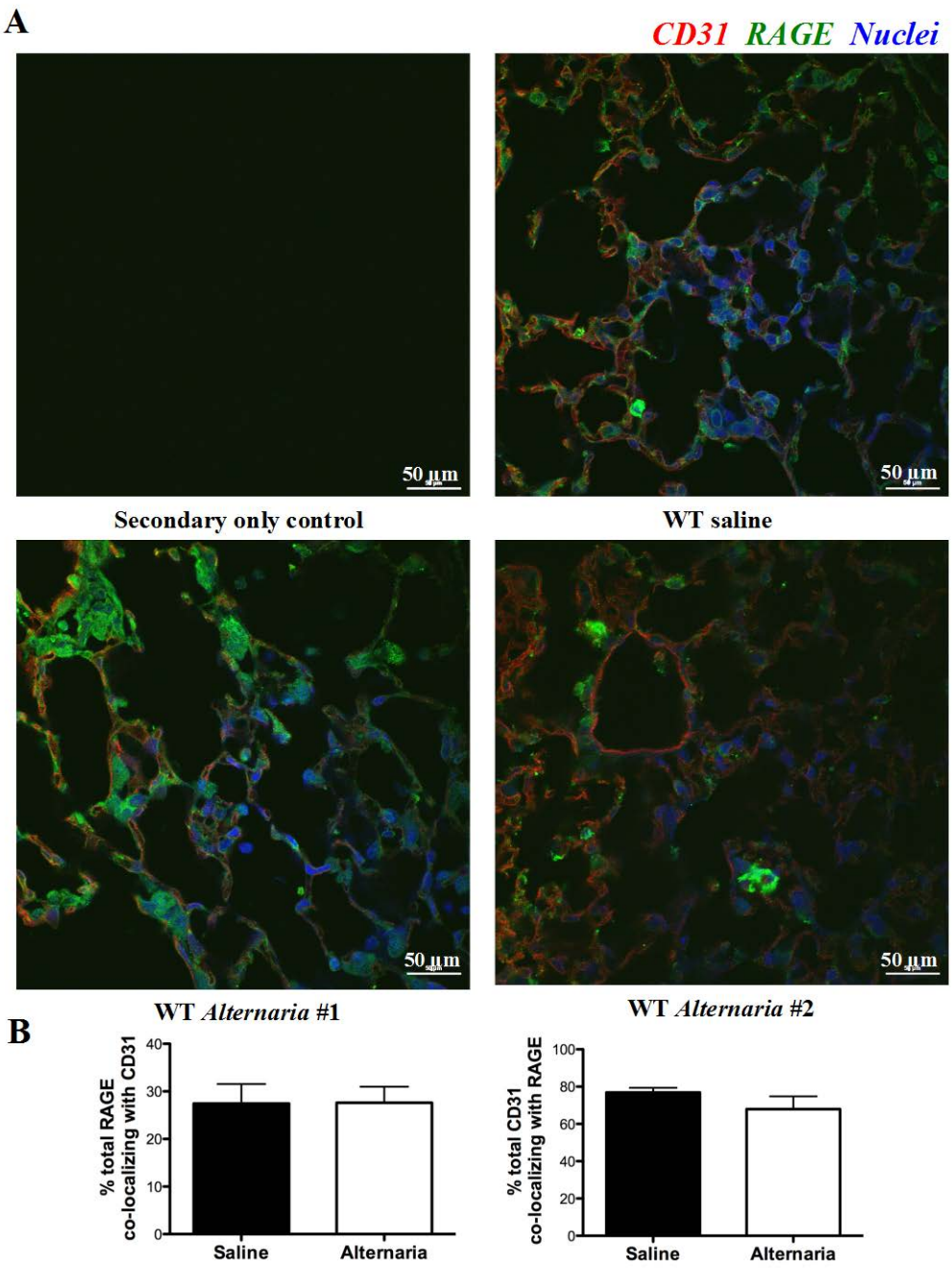


**Figure 34. RAGE is expressed on endothelial cells in the mouse lung.**

*Red: CD31, green: RAGE, blue: nuclei* (A) Representative confocal images of clean secondary control, wild-type (WT) saline-treated, and WT *Alternaria*-treated samples. Co-localization is difficult to assess by eye. Scale bars = 50 µm in all images. (B) Graphical representation of RAGE-CD31 co-localization by quantifying binary areas and comparing. Results are graphed as mean ± SEM. *p < 0.05 versus comparison. *n* = 1-2 animals per treatment group, with 8-17 representative images analyzed per treatment group.

Lung samples of mice treated with *Alternaria* were also analyzed to assess whether allergen treatment and induction of a type 2 immune response changed RAGE expression on endothelial cells (Figure 34). The total areas of RAGE and CD31 signals were increased by 55% and 57%, respectively, and the RAGE:CD31 ratio remained the same as in saline-treated animals: 2.7. In other words, both RAGE and CD31 expression increased after *Alternaria* treatment, but they increased proportionally and therefore remained at similar ratios. Additionally, about 26% of RAGE was expressed in endothelial areas, and 70% of the total endothelial area expressed RAGE after *Alternaria* exposure (Figure 34). Therefore, despite the fact that both RAGE and CD31 increase proportionally after *Alternaria* treatment, the data shows a trend towards a lower number of endothelial cells that are expressing RAGE (although this result is not statistically significant).

As a second way to assess RAGE expression on endothelial cells, two human endothelial cell lines were analyzed by Western blot for RAGE expression. Human umbilical vein endothelial cell (HUVEC) lysates and human lung microvascular endothelial cell (HLMVEC) lysates both expressed RAGE, though in relatively small amounts (Figure 35). RAGE expression in HLMVECs was also confirmed with qRT-PCR (see Section 4.11, Figure 43).

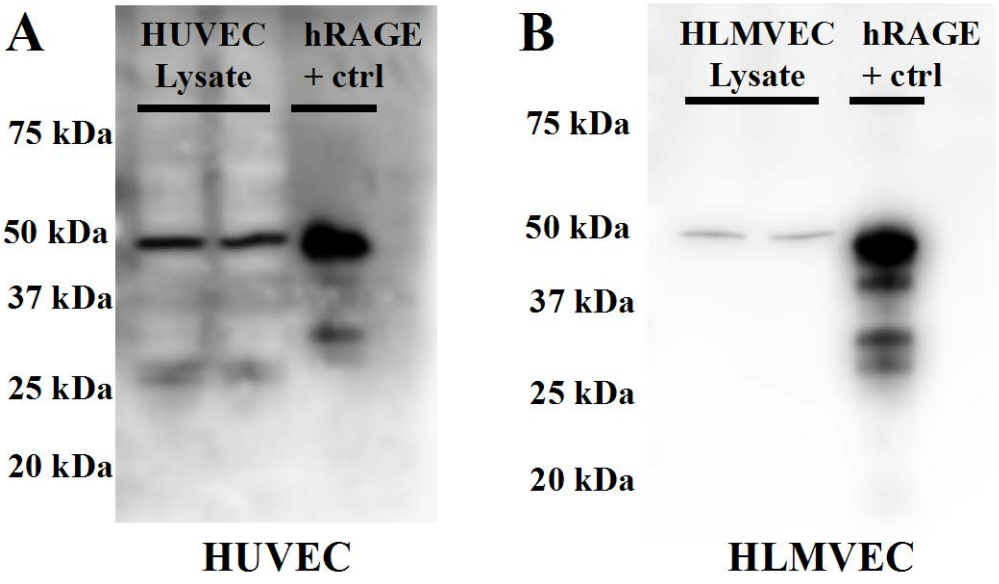


**Figure 35. Human endothelial cells express RAGE.**

(A) Human umbilical vein endothelial cell (HUVEC) and (B) human lung microvascular endothelial cell (HLMVEC) culture lysates express small amounts of RAGE (~46 kDa). *n* = 1 sample, run in duplicate per blot. *hRAGE* = human RAGE.

In summary, both human and mouse endothelial cells were shown to express RAGE. It is possible then that endothelial RAGE may be playing a role in the development of AAI.

## 4.10 VCAM-1 EXPRESSION MAY INCREASE IN WILD-TYPE LUNG TISSUE AFTER TREATMENT WITH *ALTERNARIA*

A 1995 study examining RAGE's role in diabetes showed that RAGE signaling in endothelial cells was able to promote expression of the adhesion molecule, VCAM-1, on the cell surface.[138] Importantly, VCAM-1 binds to and recruits leukocytes that express the β7 integrin into the

lung.[304] This integrin is highly expressed in ILC2s.[199] Therefore, it was hypothesized that RAGE on endothelial cells in the lung promotes recruitment of ILC2s in AAI by upregulating expression of VCAM-1.

To explore this possibility further, Western blots were carried out on lung homogenate from wild-type and RAGE KO mice that had been subjected to either the IL-33 or *Alternaria* models (Figure 2) to probe for VCAM-1 and another adhesion molecule known to be increased by RAGE expression, ICAM-1.[117] After IL-33 exposure, VCAM-1 was not detected in any samples (Figure 36A). However, no positive control was run so this may just be an antibody issue. ICAM-1 in these IL-33 animals was constitutively expressed, with a possible slight increase in the RAGE KO mice at baseline (Figure 36B). After *Alternaria* challenge, a new antibody was used to probe for VCAM-1 and again, VCAM-1 was barely detected, if at all (Figure 36C). The blot was very messy, though a possible band did appear around 100 kDa. ICAM-1 expression in *Alternaria*-treated mice was again seemingly unremarkable, with a possible increase in the RAGE KO mice treated with *Alternaria* (Figure 36D). These results were all inconclusive and it was decided that Western blot of whole lung homogenate was not the best tool for assessing endothelial adhesion molecule expression.

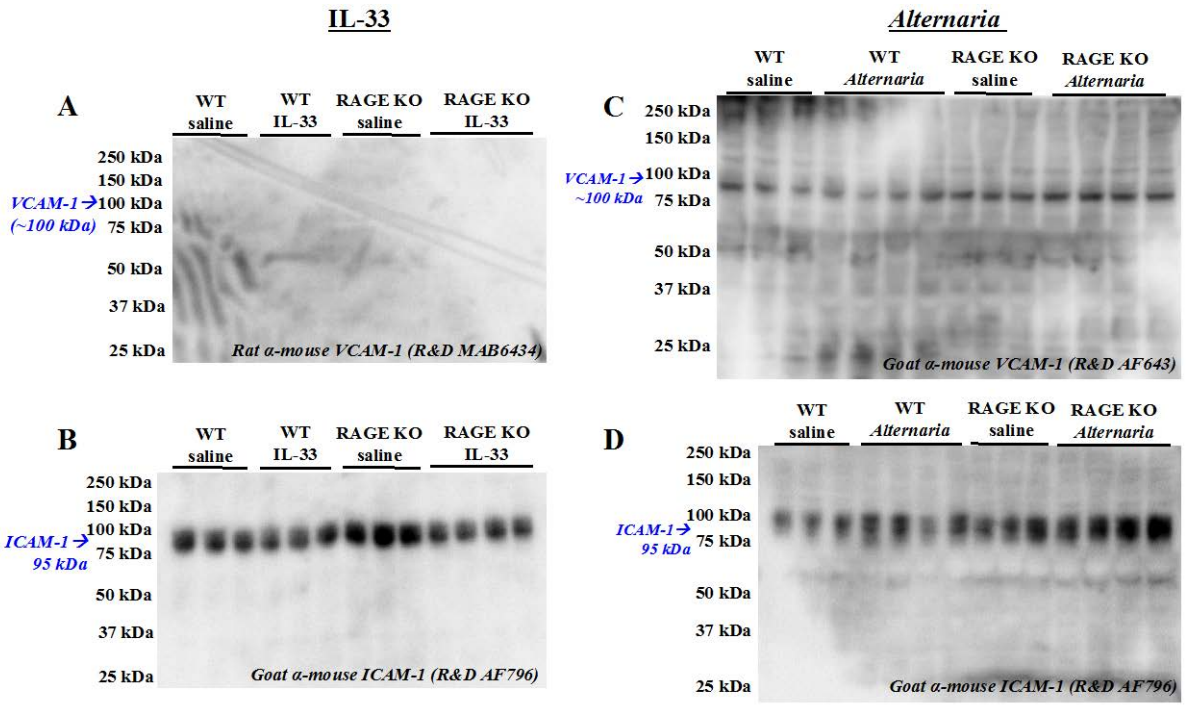


**Figure 36. VCAM-1 and ICAM-1 expression in mouse whole lung homogenate after IL-33 or *Alternaria* challenge is not well assessed by Western blot.**

(A) Immunoblot probing for VCAM-1 (R&D MAB6434; ~100 kDa) expression in wild-type (WT) and RAGE KO whole lung homogenate after four days of IL-33 treatments. No signal was detected. (B) Immunoblot probing for ICAM-1 (R&D AF796; ~95 kDa) expression in WT and RAGE KO whole lung homogenate after four days of IL-33 treatments. (C) Immunoblot probing for VCAM-1 (R&D AF643; ~100 kDa) expression in WT and RAGE KO whole lung homogenate after ten days of *Alternaria* treatment. (D) Immunoblot probing for ICAM-1 (R&D AF796; ~95 kDa) expression in WT and RAGE KO whole lung homogenate after ten days of *Alternaria* treatment. $n$ = 3-4 mice per strain per treatment group.

Because the Western blot results were inconclusive, a new method was employed, and immunohistochemical staining for VCAM-1 and ICAM-1 was carried out on frozen mouse lung tissue sections. Wild-type and RAGE KO mice had been treated with either saline or *Alternaria* over a period of ten days. VCAM-1 expression was seen in both large and small blood vessels

throughout the lung tissue (Figure 37A). By eye, it appeared that VCAM-1 expression increased in wild-type *Alternaria*-treated animals, and several blood vessels in each section appeared to be thickened. VCAM-1 expression did not appear to change between saline-treated and *Alternaria*-treated RAGE KO mouse lungs. However, quantification of VCAM-1 levels revealed no difference among any of the groups tested (Figure 37B). ICAM-1 was diffusely expressed throughout the lung tissue, but was absent around the lining of large airways (Figure 38). ICAM-1 expression was not quantified due to its diffuse expression. By eye, it does not appear that there is any noticeable difference between ICAM-1 levels in any of the observed treatment groups.

