Mechanisms Regulating Allergic Sensitization to Cockroach Allergen

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Abstract

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Allergic asthma is an inflammatory disease of the airways driven by an overactive immune response against allergens. Allergen-specific T_H2 cells are critical to the pathogenesis of asthma but factors controlling the initiation of T_H2 responses are not well understood. The emerging picture is one that pinpoints a role for airway epithelial cells and epithelial-derived cytokines in promoting T_H2 immunity to allergens. However, allergens are structurally diverse and can elicit different epithelial-derived cytokines. Thus, there are multiple pathways that lead to T_H2 immunity and mechanisms of sensitization that are unique to individual allergens remain to be elucidated. Cockroach allergen (CRA) is an environmental allergen that is strongly associated with the incidence of allergic asthma. Despite its clinical importance, very little is known about the molecular and cellular pathways involved in allergic sensitization to CRA. To determine the mechanisms that regulate allergic sensitization to CRA, we intranasally sensitized and challenged mice with CRA to induce allergic airway inflammation with hallmark features of asthma. Using this model, we demonstrate that allergic sensitization to CRA requires activation of airway epithelial cells. MyD88 deletion in airway epithelial cells reduced allergic airway response to CRA as well as production of GM-CSF during sensitization. Using a neutralizing antibody against GM-CSF, we showed that GM-CSF is essential for T_H2 priming and allergic sensitization to CRA, likely through activation of dendritic cells. When GM-CSF was intranasally delivered during sensitization, T_H2-mediated eosinophilia was restored in the absence of MyD88. Thus, airway epithelial cells promote allergic sensitization to CRA through production of GM-CSF.

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Chapter 1. Introduction

Overview

Asthma is a chronic lung disease that affects over 300 million people. It is defined on the basis of clinical characteristics that include airway hyperresponsiveness, airway inflammation, and mucus overproduction. Allergic asthma is a subgroup of asthma in which patients are allergic to known allergens such as cockroach, house dust mite (HDM), pollen, or animal dander. This response attributes a role for environmental allergens in the development and exacerbation of allergic asthma. In particular, a large percentage of asthmatics are allergic to cockroach allergen (CRA). Importantly, exposure to CRA is significantly associated with both allergic sensitization and occurrence of asthma. Additionally, children who are both allergic to cockroach and exposed to high levels of this allergen have significantly higher asthma-related morbidity.

Despite the clinical importance of CRA, the immune response to CRA is not well understood. Although several studies have identified pattern recognition receptors (PRRs) that are crucial for the recognition of CRA, the downstream mechanisms that drive allergic sensitization to CRA remain largely unknown. Several studies have reported genetic polymorphisms that are specifically associated with allergic sensitization to CRA, but not other allergens. This suggests that there are unique pathways associated with allergic sensitization to CRA. The focus of my thesis is to determine how exposure to CRA leads to the development of allergic asthma.

The underlying process driving allergic asthma is an inappropriate immune response against allergens, and the role of the adaptive immune system has been well documented in the pathogenesis of asthma. Allergen-specific T_H2 cells produce key cytokines that mediate the symptoms associated with asthma. However, the mechanisms that drive this T_H2 response during allergic sensitization are still unclear. Specifically, our understanding of the cell types and cytokines that initiate this allergic response is still incomplete. The emerging picture is one that emphasizes a critical role for airway epithelial cells in recognizing allergens and coordinating the subsequent immune response. Airway epithelial cells are equipped with PRRs that bind to specific motifs on allergens. Additionally, activation of airway epithelial cells results in the release of cytokines that promote T_H2 responses, in part by providing instructive signals to dendritic cells (DCs). However, this process is complicated by the fact that individual allergens can induce T_H2 responses through different pathways, and very little is known about the mechanisms that are unique versus common to all allergens. The primary goal of my thesis is to determine the role of airway epithelial cells and epithelial-derived cytokines during allergic sensitization to CRA. The following sections of this chapter will discuss our current understanding of: (1) Asthma and the allergic immune response; (2) Cockroach allergen; (3) Pattern recognition receptors; and (4) GM-CSF.

Asthma

Asthma is a chronic lung disease that causes reversible airflow obstruction, airway inflammation, mucus overproduction, and airway remodeling (Wenzel 2012). It manifests as recurrent episodes of wheezing, breathlessness, and chest tightness. In the United States, about 1

in 10 children and 1 in 12 adults have asthma (Vital Signs, May 2011) and approximately 334 million people are affected worldwide (The Global Asthma Report 2014, 2014). Asthma is associated with a large socioeconomic burden; the cost of asthma due to medical expenses, loss of productivity, and premature death is estimated to be $56 billion in 2007 (Vital Signs, May 2011). In the past, asthma was considered a disease of affluence but it is becoming more common in developing countries where its prevalence is rapidly rising (Pearce et al. 2007). Currently, anti-inflammatory drugs, such as corticosteroids, are the mainstay in asthma therapy. Corticosteroids act in a broad, nonspecific manner to reduce the overall inflammation in the airway. In addition, bronchodilators, such as β2-agonists, relax the muscles around the airway and are also used to control asthma. These therapies are effective in controlling most but not all asthma cases. Unfortunately, about 10% of asthma cases are refractory to corticosteroid treatment, which highlights the need for more effective therapeutics and better understanding of the underlying causative mechanisms (Trevor and Deshane 2014).

Asthma is a heterogeneous disease that is often classified as allergic (extrinsic) or non-allergic (intrinsic) asthma. As the name suggests, allergic asthma is associated with allergic sensitization in which patients are allergic to a known allergen and have detectable levels of allergen-specific IgE antibodies in their serum. People with allergic asthma tend to develop the disease early in life and have a history of allergic diseases (Wenzel 2012). On the other hand, non-allergic asthma develops later in life and is not associated with allergic sensitization (Wenzel 2012). The main focus of my thesis is to understand the role of the immune system in the initiation of allergic sensitization and the development of allergic asthma.

Allergic asthma is an inflammatory disease driven by exposure to allergens. The allergic cascade that leads to the development of allergic asthma occurs in multiple stages: (1) sensitization to an allergen; (2) early-phase response upon re-exposure; and (3) late-phase response to an allergen. During the sensitization phase, initial exposure to an allergen results in activation of airway epithelial cells and production of epithelial-derived cytokines. Meanwhile, lung DCs take up allergens, process them into peptides, and migrate to the lung-draining lymph node. In the lymph node, DCs present allergen peptides in the context of MHCII to naïve T cells and prime T_H2 cells. The ability of DCs to prime T_H2 cells is heavily influenced by the cytokine milieu in the lung. Induction of T_H2 responses is followed by production of allergen-specific IgE antibodies by B cells. These antibodies bind to IgE receptors on mast cells in the lung, which leads to the next stage in the allergic cascade.

Upon re-exposure, inhalation of allergens causes early-phase bronchoconstriction due to allergen binding to IgE antibodies and crosslinking of the IgE-receptor complex on mast cells (Bloemen et al. 2007). This causes mast cells to release mediators of bronchoconstriction, such as leukotrienes, as well as proinflammatory cytokines, such as TNFα. Exposure to allergens also causes late-phase bronchoconstriction which is driven by airway inflammation (Bloemen et al. 2007). This inflammatory response is mediated by allergen-specific $CD4^+$ T_H2 cells that are activated upon allergen exposure. Activated T_H2 cells produce IL-4, IL-5, and IL-13 which cause many of the symptoms associated with asthma. Additionally, eosinophils are recruited to the airway where they serve as important effector cells (Akuthota, Xenakis, and Weller 2011). Chronic inflammation also results in airway remodeling such as goblet cell metaplasia, subepithelial fibrosis, and increased airway smooth muscle mass (Bloemen et al. 2007; Cohn,

Elias, and Chupp 2004). Overall, allergic asthma develops due to an inappropriate immune response against allergens. The role of airway epithelial cells, DCs, T cells, and eosinophils in this allergic response will be reviewed below.

Airway Epithelial Cells

Every day, airway epithelial cells that line the mucosal surface of the airway are exposed to approximately 10,000 liters of air that contain potentially harmful substances (Seaton et al. 1995). As the first line of defense against inhaled pathogens, airway epithelial cells provide a physical barrier against the external environment by forming tight junctional complexes between neighboring cells. Airway epithelial cells also remove inhaled particles from the airway through a process called mucociliary clearance, where airway mucus traps the particles and the beating action of cilia on airway epithelial cells pushes them back up the trachea. In addition to these passive mechanisms, airway epithelial cells actively respond to infections by secreting chemokines and cytokines that recruit and activate cells of the immune system. Over the years, it has become increasingly clear that airway epithelial cells also play a role in the aberrant immune response against environmental allergens during allergic asthma. One focus of my thesis is to study the role of airway epithelial cells in the development of allergic sensitization.

Substantial evidence shows that reduced epithelial barrier integrity is associated with the development of allergic diseases. For example, absence of filaggrin, a key intracellular structural protein in keratinocytes, results in epidermal barrier dysfunction, and a loss of function mutation in the encoding *FLG* gene is a significant risk factor for atopic dermatitis (Morar et al. 2007; Palmer et al. 2006). Reduced airway epithelial barrier function has also been reported in asthmatic patients, which correlates with lower expression of tight junction proteins (Blume et al.

2013; de Boer et al. 2008; Xiao et al. 2011). Although there is no direct evidence showing that airway leakiness promotes allergic sensitization, disrupted barrier integrity could allow for greater entry of inhaled allergens into the subepithelial space and increased activation of lung resident immune cells. Among the different factors that can reduce barrier function, allergens that contain proteases can directly cleave epithelial tight junctions to disrupt barrier structures (Jeong et al. 2008a; Leino et al. 2013; Runswick et al. 2007; Wan et al. 1999). Additionally, T_H2 cytokines, which are elevated during an allergic response, can induce barrier disruption by inhibiting surface expression of tight junction proteins (Saatian et al. 2013; Soyka et al. 2012). This leads to a cycle of barrier damage in which the T_H2 allergic response reduces epithelial barrier integrity and allows for even greater penetration of inhaled allergens.

Airway epithelial cells can directly recognize inhaled allergens through PRRs such as protease-activated receptors (PARs), C-type lectin receptors, and toll-like receptors (TLRs). Multiple allergens have been reported to trigger both TLR2 and TLR4 on airway epithelial cells (Hammad et al. 2009; Page et al. 2008). Additionally, airway epithelial cell expression of dectin-1, a C-type lectin receptor, mediates recognition of β-glucan in multiple allergens (Nathan et al. 2009; Sun et al. 2012). Binding of allergens to these receptors leads to activation of airway epithelial cells and production of chemokines that recruit immune cells to the airway. For example, airway epithelial cells produce CCL2 and CCL20 in response to HDM, which attracts monocytes and dendritic cells to the lung (Hammad et al. 2009; Nathan et al. 2009). Airway epithelial cells also express CCL17 and CCL22 to recruit $CCR4^+$ T_H2 cells as well as eotaxin to recruit eosinophils (Sekiya et al. 2000; S Ying et al. 1997).

In addition to chemokines, airway epithelial cells secrete a set of prototypical cytokines – TSLP, IL-25, and IL-33 – that are elevated in the lungs of asthmatics (Cheng et al. 2014; Prefontaine et al. 2009; Sun Ying et al. 2005). Additionally, genetic polymorphisms in the *TSLP*, *IL25*, and *IL33* genes are strongly associated with asthma (Byers 2014). Although each cytokine belongs to a different cytokine family, they share the ability to promote T_H2 allergic responses. For example, all three cytokines promote T_H2 differentiation by instructing dendritic cells to induce OX40L expression while downregulating the production of IL-12p40 (Besnard et al. 2011; Chu et al. 2012). TSLP, IL-25, and IL-33 can also act directly on T cells to promote the differentiation and maintenance of T_H2 cells (Omori and Ziegler 2007; Schmitz et al. 2005; Wang et al. 2007). However, there are some key differences in the cell types that respond to each cytokine. For example, TSLP acts directly on bone-marrow resident progenitors to promote basophil hematopoiesis (Noti et al. 2013; Siracusa et al. 2011). On the other hand, both IL-25 and IL-33 can stimulate the production of IL-5 and IL-13 by group 2 innate lymphoid cells (ILC2s) (Moro et al. 2010). Recently, IL-25 was also reported to elicit multipotent progenitor type 2 cells which are distinct from ILC2s (Saenz et al. 2013). These differences might partially account for the relative importance of each cytokine in different T_H2 allergic models; such as the requirement for IL-33, but not IL-25 or TSLP, in allergic airway response to HDM (Willart et al. 2012). It is also likely that different allergens preferentially elicit different epithelial-derived cytokines, which might contribute to these differences in cytokine requirement.

To determine the contribution of airway epithelial cells to the development of asthma, bone marrow chimera studies have been used to distinguish the role of structural cells versus radiosensitive hematopoietic cells. These studies reported that TLR4 signaling in lung structural

cells is critical for the initiation of allergic responses to inhaled antigens (Hammad et al. 2009; Tan et al. 2010). However, bone marrow chimera studies do not directly address the role of airway epithelial cells. This led to the use of the Cre/*loxP* approach to selectively target airway epithelial cells. One such study confirmed that TLR4 expression on airway epithelial cells is important for eosinophilic airway inflammation (McAlees et al. 2015). Using the same approach, other studies have shown that activation of airway epithelial cells is important for the development of allergic airway disease. Selective ablation of NF-κB signaling through IKKβ in the airway epithelium led to reduced airway inflammation and peribronchial fibrosis after repetitive ovalbumin challenge in mice (Broide et al. 2005). A complementary study showed that transient activation of NF-κB specifically in airway epithelial cells is sufficient to promote sensitization to inhaled ovalbumin (Ather et al. 2011). Overall, these studies demonstrate that activation of airway epithelial cells is a critical step in the initiation of allergic airway responses.

Dendritic Cells

Dendritic cells (DCs) are professional antigen-presenting cells that present allergens in the context of MHCII to allergen-specific T cells. When DCs encounter allergens, they acquire and internally process the allergen to present it as a peptide on MHCII. During this maturation process, inflammatory signals induce the upregulation of costimulatory molecules, such as CD80, CD86, and CD40, that are necessary for activation of T cells. Mature DCs then migrate to the draining lymph node where they present antigen and prime naïve T cells to undergo T_H2 differentiation. Thus, DCs are critical for the initiation of T_H2 allergic responses. Initial studies reported that airway inflammation fails to develop when $CD11c^{hi}$ DCs are depleted before allergic sensitization (Hammad et al. 2010). During the challenge phase of the allergic response,

DCs interact with effector T_H2 cells in the lung and are required for this stage of the response (van Rijt et al. 2005). Additionally, intratracheal delivery of antigen-pulsed BMDCs is sufficient to induce allergic airway disease after aerosolized challenge with an antigen (Lambrecht et al. 2000). Overall, these findings demonstrate an integral role for DCs in the initiation of allergic airway inflammation.

There are two subsets of DCs in the lung that express either CD11b or CD103 integrin, each with unique functions. $CD103^+$ DCs mainly specialize in cross-presentation of viral antigens to CD8 T cells (Desch et al. 2011). On the other hand, there is strong evidence to suggest that $CD11b^+$ DCs are required for T_H2 priming. During sensitization to HDM, $CD11b^+$ DCs carry antigen to the draining lymph node. In this study, allergic airway response to HDM is not affected by the absence of $CD103^+$ DCs (Plantinga et al. 2013). In contrast, allergic inflammation is markedly reduced in mice lacking both $CD11b^+$ and $CD103^+$ DCs, suggesting that $CD11b^+$ DCs but not $CD103^+$ DCs are necessary for the T_H2 allergic response. Interestingly, when high doses of HDM are administered, monocyte-derived DCs are able to function as the primary APC in the absence of both $CD11b^+$ and $CD103^+$ DCs.

Although $CD11b^+$ DCs can promote T_H2 differentiation, the exact mechanism by which they induce T_H2 responses is still unclear. A recent study has shed some light on this topic by describing a subset of DCs that depends on the transcription factor IRF4 for promoting T_H2 responses (Williams et al. 2013). They showed that lack of IRF4 in $CD11c^+$ DCs reduces T_H2 responses but not viral-induced T_H1 responses in the lung. In DCs, IRF4 specifically targets and activates the genes encoding Il-10 and Il-33 to promote optimal T_H2 differentiation. An IRF4-

dependent DC subset that can control T_H2 responses was recently identified in the skin- and mesenteric-draining lymph nodes as well (Y. Gao et al. 2013).

T Lymphocytes

Initial studies of T cells in the asthmatic airways showed a strong predominance of T_H2 cells (Robinson et al. 1992). Since then, overwhelming evidence supports a central role for T_H2 cells in the pathogenesis of asthma. A role for T cells in asthma was established by demonstrating that allergic airway inflammation does not develop when CD4 T cells are depleted before allergen challenge (Gavett et al. 1994). Additional studies showed that the T_H2 cytokines, IL-4, IL-5, and IL-13, are responsible for many of the inflammatory features associated with asthma. On the other hand, the role of T_H1 cells in asthma remains unclear but is generally believed to oppose T_H2 responses. Treatment of mice with IFNɣ prior to sensitization prevents airway inflammation and development of AHR (Nakajima, Iwamoto, and Yoshida 1993). In contrast, T_H17 cells have been implicated in severe asthma. Mouse models of allergic airway disease have shown that IL-17 can mediate neutrophilia and AHR (Mckinley et al. 2008; Wilson et al. 2009; Zhao, Lloyd, and Noble 2013). As allergic asthma is strongly associated with a T_H2 response, the individual role of IL-4, IL-5, and IL-13 will be further discussed below.

