The role of IL-33 cytokine in allergic sensitization and inflammation

Stephanie Varela

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Reading Committee:

Steve Ziegler, Chair

Adam Lacey-Hulbert

Jakob Moltke

Program Authorized to Offer Degree:

Department of Immunology

University of Washington

Abstract

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Stephanie Varela

Chair of the Supervisory Committee:

Steve Ziegler

Department of Immunology

In this study, we sought to understand the role of interleukin-33 (IL-33) in the context of allergic sensitization and inflammation. To achieve this, we developed two novel models of IL-33 driven allergic sensitization and inflammation. Genetically modified mouse strains with cell specific IL-33 receptor knockouts, hyperactivating receptor variants, and cytokine knock outs were used to characterize these models and determine important responding cell types. Using an intradermal sensitization model with IL-33 and ovalbumin on WT C57BL/6 mice, we observed an increase in draining lymph node cellularity, antigen-specific IgE titers, and Th2 cell generation within skin-draining lymph nodes demonstrating evidence of classic allergic sensitization. Using CD4 T and dendritic cell specific ST2 deficient mouse lines we found that direct IL-33/ST2 signaling on CD4 T cell is necessary for a complete type 2 response. Without this signaling, we found an overall dampened type 2 sensitization response with a significant reduction in total effector Th2 cells and IgE titer. Our findings also suggest a possible role of CD11c+ dendritic cells in responding to IL-33 as we found an increased overall frequency of type 2-skewed CD4 T cells. To expand on our sensitization protocol we included several multi-day oral challenges with ovalbumin to induce antigen dependent eosinophil-rich allergic inflammation within the esophagus of mice. This established an IL-33 driven model of eosinophilic esophagitis (EoE). In characterizing the model we measured esophageal eosinophilia levels over a 7 day time course with a peak occurring after

7x challenges. We also determined that the model was antigen specific and IL-5 dependent. We also found that mice carrying a hyperactive ST2 SNP, rs10204137, showed no difference in eosinophilia levels. Collectively, these studies underscore the significance of IL-33 in promoting allergic sensitization, with CD4 T cells identified as a critical responding cell type in skin-draining lymph nodes, alongside the establishment of an IL-33 driven model for EoE.

Dedication

For my Mom, Julia Varela.

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Chapter 1: Introduction

1.1 Allergy and Alarmins

Human allergic disease accounts for a significant portion of global morbidity impacting nearly 30% of the world's population. [1] The overall worldwide burden of allergies is increasing, and with the rapid changes in the Earth's climate there is growing concern that such changes could exacerbate existing allergic conditions. [2,3] It is therefore both necessary and important to focus on elucidating both the etiology and progression of allergic disease pathology to further the development of potential therapeutic interventions. Additionally, such studies will allow us to understand how chronic allergic diseases lead to associated downstream co-morbidities. For example, chronically triggered asthma or food allergies can lead to deteriorating lung capacity or the development of irreversible esophageal strictures over time, respectively. [4–7] Consequently, there is a pressing need to not only study allergic inflammatory diseases but also understand the mechanisms underlying the initiation of allergic responses.

The concept of allergy or ‘the allergic response’ emerged in the early 1900s. Patients administered with a second dose of horse serum-derived smallpox vaccines were observed having severe hypersensitivity reactions. This lead to the coining of the term 'allergies' to describe this response. [8] Allergies can be considered a hypersensitive immune response to typically harmless environmental substances. Once sensitized to a particular allergen, a person can later be subject to allergen triggered exacerbated allergic inflammation in the form of itching, runny nose, and rashes. Allergies are commonly linked to airborne particles such as dust, animal dander, and pollen resulting in clinically diagnosable diseases like allergic rhinitis and asthma. Food related allergies to peanuts, eggs, wheat, and dairy can elicit symptoms such as diarrhea, bloating, nausea, vomiting, and, in severe cases, anaphylactic shock. [2,9–12] Despite the discomfort

caused by the symptoms alone, the chronic inflammation underlying these processes can lead to long-term damage and more serious co-morbidities. For instance, severe asthma can result in irreversible pulmonary hypertension while chronically inflamed eosinophilic esophagitis can lead to strictures resulting in life threatening esophageal rupture.[13,14] For these reasons, it is important to understand how the human allergic system functions.

The type 2 immune response was thought to have originally evolved specifically to combat helminth infection but has now become a hyperreactivity response. The type 2 response is characterized by IgE antibody generation, increased mucous production, and a robust histamine-rich granulocyte response involving eosinophils, mast cells, and basophils. This response also generates memory helper CD4 T cells (Th2 cells) producing key type 2 programming cytokines like IL-4, IL-5, and IL-13. IL-4 supports a feed-forward loop for type 2 programming, including self-promoting allergic effector Th2 cell creation, while IL-5 is important for eosinophil survival, and IL-13 acts on epithelial cells to induce mucus production.[7,15] In the context of helminth infection, the type 2 immune response initiates an adaptive response resulting in antigen-specific IgE and Th2 cells producing type 2-promoting cytokines. These cytokines trigger B cell class switching and IgE production, coating granulocytes for massive degranulation against invading worms. This process readies eosin-rich eosinophils, enhances mucus production to impede helminth spread, and equips granulocytes to disintegrate encountered worms.[16] Initially designed to combat helminth infections, this mechanism has potentially evolved into a maladaptive response against everyday environmental triggers, causing inappropriate inflammation and excess mucus production associated with allergic rhinitis and asthma.

One crucial factor that mediates the difference between an allergic response versus one of immune tolerance, is whether the offending antigen passes through a protective membrane barrier, such as within the skin or lungs. In response to such invasion, there are barrier proteins/peptides known as alarmins that act as alarm signals in case of foreign invaders. These

alarmins are typically expressed within barrier tissues like the skin, lungs, and gastrointestinal tracts, and are poised to respond to a number of environmental triggers.[18] Alarmins are endogenous, constitutively expressed proteins/peptides that are chemotactic and immune activating, released due to degranulation, cell injury, death, or in response to immune induction. Their overarching goal is to alert the immune system and help it prepare to defend against external dangers. Examples include granule-derived alarmins (Defensins, EDN, granulysin), Nuclear localized alarmins (HMGB1, IL-1alpha, and IL-33), and cytoplasmic alarmins (HSPs, S100 proteins, ATP, uric acid). [17] Alarmins achieve this by triggering release and activation, subsequently programming nearby cells to organize and mount a specific type of immune response. [18]

In the context of allergy, the alarmins most recognized for promoting type 2 allergy are the epithelial-derived cytokines: TSLP, IL-25, and IL-33. These cytokines play a pivotal role in initiating the type 2 response, demonstrated through both direct and indirect promotion of the adaptive immune response. They achieve this by recruiting and activating Antigen Presenting Cells (APCs), such as Dendritic Cells (DCs) and ILC2s. Although these cytokines are strongly associated with orchestrating allergic responses, the cellular mechanisms underlying their actions warrant further investigation.[18,19]

1.2 IL-33 and Allergy

IL-33 Cytokine Background

IL-33 cytokine is an IL-1 family alarmin pro-inflammatory cytokine. Its distinct nuclear localization and non-canonical secretion pattern make it appropriately deemed an alarmin. Notably, IL-33 is released solely in response to specific types of damage, adding to its intriguing nature. Once released and activated, IL-33 triggers various immune responses, with the most widely recognized being the induction of type 2 immunity.[20] Moreover, extensive research has

established IL-33's pathogenic role in numerous allergic diseases including asthma, allergic rhinitis, and food allergy. [11] This assertion is supported by genetic studies, mouse models, and human clinical studies. Genome-wide association studies on a large scale have identified multiple variants within IL-33 and its receptor associated with various allergic diseases. [20–22] Both human and experimental mouse model studies have highlighted the critical role of the IL-33/IL-33R signaling axis in allergic diseases. For instance, mouse studies have demonstrated that the absence of ST2, a receptor for IL-33, leads to significant reduction in allergic pulmonary inflammation.[23] Notably, IL-33-deficient mice failed to develop HDM-induced allergic rhinitis and exhibited unresponsiveness to ragweed pollen challenges.[79]

The IL-33 protein was initially discovered in 1999 while researchers were investigating its expression in canine arteries. [24,25] In 2003, it was later renamed nuclear factor from high endothelial venules (NF-HEV) due to its abundant expression in human endothelial cells [26–28]. Subsequently, in 2005, Schmitz and Kastelein rediscovered IL-33 using computational sequence searches and identified it as the ligand for an orphan receptor. [29] This receptor turned out to be a heterodimer of the suppression of tumorigenicity 2 (ST2) protein and IL-1-receptor accessory protein (IL-1RAcP). [30] Since its discovery, accumulating evidence has demonstrated IL-33's involvement in various disease pathogenesis processes, underscoring its significance as a crucial target for research. [11,31–35] These findings collectively emphasize IL-33's importance as a cytokine worthy of in-depth study.