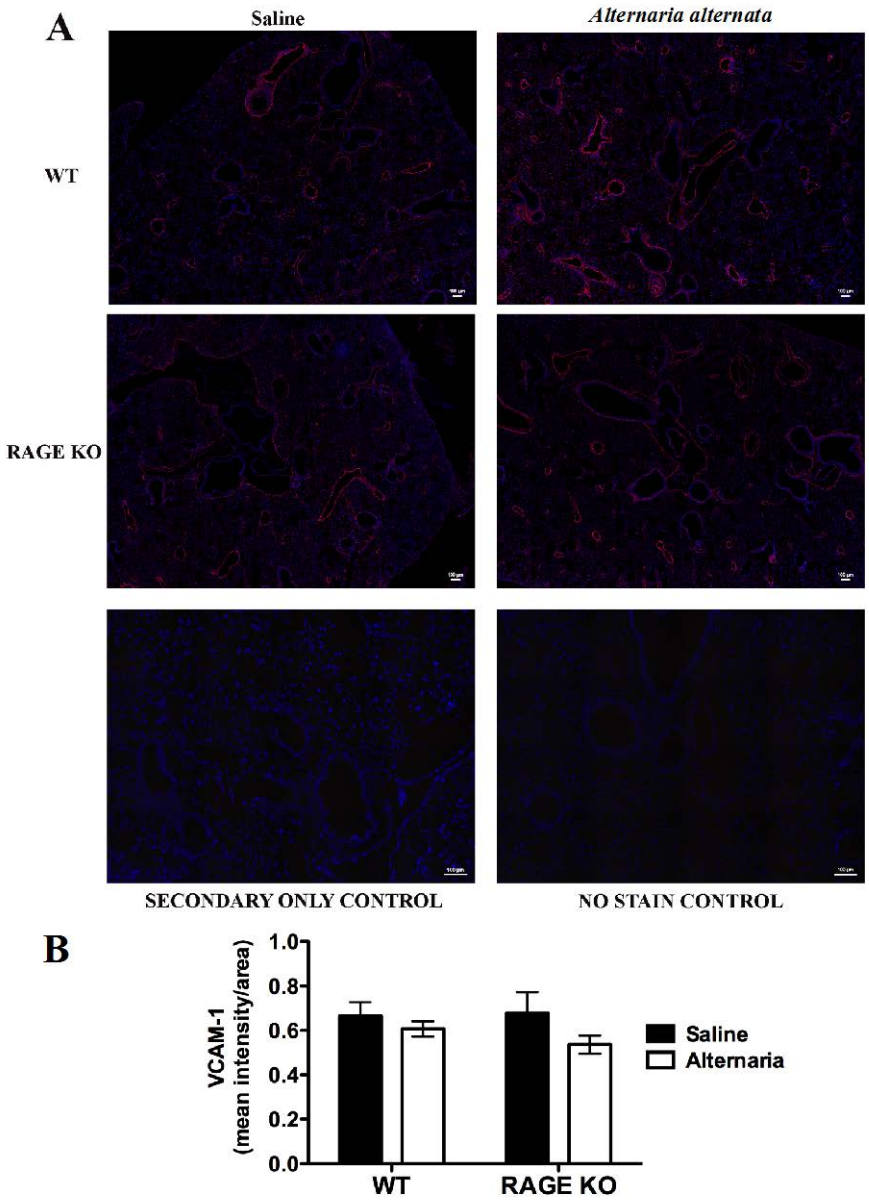


**Figure 37. VCAM-1 expression, measured using immunofluorescence, is unchanged in wild-type and RAGE KO mice after *Alternaria* challenge.**

*Red: VCAM-1, blue: nuclei.* (A) Representative 90i images of saline- or *Alternaria*-treated wild-type (WT) and RAGE KO mouse lung tissue samples showing VCAM-1 signal around blood vessels. "Secondary only" and "no stain" controls for the VCAM-1 experiment are clean and show no background fluorescence. (B) Quantification of VCAM-1 levels in WT and RAGE KO mouse lungs after treatment with *Alternaria* or saline. No difference in VCAM-1 expression is noted. Results are graphed as mean ± SEM. Results with $p < 0.05$ were considered significant (*). $n$ = 4-5 animals per strain/treatment group. Scale bars = 100 μm in all images.

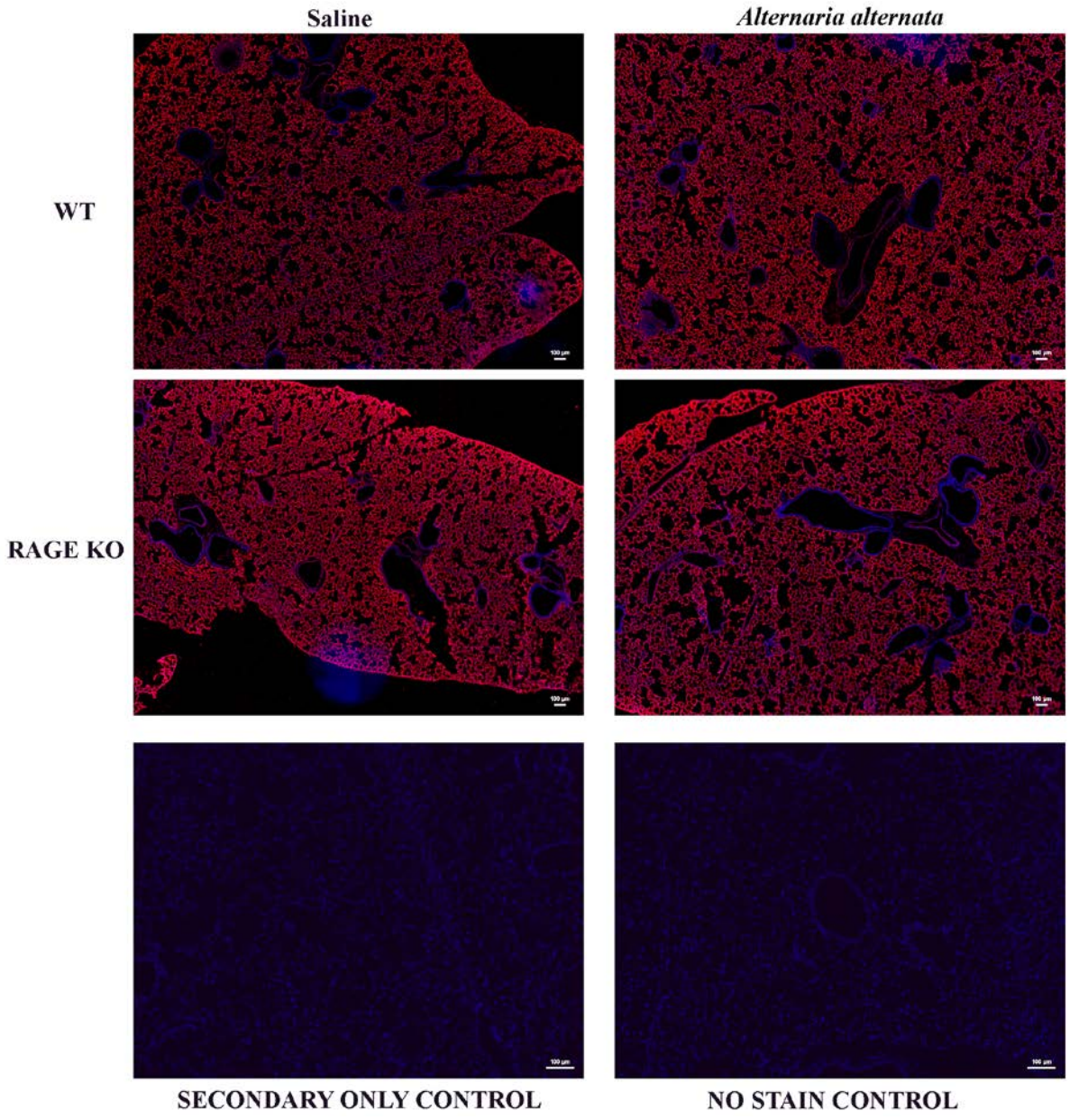


**Figure 38. ICAM-1 is diffusely expressed in mouse lung tissue, but does not appear to change with *Alternaria* exposure in wild-type or RAGE KO mice.**

*Red: ICAM-1, blue: nuclei*. Representative 90i images of saline- or *Alternaria*-treated wild-type (WT) and RAGE KO mouse lung tissue samples showing diffuse ICAM-1 signal. "Secondary only" and "no stain" controls are clean and show no background fluorescence. *n* = 4-5 animals per strain/treatment group. Scale bars = 100 μm in all images.

## 4.11 HMGB1 AND IL-33 PROMOTE VCAM-1 EXPRESSION IN ENDOTHELIAL CELLS, BUT DO NOT SEEM TO TRIGGER NF-KB SIGNALING

A series of *in vitro* studies using both human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HLMVECs) were carried out to assess the complex relationships between IL-33, RAGE signaling, and VCAM-1 expression. Cells were incubated with RAGE ligands, IL-33, or TNF-α (positive control) for 5, 15, 30, 60, 120, or 180 minutes. HUVEC cells were lysed and the cell lysate was used to assess protein levels of p-IκB, total IκB, and VCAM-1. RNA was extracted from HLMVECs for qRT-PCR to assess levels of VCAM-1, ST2, and RAGE message.

HUVECs treated with TNF-α activated NF-κB signaling, as expected, within five minutes (Figure 39). HUVECs treated with HMGB1, AGEs, or IL-33 showed no NF-κB response at any of the time points tested. This was unexpected. HUVECs, as shown above, do express RAGE (Figure 35), and RAGE ligands were expected to activate NF-κB signaling by binding to RAGE. The HUVECs and the HLMVECs did not appear to express ST2 at baseline, but this may have also been due to an antibody problem since positive control was not available to confirm that the ST2 antibody was working optimally (Figure 40). IL-33 was hypothesized to activate NF-κB signaling through ST2 or through RAGE. This study must be repeated, as ligand dosages may not be ideal. It may also take longer than three hours to see activation of the NF-κB pathway in these cells after addition of RAGE ligands or IL-33 to the media.

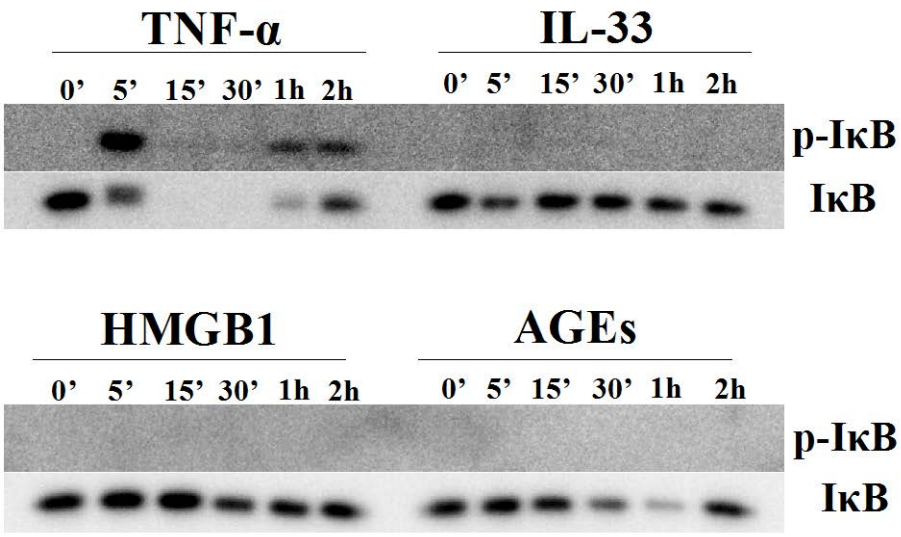


**Figure 39. IL-33, HMGB1, and AGEs do not activate NF-κB signaling in HUVECs.**

Immunoblots probing phosphorylated-IκB (p-IκB) and total IκB in human umbilical vein endothelial cell (HUVEC) lysates after stimulation with TNF-α, IL-33, HMGB1, or advanced glycation endproducts (AGES). Lysates were analyzed at six time points from time zero to two hours after addition of the stimulating molecule. TNF-α, the positive control, activates NF-κB within five minutes (depicted as phosphorylation of IκB). No signal for p-IκB was detected in any of the other lysates. *n* = 1 sample per time point per stimulating molecule.

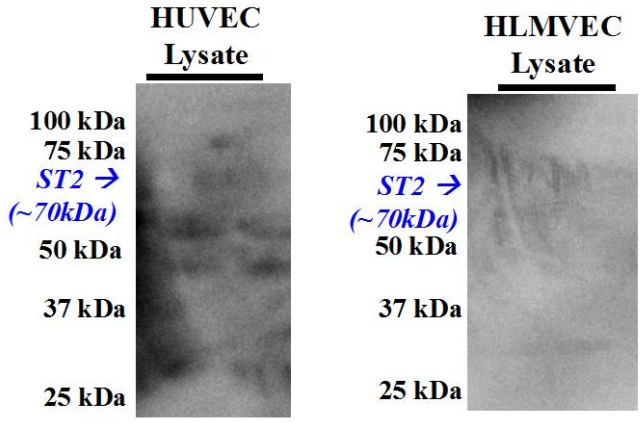


**Figure 40. ST2 expression is not observed in human endothelial cells when analyzed by Western blot.**

Immunoblot probing for ST2 (~70 kDa) in human umbilical vein endothelial cell (HUVEC) and human lung microvascular endothelial cell (HLMVEC) lysates. No signal was detected, but no positive control was available either. *n* = 1 sample, run in duplicate for each cell line.

VCAM-1 expression was also not detected in HUVECs (Figure 41). The positive control was visualized on the Western blot, yet none of the samples expressed the protein. VCAM-1 antibodies have been difficult to work with, so testing of alternative antibodies will be needed before conclusions can be drawn from these experiments.