IL-4

IL-4 signals through the IL-4 receptor complex, which is composed of the common ɣ-chain and the IL-4Rα chain. Ligation of the IL-4 receptor complex results in activation of the STAT6 signaling pathway. Initial studies showed that airway inflammation and AHR are strongly reduced after allergen challenge in IL-4-deficient mice (Brusselle, Kips, and Joos 1995). Additional studies showed that IL-4 is required during sensitization but not during the challenge

phase of the allergic response (Coyle et al. 1995). These studies demonstrate an important role for IL-4 in the induction of T_H2 allergic responses. IL-4 is an important cytokine for the differentiation of T_H2 cells (Le Gros et al. 1990). Stimulation with IL-4 leads to upregulation of GATA3, a master regulator of T_H2 cell differentiation, that is both necessary and sufficient to drive T_H2 cytokine production in T cells (Zheng and Flavell 2015). However, the initial cellular source of IL-4 remains unclear. For example, NKT cells can produce IL-4 after TCR engagement (T. Yoshimoto and Paul 1994), but mice lacking NKT cells are still capable of mounting a T_H2 response (Brown et al. 1996), suggesting other cells produce this cytokine. When activated, basophils are another cell type that produces IL-4. Recent studies have reported that basophils can migrate to the lymph node where they might interact with naïve T cells and that they are required for T_H2 response to papain (Tomohiro Yoshimoto et al. 2009). Finally, naïve T cells can make IL-4 when activated with peptide in vitro but it is unclear if autocrine and paracrine IL-4 production drives T_H2 differentiation in vivo

IL-4 is also critical for the production of allergen-specific IgE antibodies. Specifically, IL-4 induces class switching from IgM to IgE through somatic recombination of germline genes in B cells (Finkelman et al. 1988; Gould et al. 2003). Early research into asthma identified IgE antibodies as crucial for initiating an allergic reaction. In 1921, two physicians demonstrated that passive transfer of serum from an allergic individual could induce an allergic reaction when transferred to a healthy person (Frankland 2004). We now know that it is the transfer of IgE antibodies that is responsible for this reaction. When IgE is produced after sensitization, it binds to the IgE receptor expressed on the surface of mast cells and basophils. Upon allergen binding,

the IgE-IgE receptor complex crosslinks and activates mast cells and basophils to release allergic mediators such as histamine.

IL-5

IL-5 promotes the terminal differentiation of eosinophil bone marrow precursors (Yamaguchi et al. 1988), prevents apoptosis of eosinophils (Simon et al. 1997), and enhances cytotoxicity and mediator release by eosinophils (Lopez et al. 1988). Eosinophils are increased in the asthmatic airway and are important effector cells in the pathogenesis of asthma. A role for IL-5 in asthma was established early by showing that IL-5 deficient mice do not develop eosinophilic inflammation or AHR (Foster et al. 1996; Hamelmann et al. 1999). Successful outcome of anti-IL-5 therapeutics in asthmatic patients further support these findings in mice. Results from a phase 3 clinical trial for an anti-IL-5 monoclonal antibody (mAb) from GlaxoSmithKline reported that blocking IL-5 reduces the rate of exacerbations by 53% in patients with severe eosinophilic asthma (Ortega et al. 2014). Recently, the FDA approved this anti-IL-5 mAb for the treatment of adults with severe asthma.

IL-13

IL-13 is a T_H2 cytokine that signals through the IL-13 receptor complex which is comprised of IL-13Rα1 and the IL-4Rα chain. Similar to the IL-4 receptor complex, ligation of the IL-13 receptor complex results in activation of the STAT6 signaling pathway. Early studies uncovered a critical role for IL-13 in mediating different features of asthma. In the absence of IL-13, mice do not develop AHR and mucus hypersecretion in response to allergen challenge (Walter et al. 2001). Adoptive transfer of T_H2 cells from IL-13 deficient mice also fails to induce AHR, despite the production of IL-4 and IL-5 by IL-13-/- T_H2 cells. Additional studies

showed that overexpression of IL-13 in the lung or administration of IL-13 is sufficient to induce AHR, mucus cell metaplasia and subepithelial airway fibrosis (Wills-Karp et al. 1998; Zhu et al. 1999).

Although IL-13 promotes the asthmatic response, the effector mechanisms through which IL-13 mediates these features is still unclear. Several studies have suggested that IL-13 might induce AHR through direct effects on airway smooth muscle cells. For example, IL-13 can increase Cys-LT1 expression on smooth muscle cells (Espinosa et al. 2003) as well as enhance histamine- and acetylcholine-induced intracellular calcium fluxes in isolated smooth muscle cells (Laporte et al. 2001). While IL-13 does not directly stimulate the contraction of airway smooth muscle cells, these studies show that IL-13 enhances the response to other contractile agonists. On the other hand, IL-13 directly induces mucus production in airway epithelial cells cultured at the air-liquid interface (Alevy et al. 2012). Finally, IL-13 regulates subepithelial fibrosis by stimulating the production of the profibrotic mediator TGF-β2 by epithelial cells (Wen et al. 2002) as well as the proliferation of myofibroblasts (Ingram et al. 2003), a cellular mediator of interstitial fibrosis.

Eosinophils

In patients with asthma, there are increased numbers of eosinophils in the peripheral blood and BAL fluid, which correlates with the severity of asthma (Bousquet et al. 1990). However, whether eosinophils merely serve as a marker of allergic inflammation or are true mediators of allergic disease is a topic of much debate. Recent studies have provided evidence to support that eosinophils play an important role in the pathogenesis of asthma. Specifically, the role of eosinophils in allergic airway inflammation was examined in two different strains of

eosinophil-deficient mice. The Δdbl GATA mice specifically lack eosinophils due to the absence of a high-affinity GATA binding site in the GATA-1 promoter. *PHIL* mice are selectively deficient in eosinophils due to the expression of diphtheria toxin A under the control of the promoter for eosinophil peroxidase. In both strains of mice, there is a reduction in AHR and mucus production after allergen challenge (J. J. Lee et al. 2004; Walsh et al. 2008). Another study showed that in the absence of eosinophils, adoptive transfer of OVA-specific T_H2 effector cells is not sufficient to induce T_H2 responses in the lung. Instead, transfer of both T_H2 cells and eosinophils are required to restore T_H2 responses in *PHIL* mice (Jacobsen et al. 2008). The mechanism by which eosinophils mediate these responses is not well understood. Eosinophils can induce massive tissue damage by secreting cytotoxic granules that contain major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO). One study reported that intratracheal instillation of MBP and EPO can directly induce bronchoconstriction in primates (Gundel, Letts, and Gleich 1991). Overall, these studies demonstrate an integral role for eosinophils in the development of AHR and recruitment of T_H2 cells during the allergic airway response.

Cockroach Allergens

Over 4,000 cockroach species exist but only two domestic species are most commonly associated with allergies. The German cockroach *Blattella germanica* is a small cockroach that is approximately 1.9 cm in length and most commonly found in the United States and Europe (Pomés et al. 2007). The American cockroach *Periplaneta americana* is larger at 5 cm in length and is more suited to warm, tropical environments such as South America and some Asian

countries (Pomés et al. 2007) and is widely considered to be a truly disgusting insect. Cockroach allergens are dispersed into the environment as debris from cockroach secretions, feces, and exoskeleton. Major allergens from these two cockroach species have been identified as a group of proteins with diverse structure and biological function.

The Group 1 cockroach allergens, which consists of the proteins Bla g 1 and Per a 1, are secreted in the digestive tracts of cockroaches. Recently, Bla g 1 was revealed to contain a hydrophobic cavity that binds readily to lipids, which might facilitate the nonspecific transport of lipid molecules in cockroaches (Mueller et al. 2013). Bla g 2 and Per a 2 represent another group of proteins that are also secreted in the digestive tract. Although they show sequence homology to aspartic proteases, they are inactive due to a critical substitution in the enzymatic active site (L. K. Arruda et al. 1995). In contrast, Per a 3 is found in the hemolymph of cockroaches and shares structural homology with the immunogenic protein, hemocyanin (Mindykowski et al. 2010). Bla g 4 belongs to a family of proteins called lipocalins that binds to small hydrophobic ligands. This protein is only expressed in the adult male reproductive system of the cockroach and is believed to be secreted during copulation (Fan et al. 2005). Bla g 5 shows sequence homology to the glutathione S-transferase superfamily (GST) of proteins that are involved in the detoxification of toxic compounds (L. Karla Arruda et al. 1995). Expression of GST proteins is associated with insecticide resistance, which has implications for the use of insecticides to remove cockroaches from the home (Enayati, Ranson, and Hemingway 2005). Finally, Groups 6, 7, and 8 of cockroach allergens show homology to proteins involved in muscle contractions: troponin C, tropomyosin, and myosin light chain. Considering not all cockroach allergens have

been identified, our understanding of cockroach allergens is incomplete and studies are underway to determine additional cockroach allergens that are recognized by IgE.

Cockroach Allergen and Asthma

Exposure to cockroach allergen is an important risk factor for the development of allergic sensitization and allergic asthma. Over 50 years ago, the first study to survey skin sensitivity to cockroach allergen reported that 32 out of 114 allergic patients (28%) have a positive skin response to cockroach extracts (Bernton 1964). This was followed by an important study showing that inhalation of cockroach allergen results in early and late-phase bronchoconstriction, only in asthmatics that were allergic to cockroach allergen (Kang, Vellody, and Hornburger 1979). This study demonstrates a role for cockroach allergen in allergic asthma and further shows that cockroach allergen-induced asthma exacerbation is antigen specific.

Since then, various studies have reported the prevalence of cockroach allergy to be 17-41% in the United States, with higher prevalence in inner-city areas (Matsui et al. 2003; Pollart et al. 1989; Sporik et al. 1999). As one might expect, exposure to cockroach allergen is significantly associated with sensitization to cockroach allergen, and high levels of cockroach allergen are found in the homes of asthmatic children from inner-city areas (Rosenstreich et al. 1997). Several longitudinal studies also found a significant association between the indoor level of cockroach allergen and occurrence of asthma and recurrent wheeze among asthmatic children (Gold et al. 1999; Litonjua et al. 2001). Importantly, a study found that a combination of cockroach allergy and exposure to high levels of this allergen results in significantly higher hospitalization rates, more days of wheezing, and more unscheduled medical visits compared to mite and cat allergens (Rosenstreich et al. 1997). Overall, these studies show that cockroach

allergen is clinically relevant and poses a significant risk to the development of allergic sensitization and allergic asthma.

Genetic Basis for Cockroach Sensitization

Not all individuals who are exposed to high levels of CRA develop allergic sensitization, suggesting a role for genetic and environmental factors in sensitization. In fact, several genetic polymorphisms have been associated with a greater risk of cockroach sensitization. For example, a genome screen identified a significant association between HLA class II DRB1*01 alleles and CRA sensitization (Donfack et al. 2000), which indicates that certain HLA class II molecules have a higher affinity for cockroach allergens. In another study, a genome wide linkage analysis of CRA-specific IgE was conducted in Costa Rican children with asthma and their families. Sex-stratified analysis showed significant linkage to CRA-specific IgE on chromosome 5q23 in female subjects (Hunninghake et al. 2008). A candidate gene located within the interval of the linkage peak is TSLP and specifically, the T allele of SNP rs2289276 in TSLP is associated with reductions in CRA-specific IgE and total IgE. Another study reported that SNPs in *IL12A* are associated with increased risk of sensitization CRA (Pistiner et al. 2008). Interestingly, they did not find an association between any SNPs in *IL12A* and sensitization to dust mite. Finally, a recent study reported that cockroach sensitization is strongly associated with SNPs in *JAK3*, *JAK1*, *IL5RA*, *FCER1A*, and *ADAM33*. In contrast, allergic sensitization to HDM is associated with SNPs in a different set of genes: *JAK2*, *MAML1*, and *NOD1* (Tripathi et al. 2014). These last two studies demonstrate that there are unique pathways associated with allergic sensitization to individual allergens. To fully understand the initiation of allergic responses, it will be important to study how different allergens interact with the immune system.

Immune Response to Cockroach Allergen

Despite the clinical importance of CRA, the immune response to CRA is not well understood. After detecting proteolytic activity in commercially available whole body cockroach extract, most studies focused on the role of proteases in the immune response to CRA. It is important to note that none of the major allergens from German cockroach possess protease activity and only Per a 10 from American cockroach is a serine protease. This suggests that protease activity from CRA might be due to allergens yet to be identified or possibly derived from microbial components associated with cockroaches. In general, proteases activate protease-activated receptors (PAR) by cleaving the N-terminus of the receptor to liberate a ligand that then binds to the same receptor (Kawabata and Kuroda 2000). Several studies have demonstrated that PAR-2 mediates allergic sensitization to CRA (Arizmendi et al. 2011; Page et al. 2010). However, PAR-2 is not required for allergic inflammation when mice are sensitized with CRA systemically, suggesting that proteolytic activity is specifically required for mucosal sensitization (Page et al. 2010). Currently, pathways downstream of PAR-2 activation that mediate sensitization to CRA are still unknown. In vitro studies have shown that CRA proteases can activate PAR-2 to stimulate cytokine production in alveolar macrophages, dendritic cells, neutrophils, and eosinophils (Day et al. 2012; Kim et al. 2012; Lutfi et al. 2012; Wada et al. 2010). CRA proteases can also activate non-hematopoietic cells such as airway epithelial cells and fibroblasts (Kondo et al. 2004; K. E. Lee et al. 2007; Page, Strunk, and Hershenson 2003). Because PAR-2 is expressed by a wide variety of cells, it will be important to determine the relative importance of PAR-2 expression in immune cells versus structural cells for initiation of allergic responses to CRA.

CRA also contains ligands that can bind and activate TLRs. Specifically, CRA can activate TLR2 on neutrophils to induce the release of MMP-9, a protease with a range of potential immunologic functions (Page et al. 2008). In this study, TLR2 and MMP-9 were shown to have a protective effect on the development of CRA-induced allergic inflammation. Additionally, CRA contains lipopolysaccharide (LPS) that can activate TLR4. Whether LPS is truly a component of CRA is a matter of debate. However, considering that low levels of LPS are present in the environment, the role of LPS in CRA sensitization deserves some consideration. One study showed that removal of LPS from CRA does not reduce airway inflammation, which might have been due to the incomplete removal of LPS (Natarajan et al. 2011). In contrast, allergic inflammation to CRA is significantly reduced in TLR4-deficient mice (Arizmendi et al. 2011; Ullah et al. 2014). In addition to LPS, TLR4 can also bind to several endogenous ligands. High mobility group box 1 (HMGB1) is an endogenous ligand for TLR4 that is released in response to CRA. HMGB1 can also bind to the receptor for advanced glycation end products (RAGE) and a recent study showed that both TLR4 and RAGE can mediate allergic inflammation to CRA (Ullah et al. 2014).

CD206 is a C-type lectin receptor that recognizes mannose residues. CD206 is expressed on monocyte-derived DCs and mediates internalization of allergens including CRA (Royer et al. 2010). Recently, it was reported that Bla g 2 contains small, mannose-terminated glycans that can bind directly to CD206. Furthermore, human fibrocytes show expression of CD206 that mediates uptake of Bla g 2 and results in production of IL-6 and TNFα (Tsai et al. 2013). Currently, the role of CD206 in allergic sensitization to CRA is unknown. Taken together, these studies improve our understanding of the receptors that recognize CRA. However, the cellular

and molecular mechanisms that regulate sensitization to CRA remain largely unknown. The primary focus of this thesis is to shed some light on these mechanisms.

TLR/IL1R Superfamily and Asthma

Toll-like receptors (TLRs) and IL-1 receptors (IL1Rs) are members of a superfamily that share homology in the cytoplasmic region called the TIR domain. TLRs are a family of pattern recognition receptors (PRRs) that play an important role in recognizing foreign pathogens such as bacteria and viruses. These receptors bind to pathogen associated molecular patterns (PAMPs), which are evolutionarily conserved molecules expressed by microorganisms. TLR2 forms a heterodimer with TLR1 and TLR6 to recognize a diverse set of motifs including: peptidoglycan, lipoproteins, lipoteichoic acid from Gram-positive bacteria, and zymosan from fungi (Akira and Takeda 2004). TLR4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria while TLR5 binds to bacterial flagellin (Hayashi et al. 2001; Poltorak 1998). In contrast, TLR3, TLR7, and TLR9 play an important role in anti-viral immunity through recognition of intracellular viral RNA and DNA (Alexopoulou et al. 2001; Hemmi et al. 2000).

Among the TLR genes, expression of TLR2 and TLR4 is upregulated in asthma and allergic rhinitis (Ferreira et al. 2012; Fransson et al. 2005). Additionally, a polymorphism in the *TLR2* gene is strongly associated with asthma and allergies in children of European farmers (Eder et al. 2004). A role for TLR2 is further supported by studies in mice in which sensitization with a TLR2 ligand and ovalbumin (OVA) results in airway inflammation and AHR (Chisholm et al. 2004; Redecke et al. 2004). On the other hand, the dose of LPS determines the type of immune response generated in response to inhaled OVA. Specifically, a high dose of LPS (100

μg) induces a T_H1 response whereas a low dose of LPS (0.1 μg) is necessary for promoting a T_H2 response against OVA (Eisenbarth et al. 2002). The effects of LPS also depends on the timing of administration because chronic preexposure to low dose of LPS protects mice from the development of HDM-induced allergic asthma (Schuijs et al. 2015). Because allergic inflammation to HDM requires TLR4 signaling, it is unclear if the protective effects of LPS are exclusive to HDM-induced allergic asthma or are widely applicable to other allergen models. Interestingly, multiple studies have reported that allergens can facilitate TLR4 signaling. Der p 2, a HDM allergen, is functionally homologous to MD-2, a component of the TLR4 signaling complex which facilitates TLR4 signaling (Trompette et al. 2009). Similarly, a hydrophobic cavity in Bla g 1 from CRA can bind lipids and might also facilitate TLR4 signaling (Mueller et al. 2013). Whether TLR2 and TLR4 are required for recognition of other allergens and the subsequent allergic airway response is not fully understood and requires further investigation.

In contrast to TLRs, IL1Rs bind to members of the IL-1 cytokine family which includes IL-1α, IL-1β, IL-18, and IL-33. IL-1α and IL-1β are proinflammatory cytokines that have been reported to be involved in the development of asthma. Studies have shown that IL-1 is required for AHR and T_H2 cytokine production in an OVA/alum model (Johnson, Yucesoy, and Luster 2005; Nakae et al. 2003). Recently, it was shown that IL-1α , not IL-1β, is required for allergic sensitization to HDM (Willart et al. 2012). On the other hand, the role of IL-18 in asthma is controversial because IL-18 is considered an IFNɣ inducing factor. Overexpression of IL-18 in the lung induces both type 1 and type 2 responses in the airway as well as airway inflammation to OVA (Sawada et al. 2013). However, IL-18-/- mice do not show any reduction in allergic inflammation in an OVA-induced asthma model (Hartwig et al. 2008). Finally, IL-33 was

described earlier as an epithelial-derived cytokine that can promote T_H2 allergic responses. IL-33 was shown to be required in many models of allergic disease including HDM-induced allergic airway disease and food allergy to peanuts (Chu et al. 2012; Willart et al. 2012).