Genetics and Protein Biology. The human IL-33 gene is located on chromosome 9p24.1, whereas the mouse IL-33 gene is located on chromosome 19qC1. [26,27,29] The IL-33 loci comprise a nuclear domain, central domain, and IL-1-like cytokine domain. The full-length human protein is 270 amino acids long and weighs approximately 31 kDa.[26,27,29] Chromatin binding motifs within the nuclear domain anchor IL-33 to chromatin. The central domain contains cleavage sites susceptible to action by serine proteases, functioning as a 'sensor' domain. Lastly, the cytokine

domain possesses cleavage sites for caspase 3 and 7, facilitating IL-33 inactivation in the context of apoptosis. [27,30,36,37]

Sources and Responders. IL-33 exhibits its highest expression within epithelial cells, fibroblasts, and endothelial cells. [27] Among immune cell sources, Macrophages, mast cells, and DCs are most well-known for expressing IL-33 protein. However, tissue-derived cells appear to be more abundant sources of IL-33 than immune cells. When it comes to responding cell types, ILC2s, mast cells, and Tregs constitutively express ST2 and are recognized as major responders to IL-33. Th2 cells, Basophils, eosinophils, M2 macrophages, and DCs also express moderate levels of ST2 protein. NK cells, iNKt cells, and neutrophils constitutively express ST2 but at lower levels than the previously mentioned cells. Additionally, some cell types, such as Th1s and CD8 T cells, can be induced to express ST2. [27,30,33,38] In summary, IL-33 appears to exert a potent pleiotropic effect with a wide range of sources and responding cell types.

IL-33 release and activation. IL-33's non-canonical release is regulated by environmental cues and due to its nuclear localization it does not follow the traditional secretory pathway but rather is released in response to specific forms of tissue injury. Direct cell damage releases IL-33 from the nucleus, allowing cleavage by serine and cysteine proteases released from recently recruited neutrophils and mast cells. The presence of selective cleavage sites restricts IL-33 activation in autophagic inflammation yet promotes it in the context of white blood cell rich inflammation. IL-33 can act as a sensor for proteolytic allergens by being directly activated by invading allergens. Moreover, the direct cleavage of IL-33 by these proteases results in the production of multiple isoforms of varying lengths of IL-33 depending on the type of allergen. Additionally, it was shown that some classes of allergens generate IL-33 variants that more potently promote allergic disease. [27,30,36,39,40]

IL-33/ST2 Signaling Pathway. Once activated, IL-33 can bind to its cognate receptor on any of its many responding cell types. The IL-33/ST2 signaling pathway is regulated by the expression of its two main isoforms, the membrane-bound ST2 and its free soluble ST2. The ratio between these isoforms may influence the overall outcome, and the ubiquitin-proteasome system could play a role in influencing this ratio as well. During signaling, MyD88 is recruited, followed by IRAK4, which then activates TNF receptor-associated factor 6 (TRAF6). TRAF6 can subsequently activate mitogen-activated protein kinase (MAPK) and the inhibitor of nuclear factor-kb (NF-κB) kinase complex. [41] MAPK then activates activator protein 1 (AP-1). IKK then frees NF-κB, leading to the upregulation of Type 2 cytokines. [19,27,42]

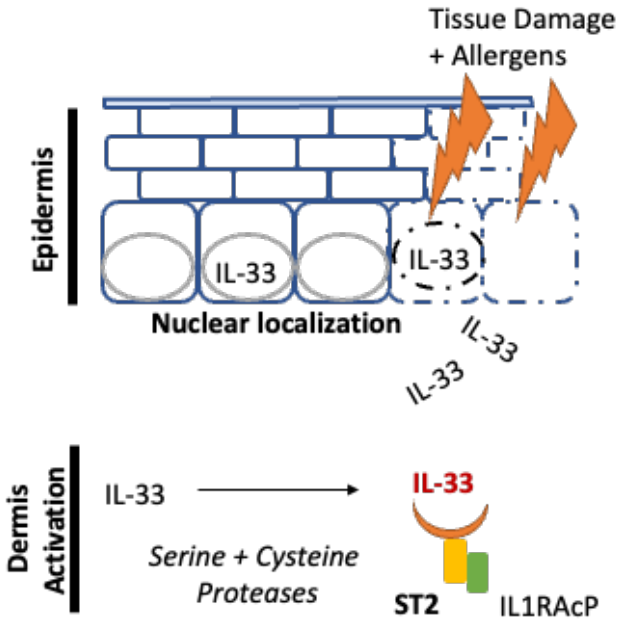

Figure 1. Interleukin-33 (IL-33) is a member of the IL-1 family of cytokines. Tissue damage leads to the release of Il-33 from the nuclear of epithelial cells. Once released, IL-33 is then activated by serine and cysteine proteases. From there, an activated IL-33 cytokine can then bind to its cognate ST2/IL1RAcP heterodimeric receptor.

IL-33 known functions in innate and adaptive immunity. IL-33 cytokine possesses the ability to directly influence both adaptive and innate immune cells, promoting inflammation. It plays a role in various immune responses, including type 2 immunity, allergic inflammation, non-allergic inflammation, homeostasis, viral infection, and cancer. Within the innate immune system, IL-33 fosters inflammation by acting on ILC2s, basophils, and NK cells, inducing them to secrete Type 2 cytokines such as IL-4, IL-5, and IL-13. [19,26,31,38] These cytokines, in turn, activate the adaptive immune system, recruit eosinophils, and initiate the process of inflammation and remodeling [19]. Within the adaptive immune system, IL-33 has been shown to induces chemotaxis of Th2 cells and direct Th2 cell signaling has been shown to promote their proliferation and enhance their survival *in vitro.* [41,43]

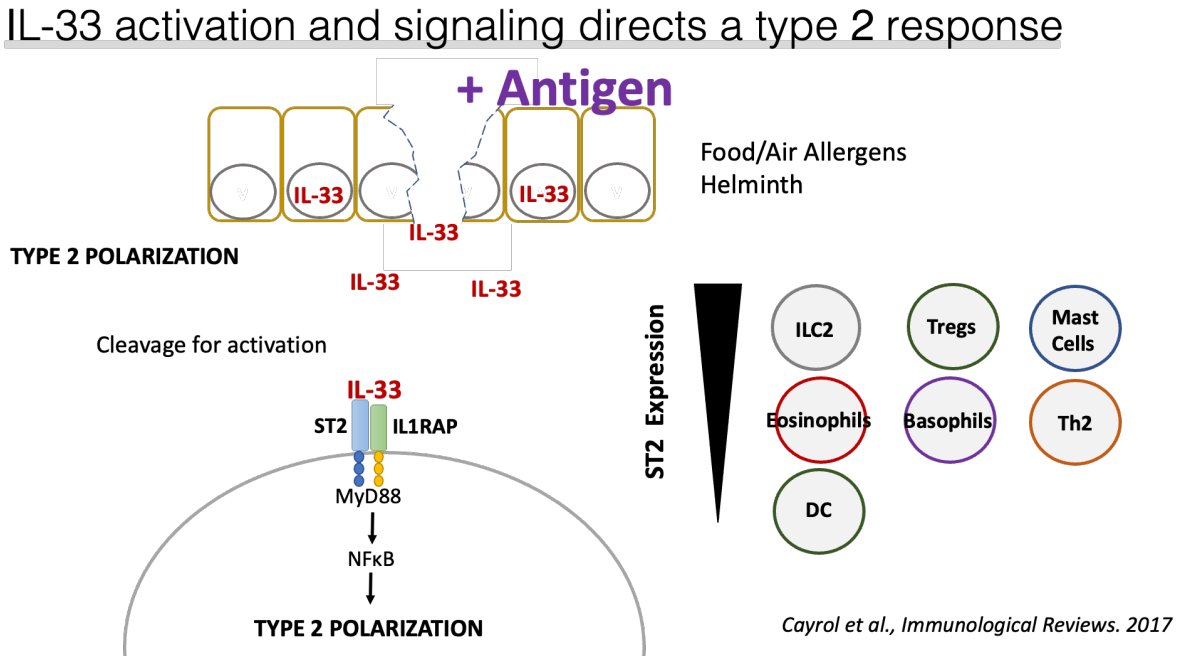

Figure 2. Diagram of cell types responding to activated IL-33. Once activated, IL-33 can bind to its cognate receptor to activate MyD88 dependent signaling pathway in a number of responding immune cells. Adapted from Cayrol et al., 2017.

The process of allergic sensitization begins when foreign invaders cross barriers, triggering the alarmin system. Antigen-presenting cells (APCs) then break down the invader, transporting the allergen fragments to local lymph nodes. In these nodes, T and B cells are presented with allergen pieces, initiating the adaptive immune system. Messenger cytokines further signal to steer the immune response toward a type 2 profile. The ultimate outcome is the activation of the antigen-specific adaptive immune system, leading to the production of antigen-specific IgE, T cells, and B cells. IgE can coat mast cells (and sometimes basophils), primed for a type 1 hypersensitivity reaction upon challenge with the offending allergen. Additionally, memory T and B cells can exacerbate the allergic inflammatory process by recruiting more granulocytes, such as eosinophils, and other white blood cells that contribute to allergic inflammation [7,15,44–46].