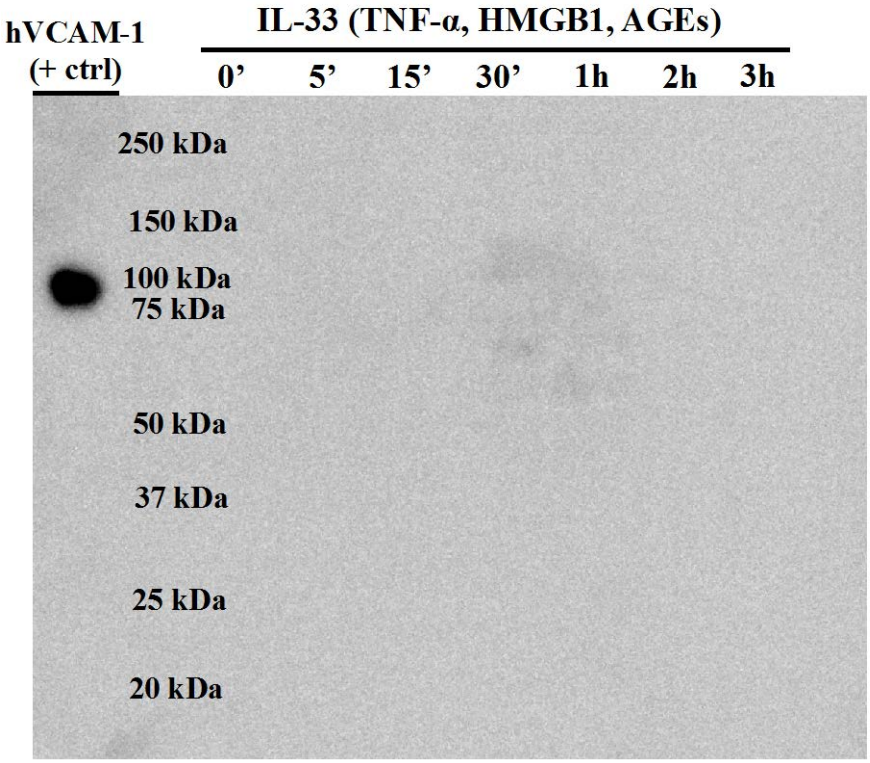


**Figure 41. VCAM-1 expression, as assessed by Western blot, is not induced in HUVECs.**

Representative immunoblot probing for human VCAM-1 (hVCAM-1) in human umbilical vein endothelial cell (HUVEC) lysates in non-reducing conditions. HUVECs were analyzed at various time points after addition of a stimulating molecule. Here, IL-33 was used to stimulate the cells. This blot is identical to the blots analyzing TNF-α-, HMGB1-, or AGE-stimulated HUVECs. No VCAM-1 was detected in any of the samples. *n* = 1 sample per time point per stimulating molecule.

To approach questions about endothelial activation via IL-33 or RAGE ligands using a different method, a series of qRT-PCR studies were carried out. In this case, HLMVECs were used since they provided a lung-specific cell to more accurately model the cell type of interest. First, the results showed that HLVECs do, in fact, express mRNA for VCAM-1, RAGE, and ST2 (Figure 42, Figure 43, Figure 44).

Secondly, as expected, TNF-α caused upregulation of VCAM-1 mRNA (Figure 42A). Levels were 15-fold higher than baseline two hours after addition of TNF-α to the media, and levels increased to almost 43-fold greater than baseline three hours after addition of TNF-α. Comparatively, after three hours, HMGB1 was able to increase VCAM-1 expression by 6-fold (Figure 42B) and IL-33 increased VCAM-1 expression about 3-fold from baseline (Figure 42C). Therefore, both HMGB1 and IL-33 can increase VCAM-1 expression on HLMVECs within two to three hours, but to a lesser degree than known potent activators such as TNF-α (Figure 42D).

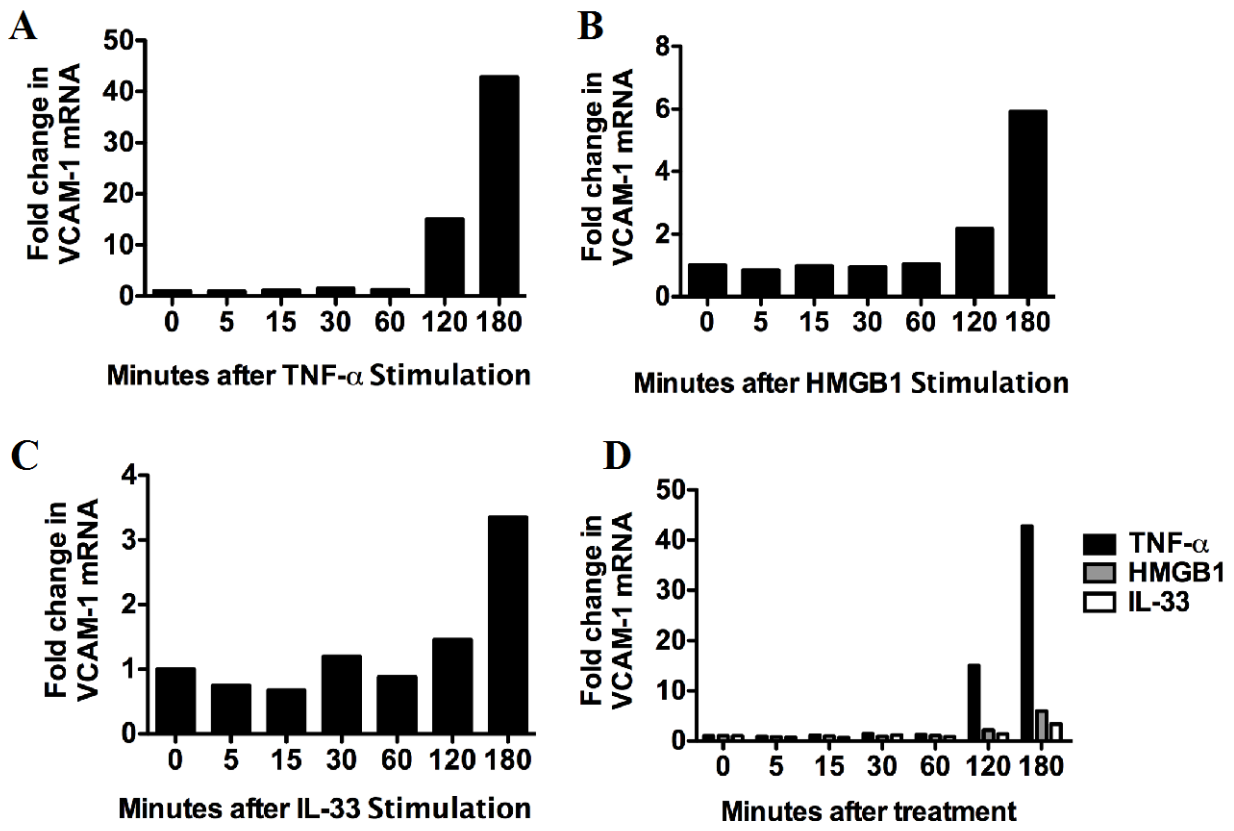


**Figure 42. VCAM-1 mRNA is upregulated in HLMVECs after stimulation with TNF-α, HMGB1, or IL-33.**

qRT-PCR analysis of RNA isolated from human lung microvascular endothelial cells (HLMVECs) after stimulation with (A) TNF-α, (B) HMGB1, or (C) IL-33 over the course of three hours (180 minutes) to assess changes in VCAM-1 expression. (D) All three sets of data (A-C) were combined to highlight differences in the fold-change values between groups. All starting points (0 minutes) were set to 1.0 and fold-change values were calculated against this. *n* = 1 sample per time point per treatment group, run in triplicate on the PCR plate.

RAGE expression levels were also analyzed on HLMVECs. Surprisingly, all three molecules (TNF-α, HMGB1, and IL-33) decreased RAGE expression in these cells (Figure 43A, B, C). The most surprising result was that HMGB1 stimulation decreased RAGE expression levels to about one-tenth of the expression at baseline. This is in contrast to what has previously

been published.[121] Obviously this was only one trial, however, so the experiment will have to be rerun to see if this result is consistent among trials.

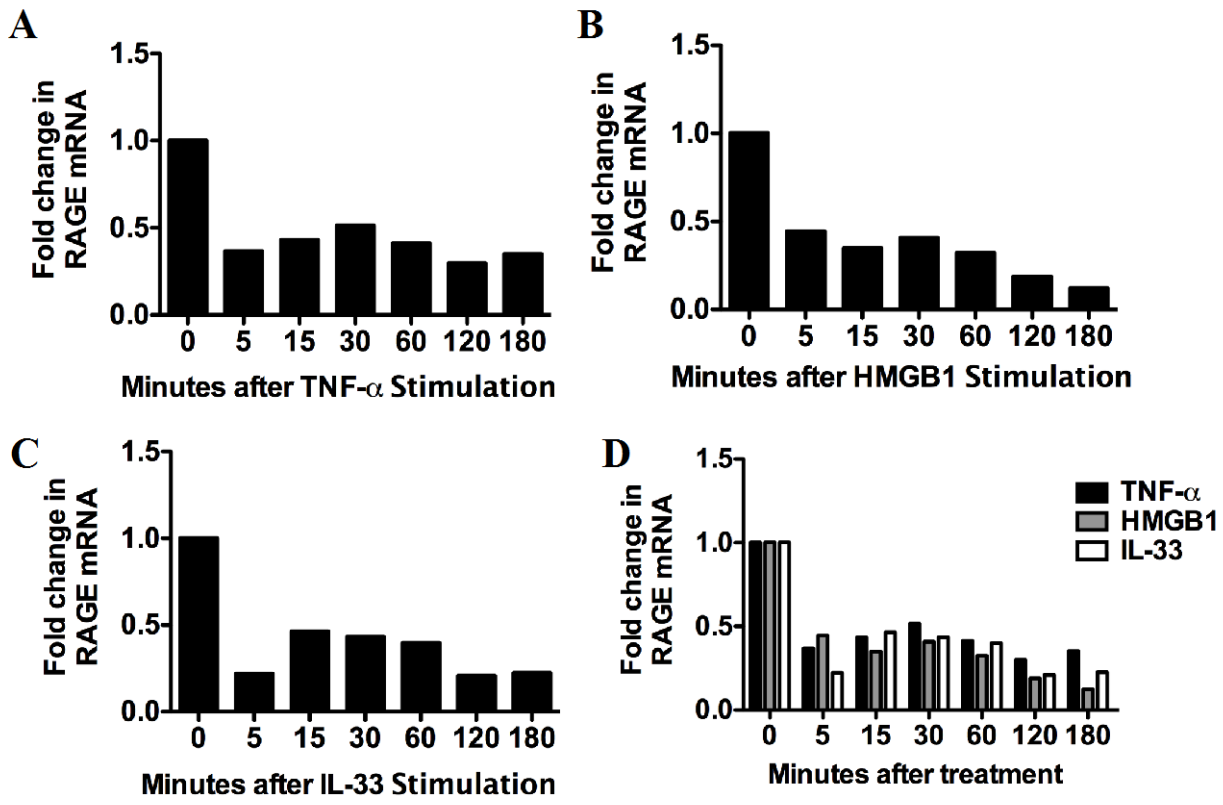


**Figure 43. RAGE mRNA expression is decreased in HLMVECs after stimulation with TNF-α, HMGB1, or IL-33.**

qRT-PCR analysis of RNA isolated from human lung microvascular endothelial cells (HLMVECs) after stimulation with (A) TNF-α, (B) HMGB1, or (C) IL-33 over the course of three hours (180 minutes) to assess changes in RAGE expression. (D) All three sets of data (A-C) were combined to highlight differences in the fold-change values between groups. All starting points (0 minutes) were set to 1.0 and fold-change values were calculated against this. *n* = 1 sample per time point per treatment group, run in triplicate on the PCR plate.

Lastly, expression of the IL-33 receptor ST2 was analyzed on HLMVECs. Expression of this receptor will be important for assessing RAGE-IL-33 signaling pathways in these cells in future experiments. As opposed to Western blot results with HUVECs (Figure 40), mRNA analysis revealed that HLMVECs do, in fact, express ST2. TNF-α increased ST2 expression about 3-fold after three hours (Figure 44A) and IL-33 increased ST2 mRNA expression approximately 1.7-fold after three hours (Figure 44B). HMGB1 had a minimal effect on ST2 expression, showing only a 1.2-fold increase in receptor expression after three hours (Figure 44C). Therefore, it seems that IL-33 can slightly upregulate expression of its receptor, while HMGB1 has little to no effect on ST2 expression (Figure 44D).

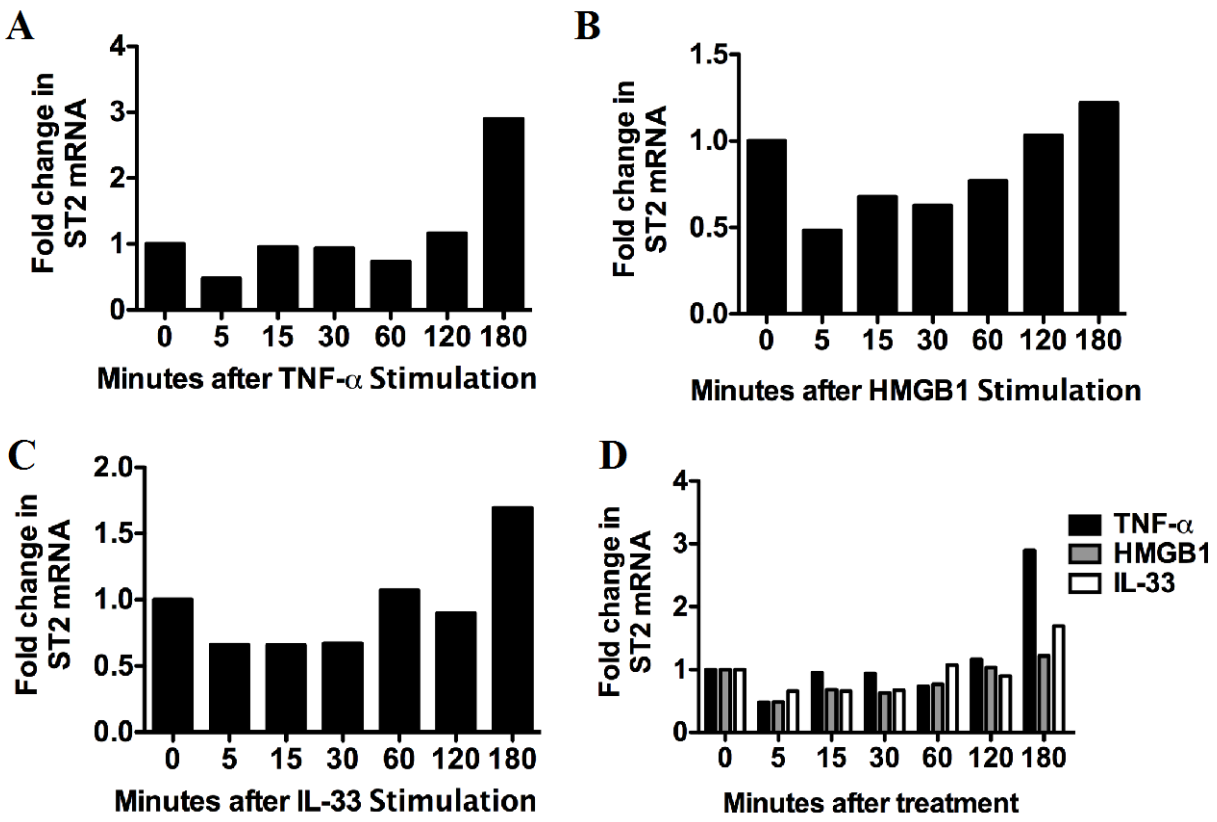


**Figure 44. ST2 mRNA is slightly upregulated in HLMVECs after stimulation with TNF-α or IL-33, but not RAGE.**

qRT-PCR analysis of RNA isolated from human lung microvascular endothelial cells (HLMVECs) after stimulation with (A) TNF-α, (B) HMGB1, or (C) IL-33 over the course of three hours (180 minutes) to assess changes in ST2 expression. (D) All three sets of data (A-C) were combined to highlight differences in the fold-change values between groups. All starting points (0 minutes) were set to 1.0 and fold-change values were calculated against this. *n* = 1 sample per time point per treatment group, run in triplicate on the PCR plate.