TLR/IL1R Signaling and MyD88

Activation of TLRs and IL1Rs induces NF-κB transcription factors which control expression of inflammatory cytokine genes. These transcription factors are kept inactive in the cytosol due to their association with IκB proteins. Activation of NF-κB requires phosphorylation of IκB by the Iκκ complex which results in proteasome-mediated degradation of IκB (Karin and Ben-Neriah 2000). Signaling pathways downstream of TLRs and IL1Rs result in activation of the Iκκ complex and NF-κB. MyD88 is an important adaptor molecule in this signaling pathway. Upon ligand binding to the receptor, MyD88 is recruited and interacts with the cytoplasmic region of the receptor through TIR-TIR domain interactions. Meanwhile, the N-terminal death domain allows MyD88 to interact with the serine/threonine kinase, IRAK4 (Wesche et al. 1997). This interaction leads to phosphorylation of IRAK1 and recruitment of additional adaptor proteins and kinases that ultimately results in activation of the Iκκ complex and NF-κB. It is important to note that TLR3 and TLR4 can activate MyD88-independent pathways in cells. However, TLR4 signaling through MyD88-independent pathway mainly stimulates IFN-inducible genes (Kawai et al. 2001).

GM-CSF

GM-CSF is a 23-kDa glycosylated cytokine that is similar in structure to other growth factors such as IL-3 and IL-5. It was first recognized as a cytokine that promotes differentiation

of both granulocyte and macrophage colonies from mouse bone marrow progenitor cells (Burgess, Camakaris, and Metcalf 1977). GM-CSF binds to a heterodimeric receptor complex that consists of a cytokine-specific α-chain and a common β-chain that is shared with receptors for IL-3 and IL-5. By itself, the α-chain has low affinity for GM-CSF and requires the heterodimeric complex to bind to GM-CSF with high affinity (Gearing et al. 1989). The β-chain is required for signal transduction due to constitutive binding of the tyrosine kinase Jak2 to the cytoplasmic domain. At low concentrations of GM-CSF (0.1-10 pM), phosphorylation of serine^{585} in the β-chain leads to activation of the PI3K/Akt signaling pathway which results in cell survival without proliferation (Guthridge et al. 2006). At high concentrations, phosphorylation of tyrosine^{577} in the β-chain activates the STAT5 signaling pathway which promotes cell survival, proliferation, and activation (Guthridge et al. 2006). Until recently, it was unclear how transphosphorylation of Jak2 occurs upon GM-CSF binding due to the distance between βc dimers (120 Å). A recent study revealed that the GM-CSF receptor complex assembles into a dodecamer, which brings the βc subunits close enough to allow for transphosphorylation and initiation of signal transduction (Hansen et al. 2008).

GM-CSF and Pulmonary Alveolar Proteinosis

GM-CSF is constitutively expressed in the lung and is essential for pulmonary surfactant homeostasis. In humans, absence of GM-CSF signaling due to autoantibodies against GM-CSF or a hereditary mutation in the GM-CSF receptor results in a disease called pulmonary alveolar proteinosis (PAP). In GM-CSF-deficient mice, a similar lung pathology develops in which there is accumulation of excess surfactant in the alveolar space. To maintain the optimal amount of lung surfactant for proper lung function, it is necessary to balance the production, secretion,

reuptake, and clearance of surfactant. Alveolar type II cells and alveolar macrophages are essential in the clearance of surfactant (Huffman et al. 1996). In the absence of GM-CSF, surfactant catabolism in alveolar macrophages is disrupted, leading to the buildup of excess surfactant in the alveoli. Additionally, the effects of GM-CSF extend beyond surfactant catabolism as it also regulates the terminal differentiation, survival, and activation of alveolar macrophages via the transcription factor PU.1.

GM-CSF and Asthma

There is mounting evidence to suggest that GM-CSF plays an important role in the pathogenesis of asthma. In patients with asthma, increased production of GM-CSF is detected at both the RNA and protein level in epithelial cells, bronchoalveolar lavage (BAL), and sputum (Broide and Firestein 1991; Park et al. 1998; Sousa et al. 1993). Furthermore, GM-CSF levels correlate with eosinophilia and disease severity (Woolley et al. 1995). Conversely, treatment with corticosteroids, which improves pulmonary function in asthmatics, also inhibits GM-CSF synthesis, suggesting a role for GM-CSF in mediating allergic inflammation (Cotter et al. 1999; Sousa et al. 1993). These findings were further supported by studies reporting that genetic polymorphisms in the GM-CSF gene are associated with an increased risk for asthma and atopy (He et al. 2003; Kabesch et al. 2007).

In mice, overexpression of GM-CSF in the lung permits allergic sensitization to an otherwise innocuous antigen, ovalbumin (Stämpfli et al. 1998). Additionally, overexpression of GM-CSF in the lung substantially reduces the dose of HDM allergen necessary for the development of allergic inflammation (Llop-Guevara et al. 2014). These results suggest that increased levels of GM-CSF in the lung enhances the risk of allergic sensitization. Interestingly,

upregulation of GM-CSF occurs in response to a wide variety of factors such as cigarette smoke, diesel exhaust particles, allergens, viruses, and fungi (Ohta et al. 1999; Schelenz, Smith, and Bancroft 1999; Subauste 1995; Trimble et al. 2009). As a result, GM-CSF is required for allergic inflammation in mice exposed to cigarette smoke and ovalbumin (Trimble et al. 2009). Similarly, blocking GM-CSF reduces AHR caused by exposure to diesel exhaust particles (Ohta et al. 1999). In response to allergens, neutralizing GM-CSF prior to sensitization with house dust mite and *Blomia tropicalis* dust mite allergen significantly reduces allergic inflammation after allergen challenge (Willart et al. 2012; Zhou et al. 2014). The role of GM-CSF in allergic sensitization is limited to these studies with mite allergens. It is unknown if GM-CSF is induced by other allergens such as CRA and whether it is required for sensitization to other allergens. In addition to playing a role during sensitization, GM-CSF might also be required during the challenge phase. In mice that were sensitized with OVA and alum intraperitoneally, blocking GM-CSF before challenging mice with OVA reduces allergic airway inflammation. These studies have implications for the design of asthma therapeutics that can target GM-CSF.

GM-CSF and Dendritic Cells

GM-CSF can efficiently promote the development of dendritic cells (DCs) in vitro and is commonly used to grow dendritic cells from human monocytes and bone marrow precursor cells (Inaba et al. 1992). However, there is only a slight reduction in the development of DCs in lymphoid organs of GM-CSF deficient mice (Vremec et al. 1997). In the lung, absence of GM-CSF signaling dramatically reduces the number of $CD103^+$ DCs and to a lesser extent, the number of $CD11b^+$ DCs (Greter et al. 2012). These results demonstrate that during homeostasis, GM-CSF controls the development of DCs in the lung.

Under inflammatory conditions, GM-CSF promotes the maturation of dendritic cells and induces upregulation of costimulatory molecules as potently as TLR agonists (Min et al. 2010). Blocking GM-CSF during allergic sensitization reduces the number of DCs that carry antigen to the draining lymph node and prevents upregulation of costimulatory molecules (Willart et al. 2012). This has direct effects on the ability of DCs to prime T cells since DCs from anti-GM-CSF treated mice show reduced ability to prime T_H2 cells (Zhou et al. 2014).

In response to immune stimuli, GM-CSF is produced by both hematopoietic and non-hematopoietic cells such as lymphocytes (Broide and Firestein 1991), alveolar macrophages (Broide and Firestein 1991), eosinophils (Gauvreau et al. 1998), airway epithelial cells (Sousa et al. 1993), and fibroblasts (Fitzgerald et al. 2003). During allergic sensitization, airway epithelial cells are believed to be the main source of GM-CSF but this has not been proven definitively in vivo. However, it is easy to speculate that crosstalk between airway epithelial cells and dendritic cells in the form of GM-CSF might regulate allergic sensitization.

Summary

Type 2 responses are triggered by diverse stimuli such as helminths, allergens, venoms, and vaccine adjuvants. The focus of my thesis is to understand how type 2 responses are initiated in response to environmental allergens. Due to the heterogeneity in the pathways that lead to type 2 immune responses, the cellular and molecular networks that orchestrate these responses are not well understood. Specifically, there is a need to better understand the mechanisms that promote sensitization to specific allergens versus mechanisms that are employed by all allergens. In particular, we are interested in cockroach allergen, due to its

association with the development and exacerbation of allergic asthma. Despite its clinical importance, little is known about the initiation of type 2 responses to cockroach allergen. In this thesis, we explore the role of immune cells and airway epithelial cells during allergic sensitization to cockroach allergen. In addition, we identify the molecular mediators that are induced in response to cockroach allergen and address the role of epithelial-derived cytokines in promoting allergic sensitization to cockroach allergen.

Chapter 2: Airway epithelial cells promote allergic sensitization to cockroach allergen through GM-CSF production

Introduction

Allergic asthma is a chronic lung disease characterized by reversible airflow obstruction and mucus hypersecretion (Wenzel 2012). The underlying cause is an abnormal immune response directed against harmless allergens that results in infiltration of eosinophils and T_H2 cells into the airways (Wenzel 2012). Over the past 50 years, the global prevalence of asthma has steadily risen, and there is increasing evidence that environmental factors contribute to this alarming rise in asthma cases. In particular, exposure to cockroach allergen (CRA) is a major risk factor for the development and exacerbation of allergic asthma. As one of the most potent allergens associated with asthma, sensitization to CRA occurs at levels of exposure that are 10-100 fold lower than dust mite and cat allergens (Eggleston et al. 1998; Olmedo et al. 2011; Sporik et al. 1999). Furthermore, the indoor level of CRA is significantly associated with the occurrence of asthma and recurrent wheeze among asthmatic children (Gold et al. 1999; Litonjua et al. 2001). Unfortunately, children who are both allergic to CRA and exposed to high levels of this allergen also have a higher rate of hospitalization and unscheduled medical visits than children allergic to dust mite or cat dander (Rosenstreich et al. 1997).

Despite the importance of CRA as a clinically relevant allergen, little is known about the cellular and molecular mechanisms involved in sensitization to CRA. Sensitization to CRA occurs through inhalation of airborne particles composed of debris from cockroach secretions, feces, and exoskeletons (De Lucca et al. 1999). As a complex mixture of proteins and lipids,

CRA extract contains proteases that can disrupt the integrity of the airway epithelium and activate protease-activated receptor 2 on a wide variety of cells (PAR-2) (Arizmendi et al. 2011; Jeong et al. 2008b; Kondo et al. 2004; Lewkowich et al. 2011; Ossovskaya and Bunnett 2004). The role of proteases in the immune response to CRA has been the focus of many studies, and several studies have demonstrated that PAR-2 mediates allergic sensitization to CRA (Arizmendi et al. 2011; Page et al. 2010). CRA also contains ligands that can bind and activate toll-like receptors (TLRs). Specifically, CRA can activate TLR2 on neutrophils to induce the release of cytokines (Page et al. 2008, 2009). In addition, CRA contains low levels of lipopolysaccharide (LPS) that can directly activate TLR4 and induce the release of high mobility group box 1 (HMGB1), an endogenous TLR4 ligand, (Ullah et al. 2014; Yang et al. 2015). Although these studies improve our understanding of the receptors that recognize CRA, the downstream mechanisms leading to allergic sensitization remain largely unknown.

During allergic sensitization, DCs prime allergen-specific naïve T cells to induce T_H2 cell differentiation in the draining lymph node. However, the molecular mechanisms that determine T_H2 cell polarization by DCs have not been well-defined. In recent years, it has become increasingly clear that the local cytokine milieu influences the function of DCs. Specifically, the interplay between epithelial cells and DCs plays an important role in directing the immune response to allergens. Airway epithelial cells are constantly sampling the inhaled environment and are equipped with an arsenal of receptors that recognize allergens. Upon allergen stimulation, activated airway epithelial cells produce and release chemokines that recruit immune cells into the lungs. In response to allergens, airway epithelial cells also produce cytokines that promote T_H2 immunity such as TSLP, IL-25, and IL-33. However, different allergens elicit

different epithelial-derived cytokines, suggesting that there are a number of diverse pathways that lead to T_H2 immunity. Factors that are uniquely elicited by individual allergens versus common to allergic sensitization in general remain to be elucidated.

To determine the role of airway epithelial cells in mucosal sensitization to CRA, we established a model of CRA-driven allergic airway disease. Mice were sensitized and challenged mice with CRA intranasally, resulting in the development of airway inflammation with hallmark features of asthma. Using Cre-Lox technology to selectively delete MyD88 in specific cell populations, we showed that MyD88 signaling was required in airway epithelial cells, but not immune cells, for allergic sensitization to CRA. CRA stimulation of airway epithelial cells directly induced production of GM-CSF in a MyD88-dependent manner. Among the epithelial-derived cytokines, we demonstrated that GM-CSF, but not TSLP or IL-25, is integral to the initiation of T_H2 allergic response to CRA. We found that epithelial cell-derived GM-CSF promoted DC function and T_H2 priming at sensitization. Overall, we report mechanistic detail describing how allergic sensitization to CRA is regulated by the interplay between epithelial cells and dendritic cells.

Materials and Methods

Mice. C57BL/6 mice were purchased from Jackson or Charles River Laboratories. TLR2-/- mice were provided by John Gebe (Benaroya Research Institute). $TLR4^{lps\text{-}del}$ mice on the C57BL/6 background were obtained from The Jackson Laboratory; MyD88-/- mice were provided by Tobias Hohl (Memorial Sloan Kettering Cancer Center); $MyD88^{flox/flox}$ mice were provided by Mohamed Oukka (Seattle Children's Hospital); Casp1-/- mice were provided by

Michael Gale (University of Washington); IL-33-/- mice were provided by Dirk Smith (Amgen); SPC-Cre mice were provided by Brigid Hogan (Duke University); IL17RB-/- mice were provided by Xiaoxia Li (Cleveland Clinic Lerner Research Institute). All animals were housed in specific pathogen-free conditions in the Benaroya Research Institute animal facility and all experiments were approved by the Benaroya Research Institute Animal Care and Use Committee.

Reagents. Lyophilized whole body German cockroach extract was obtained from Greer laboratories and resuspended in PBS to a concentration of 2 mg/ml. Low endotoxin ovalbumin was purchased from Worthington Biochemical Corporation (LS003061). LPS from E coli 026:B6 was purchased from Sigma (L2654). Human IL-1ra (anakinra) was purchased from the Virginia Mason hospital pharmacy. Recombinant GM-CSF was purchased from Peprotech (315-03).

Cockroach allergen induced allergic airway model. On days 0-2, mice were anesthetized with isoflurane and sensitized intranasally with 40 μg of CRA (Greer, B46) in a total volume of 20 μl PBS. After 15 days, mice were challenged intranasally with 40 μg of CRA on two consecutive days and airway inflammation was assessed 72 hours later. Control mice were treated with PBS only, under the same protocol. For neutralization experiments, anti-GM-CSF antibody (Clone MP1-22E9, Biolegend) was injected 5 hours before the first sensitization or challenge dose. 120 μg of antibody was administered i.p. and 30 μg of antibody was administered intranasally. Anakinra, IL-1ra, was i.p. administered at 25 mg/kg with each dose of CRA for a total of five times. To deplete CD4 T cells, 150 μg of anti-CD4 antibody (GK1.5) was i.p. administered on

day -1, 2, and 16. To determine if GM-CSF can recover T_H2 allergic inflammation in MyD88-/- mice, mice were intranasally treated with 50 ng of GM-CSF plus CRA during sensitization and were only challenged with CRA.

Evaluation of airway inflammation. Mice were euthanized by i.p. injection with 1 ml of 2.5% Avertin. Bronchoalveolar lavage (BAL) was performed four times, each time with 1 ml of PBS. The first ml of BAL fluid was collected for ELISA. Next, BAL was centrifuged at 250 x g for 5 min to collect the cell pellet. Cells were resuspended in FACS buffer and counted using a hemocytometer. Differential cell counts were performed by flow cytometry.

After BAL, lungs were perfused by injecting 3 ml of PBS into the right ventricle of the heart until the lungs turned white. The right upper lobe of the lung was placed in 1 ml of RNALater and stored at -80°C for later analysis by qPCR. The remaining lung tissue was fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned, and stained with H&E and periodic acid-Schiff. Serum was collected and analyzed for CRA-specific antibodies by ELISA.

To determine differential cell counts in the lung, single cell suspensions were prepared by cutting the lung into fine pieces using a scissor. Next, lung tissue was digested in 50 μg/ml of Liberase TM (Roche) and 10 U/ml of DNase I in RPMI-1640 at 37°C for 30 min. Digested lung samples were filtered through a 100-μm cell strainer and washed with equal volumes of RPMI plus 20% FBS. Red blood cells were lysed with ACK lysis buffer and lung cells were centrifuged at 250 x g for 5 min. Cells were resuspended in FACS buffer and counted using a hemocytometer. Differential cell counts were performed by flow cytometry.

A647-CRA and Migration of DCs. A647 was conjugated to CRA using the Alexa Fluor 647 microscale protein labeling kit per manufacturer's instructions (Life Technologies). DC uptake of A647-CRA and migration to the draining lymph node was assessed by administering 11 μg of A647-CRA plus 39 μg of unconjugated CRA intranasally. Twenty four hours later, lung draining lymph node was collected and single cell suspension was prepared. Lymph nodes were teased apart using needles and digested in 1 ml of digest solution containing 50 μg/ml of Liberase TM (Roche) and 10 U/ml of DNase I in RPMI-1640. After digesting for 25 minutes at 37°C, 100 μl of 0.1 M EDTA was added to each sample and digested for five additional minutes at 37°C. Next, digested lymph nodes were crushed with a 1 ml syringe and filtered through a 100-μM cell filter. Cells were resuspended in FACS buffer and analyzed by flow cytometry.

Evaluation of sensitization to CRA. On days 0-2, mice were sensitized with 40 μg of CRA intranasally. On day 3, lung draining lymph nodes were collected and single cell suspensions were prepared as described above. Cells were plated in a 96-well flat bottom plate at $5x10^6$ cells/ml and cultured with 0 or 10 μg/ml of CRA. The cell culture supernatant was collected on day 7 and cytokine levels were analyzed by ELISA.