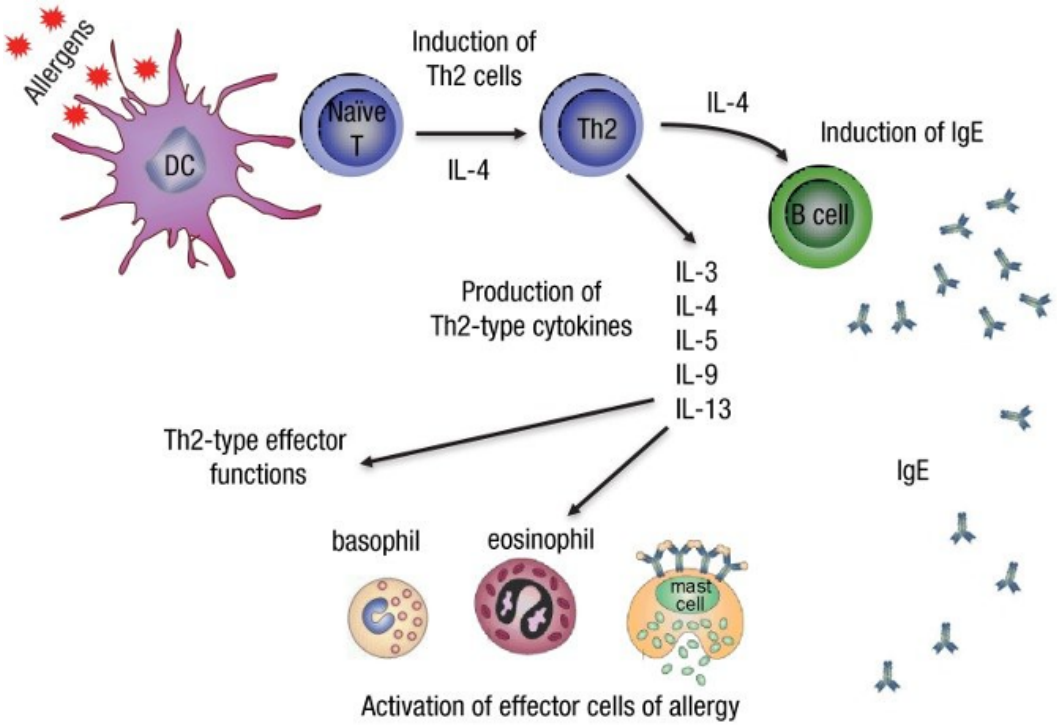

Figure 3. Diagram of type 2 sensitization biology. During allergic sensitizing, antigen presenting cells like dendritic cells migrate to the draining lymph node in order to present antigens to naive CD4 T cells. Adapted from Galli et al, 2008.

The allergen, upon crossing the barrier, damages multiple cells that constitutively express the IL-33 cytokine, thereby triggering the alarmin system and causing the non-canonical release of IL-33 from the nucleus. Serine and cysteine proteases from infiltrating white blood cells then cleave IL-33, activating it. IL-33 subsequently signals on the ST2 heterodimer, activating the MyD88/IRAK4 signaling cascade within its target cells. The responding cell types are then activated to carry out their functions. In the context of allergic sensitization, this process leads to the sensitization of the adaptive immune system, resulting in the production of memory B and T cells. Upon challenge with the allergen, the system generates a predominantly eosinophilic response at the tissue site being challenged. In summary, this outlines how IL-33 cytokine acts as an adjuvant, inducing allergic sensitization against an allergen. Once again, the pleiotropic or broad effects of IL-33 serve to underscore its importance. [27,30,42]

Given the pivotal role of IL-33, several important questions remain to be addressed. Firstly, there is a need to develop innovative tools that can effectively probe IL-33's involvement in both allergic sensitization and challenge-dependent disease and inflammation. Secondly, a better understanding of the cellular mechanisms behind IL-33's ability to promote allergic responses is essential. Specifically, inquiries into whether IL-33 can directly impact CD4 T cells, dendritic cells (DCs), and innate lymphoid cells type 2 (ILC2s) and whether signaling on these cell types is indispensable for the allergic response.

Our overarching goals encompass two key objectives: 1) The creation of tools dedicated to investigating IL-33's role in allergic sensitization and disease, and 2) The determination of which cell types respond to IL-33 in the context of allergic sensitization. Addressing these questions will undoubtedly contribute to a more comprehensive understanding of IL-33's intricate involvement in allergic processes.

Chapter 2: IL-33-mediated epicutaneous sensitization requires ST2+ CD4 T cells

Sections of text in this chapter have been modified slightly from the following manuscript:

SVarela and SF Ziegler. 2023. IL-33-mediated epicutaneous sensitization requires ST2+ CD4 T cells. *In revision. ImmunoHorizons.*

2.1 Abstract

2.2 Introduction

Human allergic disease is responsible for a significant proportion of morbidity worldwide, affecting nearly 30% of the world's populations [1]. Interleukin-33 (IL-33) is an IL-1 family pro-inflammatory cytokine that has been shown to have a functional role in allergic disease [11,48,49]. Both human and experimental mouse model studies have demonstrated that the IL-33/IL-33R signaling axis is critical to allergic disease [29]. While IL-33 has been identified as a highly attractive therapeutic target for allergic disease, the cellular mechanisms underlying its ability to promote allergic disease are not well understood.

The decision to focus on IL-33 is supported by clinical and mouse model evidence. Aeroallergen sensitized patients are frequently associated with atopic dermatitis and other epithelial barrier disorders. Mouse models have further demonstrated that epicutaneous and respiratory sensitization can also lead to allergic sensitization culminating in EoE [50–53]. Clinical studies have shown increased IL-33 transcript and protein levels within the esophagus during EoE inflammation while mouse models of EoE have additionally highlighted the pivotal role of IL-33 in the pathogenesis of this allergic disease [54]

Allergic sensitization begins with uptake of antigen by antigen presenting cells, such as dendritic cells (DCs), at the site of damage. DAMPS, PAMPS, and Alarmins deliver 'programing' signals to resident innate immune cells (e.g., DCs) which then migrate to the draining lymph node to 'sensitize' naïve T cells via antigen presentation. During this process, T cells receive both antigen and molecular signals to differentiate into Type 2 helper CD4 T cells, termed Th2 cells, which can be defined by their expression of GATA3 transcription factor and Type-2 cytokine secretion. This Th2 cell programming also promotes B cell isotype class switching to generate antigen specific IgE antibodies that mediate ensuing allergic inflammation. When stimulated by either antigen or cytokine signaling, Th2 cells can become effector Th2 cells that secrete IL-13 and IL-5 cytokines, both of which carry out Type 2 effector functions responsible for classic clinical allergic symptoms [7,9].

As a barrier Alarmin IL-33 has been proposed to both program APCs and promote Th2 cell proliferation during the sensitization process [27]. Large-scale Genome Wide Association Studies have identified multiple variants within IL-33 and its receptor that associate with an increased risk for a range of allergic disease [21,22,27,39,55]. At steady state, full-length IL-33 is constitutively expressed at barrier tissues within the nucleus of epithelial cells. In response to tissue damage IL-33 is released into the intra- and sub-dermal space while local pro-inflammatory proteases activate it via cleavage. The activated form of IL-33 is then free to bind to its cognate receptor, an ST2 and IL1RAcP protein heterodimer (ST2) to drive a pro-inflammatory response via IL-33 responding cell types [20,27,33,48]. There are a number of ST2 expressing innate and adaptive cells poised and ready to respond to IL-33; however, which cells directly respond to IL-33 during allergic sensitization is less understood.

Previous mouse models have studied the effects of IL-33 by broadly inducing epithelial barrier damage (tape stripping, MC903) that also lead to the expression of other inflammatory mediators [56–58]. In this study, we developed an *in vivo* sensitization model that *isolates* the effects of IL-33 administration from physical damage inflicted to skin by intradermally administering activated recombinant IL-33 in the presence and absence of antigen. This model of direct IL-33-mediated sensitization leads to increased dLN cellularity, production of IgE, and the induction of GATA3, IL-13 and/or IL-5 expressing Th2 cells. Using cell-specific deletion of ST2 we found that IL-33 signaling in both CD4 T cells and DCs is involved in this response, demonstrating the multi-faceted role of IL-33 in type-2 sensitization in the skin.

2.3 Results

2.4 List of Figures and Tables

2.5 Discussion

DISCUSSION

Despite its established importance within allergic disease, the cellular mechanisms underlying IL-33-mediated allergic sensitization remains unclear. Previous mouse models of IL-33-dependent allergic disease have used skin damage (e.g., tape-stripping) [56–58]. These manipulations lead to IL-33 release, but also induce the expression of other factors that can contribute to responses obscuring the direct role of IL-33. In this study we determined the direct effects of IL-33, distinct from damage-induced inflammation, through intradermal administration of recombinant IL-33 in the presence or absence of antigen (ovalbumin; OVA). These studies allowed us to directly determine the role of IL-33 in sensitization through a comprehensive analysis of the cellular responses to this treatment regimen.

Following immunization with IL-33 + Ova we observed an increase in dLN cellularity, increased CD4 T cell accumulation in the dLN, and elevated circulating IgE (both total and OVA-specific). Administration of IL-33 alone led to an increase in dLN cellularity and CD4 T cell recruitment; however, it did not significantly increase IgE levels over controls. Importantly, there was no increase in effector Th2 cells in IL-33-treated mice. This suggested that IL-33 alone could promote aspects of activation, but only the combination of IL-33+OVA induced allergic sensitization with CD4 T cell proliferation/accumulation and activation including expression of the cytokines IL-13 and IL-5 cytokines, both of which are important for allergic effector functions.