While preliminary, these data show important impacts of RAGE ligands and IL-33 on endothelial cells. These experiments also demonstrate the use of this system for further studies to better understand the signaling pathways among RAGE, IL-33, VCAM-1, and ILC2s.

## 4.12 BLOCKING VCAM-1 DURING ALLERGEN EXPOSURE ATTENUATES AAI

To further test the hypothesis that VCAM-1 may be involved in ILC2 recruitment to the lung during AAI, *in vivo* studies in which a VCAM-1 blocking antibody was administered to wild-type mice in conjunction with IL-33 or *Alternaria* were carried out. IL-33 was given once a day for four consecutive days, and *Alternaria* was given every three days over the course of a ten-day period as previously described (Figure 2). Mice were injected with the blocking antibody or an isotype control antibody intravenously through the tail vein one hour before each intranasal dose of cytokine or allergen was given.

In the IL-33 studies, total BALF cell counts and eosinophil numbers were increased after IL-33 treatment (Figure 45). IL-5 and IL-13 levels in the BALF were also increased after IL-33 treatment alone (Figure 46). When the VCAM-1 blocking antibody was given though, cellular inflammation, eosinophil numbers, and type 2 cytokine levels in the BALF were decreased, though not significantly so (Figure 45, Figure 46). This suggests that loss of VCAM-1 receptor ability could lead to a decreased inflammatory response. Unfortunately, the isotype control antibody also caused decreased cellular inflammation and type 2 cytokine levels, suggesting that the results observed with the VCAM-1 antibody may be due to non-specific effects via Fc receptors. Similar results were also seen using the *Alternaria* model to induce AAI (Figure 47). There was also a great deal of variation in the severity of AAI that developed in mice after using either IL-33 or *Alternaria*. More consistent baseline treatments will be needed to see if there really is an effect on AAI after VCAM-1 blocking antibody administration.

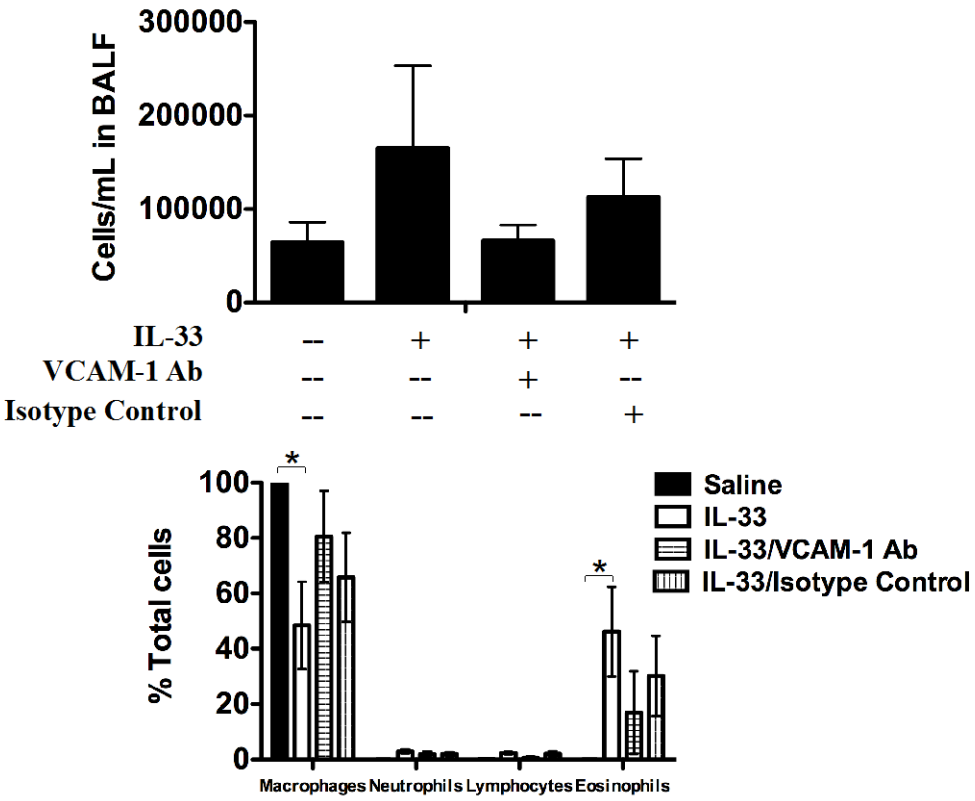


**Figure 45. Use of a VCAM-1 blocking antibody attenuates eosinophil influx in the BALF after intranasal IL-33 challenge.**

Cellular concentration and percentage of eosinophils in the BALF of wild-type mice increased with intranasal IL-33 administration. This effect was somewhat blunted when a VCAM-1 blocking antibody (Ab) was administered one hour before intranasal cytokine treatments. All data was collected on Day 4 of the IL-33 model. Results are presented as means ± SEMs. *$p < 0.05$ versus comparison. *n* = 3-5 mice per treatment group.

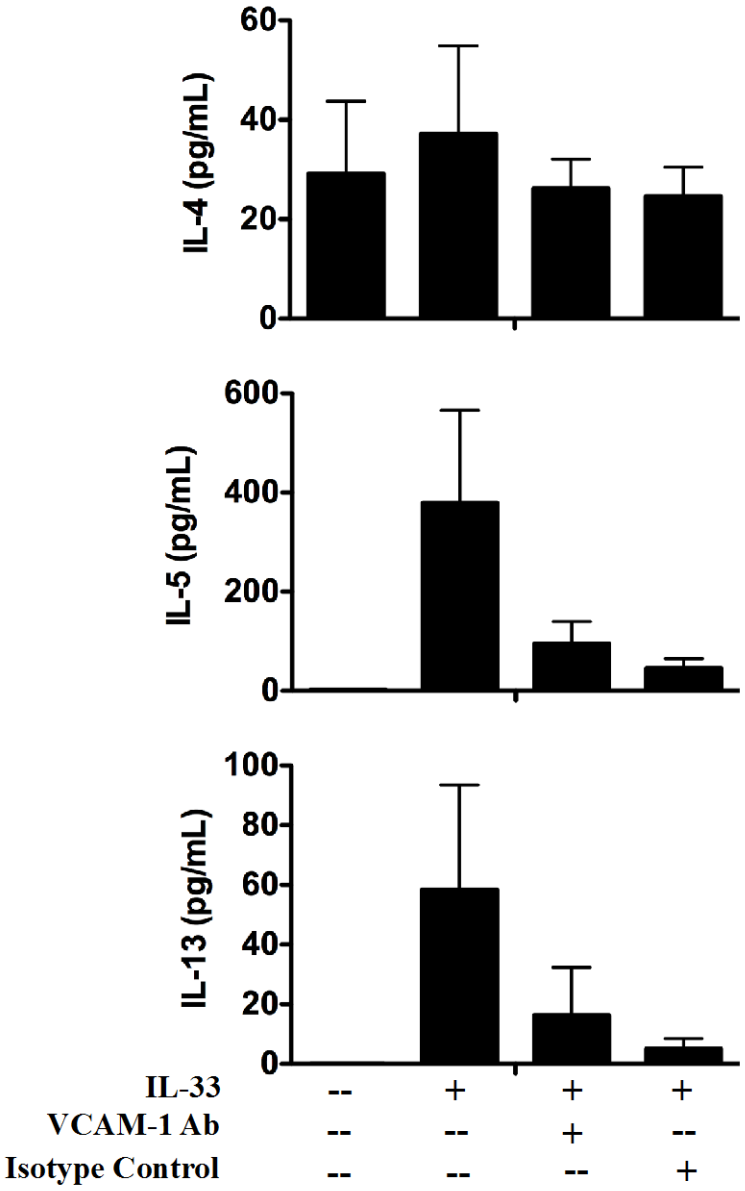


**Figure 46. A VCAM-1 blocking antibody can repress increases in IL-5 and IL-13 cytokine levels in response to intranasal IL-33 (though so can an isotype control).**

ELISA analyses for IL-4, IL-5, and IL-13 cytokine concentrations in undiluted BALF samples collected on Day 4 of the IL-33 model. IL-5 and IL-13 levels in the BALF of wild-type mice increased with intranasal IL-33 administration, and this effect was attenuated when a VCAM-1 blocking antibody (Ab) was administered one hour before intranasal cytokine treatments. An isotype control antibody also diminished the IL-5 and IL-13 responses after IL-33 treatments. IL-4 levels were relatively unchanged among all groups. Results are presented as means ± SEMs. *$p < 0.05$ versus comparison. $n$ = 3-5 mice per treatment group.

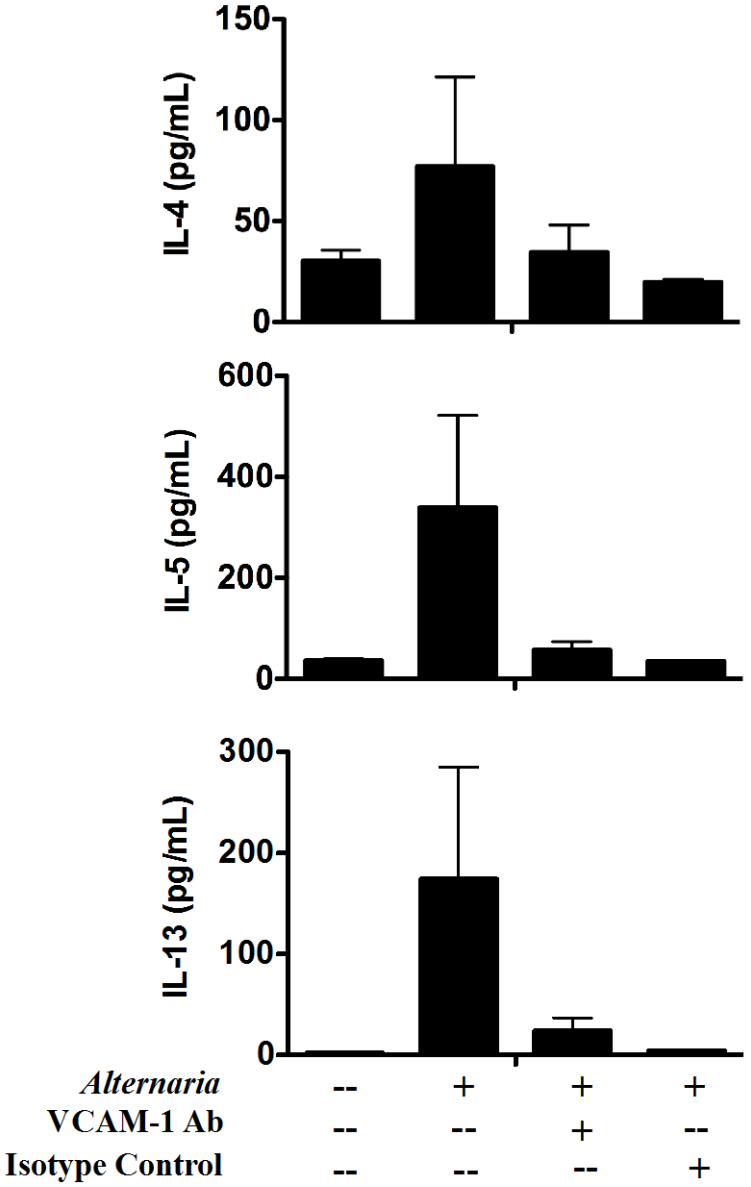


**Figure 47. A VCAM-1 blocking antibody can repress increases in IL-4, IL-5, and IL-13 levels in response to *Alternaria* (though an isotype control antibody can do the same).**

ELISA analyses for IL-4, IL-5, and IL-13 cytokine concentrations in undiluted BALF samples collected on Day 10 of the *Alternaria* model. IL-4, IL-5, and IL-13 levels in the BALF of wild-type mice increased with intranasal *Alternaria* administration, and this effect was attenuated when a VCAM-1 blocking antibody (Ab) was administered one hour before intranasal allergen treatments. An isotype control antibody also diminished the IL-4, IL-5, and IL-13 responses after *Alternaria* exposure. Results are presented as means ± SEMs. *$p < 0.05$ versus comparison. $n$ = 4-5 mice per treatment group.

# 5.0 DISCUSSION

## 5.1 DIFFERENCES IN ACUTE AND CHRONIC ALLERGEN MODELS

When RAGE was first linked to development of asthma pathogenesis, it was in a chronic HDM allergen model.[144] Because ILC2s are early initiators of AAI, an acute asthma model using *Alternaria alternata* was utilized in these studies to better identify RAGE's role in ILC2-mediated type 2 responses. Studies have shown that there are differences in ILC2 and Th2 cell responses in acute and chronic allergen models.[206, 273] There is additional variability among allergens in the mechanisms they use to trigger inflammation. For example, while HDM responses are mostly mediated by papain cysteine proteases, *Alternaria* responses are controlled by serine proteases.[23, 177, 186] The results presented here indicate that RAGE plays a similar role in both *Alternaria* and HDM models, though subtle differences did emerge from the data.

First, in the development of AHR, *Alternaria* exposure appeared to cause a more robust increase in airway resistance (Rn) than HDM challenge in wild-type mice (Figure 7).[144] In contrast, *Alternaria* did not cause any change in tissue damping (G) in wild-type or RAGE KO mice, while HDM caused a significant increase in tissue damping in wild-type, but not RAGE KO mice. Both *Alternaria* and HDM induced significant increases in tissue elastance (H) in wild-type, but not RAGE KO mice. The differences in these pulmonary function parameters suggest that acute *Alternaria* allergen exposure affects the lungs in a different way than chronic

HDM allergen exposure. One possible reason for this is that extensive remodeling takes place in the airway during chronic allergen exposures.[305] IL-13[306] and eosinophils[307] contribute to airway smooth muscle thickening and peribronchial collagen depositions. These changes in the airway may contribute to the differences in pulmonary function parameters that are seen between the ten-day *Alternaria*-treated mice and the seven-week HDM-treated mice.