Bone marrow chimeras. Mice were irradiated twice with 4.5 Gy and at least 2×10^6 bone marrow cells were transferred i.v. by retro-orbital injection. Mice were used for experiments after 8-10 weeks to allow for bone marrow reconstitution.

Flow Cytometry. To prevent nonspecific binding of antibodies, cells were incubated with anti-CD16/CD32 (Clone: 2.4G2) in FACS buffer for 15 minutes at room temperature. The following antibodies were purchased: anti-Ly6G FITC (1A8), anti-IFNɣ FITC (XMG1.2), anti-Ly6C

PerCpCy5.5 (HK1.4), anti-CD103 Pacific Blue (2E7), anti-Foxp3 e450 (FJK-16s), anti-CD4 BV605 (RM4-5), anti-CD80 BV605 (16-10A1), anti-CD45R BV650 (RA3-6B2), anti-IL-5 APC (TRFK5), anti-MHCII A700 (M5/114.15.2), anti-CD44 A700 (IM7), anti-CD11b APC-e780 (M1/70), anti-TCRβ APC-e780 (H57-597), anti-Siglec F PE (E50-2440), anti-IL-13 PE (eBIo13A), anti-CD3 PECy5 (145-2C11), anti-CD11c PE-Cy7 (N418), anti-IL-4 PECy7 (BVD6-24G2). Cells were stained with surface antibodies for 12 minutes at room temperature. For intracellular staining of cytokines, cells were fixed, permeabilized, and stained using the Foxp3 fix/perm buffer set (Biolegend) according to manufacturer's instructions. Samples were analyzed with BD LSR II flow cytometer (BD Biosciences).

Real-time PCR. Lung tissues were homogenized in RNA lysis buffer and RNA was isolated using the Nucleospin RNA kit (Clontech). RNA was reverse-transcribed into cDNA using PrimeScript Reverse Transcriptase (Takara) according to the manufacturer's instructions. cDNA was amplified in a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR *Premix Ex Taq* II (Takara). The following primer pairs were used:

Air-liquid interface cultures. Primary airway epithelial cells were cultured as previously described (You et al. 2002). Mice were euthanized by CO_2 and the trachea was collected and digested in 0.2% pronase at 4°C overnight. Mouse tracheal epithelial cells (MTEC) were isolated and cultured on collagen-coated transwells. After one week, MTEC cultures were transitioned to air-liquid interface (ALI) by removing medium from the upper compartment and maintained at ALI for 2 weeks. Cells were exposed to 15 μg of CRA for 24 hours and medium

from the basal compartment was collected for ELISA. RNA was isolated from cells that were lysed with RNA lysis buffer.

ELISA. To detect CRA-specific IgE, plates were coated overnight with 6.25 μg/ml CRA in PBS at 4°C. Plates were washed and blocked with 1% BSA in PBS for 2 hours at room temperature. Serum samples were added to the plates and incubated for 2 hours at room temperature. Plates were washed and biotin-conjugated anti-mouse IgE was added at 1:250 dilution and incubated for 2 hours at room temperature. Plates were washed and incubated with streptavidin-HRP for 30 minutes. 1-Step Ultra TMB-ELISA substrate (Pierce) was added and incubated at room temperature in the dark. The reaction was stopped with 2 N H_2SO_4 and OD was measured at 450 nm.

To detect cytokines, the following capture and detection antibody pairs were used: anti-IL-4 Purified (11B11) and anti-IL-4 Biotin (BVD6-24G2), anti-IL-5 Purified (TRFK5) and anti-IL-5 Biotin (TRFK4), anti-IL-13 Purified (eBio13A) and anti-IL-13 Biotin (eBio1316H), anti-IL-17A Purified (eBio17CK15A5) and anti-IL-17A Biotin (eBio17B7), anti-GM-CSF Purified (MP1-22E9) and anti-GM-CSF Biotin (MP1-3G6). The concentration of cytokines in the BAL fluid were detected using these antibodies and the protocol described above.

Statistics. Statistical analysis was performed using GraphPad Prism 5. Comparisons were made using either unpaired Student's *t* test or one-way ANOVA with a Tukey posthoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Intranasal sensitization and challenge with CRA results in airway inflammation with features of allergic asthma.

To study the mechanism of allergic sensitization to CRA, we established a model of allergic airway disease in which mice were intranasally sensitized and challenged with CRA, in the absence of adjuvants. After CRA challenge, mice developed airway inflammation with eosinophils representing the dominant infiltrating cell type, with recruitment of CD4 T cells, DCs, and neutrophils as well (Figure 2.1, A). Consistent with an allergic response, histological analysis of lung sections showed extensive mucus production by airway goblet cells (Figure 2.1, B). In addition, we detected elevated levels of CRA-specific IgE antibodies in the serum (Figure 2.1, C). Analysis of T cell responses revealed increased lung mRNA expression of T_H2 and T_H17 cytokines, whereas IFNɣ was not upregulated (Figure 2.1, D). To determine the role of CD4 T cells in the response, mice were treated with the depleting anti-CD4 antibody (GK1.5). In the absence of CD4 T cells, mice failed to develop features of allergic inflammation in response to CRA (Figure 2.2). These results demonstrate that sensitization and challenge with CRA leads to the development of airway inflammation that closely resembles allergic asthma.

MyD88 is required for the development of allergic airway inflammation to CRA.

Previous work had suggested that toll-like receptors (TLRs) may play a role in the response to CRA in the airways (Arizmendi et al. 2011; P. Gao and Grigoryev 2010; Natarajan et al. 2011; Page et al. 2008, 2011). To assess their role in this model, *Myd88*-/- mice were sensitized and challenged with CRA as described above. Following CRA challenge, *Myd88*-/-

mice developed markedly less airway inflammation compared to WT mice. The numbers of eosinophils and CD4 T cells were strongly reduced in the BAL, and histological analysis confirmed this reduction in immune cell infiltrates and revealed less mucus production in the lung (Figure 2.3, A-B). Furthermore, T_H2 cytokine levels were significantly lower in the BAL fluid of *Myd88*-/- mice (Figure 2.3, C). When we investigated the role of individual TLRs in sensing CRA, we found no significant differences in airway eosinophilia between WT and *Tlr2*-/- mice (Figure 2.4). On the other hand, TLR4-deficient mice ($Tlr4^{lps\text{-}del}$ mice) exhibited significantly reduced airway eosinophilia and T_H2 cytokine production, similar to *Myd88-/-* mice (Figure 2.5). As LPS is a TLR4 agonist that can promote T_H2 responses at low doses (Eisenbarth et al. 2002), we asked if the low level of LPS (11 ng) in CRA was sufficient to drive sensitization to CRA. In contrast to CRA-treated mice, mice sensitized and challenged with 11 ng of LPS plus ovalbumin failed to develop airway eosinophilia (Figure 2.6). This suggests that, while LPS might contribute, it alone does not drive sensitization to CRA and supports a role for other components of CRA in the development of airway inflammation

In addition to TLRs, MyD88 is also required for signaling pathways downstream of the IL-1 receptor family. Members of the IL-1 cytokine family, such as IL-1α and IL-33, have been shown to be important for sensitization to some allergens (Willart et al. 2012). However, the role of IL-1 cytokines in allergic airway response to CRA remains unknown. Blocking IL1R signaling with IL-1ra (Anakinra) during sensitization and challenge with CRA resulted in airway eosinophilia similar to that of the control group, demonstrating that IL-1α and IL-1β are not required (Figure 2.7, A). Next, we compared WT and *casp1-/-* mice to determine the role of IL-18 and IL-1β. Caspase 1 is required for processing pro-IL-1β and pro-IL-18 into their active

forms. We found no significant differences in eosinophil numbers between WT and *casp1*-/- mice after CRA challenge (Figure 2.7, B). However, when IL-33-deficient mice were sensitized and challenged with CRA, we found significantly less airway eosinophilia compared with WT mice, but the numbers of CD4 T cells, DCs, and neutrophils remained elevated (Figure 2.8). These results show that among the IL-1 cytokines, only IL-33 played a role in CRA-induced airway eosinophilia.

MyD88 is required in airway epithelial cells for allergic inflammation to CRA.

MyD88 is expressed broadly, in both immune and structural cells. To determine which of these cellular compartments were responsible for MyD88-dependent responses to CRA, we established reciprocal bone marrow (BM) chimeras in which WT or *Myd88*-/- BM cells were transferred into irradiated WT or *Myd88*-/- hosts. WT → WT BM chimeric mice developed a robust allergic response to CRA. In contrast, *Myd88*-/- → *Myd88*-/- BM chimeric mice developed reduced airway eosinophilia, which confirmed the critical role of MyD88 in CRA-induced allergic inflammation (Figure 2.9). A similar reduction in airway eosinophilia was observed when MyD88 was absent in structural cells (WT → *Myd88*-/-). In contrast, absence of MyD88 in the radiosensitive, hematopoietic compartment (*Myd88*-/- → WT) did not affect the degree of allergic inflammation to CRA (Figure 2.9). These results indicate that MyD88 expression is crucial in lung radioresistant cells for coordinating a T_H2 allergic response against CRA.

MyD88 is expressed by various structural cells such as epithelial cells and fibroblasts. As airway epithelial cells are uniquely positioned to sense allergens and can initiate T_H2 allergic responses, we hypothesized that MyD88 expression is required in this population to promote

responses to CRA. We tested this hypothesis in mice lacking MyD88 specifically in airway epithelial cells. SPC-Cre/*Myd88*$^{fl/fl}$ mice express Cre recombinase under the control of the surfactant protein C promoter, which results in specific deletion of MyD88 in distal airway epithelial cells (Okubo and Hogan 2004). When SPC-Cre/*Myd88*$^{fl/fl}$ mice were sensitized and challenged with CRA, they developed significantly reduced airway eosinophilia (Figure 2.10, A). In addition, restimulation of cells isolated from the lungs of these mice revealed a significant decrease in the percentage and number of IL-5^{+} and IL-13^{+} CD4 T cells (Figure 2.10, B). On the other hand, deletion of MyD88 in immune cells (CD4-Cre/*Myd88*$^{fl/fl}$ and CD11c-Cre/*Myd88*$^{fl/fl}$) had no effect on the degree of airway inflammation following sensitization and challenge with CRA (Figure 2.11). Overall, these results demonstrate that MyD88 signaling in airway epithelial cells is crucial for CRA-driven allergic airway disease.

GM-CSF is produced by airway epithelial cells in a MyD88 dependent manner.

Following allergen exposure, airway epithelial cells can initiate and regulate T_H2 responses through production of cytokines such as TSLP, IL-25, and IL-33. Although we found a role for IL-33 in CRA-driven airway eosinophilia, we were interested in investigating pathways that were downstream of MyD88 signaling in airway epithelial cells. However, we found no significant differences in CRA-induced airway eosinophilia between WT, *Tslp-/-*, and *Il17rb-/-* mice (Figure 2.12).

Among the additional epithelial-derived cytokines that were tested, we found an increase in GM-CSF expression resulting in elevated BAL levels as early as two hours post-sensitization, which returned to baseline by 24 hours (Figure 2.13, A). To assess if upregulation of GM-CSF was dependent on MyD88 expression in airway epithelial cells, we analyzed the expression of

GM-CSF in SPC-Cre/*Myd88*$^{fl/fl}$ mice after sensitization with CRA. Similar to WT mice, GM-CSF expression was increased in *Myd88*$^{fl/fl}$ mice, whereas SPC-Cre/*Myd88*$^{fl/fl}$ mice failed to upregulate GM-CSF in response to CRA (Figure 2.13, B).

To confirm that GM-CSF was produced by airway epithelial cells in response to CRA, we established organotypic air-liquid interface (ALI) cultures of primary epithelial cells from WT and *Myd88*-/- tracheas. When WT ALI cultures were stimulated with CRA, there was a significant increase in GM-CSF levels in the culture medium on the basolateral side (Fig. 4C). In contrast, ALI cultures established from *Myd88*-/- mice failed to upregulate GM-CSF after stimulation with CRA (Figure 2.13, C). These results demonstrate that airway epithelial cells produce GM-CSF in a MyD88-dependent manner following exposure to CRA.

GM-CSF is required during allergic sensitization, but not challenge, in CRA-driven allergic airway disease.

GM-CSF plays an important role in the development of allergic inflammation to mite allergens (Willart et al. 2012; Zhou et al. 2014). To investigate the role of GM-CSF in regulating CRA-driven allergic airway disease, mice were treated with neutralizing αGM-CSF antibody at either sensitization or challenge. Blocking GM-CSF at sensitization strongly reduced airway inflammation after CRA challenge, with eosinophil numbers in the BAL significantly lower by 88% compared to isotype control treatment (Figure 2.14, A). There was also a reduction in the number of CD4 T cells, DCs, and neutrophils (Figure 2.14, A). Furthermore, mRNA expression of T_H2 cytokines and serum CRA-specific IgE levels were also reduced (Figure 2.14, B-D). In contrast, blocking GM-CSF at challenge failed to reduce airway eosinophilia in mice sensitized

and challenged with CRA (Figure 2.15). These results reveal a crucial role for GM-CSF during sensitization, but not challenge, in CRA-induced allergic airway disease.

GM-CSF is required for optimal DC function and T cell priming.

Given that the allergic response to CRA is CD4 T cell driven, we postulated that GM-CSF played a role in DC function during priming. To explore this possibility, we asked how neutralization of GM-CSF affected the function of DCs in the lung draining lymph node. Mice were sensitized with fluorescently-labeled CRA in the presence or absence of the blocking GM-CSF antibody, and the number of CRA+ DCs in the draining lymph node was determined by flow cytometry. Neutralization of GM-CSF significantly reduced the delivery of fluorescently labelled CRA to the draining lymph node. There was a reduction in both the percentage and total number of A647-CRA^{+} DCs in the lung-draining lymph node 24 hours after CRA administration (Figure 2.16, A). Additionally, surface expression of the costimulatory molecule CD80 was markedly decreased on A647^{+} DCs (Figure 2.16, B). These findings suggest that GM-CSF regulates the ability of DCs to acquire antigen, upregulate costimulatory molecules, and migrate to the draining LN during sensitization. To examine if GM-CSF blockade affected the ability of DCs to prime T_H2 cells, we sensitized mice with CRA and collected lung-draining lymph node cells on day 3. When these cells were restimulated with CRA, there was a significant reduction in the production of T_H2 cytokines from αGM-CSF treated mice (Figure 2.16, C). Overall, these results demonstrate that GM-CSF is required for optimal function of DCs and T cell priming during CRA sensitization.

GM-CSF rescues T_H2 allergic airway response to CRA in Myd88-/- mice.

To determine the effects of GM-CSF downstream of MyD88 signaling, we intranasally administered GM-CSF to *Myd88*-/- mice during CRA sensitization. Treatment with GM-CSF largely restored airway inflammation in *Myd88-/-* mice to levels observed in WT mice. This was due to a significant increase in the number of eosinophils and CD4 T cells, whereas recruitment of neutrophils to the BAL was not recovered by GM-CSF treatment (Figure 2.17, A). Furthermore, GM-CSF selectively restored T_H2, but not T_H17 responses, in *Myd88*-/- mice. In vitro restimulation of lung cells with PMA/ionomycin revealed a significant increase in the number of IL-5^+ and IL-13^+ CD4 T cells, but not IL-$17A^+$ or IFNγ^+ CD4 T cells (Figure 2.17, D). Consistent with the recovery of T_H2 responses, CRA-specific IgE levels were also restored by GM-CSF treatment (Figure 2.17, C). These results demonstrate that GM-CSF, downstream of MyD88 signaling in airway epithelial cells, specifically promotes T_H2 eosinophilic inflammation following exposure to CRA.

Discussion

The factors that control T_H2 responses to allergens in the airways remain elusive. In this study, we show that intranasal sensitization and challenge with CRA leads to the development of allergic airway disease with hallmark features of allergic asthma. During sensitization, CRA-stimulated airway epithelial cells displayed a marked increase in GM-CSF expression that was dependent on MyD88 signaling. Loss of GM-CSF, either via antibody blockade or MyD88 deficiency, reduced allergic response to CRA, while providing GM-CSF specifically promoted T_H2-mediated eosinophilia in the absence of MyD88. We determined that epithelial cell-derived

GM-CSF promoted sensitization to CRA by enhancing DC maturation and T_H2 priming. These results demonstrate that airway epithelial cells regulate allergic sensitization to CRA through production of GM-CSF.

In response to CRA, cytokine production by BMDCs requires MyD88, an essential adaptor molecule in the TLR signaling pathway (Page et al. 2011). Although this suggests a role for TLRs in the recognition of CRA, the role of MyD88 in CRA-driven allergic airway disease has not been directly examined. In this study, we show that both MyD88 and TLR4 were required for the development of an allergic airway response to CRA. Because stimulation of TLR4 with low levels of LPS can induce T_H2 responses against ovalbumin (Eisenbarth et al. 2002), we examined the role of LPS in this TLR4-mediated allergic response. We found that the amount of LPS in CRA was 10 fold lower than the dose of LPS necessary to promote T_H2 responses. As a result, we showed that the low level of LPS in CRA was not sufficient to drive a T_H2 allergic response. These results demonstrate that, while LPS might contribute, it alone does not drive sensitization to CRA. In fact, it raises the possibility that other components of CRA are involved in promoting allergic sensitization.

Bla g 1 is a major cockroach allergen that is secreted in the digestive tract and found in fecal particles. Recently, the structure of Bla g 1 was revealed to contain a hydrophobic cavity that can bind lipids (Mueller et al. 2013). Many of the lipids associated with Bla g 1 are known to activate TLR2 and TLR4 (Mueller et al. 2013). Thus, Bla g 1 might enhance allergic sensitization by facilitating lipid binding to TLRs. One possibility is that Bla g 1 promotes LPS binding to TLR4 and this interaction lowers the threshold of LPS required to generate T_H2 allergic responses. This ability to bind lipids is also shared by major allergens from pollen, fruit,

house dust mite, cat, and dog, and suggests that binding lipids might be a common mechanism employed by allergens to induce sensitization (Bublin, Eiwegger, and Breiteneder 2014).