To determine the cellular tropism of IL-33 in our model of allergic sensitization we utilized mouse strains in which the IL-33 receptor, ST2, was ablated in CD4 T cells (CD4-Cre/ST2$^{fl/fl}$) and dendritic cells (CD11c-Cre/ST2$^{fl/fl}$). We hypothesized that IL-33/ST2 signaling by CD4 T cells and/or dendritic cells would be required for sensitization. Indeed, in the absence of IL-33 signaling by CD4 T cells we found reduction in dLN cellularity, circulating IgE levels, and allergic effector T

cells with IL-33 + Ova sensitization. This suggested cell specific ablation of ST2 in CD4 T cells, and thus ability to respond to IL-33, reduced the overall sensitization response. The finding that there were some $ST2^+$ Th2 cells in the CD4-Cre/ST2 mice, likely reflects inefficient deletion of ST2, supports our finding that only ST2+ CD4 T cells respond to this sensitization regimen. IL-33 is known to increase the effector function of $ST2^+$ CD4 T cells, leading to increased expression of the type-2 effector cytokines.[22] Overall, these data suggest that IL-33 has a critical role in pushing $ST2^+$ Th2 cells to make IL-13 and IL-5 within the dLN as CD4-Cre/$ST2^{fl/fl}$ mice were able to generate similar levels of $GATA3^+$ Th2 cells, there was a reduction in IL-13+/IL-5+ T cells. It's likely that CD4-Cre/$ST2^{fl/fl}$ mice have a reduction in effector cytokine production, and not total Th2 cells, because ST2 expression does not occur until after the Th2 cell differentiation [46,59]. However, once generated, our data suggests Th2 cells may require additional IL-33 signaling in order to continue to support their own propagation and proliferation through the expression of effector cytokines.

We also examined whether IL-33 signaling by CD11c+ dendritic cells (DCs) played role in promoting sensitization and found only a very minor contribution (data not shown). This finding is interesting as previous studies have found that IL-33-treated DCs can promote Th2 differentiation of naïve CD4 T cells [60]. However, those studies were performed in vitro, and may demonstrate what IL-33 signaling can do, not necessarily what it does do in vivo.

While we know that IL-33 has a crucial role in promoting allergic disease the underlying mechanisms remain unclear. Specifically, an understanding of these mechanisms within the context of IL-33-driven allergic sensitization in the skin is critical for understanding how allergic disease is initiated. For example, sensitization to peanut is believed to be via epicutaneous exposure, and recent studies have suggested a role for IL-33 in this process [31,49,61,62]. Here, we demonstrate that IL-33 has an important role in directly promoting Th2 cell development within skin draining lymph nodes following sensitization. We also show that direct IL-33 signaling on Th2

cells may be required to encourage intra-lymph node T cell proliferation, a crucial step in the sensitization process.

Another important issue is the source of IL-33 in the skin. Previous work has shown that keratinocytes are a source of IL-33, but certainly other cellular sources (e.g., fibroblasts and endothelial cells) are likely to be involved in sensitization, as well as cells that migrate into the dLN during the response [61,63–65].

In closing, we have shown that IL-33 signaling by CD4 T cells is critical for allergen sensitization. Overall, our studies support the importance of IL-33 in driving an allergic sensitization response and identify CD4 T cells as an important responding cell type within the skin dLNs. Information derived from these studies will help inform future therapies that seek to block IL-33 and significantly modulate the allergic immune cell response.

Chapter 3: IL-33 sensitization induces model eosinophilic esophagitis

3.1 Introduction

It is essential to improve our understanding of IL-33 cytokine and its association with allergic disease and inflammation. Individuals who develop increasingly severe or multi-trigger allergic conditions often initially start with allergic skin conditions like atopic dermatitis. The progression from a single allergic disease to further development of worsening allergies is believed to involve the phenomenon known as the atopic march [12]. The skin represents a well-established common route for patients to become sensitized to environmental antigens [12,49,62]. Therefore, our focus in this chapter is on studying how skin-derived IL-33 drives allergic sensitization through the skin and ultimately leads to downstream allergic inflammation following oral challenge.

Our IL-33 + Ova sensitization protocol effectively primed, or sensitized, the mouse's adaptive immune system. This priming was sufficient so that upon challenging other target organs (esophagus and lungs), the adaptive memory system could induce an allergic inflammatory response. The investigation aims to uncover how the versatile alarmin IL-33 cytokine influences the development of various allergic disease models, such as EoE, anaphylaxis, and asthma. Expanding our sensitization model, we took additional steps to develop and characterize several allergic inflammatory models of challenge dependent allergic disease. Furthermore, as we characterized these models, we gained further insights into IL-33 biology in the context of eosinophil-dominant allergic inflammation.

To make meaningful progress in understanding allergic diseases, it is important to address the knowledge gap surrounding IL-33. Specifically, we needed a system that mimics adaptive-dependent sensitization and an understanding of how IL-33 within the skin can promote type 2

sensitization leading to EoE and other allergic diseases like asthma and anaphylaxis. The study aims to elucidate the role of IL-33 in this system during allergic inflammation.

3.2 Eosinophilic Esophagitis

Eosinophilic Esophagitis (EoE) is a severe allergic inflammatory disease signified by eosinophilic inflammation and esophageal dysfunction in response to specific allergens [66]. Ranking as the second most common cause of esophagitis, EoE affects 1 to 5 individuals per 10,000 in the United States and Europe.[66] As EoE progresses, symptoms can evolve from nausea to more severe complications including esophageal dysfunction, food impaction, and potentially deadly esophageal rupture. While restrictive elemental diets and corticosteroids effectively treat EoE, patient adherence to such diets is challenging, and corticosteroids carry well-known long-term health consequences [4,5]. Understanding the mechanisms underlying EoE progression is crucial for developing more effective and targeted therapeutic interventions.

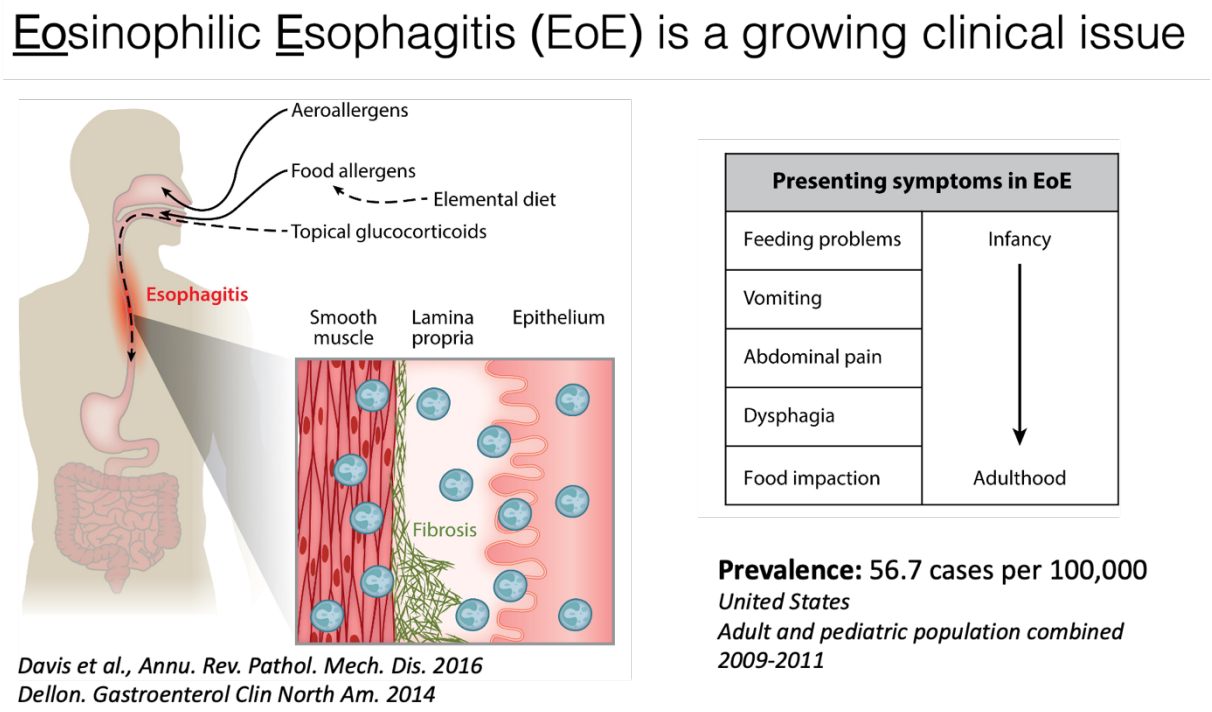

Figure 9. Eosinophilic Esophagitis is a growing clinical issue. Adapted from Davis et al. 2015.[66] Eosinophilic esophagitis is an allergic disease of the esophagus. Once sensitized, an individual can experience allergic inflammation in response to subsequent trigger by offending allergen. Chronic inflammation can lead to a progression of worsening symptoms over time.

Approximately 50-80% of EoE patients also present with other atopic comorbidities, such as atopic dermatitis, asthma, and food allergies [66]. The 'atopic march,' a developmental progression of atopic disorders, is suspected to initiate type 2 dysregulation through barrier dysfunction, leading to the development of additional atopic diseases. Mouse models disrupting skin barrier function show that epicutaneous sensitization is sufficient to induce an EoE-like phenotype [53,67–70]. The association of EoE with 'atopic march' diseases and overlapping genetic factors implicates both barrier dysfunction and type 2 inflammation in EoE pathogenesis, emphasizing the critical need to investigate how sensitization in the skin leads to esophageal inflammation.