An interesting finding that was uncovered in the course of the pulmonary function studies was that naïve RAGE KO mice have inherently lower lung compliance than naïve wild-type mice (Figure 7D). Compliance is the ability of the lungs to stretch. Since RAGE is important for the spreading of AT1 cells and because it makes important connections with the basement membrane,[84, 113, 114] it is possible that a lack of RAGE decreases baseline compliance in the lung due to AT1 cell clumping and decreased surface area. Notably, RAGE KO mice can spontaneously develop pulmonary fibrosis, which is a disease primarily characterized by a decrease in lung compliance.[84]

Another difference noted between the acute and chronic models of AAI was the release of IL-4. As shown in Figure 8, *Alternaria*-treated RAGE KO mice had attenuated IL-4 responses. HDM-treated RAGE KO mice, on the other hand, had intact IL-4 responses.[144] Because HDM challenges occurred over the course of seven weeks, it is possible that alternative mechanisms of adaptive immune system activation were stimulated. The ten-day *Alternaria* model, on the other hand, may not allow sufficient time for these mechanisms to be induced. Basophils are potent producers of IL-4 and have been implicated in the type 2 immune response to allergens and helminthes.[283, 308] It is possible that these cells participate in compensatory IL-4 production in the face of chronic allergen exposure. Dendritic cells are also initiators of allergen-induced type 2 immune responses.[31] Dendritic cells must process antigen, travel to the lymph

node, interact with naïve T cells, and promote differentiation of Th2 cells. Th2 cells then have to migrate back to the lung to begin producing type 2 cytokines. It is possible that all of this does not occur quickly enough to induce a potent IL-4 response in the *Alternaria* model, but it may be an ILC2-independent mechanism by which IL-4 is produced in the chronic HDM model.

Another striking difference between the acute and chronic models of AAI was found in the forms of IL-33 that predominated in the lung tissue of wild-type mice after allergen challenge (Figure 13).[162] In both models, IL-33 appears as two bands, one at ~30 kDa and one at ~21 kDa. These lengths are consistent with previous reports of full-length and protease-cleaved IL-33.[192, 193] However, the top band (full-length IL-33) is more prominently expressed in HDM-treated mice, while the cleavage product is expressed more intensely in the *Alternaria*-treated mice. This might be due to different protease activity in the *Alternaria* and HDM allergens, or it might be a reflection of the differences that occur in IL-33 cleavage events over time (days versus weeks of exposure). Alternatively, in chronic asthma, other cell types may begin to release IL-33 in a full-length form as more damage is done to the pulmonary tissue. For example, human primary airway smooth muscle cells isolated from severe asthmatics have been shown to produce full-length, 30 kDa IL-33 *in vitro*. No smaller band was appreciated on Western blots in these studies.[180]

Similar differences in banding patterns were also seen in the analysis of HMGB1 levels in the lung tissue after acute or chronic allergen exposure in wild-type mice (Figure 28, Figure 30). In the paper examining the different redox forms of HMGB1, the lower-molecular weight disulfide HMGB1 band only appeared in the absence of DTT.[302] Since the band appeared even in reducing conditions, it makes sense that the lower band that is seen in wild-type mouse lungs after *Alternaria* treatment is not HMGB1 in an oxidized state (Figure 29). Therefore, the lower

band is more likely to be a cleaved form of HMGB1. Cleavage forms of HMGB1 have not been well explored, but there is evidence in the literature that they exist. One paper describes a cleavage of the N-terminal of HMGB1 between arginine 10 and glycine 11 by a thrombin-thrombomodulin complex, leading to a decrease in HMGB1 inflammatory activity and a banding pattern on Western blot that is very similar to what was observed in these experiments.[301] It is currently unclear why HMGB1 would be cleaved in the acute asthma model but full-length in the chronic model.

A minor difference, but one worth noting, was the effect of allergen on RAGE expression itself, though this must be more carefully explored in the *Alternaria* model. In the HDM model, RAGE expression did not increase after HDM challenge.[144] In *Alternaria*-treated wild-type lung tissue, RAGE immunofluorescence levels were shown to increase by 55% over those seen in saline-treated wild-type lung tissue (Figure 34). This may represent an initial increase in RAGE expression in the lung early on in allergic inflammatory responses that may resolve to baseline levels if the allergen exposure continues chronically.

Finally, sRAGE was able to inhibit AAI in wild-type mice treated chronically with HDM,[144] but it was unable to prevent development of AAI in wild-type mice after *Alternaria* or IL-33 treatments (Figure 31, Figure 32). Chronic administration of sRAGE may be needed in order for it to have an effect (in other words, four treatments may not be sufficient to impair AAI, but twenty-eight doses is sufficient). Additionally, *Alternaria* seems to be a more potent allergen when it comes to inducing AAI in these mice. A higher dose of sRAGE may be needed to see the same effect in the *Alternaria* model that is seen in the HDM model. sRAGE functions by sequestering RAGE ligands and preventing them from binding mRAGE to activate downstream signaling events. RAGE has been shown to work both upstream and downstream of

IL-33. Therefore, sRAGE may only block the upstream RAGE-induced release of IL-33 and may not have any effect on events downstream of IL-33. sRAGE, when co-administered with HDM, prevents initial IL-33 release, thus shutting down the entire type 2 immune response. In the case of IL-33 being co-administered with sRAGE though, IL-33 may still be able to carry out its downstream effects via RAGE in wild-type mice. In RAGE KO mice, this effect cannot occur after exogenous IL-33 administration due to a lack of RAGE downstream of IL-33, but in wild-type mice this mechanism remains intact. The *Alternaria* results are difficult to fit into this scenario, however, as one would expect it to be similar to the HDM results. It possible though that IL-33 and *Alternaria* activate type 2 responses using a different mechanism than HDM does; sRAGE may be helpful in preventing only those mechanisms related to HDM-triggered allergen challenges.

Despite differences between some aspects of acute and chronic models of AAI, both are dependent on the presence of RAGE for the promotion of asthma-like pathogenesis in mouse lungs.

## 5.2 RAGE-DEPENDENT ILC2 ACCUMULATION IN THE LUNG

It has been shown here that RAGE is necessary for the accumulation of ILC2s in the lung in AAI (Figure 10, Figure 16). Without RAGE, pulmonary ILC2s do not accumulate in the lungs after allergen challenge, leading to loss of the IL-5 and IL-13 response and a dramatic lack of eosinophilia, mucus hypersecretion, and AHR. Closer examination of the ILC2 populations in the wild-type lung after stimulation of a type 2 response revealed two ILC2 populations: one that was positive for ST2 and one that was negative for ST2. It was proposed that the ST2-

population was an immature precursor to the ST2+ ILC2 since it produced less IL-5 and IL-13 (Figure 11). This hypothesis is in line with a very recent study identifying two distinct ILC2 populations: iILC2s and nILC2s.[247] iILC2s do not express ST2 while nILC2s do. iILC2s were found to be IL-25-responsive precursors to nILC2s. Because iILC2s can transition to nILC2s faster than nILC2s can proliferate, they are crucial for the rapid accumulation of ILC2s in the lung after allergen challenge.

This brings up an important question about ILC2 accumulation in the lung: is RAGE important for proliferation of resident ILC2s in the lung, or is it important for recruitment of ILC2s from the bone marrow to the lung? Debate about the expansion of resident ILC2 populations versus recruitment of new ILC2s to the lung in the face of allergen challenge has been rampant in the ILC2 field. It is likely that both in-organ expansion and recruitment of ILC2s contribute to the increase in ILC2 numbers during inflammatory responses.

Resident ILC2s have been shown to exist in both naïve mouse and human lung tissue.[33, 35, 202, 208, 259, 271] In the experiments presented here (Figure 10, Figure 16), baseline ILC2 numbers were significantly lower in untreated C57BL/6 wild-type mice compared to those which have been previously published.[35, 256] The baseline numbers also varied among experiments. For example, wild-type saline-treated mice expressed ~1,500 ILC2s in one experiment (Figure 10) and only ~100 cells in another (Figure 16). In contrast, other publications show the presence of about ~10,000 ILC2s at baseline.[35, 256] Cell loss during the process of obtaining a single cell suspension for flow cytometry may be a problem in the experiments presented here, so future studies will aim to streamline this procedure according to recently published standardized protocols.[309] Successful isolation of ILC2s from the lung will be crucial for establishing if wild-type and RAGE KO mice have similar levels of resident ILC2s at baseline. It has been shown

that neonatal mouse lungs express very few ILC2s, but that levels increase to normal adult levels within a week after birth.[258] RAGE is also differentially expressed in neonates: embryos constitutively express RAGE, but levels are downregulated in all organs except the lung after birth.[81] It is possible then that RAGE is important for initial recruitment of ILC2s into the lung soon after birth. A recent study supporting this idea showed the importance of RAGE in leukocyte recruitment in preterm infants.[137] A complete lack of ILC2 accumulation in the lungs of RAGE KO mice in response to allergen may be a result of ILC2s simply not being present to begin with in global RAGE KO mice. A preliminary first attempt to quantify baseline wild-type and RAGE KO ILC2 levels using the new standardized ILC2 flow protocol resulted in low cell yields, but similar numbers of Lin-CD45+ cells in both strains (data not illustrated).

Studies have shown a role for hematopoietic RAGE in the proliferation and activation of various immune cells.[92, 95, 96, 143] Therefore, it is possible that RAGE is needed directly on ILC2s for their development or activation. However, the finding that mice with global RAGE deletion can induce ILC2 numbers and functional profiles in the alimentary tract comparable with those seen in wild-type mice (Figure 18, Figure 19, Figure 20) strongly suggests that RAGE is not needed for ILC2 expansion or maturation *in vivo*. Furthermore, chimeric studies indicate that hematopoietic RAGE does not contribute to the development of eosinophilic inflammation in the HDM model.[162] Thus, the presence of RAGE directly on the surface of ILC2s is probably not necessary for these cells to proliferate or properly function *in vivo*. Data show that RAGE is either minimally or not expressed on ILC2s (Figure 24) or Th2 cells (Figure 23), though more careful studies will be needed to confirm these assumptions.

Together, the bone marrow chimera studies[162] and alimentary tract data (Figure 18, Figure 19, Figure 20) make a strong case that RAGE in pulmonary stromal cells is important for

allergic pulmonary inflammation. The chimera studies show the importance of overall stromal RAGE, while the gut studies support the idea that RAGE in non-pulmonary stromal tissues is not crucial for the development of allergic inflammation. RAGE is expressed in both pulmonary epithelial cells and endothelial cells.[82, 88] Studies of RAGE on endothelial cells have shown that RAGE can recruit immune cells into the lung directly via interactions with Mac-1[117] or indirectly through upregulation of adhesion molecules on endothelial cells.[138]

Mac-1 contains a β2 integrin that facilitates interactions with RAGE for leukocyte recruitment. Notably, ILC2s also express mRNA for β2 integrin.[199] Therefore, RAGE may directly bind and facilitate ILC2 migration into the lung after allergen exposure. The increase in levels of RAGE after *Alternaria* exposure (Figure 34) may indicate that RAGE increases on endothelial cells early on in the allergic response to facilitate ILC2 recruitment into the lung. Once this has occurred, RAGE may revert to baseline levels and thus does not appear increased in chronically HDM-treated mouse lungs.[144]

Alternatively, RAGE might not directly recruit ILC2s into the lung but might promote expression of a downstream mediator to facilitate ILC2 recruitment. RAGE activation induces NF-kB nuclear translocation and amplification of downstream signaling pathways,[124] including a number of which that promote expression of adhesion molecules, such as VCAM-1[138] and ICAM-1.[310] Two studies exploring the relationship between RAGE and ICAM-1 expression on endothelial cells demonstrated complementary roles for ICAM-1 and RAGE in mediating leukocyte adhesion to endothelium.[135, 136] Thus it is possible that RAGE mediates ILC2 accumulation not directly, but by regulating other downstream mediators, such as vascular adhesion molecules (described in Section 5.3 below). Preliminary experiments examining levels of IL-5 and IL-13 in the lung following blockade of VCAM-1 in the *Alternaria* and IL-33

models of AAI (Figure 45, Figure 46, Figure 47) show a decrease in type 2 cytokines with VCAM-1 blockade. However, this same result was found with administration of the isotype control antibody. Switching to a new VCAM-1 antibody (i.e. BD Pharmigen, clone 429)[304], using VCAM-1 Fab fragments, or blocking β7 integrin instead may be potential alternative directions for future experiments. These experiments will help to show if VCAM-1 is important in the recruitment of ILC2s to the lung.

At this time, it appears that RAGE is important for recruitment of ILC2s to the lung during inflammation, but further experiments will be needed to define exactly how this occurs.

## 5.3 RAGE AND VASCULAR ADHESION MOLECULE EXPRESSION

As discussed above, it is possible that RAGE promotes ILC2 recruitment to the lung by mediating the upregulation of adhesion molecules in pulmonary endothelial cells. RAGE has been shown to increase expression of VCAM-1,[138] and VCAM-1 is known to bind with the α4β7 integrin on leukocytes to facilitate their recruitment into the lung.[304] ILC2s express significant amounts of the β7 integrin.[199] Both VCAM-1 and mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1) can interact with α4β7 integrin to mediate cell recruitment into the intestine,[311] but only VCAM-1 was found to be important for β7-mediated recruitment of cells to the lung,[304] suggesting that different mechanisms exist in different organs for leukocyte recruitment. This is complementary to data demonstrating that ILC2-mediated type 2 responses in the GI tract do not rely on RAGE. While GI endothelial cells can recruit ILCs via MadCAM-1,[312] lung may require RAGE for VCAM-1 expression and subsequent ILC2 recruitment. The

fact that RAGE expression is almost exclusively limited to the lung in healthy adults provides support that RAGE may function in a lung-specific manner to recruit ILC2s.