Next, we investigated how activation of MyD88 results in CRA sensitization, starting with the cell types involved. MyD88 is not only expressed in hematopoietic cells such as DCs, T cells, and eosinophils, but also on structural cells such as fibroblasts and epithelial cells. Bone marrow chimera experiments showed that MyD88 expression in radioresistant structural cells was required and sufficient for allergic airway response to CRA. These results are consistent with studies on house dust mite (HDM) where TLR4 expression is also required in lung structural cells for allergic inflammation (Hammad et al. 2009; Tan et al. 2010). However, in addition to structural cells, there are radioresistant hematopoietic cells, such as alveolar macrophages and mast cells that could potentially play a role in the response to CRA. To test the hypothesis that MyD88 signaling in airway epithelial cells is crucial for allergic sensitization, we used the Cre/lox system to selectively delete MyD88 in airway epithelial cells. We showed that mice lacking MyD88 in airway epithelial cells, but not in $CD11c^+$ or $CD4^+$ cells, developed significantly reduced CRA-driven allergic disease. Our findings demonstrate that MyD88 signaling in airway epithelial cells is critical for the initiation of allergic responses against CRA.

It is commonly believed that airway epithelial cells promote T_H2 immunity at mucosal surfaces by producing cytokines such as TSLP, IL-25, and IL-33. However, allergens can initiate T_H2 responses through different pathways and the requirement for individual cytokines depends on the specific allergen-driven model. For allergic airway response to CRA, we found that TSLP and IL-25 were not required for sensitization. These findings are consistent with a recent study showing that absence of TSLP does not impair the development of CRA-induced

allergic response (Jang, Morris, and Lukacs 2013). On the other hand, we found that IL-33 was only required for certain aspects of the response, such as accumulation of eosinophils in the airway after CRA challenge. These results differ from studies in which IL-33 was reported to be required for the complete T_H2 allergic response to HDM and peanut, rather than just the recruitment of eosinophils (Chu et al. 2012; Willart et al. 2012). Given that IL-33 deficient mice did not fully recapitulate the loss of inflammation seen in *Myd88*-/- mice, we predict that IL-33 might play a role during allergen challenge. IL-33 can act directly on eosinophils to promote their survival, as well as indirectly through IL-5-induction from ILC2s (Cherry et al. 2008; Gorski, Hahn, and Braciale 2013; Molofsky et al. 2013; Stolarski et al. 2010). Additional studies are required to investigate if these mechanisms are involved in IL-33 dependent airway eosinophilia during allergic airway response to CRA.

As we continued to investigate the role of airway epithelial cells during sensitization, we found that airway epithelial cells produced GM-CSF in response to CRA. Although GM-CSF is produced by a variety of cell types, it has generally not been considered to be an epithelial-derived cytokine. Nonetheless, we show that MyD88 deletion in airway epithelial cells abrogated upregulation of GM-CSF after stimulation with CRA, both in vitro and in vivo. These results led to the hypothesis that airway epithelial cells promote allergic sensitization to CRA by producing GM-CSF.

In asthmatics, increased expression of GM-CSF is consistently observed in airway epithelial cells as well as lymphocytes and alveolar macrophages in the BAL (Broide D and Firestein 1991; Park et al. 1998; Sousa et al. 1993). Studies have identified several polymorphisms in the GM-CSF gene (*CSF2*) as risk factors for the development of asthma and

atopy (He et al. 2003; Kabesch et al. 2007). In mouse models of allergic airway disease, neutralization of GM-CSF reduces airway inflammation to HDM and *Blomia Tropicalis* allergens (Willart et al. 2012; Zhou et al. 2014). However, aside from mite allergens, it is unclear if GM-CSF plays a role in allergic sensitization to other allergens. In this study, we show that GM-CSF was required for allergic sensitization to CRA. Blockade of GM-CSF at sensitization significantly reduced airway inflammation upon allergen challenge. Our results support the idea that multiple allergens might elicit GM-CSF as a common pathway of allergic sensitization and additional studies are needed to examine this possibility.

In addition to allergens, GM-CSF is produced in response to viruses, fungi, cigarette smoke, and diesel exhaust particles (Ohta et al. 1999; Schelenz, Smith, and Bancroft 1999; Subauste 1995; Trimble et al. 2009). Enhanced levels of GM-CSF in the lung due to these environmental stimuli could increase the overall risk of allergic sensitization. In fact, multiple studies have reported that overexpression of GM-CSF in the lung can promote sensitization to ovalbumin and sub-threshold levels of HDM (Llop-Guevara et al. 2014; Stämpfli et al. 1998). As a result, GM-CSF might play a more pervasive role in allergic sensitization than previously thought.

To study the role of GM-CSF during CRA sensitization, we asked how blockade of GM-CSF affected functional maturation of DCs and T_H2 priming at sensitization. As a growth factor, GM-CSF regulates the development and maturation of DCs. In this study, we show that GM-CSF was required for delivery of antigen to the draining lymph-node and upregulation of costimulatory molecules. These findings are consistent with a study reporting that neutralization of GM-CSF reduced the migration of DCs to the draining lymph node and expression of

maturation markers after administration with IL-1α plus OVA (Willart et al. 2012). In contrast, blockade of GM-CSF did not affect the migration of DCs or their costimulatory molecule expression during sensitization to Blomia Tropicalis mite allergen (Zhou et al. 2014). Additional studies are necessary to show that GM-CSF directly targets DCs during CRA sensitization. Ultimately, we found that blockade of GM-CSF significantly reduced T_H2 priming which decreased airway inflammation after CRA challenge. GM-CSF can also enhance the function and survival of eosinophils, which accounts for 70% of the cells in the BAL during allergen challenge. However, it is unlikely that GM-CSF is required for the survival of eosinophils because blockade of GM-CSF at challenge did not reduce airway inflammation. This might be due to overlapping roles for GM-CSF and IL-5 during allergen challenge since IL-5 can also promote the survival of eosinophils.

Finally, we provide evidence that GM-CSF acts downstream of the MyD88 signaling pathway to promote allergic sensitization. We show that intranasal delivery of GM-CSF during sensitization recovered T_H2-mediated airway eosinophilia in the absence of MyD88. Surprisingly, GM-CSF failed to rescue both T_H17 responses and neutrophilia in the absence of MyD88. These findings suggest that there are distinct pathways that control the recruitment of eosinophils versus neutrophils during allergic airway disease, likely due to differential regulation of T_H2 versus T_H17 responses. Furthermore, these results reveal a crucial role for GM-CSF in specifically promoting the T_H2 arm of the immune system. Additional studies are necessary to identify the pathways that control neutrophilia. These results suggest that therapeutics can be tailored to specifically target eosinophilic vs. neutrophilic asthma by blocking these different pathways.

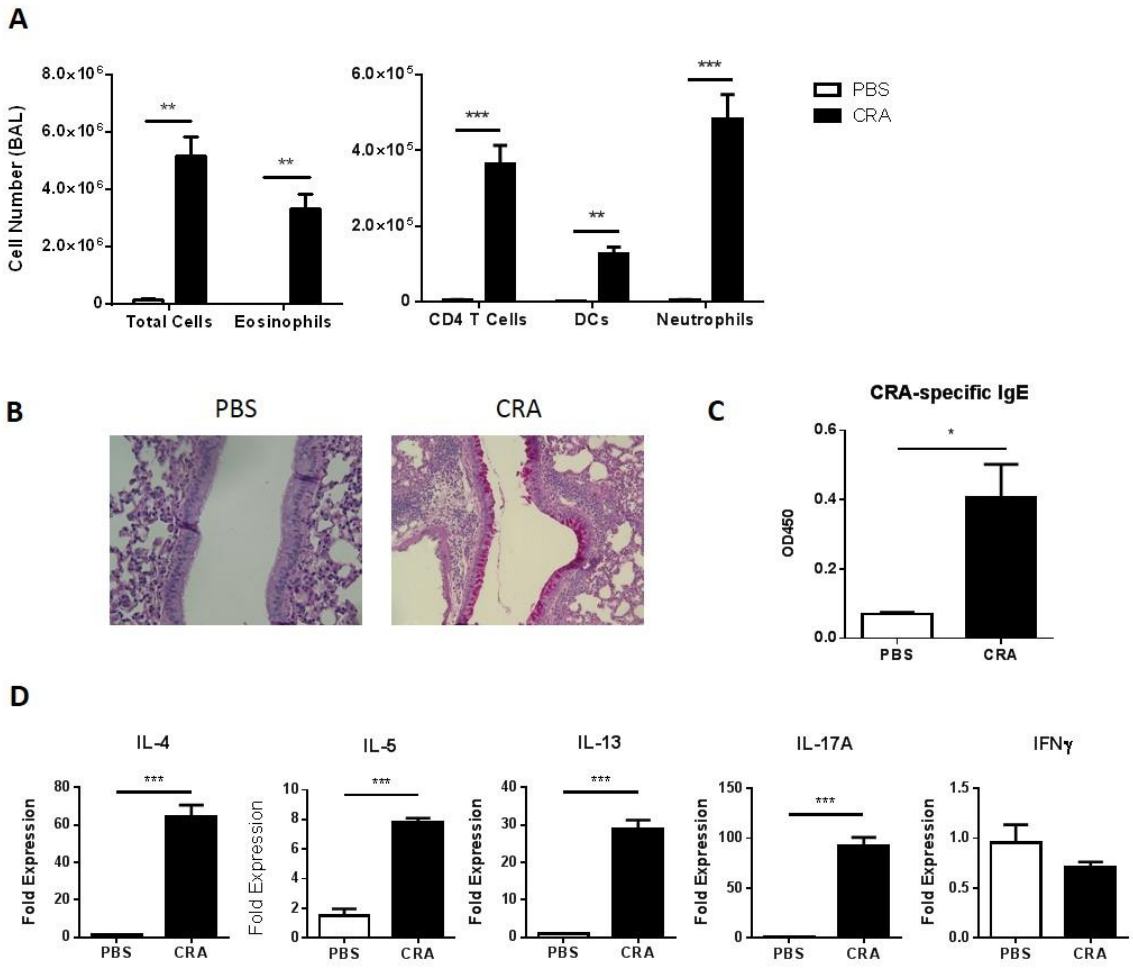


Figure 2-1: Intranasal sensitization and challenge with CRA results in TH2 allergic airway inflammation.

On days 0-2, C57BL/6 mice were intranasally sensitized with CRA and challenged with CRA on days 17-18. Allergic airway inflammation was assessed 72 hours after the last challenge. (A) BAL differential cell counts were determined by flow cytometry. (B) PAS staining of formalin-fixed and paraffin-embedded lung sections. Purple staining cells for PAS represent mucus producing goblet cells. (C) Serum levels of CRA-specific IgE were tested by ELISA. (D) RNA was isolated from the lungs of PBS- versus CRA-challenged mice and mRNA expression of IL-4, IL-5, IL-13, IL-17A, and IFNɣ was determined by qPCR. All expression values are relative to PBS control samples and normalized to the housekeeping gene GAPDH. Values are mean ± SEM. Comparisons were made using unpaired Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

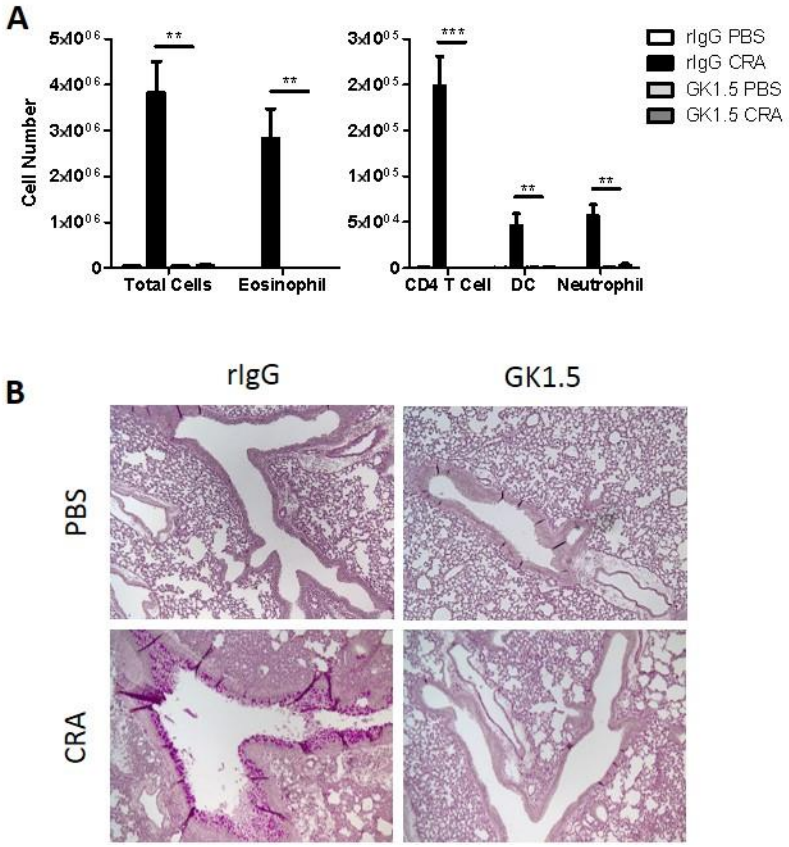


Figure 2-2: CD4 T cells are required for allergic airway inflammation to CRA.
C57BL/6 mice were treated i.p. with rIgG or anti-CD4 antibody (GK1.5) to deplete CD4 T cells during the course of sensitization and challenge to CRA. (A) BAL differential cell counts were determined by flow cytometry (B) PAS staining of formalin-fixed and paraffin-embedded lung sections. Values are mean ± SEM. Comparisons were made using unpaired Student's *t* test. ** $P < 0.01$, *** $P < 0.001$.

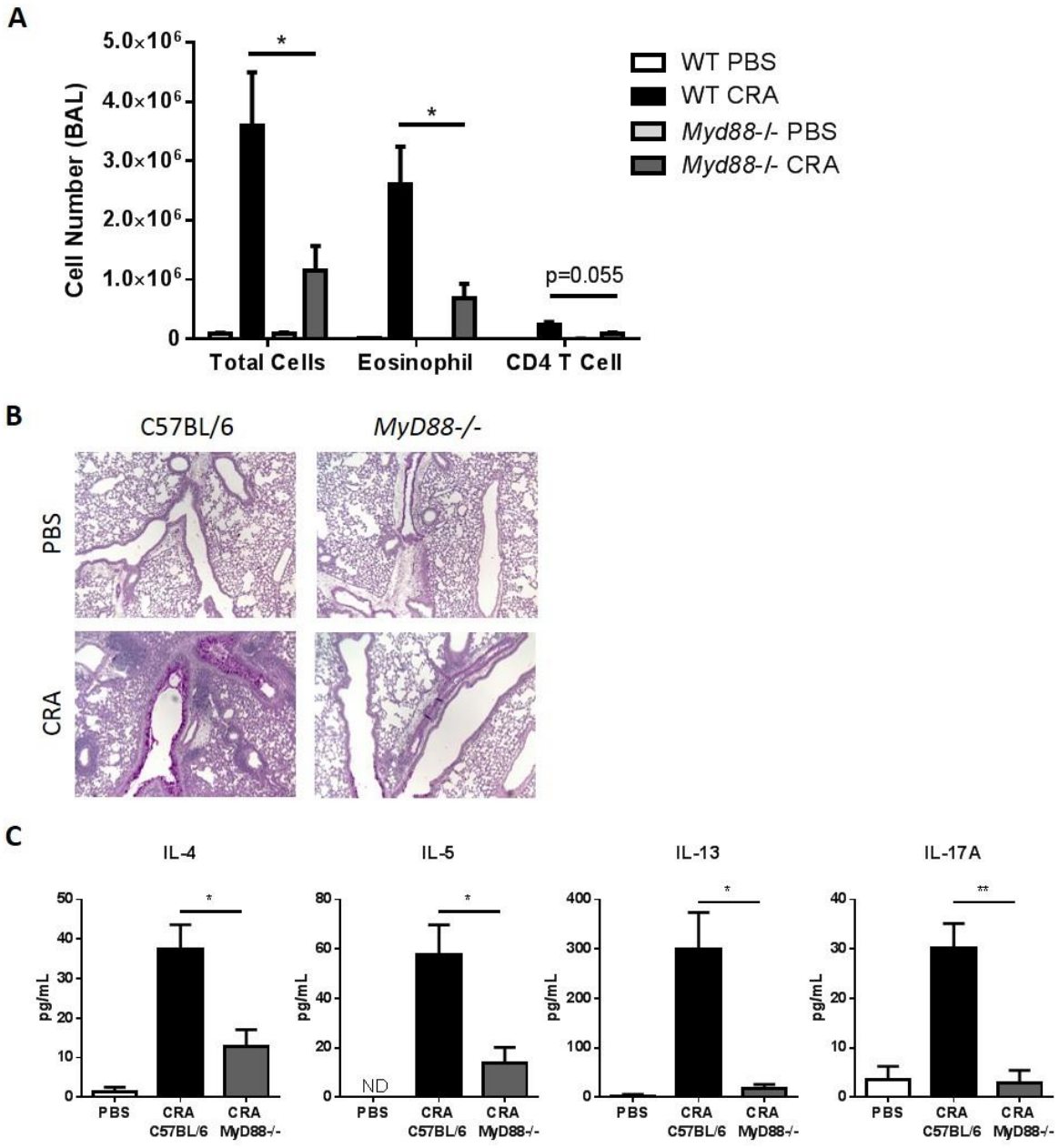


Figure 2-3: MyD88 is required for the development of allergic airway inflammation to CRA.

C57BL/6 and *Myd88*-/- mice were sensitized and challenged with CRA as described in the Methods. (A) BAL differential cell counts were determined by flow cytometry (B) PAS staining of formalin-fixed and paraffin-embedded lung sections. (C) Concentration of IL-4, IL-5, IL-13, and IL-17A from BAL fluid was determined by ELISA. Values are mean ± SEM. Comparisons were made using unpaired Student's *t* test. * $P < 0.05$, ** $P < 0.01$.