EoE is a particularly severe condition with limited treatment options. Characterized by eosinophil infiltration in response to specific allergens, EoE can progress into chronic esophageal dysfunction, leading to dysphagia and potentially life-threatening impactions [4,5]. Although current treatments, such as highly restrictive diets and chronic corticosteroid use, alleviate acute symptoms, they come at a significant cost to the long-term quality of life and health of patients [71,72]

EoE is intricately linked to IL-33 and skin barrier damage, highlighting the importance of understanding IL-33's role in allergic inflammation for elucidating EoE's pathogenesis. Sensitization to allergens is highly associated with barrier disruption and the IL-33 cytokine. IL-33 is constitutively expressed within the skin and is known for promoting type 2 responses by priming the adaptive immune system. In the context of EoE, IL-33 induces T cells to produce type 2 cytokines (IL-5 and IL-13), driving allergic inflammation and eosinophilia. Within adaptive-

dependent allergy, T cells become sensitized against antigens, prompting B cells to produce antigen-specific IgE. Upon challenge with the sensitized antigen, allergic inflammation characterized by a robust eosinophilic infiltration ensues within challenged tissue. Additionally, EoE patients on restrictive diets promptly experience EoE recurrence upon deviation, underscoring the adaptive antigen-dependent allergic nature of the disease and the difficulty in managing EoE and its impact on patient quality of life.

Type 2 immunity, resulting from a combination of innate and adaptive immune responses, plays a crucial role in priming the immune system in allergic reactions. Type 2 Innate Lymphoid Cells (ILC2s) are critical among innate immune cells, producing IL-5 and IL-13 when activated in tissues. The adaptive component initiates when Antigen Presenting Cells (APC) prime CD4+ T helper cells with antigen, inducing their differentiation into Th2 effector and memory cells. Upon challenge with cognate antigen, Th2 effector cells secrete IL-4, IL-5, IL-9, and IL-13.[15] IL-4 amplifies the type 2 response, IL-5 promotes eosinophil differentiation, survival, and proliferation, and IL-13 signals on epithelial cells to release eotaxin-3, an eosinophil recruitment chemokine [73].

IL-33 emerges as a key promoter of type 2 sensitization and allergic inflammation in EoE pathogenesis. Highly expressed in endothelial, epithelial, and fibroblast-like cells during homeostasis and inflammation [40], IL-33 signals through the heterodimeric protein, ST2, highly expressed on ILC2s and many other innate and adaptive immune cells [31,40]. IL-33 serves as a crucial mediator of allergic disease and is sufficient to induce eosinophilia when administered at various tissue sites. [10,31,74,75] Esophageal IL-33 mRNA and protein expression are elevated in EoE patients with active disease, and ST2 deficient mice do not develop esophageal eosinophilia in an epicutaneous sensitization model of EoE .[47,74,75] Despite this, there are no existing models of IL-33-driven esophageal eosinophilia, and previous EoE models fail to dissect the cellular mechanism by which IL-33 cytokine mediates esophageal eosinophilia. Understanding these mechanisms is essential for developing targeted therapeutic interventions for EoE.

3.2 Results

Development of an IL-33 initiated OVA sensitized murine model of Eosinophilic Esophagitis model

In order to create a model of EoE, we adapted our sensitization protocol by including multiple daily oral challenges with ova. Mice were sensitized with either IL-33 + Ova or Ova alone with 7 days of rest, as before. However, starting on Day 12 mice are intragastrically challenged with 5mg of Ova each day for 7 days. Analysis of the esophagus, spleen and serum was performed 24 hours following the final challenge (**Figure 10a**). In order to measure for EoE, esophageal levels of eosinophil accumulation was measured by H&E staining and flow cytometry of digested tissue. Eosinophil populations were identified by their high side scatter and high siglec-F profile on flow cytometry. Also, the levels of total serum IgE following 7 days of challenges was measured by IgE ELISA. Mice that have significantly increased eosinophil accumulation and type 2 inflammation as compared to control mice are considered 'positive' for an EoE-like phenotype.

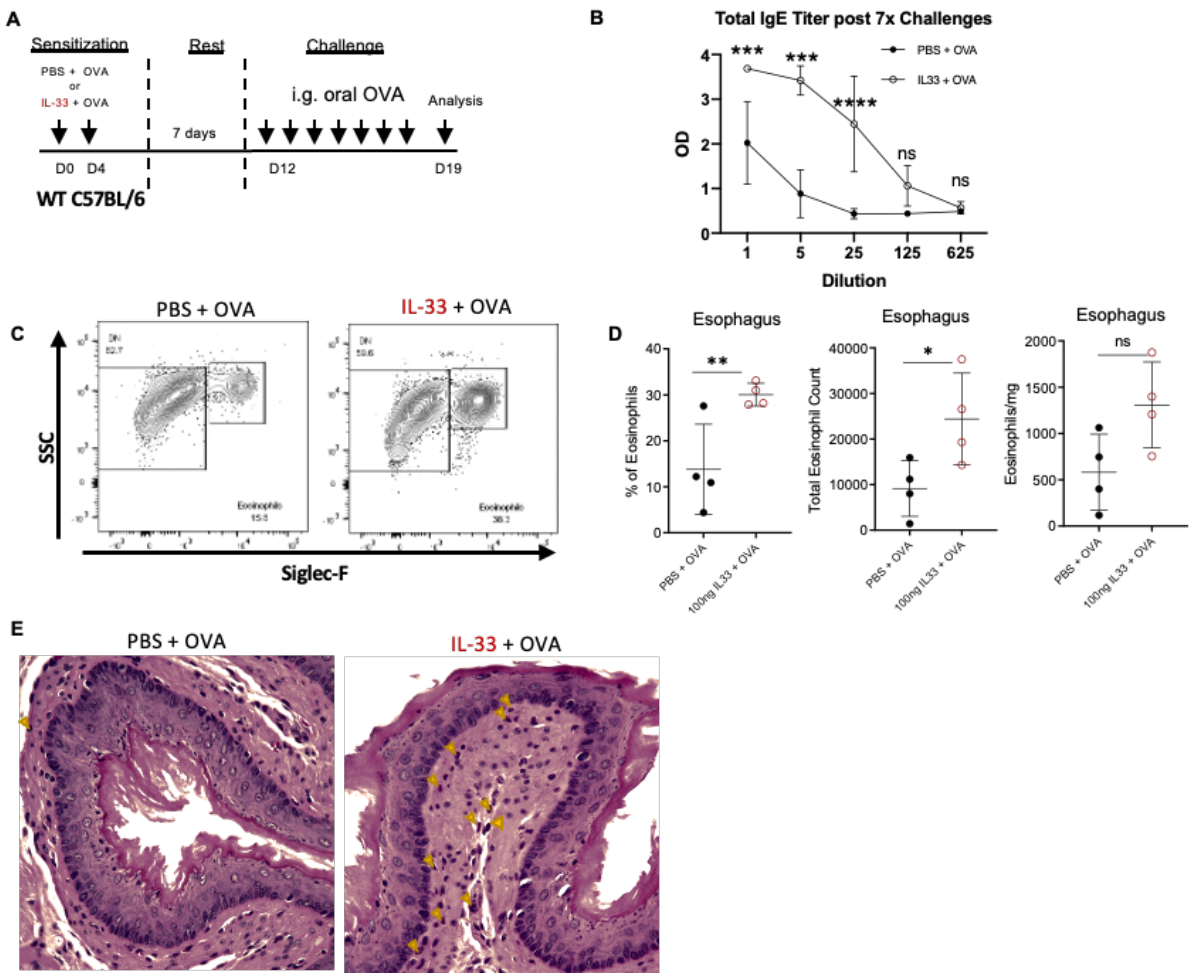

Figure 10. Intradermal administration of IL-33 with OVA antigen allergically predisposes mice to develop Eosinophilic Esophagitis. A) Schematic of sensitization, rest and challenge phase of EoE model. **B**) dot plot of serum levels of total IgE titer done by ELISA **C**) Representative flow plots of eosinophil frequencies within esophageal tissue. Mouse eosinophils were identified as live, $CD45^{+}$, Lin^{-} (TCRbeta, CD19, CD11c), Siglec-F^{+} side-scatter high. **D**) Dot graphs showing frequency of eosinophils per CD45+ cells and total cells per milligram (mg) of esophageal tissue. **E)** Representative hematoxylin and eosin-stained sections of mouse esophageal cross-sections. Yellow arrows indicate tissue infiltrating eosinophils. **F**) Dot graph of eosinophils per high power field (hpf).

The objective of this experiment was to identify the most effective range for challenging EoE mice to elicit maximum eosinophilia. Following sensitization on the same day as previous procedures, subsets of mice were either left unchallenged and subsequently harvested or subjected to intragastric challenges with ova antigen at varying frequencies (1x, 5x, or 7x). Esophageal tissue was then examined for eosinophil frequency, total eosinophils, and total eosinophils per mg of tissue. Our findings revealed a progressive increase in total eosinophils and total eosinophils per mg of tissue with subsequent ova challenges (**Figure 11B-C**). Notably, the 7x challenge resulted in the highest levels of eosinophils in terms of both total count and total count per mg of tissue (**Figure 11C**).