Unpublished immunofluorescence data from our lab show an increase in VCAM-1 expression in wild-type, HDM-treated mice that is absent in RAGE KO, HDM-treated mice (data not illustrated). This result could not be recapitulated in immunofluorescence studies in mice subjected to the *Alternaria* model (Figure 37). It may be that the method used to quantify the data after *Alternaria* treatment masked the true results since VCAM-1 staining in large vessels can skew the data. Analyzing these slides on a fluorescent or a confocal microscope using a higher power objective to focus on smaller tissue areas, smaller vessels, and areas of eosinophilic inflammation may help to enhance the clarity of the data. Another issue could be that the acute model does not allow enough time for upregulation of VCAM-1 and that VCAM-1 is only upregulated at later stages in inflammation. If this is the case, then alternative mechanisms (such as direct RAGE recruitment of ILC2s) must be in place to facilitate rapid, early ILC2 migration into the tissue. qRT-PCR data demonstrated increased VCAM-1 expression within two to three hours in human endothelial cells after stimulation with TNF-α, HMGB1, or IL-33 (Figure 42), indicating that delayed VCAM-1 upregulation is likely not the issue with the immunofluorescence data. Immunofluorescence staining for ICAM-1 was also inconclusive due to the widely distributed expression of the protein in pulmonary tissue (Figure 38). Additionally, Western blots attempting to quantify VCAM-1 levels in lung or human endothelial cells were unsuccessful, likely due to a paucity of good VCAM-1 antibodies (Figure 36, Figure 41). New antibodies will be utilized in future experiments to better understand changes in VCAM-1 expression in pulmonary endothelial cells after allergen treatment. Studies have also shown that endothelial cell apical membrane proteins can be isolated from the rest of the lung tissue via

cationic colloidal silica.[313, 314] This method will be useful for specific examination of adhesion molecules and RAGE on endothelial cell surfaces by Western blot. The only seemingly useful tool that was employed in these experiments for VCAM-1 quantification was qRT-PCR (Figure 42). Implementing the use of more helpful tools is needed to better study RAGE influences on VCAM-1 expression.

## 5.4 RAGE, ILC2S, AND OTHER TYPE 2 IMMUNE CELL RESPONSES

Although this study focuses on RAGE's role in ILC2 accumulation in allergen responses, a lack of RAGE might also inhibit the responses of other IL-5– and IL-13–producing cells, such as T cells and natural killer T cells. In fact, numbers of CD4+ Th2 cells that express IL-5 and IL-13 were lower after *Alternaria* exposure in RAGE KO mice when compared with numbers in wild-type mice (Figure 12). Previous studies have shown that ILC2s can be the major source of IL-5 and IL-13 in models of AAI.[271] Furthermore, decreased T cell responses in this model are not surprising because recent studies have shown that ILC2s and T cells are highly interdependent. T cell responses are diminished when ILC2s are not present, and ILC2s are reliant on T cells for the maintenance of active Th2 cytokine secretion.[36, 285, 315] Another recent study has also shown that one of the primary roles of T cells in allergic responses is to magnify the inflammation and AHR that first result from ILC2 activation.[273]

Dendritic cells are also important players in type 2 immune responses and have not yet been examined in models of AAI in RAGE KO mice. Studies have shown that *Alternaria* exposure potently activates dendritic cells to polarize cells to the Th2 phenotype.[316] IL-13 produced from ILC2s has been shown to mediate dendritic cell migration to the lymph nodes for

T cell priming,[36] suggesting that dendritic cell responses are likely altered as well in RAGE KO mice. A recently published study also uncovered a method by which bronchial epithelial cells directly interact with localized dendritic cells to regulate the inflammatory response.[317] This "imprinting" method has been shown to decrease dendritic cytokine production and activity. Interestingly, dendritic cell allergen uptake and downstream effects were dictated by the health of the epithelium: healthy epithelium caused attenuated dendritic cell responses while damaged epithelium promoted dendritic cell polarization of T cells.[317] These interactions between dendritic cells and the epithelium would be interesting to explore in RAGE KO mice as well.

## 5.5 RAGE AND IL-33

RAGE appears to function both upstream and downstream of IL-33 to promote AAI. As discussed, RAGE's role downstream of IL-33 release may involve its function on endothelial cells to recruit IL-33-activated ILC2s to the lung (discussed in Section 5.2). RAGE's role upstream of IL-33 release is also not well understood, but may involve RAGE on epithelial cells. RAGE is highly expressed on AT1 epithelial cells[86-89] and IL-33 has been localized to AT2 epithelial cells in the lung.[183-186] It is therefore possible that RAGE signaling on AT1 cells promotes release of IL-33 in AT2 cells (explored further in Section 6.1.1).

The idea that asthma and AAI are controlled by alveolar epithelial cells is somewhat surprising, but not entirely impractical. One of the most common symptoms in asthma exacerbations is constriction of the bronchial smooth muscle cells. Because alveolar epithelial cells are considered to be distal to the bronchi, are not surrounded by smooth muscle cells, and function primarily in gas exchange, many believe that they do not play a role in asthma

pathogenesis. However, upon histological examination of lung tissue, one can appreciate that alveolar cells abut the adventitia of larger airways and vessels in both mice and humans (Figure 48). While bronchial epithelium and pulmonary endothelium reside on the luminal side of airways and vessels, respectively, the outer circumference of these structures is completely surrounded by alveoli. Therefore, alveolar epithelial cells are poised to modulate pulmonary airways and vessels just as well as bronchial epithelium and pulmonary endothelium are. This seems to be a very practical setup since alveolar cells, at the forefront of gas exchange, must communicate with airways and vessels to control ventilation and profusion matching.

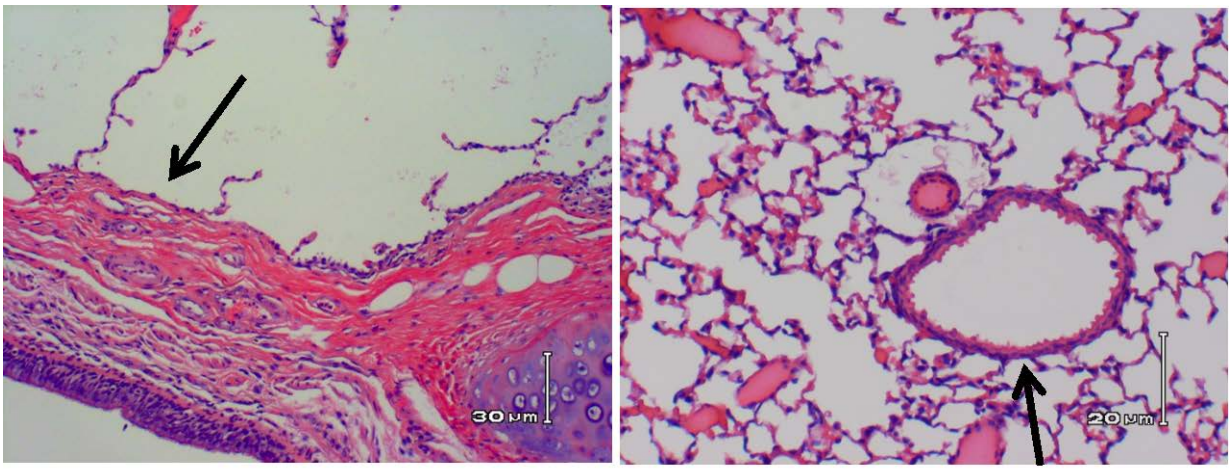


**Figure 48. Alveolar epithelial cells surround airways and vessels in both human and mouse lungs.**

Representative H&E stains of human (left) and mouse (right) lung tissue. Arrows point to alveolar epithelial cells surrounding the adventitia of larger airways and vessels. Human tissue imaged at 100X, scale bar = 30 µm. Mouse tissue imaged at 200X, scale bar = 20 µm.

## 5.6 RAGE LIGANDS THAT TRIGGER AAI

RAGE is activated by many ligands, yet it is unclear which ligand or ligands promote type 2 inflammatory responses in these allergen models. Two known RAGE ligands, S100A8/A9 and HMGB1, are associated with asthma pathogenesis in human subjects.[158, 159] A recent mouse study has shown that HMGB1-RAGE signaling acts upstream of IL-33 in an HDM model to promote allergic inflammation.[156] However, administration of HMGB1 directly to mouse lungs induces a large neutrophilic (not eosinophilic) response,[318] likely through interactions with Toll-like receptor 4.[319] Examination of HMGB1 in BALF after *Alternaria* treatment revealed no specific expression pattern. HMGB1 was found in both a full-length form and a (probable) cleaved form in the lung homogenate of *Alternaria*-treated wild-type mice (Figure 28, Figure 29). RAGE KO mice treated with *Alternaria* did not express this lower molecular weight band, suggesting that this cleaved version of HMGB1 is associated with development of AAI. S100A8/A9 was not examined in any of these models, but is worth examining in future studies. A recent publication focusing on atopic dermatitis showed that S100A9 could stimulate keratinocytes to produce IL-33 via RAGE signaling to promote the type 2 inflammatory response.[109]

Because of IL-33's similarities to the known RAGE ligand HMGB1, the ability of RAGE to bind IL-33 was explored. While a hybrid ELISA binding study hinted that sRAGE might be able to bind IL-33 *in vitro* (Figure 25), ForteBIO Octet studies suggested that IL-33 was not a RAGE ligand (Figure 26). The fact that sRAGE co-administration with IL-33 to mice *in vivo* did not prevent development of AAI (Figure 32) is additional evidence to support the idea that IL-33 is not a RAGE ligand. If IL-33 bound RAGE, sRAGE would have sequestered all of the IL-33 in the treatment solution, thus preventing IL-33 from triggering AAI to its maximum level in wild-

type mice. Functional studies examining IL-33's ability to signal through RAGE in endothelial cell cultures will be helpful for understanding more about this potential interaction.

Overall, further studies are needed to determine whether known, new, or multiple RAGE ligands are involved in promoting RAGE-dependent allergic responses (see Section 6.2).

# 6.0 FUTURE DIRECTIONS

While the data presented here have given some insight into the mechanism by which RAGE promotes AAI, many questions still remain to be answered in order to have a more complete understanding of this process. Several experiments presented below will expand upon current studies, while others will open new areas of exploration in this field.

## 6.1 ON WHAT CELL TYPE IS RAGE EXPRESSION CRUCIAL FOR DEVELOPMENT OF AAI?

### 6.1.1 RAGE on type 1 alveolar epithelium vs. RAGE on vessel endothelium

It seems that RAGE in pulmonary parenchymal, not hematopoietic, cells is important for development of AAI.[162] RAGE is expressed in AT1 epithelial cells and pulmonary endothelial cells (Figure 34, Figure 35).[82, 88] It is unclear if RAGE is important on epithelial cells, endothelial cells, or perhaps both for development of a type 2 response.

To differentiate the importance of RAGE on these two cell types, tissue-specific inducible RAGE KO mice could be used. Allergen treatments can be administered to wild-type mice, global RAGE KO mice, mice with RAGE knocked out only in AT1 epithelial cells (via an aquaporin-5 (*Aqp5*) AT1-epithelial-cell-specific promoter),[320] and mice with RAGE knocked out

only in endothelial cells (via a Tie2 endothelial-specific promoter).[321] Type 2 cytokine levels, eosinophilic inflammation, IL-33 levels, and ILC2 numbers in the lung can be measured to assess differences in AAI in these mice. It is expected that if RAGE is necessary for development of AAI in the specific cell type of interest, then tissue-specific knockout of RAGE in those cells will result in no development of AAI in response to allergen. If RAGE is not necessary in those cells for AAI, then AAI should develop normally in mice lacking RAGE in those specific cells. The answer may be more complicated than expected, however. For example, loss of RAGE in the alveolar epithelium may attenuate IL-33 production/release, but alternative mechanisms may be in place to activate ILC2s, which may still be able to migrate into the lung via endothelial RAGE. The possibilities are many, but use of tissue-specific RAGE KO mice will be an excellent tool for uncovering some of these complicated interactions.

Additionally, *in vitro* signaling studies using primary AT1 and AT2 epithelial cells will be helpful to better understand how RAGE promotes AAI. Since RAGE is known to be expressed on AT1 epithelial cells and IL-33 is known to be highly expressed in AT2 epithelial cells, it would be interesting to investigate if there are paracrine signals between the two cell types through which RAGE signaling on AT1 cells promotes IL-33 release from AT2 cells. AT1 cell cultures[322, 323] can be treated with RAGE ligands (i.e. HMGB1, AGES, S100 proteins), and changes in gene expression and signaling proteins (i.e. NF-κB-related proteins, ERK 1/2, etc.) can be assessed via microarrays, qRT-PCR, Western blot, and ELISA. Media from the RAGE-stimulated AT1 epithelial cells can also be collected and placed onto cultures of AT2 epithelial cells[324] to see if release of IL-33 can be induced from AT2 cells. Further studies would then be needed to identify what specific molecule(s) within the AT1 cell media induces IL-33 release from AT2 cells. This could be done by comparing media from unstimulated AT1 cells with

media from RAGE-stimulated AT1 cells using mass spectrometry. Proteins that are increased in the RAGE-stimulated media would be an excellent starting point and may provide some insight into if and how RAGE on AT1 cells can induce IL-33 release from AT2 epithelial cells. Uric acid and ATP are two molecules that have previously been shown to promote IL-33 release from cells and may also be worth investigating more closely in these studies.[176, 177]

### 6.1.2 RAGE on ILC2s

While ILC2 qRT-PCR (Figure 24) and studies of ILC2 reactions in the gut of RAGE KO mice (Figure 18, Figure 19, Figure 20) suggest that RAGE directly on ILC2s is not important for their development or function, more precise examination of RAGE expression on ILC2s is important. First, since ILC2s have now been shown to be expressed in the spleen of RAGE KO mice at high levels (Figure 20, Figure 21, Figure 22**)**, RAGE KO ILC2s can be more easily isolated. Prior to these studies, very low ILC2 numbers were seen in the lung and thus RAGE KO ILC2s could not be successfully isolated. RAGE KO ILC2s will be a much-needed proper negative control to assess RAGE expression in these cells. Once enough wild-type and RAGE KO ILC2s can be isolated from mouse spleens, qRT-PCR and Western blots can be carried out to look at both RAGE mRNA and protein expression. Development of a specific RAGE antibody for flow cytometry studies on ILC2s would also be extremely helpful in future studies.

As discussed previously, an absence of ILC2s accumulating in the lungs of RAGE KO mice in response to allergen could be due to decreased recruitment or proliferation of ILC2s. Another way to test if RAGE expression on ILC2s directly is important for their proliferation is to activate the isolated ILC2s *in vitro* as depicted in Figure 22. If wild-type ILC2s proliferate normally and RAGE KO ILC2s do not, then RAGE may be important for ILC2 proliferation *in*

*vivo* as well. Wild-type ILC2s can also be cultured in the presence of a RAGE-blocking antibody or a small molecule inhibitor or RAGE to see if this can block normal ILC2 proliferation. qRT-PCR studies on activated wild-type ILC2s in culture may also reveal changes in RAGE expression over time in these cells. For example, RAGE may not be expressed on ILC2s until they are activated.