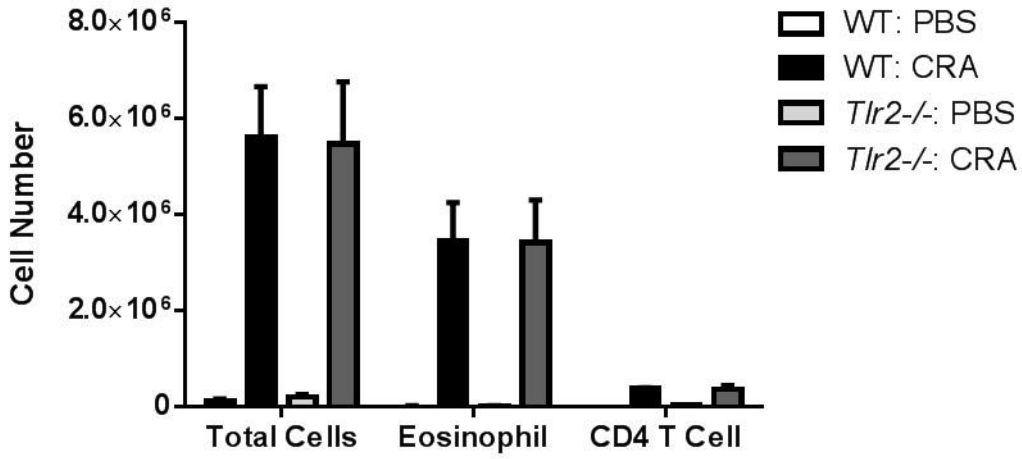


Figure 2-4: TLR2 is not required for allergic airway response to CRA.

C57BL/6 and *Tlr2*-/- mice were sensitized and challenged with CRA as described in the Methods. (A) BAL differential cell counts were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using unpaired Student's *t* test.

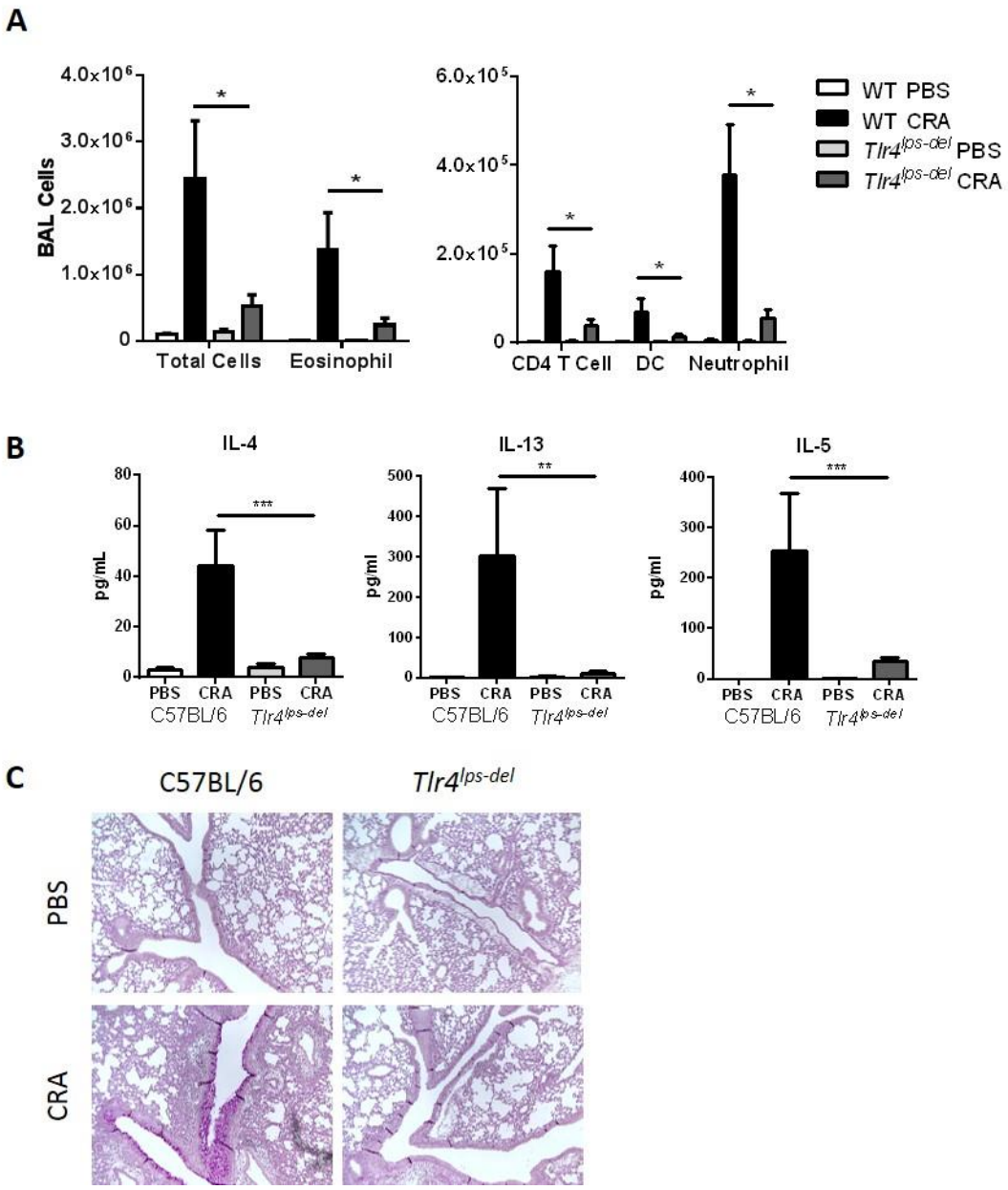


Figure 2-5: TLR4 plays a role in the allergic airway response to CRA.

C57BL/6 and TLR4-deficient (*Tlr4*$^{lps\text{-}del}$) mice were sensitized and challenged with CRA as described in the Methods. (A) BAL differential cell counts were determined by flow cytometry (B) Concentration of IL-4, IL-5, IL-13, and IL-17A from BAL fluid was determined by ELISA. (C) PAS staining of formalin-fixed and paraffin-embedded lung sections. Values are mean ± SEM. Comparisons were made using unpaired Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

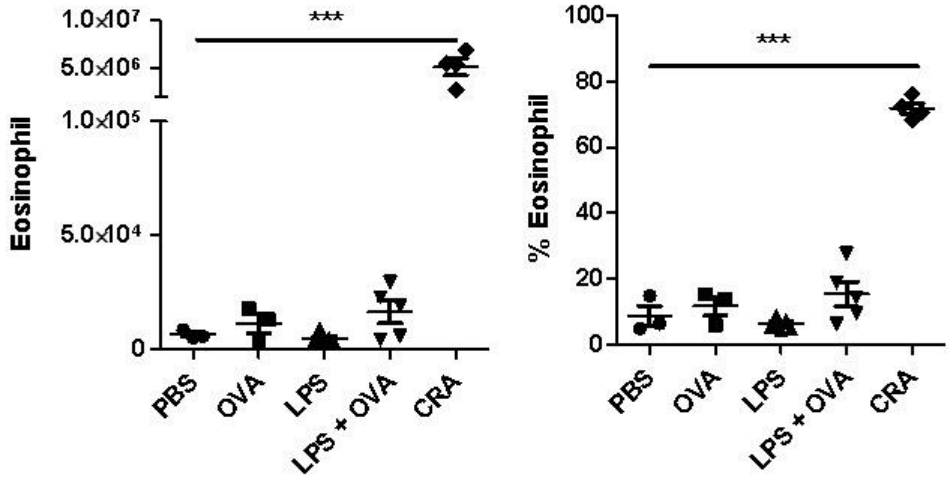


Figure 2-6: Low level of LPS in CRA is not sufficient to drive a T_H2 allergic response to inhaled ovalbumin.

C57BL/6 mice were sensitized and challenged with PBS, 40 μg of OVA, 11 ng of LPS, 11 ng of LPS plus 40 μg of OVA, or 40 μg of CRA which contains 11 ng of LPS. (A) The number and percent of eosinophils were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using one-way ANOVA with a Tukey posthoc test. *** $P < 0.001$.

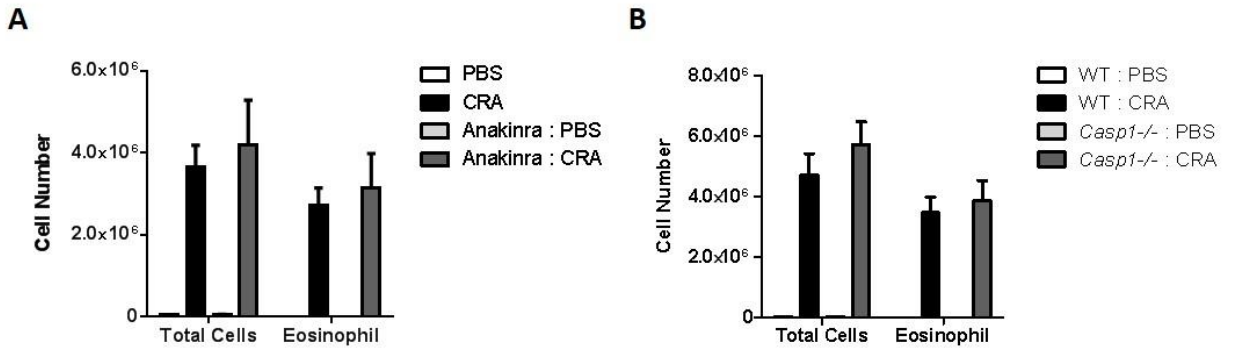


Figure 2-7: IL-1α, IL-1β, and IL-18 are not required for allergic airway response to CRA. C57BL/6 mice were i.p. injected with PBS or an IL-1R antagonist (Anakinra) at the time of CRA administration and were sensitized and challenged with PBS or CRA as described in the Methods. BAL cell differentials were determined by flow cytometry. (B) C57BL/6 and *casp1*-/- mice were sensitized and challenged with PBS or CRA as described in the Methods. BAL cell differentials were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using Student's *t* test.

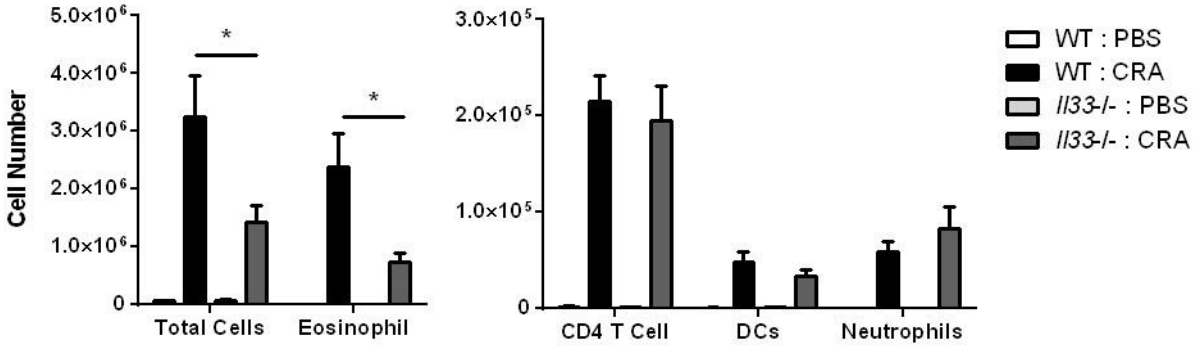


Figure 2-8: IL-33 is required for CRA-induced airway eosinophilia.
C57BL/6 and IL-33 deficient mice were sensitized and challenged with PBS or CRA as described in the Methods. BAL cell differentials were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using Student's *t* test. * $P < 0.05$.

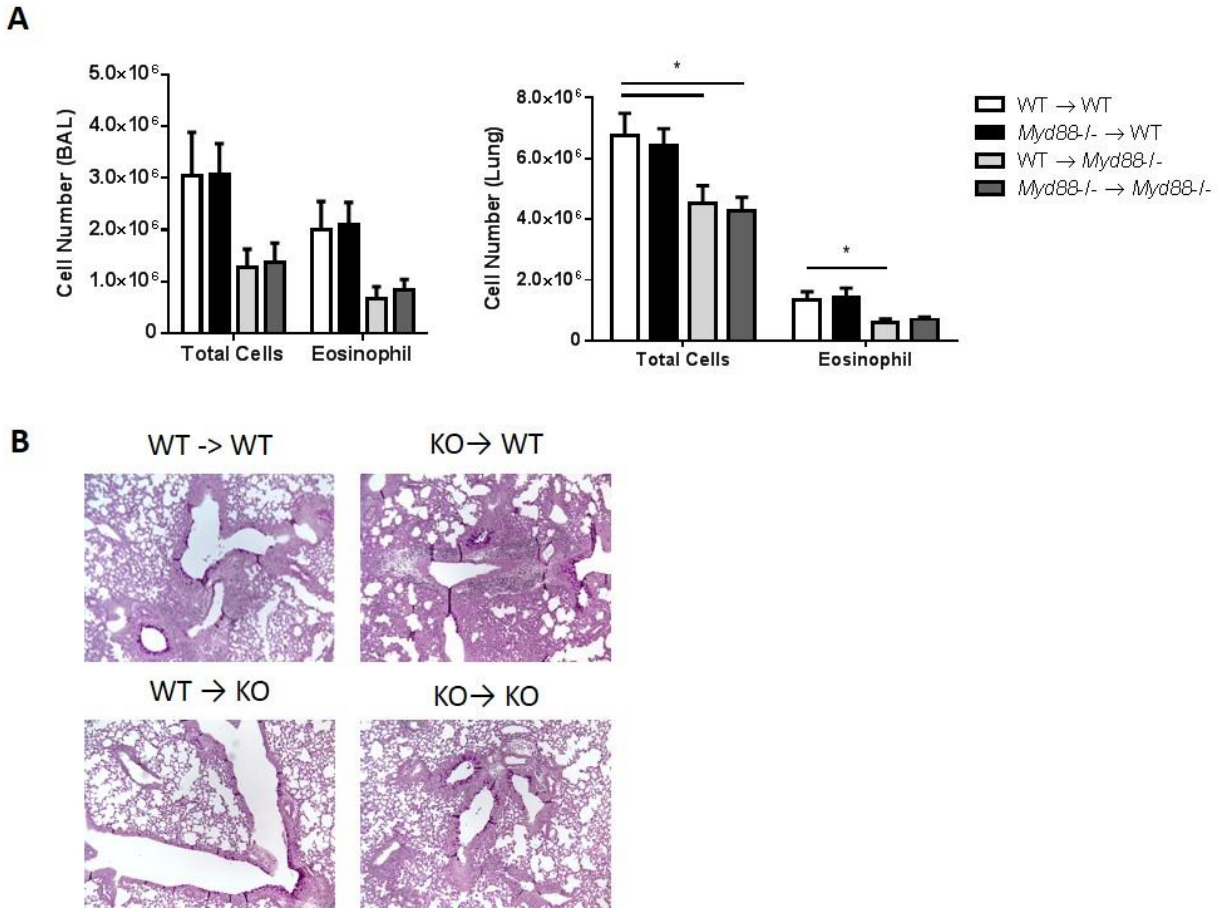


Figure 2-9: MyD88 expression is required in radioresistant cells to drive allergic inflammation to CRA.

C57BL/6 and *Myd88*-/- mice were irradiated twice with 4.5 Gy and either WT or *Myd88*-/- bone marrow cells were transferred into the irradiated host. After resting the mice 8-10 weeks to allow for bone marrow reconstitution, mice were sensitized and challenged with CRA. (A) BAL and lung cell differentials were determined by flow cytometry. (B) PAS staining of formalin-fixed and paraffin-embedded lung sections. Values are mean ± SEM. Comparisons were made using one-way ANOVA with Tukey posthoc test. * $P < 0.05$.

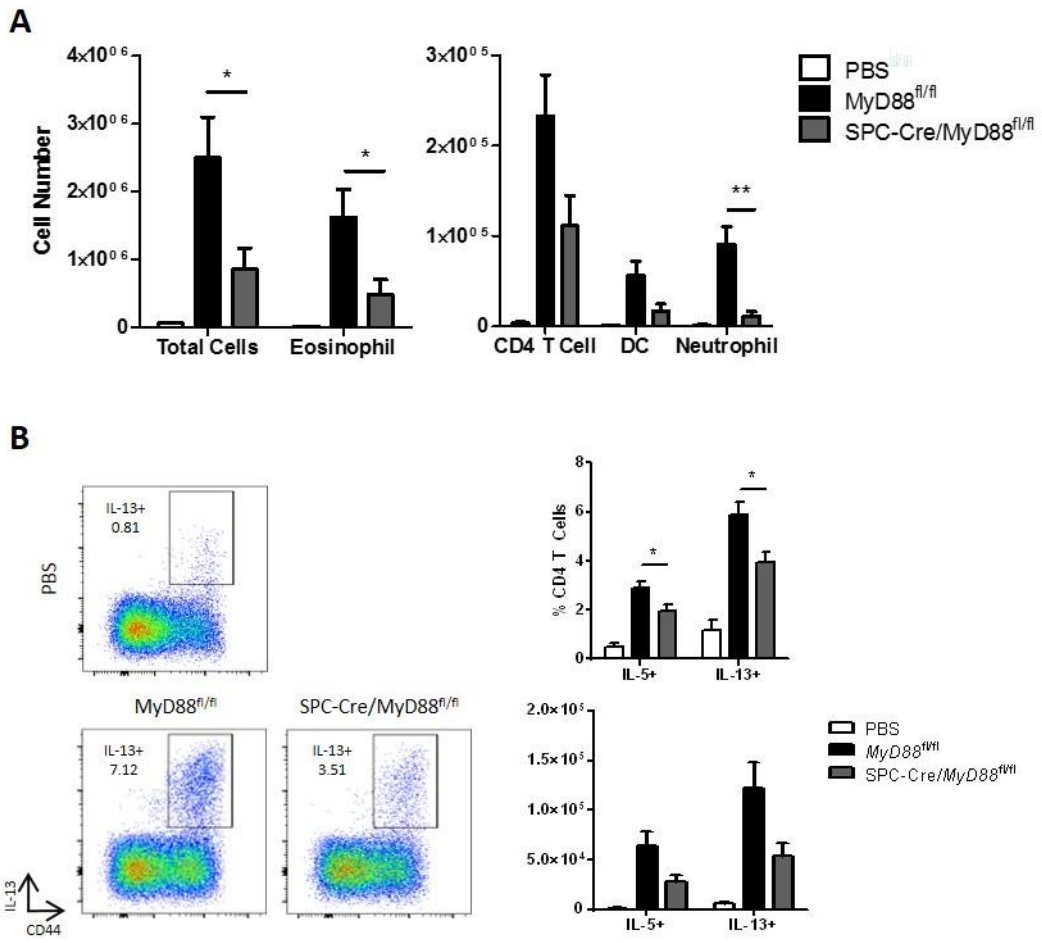


Figure 2-10: MyD88 expression is required in airway epithelial cells for allergic inflammation to CRA.