However, it is important to note that the frequency of eosinophils per live CD45+ cells did not exhibit a significant increase compared to the PBS + Ova control groups (**Figure 11A**). This is likely attributed to the increasing number of CD45+ immune cells infiltrating into esophageal tissue with each challenge. Moreover, the analysis indicates that eosinophils make up approximately 20-30% of the infiltrating CD45 cells. Consequently, while it may appear that there is no increase in eosinophils based on frequency, the total numbers provide a more accurate representation of the overall eosinophil burden.

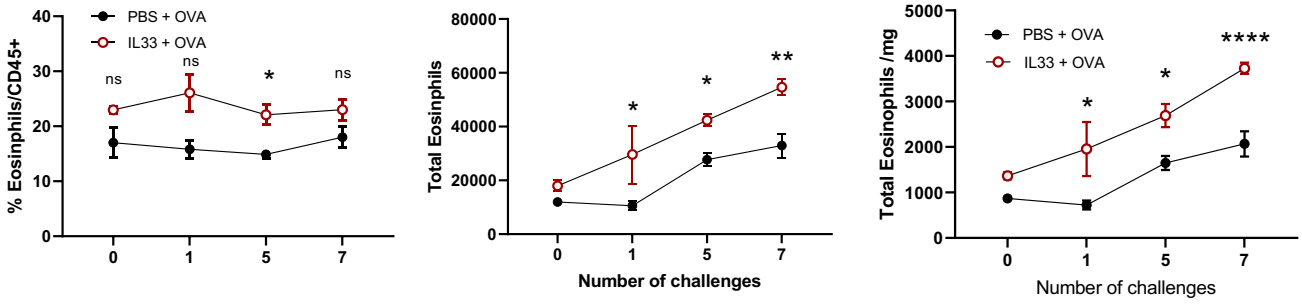

Figure 11. Time course of esophageal eosinophilia post Ova challenges. A) Line graph showing total eosinophils per $CD45^{+}$ cells, **B**) Total eosinophils and **C**) eosinophils per mg of

esophageal tissue 0, 1, 5, and 7 days post challenge with 5 mg of OVA. Two-way Analysis of Variance (ANOVA).

In order to characterize and identify the most effective range for challenging EoE mice to elicit maximum eosinophilia, we performed a time course of esophageal challenges. Following sensitization on the same day as previous procedures, subsets of mice were either left unchallenged and subsequently harvested or subjected to intragastric challenges with ova antigen at varying frequencies (1x, 5x, or 7x). Esophageal tissue was then examined for eosinophil frequency, total eosinophils, and total eosinophils per mg of tissue. Our findings revealed a progressive increase in total eosinophils and total eosinophils per mg of tissue with subsequent ova challenges (**Figure 11B-C**). Notably, the 7x challenge resulted in the highest levels of eosinophils in terms of both total count and total count per mg of tissue (**Figure 11C**).

In conclusion, the data reveals a dose-dependent increase in infiltrating eosinophils within esophageal tissue with repeated intragastric ova challenges over a 7 day period. Notably, the data highlights that Day 7, following 7x challenges, represents the peak level of eosinophilia compared to the control. This identifies day 7 as an optimal time point for further investigation. The dose-response pattern suggests that eosinophilia resulted from repeated challenges, indicating that the mice are sensitized and exhibiting an allergic response to the sensitized antigen. The observed slight increase in eosinophils on Day 0, without any challenges, further supports the conclusion that the eosinophil response is specifically tied to the challenging antigen to which the mice are sensitized.

In order to determine if the esophageal eosinophilia observed was an antigen specific response, we decided to challenge the mice with an alternate protein, Bovine Serum Albumin (BSA). The idea was to challenge the mice with another albumen type of protein distinctly different from ovalbumin. In this case, mice were sensitized with either Ova alone or IL-33 + Ova as normal.

They were rested for the same amount of time. However, this time, we either intragastrically challenged the mice with 5 mg of Ova or 5 mg of BSA. Esophagus and spleen were harvested 24 hours following the final challenge. We found that mice sensitized with IL-33 + Ova and challenged with Ova exhibited increased levels of esophageal eosinophils compared to those sensitized with Ova alone and also challenged with Ova (**Figure 12A**). Additionally, mice sensitized with IL-33 + Ova and challenged with Ova display higher levels of eosinophilia compared to IL-33 + Ova sensitized mice challenged with BSA. This indicated that mice sensitized with IL-33 + Ova specifically induce esophageal eosinophilia in response to their sensitizing antigen, thereby characterizing the model as antigen-specific. Furthermore, in the spleen, we observed significantly higher levels of eosinophilia in mice sensitized with IL-33 + Ova, regardless of the challenge, in comparison to mice sensitized with Ova alone (**Figure 12B**). This outcome somewhat aligns with expectations, considering that sensitization with recombinant IL-33 often results in a low level of eosinophilia within the spleen.

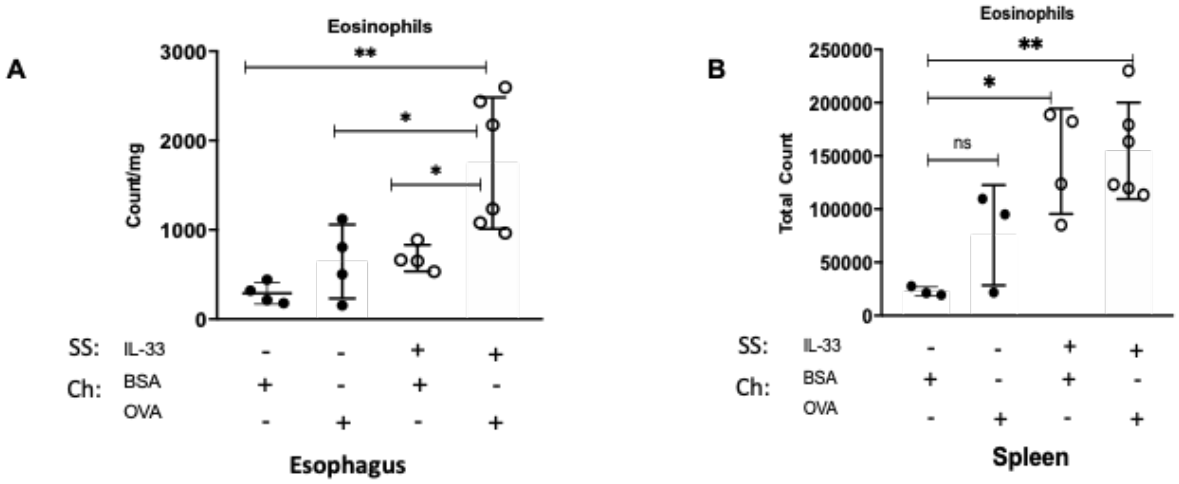

Figure 12. Challenge dependent esophageal eosinophilia is antigen specific. Mice were sensitized with 2ug of OVA in PBS, with or without 0.1ug of recombinant IL-33. Mice were either challenged with intragastric administration 5mg of OVA or 5mg of BSA (n = 4-6). **A)** Dot plots demonstrating number of eosinophils per mg of esophageal tissue and **B)** total number of eosinophils within spleen following challenge. One-way ANOVA.

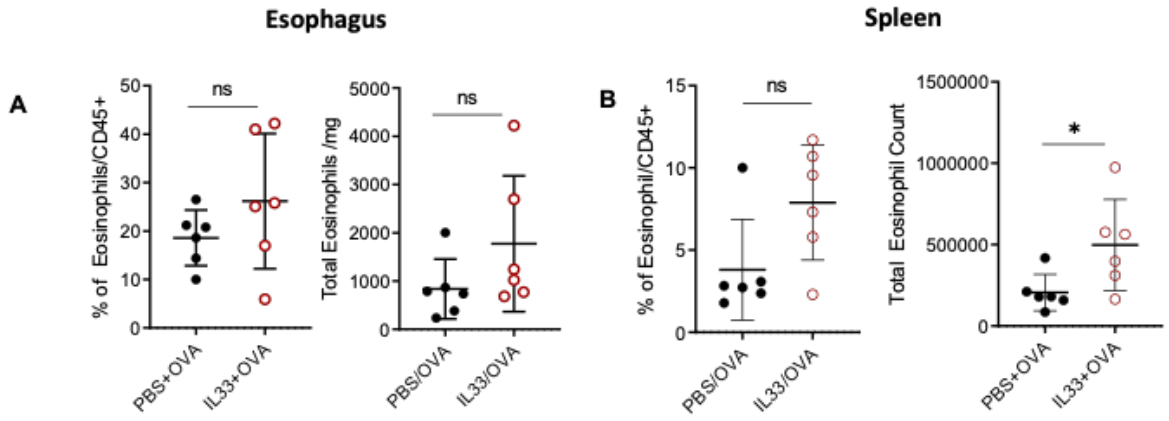

Figure 13. Esophageal eosinophilia requires adaptive immunity. The EoE model was tested on Rag-/- mice. **A)** Dot plots demonstrating number of eosinophils per CD45+ cells, total count, and per mg of esophageal tissue. **B**) Dot plots demonstrating frequency of eosinophils per CD45+ Cells and total number eosinophils within the spleen (n=4-6).