Examination of RAGE on specific cell types using tissue-specific knockout mice and *in vitro* studies will help to focus future studies on the cell types that are important for RAGE-driven AAI.

## 6.2 WHAT LIGAND(S) ACTIVATES RAGE IN AAI?

As a PRR, RAGE can bind to many molecules without having to recognize a specific amino acid sequence in the ligand. It is unknown what ligand binds to RAGE in models of AAI.

In both the HDM[162] and *Alternaria* (Figure 14) models, IL-33 is not upregulated in response to allergen when RAGE is absent, suggesting that RAGE is upstream of IL-33 release. The question then arises: what is upstream of RAGE that causes release of IL-33? An examination of known RAGE ligands that have been linked to asthma, such as HMGB1 and S100A8/S100A9, may yield some answers to this question. However, preliminary studies looking at HMGB1 levels in AAI models did not seem to provide any clear answers (Figure 27, Figure 28, Figure 29, Figure 30). A very recent study showed that natural killer cells (group 1 ILCs) are activated in a tumor environment by S100A8/S100A9 signaling through RAGE.[325] Allergen-triggered S100A9 has also been shown to signal through RAGE on keratinocytes to promote IL-33 release in atopic dermatitis.[109] A closer examination of S100A8/S100A9 levels

may be important for understanding RAGE signaling in AAI, and may even elicit activatation ILC2s. ELISAs and Western blots to look for S100A8/S100A9 protein levels along with qRT-PCR to examine S100A8/A9 mRNA in the lung tissue and BALF of wild-type and RAGE KO animals after HDM or *Alternaria* treatment would be an excellent starting point. Recombinant S100A8/S100A9 can also be purchased commercially. This ligand can be administered to wild-type mice to see if RAGE signaling is activated and IL-33 levels go up as a result.

Besides focusing on known RAGE ligands that the literature suggests may be involved in AAI, it may also be useful to explore the discovery of novel RAGE ligands. This was briefly investigated in the IL-33/sRAGE binding studies presented in Figure 25 and Figure 26. At this time, it is unclear if IL-33 is RAGE ligand, but it does not seem likely. Signaling studies using pulmonary endothelial or alveolar epithelial cell culture systems and RAGE-blocking antibodies will help to functionally analyze whether IL-33 can signal through RAGE.

Binding studies using Biacore or the ForteBIO Octet systems (similar to those carried out with sRAGE and IL-33) could be useful once promising new RAGE ligands are identified. First, however, the list of molecules that can bind RAGE must be narrowed down. One way to do this is to run allergen extract over an sRAGE-binding column and then run mass spectrometry to identify the molecules that bind to the column. This method was attempted by a previous student in the Oury Lab, Dr. Pavle Milutinovic, and he found that HDM extract did not contain any promising potential RAGE binding partners.[326] He then ran lung homogenates from HDM-treated wild-type and RAGE KO mice over the sRAGE binding column. Several endogenous RAGE ligands were differentially expressed in wild-type allergen-treated animals when compared to saline-treated controls. The expression of these binding partners was not the same in RAGE KO mice treated with HDM. These experiments should be repeated with *Alternaria*

extract and with lung homogenate from *Alternaria*-treated mice to see if RAGE binding partners are in *Alternaria* extract and if expression of endogenous ligands changes in response to *Alternaria* the same way it does in response to HDM. Furthermore, more specific binding studies (Biacore and FortBIO Octet) and *in vitro* and *in vivo* signaling studies can be carried out on several of the most promising candidates that may emerge from the binding column experiments.

The fact that administration of IL-33 does not induce AAI in RAGE KO mice (Figure 15) suggested that RAGE also plays a role downstream of IL-33. Since IL-33 does not seem to be a RAGE ligand, it is important to explore what could be activating RAGE downstream of IL-33 signaling (if RAGE is not acting as a recruiter of ILC2s directly). In addition to the mass spectrometry method described above, another useful method for identifying possible RAGE ligands would be a microarray. This could be used to examine differences in the mRNA levels of a variety of molecules involved in type 2 responses or in RAGE signaling in wild-type and RAGE KO mice after saline or IL-33 treatment. These studies may offer some insight into what ligands are involved in RAGE signaling downstream of IL-33 and which ones are differentially expressed in wild-type and RAGE KO mice.

## 6.3 ARE ILC2S RECRUITED TO THE LUNG, AND IF SO, HOW IS RAGE INVOLVED?

As discussed previously, it is unclear if ILC2 accumulation in the lung is dependent on RAGE for recruitment or proliferation of these cells. The next several experiments presented will explore some of these possibilities.

### 6.3.1 RAGE directly recruits ILC2s

RAGE has been shown to bind to the αMβ2 integrin, Mac-1, on leukocytes to recruit these cells to inflamed tissues.[117] ILC2s are said to express mRNA for the β2 integrin.[199] First, qRT-pCR on isolated splenic ILC2s should be carried out to confirm that they do, in fact, express β2 integrin (Mac-1). To test the hypothesis that pulmonary endothelial RAGE can directly bind to and recruit ILC2s into the lung, a preliminary binding study can be carried out: various concentrations of sRAGE can be loaded into a high-binding 96-well plate and fluorescently-labeled-ILC2s can be added on top of the sRAGE.[117] The total fluorescent signal in each well can be measured to assess how many ILC2s bind to RAGE. This experiment can be repeated with a slight variation: RAGE-expressing endothelial cells can be cultured in a 96-well plate and again, fluorescently labeled ILC2s can be added to each well. A RAGE-blocking antibody can also be added in some wells to see if inhibition of RAGE prevents ILC2 binding. Other wells could also contain a Mac-1 blocking antibody[117] to see if inhibiting this molecule prevents ILC2 binding.

*In vivo* studies can also be carried out to assess if RAGE is directly recruiting ILC2s via Mac-1. A Mac-1-blocking antibody can be administered directly to wild-type mice treated with allergen and levels of ILC2s in the lungs can be assessed and compared to wild-type, allergen-treated mice that did not receive the blocking antibody.

These studies will help to decipher RAGE's role as a direct recruiter of ILC2s in AAI.

### 6.3.2 RAGE indirectly promotes ILC2 recruitment

A focus of several experiments in this dissertation examined RAGE's role in upregulation of VCAM-1, which could bind to the β7 integrin on ILC2s. In future studies, the VCAM-1 blocking antibody study (Figure 45, Figure 46, Figure 47) should be repeated using only Fab fragments of the VCAM-1 antibody. This can easily be done by first cleaving the Fc region off of the antibody with pepsin. This will hopefully remove non-specific binding effects that can be caused by Fc receptor binding interactions. AAI and ILC2 numbers in the lungs will be examined after treatment with VCAM-1 antibodies and allergen in wild-type mice. These studies seem promising already, and the use of Fab fragments will hopefully improve the clarity of the results in future studies. These experiments can also be repeated using a β7-blocking antibody to see if similar results are found.

Additionally, expansion of the current *in vitro* studies will be carried out. Because IL-33 can slightly increase VCAM-1 expression in endothelial cells (Figure 42), it would be interesting to carry this study out again with the addition of a RAGE blocking antibody, an ST2 blocking antibody, and a combination of the two. This will help to determine if IL-33 can signal through RAGE to promote VCAM-1 expression. Use of alternative RAGE ligands such as AGEs and S100A8/S100A9 will also be useful to see if these ligands can promote VCAM-1 expression, ST2 expression, or IL-33 release in pulmonary endothelial cells.

Finally, the cell culture studies can be repeated using primary pulmonary endothelial cells that can be isolated from wild-type and RAGE KO mice as previously described.[327]

### 6.3.3 RAGE promotes ILC2 proliferation in the lung

Another hypothesis that has not been fully explored is that RAGE may be needed to promote proliferation of resident ILC2 populations in the lung. There is a controversy in the field as to whether the lung has resident ILC2s that proliferate in response to inflammatory signals, if new ILC2s are recruited from the bone marrow to inflamed lungs, or a combination of both.

First, it would be best to quantitate the number of ILC2s that reside in wild-type and RAGE KO lungs at baseline. RAGE KO mice may simply have no ILC2s in the lung to begin with, thus there is no starting material for cellular proliferation. Flow cytometry can be carried out on naïve wild-type and RAGE KO mice to assess baseline ILC2 levels in the two strains. A newly revised, standardized flow cytometry protocol has recently been published[309] and may provide better ILC2 yields and more accurate baseline results than those illustrated in this dissertation.

One way to explore RAGE's role in proliferation of ILC2s has been discussed in Section 6.1.2: stimulate isolated wild-type and RAGE KO ILC2s in culture with IL-2, IL-7, and IL-33 and observe their proliferation, or stimulate wild-type ILC2s in both the presence and absence of a RAGE blocking antibody and observe differences in proliferation.

ILC2s require IL-2 and IL-7 for proliferation *in vitro*. A recently published study has also demonstrated that IL-2 is crucial for ILC2 survival, proliferation, and cytokine secretion during pulmonary inflammatory responses in mice.[328] IL-2 and IL-7 cytokines have not been investigated extensively in wild-type and RAGE KO mice after treatment with *Alternaria* or HDM. The amounts of these cytokines are undetectable in BALF (data not shown), so qRT-PCR, ELISA, or ELISPOT of whole lung homogenates would be better ways to analyze levels of these

cytokines. If the cytokine levels are altered in RAGE KO mice, this may be the reason why ILC2s cannot proliferate in response to allergen.

Finally, the IL-33 receptor, ST2, is known to require an accessory protein for signaling. To examine if RAGE can act as an accessory protein for the ST2 receptor on ILC2s, isolated ILC2s from wild-type and RAGE KO mice can be isolated and stimulated with IL-33 *in vitro*. If RAGE is required for IL-33 binding and activation of ST2, then RAGE KO ILC2s should not be activated by IL-33.

It should be noted that most of these experiments assume that RAGE is expressed on ILC2s. Experiments outlined in Section 6.1.2 should be able to identify if RAGE is expressed on these cells or not, and if some of the experiments outlined here are worth pursuing. Data presented from the GI studies (Figure 18, Figure 19, Figure 20) demonstrate that ILC2s function normally in RAGE KO mouse GI tracts during type 2 immune responses. This suggests that RAGE is not important on ILC2s themselves. Therefore, RAGE is probably more important for ILC2 homing to the lung during inflammation or development. Examination of resident ILC2 populations in the lung as well as observations about IL-2 and IL-7 developmental signals for ILC2s will still be important questions to answer about RAGE's role in ILC2 proliferation in the lung, regardless of whether RAGE is expressed directly on ILC2s or not.

## 6.4 WHAT OTHER TYPE 2 RESPONSE ELEMENTS ARE INHIBITED BY LOSS OF RAGE IN AAI?

The absence of ILC2s in the lungs of RAGE KO mice after allergen challenge shows that the response of these major early players in the type 2 response to allergens is defective when RAGE

is absent. Studies shown here, however, have also demonstrated that CD4+ Th2 cell responses are impaired in RAGE KO mice as well (Figure 12), suggesting that ILC2s are not the only immune cells affected by loss of RAGE. Indeed, one study has even shown that ILC2s, while important for locally induced type 2 responses, are not necessary for systemic, antigen-induced type 2 immune responses.[329] Therefore, other mechanisms involving other immune cells are important for mounting system type 2 immune responses.

Mast cells are important sources of IL-33,[330] though there is conflicting evidence in the literature about RAGE expression on mast cells themselves (though more studies point to a lack of RAGE expression on mast cells).[100, 160, 331] Mast cell proteases have recently been shown to be important for cleavage of full length IL-33 into its more active form.[193, 332] Exploration of mast cell populations in RAGE KO mice would be interesting to see if they are contributing to an impaired type 2 immune response in these mice.

Dendritic cells (DCs) are also important cells in the type 2 immune response and interact closely with pulmonary epithelial cells to mediate immune responses.[317] These cells are known to express and even rely on RAGE for certain functions.[92, 143] ILC2 function has also been shown to be important for dendritic cell migration to lymph nodes for T cell priming.[36] Exploration of DC populations in RAGE KO mice may provide additional insight into the broader mechanisms that are at work for RAGE to promote AAI.

## 6.5 WHY ARE THERE DIFFERENCES IN IL-33 BANDING PATTERNS IN THE HDM AND *ALTERNARIA* MODELS?

As discussed previously, the banding pattern for IL-33 in the chronic HDM model is different from that seen in the acute *Alternaria* model (Figure 13).[162] Further exploration into these banding patterns may be helpful in understanding the role that IL-33 is playing and also how different allergen proteases affect IL-33 cleavage.

First, the two bands could be excised from a protein gel and submitted for mass spectrometry analysis to identify where exactly the cleavage of full length IL-33 occurred. This may provide some insight as to why *Alternaria*-treated mice have more cleaved IL-33 in the lung and HDM has more full-length IL-33.

IL-33 can also be incubated with *Alternaria* or HDM extract directly to see how proteases in each of these allergen extracts affects IL-33 cleavage. This would require purifiying or obtaining full length IL-33 to use as starting material, which has previously been carried out by another group.[192] This group also has very specific antibodies to IL-33 that may be better than the commercially available one (R&D) used in these experiments. This experiment may help to confirm the IL-33 expression pattern that is seen in mice during AAI.

## 6.6 CAN RAGE BE TARGETED AS A THERAPEUTIC FOR ASTHMA?

Ultimately, the hope of understanding how RAGE promotes AAI would be to find a novel therapeutic to target this pathway in human asthma sufferers. RAGE appears to be acting very early on in the development of AAI, as it is needed for IL-33 release. Administration of sRAGE

to mice in the HDM model was promising as sRAGE diminished AAI.[144] These results did not seem to be consistent in other models of AAI (Figure 31, Figure 32). Experiments in which sRAGE is administered with *Alternaria* or IL-33 should be repeated, possibly with different dosages or treatment regimens. IL-33 release following treatment with sRAGE should also be closely monitored.

It also will be important to see if sRAGE can attenuate AAI after it has already been established. In this case, HDM or *Alternaria* should be given to wild-type mice to establish AAI before sRAGE is administered, and then effects on AAI can be measured. These experiments can also be repeated with small molecule inhibitors of RAGE such as FPS-ZM1 (now available commerically from Calbiochem/Millipore).[151]

## 6.7 WHAT ROLE, IF ANY, DOES RAGE PLAY IN OTHER TYPES OF ASTHMA?

Allergic asthma is just one of the many phenotypic types of asthma that are observed in the general population.[25] Another growing type of asthma is obesity-associated asthma, which greatly differs from allergic asthma in that it tends to be later-onset, less Th2-driven, and highly refractory to standard asthma therapies.[25, 333-336] Importantly, a recent study has linked IL-17-producing ILC3s to obesity-associated asthma in mouse models.[238] In a future study, it would be interesting to feed both wild-type and RAGE KO mice a high-fat diet to see if RAGE KO mice are protected from obesity-induced changes in AHR and AAI. This study would show that RAGE is important for several different types of asthma, or, it will show that RAGE is a specific mediator of allergic, Th2-driven asthma.