MyD88$^{fl/fl}$ and SPC-Cre/MyD88$^{fl/fl}$ mice were sensitized and challenged with CRA. (A) BAL cell differentials were determined by flow cytometry. (B) Lung cells were restimulated with PMA and ionomycin with GolgiPlug and intracellular cytokines were stained. Values are mean ± SEM. Comparisons were made using Student's *t* test. * $P < 0.05$, ** $P < 0.01$.

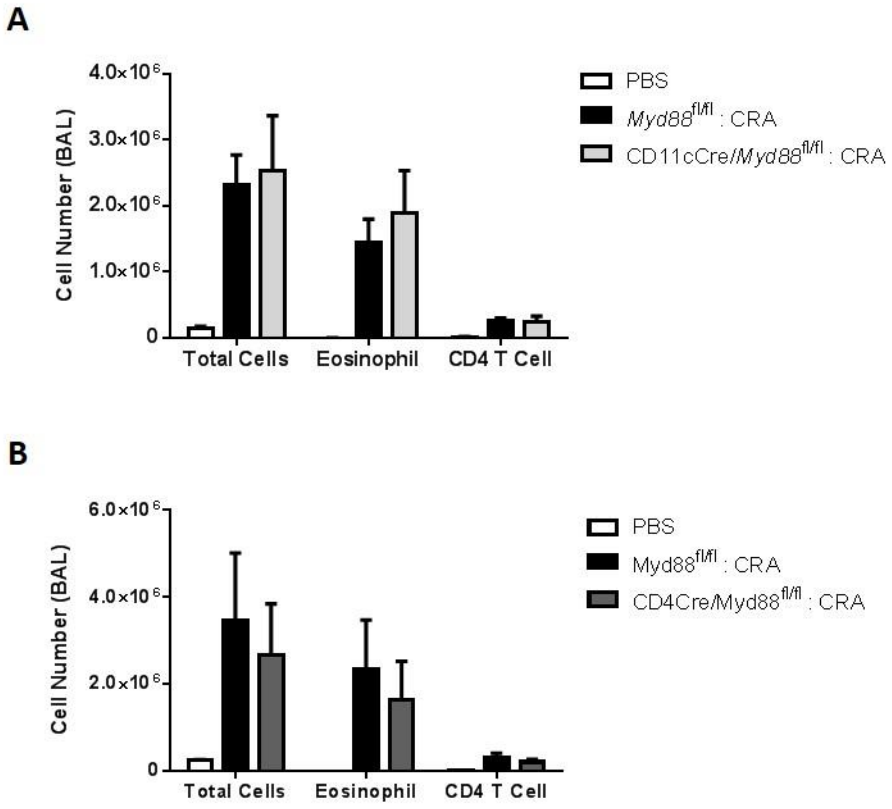


Figure 2-11: MyD88 expression is not required in CD11c+ and CD4+ immune cells for allergic inflammation to CRA.

(A) MyD88$^{fl/fl}$ and CD11c-Cre/MyD88$^{fl/fl}$ mice were sensitized and challenged with CRA. BAL cell differentials were determined by flow cytometry. (B) MyD88$^{fl/fl}$ and CD4-Cre/MyD88$^{fl/fl}$ mice were sensitized and challenged with CRA. BAL cell differentials were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using Student's *t* test.

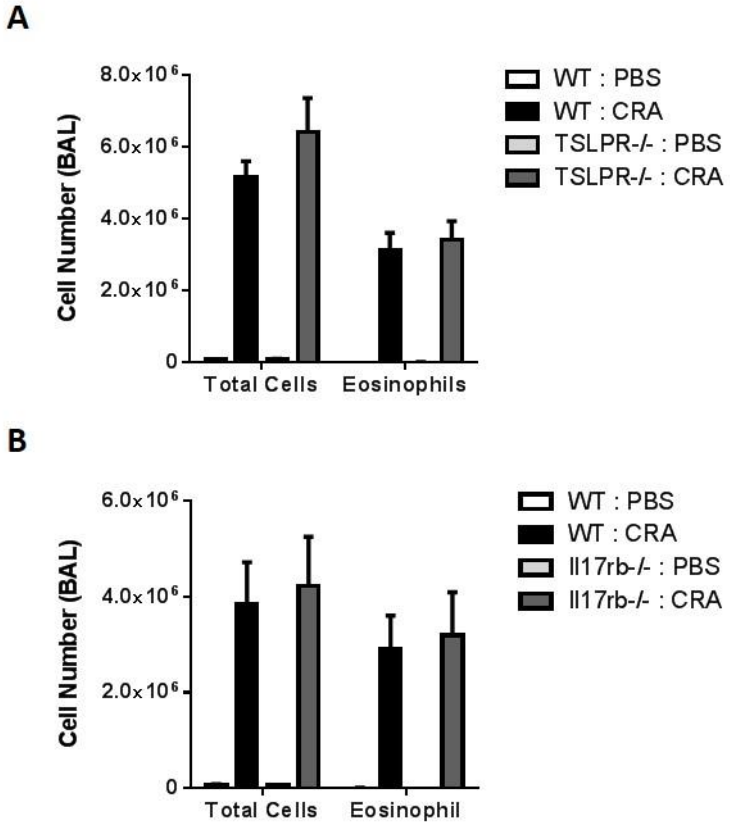


Figure 2-12: TSLP and IL-25 are not required for allergic airway response to CRA.
(A) C57BL/6 and TSLPR-/- mice were sensitized and challenged with CRA. BAL cell differentials were determined by flow cytometry. (B) C57BL/6 and *Il17rb*-/- mice were sensitized and challenged with CRA. BAL cell differentials were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using Student's *t* test.

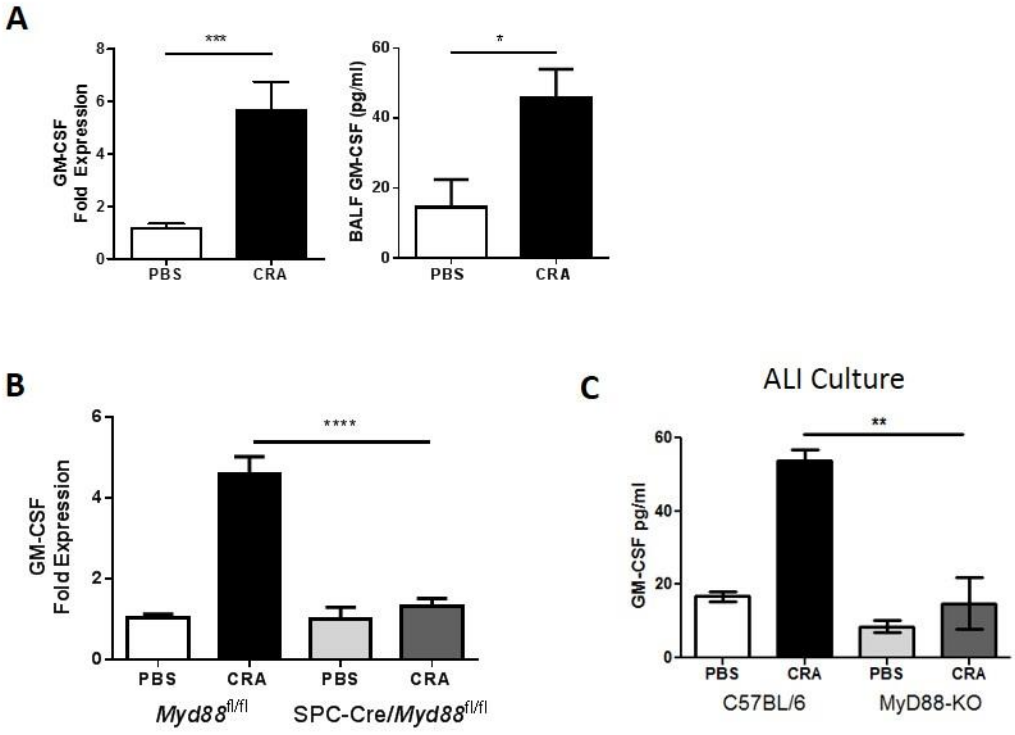


Figure 2-13: GM-CSF is produced by airway epithelial cells in a MyD88 dependent manner.

(A) C57BL/6 mice intranasally treated with CRA. Two hours later, lung tissue was collected and mRNA expression of GM-CSF was determined by qPCR. BAL fluid was also collected and GM-CSF protein level was measured by ELISA. (B) $MyD88^{fl/fl}$ and SPC-Cre/$MyD88^{fl/fl}$ mice were intranasally treated with CRA. Two hours later, lung tissue was collected and mRNA expression of GM-CSF was determined by qPCR. (C) Tracheas C57BL/6 and *Myd88*-/- mice were harvested and tracheal epithelial cells were cultured at air-liquid interface. Cells were exposed to 15 μg of CRA for 24 hours and medium from the basal compartment was collected and GM-CSF levels were measured by ELISA. Values are mean ± SEM. Comparisons were made using Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

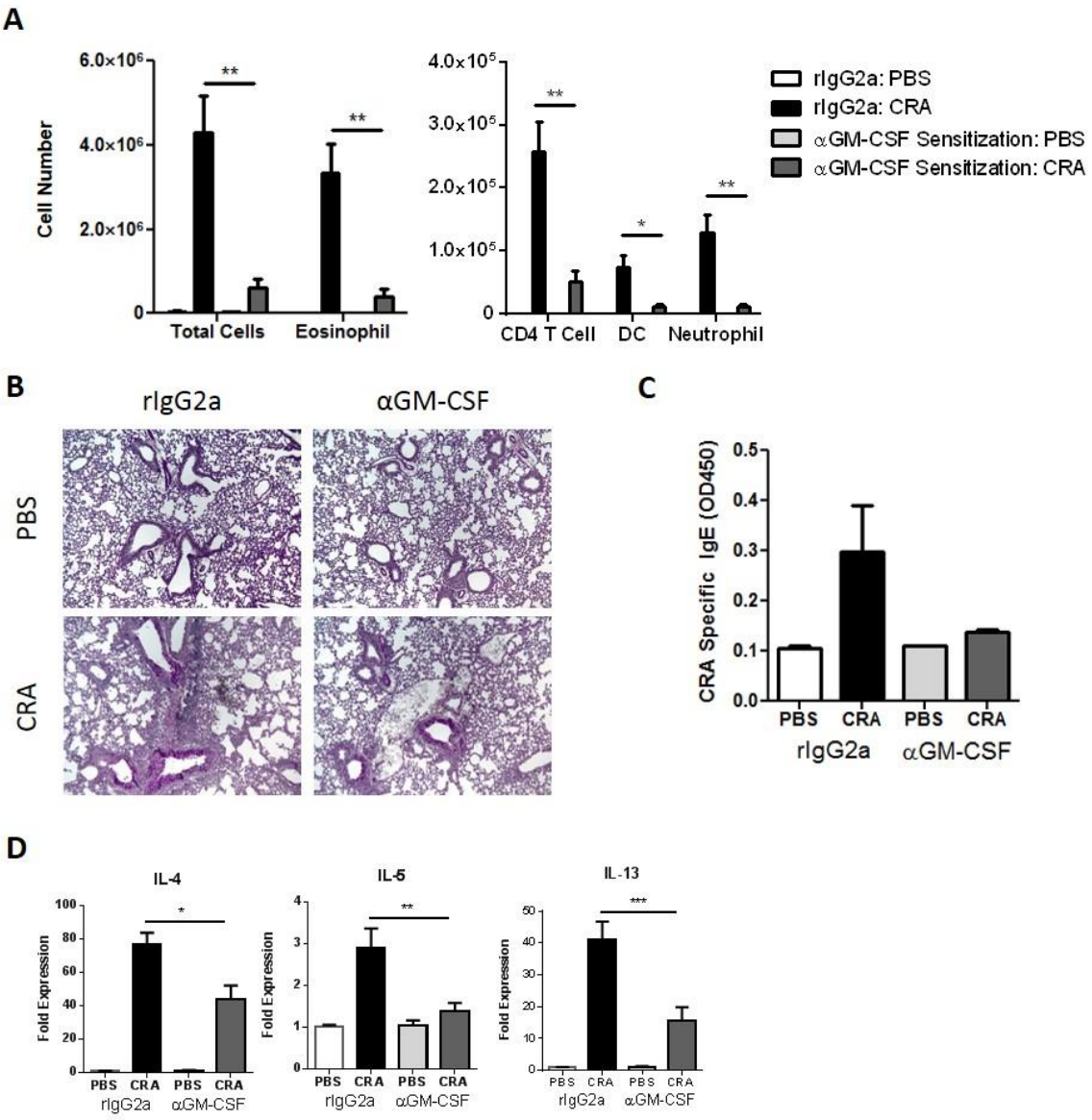


Figure 2-14: GM-CSF is required for allergic sensitization to CRA.

(A) C57BL/6 mice were treated with rIgG2a or a neutralizing anti-GM-CSF antibody, five hours prior to sensitization. Mice were then sensitized and challenged with CRA and airway inflammation was analyzed 72 hours later. (A) BAL differential cell counts were determined by flow cytometry. (B) PAS staining of formalin-fixed and paraffin-embedded lung sections. (C) Serum levels of CRA-specific IgE. (D) RNA was isolated from the lung and mRNA expression of IL-4, IL-5, and IL-13 was determined by qPCR. All expression values are relative to PBS control samples and normalized to the housekeeping gene GAPDH. Values are mean ± SEM. Comparisons were made using Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

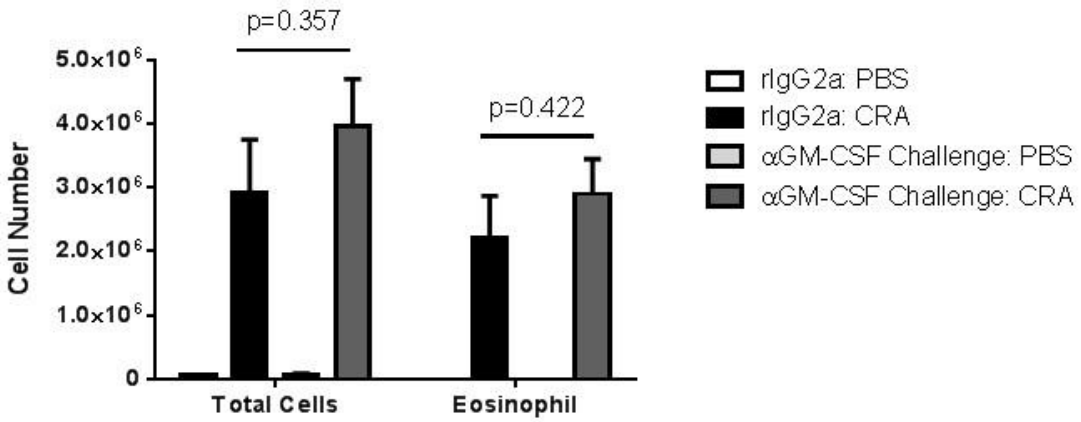


Figure 2-15: GM-CSF is not required during the challenge phase of CRA-induced allergic airway disease.

(A) C57BL/6 mice were treated with rIgG2a or a neutralizing anti-GM-CSF antibody, five hours prior to challenge. (A) After allergen challenge, BAL differential cell counts were determined by flow cytometry. Values are mean ± SEM. Comparisons were made using Student's *t* test.