Additionally, to assess the dependence of our EoE model on the adaptive immune system, we tested it on a Rag-/- background. These mice are genetically modified to lack a functional Recombination Activating Genes (RAG) gene. Both RAG1 and RAG2 are important for directing VDJ recombination and when knocked out both T and B cells are unable to develop normally.[76] To determine if the adaptive immune system was required within this model, we sensitized Rag-/- mice according to the protocol with Ova alone or IL-33 + Ova. We then measured the frequency and number of eosinophils within the esophagus and spleen following Ova i.g. challenge. Our findings indicated that mice sensitized with IL-33 + Ova did not exhibit significantly increased levels of eosinophilia within the esophagus compared to Ova alone controls. IL-33 + Ova sensitized mice were unable to induce esophageal eosinophilia on the Rag-/- background. This suggests that the presence of esophageal eosinophilia following challenge likely requires adaptive immunity.

In summary, based on the antigen specific and Rag-/- data, we are convinced that our model is dependent on the adaptive immune system. This is significant as it closely mimics the human condition of EoE. It suggests that the eosinophil response we are observing is due to the adaptive immune system and supports the data demonstrating its challenge-dependent disease model.

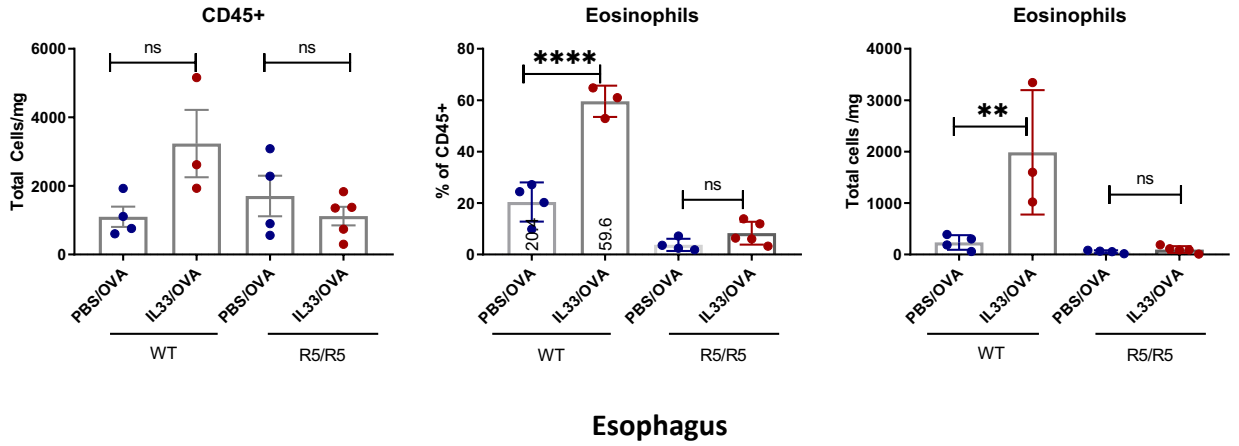

Figure 14. IL-5 is required for esophageal eosinophilia. A) CD45+ immune cells **B)** % of eosinophils/CD45+ within esophagus. **C)** Total # of eosinophils per mg of esophageal tissue

In order to determine if esophageal eosinophilia accumulation within this model required IL-5, we tested the model on a IL-5-/- mouse strain. Eosinophil survival relies heavily on IL-5 production and direct signaling on eosinophils.[20,77] To investigate this, we employed the use of a Red5 reporter mice (recombinase-expressing detector for IL-5). In these mice, the translation initiation site for endogenous IL-5 has been disrupted by a tdTomato expression cassette.[78] As a result, cells that would normally express IL-5 emit a red fluorescence, simultaneously eliminating endogenous IL-5 production in homozygous Red5 mice. Heterozygous Red5 mice (R5/+) possess cells expressing both tdTomato and actual IL-5 cytokine, whereas homozygous Red5

(R5/R5) mice serve as IL-5-/- models. If IL-33 treated mice fail to induce esophageal eosinophilia on an IL-5-/- background, it would indicate a critical requirement of IL-5 for EoE within this particular system. Specifically, this would manifest as a decrease in both the percentage and total burden of eosinophils within the esophagus.

We found that esophageal eosinophilia in our IL-33-driven model is indeed dependent on IL-5. This outcome aligns with expectations considering the crucial role of IL-5 as a survival cytokine for eosinophils. In contrast to wild-type (WT) counterparts, the R5/R5 homozygous IL-5 knockout (IL-5KO) mice exhibit no significant difference between their IL-33 + Ova sanitized and ova alone controls. This observation strongly implies that eosinophilic esophagitis (EoE) could not be induced in the absence of IL-5.

In conclusion, the absence of eosinophils within the esophagus due to the lack of IL-5 suggests a heavy reliance on IL-5's survival signals for their accumulation. This challenges the idea that the accumulation is solely attributed to an increase in eosinophil infiltration. Although both processes may occur simultaneously, the indispensability of IL-5 to observe the phenotype strongly underscores its significant role in the observed eosinophil accumulation in the esophagus. It is plausible that eosinophils accumulate due to an extended lifespan, rather than solely because of increased infiltration or decreased emigration.

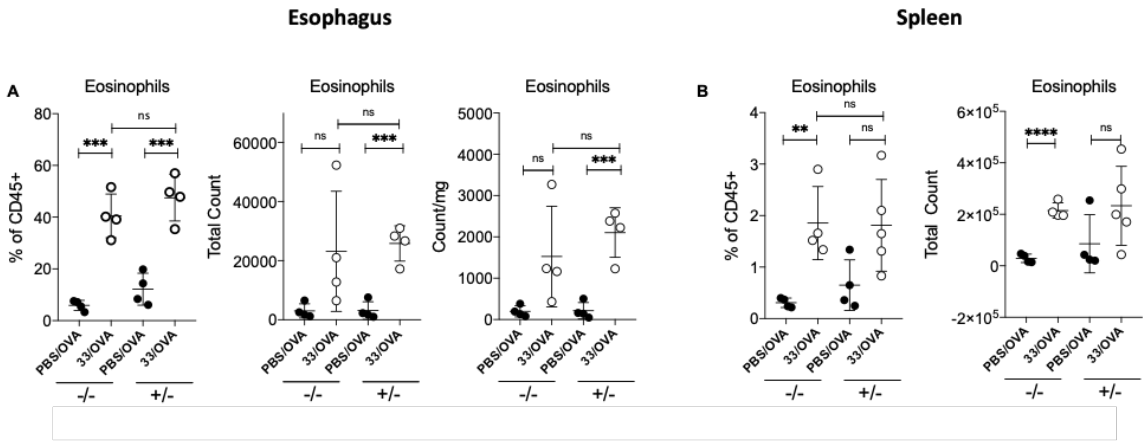

Figure 15. Hyperactivating ST2 SNP has no effect on esophageal eosinophilia. Mice were sensitized with 2ug of OVA in PBS, with or without 0.1ug of recombinant IL-33. Mice were challenged with intragastric administration of 5mg of OVA. (n = 4-6). **A**) Dot plots demonstrating number of eosinophils per CD45+ cells, total count, and per mg of esophageal tissue. **B**) Dot plots demonstrating frequency of eosinophils per CD45+ cells and total number eosinophils within the spleen

Furthermore, in collaboration with Dr. Paul Bryce Laboratory of Northwestern University, a novel transgenic mouse strain was created to mimic the effects of a polymorphism, rs10204137, within the ST2 protein. Using a CRISPR targeting approach, gRNA was designed to change CAG to AGG, thereby mimicking the amino acid change from a glutamine to arginine (Q501R). This variant was found to enhanced IL-33 responsiveness within an in vitro system, and indeed our own preliminary data show that mice heterozygous for the SNP shower higher levels of eosinophilia when injected with IL-33 alone **(**Data not shown). There is also published evidence that this variant was found to enhance IL-33 responsiveness with an *In Vitro* system.[55]

The aim of this experiment was to assess whether the known hyperactivating single nucleotide polymorphism (SNP) within the ST2 protein has any promotional impact on esophageal

eosinophilia levels in our mouse model of eosinophilic esophagitis (EoE). To test this, mice were bred heterozygous to heterozygous to generate SNP negative littermate controls. Both ST2 SNP-negative littermates and heterozygous SNP-positive mice underwent the same sensitization and challenge procedures as before. The data indicates no discernible difference between normal Wt mice and those with the ST2 SNP. WT mice and ST2 SNP mice exhibit comparable frequencies and levels of eosinophilia following intragastric challenge. Additionally, no variation was observed in eosinophilia levels within the spleen. These findings suggest that the hyperactivating role previously associated with this SNP may not manifest in this specific challenge model.

In conclusion, the ST2 SNP does not exhibit hyperactivating characteristics in this context. It raises the possibility that such characteristics might be evident in other diseases. This could be linked to the specific cell types expressing ST2 that play a crucial role in mediating the EoE phenotype. For instance, IL-33 responding ILC2s might be significant mediators in allergic asthma, but in EoE, they might not play as prominent a role. Therefore, ILC2s with SNP+ ST2 may have a more pronounced effect in an asthma model than in EoE.