# 7.0 FINAL THOUGHTS

Organ-specific innate responses have evolved based on the physiology and environmental interactions of each organ.[337] These responses help to maintain organ tissue homeostasis, and unbalanced innate responses result in tissue pathology. RAGE's high expression in the lung and involvement in the initiation of innate immune responses as a PRR make it an attractive potential mediator of organ-specific innate immunity in the lung.

The experiments presented here are the first evidence of a parenchymal factor, RAGE, that modulates lung-specific recruitment of ILC2s. Release of the ILC2-stimulating cytokine, IL-33, as well as downstream type 2 immune responses mediated by IL-33, are pulmonary RAGE-dependent. Because RAGE is expressed on both alveolar epithelial cells and vascular endothelial cells in the lung, it has been proposed that RAGE functions in both of these cellular compartments to promote AAI through different mechanisms. Epithelial RAGE may be involved in IL-33 release from AT2 epithelial cells, while endothelial RAGE facilitates ILC2 recruitment downstream of IL-33 release either directly or through upregulation of adhesion molecules (Figure 49).

Further investigations will focus on identifying the cell-specific roles of RAGE in the lung, with an emphasis on defining the mechanisms by which RAGE promotes IL-33 release and the mechanisms by which RAGE facilitates ILC2 recruitment into the lung. Understanding these

RAGE-mediated pathways in the early initiation of allergic airway responses may elicit new therapeutic targets for the treatment and prevention of asthma exacerbations.

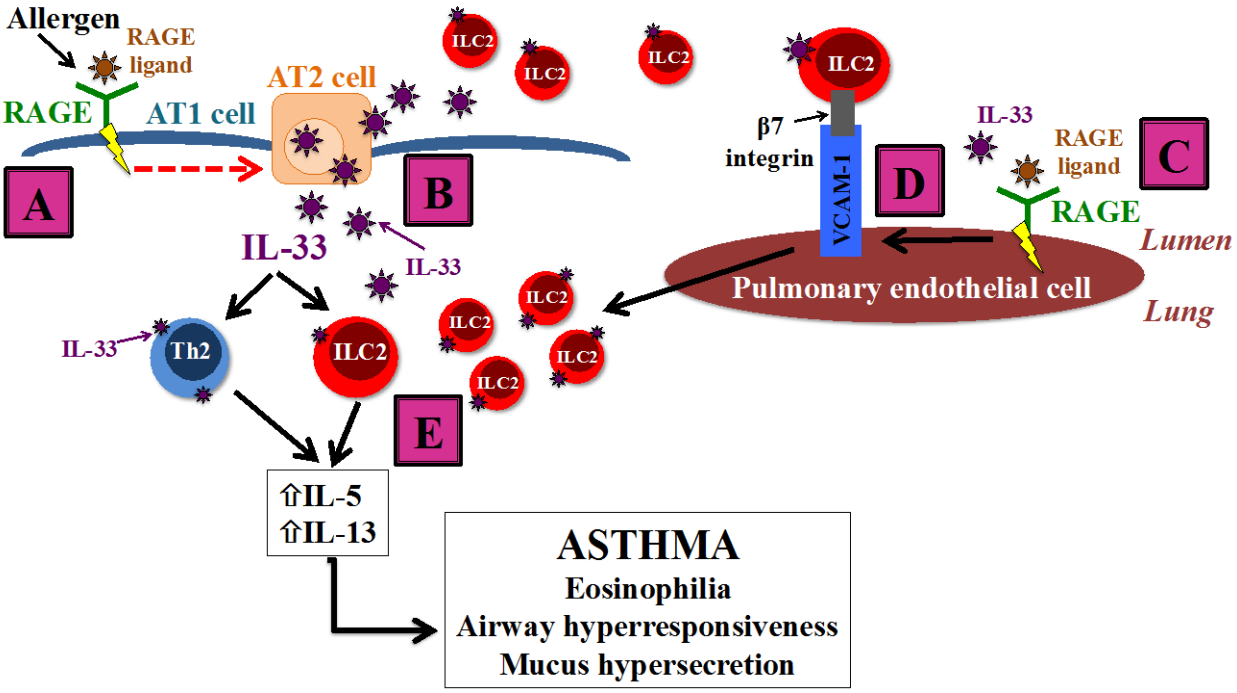


**Figure 49. Summary of RAGE's possible roles in allergic airway inflammation.**

(A) Allergens trigger release of RAGE ligands, which bind to and activate RAGE on type 1 alveolar (AT1) epithelial cells. (B) This signaling is suspected to trigger release of IL-33 from type 2 alveolar (AT2) epithelial cells. IL-33 can activate resident immune cells in the lung and is also released into the circulation to activate ILC2s in the bone marrow. (C) Some ligand (possibly IL-33 itself) binds RAGE on pulmonary endothelial cells and activates downstream signaling pathways. (D) Activated RAGE promotes expression of VCAM-1 on pulmonary endothelial cells. VCAM-1 can bind to activated ILC2s in the blood stream via interactions with β7 integrin. (E) ILC2s accumulate in the lung as a result of extracellular recruitment and expansion of resident ILC2 populations. Together with Th2 cells, ILC2s produce large amounts of IL-5 and IL-13 to exacerbate allergic airway inflammation and airway hyperresponsiveness.

# APPENDIX

## CURRICULUM VITAE

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**Education:** **University of Pittsburgh School of Medicine**, Pittsburgh, PA
June 2010-present
Medical Scientist Training Program, MD/PhD Candidate, May 2017
PhD: Cellular and Molecular Pathology, Adivsor: Tim D. Oury, MD, PhD

**University of Pittsburgh**, Pittsburgh, PA
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Bachelor of Science in Molecular Biology (Biochemistry Track)
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| | | |
|---|---|---|
| **T32 GM008208-22** | Clayton Wiley, M.D., Ph.D. (PI) | 2010-2011 |
| MSTP Training Grant | | |
| **T32 HL094295** | Wendy Mars, Ph.D. (Director) | 2012-2014 |
| Angiopathy Training Grant | Tim Oury, M.D., Ph.D. (Mentor) | |
| **1F30ES024045** | Elizabeth Oczypok, B.S. (PI) | 2014-2017 |
| NIEHS NRSA | | |

RAGE as an upstream activator of the Th2 inflammatory immune response in asthma

**Academic Positions and Honors:**

Memberships in Professional and Academic Societies

| | |
|---|---|
| 2007- | Student Member, C.F. Reynolds Medical History Society |
| 2007-2010 | Member, Alpha Epsilon Delta, Pre-professional Health Honors Society |
| 2007-2010 | Member, Sigma Alpha Lambda, Honor Society |
| 2008-2012 | Member, The Biochemical Society |
| 2010- | Medical Student Member, American Medical Association |
| 2012-2013 | Student Member, Society for Free Radical Biology and Medicine |
| 2013- | Trainee Member, American Thoracic Society |
| 2014-2015 | Trainee Member, American Association of Immunologists |

Leadership Positions

| | |
|---|---|
| 2011-2012 | Co-chair, Pitt MSTP Second Look Committee |
| 2011-2012 | Co-chair, Pitt MSTP Welcoming and Peer Mentorship Committee |
| 2011-2012 | President, University of Pittsburgh School of Medicine St. Luke Society |
| 2011-2012 | Student Advisor, Faculty and Students Together (FAST) |
| 2012-2013 | Chair, Pitt MSTP Hosting Committee |
| 2013 | Student co-chair, Pitt Cellular and Molecular Pathology Retreat Committee |
| 2013-2014 | Co-chair, Pitt MSTP Student Committees |
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Honors and Awards

| | |
|---|---|
| 2006 | University Honors College Tuition Scholarship |
| 2007 | Pennsylvania Space Grant Consortium Undergraduate Research Award |
| 2007 | Alison Bentley Kephart Award, for outstanding work as a first-year undergraduate in the biological sciences |
| 2008 | Chancellor's Undergraduate Research Fellowship |
| 2008 | The Biochemical Society Summer Vacation Studentship |
| 2008 | European Space Agency Young Researcher Award |
| 2008 | Pennsylvania Space Grant Consortium Fall Term Fellowship |
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| 2014 | Third Place Poster Award, UPMC Pathology Department Annual Retreat |
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**Speaking Engagements:**

**Invited instructor**, March 19, 2015
Molecular Pathobiology Graduate Student Course, Department of Pathology, MSCMP 2470
"Pulmonary receptor for advanced glycation endproducts, group 2 innate lymphoid cells, and asthma"

**Selected Graduate Student Speaker**, October 28, 2014
Biomedical Graduate Student Association Annual Symposium
"Pulmonary RAGE Mediates IL-33 Release and Lung-specific Accumulation of Group 2 Innate Lymphoid Cells in Allergic Airway Inflammation"

**Invited Speaker**, June 2013 and June 2014
Summer Undergraduate Research Program
University of Pittsburgh School of Medicine
"Becoming a Physician-Scientist"

**Invited Speaker/Instructor**, June 2013
Howard Hughes Medical Institute Summer Undergraduate Program Journal Club
University of Pittsburgh

**Selected Graduate Student Speaker**, May 2013
University of Pittsburgh Annual Department of Pathology Retreat
"Pulmonary receptor for advanced glycation end products (RAGE) is essential for nuocyte activation in asthma"

**Reviewer Participation:**
*PLoS One*
*AJRCMB*

**Publications:**

Peer reviewed publications:

**Oczypok EA**, Sanchez MS, Van Orden DR, Berry GJ, Pourtabib K, Gunter ME, Roggli VL, Kraynie AM, Oury TD. Erionite-associated malignant pleural mesothelioma in Mexico. Environ Res. 2015. (Submitted, revisions requested)

**Oczypok EA***, Milutinovic PS*, Alcorn JF, Khare A, Crum LT, Manni ML, Epperly MW, Pawluk AM, Ray A, Oury TD. Pulmonary receptor for advanced glycation endproducts promotes asthma pathogenesis via IL-33 and accumulation of group 2 innate lymphoid cells. J Allergy Clin Immunol. 2015 Apr 27 pii: S0091-6749(15)00409-1. [Epub ahead of print] PMID: 25930197
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**Oczypok EA**, Oury TD, and Chu CT. It's a cell-eat-cell world: autophagy and phagocytosis. Am J Pathol. March 2013;182: 612-622. Review. PMID: 23369575

Etheridge T*, **Oczypok EA***, Lehmann S, Fields BD, Shephard F, Jacobson LA, Szewczyk NJ. Calpains mediate integrin attachment complex maintenance of adult muscle in Caenorhabditis elegans. PLoS Genet. 2012 Jan;8(1):e1002471. Epub 2012 Jan 12. PMID: 22253611
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**Oczypok EA**, Etheridge T, Freeman J, Stodieck L, Johnsen R, Baillie D, Szewczyk NJ. Remote automated multi-generational growth and observation of an animal in low Earth orbit. J R Soc Interface. 2012 Mar 7;9(68):596-9. Epub 2011 Nov 30. PMID: 22130552.

Abstracts:

Kraynie, AM, **Oczypok EA**, Sanchez MS, Van Orden DR, Berry GJ, Gunter ME, Roggli VL, Oury TD. (2015) Erionite-associated malignant pleural mesothelioma in Mexico. Pulmonary Pathology Society Biennial Meeting. San Francisco, CA, USA, Jun 2-5, 2015. (Abstract)

**Oczypok, EA**, Milutinovic, PS, Manni, ML, Khare, A, Alcorn, JF, Ray, A, Oury, TD. (2015). "Pulmonary receptor for advanced glycation end products (RAGE) mediates lung-specific recruitment of group 2 innate lymphoid cells in allergic airway inflammation." American Thoracic Society International Conference. Denver, CO. Am J Respir Crit Care Med 191;2015:A5166. (Abstract/Poster)

**Oczypok, EA**, Milutinovic, PS, Manni, ML, Khare, A, Alcorn, JF, Ray, A, Oury, TD. (2014). "Pulmonary receptor for advanced glycation end products (RAGE) mediates lung-specific recruitment of type 2 innate lymphoid cells in allergic airway inflammation." EMBO Conference on Innate Lymphoid Cells. Paris, France. (Abstract/Poster)

**Oczypok, EA**, Milutinovic, PS, Manni, ML, Khare, A, Alcorn, JF, Ray, A, Oury, TD. (2014). "Receptor for advanced glycation end products is an early upstream activator of the Th2 inflammatory immune response in allergic asthma." American Thoracic Society International Conference. San Diego, CA. Am J Respir Crit Care Med 189;2014:A4200. (Abstract/Poster)

Milutinovic PS, Alcorn JF, Crum LT, Manni, ML, **Oczypok, EA**, Schmid, RJ, Oury TD. (2013) "Pulmonary Receptor For Advanced Glycation End Products Promotes Asthma Pathogenesis Via

An Il-33-Dependent Pathway." American Thoracic Society International Conference, Philadelphia, PA, USA. Am J Respir Crit Care Med 187;2013:A2640. (Abstract)

**Oczypok, E**., Manni, M., Crum, L., Schmid, R., Oury, T. (2012). "Extracellular Superoxide Dismutase Promotes Phagocytosis During Acute Pulmonary Infection." Society for Free Radical Biology and Medicine Annual Meeting, San Diego, CA. (Abstract/Poster)

**Oczypok, E**., Manni, M., Crum, L., Schmid, R., Oury, T. (2012). "Extracellular Superoxide Dismutase Promotes Phagocytosis During Acute Pulmonary Infection." Pittsburgh International Lung Conference, "Acute Lung Injury/ New Mechanisms, Future Therapies, and the Translation to Clinical Care," Pittsburgh, PA. (Abstract/Poster)

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**Oczypok, E**. and Szewczyk, N. (2007). "An integrin-containing muscle adhesion complex opposes muscle protein degradation in C. elegans." The Biochemical Society Annual Symposium, Manchester, UK. (Abstract)

Orions Quest Schools, **Oczypok, E**., Szewczyk, N., Drummond, T., Lawrie, P., Freeman, J., Stodieck, L., Johnsen, R., Baillie, D., and Othman, M. (2007). "Normal growth, development and behavior of Caenorhabditis elegans through ten generations of autonomous culturing onboard the International Space Station (ISS)." Mountain and Wilderness Medicine World Congress, Aviemore, Scotland. (Abstract)

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