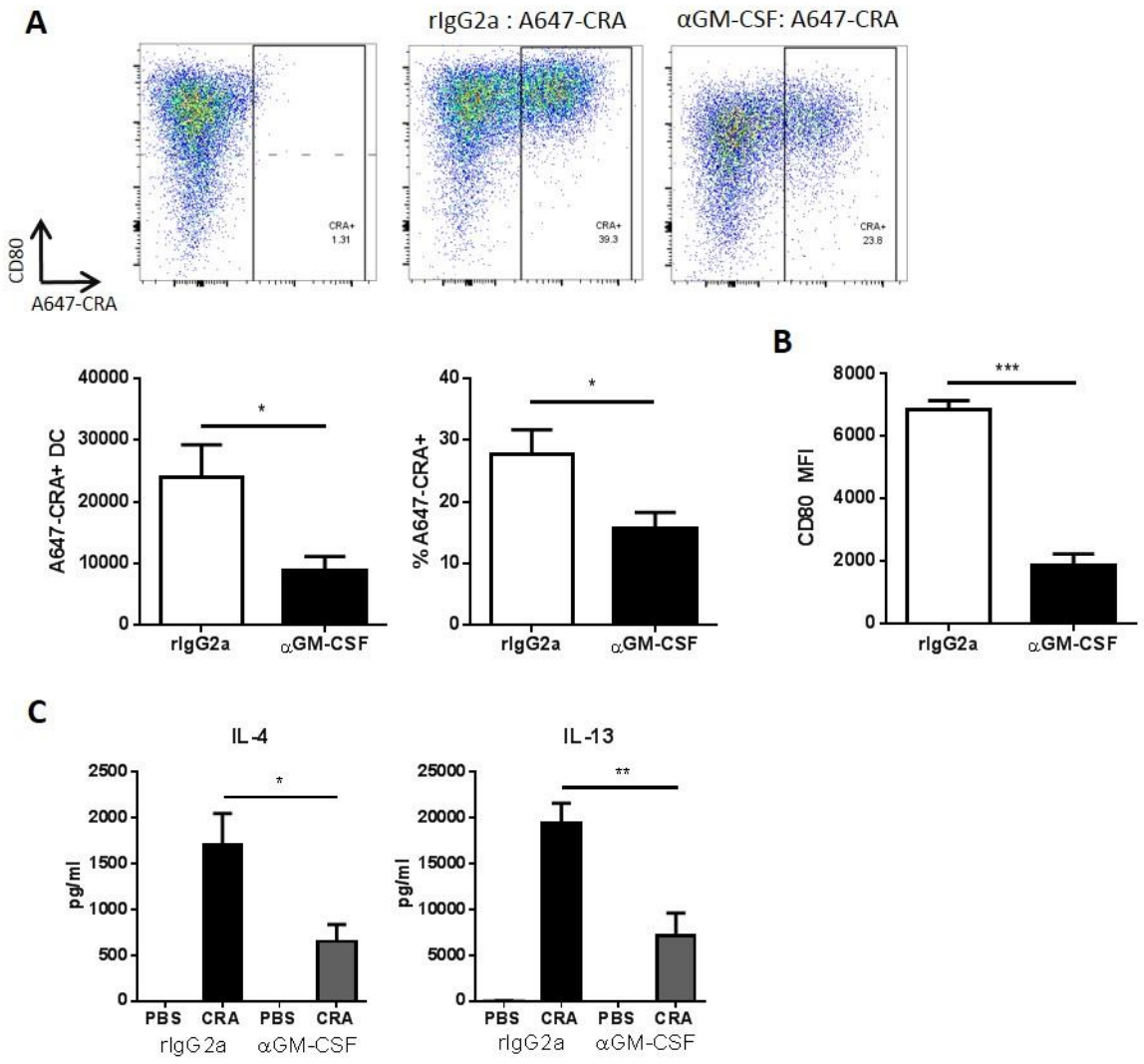
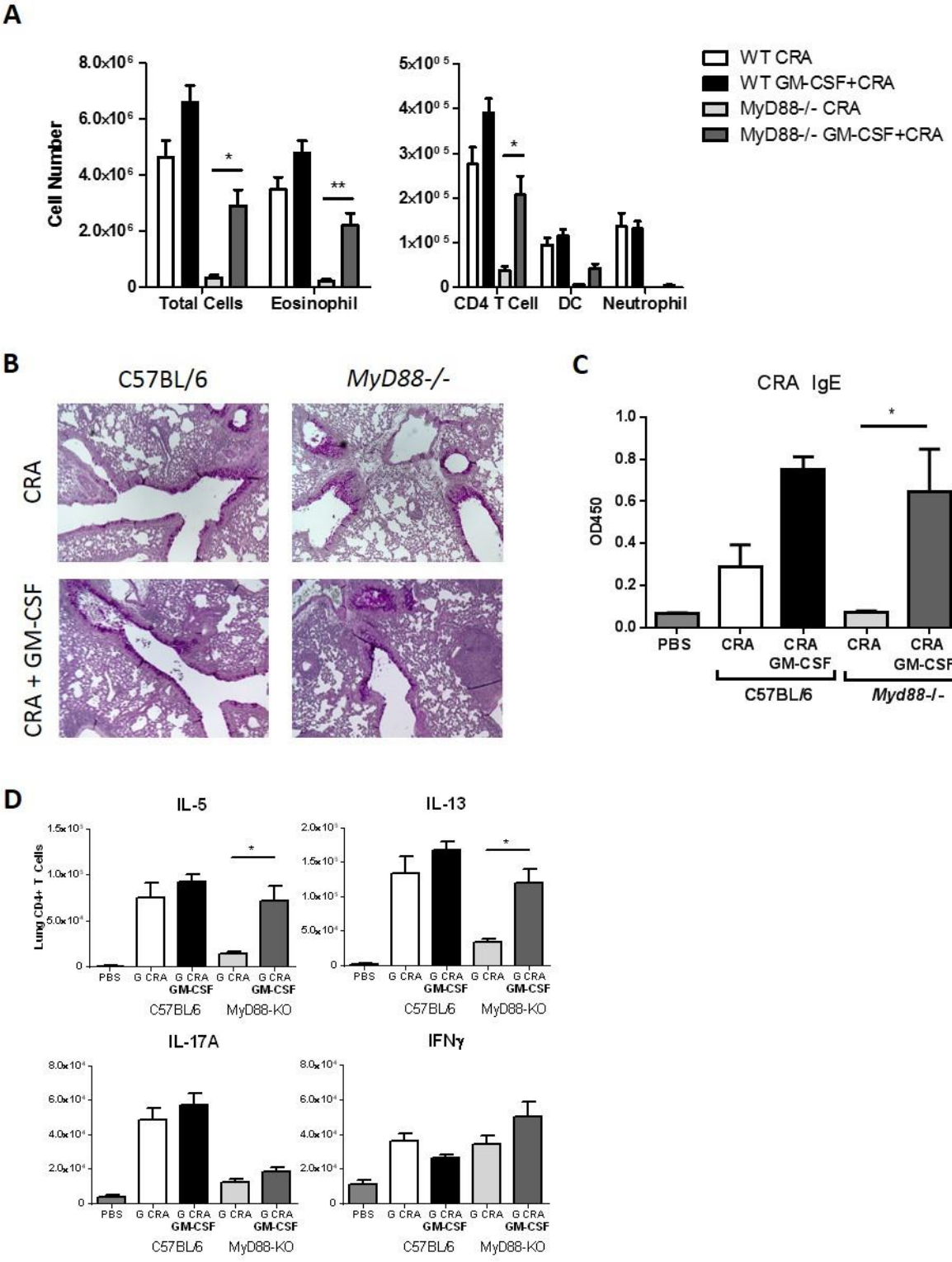


Figure 2-16: GM-CSF is required for optimal DC function and T cell priming.

(A) C57BL/6 mice were intranasally treated with A647-CRA and the draining lymph node was collected 24 hours later. Cells were analyzed by flow cytometry to assess the uptake of CRA and maturation of DCs. DCs were gated as CD11c+ and MHCII+. (A) Total number of A647+ DCs was determined. Within the DC population, the percentage of A647+ DCs was determined. (B) Mean fluorescent intensity (MFI) of costimulatory molecule CD80 on A647+ DCs. (C) On days 0-2, C57BL/6 mice were sensitized with CRA. On day 3, the lung draining lymph node was collected and cells were restimulated with CRA. On day 7, culture supernatant was collected and concentration of IL-4 and IL-13 were determined by ELISA. Values are mean ± SEM. Comparisons were made using Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.


A
Cell Number
8.0x10^6
6.0x10^6
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0
Total Cells
Eosinophil
5x10^0 5
4x10^0 5
3x10^0 5
2x10^0 5
1x10^0 5
CD4 T Cell
DC
Neutrophil
WT CRA
WT GM-CSF+CRA
MyD88-/- CRA
MyD88-/- GM-CSF+CRA
B
C57BL/6
MyD88-/-
CRA
CRA + GM-CSF
C
CRA IgE
OD450
1.0
0.8
0.6
0.4
0.2
0.0
PBS
CRA
CRA GM-CSF
C57BL/6
Myd88-/-
D
IL-5
IL-13
IL-17A
IFNγ
Lung CD4+ T Cells
PBS
G CRA
G CRA GM-CSF
C57BL/6
MyD88-KO

Figure 2-17: GM-CSF rescues T_H2 allergic airway response to CRA.

C57BL/6 and *Myd88*-/- mice were intranasally treated with or without 50 ng of GM-CSF during sensitization which was administered along with CRA. Mice were challenged with CRA as usual. (A) BAL differential cell counts were determined by flow cytometry (B) PAS staining of formalin-fixed and paraffin-embedded lung sections. (C) Lung cells were restimulated with PMA and ionomycin with GolgiPlug for 4 hours at 37° C and intracellular cytokines were stained. (D) Serum CRA-specific IgE levels were determined by ELISA. Values are mean ± SEM. Comparisons were made using one-way ANOVA with Tukey posthoc test. * $P < 0.05$, ** $P < 0.01$.

Chapter 3: Concluding Remarks

The underlying cause of allergic asthma is an aberrant immune response directed against allergens such as CRA. Although allergen-specific T_H2 cells are considered central to the pathogenesis of allergic asthma, it is unclear how T_H2 responses are initiated during sensitization. The emerging picture suggests that the local cytokine milieu heavily influences the function of DCs and the type of T cell response that is initiated in response to allergens. Airway epithelial cell-derived cytokines are key components in the cytokine milieu of the lung environment. Activation of airway epithelial cells by inhaled allergens results in the release of cytokines such as TSLP, IL-25, and IL-33. Individually, each cytokine can directly promote T_H2 allergic responses against the innocuous antigen ovalbumin (Fort et al. 2001; Headley et al. 2009; Schmitz et al. 2005). However, the role of each cytokine in the initiation of T_H2 immunity to specific allergens is complex. For example, IL-33, but not TSLP or IL-25, is required for allergic sensitization to house dust mite and peanut allergens (Chu et al. 2012). In fact, increasing evidence suggests that different allergens can elicit unique pathways that lead to T_H2 immunity. To better understand how allergen exposure leads to the development of allergic asthma, it is important to identify mechanisms that are uniquely employed by individual allergens versus those that might be common among all allergens.

In particular, the molecular and cellular pathways involved in mucosal sensitization to CRA remains to be elucidated. To study the mechanisms that regulate allergic sensitization to CRA, we began by examining if TLRs are important for the recognition of CRA. We found that allergic airway response to CRA is dependent on MyD88, an essential adaptor molecule in signaling pathways downstream of TLRs and IL1Rs. We determined that TLR4, but not TLR2,

is required for allergic sensitization to CRA. These results add to a number of studies that have also reported an important role for TLR4 in allergic sensitization to house dust mite, fungal protease, short ragweed allergen, and nickel (Hammad et al. 2009; Li et al. 2011; Millien et al. 2013; Schmidt et al. 2010). This leads us to the question of why TLR4 is required for sensitization to these different allergens. One possibility is that these allergens facilitate binding of LPS to TLR4. For example, Der p 2, a house dust mite allergen, is functionally homologous to MD-2, a component of the TLR4 signaling complex (Trompette et al. 2009), and Fel d 1, a cat allergen, can also bind directly to LPS (Herre et al. 2013). Recently, it was reported that Bla g 1, a major cockroach allergen, contains a hydrophobic cavity that can bind to lipids. These results suggest that allergens might promote triggering of TLR4 as a common mechanism to initiate sensitization.

Among the main findings presented in this thesis is that activation of airway epithelial cells is crucial for sensitization to CRA. To the best of our knowledge, this is the first study to directly show that allergic sensitization to CRA requires MyD88 expression in airway epithelial cells, but not hematopoietic cells. This was demonstrated in both reciprocal bone marrow chimera studies and experiments using the Cre/lox system to selectively delete MyD88 in airway epithelial cells. Next, we set out to unravel the mechanism by which airway epithelial cells mediate allergic sensitization to CRA. Because it is widely believed that airway epithelial cells promote T_H2 immunity through production of TSLP, IL-25, and IL-33, we investigated the role of these epithelial-derived cytokines in allergic sensitization to CRA. Surprisingly, we found that TSLP and IL-25 are not required for allergic airway response to CRA. In contrast, we found that IL-33 is required for only one aspect of the allergic response, airway eosinophilia.

Therefore, our data challenges the idea that allergic sensitization requires TSLP, IL-25, and IL-33 for initiation of T_H2 responses to allergens. Instead, it reemphasizes the idea that structurally diverse allergens can elicit unique pathways that lead to T_H2 immunity. Currently, there are several clinical trials investigating the benefit of targeting these cytokines for the treatment of asthma. Our study suggests that these therapeutics might not be effective for all asthmatic patients, particularly for those that are mainly allergic to CRA.

In continuing to investigate the role of airway epithelial cells during sensitization to CRA, we uncovered that airway epithelial cells produced GM-CSF in response to CRA. Although GM-CSF can be produced by a variety of cell types, we showed that MyD88 expression in airway epithelial cells is required for production of GM-CSF after inhalation of CRA. ALI cultures confirmed that airway epithelial cells produce GM-CSF after direct stimulation with CRA in a MyD88-dependent manner. Furthermore, we demonstrate that GM-CSF is required for allergic sensitization to CRA. Using a neutralizing antibody against GM-CSF, we showed that blocking GM-CSF during sensitization, but not challenge, dramatically reduces the allergic airway response to CRA. Blocking GM-CSF during sensitization also reduces T_H2 priming in the lymph node. This correlates with a reduction in the number of A647-CRA^+ DCs in the lung draining lymph node, as well as lower expression of the costimulatory molecule CD80 on CRA^+ DCs. Although we do not directly show that airway epithelial cells are the main source of GM-CSF in vivo, these results strongly suggest that epithelial cells mediate allergic sensitization to CRA through production of GM-CSF.

Finally, we demonstrated that GM-CSF acts downstream of MyD88 during allergic sensitization to CRA. Specifically, we showed that intranasal delivery of GM-CSF during

sensitization selectively restores T_H2-mediated airway eosinophilia and CRA-specific IgE production in the absence of MyD88. In contrast, GM-CSF fails to recover both T_H17 responses and neutrophilia in the absence of MyD88. This data suggests that MyD88 signaling initiates several molecular programs that have very distinct functions during sensitization to CRA. In particular, GM-CSF appears to be part of a pathway that selectively promotes T_H2 responses downstream of MyD88. On the other hand, we have not determined the molecular mechanism responsible for T_H17-mediated neutrophilia. This has clinical implications for the treatment of T_H2- versus T_H17-associated asthma, underscoring the need to develop therapeutics that can target these different subtypes of asthma.

In this thesis, we describe a detailed mechanism of how airway epithelial cells regulate allergic sensitization to CRA. During sensitization, inhalation of CRA activates MyD88 signaling in airway epithelial cells, which results in the release of a key cytokine GM-CSF. GM-CSF specifically promotes T_H2 priming, most likely by regulating the functional maturation of DCs. Overall, studies outlined in this thesis demonstrate that airway epithelial cells modulate the local cytokine environment in the lung to instruct immune cells and direct the immune response against CRA.

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Curriculum Vitae

ALYSSA SHEIH

EDUCATION:

Ph.D. Candidate, Molecular & Cell Biology, University of Washington
- National Science Foundation Graduate Research Fellowship
- Cumulative GPA: 3.76/4.00

B.S. Bioengineering, University of Washington, June 2010
- Graduated with Departmental Honors
- Cumulative GPA: 3.95/4.00

RESEARCH EXPERIENCE:

Graduate Student, Department of Immunology
Advisor: Steven Ziegler, 09/11 – Present
- Characterized a mouse model of allergic asthma to study how mucosal exposure to cockroach allergen leads to the development of asthma.
- Designed experiments to determine the receptors involved in the recognition of cockroach allergen by airway epithelial cells and innate immune cells.
- Examined the mechanisms by which the cytokine, GM-CSF, promotes sensitization to cockroach allergen.
- Collaborated with other research groups to explore new ideas in the asthma research field.

Research Assistant, Department of Chemical Engineering
Advisor: Hong Shen, 06/08 – 09/10
- Modified the surface property of nanoparticles by conjugating varying densities of polyethylene glycol (PEG) to study how PEGylation affects the uptake of nanoparticles by the immune system to improve the design of vaccine delivery vehicles.
- Quantified the uptake of particles using flow cytometry and determined the uptake mechanism using chemical inhibitors.
- Wrote a research plan and a detailed budget that received funding for research project through the Levinson Emerging Scholars Program.

Research Assistant, Department of Bioengineering
Advisor: Daniel Ratner, 06/09 – 09/09
- Generated a carbohydrate vaccine against the intestinal parasite, *Entamoeba histolytica*, for pre-clinical testing in animals.
- Presented a poster at the Molecular Parasitology Meeting.

Research Assistant, Department of Biology
Advisor: Benjamin Kerr, 06/07 – 06/08

- Developed a computational model to explore how host sociality and pathogen virulence coevolve.
- Designed computer simulations to test the outcomes of this model using object-oriented programming in Java.
- Published research findings in the Journal of Theoretical Biology.

PUBLICATIONS & PRESENTATIONS:

- **Sheih** A, Ziegler SF. (2015). Allergic sensitization to cockroach allergen requires production of GM-CSF downstream of the TLR4/MyD88 pathway. Presented at the RIKEN IMS Summer Program, June 17, 2015; Oral Presentation.
- **Sheih** A, Ziegler SF. (2014). Mechanisms regulating the development of allergic airway inflammation to cockroach allergen. Presented at the Abcam Immunoregulatory Networks Meeting, June 17, 2014; Poster.
- **Sheih** A, Ziegler SF. (2013). Allergic airway response to cockroach allergen is dependent on thymic stromal lymphopoietin. Presented at the American Association of Immunologists Meeting, May 5, 2013; Poster.
- Ratner DM, **Sheih A**. (2009). Targeting *Entamoeba histolytica* Asparagine-Linked Glycans. Presented at the Molecular Parasitology Meeting, September 16, 2009; Poster 252C.
- Prado F, **Sheih A**, West JD, Kerr B. "Coevolutionary Cycling of Host Sociality and Pathogen Virulence in Contact Networks." Journal of Theoretical Biology. 261. 4 (2009): 561-9.

TEACHING EXPERIENCE:

Pacific Science Center Science Communication Fellow, 2014-Current

- Developed a hands-on activity that teaches how the immune system recognizes harmful pathogens.
- Facilitated this activity as part of the museum's public outreach programs.
- Attended professional development workshops on science communication.

Graduate Teaching Assistant, Biology 220, University of Washington, 01/12 – 03/12

- Instructed lab classes on plant and animal physiology.
- Interacted with students during class and office hours to explain difficult concepts and how lab activities related to the lecture material.

Mentor, Seattle Biomed BioQuest Outreach Program, 07/11

- Mentored a small group of high school students with a research project on HIV, using biological samples from Seattle Biomedical Research Institute.
- Explained basic concepts in immunology and taught lab techniques necessary to complete the project.

Bioengineering Outreach Program, University of Washington, 09/09 – 12/09

- Designed a teaching module on nanotechnology with a hands-on activity demonstrating the synthesis of gold nanoparticles.
- Developed a teaching module about muscles and electrical signals by demonstrating how to conduct an electromyography test.

Peer Teaching Assistant, Experimental Evolutionary Ecology, 09/08 – 12/08

- Assisted students with basic sterile techniques, pipetting, and plating bacteria.
- Set up weekly labs by preparing materials such as liquid culture tubes and agar plates.
- Answered questions and helped students understand the material being emphasized.

HONORS & AWARDS:

- National Science Foundation Graduate Research Fellowship, 2010
- Levinson Emerging Scholarship, University of Washington, 10/2009
- Gates Millennium Scholarship, Bill & Melinda Gates Foundation, 09/2006
- Washington NASA Space Grant Scholarship, University of Washington, 09/2006
- Nordstrom Cultural Diversity Scholarship, Nordstrom, 09/2006
- Washington Award for Vocational Excellence Scholarship, 09/2006

RESEARCH PROGRAMS:

- Clinical Research Experience for Engineers, 06/09 - 09/09
- University of Washington Amgen Scholars Program, 06/08 - 08/08
- Washington Space Grant Summer Undergraduate Research Program, 06/06 - 08/ 08