3.3 List of Figures and Tables

3.4 Discussion

The experiments detailed above aimed to establish an effective model for inducing eosinophilic esophagitis (EoE) and to investigate various factors influencing esophageal eosinophilia in mice. Following an adapted sensitization protocol with multiple daily oral challenges of ovalbumin (Ova), mice were subjected to intragastric challenges, leading to increased eosinophil accumulation in the esophagus. The optimal time point for investigation was identified as Day 7 post 7x Ova challenges, revealing a dose-dependent increase in eosinophils. Antigen specificity was confirmed by challenging Ova sensitized mice with bovine serum albumin (BSA), demonstrating that IL-33 sensitized mice exhibited increased esophageal eosinophilia specifically in response to their sensitizing antigen. The dependence on the adaptive immune system was supported by testing the model on Rag-/- mice, where IL-33 sensitized mice failed to induce esophageal eosinophilia on the Rag-/- background, indicating a requirement for adaptive immunity.

Subsequent experiments demonstrated that IL-5 is important for esophageal eosinophilia, as IL-33-driven models exhibited dependency on IL-5, aligning with expectations due to the survival role of IL-5 for eosinophils. Notably, the hyperactivating ST2 SNP, rs10204137, did not exhibit an effect on esophageal eosinophilia, challenging previous expectations. The study concludes that the absence of eosinophils in the esophagus without the presence of IL-5 cytokine, suggests a reliance on IL-5's survival signals for accumulation. This challenges the idea that accumulation is solely due to increased infiltration, with the indispensability of IL-5 highlighting its significant role. The potential variation in the hyperactivating role of the ST2 SNP in different diseases is suggested, possibly related to specific cell types expressing ST2 that play varying roles in different conditions.

Overall, the EoE model presents a number of advantages. It effectively replicates human disease, showing its antigen-dependent and specific nature, also evident in patients entering remission by eliminating triggering factors with a specified elimination diet. The model's dependence on the adaptive immune system underscores its relevance to human disease. This is highlighted by the sensitization of mice to the introduced ova with IL-33, leading to the development of allergic inflammation following challenges and establishing it as a truly allergic system.

Moreover, the ST2 SNP data proved interesting as it did not yield the anticipated hyperactivating response. Our EoE data specifically indicates no discernible difference in esophageal eosinophilia associated with the hyperactivating SNP. In speculating as to why signs of hyperactivity are not evident in SNP+ groups we hypothesized that the hyperactivating mechanism used by this SNP involves elevating ST2 expression, resulting in increased soluble ST2 in the bloodstream of SNP+ mice. Although this heightened soluble ST2 may influence the sensitization phase, its impact is not manifest in the EoE challenge model. The observed disparity between sensitization and challenge results points to a nuanced role for the SNP. Additionally, the lack of observable effects

in our challenge models may be attributed to the significance of specific mediating cells in the process.

Moving forward, our future directions involve refining characterizations through more complex flow tables to measure various cell populations, with particular attention to infiltrating T cells, DCs, and ILC2s. We planned to evaluate serum levels of IL-33 cytokine and quantify eosinophils and other white blood cells in the esophagus, spleen, lungs, and blood. Additionally, we aim to scrutinize ST2 expression levels on all relevant cell types to understand variations in their IL-33 responsiveness across treatment groups. To further delve into sensitization data, we intend to test our challenge models on CD4 and CD11c knockout strains, exploring the potential impact on reducing allergic disease.

Chapter 4: Material and Methods

Ethics approval

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Benaroya Research Institute, and were performed in accordance with the approved guidelines for animal experimentation at the Benaroya Research Institute.

Mice and Intradermal Sensitization

All mice used were between 8 to 15 weeks of age. C57BL/6 and CD4-Cre/ST2$^{fl/fl}$ mice were obtained from Jackson Laboratories and further bred in-house. All mice were certified to be specific pathogen–free and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee at Benaroya Research Institute (Seattle, WA). Intradermal injections of 100ng of recombinant carrier-free IL-33 (Cat#580508; BioLegend) with and without 2ug OVA (A7642; Sigma-Aldrich) in 50ul of sterile PBS were administered twice, separated by 4 days. Mice were euthanized on Day 12. All experiments used age-gender- and genetic strain-matched controls in order to control for cross-experiment variation within data sets.

EoE, Asthma and anaphylaxis Challenge

Intradermal injections were performed as previously describe (Han & Ziegler, 2017). Briefly, 100ng of recombinant IL-33 (company) with 2ug OVA (A7642; Sigma-Aldrich) in a 50ul volume of sterile PBS 2 times, separated by 4 days. Intragastric challenges with 5mg OVA (A7642; Sigma-Aldrich) or low endotoxin BSA (sigma-aldrich) were administer for 7 days. Mice were intranasally challenged with 25ug of OVA within 25ul of sterile PBS, once per day for a maximum of two days.

Total IgE and Ag-specific IgE ELISA

High-binding 96-well ELISA plates (Costar, Corning) were coated with either 10ug/ml of anti-mouse IgE ab (MT56E, Mabtech) or 20ug/ml of OVA (A7642; Sigma-Aldrich) in PBS overnight at 4C. The plates were washed 3 times with 0.5% PBS Tween 20 and blocked with 5% FCS Serum for 1 hour at RT. Blocking buffer was removed and 50ul of sera (diluted 1:2 with 2x serial dilution from each sample was incubated with plate either overnight at 4C or 2hrs at 37C. Plates were washed and 1:500 of biotinylated anti-IgE ab (R35-72; BD Biosciences) for 0.5hr at 37C. Plates were washed and incubated for 0.5hr at 37C with 1:250 dilution of Streptavidin HRP (Sigma-Aldrich). 100ul of Tetramethylbenzidine substrate per well was incubated at RT for 10 minutes max. The reaction was stopped with 2 N H_2SO_4 and OD was measured at 450nm on a plate reader.

In Vitro Stimulation assay

Inguinal, brachial and axial lymph node cells were isolated from mice immunized with either Ova Alone or rIL-33 + Ova. Cells were then counted directly ex vivo with trypan blue and a cell counter. 250 thousand cells/well were incubated in a 96-well U bottom plate with PMA, Ionomycin for 1 hr. 1:1000 of Monensin was added to culture and cells were incubated for another 4-5 hours. Finally, cells were washed with PBS and then underwent live/dead and antibody staining.

Flow Cytometry

The antibodies used for flow cytometry analysis are shown in supplemental **Table 1**. Abs for flow cytometry were purchased from BD Biosciences, eBioscience, and BioLegend. 2% FACs buffer was prepared as follows: 2% [v/v] BSA and in PBS, pH7.5. Cells were incubated with Zombie Aqua Fixable Viability Dye (Cat# 423102; BioLegend) and Purified Rat Anti-Mouse CD16/CD32 FC block (Cat#553142; BD Biosciences) diluted in sterile PBS for 20 minutes at 4C. Cells were

washed with twice with PBS. BD Cytofix/Cytoperm™ kit was used to fix and permeabilize cells for intracellular staining. Cells were incubated for 20 minutes at 4C and then washed twice with BD Fix/Perm wash buffer Abs diluted in BD Fix/Perm wash were incubated with cells for 30 min, washed twice times with BD Fix/Perm wash, and then once with 2% FACs buffer. Data was acquired on a FACSymphony™ A5.

Digestion of Esophageal tissue

Immediately after Avertin euthanasia, mice are perfused with cold PBS, and the esophagus is excised, minced, and digested enzymatically with digestion solution (PBS, 1mg/ml Liberase TM, and 20 U/ml DNase) at 37°C for 30 min. The suspension was strained with a 100-µm cell strainer, washed twice with/and resuspended in 0.5% FACS buffer.

Histology

At necroscopy, the distal portion of the esophagus was fixed in 4% paraformaldehyde and embedded in paraffin and 5 µm sections were cut and stained with hematoxylin and eosin (H&E). For immunofluorescence, sections were dewaxed and stained with anti-siglec F mAB (Biolegend), followed by a secondary staining and counterstaining with DAPI. Analysis of the histology slides was done by counting the number of siglec F+ eosinophils within a high power field (HPF).

Statistical Analysis

One-way Analysis of Variance (ANOVA) with Tukey's multiple comparisons tests was performed on normally distributed data. Student t tests were also performed on normally distributed data. *P≤0.05;**P≤0.01; ***P≤0.001; ****P≤ 0.0001. All data are representative of at least two independent experiments analyzed with 3-6 mice per group. Error bars indicate the mean ±s.d. Data were analyzed with Prism 8 (Graphpad Software, La Jolla, California).

Chapter 5: List of Figures and Tables

Supplemental Table 1

Type	Marker	Clone	Fluorophore	LSR settings	Dilution
Fc Block	FC Block		-	-	1:100
Surface	Live/Dead - Aqua	n/a	AmCyan	V510	1:1000
ICS	CD44	IM7	BV605	V595	1:600
ICS	CD4	RM4-5	PE/Dazzle 594	YG602	1:400
ICS	TCRb	H57-597	PerCp/Cy5.5	B710	1:300
ICS	PD-1	29F.1a12	APC-Fire 750	R780	1:200
ICS	CD90.2	30-H12	BV785	V785	1:200
ICS	IL-5	B328753	APC	R675	1:150
ICS	ST2	U29-93	BV421	V427	1:100
ICS	IL-4	BVD6-24G2	PE-Cy7	YG780	1:100
ICS	IL-13	ebio13A	PE	YG585	1:100
ICS	GATA3	L50-823	FITC	B510	1:50

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